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1           **Do the *Escherichia coli* European Union shellfish safety**  
2           **standards predict the presence of *Arcobacter* spp., a potential**  
3                                 **zoonotic pathogen?**

4  
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11   Running title: Enumeration and correlation of *E. coli* and *Arcobacter* spp. in shellfish.

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16

17   **Abstract**

18   The genus *Arcobacter* comprises *Campylobacter*-related species, considered zoonotic  
19   emergent pathogens, the presence of which in water has been associated with faecal  
20   pollution. Discharges of faecal polluted water into the sea have been considered as one of  
21   the main reasons for the presence of *Arcobacter* in shellfish, and this may represent a risk  
22   for public health. In this study, the European Union shellfish food safety criteria based on

23 levels of *Escherichia coli* were studied in relation to their capacity to predict the presence  
24 of *Arcobacter* species. In addition, the accumulation factor (AF) that measures the  
25 concentration ratio between the microbes present in the shellfish and in the water, was  
26 also studied for both bacteria. The results show that the presence of *E. coli* correlated with  
27 the presence of the potentially pathogenic species *A. butzleri* and *A. cryaerophilus*.  
28 However, in 26.1% of the shellfish samples (corresponding to those taken during summer  
29 months) *E. coli* failed to predict the presence of, for instance *A. butzleri* and *A. skirrowii*,  
30 among other species. In the rest of the samples a significant correlation between the  
31 concentration of *E. coli* and *Arcobacter* spp. (mussels and oyster;  $R^2= 0.744$ ) was found.  
32 This study indicates that the presence of *E. coli* can predict the presence of pathogenic  
33 *Arcobacter* species in shellfish samples harvested from water with temperatures lower  
34 than 26.2 °C. Consumption of shellfish collected at higher temperatures which may not  
35 be permissive to the growth of *E. coli* but does allow growth of *Arcobacter* spp., may  
36 represent a risk for consumers.

37

38 **Keywords: Risk assessment, Most Probable Number, seafood, marine water,**  
39 **accumulation factor.**

#### 40 **Nomenclature**

41 EU - European Union

42 MPN - Most Probable Number

43 AF - Accumulation Factor

44 AB - Alfacs Bay

45 PNC - Poble Nou Channel

46 CAT - Cefoperazone, Amphotericin B, and Teicoplanin

47 GM - Geometric Mean

## 48 **1 Introduction**

49 The genus *Arcobacter* includes species that are capable of causing diarrhoea and  
50 bacteremia in humans (Collado and Figueras, 2011; Van den Abeele et al., 2014; Hsu and  
51 Lee, 2015) and, more specifically, *Arcobacter butzleri* has been considered a zoonotic  
52 agent and an emergent pathogen by the International Commission on Microbiological  
53 Specifications for Foods (ICMSF, 2002). These microorganisms can be transmitted to  
54 humans and animals through the consumption of water and food products contaminated  
55 with sewage (Ho et al., 2006; Fong et al., 2007; Miller et al., 2009; Collado and Figueras,  
56 2011; Hsu and Lee, 2015; Ferreira et al., 2016). In fact, *Arcobacter* spp. have been  
57 associated with the fecal contamination of water samples and are persistently found in  
58 wastewater because they are considered to be able to grow in this environment (Collado  
59 et al., 2008; McLellan et al., 2010; Fisher et al., 2014). Several studies have demonstrated  
60 a high worldwide prevalence of *Arcobacter* in shellfish ranging from a 14.7% found in  
61 India, to a 73.3% found in Spain (Fernández et al., 2001; Collado et al., 2009; Nieva-  
62 Echevarria et al., 2013; Levican et al., 2014; Collado et al., 2014; Mottola et al., 2016;  
63 Laishram et al., 2016; Salas-Massó et al., 2016; Leoni et al., 2017). These differences in  
64 prevalence may depend on the methods used for the detection and isolation of these  
65 microbes and also on the different environmental conditions of the water in relation to the  
66 degree of fecal contamination (Collado et al., 2008; Collado and Figueras, 2011; Levican  
67 et al., 2016; Salas-Massó et al., 2016; Leoni et al., 2017). Many studies consider shellfish  
68 as reservoirs for *Arcobacter* species and, in fact, 8 of the 27 species that are included in  
69 the genus *Arcobacter* have been described from shellfish (Collado et al., 2009; Figueras

70 et al., 2011a; Figueras et al., 2011b; Levican et al., 2012; Levican et al., 2014; Diéguez et  
71 al., 2017; Figueras et al., 2017; Tanaka et al., 2017). The high prevalence of *Arcobacter*  
72 in shellfish may pose a potential health risk for consumers as they are usually consumed  
73 raw or lightly cooked (Collado et al., 2009).

74 Food safety regulations governing the production and sale of shellfish have been  
75 developed throughout the world. Within the European Union (EU), the shellfish  
76 harvesting areas are classified into four categories (A, B, C and D) following the 2004  
77 EU regulation (Anon, 2004) updated in 2015 (Anon, 2015). These categories designate  
78 increasing concentrations of the fecal indicator bacteria *Escherichia coli* that should  
79 predict the presence of pathogenic microbes in flesh and intervalval liquid. In category  
80 A, shellfish do not require depuration before placing them on the market. This is because  
81 at least 80 % of the samples, collected as part of a regular monitoring program, do not  
82 exceed 230 Most Probable Number (MPN) *E. coli*/100 g and the remaining 20 % do not  
83 exceed 700 MPN *E. coli*/100 g. The other categories (B-D) that have equal requirements  
84 in the updated and earlier version of this regulation involve higher concentrations of *E.*  
85 *coli* and therefore, shellfish require depuration to reach the values of category A before  
86 consumption (Anon, 2004, 2015). For category B, 90% of samples must have  $\leq 4,600$   
87 MPN *E. coli* /100 g and the remaining 10% should not exceed 46,000 MPN *E. coli* /100  
88 g; category C, all samples are  $\leq 46,000$  MPN *E. coli* /100 g; and category D 100% of the  
89 samples show values  $\geq 46,000$  MPN *E. coli* /100 g. As indicated shellfish obtained from  
90 categories B-D cannot be placed directly on the market. Thus, samples of B category  
91 require 24h of depuration, while samples of category C must be maintained in a clean  
92 water area for at least one month to reach category A, and harvesting of shellfish is  
93 prohibited for category D (Anon, 2004, 2015).

