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2 **Fermentative hydrogen production under moderate halophilic conditions**

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10

11 **Abstract**

12 Dark fermentation is an intermediate microbial process occurring along the anaerobic biodegradation  
13 of organic matter. Saline effluents are rarely treated anaerobically since they are strongly inhibited by  
14 high salt concentrations. This study deals with the characterization of microbial communities  
15 producing hydrogen under moderate halophilic conditions. A series of batch experiments was  
16 performed under anaerobic conditions, with glucose as substrate (5 g L<sup>-1</sup>) and under increasing NaCl  
17 concentrations ranging from 9 to 75 g<sub>NaCl</sub> L<sup>-1</sup>. A saline sediment of a lagoon collecting salt factory  
18 wastewaters was used as inoculum. Interestingly, a gradual increase of the biohydrogen production  
19 yield according to NaCl concentration was observed with the highest value (0.9 ± 0.02 mol<sub>H<sub>2</sub></sub> mol<sub>Glucose</sub><sup>-1</sup>)  
20 obtained for the highest NaCl concentration, ie. 75 g<sub>NaCl</sub> L<sup>-1</sup>, suggesting a natural adaptation of the  
21 sediment inoculum to salt. This work reports for the first time the ability of mixed culture to produce  
22 hydrogen in moderate halophilic environment. In addition, maximum hydrogen consumption rates  
23 decreased while NaCl concentration increased. A gradual shift of the bacterial community structure,  
24 concomitant to metabolic changes, was observed with increasing NaCl concentrations, with the  
25 emergence of bacteria belonging to *Vibrionaceae* as dominant bacteria for the highest salinities.

26

27 **Key words:** Biohydrogen, CE-SSCP, Dark fermentation, Pyrosequencing, Salinity, *Vibrionaceae*,

28

## 29 1. Introduction

30 The actual increasing interest in hydrogen (H<sub>2</sub>) as a promising clean and sustainable energy  
31 carrier is mainly due to its high energy density (122 kJ g<sup>-1</sup>) as well as the high efficiency of fuel cells  
32 to convert H<sub>2</sub> to electricity for transportation purposes [1–3]. Nowadays, most of the hydrogen  
33 produced worldwide is generated by natural gas reforming. However, producing hydrogen from fossil fuel  
34 produce more than twice carbon oxide equivalent compared to biohydrogen and contribute to resource depletion  
35 [4,5]. Developing alternatives technologies to produce hydrogen from renewable energy sources and  
36 minimizing their environmental impact are therefore of high priority. Producing hydrogen in biological  
37 processes is considered more environmentally friendly and sustainable than from conventional techniques.  
38 Particularly, dark fermentation is a process that aims at producing biohydrogen and treating organic  
39 waste at the same time [1–3,6–9]. During anaerobic digestion of those compounds, several microbial  
40 metabolism pathways outcompete for hydrogen which is a key intermediate in the trophic chain, as  
41 electron carrier (Fig. 1 – General pathways for hydrogen production. Bold arrows are represented for  
42 hydrogen production pathways, dotted arrows for hydrogen consumption pathways and dashed arrows  
43 for hydrogen production concurrent routes (adapted from Guo et al [3]).

44 ). When applying specific operating conditions (low pH, high substrate/inoculum ratio), consumption  
45 and concurrent routes for hydrogen production can be avoided [3].

46 Moreover, saline wastewaters, that can be generated by fish, seafood, petroleum and leather  
47 industries, may contain large amounts of organic matter that have to be treated [10,11]. Wastewaters  
48 discharged from drinking water treatment plants using ion-exchange membrane and reverse-osmosis  
49 processes may also contain high amounts of salts [11]. Overall, saline wastewaters represent more than  
50 5% of the worldwide effluent treatment requirements [12]. Discharging those saline wastewaters  
51 directly to the environment leads to high risks of soil, surface water and groundwater salinization. In  
52 most cases, high salinity wastewaters have to be diluted before any biological treatment to reduce their  
53 salinity. Indeed, a high salinity can disturb the osmotic balance across microbial cell walls and cause  
54 plasmolysis of microbial cells, making unefficient any biological treatment [13,14]. Dilution of saline

55 wastewaters implies a high increase in water consumption of the treatment bioprocess and, as a  
56 consequence, its operating costs.

57 Although  $\text{Na}^+$  concentration has a strong inhibitory effect on anaerobic digestion processes [12,14,15],  
58 natural microbial communities can nevertheless well adapt to high salt concentrations to finally exhibit  
59 efficient activity for anaerobic treatment of saline wastewaters [10]. Some anaerobic microbial  
60 communities were reported as halophilic [16,17], whereas some others were non-halophilic and had to  
61 be adapted by increasing salt concentrations prior to using them as inoculum [12,18,19].

62 While many species from *Clostridium*, *Enterobacter* and *Escherichia* genera have been described in  
63 the literature as hydrogen-producing bacteria in mixed cultures in non halophilic environment  
64 [2,3,8,9,20–23], only few studies have dealt with dark fermentation of saline effluents in mixed  
65 cultures [24,25]. Regarding fermentative hydrogen production under moderate halophilic conditions  
66 with pure cultures, only *Bacillus megaterium* [26], *Halocella cellulolytica* [27] and *Clostridium*  
67 *acetobutylicum*[28] were reported previously to produce  $\text{H}_2$  at pH7 and under saline conditions up to  
68 2% w/v NaCl [26–28]. Interestingly, Alshiyab et al. [28] showed that hydrogen productivity of  
69 *Clostridium acetobutylicum* decreased by 18% while increasing NaCl concentration from 0 to 5  
70  $\text{g}_{\text{NaCl}} \text{L}^{-1}$ . Simankova et al. [27] reported that a halophilic bacteria, *Halocella cellulolytica*, isolated  
71 from hypersaline lagoons with high NaCl concentrations (50 to 200  $\text{g}_{\text{NaCl}} \text{L}^{-1}$ ), was also capable of  
72 hydrogen production of about 4  $\text{mmol}_{\text{H}_2} \text{L}^{-1}$  during microcrystalline cellulose breakdown. Liu et al. [26]  
73 studied the capability of *Bacillus megaterium* (*Bacillus sp B2*) to produce hydrogen within a range of 4  
74 to 70%  $\text{g}_{\text{NaCl}} \text{L}^{-1}$ . They observed a maximum hydrogen production of 1.65  $\text{mol}_{\text{H}_2} \text{mol}_{\text{Glucose}}^{-1}$  in marine  
75 conditions (30  $\text{g}_{\text{NaCl}} \text{L}^{-1}$ ). Kivisto et al. [24] showed that *Halanaerobium saccharolyticum* spp.  
76 *saccharolyticum* (Hssa) and *senegalensis* (Hsse) produced respectively 0.6 and 1.6  $\text{mol}_{\text{H}_2} \text{mol}_{\text{Glycerol}}^{-1}$ ,  
77 at pH7 and 150  $\text{g}_{\text{NaCl}} \text{L}^{-1}$ . Similarly, Brown et al. [29] described *Halanaerobium hydrogeniformans* as a  
78 fermentative hydrogen producer in haloalkaline conditions at pH11 and 70  $\text{g}_{\text{NaCl}} \text{L}^{-1}$ . Those results  
79 emphasize that hydrogen producers do exist and can produce hydrogen efficiently in pure cultures  
80 under halophilic conditions, mainly in neutral or alkaline conditions unlike classical dark fermentation  
81 processes that are operated under acid conditions.

82 The aim of the present study is to evaluate the capability of mixed cultures to produce biohydrogen by  
83 dark fermentation under increasing NaCl concentrations. Experiments were performed in mixed  
84 culture, inoculated with a microbial ecosystem adapted to saline conditions, to provide new insights about  
85 using dark fermentation in moderate halophilic condition for producing hydrogen and treating saline  
86 waste streams at the same time, that cannot be feasible with pure cultures. A series of batch  
87 experiments was thus carried out with increasing NaCl concentrations from 9 to 75 g.L<sup>-1</sup> at pH 8, and  
88 using a saline sediment well adapted to halophilic conditions as inoculum.

## 89 **2. Materials and Methods**

### 90 **2.1. Source of inoculum**

91 The seed sediment used for hydrogen production was sampled in a lagoon collecting wastewaters  
92 from a salt factory. The sediments were filtrated through a 2 mm sieve and stored at lab temperature  
93 before inoculation. The initial pH of the sediments was 8.5 and the salinity 67.4 g L<sup>-1</sup>.