94 It has been proven that *E. coli* is not suitable for predicting the presence of some additional  
95 pathogens such as species of *Vibrio* which naturally occur in marine environments and  
96 are not related to fecal pollution (Roque et al., 2009; Oliveira et al., 2011). Regarding this  
97 problem the National Shellfish Sanitation Program (NSSP, USA) has included among  
98 others, the evaluation of levels of *V. parahaemolyticus* and *V. vulnificus* in their standards  
99 for harvesting shellfish (NSSP, 2013). Human viruses (mainly enteroviruses, Noroviruses  
100 and Hepatitis A viruses) can persist after being released into seawater for longer periods  
101 than *E. coli* (from weeks to months), thus the latter is neither a suitable proxy for the  
102 presence of viruses (Formiga-Cruz et al., 2002, DePaola et al., 2010; Manso and Romalde  
103 2013; Brake et al., 2014; Rodríguez-Manzano et al., 2014).

104 The capacity of *E. coli* to predict the presence of *Arcobacter* in water has been  
105 demonstrated in some studies (Collado et al., 2008; Collado et al., 2010). However, the  
106 information about this relationship in shellfish derives from only one very recent study  
107 which demonstrated that concentrations of *E. coli* >230 MPN/100 g in the shellfish were  
108 associated with a higher number of positive samples for *A. butzleri* (Leoni et al., 2017).  
109 Nevertheless, the latter study did not investigate the concentration of *Arcobacter* in  
110 shellfish or in the surrounding ambient water. Therefore, the objective of the present study  
111 was to quantify *Arcobacter* in shellfish and their surrounding water by means of the MPN  
112 and to correlate these values with those of *E. coli* in two scenarios with different levels of  
113 fecal pollution. Thus, the primary objective is to evaluate if the presence of *E. coli* is able  
114 to predict the presence of *Arcobacter* in water and shellfish. In addition, the accumulation  
115 factor (AF), which is the ratio between the MPN of the bacteria in the shellfish and in the  
116 water (Shieh et al., 2003; Martins et al., 2006, Derolez et al., 2013), was also evaluated  
117 for *E. coli* and *Arcobacter*.

118

## 119 **2 Experimental procedures**

### 120 **2.1 Location and sampling**

121 Sampling was performed at two sampling sites once a month between March 2013 and  
122 June 2014, except in July and August 2013 when the samples were collected fortnightly.  
123 The two sampling sites were Alfacs Bay (AB) which is a shellfish harvesting area situated  
124 at the Ebro River Delta, Spain (40° 34' 22.43" N, 0° 39' 12.96" E), and classified as B  
125 category according to the Annex II criteria of EU Regulation 854/2004 (Order  
126 APA/3228/2005), and a channel that receives untreated sewage from the village of Poble  
127 Nou (40° 38.515N'; 00° 41.617'E), designated as PNC in this study. In each sampling  
128 occasion the bivalve mollusks taken from AB consisted of 1.5-2 kg of mussels (*Mytilus*  
129 *galloprovincialis*) and 20-25 individual oysters (*Crassostrea gigas*), to provide a  
130 minimum weight of 100 g of flesh, with the exception of November 2013 and December  
131 2013 when mussels did not have the recommended commercial size and only oysters were  
132 collected. In addition, 2 L of the surrounding water were also sampled each time. Half of  
133 the amount of the collected shellfish and all the water samples were directly studied for  
134 the presence of *E. coli* and *Arcobacter* spp. The remaining half of the shellfish, i.e.  
135 approximately 1 kg of mussels and 10-15 oysters, were placed in a cage in the PNC to be  
136 exposed to its fecal contaminated water. Three exposure times were preliminarily tested  
137 24, 48 and 72h, but no differences were observed in the MPN of *E. coli* and *Arcobacter*  
138 found in the oysters and mussels (data not shown). Most of the samples were exposed for  
139 72h with the exception of the samples of July and August that were exposed for shorter  
140 periods of 24 and 48h because a more extended exposure to the high water temperatures  
141 of summer could affect the survival of the shellfish. After that, the mussels and oysters

142 were removed, along with 2 L of the PNC water, to perform the same analyses as that  
143 from the AB samples. A total of 75 samples were analyzed i.e. 33 from water (21 from  
144 AB and 12 from PNC) and 42 from shellfish (11 mussel samples from AB and 8 from the  
145 PNC; 12 oyster samples from AB and 11 from the PNC). Four samples of shellfish  
146 exposed to the PNC were not available for analysis because in two of them, the shellfish  
147 died and the other two were lost. Water temperature (°C) and salinity (parts per thousand,  
148 ‰) were recorded at each site during sampling by means of a portable multi- parameter  
149 probe (YSI professional, Ohio, US).

## 150 **2.2 Analyses of *E. coli* and *Arcobacter* spp.**

### 151 **2.2.1. Quantification of *E. coli* and *Arcobacter* spp.**

152 Quantification of *E. coli* from water and shellfish was performed using the two step MPN  
153 method involving a presumptive and a confirmatory step, according to ISO/TS 16649-  
154 3:2005. Briefly, 100 mL of water or 100 g of shellfish flesh and intervalval liquid were  
155 mixed thoroughly and homogenized in a stomacher (Lab·Blender 400, West Sussex, UK),  
156 respectively, with peptone water. The homogenate was used for preparing 3 dilutions (i.e.  
157 1, 0.1 and 0.01 mL or g of the original sample) that were each inoculated into 5 tubes  
158 containing Glutamate broth (OXOID, Basingstoke, UK) that were incubated for 24h at  
159 37°C ( $\pm 1^\circ\text{C}$ ). Tubes in which the color of the media changed from purple to yellow  
160 indicated the presence of coliforms and were then confirmed for the presence of *E. coli*.  
161 The confirmation was performed by subculturing cells from the yellow Glutamate broth  
162 tubes in Tryptone Bile X-glucuronide Agar medium (TBX, OXOID, Basingstoke, UK) at  
163 44°C,  $\pm 1^\circ\text{C}$ , for 24h. Colonies showing the typical greenish-blue color were considered  
164 to belong to *E. coli*. The number of positive confirmed tubes per dilution were counted



165 and used to derive the MPN results of *E. coli* (per 100 mL or 100 g) using the CEFAS  
166 MPN tables (Appendix 2 CEFAS issue No. 11, 2015).

167 The same original dilutions prepared in peptone water were used for investigating the  
168 MPN of *Arcobacter* as described by Collado et al. (2008). However, for comparison  
169 purposes with *E. coli*, the volume of the initial sample used in our study was 100 mL or  
170 100 g instead of the 10 g used in Collado's protocol. Dilutions were performed in  
171 *Arcobacter* broth supplemented with Cefoperazone, Amphotericin B and Teicoplanin,  
172 i.e. *Arcobacter*-CAT broth (OXOID, Basingstoke, UK); and incubation was performed  
173 at 30°C for 48 h. Confirmation of the presence of *Arcobacter*, in tubes which presented  
174 turbidity, was done as described by Collado et al. (2008). The confirmation consisted on  
175 the detection of the typical small, beige to off-white, translucent and convex colonies  
176 obtained after having inoculated and cultured at 30 °C for 48 h under aerobic conditions  
177 100µl of the enrichment tubes by passive filtration (0.45µm nitrocellulose filters;  
178 Millipore) on Blood Agar (BA) plates (Tryptone Soy Agar supplemented with 5% sheep  
179 blood BD Difco, Le Pont de Claix, France). Presumptive colonies were confirmed as  
180 *Arcobacter* spp. by Gram staining. The MPN final values from 100 mL or 100 g were  
181 obtained using the software MPN Build 23 (Mike Curiale software;  
182 <http://i2workout.com/mcuriale/mpn/index.html>). When processing samples of the PNC,  
183 up to 6 dilutions were performed because higher bacterial counts were expected.

### 184 **2.2.2 Detection of *Arcobacter* spp.**

185 Additionally, all the samples were analyzed for the presence of *Arcobacter* species using  
186 two methods. The conventional one, described in previous studies (Collado et al., 2008;  
187 Levican et al., 2014; Salas-Massó et al., 2016), involved the use of a pre-enrichment in  
188 *Arcobacter*-CAT broth followed by subculturing by passive filtration on BA. The second

189 method included enrichment in *Arcobacter*-CAT broth supplemented with 2.5% NaCl  
190 (w/v) and subculturing was done on Marine Agar (MA, Scharlab, Barcelona, Spain)  
191 where the presumed *Arcobacter* showed pale yellow to orange colonies. When present,  
192 eight presumptive colonies were obtained from each media that were first genotyped with  
193 Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) in order to eliminate  
194 clonal redundant isolates. The different ERIC genotypes or strains were identified to  
195 species level by the 16S rRNA gene Restriction Fragment Length Polymorphism (16S  
196 rRNA-RFLP) method described by Figueras et al. (2012). When necessary identification  
197 was confirmed using the partial sequences of the *rpoB* (621bp) gene using primers and  
198 PCR conditions described by Salas-Massó et al. (2016).

### 199 **2.3 Data analysis**

200 The geometric mean (GM) and standard deviation of the MPN results were used for the  
201 statistical analyses. Counts < 10 *E. coli* and < 20 *Arcobacter* MPN/100 mL or 100 g, which  
202 were the limits of detection of the method, were assigned a value of 1 to allow log  
203 transformation. All the statistical analyses were performed with the IBM SPSS Statistics  
204 22.0. Normality distribution of the data was assessed using the Shapiro-Wilk and  
205 Kolmogorv-Smirnov test. For those data that did not follow a normal distribution the non-  
206 parametric Spearman's rho (correlation coefficient) tests was used for the analyses. To  
207 calculate the regression between the concentration of *E. coli* and *Arcobacter*, a linear  
208 regression model (SigmaPlot 9.0 software) was used with log-transformed data and  
209 statistical significance was established at  $P < 0.05$ . A t-test for equality of means was  
210 performed to evaluate significant differences between the MPN geometric means of *E.*  
211 *coli* and *Arcobacter* found in water and shellfish samples from both the AB and PNC  
212 origins.

213 The AF of each microorganism (*E. coli* and *Arcobacter*) within the shellfish was  
214 calculated by dividing the GM of the MPN obtained from the shellfish by the one obtained  
215 from the water (GM MPN shellfish/ GM MPN water) as described by Burkhardt and  
216 Calci (2000). We also analyzed during the study period the AF data of *E. coli* and  
217 *Arcobacter* to determine if a hyperaccumulation occurred. The latter have been defined  
218 by Burkhardt and Calci (2000) as the accumulation factor of a particular organism greater  
219 than the mean for the entire data + 1 standard deviation ( $\bar{x} + 1SD$ ).

### 220 **3 Results**

#### 221 **3.1 Presence of *E. coli* and *Arcobacter* in water and shellfish samples**

222 In AB, 6/21 water samples (28.6%) were exclusively positive for *E. coli*, 4/21 (19.0%)  
223 for *Arcobacter* and 1 (4.8%) sample was simultaneously positive for both microbes  
224 (Table 1). Of the 23 shellfish samples, only 12 (43.5%) were positive for *E. coli* (7 alone  
225 and 5 in combination with *Arcobacter*, Table 1). The shellfish samples presented the same  
226 GM ( $1.2 \times 10^2$ ) for *E. coli* and *Arcobacter*, while in the water the density of *Arcobacter*  
227 was higher (Table 1).

228 The Alfacs Bay samples (water and shellfish) that were positive for *Arcobacter* presented  
229 a statistically higher ( $P=0.001$ ) mean water temperature ( $26.2\text{ }^{\circ}\text{C}$ ) than those that were  
230 only positive for *E. coli* ( $18.9\text{ }^{\circ}\text{C}$ ) and those positive for both microbes ( $19.4\text{ }^{\circ}\text{C}$ ; Table 1).  
231 As shown in Table 1, a similar number of positive samples for *E. coli* and *Arcobacter*  
232 were obtained by the MPN from water (i.e. 7/21 and 5/21, respectively) and shellfish (i.e.  
233 12/23 and 11/23, respectively). In addition, the same GM value ( $1.2 \times 10^2 \pm 2$ ) was obtained  
234 from the shellfish for both microbes, while in the water the values were slightly different  
235 i.e.  $5.6 \times 10^1 \pm 2.2$  for *E. coli* and  $1.0 \times 10^2 \pm 3.1$  for *Arcobacter* (Table 1). The higher

236 number of positive samples for *Arcobacter*, 81% (17/21) in water and 69.6% (16/23) in  
237 shellfish, were obtained with the culture approach that used enrichment in Arcobacter-  
238 CAT broth supplemented with salt followed by isolation on Marine Agar (Table 1). In  
239 contrast, the enrichment in Arcobacter-CAT followed by isolation on Blood Agar yielded  
240 a low number of positive samples i.e. 19% (4/21) from water and 26.1% (6/23) from  
241 shellfish (Table 1).

242 From PNC all the samples of water and shellfish were positive for both bacteria with the  
243 MPN method, while with both culture approaches the positive samples for *Arcobacter*  
244 ranged between 66.7% and 78.9% (Table 2). The densities of *E. coli* ( $GM=6.6 \times 10^4 \pm 5.1$ )  
245 and *Arcobacter* spp. ( $GM=5.4 \times 10^5 \pm 7.8$ ) in the shellfish exposed for 72h to the PNC  
246 contaminated water were slightly higher than the densities of these bacteria found in water  
247 (Table 2). When comparing the *Arcobacter* and the *E. coli* MPN values obtained from  
248 both water and shellfish in the PNC, the former had significantly higher MPN than the  
249 latter in both matrices (Table 2).