### 94 **2.2. Hydrogen production in batch tests**

95 Hydrogen production experiments were performed in 600 mL glass bottles in batch conditions. About  
96 1.5 g of the seed sediment was added to the culture medium to obtain a final concentration of  
97 300 mg<sub>VS</sub> L<sup>-1</sup> (final working volume of 200 mL). The culture medium was composed of 100mM  
98 phosphate buffer, 5 g L<sup>-1</sup> glucose and a solution oligoelements with the following final concentrations  
99 : 7.50 g L<sup>-1</sup> FeCl<sub>2</sub>, H<sub>2</sub>O, 0.30 g L<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, H<sub>2</sub>O, 0.59 g L<sup>-1</sup> MnSO<sub>4</sub>, H<sub>2</sub>O, 0.13 g L<sup>-1</sup> CoCl<sub>2</sub>, 6H<sub>2</sub>O,  
100 0.35g L<sup>-1</sup> ZnCl<sub>2</sub>, 0.13 g L<sup>-1</sup> NiCl<sub>2</sub>,6H<sub>2</sub>O, 0.075 g L<sup>-1</sup> CuCl<sub>2</sub>,2H<sub>2</sub>O, 0.13 g L<sup>-1</sup> NaMoO<sub>4</sub>,2H<sub>2</sub>O. The initial  
101 pH was adjusted to 8 using NaOH (1M). All batch tests were carried out in triplicate. To ensure  
102 anaerobic conditions, each bottle headspace was flushed and purged with nitrogen gas after  
103 inoculation for 5 minutes. Composition of headspace gas was checked and oxygen content was less  
104 than 0.5% in all bottles. Then, the bottles were capped with a rubber stopper and incubated at 35°C for  
105 more than 20 days. Two-milliliter aliquots were periodically collected and centrifuged (20,000g, 10  
106 min). Supernatants and pellets were stored at -20°C. Supernatants were used for further chemical  
107 analysis and pellets for DNA extraction.

### 108 **2.3. Chemical analyses**

109 Volatile fatty acids (VFA) composition, *ie.* acetic (C2), propionic (C3), butyric and iso-butyric (C4  
110 and iC4), valeric and iso-valeric (C5 and iC5) and caproic (C6) acids was determined with a gas  
111 chromatograph (GC-3900 Varian) equipped with a flame ionization detector. The concentrations of  
112 non-VFA metabolic products such as ethanol, lactate and formate were measured by HPLC analysis  
113 and refractometric detection, as previously described [21].

114 Biogas production volume was periodically estimated by measuring the gas pressure in headspace.  
115 Biogas composition (CH<sub>4</sub>, CO<sub>2</sub>, H<sub>2</sub> and N<sub>2</sub>) was determined using a gas chromatograph (Clarus 580,  
116 Perkin Elmer) coupled to Thermal Catharometric detection (TCD), as described elsewhere [21].

### 117 **2.4. Data analysis**

118 To assess accurately H<sub>2</sub> production performances, cumulative H<sub>2</sub> production curves were fitted to a  
119 modified Gompertz equation for each batch experiment, as proposed by Quéméneur et al. [20] :

$$120 \quad H_2(t) = H_{2\max} \cdot \exp(-\exp(V_{\max} \cdot \exp(1) / H_{2\max} (\lambda - t) + 1))$$

121 where H<sub>2max</sub> corresponds to the maximum experimental H<sub>2</sub> yield (in mol H<sub>2</sub> mol<sub>Glc</sub><sup>-1</sup>), V<sub>max</sub> : the  
122 maximum H<sub>2</sub> production rate (in mol<sub>H2</sub> mol<sub>Glc</sub><sup>-1</sup> day<sup>-1</sup>), λ the lag phase (in days), and t the incubation  
123 time (in days) (see Fig. 1 – General pathways for hydrogen production. Bold arrows are represented  
124 for hydrogen production pathways, dotted arrows for hydrogen consumption pathways and dashed  
125 arrows for hydrogen production concurrent routes (adapted from Guo et al [3]).

126 Fig. 2).

127 Specific H<sub>2</sub> consumption rates R<sub>c</sub> (in d<sup>-1</sup>) (Fig. 12) were estimated from the H<sub>2</sub> decrease in cumulative  
128 H<sub>2</sub> values at H<sub>2max</sub> time and at the end of the experiment, that was then normalized according to the  
129 corresponding H<sub>2max</sub> value.

### 130 **2.5. DNA extraction, PCR amplification and CE-SSCP fingerprinting**

131 Molecular fingerprinting of the bacterial communities were performed for the samples taken at the  
132 time of maximal hydrogen production (H<sub>max</sub>) for each reactor. After centrifugation (20,000g, 10 min.)  
133 of 2mL sample aliquots, genomic DNA was extracted and purified from the pellets using a  
134 PROMEGA Wizard® Genomic DNA kit. The DNA amount and purity in extracts were measured by

135 spectrophotometry (Infinite NanoQuant M200, Tecan). Then, 16S rRNA genes were amplified using  
136 universal primers for bacteria, named W49 (5'-ACGGTCCAGACTCCTACGGG-3' *Escherichia coli*  
137 position 330) [30] and 5'-fluorescein phosphoramidite labeled W104 (5'-6FAM-  
138 TTACCGCGGCTGCTGGCAC-3' *Escherichia coli* position 533) [30]. Each PCR (Polymerase Chain  
139 Reaction) mixture (50 $\mu$ L) contained 5 $\mu$ L of 10x Pfu Turbo DNA buffer, 200 nMf of dNTP, 500 nMf  
140 of each primer, 2.5 U  $\mu$ L<sup>-1</sup> of Pfu Turbo DNA polymerase (Stratagene) and 10 ng of genomic DNA.  
141 Reactions were performed in Mastercycler thermal cycler (Eppendorf). The 16S rRNA genes were  
142 amplified as follows : initial denaturing at 94°C for 2 min, followed by 25 cycles performed at 94°C  
143 for 30 s, 61°C for 30 s and 72°C for 30 s, with a final elongation at 72°C for 10 min. Reactions were  
144 stopped by cooling the mixture to 4°C.

145 PCR products were then separated by capillary electrophoresis single-strand conformation  
146 polymorphism (CE-SSCP). This method allows the separation of DNA fragments that have about the  
147 same size but different base compositions [31]. For this, one microliter of the appropriate dilution of  
148 PCR products was mixed with 18.925  $\mu$ L of formamide and 0.075  $\mu$ L of internal standard GeneScan  
149 ROX (Applied Biosystems). Samples were heat-denatured at 95°C for 5 min and immediately re-  
150 cooled directly on ice for 5 min. CE-SSCP electrophoresis was performed in an ABI Prism 3130  
151 genetic analyzer (Applied Biosystems) in 50 cm capillary tubes filled with conformation analysis  
152 polymer, corresponding buffer and 10% glycerol (Applied Biosystems). Samples were eluted at 12kV  
153 and 32°C for 30 min.

154 For bacterial identification, pyrosequencing of the DNA samples using a 454 protocol was performed  
155 (Research and Testing Laboratory (Lubbock, USA).

156 The sequences of the most abundant bacteria found for each salt concentration was deposited in the  
157 NCBI genbank database under the accession number : KC905780 for the 39% abundant bacteria at  
158 9 g<sub>NaCl</sub>L<sup>-1</sup> , KC905779 for the 27% abundant bacteria at 9 g<sub>NaCl</sub>L<sup>-1</sup>, KC905778 for the 36% abundant  
159 bacteria at 19 g<sub>NaCl</sub>L<sup>-1</sup>, KC905777 for the 19% abundant bacteria at 29 g<sub>NaCl</sub>L<sup>-1</sup>; KC905776 for the 36%  
160 abundant bacteria at 38 g<sub>NaCl</sub>L<sup>-1</sup>; KC905775 for the 26% abundant bacteria at 38 g<sub>NaCl</sub>L<sup>-1</sup>; KC905774  
161 for the 22% abundant bacteria at 48 g<sub>NaCl</sub>L<sup>-1</sup>; KC905773 for the 79% abundant bacteria at 58 g<sub>NaCl</sub>L<sup>-1</sup>;  
162 KC905772 for the 92% abundant bacteria at 75 g<sub>NaCl</sub>L<sup>-1</sup>.