250

### 251 **3.2 Correlation of *E. coli* and *Arcobacter***

252 A significant positive correlation between the detection of *E. coli* and *Arcobacter* was  
253 found when comparing the concentrations of both bacteria in shellfish ( $R^2=0.744$ ,  $P$   
254  $<0.05$ ) and in water ( $R^2=0.791$ ,  $P <0.05$ ), (Figures 1 and 2, respectively). As shown in  
255 Table 1, the lower densities of both bacteria were found in the water and shellfish samples  
256 from AB. The majority of the MPN results obtained from AB corresponded to A category  
257 ( $<230$  *E. coli*/100 g) and only a few to B category, while the higher concentrations  
258 corresponded to PNC samples (Figures 1 and 2).

259 In addition, a significant positive correlation (Table S1) was obtained when considering  
260 data from AB and PNC together, not only between microorganisms, but also when  
261 comparing separately the detection of one microorganism (*E. coli* or *Arcobacter* spp.) in  
262 water versus its detection in both types of shellfish (mussel/s or oyster/s).

263 When the data from AB and PNC were analyzed separately, it was shown that in AB the  
264 presence of *E. coli* and *Arcobacter* in water predicted ( $p <0.05$ ) their presence in shellfish  
265 (Table S1). However, in PNC it was observed that the presence of *E. coli*, both in water  
266 and shellfish, correlated with the presence of *Arcobacter* in both matrices. Also, the  
267 presence of *Arcobacter* in water was positively correlated with its presence in shellfish  
268 (Table S1).

269 When investigating if levels of *E. coli*, classified according to the categories (A-D) of the  
270 EU legislation, found in the shellfish samples could predict the presence or absence of  
271 *Arcobacter* spp. in these samples (Table S2), we observed that at the lowest level of *E.*  
272 *coli* ( $<230$  MPN/100 g) oysters were more positive for *Arcobacter* than mussels (83 vs.  
273 44%). When examining the species of *Arcobacter* identified in those samples (Table S2),  
274 *A. molluscorum* was the most recovered species among mussels and *A. marinus* in oysters.

275 Shellfish from the PNC showed higher concentrations of *E. coli* and corresponded to  
276 classes C (between 4600 and 46000 MPN/100 g) and D (>46000 MPN/100 g) and  
277 presented a higher diversity of *Arcobacter* species like *A. cloacae*, *A. cryaerophilus*, *A.*  
278 *defluvii*, *A. ellisi* and *A. halophilus* (Table S2). However, the dominating species in both  
279 mussels and oysters was *A. butzleri*. Similar diversity of species was also observed in  
280 water (Table S3). Regarding the distribution of species depending on the matrix (shellfish  
281 vs. water), *A. aquimarinus* and *A. ellisii* were found in shellfish, but not in water (Table  
282 S2). On the contrary, *A. ebronensis*, *A. nitrofigilis* and *A. skirrowii* were isolated from  
283 water, but not from shellfish (Tables S2 and S3).

284

### 285 **3.3 Accumulation factor of *E. coli* and *Arcobacter* in shellfish**

286 The mean AF for *E. coli* and *Arcobacter* in mussels from Alfacs Bay in the period studied  
287 were  $72.61 \pm 122.89$  and  $38.84 \pm 112.94$  respectively (Figure 3A); and for oysters  $39.31$   
288  $\pm 80.78$  and  $35.16 \pm 54.28$ , respectively (Figure 3B). The mussels from AB presented 2  
289 hyperaccumulation (mean + 1SD) moments for *E. coli* and both occurred in June (2013  
290 and 2014), and only one for *Arcobacter* that occurred in May 2014 (Figure 3B). The  
291 oysters from AB also presented two hyperaccumulation moments for *E. coli*, one in  
292 December 2014 and one in June 2014. Hyperaccumulation of *Arcobacter* within oysters  
293 took place on 3 occasions, two in June (2013 and 2014) and one in December 2013.

294 In PNC samples, we observed that for mussels two hyperaccumulation peaks for *E. coli*  
295 occurred (in May and June 2014), whereas there were three episodes of  
296 hyperaccumulation for *Arcobacter* in August 2013, March and June 2014 (Figure 3C).  
297 The oysters from PNC showed three hyperaccumulation peaks for *E. coli* (December

298 2013, May and June 2014) and 3 for *Arcobacter* (February, May and June 2014; Figure  
299 3D).

300

#### 301 **4. Discussion**

302 In our study the relationship between presence and abundance of species from the  
303 emergent pathogen genus *Arcobacter* and the faecal indicator *E. coli* were evaluated to  
304 determine if the fecal indicator bacteria could predict the presence of *Arcobacter* spp.  
305 This relationship was determined analysing the concentration of both microbes in the  
306 shellfish and their surrounding harvesting waters. In order to increase the knowledge  
307 about the ecology of both bacteria, the relationship was studied in two completely  
308 different scenarios: a shellfish harvesting area (Alfacs Bay) and a heavily fecal polluted  
309 channel.

310 Alfacs Bay represents a commercial shellfishery officially classified as a B harvesting  
311 area, where during our study 91% (21/23) of the shellfish samples obtained from there  
312 were below the 230 *E. coli* MPN threshold that EU Regulation establishes as the limit for  
313 harvesting areas of A category (Anon, 2004, 2015). In fact, *E. coli* was not detected in  
314 48% of those samples. Moreover, the percentage of samples with *E. coli* values higher  
315 than 230 MPN, but not exceeding 700 MPN was 9% (n= 2). Our data indicates that  
316 although AB is a harvesting zone classified as B, it is close to the criteria of a category A  
317 zone.

318 As expected, a higher prevalence of positive samples for both bacteria was found in  
319 shellfish (18/23; 78.3%) than in the water (11/21; 52.4%) due to the shellfish  
320 accumulation capacity. This is to our knowledge the first study that investigates

321 simultaneously the presence of *Arcobacter* and *E. coli* both in the harvesting waters and  
322 in the shellfish.

323 The AB shellfish samples that were only positive for *Arcobacter* MPN (26.1%) were the  
324 ones collected during the summer months (July and August) when the water temperature  
325 was above 26.2°C, while those exclusively positive for *E. coli* (30.4%) showed a mean  
326 temperature of 18.9 °C (Table 1). These results would support previous findings that  
327 indicate that fecal indicator bacteria decrease when the temperature of the water increases  
328 (Chigbu et al., 2005; Leight et al., 2016). The no detection of *E. coli* in these summer  
329 samples suggests that this faecal indicator would fail to predict the presence of *A. butzleri*  
330 and *A. skirrowii* among other species found at 26.2°C (Table 1). Levican et al. (2014), in  
331 a study performed in the same area, showed that the levels of *Arcobacter* tend to decrease  
332 in colder temperatures. However, this seasonality may depend on the species, i.e. *A.*  
333 *cryaerophilus* and *A. skirrowii* are more prevalent at colder temperatures (9.8-19.8 °C)  
334 than in warmer ones (20-29.5°C), where *A. butzleri* prevail (Fisher et al. 2014, Levican et  
335 al., 2014). Recently, Leoni et al., (2017) found that *A. butzleri* is most frequently  
336 recovered from Italian shellfish in the winter-spring season, attributing this difference to  
337 geographical and climatic features and to different inputs of fecal contamination.

338 In general, no correlation between *E. coli* and the *Arcobacter* spp. was observed in the  
339 Alfacs Bay samples. However, after a deeper analysis taking into account the different  
340 *Arcobacter* species recovered from all the water and shellfish samples with different  
341 levels of *E. coli* (Tables S2 and S3) correlations with concrete species were observed. The  
342 lack of significant correlation observed between the MPN of *E. coli* and *Arcobacter* in  
343 any type of samples from the AB (Table S1) was probably due to the low levels of fecal  
344 pollution found in the water of the Bay. Only 33% (7/21) of the samples were positive for



345 *E. coli* with a GM of 56 MPN/100 mL and maximum values of 170 MPN/100 mL. In  
346 fact, a previous study has demonstrated that inputs of faecal pollution of  $4.9 \times 10^3$  CFU/100  
347 mL of *E. coli* entering the seawater were not detected at 200 m distance from the discharge  
348 point, as a consequence of an important dilution effect (Collado et al., 2008). The deeper  
349 analysis showed that in agreement with results of Leoni et al. (2017) the presence of *E.*  
350 *coli* in shellfish was associated with the presence of the dominating species *A. butzleri*,  
351 and *A. cryaerophilus* (Tables S2 and S3). These two species have been recovered from  
352 patients with intestinal illnesses (Figueras et al., 2014). However, species recovered from  
353 shellfish and seawater as *A. molluscorum* and *A. mytili* showed an inverse relationship  
354 with *E. coli* (Tables S2 and S3). When the concentration of *E. coli* in water and shellfish  
355 was low, indicating low levels of fecal contamination, the prevalence of the mentioned  
356 marine species increased. A possible explanation for this behavior is that these species  
357 are indigenous of marine environments and as such could be adapted to survive better in  
358 seawater than *E. coli* (D'Sa and Harrinson, 2005). However, other species such as *A.*  
359 *butzleri* and *A. cryaerophilus* are introduced in the seawater with the fecal pollution  
360 (Maugeri et al., 2000; Wirsen et al., 2002; Fera et al., 2004; Collado et al., 2009; Salas-  
361 Massó et al., 2016).

362 The methodology of the MPN for *Arcobacter* uses Arcobacter-CAT broth followed by  
363 subculturing on Blood Agar plates for confirmation, and this combination of media has  
364 shown to cause a bias in the detection of environmental species (Table 1). For instance,  
365 species like *A. bivalviorum*, *A. marinus*, *A. ebronensis* and *A. mytili*, previously related to  
366 shellfish and new potential *Arcobacter* species that were only recovered with the method  
367 supplemented with NaCl (Salas-Massó et al., 2016), would not be detected with the MPN  
368 method. The pathogenicity of these *Arcobacter* species to humans remains unknown.

369 However, when analyzing marine samples, culture media with at least 2.5% NaCl should  
370 be used in order to ensure enhanced recovery results (Salas-Massó et al., 2016). A bias  
371 in relation to the species detected and caused by the enrichment step has also been  
372 described in other studies (Ho et al., 2008; Levican et al., 2016). It was demonstrated that  
373 when analyzing samples directly, *A. cryaerophilus* may be the predominant species, but  
374 after the enrichment step, *A. butzleri* becomes the most prevalent one due to its faster  
375 growth capacity (Ho et al., 2008; Levican et al., 2016).

376 Although Alfacs Bay is a good representative of the western Mediterranean shellfish  
377 growing areas, its low fecal contamination levels did not provide a wide range of  
378 conditions to generate multiple scenarios where the performance of the correlation of *E.*  
379 *coli* and *Arcobacter* spp. could be compared. As a second scenario for the study, the Poble  
380 Nou Channel was chosen as the water harbored high levels of fecal pollution (geometric  
381 mean of *E. coli*  $4.1 \times 10^4$  MPN/100 mL). In this water, the concentration of *Arcobacter*  
382 spp. ( $4.5 \times 10^5$  MPN/100 mL) was one log higher ( $p = 0.05$ ) than that of *E. coli*, which  
383 agrees with the concentrations described by Collado et al., (2008) in contaminated  
384 freshwater that impacted a seawater bathing area ( $3.7 \times 10^5$  MPN/100 mL for *Arcobacter*  
385 spp. vs.  $4.9 \times 10^3$  CFU/100 mL for *E. coli*). This difference in the concentration of both  
386 bacteria has also been observed in a recent study that investigated the efficiency of a  
387 natural (biological) process of purifying wastewater by storing it in open air lagoons  
388 (Fernández-Cassi et al., 2016). The wastewater to be treated showed concentration of  
389 *Arcobacter* ( $7.51 \times 10^6$  MPN/100 mL) higher than those of *E. coli* ( $7.23 \times 10^4$  MPN/100  
390 mL) (Fernández-Cassi et al., 2016). Some authors have indicated that the high prevalence  
391 of *Arcobacter* spp. found in sewage could be associated to contamination from human  
392 feces (Moreno et al., 2003; Collado et al., 2008; Merga et al., 2014). However, the

393 prevalence found in human feces does not support this statement and therefore other  
394 studies indicate that this high abundance is related to the capacity of *Arcobacter* to  
395 multiply in the sewage system (McLellan et al., 2010; Vandewalle et al., 2012; Fisher et  
396 al., 2014). Interestingly, we found that all the shellfish samples exposed during 24, 48 and  
397 72h to the PNC tested positive for both *E. coli* and *Arcobacter* and their concentrations  
398 increased 3 and 4 logs respectively from their original concentration in AB (Tables 1 and  
399 2). The MPN of PNC water ( $10^4$  *E. coli* and  $10^5$  *Arcobacter*) were on the same log rank  
400 as the values reached in the shellfish for both microbes, respectively (Table 2). This  
401 similarity of concentrations inside the shellfish with respect to water may be related to  
402 what was suggested by Jozić et al. (2012) that bioaccumulation via filtering reaches a  
403 plateau of the maximum concentration of particles that the shellfish body can support.  
404 Moreover, the low salinity of the PNC can be stressful for the shellfish and could  
405 contribute to a lowering of the filtration rates (Gosling, 2003). However, when  
406 considering only the PNC results, a statistically positive correlation between the presence  
407 of *E. coli* and *Arcobacter* within the shellfish was found. In this case, only *Arcobacter*  
408 showed a positive correlation between its concentration in the water column and the  
409 shellfish (Table S1).