## 163 2.6. Statistical analyses of CE-SSCP fingerprints

164 CE-SSCP profiles were aligned with an internal standard, ROX, to consider the inter-sample  
165 electrophoretic variability. The CE-SSCP profiles were normalized using the Statfingerprints library  
166 [32] in R software version 2.9.2 [33], with a standard procedure described by Fromin et al. [34]. The  
167 genetic distances between bacterial communities were assessed using Euclidean distances to obtain  
168 similarity matrices. Genetic distances between samples were analyzed by principal component  
169 analysis (PCA). Linear correlations between PCA ordination of bacterial communities and  
170 environmental parameters were established using the *envfit* function of the *vegan* library [35]. Average  
171 CE-SSCP profiles were calculated for each condition, and corresponded to the average abundance of  
172 individual peaks from replicates.  
173 The Simpson diversity index was evaluated to estimate the complexity of the community by assessing  
174 the number of species (number of peaks) as well as the relative abundance (area under each peak) [36].

## 175 3. Results and Discussion

### 176 3.1. Hydrogen production performances according to NaCl concentrations

177 Throughout the batch experiments, no CH<sub>4</sub> was detected in head space, and only H<sub>2</sub> and CO<sub>2</sub> were  
178 found as gaseous products. The Gompertz model showed a good fit with the experimental H<sub>2</sub>  
179 production data, with determination coefficients  $r^2$  over 0.994 for all batch tests. Fig. 3 presents the  
180 hydrogen production parameters according to NaCl concentrations. The maximum hydrogen  
181 production yield ( $H_{2max}$ ) decreased from 0.65 ( $\pm 0.01$ ) mol<sub>H<sub>2</sub></sub> mol<sub>Glucose</sub><sup>-1</sup> to 0.42 ( $\pm 0.02$ ) mol<sub>H<sub>2</sub></sub> mol<sub>Glucose</sub><sup>-1</sup>  
182 <sup>1</sup> when NaCl concentrations increased from 9 g<sub>NaCl</sub> L<sup>-1</sup> to 19 g<sub>NaCl</sub> L<sup>-1</sup>. This result was consistent with  
183 previous studies that showed an inhibition of fermentative hydrogen production at low NaCl  
184 concentrations (less than 30 g<sub>NaCl</sub>/L) with the same substrate (glucose) [25,28]. Alshiyab et al [28]  
185 showed that *Clostridium butylicum* presented a decrease of hydrogen productivity (18% less) from 0 to  
186 5 g<sub>NaCl</sub>L<sup>-1</sup> at slight lower pH and temperature conditions (pH 7, 30°C). Similarly, Zheng et al [25]  
187 showed a continuous decrease of the hydrogen production rate from 0.597 mol<sub>H<sub>2</sub></sub> mol<sub>Sucrose</sub><sup>-1</sup> d<sup>-1</sup> to  
188 0.089 mol<sub>H<sub>2</sub></sub> mol<sub>Sucrose</sub><sup>-1</sup> d<sup>-1</sup> with the increase of NaCl concentration from 0 to 30 g<sub>NaCl</sub>L<sup>-1</sup> in a more  
189 acidic medium (pH6) with heat-treated anaerobically digested sludge. In the present study, the first



190 loss in hydrogen production (35% for 9 to 19  $\text{g}_{\text{NaCl}} \text{L}^{-1}$ ) is consistent with these results, but the use of a  
191 not heat-treated inoculum made possible the selection of microbial populations that are halophilic  
192 tolerant, fermentative and hydrogen-producing rather than sporulating *Clostridium* strains, known to  
193 be inhibited by high NaCl concentrations.

194 By increasing NaCl concentration, maximum hydrogen production ( $\text{H}_{2\text{max}}$ ) increased constantly up to  
195 a maximum value of  $0.90 (\pm 0.02) \text{ mol}_{\text{H}_2} \text{ mol}_{\text{Glucose}}^{-1}$  at  $75 \text{ g}_{\text{NaCl}} \text{L}^{-1}$ . Interestingly, this NaCl  
196 concentration ( $75 \text{ g L}^{-1}$ ) was very close to the natural salinity of the sediment used as inoculum ( $67 \text{ g}$   
197  $\text{L}^{-1}$ ). This suggested that the hydrogen-producing fermentative bacteria present in the inoculum were  
198 already adapted to such high NaCl concentrations.

199 Moreover, a decrease of the specific  $\text{H}_2$  consumption rate was observed while increasing salinity,  
200 suggesting that hydrogen-consumers were more specifically impacted by high NaCl concentrations.

201 Thus, the highest  $\text{H}_{2\text{max}}$  value was observed for the highest NaCl concentration (see Fig. 3). However,  
202 at NaCl concentrations above  $9 \text{ g}_{\text{NaCl}} \text{L}^{-1}$ , the maximum hydrogen production rates ( $\text{V}_{\text{max}}$ ) were  
203 significantly impacted with a sharp decrease from  $0.53 \pm 0.19 \text{ mol}_{\text{H}_2} \text{ mol}_{\text{Glc}}^{-1} \text{ d}^{-1}$  at  $9 \text{ g}_{\text{NaCl}} \text{L}^{-1}$  to  
204  $0.08 \pm 0.02 \text{ mol}_{\text{H}_2} \text{ mol}_{\text{Glc}}^{-1} \text{ d}^{-1}$  at  $19 \text{ g}_{\text{NaCl}} \text{L}^{-1}$  (Fig. 3). All batch tests were run at the same time. At  $9 \text{ g}_{\text{NaCl}} \text{L}^{-1}$   
205 hydrogen was much faster than the ones with NaCl concentration up to  $19 \text{ g}_{\text{NaCl}} \text{L}^{-1}$ . Since the exponential phase  
206 was shorter, the number of points on the curves was reduced when fitting the data to the Gompertz model: This  
207 implied a higher error on  $\text{V}_{\text{max}}$  estimation by the model. The maximum hydrogen production rate obtained  
208 in this work at  $9 \text{ g}_{\text{NaCl}} \text{L}^{-1}$  is in accordance with previous studies [20,23]. Indeed, Quemeneur et al. [20]  
209 obtained a maximum hydrogen production rate of  $0.57 \pm 0.15 \text{ mol}_{\text{H}_2} \text{ mol}_{\text{Glc}}^{-1} \text{ d}^{-1}$  in similar conditions  
210 with no salt addition and using a heat-treated anaerobically-digested sludge at pH 5.5. On sucrose,  
211 Quemeneur et al. [23] showed an increase of the maximum hydrogen production rate from  $0.56$   
212  $\text{ mol}_{\text{H}_2} \text{ mol}_{\text{Sucrose}}^{-1} \text{ d}^{-1}$  to  $1.31 \text{ mol}_{\text{H}_2} \text{ mol}_{\text{Sucrose}}^{-1} \text{ d}^{-1}$  due to an increase of the pH value from 4 to 6 in a  
213 non saline medium and with a heat-treated anaerobically-digested sludge.

214 Additionally, by increasing NaCl concentrations, a significant increase of the lag phase was observed  
215 from 0.70 to 3.94 days (Fig. 3). A low amount of microorganisms capable of hydrogen production in  
216 the initial inoculum as well as a longer generation time of these micro-organisms could affect the lag  
217 phase. Moreover, previous studies reported an increase in lag phase due to the time for osmotic

218 adaptation of microorganisms to maintain their osmotic pressure through the cytoplasmic membrane  
219 [14]. Indeed, the ionic strength, determined by ions species and concentrations, is an important factor  
220 for bacteria growth [28,37]. A high level of ionic strength can result in cell lysis due to a high  
221 osmolarity environment caused by NaCl or other ions that trigger a cell dehydration through the  
222 cytoplasmic membrane [14,15]. Hydrogen producing cells have to be halotolerant or capable to adapt  
223 to salt to produce hydrogen in saline environment [14].