410 As mentioned above, this study corroborates the results obtained from water by Collado  
411 et al. (2008) and Leoni et al. (2017) that demonstrated that the presence of *Arcobacter* is  
412 related to the fecal contamination. However, in those studies the correlation between the  
413 two microbes (*E. coli* and *Arcobacter*) was not quantified as has been done in the present  
414 study for the first time. The correlation values (Spearman's rho) obtained between the  
415 MPN values of *Arcobacter* and *E. coli* found in water (rho= 0.791) and those found in  
416 shellfish (rho= 0.873) (Figures 1 and 2) seem to indicate that detection of *Arcobacter* in

417 water may predict its presence in shellfish, independently of the concentration of the  
418 bacteria in water, as the correlation coefficients obtained in both AB (0.527) and PNC  
419 (0.472) were statistically significant.

420 In addition to the enumeration of both bacteria in water and within shellfish, we  
421 established for the first time the AF for *E. coli* and *Arcobacter* in mussels and oysters. It  
422 was observed that in June 2014, mussels and oysters from AB and PNC presented AF for  
423 *E. coli* and *Arcobacter* higher than the threshold established as their hyperaccumulation.  
424 This is something that could be expected because a positive correlation between the  
425 temperature and the filtration rates of bivalves has been described (Gosling, 2003; Anestis  
426 et al., 2010; Galimany et al., 2011). In fact, in June 2014, the temperature of the water  
427 was 21 °C at AB and 23.7 °C at PNC, warmer than the rest of the sampling period (mean  
428 temperatures of 19.13°C for AB and 18.01°C for PNC). Iwamoto et al. (2010) showed  
429 that seafood associated infections caused by bacteria occurred with a higher prevalence  
430 in warm months (from June to August). The hyperaccumulation of *E. coli* and *Arcobacter*  
431 that primarily occurred in June, may be considered as an extra risk for the consumer, as  
432 previously reported by Burkhardt and Calci, (2000). These authors found a relationship  
433 between the hyperaccumulation events of F<sup>+</sup> coliphages and the illness caused by  
434 Norwalk-like virus. The generally accepted mathematical models that explain the  
435 filtration rates in oysters indicate that this rate has a positive correlation with the  
436 temperature (Ehrich and Harris, 2015). However, there is another mathematical model  
437 supporting oyster's higher filtration rates in winter (Powell et al., 1992). The latter model  
438 applied to oysters is based on the size of the bivalve (i.e. juvenile and market sized).  
439 During the winter months oysters reach their adult size thus their filtration rate would

440 increase despite the lower temperatures. This explanation would support the  
441 hyperaccumulation event observed in December 2013 for oysters in our study.

442 The fact that the concentration of the bacteria (*E. coli* and *Arcobacter*) found in the water  
443 and in the shellfish, was very similar in the PNC, lowered the AF in this site in relation  
444 with what we observed for the AB site. Additionally, the exposure of the shellfish to the  
445 PNC water allowed us to see how the two types of shellfish studied behaved regarding  
446 the accumulation of both bacteria (Figure 3). In our experiment, both, oysters and mussels  
447 were equally exposed to the PNC water, while in AB we had no record when the shellfish  
448 were introduced in the system and for how long they were exposed to the surrounding  
449 water. Interestingly, mussels and oysters from the PNC did not present their  
450 hyperaccumulation episodes at the same time (Figure 3). This can be attributed to how  
451 different bivalves control their accumulation rates. Nowadays, there exist two theories,  
452 one that indicates that the filtration rate is physiologically controlled (Bayne, 1998;  
453 Hawkins et al., 1998) and the other that postulates that this depends on the capacity of the  
454 pump and the concentration of food particles in the water (Jørgensen, 1996). The most  
455 accepted is that the pumping rate and retention efficiency is a function of the nutritional  
456 needs or gut satiation of the individual bivalves (Gosling, 2003). However, given that the  
457 environmental conditions were the same, as occurred with the shellfish exposed to PNC  
458 water, the filtration rate may be controlled by the gills and also be dependent on body size  
459 (Powell et al., 1992; Gosling, 2003). In this sense the gill's structure is different in both  
460 types of shellfish (Pechenik, 1991), and the body size of oysters is bigger than for mussels.  
461 In addition to that the accumulation rates can also be affected by the different  
462 susceptibility of the mussels and the oysters to the physicochemical characteristics of the  
463 surrounding water (Gosling, 2003 and references therein).

464

## 465 **5 Conclusions**

466 This study is the first to provide comparative data of the concentration of *E. coli* and  
467 *Arcobacter* spp. from shellfish and from their surrounding water, including information  
468 about the accumulation rate of both bacteria in two different scenarios: low and high fecal  
469 pollution and in two types of shellfish (mussels and oysters).

470 The genus *Arcobacter* comprises species that are emergent pathogens like *A. butzleri*, *A.*  
471 *cryaerophilus* and *A. skirrowii* (Figueras et al., 2014; Van den Abeele et al., 2014). As  
472 shown by several studies *A. butzleri* is not only the most frequent species recovered from  
473 human samples (Figueras et al., 2014; Van den Abeele et a., 2014), but also from shellfish  
474 samples (Levican et al., 2013; Salas-Massó et al., 2016; Leoni et al., 2017). Although this  
475 prevalence may be overestimated due to the common use of a pre-enrichment step in the  
476 recovery of *Arcobacter* species (Ho et al., 2008; Levican et al., 2016). Our results show  
477 that the presence of *E. coli* correlates with the presence of two of these potentially  
478 pathogenic species, *A. butzleri* and *A. cryaerophilus*. However, *E. coli* would fail to  
479 predict the presence of *A. butzleri* and *A. skirrowii* among other species in 26.1% of the  
480 shellfish samples harvested from Alfacs Bay during the warmer months (>26.2°C) and  
481 this may have significant public health implications. The presence of *Arcobacter* in  
482 shellfish when *E. coli* was not detected would mean that this shellfish would be classified  
483 as class A which can be directly consumed without depuration (Anon, 2004, 2015). Thus,  
484 the presence of potential pathogenic *Arcobacter* species in shellfish may pose a risk to  
485 consumers. More studies need to be performed to know if the depuration process  
486 established by the European Regulation for *E. coli*, would also be enough to eliminate the  
487 burden of *Arcobacter* spp. from shellfish samples.

488

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495

## 496 **7 Authors and Contributors**

497 NSM carried out the experiments and literature review and drafted the manuscript, being  
498 the principal author; KBA, MDF evaluated results, drafted the manuscript and supervised;  
499 and MJF designed the research project evaluated results, drafted the manuscript and  
500 supervised. All the authors read and approved the final manuscript.