### 224 **3.2. Shift of microbial fermentative metabolism with NaCl concentration**

225 Fig. 4 presents the distribution of the soluble metabolites produced concomitantly with H<sub>2</sub>. At a  
226 physiological NaCl concentration of 9g<sub>NaCl</sub> L<sup>-1</sup>, the production of acetate and butyrate as major  
227 fermentation products suggests that *Clostridium spp* were the dominant bacteria in the mixed culture.  
228 Hydrogen production is also presented on Fig. 4. As the theoretical rate of H<sub>2</sub> production through the  
229 acetate and butyrate pathways is 2 moles H<sub>2</sub> per mole of acetate or butyrate, the experimental  
230 observation of this ratio reflects whether hydrogen is entirely produced by the acetate and butyrate  
231 pathways (value close to 2) or if H<sub>2</sub> is concomitantly consumed by homoacetic or other hydrogen-  
232 consuming bacteria causing an overproduction of acetate (lower than 2). Hydrogen consumption  
233 results either from homoacetogenesis producing only acetate from H<sub>2</sub> and CO<sub>2</sub> or from caproate  
234 formation from H<sub>2</sub> and equimolar amounts of acetate and butyrate. In Fig. 4, a clear consumption  
235 pathway is shown with no caproate production, meaning that hydrogen consumption was likely due to  
236 the activity of homoacetogenic bacteria already present in the inoculum.

237 Furthermore, increasing NaCl addition led to an increase of formate production, and in higher  
238 proportion above 38 gNaCl L<sup>-1</sup>. The formate metabolic pathway which produces concomitantly  
239 hydrogen occurs when enteric facultative anaerobes such as *Escherichia coli* are involved [7].  
240 However, accumulation of formate was likely due to an increase of glucose conversion to formate, and  
241 the lack in formate dehydrogenase activity or inhibition of microorganisms able to use formate for  
242 their own growth such as methanogens. Increasing salt concentration led also to an increase of the  
243 lactate concentration. Lactate pathway can be either a concurrent route for hydrogen production [38]  
244 or the result of an alternative metabolic route of hydrogen-producing bacteria under environmental  
245 stress [2]. This means that lactic bacteria were probably still active in such moderate halophilic

246 environment as it was previously shown by Roling et al. [39] with *Tetragenococcus halophila* a salt-  
247 tolerant bacteria capable of mixed acid fermentation at 100 g<sub>NaCl</sub>L<sup>-1</sup>. Furthermore, ethanol production  
248 increased from 0 to 5.04 mmole between 19 and 29 g<sub>NaCl</sub> L<sup>-1</sup>, respectively, and remained then stable  
249 for higher salinities. As a result, the ethanol route, which is a concurrent route of fermentative  
250 hydrogen production [2,3], was induced by highly moderate salt concentration, likely by modifying  
251 the overall metabolism of hydrogen-producing bacteria. Kivisto et al. [24] showed that halophilic  
252 bacteria *Halanaerobium saccharolyticum* (Hssa) produced low amount of ethanol by glycerol  
253 fermentation. Liu et Wang [26] showed that ethanol and acetate were the major soluble products of a  
254 pure culture of *Bacillus megaterium* from glucose in a dark fermentation process operated within a  
255 range of 4 to 70 g<sub>NaCl</sub> L<sup>-1</sup>. In contrast, Zheng et al. [25] showed that the ethanol fraction did not change  
256 significantly by increasing NaCl concentrations from 0 to 30 g<sub>NaCl</sub>L<sup>-1</sup> in mixed culture with glucose as  
257 substrate.

258 Nevertheless, the propionate pathway seemed to be strongly inhibited by NaCl as a decrease of  
259 propionate production from 1.33 to 0.09 mmol occurred from 9 to 75 g<sub>NaCl</sub>L<sup>-1</sup>, respectively. No  
260 inhibition of the hydrogen consumption rate was observed in previous studies even though Zheng et al.  
261 [25] showed that the propionate fractions did not change significantly with NaCl concentration from 0  
262 to 30 g<sub>NaCl</sub>L<sup>-1</sup> in mixed culture with glucose as substrate.

### 263 **3.3. Shift of the bacterial community composition towards the emergence of *Vibrio* subspecies** 264 **as main hydrogen-producing bacteria in moderate halophilic environment**

265 First, the 16S rRNA gene-based CE-SSCP fingerprint profiles of the cultures operated at different  
266 NaCl concentrations are presented in Fig. 5 and each profile correspond to the average of three  
267 replicates determined at the maximum H<sub>2</sub> production time. Overall, these profiles presented very  
268 simple communities structures with an average Simpson diversity index of 0.88±0.05, and the  
269 emergence of only one or two main peaks as main dominant bacteria for each salt concentration. A  
270 clear shift in bacterial communities was observed from 19g<sub>NaCl</sub> L<sup>-1</sup> up to the highest NaCl  
271 concentration, which is consistent with the results obtained on the distribution pattern of microbial  
272 metabolites and hydrogen production performances. According to the literature dealing with  
273 fermentative hydrogen producing bacterial community [20,21], the inoculum was more diverse than

274 the profiles obtained within the reactors, with a Simpson diversity index of 0.98 for the inoculum  
275 bacterial community.

276

277 Fig. 6 presents the principal component analysis (PCA) comparing the genetic distances between CE-  
278 SSCP profiles representing the bacterial communities at the maximum H<sub>2</sub> production time, according  
279 to the experimental conditions [21]. As shown in Fig. 6, the individual profiles were highly similar  
280 under a same condition, suggesting a high reproducibility of the experiments. Moreover, samples  
281 could be clustered according to the dominant species in association with a salinity, with one group at 9  
282 g<sub>NaCl</sub> L<sup>-1</sup>, one group for samples ranging from 19 to 48g<sub>NaCl</sub> L<sup>-1</sup>, and a last group from 58 to 75g<sub>NaCl</sub> L<sup>-1</sup>.  
283 These results suggested that salinity was an important factor to select different specific species in non  
284 saline conditions (cluster 1 at 9 g<sub>NaCl</sub> L<sup>-1</sup>), in conditions closed to marine ones (cluster 2 from 19 to  
285 48g<sub>NaCl</sub> L<sup>-1</sup>) and in high halophilic conditions (cluster 3 from 58 to 75g<sub>NaCl</sub> L<sup>-1</sup>). Changes in lag-phase  
286 (p<0.001) and maximal H<sub>2</sub> production value (H<sub>2max</sub>) (p<0.001) correlated significantly with the genetic  
287 distance between samples. These results show that genetic differences between bacterial communities  
288 can be directly correlated to their metabolic activity. This tendency can be observed in Fig. 4 where  
289 three distinct behaviours are clearly distinguished. In Fig.4, the cluster 1 presents acetate and butyrate  
290 production pathways and propionate consumption pathway. Cluster 2 shows additional formate  
291 production and ethanol consumption pathways. Cluster 3 presents acetate, butyrate and formate  
292 production pathways and ethanol and lactate consumption pathways with an interesting inhibition of  
293 propionate consumption pathway due to sodium chloride concentration.

294 One DNA sample of each triplicate was then sequenced by pyrosequencing. Table 1 presents the  
295 percentage of species representing 1% or more of the bacterial community and Fig. 7 shows the  
296 phylogenetic distribution of bacterial orders found at the maximum hydrogen . This results are in  
297 accordance with PCA results as the addition of NaCl changed substantially global bacterial  
298 community structures. Fig. 7 shows a clear shift in microbial community composition in favor of  
299 *Vibrionales* with increasing NaCl concentration. Indeed, relative abundances of *Clostridium*,  
300 *Enterobacter* and *Escherichia* species decreased as the salinity increased, and *Vibrio spp.* supplanted  
301 gradually these species (Table 1). In non-halophilic conditions, the most abundant species were

302 affiliated to genera *Clostridium*, *Enterobacter* and *Escherichia* ssp. In moderate halophilic conditions,  
303 *i.e.* 38 g<sub>NaCl</sub> L<sup>-1</sup>, bacteria belonging to *Bacteroidales* (43%) and *Vibrionales* (43%) orders were the  
304 main dominant bacteria (Fig. 7). At higher salinities, *Vibrio* spp. was the main bacteria found. The  
305 proportion of *Vibrio* spp. within the microbial community increased gradually with increasing salt  
306 concentration to reach up to 92% at 75 g<sub>NaCl</sub> L<sup>-1</sup>. In Cluster 3, a singular strain was identified which  
307 belongs to the family of *Vibrionaceae*. The sequence of this main strain presents a similarity  
308 percentage lower than 95% when compared to reference sequences.

309  
310 Species belonging to genera *Clostridium*, *Enterobacter* and *Escherichia* are well-known as highly  
311 efficient H<sub>2</sub> producers, often isolated from mixed cultures able to produce hydrogen by dark  
312 fermentation in mesophilic conditions [2,3,8,9,20–23], which corroborates the results obtained for low  
313 saline conditions (9 g<sub>NaCl</sub> L<sup>-1</sup>). However, the abundance of these bacteria was significantly lower at any  
314 concentration above 9 g<sub>NaCl</sub> L<sup>-1</sup>. This is consistent with previous reports of Alshiyab et al. [28] who  
315 studied the effect of salt addition to fermentation medium on hydrogen production. Their study was  
316 carried out at low NaCl concentrations from 0 to 5 g<sub>NaCl</sub> L<sup>-1</sup>. They showed that NaCl negatively  
317 impacted glucose degradation by *Clostridium acetobutylicum*, causing a productivity loss of 18% from  
318 0 to 5 g<sub>NaCl</sub> L<sup>-1</sup>. Zheng et al [25] showed a decrease of 64% of H<sub>2</sub> yield with heat-treated anaerobic  
319 mixed cultures when increasing the salt concentration from 0 to 8.4 g<sub>NaCl</sub> L<sup>-1</sup>. This decrease in  
320 hydrogen yield led to similar low values of biohydrogen yield from 1.70 mol<sub>H<sub>2</sub></sub> mol<sub>Glucose</sub><sup>-1</sup> to 0.61  
321 mol<sub>H<sub>2</sub></sub> mol<sub>Glucose</sub><sup>-1</sup> for respective NaCl concentration of 3 g<sub>NaCl</sub> L<sup>-1</sup> and 30 g<sub>NaCl</sub> L<sup>-1</sup> that is consistent with  
322 our observations of 0.65 (±0.01) mol<sub>H<sub>2</sub></sub> mol<sub>Glucose</sub><sup>-1</sup> at 9 g<sub>NaCl</sub> L<sup>-1</sup>. In contrast, at such low NaCl  
323 concentration, Lee et al. [40] showed a decrease of hydrogen production when Na<sup>+</sup> ion concentration  
324 increased. They studied the effect of salt concentration on metabolic pathways in dark fermentation  
325 from 0 to 12 g<sub>Na<sup>+</sup></sub> L<sup>-1</sup> using an acid-treated anaerobic sludge as inoculum. These results are not in  
326 accordance with our results, maybe because of several differences in experimental conditions. Indeed,  
327 acid pretreatment of the inoculum could have led to select spore-forming bacteria, such as members of  
328 *Clostridiales* family, which was probably detrimental to hydrogen production at higher salt  
329 concentration, as same as our results suggested. Moreover, in our study, no pretreatment was used and

330 the inoculum originating from a saline source was used under similar conditions of salinity (for 70g<sub>Na+</sub>  
331 L<sup>-1</sup> conditions) and pH (pH8).

332  
333 Poorly referenced in the literature, hydrogen production from marine bacteria was previously observed  
334 with *Bacillus megaterium* belonging to the *Bacillales* order and isolated from sludge of an intertidal  
335 zone of a bathing beach in Tianjin (China) [26]. Liu et al. [26] showed that *Bacillus megaterium* could  
336 be applied to biohydrogen production using both marine and fresh organic water. Best performances  
337 of *Bacillus megaterium* were obtained at pH 7, 37°C and 30g<sub>NaCl</sub> L<sup>-1</sup> within a range of 4 and 70 g<sub>NaCl</sub> L<sup>-1</sup>  
338 with a hydrogen production rate of 1.65 (±0.04) mol<sub>H<sub>2</sub></sub> mol<sub>Glucose</sub><sup>-1</sup>. At salt concentration higher than  
339 50 g<sub>NaCl</sub> L<sup>-1</sup>, Brown et al. [29] described a new alkaliphilic bacterium, *Halanaerobium*  
340 *hydrogeniformans*, isolated from haloalkaline anaerobic sediments of Soap Lake in Washington State.  
341 They showed that *Halanaerobium hydrogeniformans* was capable of hydrogen production in  
342 haloalkaline conditions, at pH11 and 70g<sub>NaCl</sub> L<sup>-1</sup>. Simankova et al. [27] isolated also *Halocella*  
343 *cellulolytica*, from cyanobacterials mats and anaerobic sediments of the saline lagoons of lake Sivash  
344 (Crimea) of variable salinities. *Halocella cellulolytica* was able to produce hydrogen by dark  
345 fermentation from a large variety of substrates, including glucose, and produces acetate, ethanol,  
346 lactate, H<sub>2</sub> and CO<sub>2</sub> as fermentation products with cellulose as substrate. The optimal growth  
347 parameters for this strain were pH7, 150 g<sub>NaCl</sub> L<sup>-1</sup>, 39 °C. *Halocella cellulolytica* produced more than  
348 4 mmol<sub>H<sub>2</sub></sub> L<sup>-1</sup> during microcrystaline cellulose breakdown. Oh et al. [41] showed recently that five  
349 *Vibrio spp.*, isolated from anaerobic granular sludge, produced efficiently hydrogen with 1.22 and 1.12  
350 mol<sub>H<sub>2</sub></sub> mol<sub>Glucose</sub><sup>-1</sup> with acetate and ethanol as main metabolites (Fig. 4). Those *Vibrio spp.* presented  
351 high H<sub>2</sub> conversion properties among 34 isolates but they were not studied under moderate halophilic  
352 conditions.

353 Finally, the composition of bacterial communities at 9g<sub>NaCl</sub> L<sup>-1</sup> was similar to that reported in previous  
354 dark fermentation studies [21–23,42,43]. Nevertheless, at 38g<sub>NaCl</sub> L<sup>-1</sup> *Clostridiales spp.* was in co-  
355 dominance with *Vibrio spp.* Then, the bacterial community changed with increasing NaCl  
356 concentration until *Vibrio spp.* became largely dominant (92%) at 75g<sub>NaCl</sub> L<sup>-1</sup>. At 58 g<sub>NaCl</sub> L<sup>-1</sup> and 75  
357 g<sub>NaCl</sub> L<sup>-1</sup> (Cluster 3), a singular strain was identified with a percentage of identity to references

358 sequences lower than 95%. This strain belongs to *Vibrionaceae* and was able to produce hydrogen in  
359 high halophilic environment.

#### 360 **4. Conclusion**

361 The aim of the present study was to evaluate the impact of moderate halophilic conditions on  
362 fermentative hydrogen production as well as microbial communities by increasing NaCl  
363 concentrations, with simultaneous objectives of producing biohydrogen and treating organic pollutants  
364 from saline effluents. Salt concentration presented a strong selective pressure on the structure of the  
365 bacterial communities, characterized by the emergence of a new species affiliated to the family of  
366 *Vibrionaceae*. *Vibrio* spp exhibited the highest hydrogen yields at the highest NaCl concentrations  
367 ( $0.90 \pm 0.02 \text{ mol}_{\text{H}_2}/\text{mol}_{\text{Glucose}}$  at  $75 \text{ g}_{\text{NaCl}} \text{ L}^{-1}$ ), compared to  $0.65 \pm 0.01 \text{ mol}_{\text{H}_2} \text{ mol}_{\text{Glucose}}^{-1}$  at  $9 \text{ g}_{\text{NaCl}} \text{ L}^{-1}$ ,  
368 likely due to lower hydrogen consumption rates.

369 This work reports for the first time the ability of mixed cultures to produce biohydrogen under  
370 halophilic conditions that can be found in numerous salty waste streams that have not been used so far.  
371 Interestingly, a lowering of the hydrogen consumption rates was observed precluding of further  
372 development of efficient bioprocesses for hydrogen production with low consumption rates, and  
373 therefore higher stability. Overall, these results give new possibilities of technological development  
374 for treating saline effluents and producing biohydrogen efficiently

375

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380

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492 **Fig. 1** – General pathways for hydrogen production. Bold arrows are represented for hydrogen  
493 production pathways, dotted arrows for hydrogen consumption pathways and dashed arrows for  
494 hydrogen production concurrent routes (adapted from Guo et al [3]).