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709 **9 Tables and Figures**

710 **Table 1.** Positive samples for *E. coli* and *Arcobacter* spp. from the water and shellfish of Alfacs Bay (AB).

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Sample	N of positives (%)	No (%) positive samples by MPN for <i>E. coli</i> and <i>Arcobacter</i> spp.			Geometric mean $\pm$ SD <sup>a</sup>		No (%) positive samples for <i>Arcobacter</i> spp. by culture <sup>b</sup>	
		Only <i>E. coli</i>	Only <i>Arcobacter</i>	<i>E. coli</i> + <i>Arcobacter</i>	<i>E. coli</i>	<i>Arcobacter</i>	<i>Arcobacter</i> CAT broth + BA <sup>c</sup>	<i>Arcobacter</i> CAT-NaCl broth + MA <sup>d</sup>
Mean temperature (°C)								
Water n=21	11 (52.4)	6 (28.6)	4 (19.0)	1 (4.8)	5.6x10 <sup>1</sup> $\pm$ 2.2	1.0x10 <sup>2</sup> $\pm$ 3.1	4 <sup>e</sup> (19.0)	17 <sup>f</sup> (81.0)
Shellfish n= 23	18 (78.3)	7 (30.4)	6 (26.1)	5 (21.7)	1.2x10 <sup>2</sup> $\pm$ 2.7	1.2x10 <sup>2</sup> $\pm$ 2.2	6 <sup>g</sup> (26.1)	16 <sup>h</sup> (69.6)
Total n=44	29 (65.9)	13 (29.5)	10 (22.7)	6 (13.6)	8.9x10 <sup>1</sup> $\pm$ 2.7	1.1x10 <sup>2</sup> $\pm$ 2.4	10 (22.7)	33 (75.0)
		<b>18.9 °C</b>	<b>26.2 °C<sup>i</sup></b>	<b>19.4 °C</b>				

712 <sup>a</sup> Geometric mean of the MPN/results obtained from 100 mL of water or 100 g of shellfish.

713 <sup>b</sup> Enrichment was performed in *Arcobacter* CAT (Cefoperazone, Amphotericin B, and Teicoplanin) broth.

714 <sup>c</sup> Enrichment followed by culturing on Blood Agar (BA) after passive filtration.

715 <sup>d</sup> Enrichment broth supplemented with 2.5% NaCl (w/v) and followed by culturing on Marine Agar (MA) after passive filtration.

716 <sup>e</sup> Species recovered: *A. butzleri*, *A. molluscorum*, and *A. mytili*.

717 <sup>f</sup> Species recovered: *A. bivalviorum*, *A. butzleri*, *A. cloacae*, *A. ebronensis*, *A. halophilus*, *A. marinus*, *A. molluscorum*, *A. mytili*, *A. skirrowii* and *Arcobacter* sp.

718 <sup>g</sup> Species recovered: *A. butzleri*, *A. mytili* and *Arcobacter* sp.

719 <sup>h</sup> Species recovered: *A. bivalviorum*, *A. butzleri*, *A. marinus*, *A. molluscorum*, *A. mytili*, and *Arcobacter* sp.

720 <sup>i</sup> Mean temperature of the water samples positive only for *Arcobacter* was higher (P= 0.001) than that of samples only positive for *E. coli* and higher (P= 0.005) than  
 721 those positive for *E. coli* and *Arcobacter*. Species recovered: *A. bivalviorum*, *A. butzleri*, *A. cloacae*, *A. halohilus*, *A. molluscorum*, *A. mytili*, *A. skirrowii* and *Arcobacter* sp.  
 722

723 **Table 2.** Positive samples for *E. coli* and *Arcobacter* spp. from the water of the Poble Nou Chanel (PNC) and from shellfish exposed to  
 724 this water for 3 days.

725 **Table 2.** Positive samples for *E. coli* and *Arcobacter* spp. from the water of the Poble Nou Chanel (PNC) and from shellfish exposed to this  
 726 water for 3days.

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Sample	N	Geometric $\bar{X} \pm SD^a$			No (%) positive samples for <i>Arcobacter</i> spp. by culture <sup>b</sup>	
		Mean temperature (°C)			<i>Arcobacter</i> CAT broth + BA <sup>c</sup>	<i>Arcobacter</i> CAT-NaCl broth + MA <sup>d</sup>
Water	12	Both	<i>E. coli</i>	<i>Arcobacter</i>	9 (75.0) <sup>f</sup>	8 (66.7) <sup>g</sup>
Shellfish	19	Both	<i>E. coli</i>	<i>Arcobacter</i>	15 (78.9) <sup>h</sup>	15 (78.9) <sup>i</sup>
Total	31	31 (100) <b>18.3 °C</b>	<i>E. coli</i>	<i>Arcobacter</i>	24 (77.4)	23 (74.2)

728 <sup>a</sup> Geometric mean of the MPN/results obtained from 100 mL of water or 100 g of shellfish

729 <sup>b</sup> Enrichment was performed in *Arcobacter* CAT (Cefoperazone, Amphotericin B, and Teicoplanin) broth.

730 <sup>c</sup> Enrichment followed by culturing on Blood Agar (BA) after passive filtration.

731 <sup>d</sup> Enrichment broth supplemented with 2.5% NaCl (w/v) and followed by culturing on Marine Agar (MA) after passive filtration.

732 <sup>e</sup> The *Arcobacter* MPN values obtained from PNC water (P=0.004) and shellfish (P=0.002) samples were significantly higher than those of *E. coli* from the same  
 733 samples.

734 <sup>f</sup> Species recovered: *A. butzleri*, *A. molluscorum*, and *A. mytili*.

735 <sup>g</sup> Species recovered: *A. bivalviorum*, *A. butzleri*, *A. cloacae*, *A. ebronensis*, *A. halophilus*, *A. marinus*, *A. molluscorum*, *A. mytili*, *A. skirrowii* and *Arcobacter* sp.

736 <sup>h</sup> Species recovered: *A. aquimarinus*, *A. bivalviorum*, *A. butzleri*, *A. cloacae*, *A. cryaerophilus*, *A. defluvii*, *A. ellisii* and *Arcobacter* sp.

737 <sup>i</sup> Species recovered: *A. bivalviorum*, *A. butzleri*, *A. cloacae*, *A. cryaerophilus*, *A. halophilus*, *A. marinus*, *A. molluscorum*, *A. mytili*, and *Arcobacter* sp.