495 **Fig. 2** – Representation of H<sub>2</sub> production dynamics from a mixed culture cultivated with glucose as  
496 substrate in batch. The estimated values of H<sub>2max</sub>, V<sub>max</sub> and Lag time correspond to the H<sub>2</sub> production  
497 potential (mol<sub>H<sub>2</sub></sub> mol<sub>Glc</sub><sup>-1</sup>), the maximum H<sub>2</sub> production rate (mol<sub>H<sub>2</sub></sub> mol<sub>Glc</sub><sup>-1</sup> day<sup>-1</sup>) and the lag phase  
498 time (day), respectively. The Rc value corresponds to the H<sub>2</sub> consumption rate and was estimated by  
499 the H<sub>2</sub> decrease measured experimentally between the H<sub>2max</sub> time and the end of the experiments, and  
500 then normalized according to H<sub>2max</sub> value.

501 **Fig. 3** - Hydrogen production parameters of the modified Gompertz model according to NaCl  
502 concentrations. (a) maximum hydrogen production rate, (b) hydrogen production yield and (c) lag  
503 phase (d) H<sub>2</sub> consumption rate.

504 **Fig. 4** - Metabolic end-product distribution (in mmoles) and maximal H<sub>2</sub> production (in mmoles)  
505 according to NaCl concentrations. The values correspond to the average of three replicates

506 **Fig. 5** - CE-SSCP profiles based on 16S rRNA gene fragments retrieved from H<sub>2</sub>-producing mixed  
507 cultures supplemented with different NaCl concentration. The profiles correspond to samples taken at  
508 the maximum H<sub>2</sub> production time. The X and Y axes of each peak represent respectively the relative  
509 peak electrophoresis migration distance and the relative peak intensity (Arbitrary Units).

510 **Fig. 6** - Principal Component Analysis (PCA) biplot of CE-SSCP profiles. For each salinity, triplicates  
511 were clustered with ovals. The first two principal components (Axis1 and Axis2) explained 69,6% of  
512 the genetic variation. Significant linear correlations between changes in genetic profiles and changes  
513 in environment parameters are indicated by arrows: lag phase (p<0,001), salinity (p<0,001) and  
514 H<sub>2max</sub> (p<0,001).

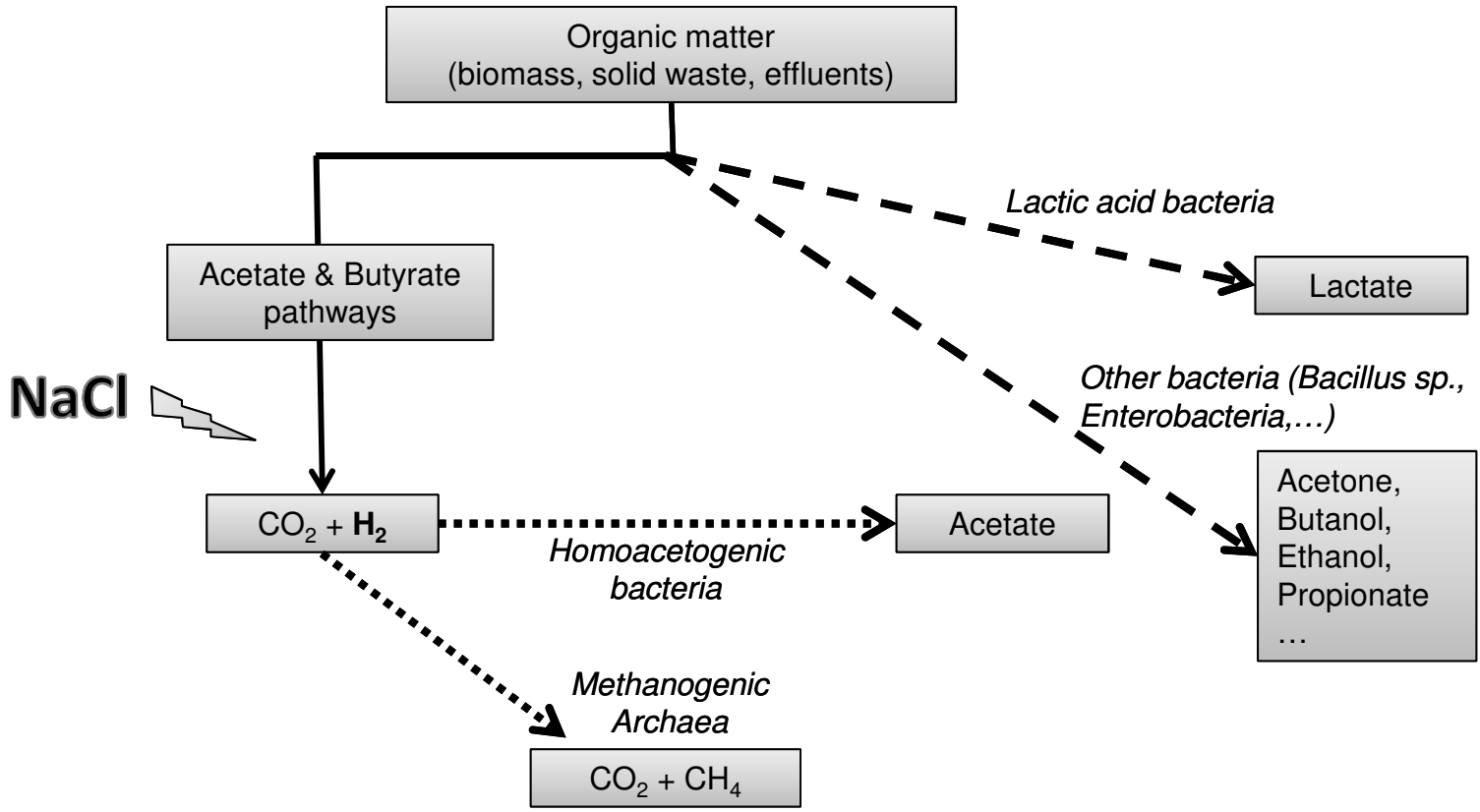
515 **Fig. 7** - Phylogenetic distribution of bacterial orders found at the maximum hydrogen production time  
516 and for different NaCl concentrations.

517 **Table 1** - Taxonomic classification of bacterial taxonomic distribution (in %) according to the salt  
518 concentration, and obtained by pyrosequencing. Bacterial orders are presented in boldcase while  
519 species or closest known phylogenetical level in italics. Names in italics correspond to the closest  
520 physiogenetical known sequence depending on the percentage of identity (I) to reference sequence  
521 (species if I > 97%; genus if 97% ≥ I > 95%; family if 95% ≥ I > 90%; order if 90% ≥ I > 85%).  
522 Relative abundance was defined as the number of sequences affiliated with that taxon divided by the  
523 total number of sequences per sample. Phyla, classes, and genera making up less than 1% of total  
524 composition in all libraries were grouped as “others”.

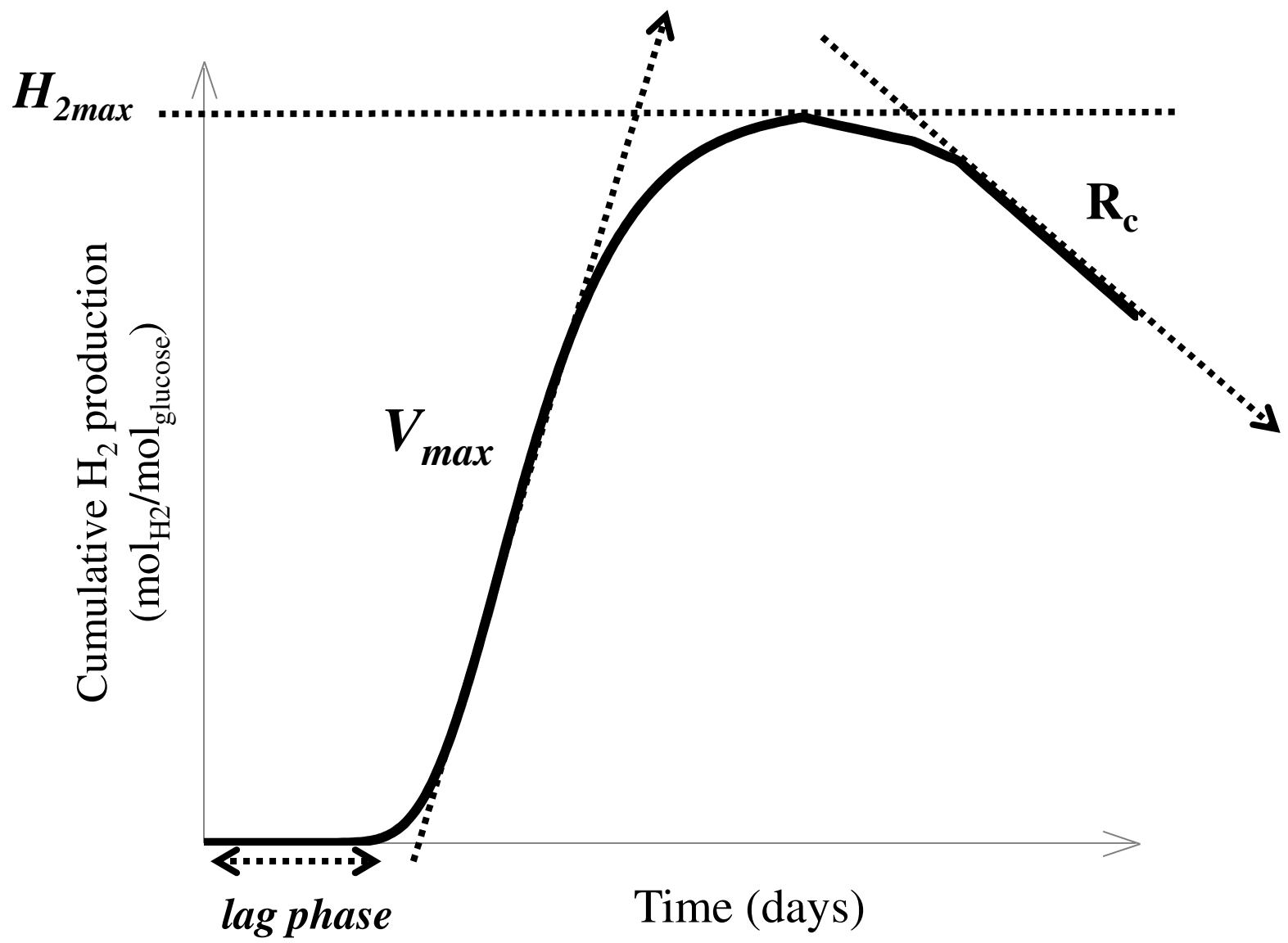
Table 1

% ORDER / species (closest physiological known sequence )	9gNaCl/L	19gNaCl/L	29gNaCl/L	38gNaCl/L	48gNaCl/L	58gNaCl/L	75gNaCl/L
<b>ALTEROMONADALES</b>	<b>1</b>	<b>19</b>	<b>6</b>	<b>2</b>	<b>1</b>	<b>0</b>	<b>0</b>
<i>Pseudoalteromonas sp</i>	1	19	6	2	1	0	0
<b>BACTEROIDALES</b>	<b>0</b>	<b>0</b>	<b>19</b>	<b>5</b>	<b>26</b>	<b>16</b>	<b>2</b>
<i>Bacteroidaceae (unk genus)</i>	0	0	1	0	4	0	0
<i>Bacteroidales (unk family)</i>	0	0	16	4	19	15	2
<i>Porphyromonadaceae (unk genus)</i>	0	0	2	1	3	1	0
<b>CLOSTRIDIALES</b>	<b>44</b>	<b>28</b>	<b>29</b>	<b>43</b>	<b>17</b>	<b>2</b>	<b>2</b>
<i>Clostridiaceae (unk genus)</i>	4	9	14	1	0	2	2
<i>Clostridiaceae unclassified (unk genus)</i>	0	0	1	36	0	0	0
<i>Clostridiales (unk family)</i>	0	0	1	6	0	0	0
<i>Clostridium (unk species)</i>	39	1	0	0	0	0	0
<i>Clostridium cochlearium</i>	0	1	1	0	0	0	0
<i>Clostridium sp</i>	1	0	0	0	0	0	0
<i>Clostridium thiosulfatireducens</i>	0	5	1	0	0	0	0
<i>Oscillibacter valericigenes</i>	0	12	11	0	17	0	0
<b>ENTEROBACTERIALES</b>	<b>47</b>	<b>2</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
<i>Enterobacter hormaechei</i>	4	0	0	0	0	0	0
<i>Enterobacter sp</i>	27	1	0	0	0	0	0
<i>Escherichia (unk species)</i>	1	0	0	0	0	0	0
<i>Escherichia coli</i>	8	0	0	0	0	0	0
<i>Escherichia sp</i>	7	1	0	0	0	0	0
<b>FUSOBACTERIALES</b>	<b>0</b>	<b>0</b>	<b>1</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
<i>Propionigenium maris</i>	0	0	1	0	0	0	0
<b>VIBRIONALES</b>	<b>3</b>	<b>47</b>	<b>38</b>	<b>43</b>	<b>47</b>	<b>79</b>	<b>92</b>
<i>Vibrio nereis</i>	0	0	13	26	22	0	0
<i>Vibrio parahaemolyticus</i>	1	11	4	1	0	0	0
<i>Vibrio proteolyticus</i>	0	0	1	0	0	0	0
<i>Vibrio sp</i>	2	36	19	16	17	0	0
<i>Vibrio tubiashii</i>	0	0	1	0	0	0	0
<i>Vibrionaceae (unk genus)</i>	0	0	0	0	8	79	92
<b>Others</b>	<b>5</b>	<b>4</b>	<b>7</b>	<b>7</b>	<b>9</b>	<b>3</b>	<b>4</b>

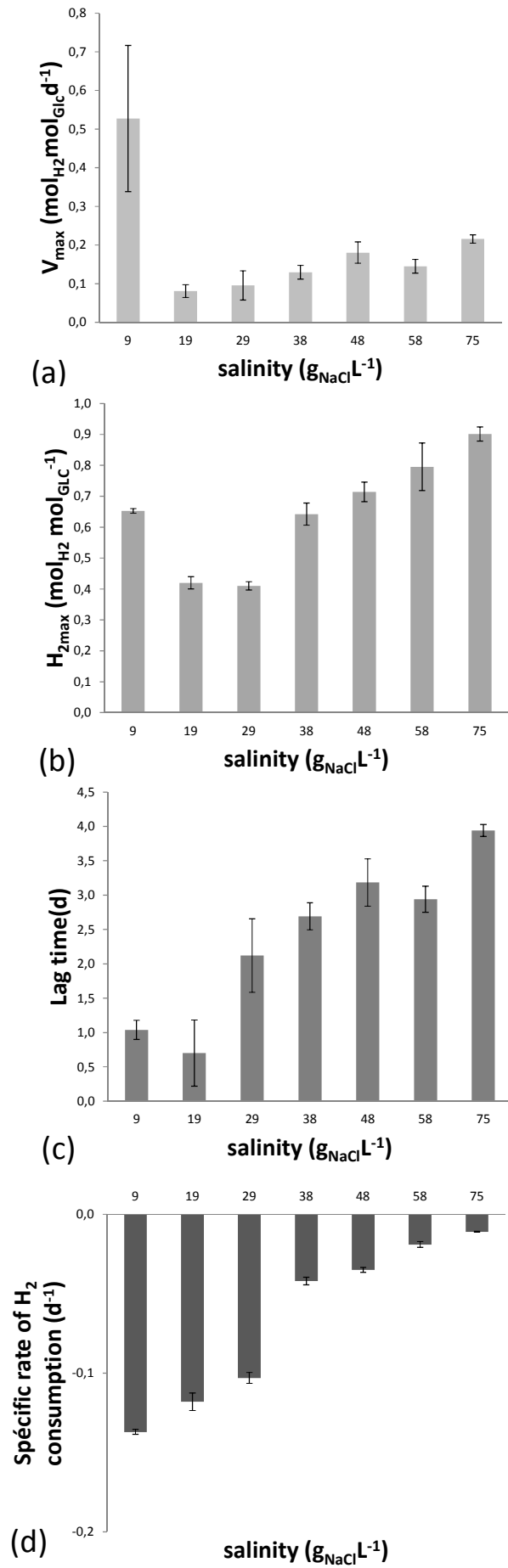
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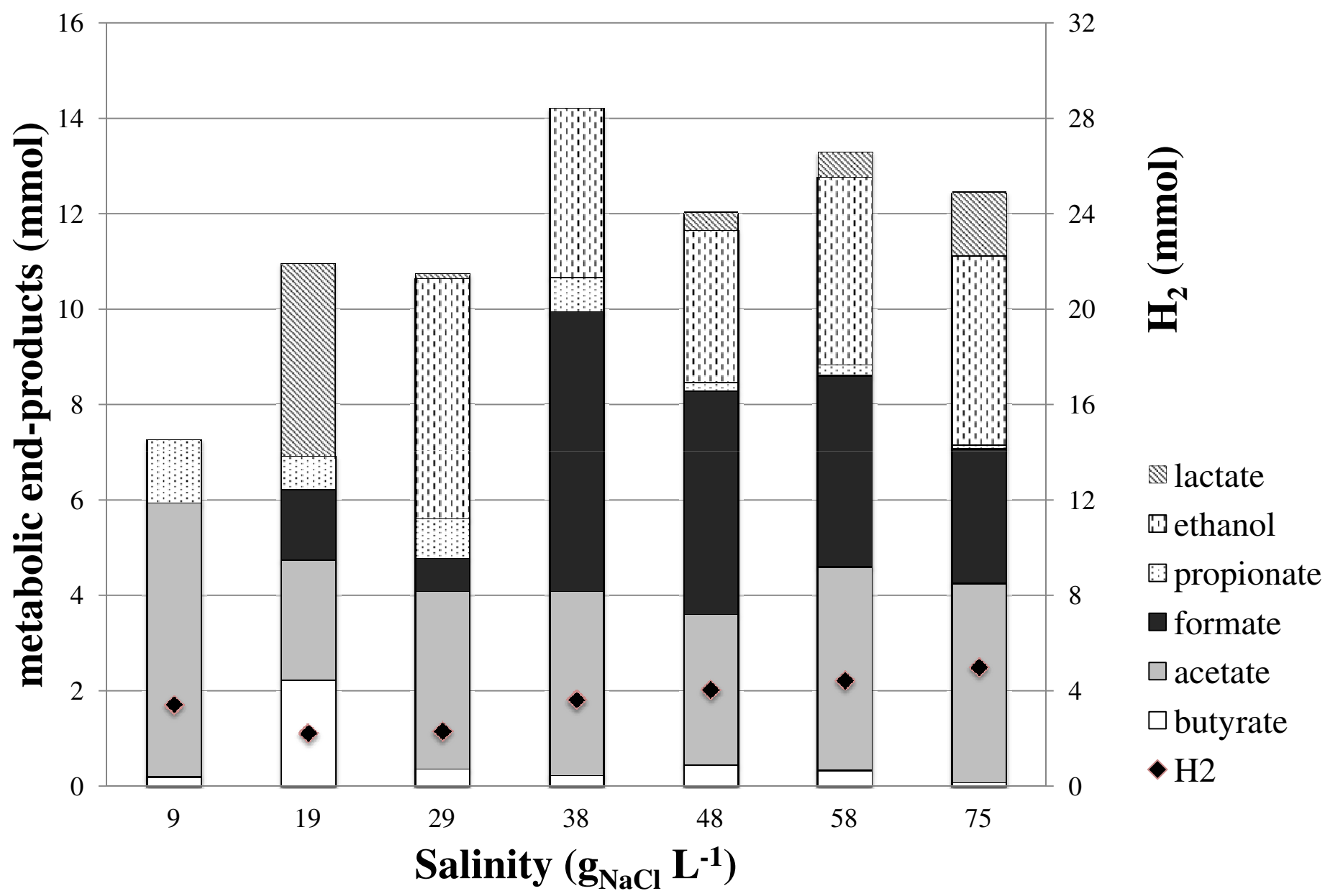
Figure



Figure

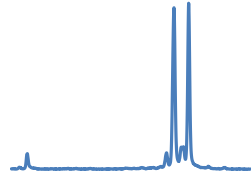
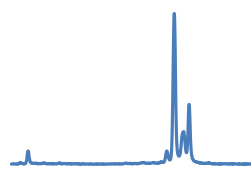
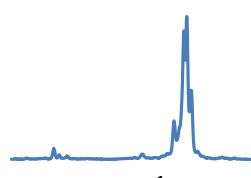
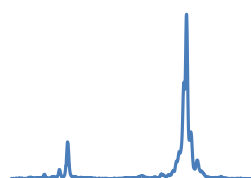
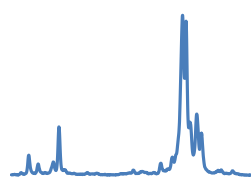
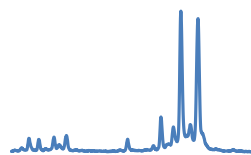
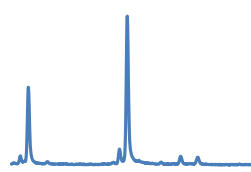


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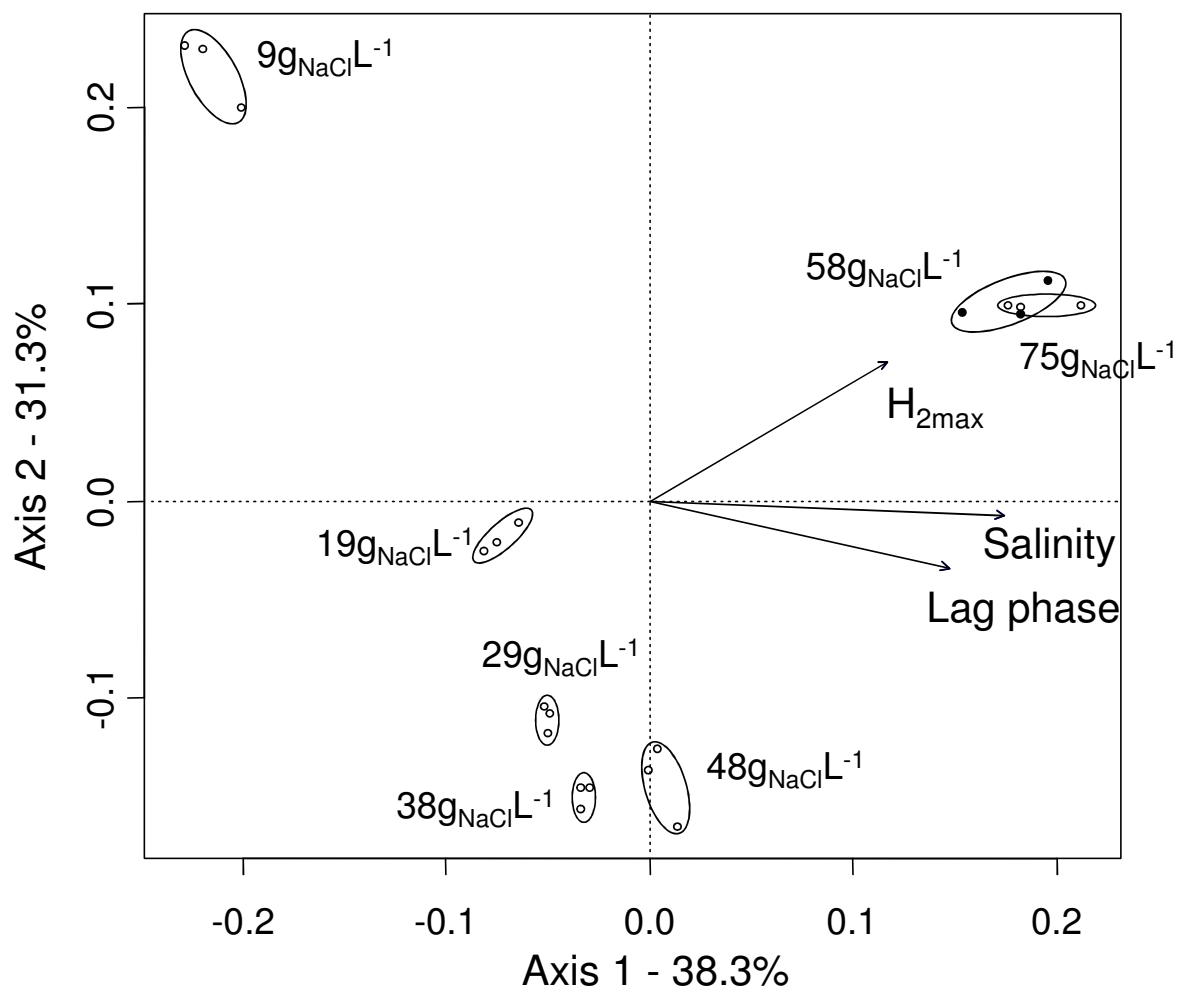




Figure



Figure



Figure