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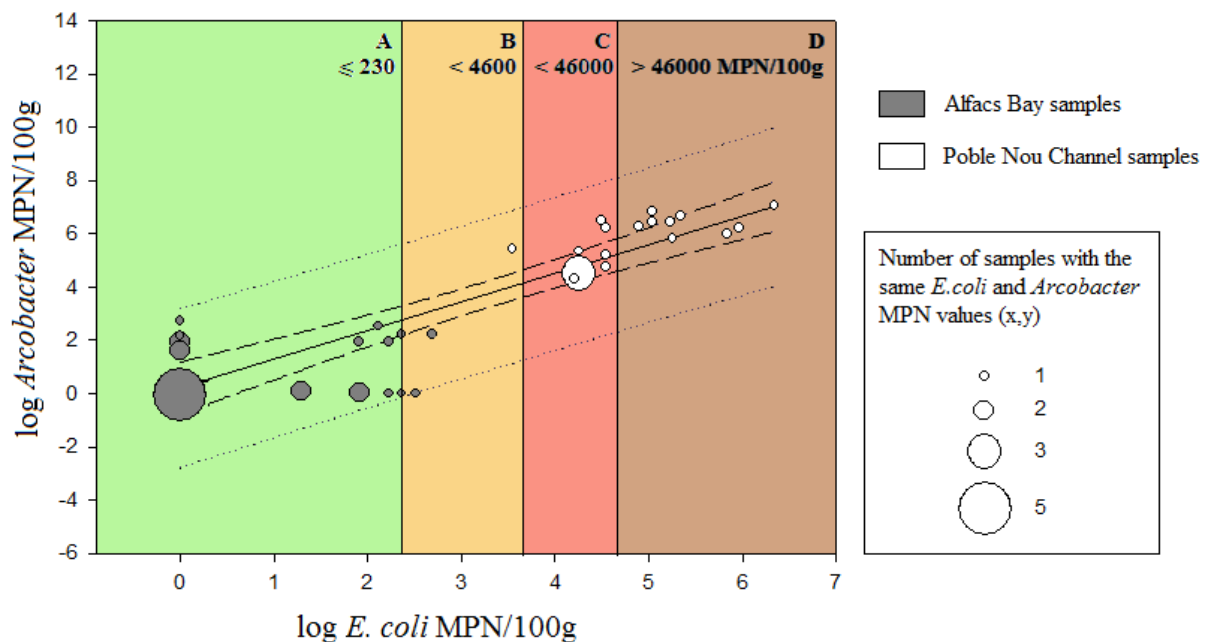
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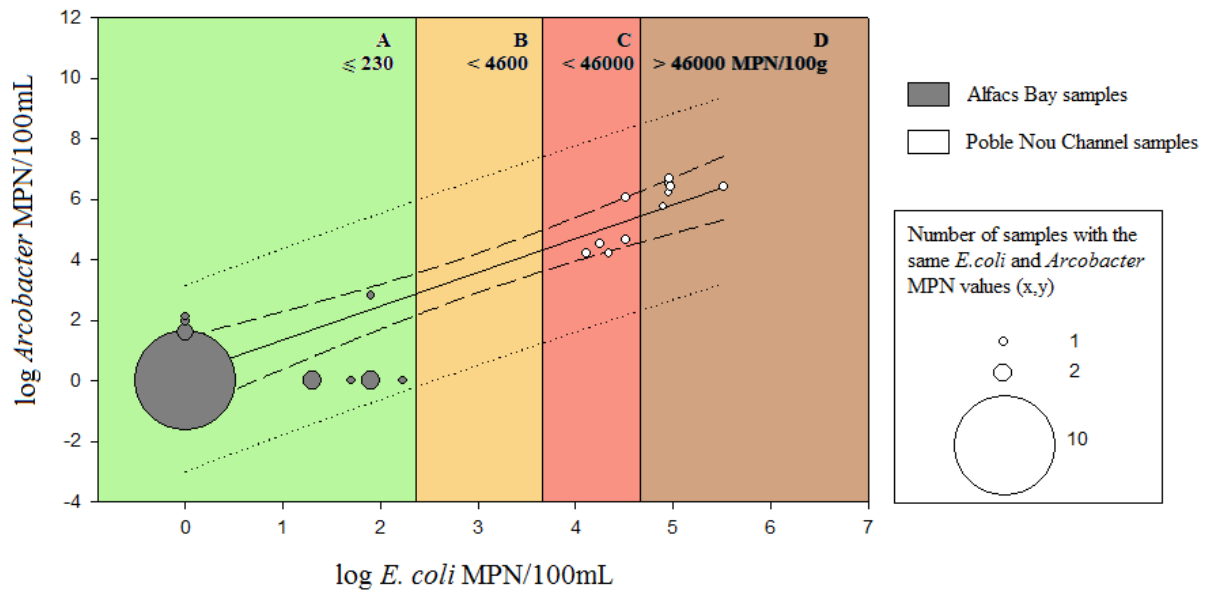
751 **Figure 1.** Linear regression showing the correlation between the MPN concentration of *E.*  
 752 *coli* and *Arcobacter* sp. in the AB (grey) and in the PNC (white) for 100 g of shellfish ( $\rho=$   
 753 0.873,  $P= 0.000$ ). The different colors indicate the standards of the four categories (A, B, C  
 754 and D) established by the European Union for the shellfish harvesting areas on the basis of  
 755 the MPN results of *E. coli*/100g (Anon, 2004, 2015): class A (green), shellfish do not require  
 756 depuration and can go direct to the market; class B (orange), 24h of depuration is needed;  
 757 class C (red), shellfish have to be placed in a clean water for at least one month and class D  
 758 (brown), these shellfish are prohibited for consumption. The size of the circles represents  
 759 how many samples presented the same MPN values for *E. coli* and *Arcobacter*. Line — =  
 760 linear regression; lines --- = 95% confidence interval; lines ... = 95% predictive concentration  
 761 interval.



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763 **Figure 2.** Linear regression showing the correlation between the MPN concentration of *E.*  
 764 *coli* and *Arcobacter* sp. in the AB (grey) and in the PNC (white) for 100 mL of water ( $\rho=$   
 765 0,791,  $P= 0.000$ ). The different colors indicate the standards of the four categories (A, B, C  
 766 and D) established by the European Union for the shellfish harvesting areas on the basis of

767 the MPN results of *E. coli*/100g (Anon, 2004, 2015): class A (green), shellfish do not require  
 768 depuration and can go direct to the market; class B (orange), 24h of depuration is needed;  
 769 class C (red), shellfish have to be placed in a clean water for at least one month and class D  
 770 (brown), these shellfish are prohibited for consumption. The size of the circles represents  
 771 how many samples presented the same MPN values for *E. coli* and *Arcobacter*. Line — =  
 772 linear regression; lines --- = 95% confidence interval; lines ... = 95% predictive concentration  
 773 interval.



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775 **Figure 3.** Accumulation factor (AF; GM MPN shellfish/ GM MPN water) of *E. coli* and  
 776 *Arcobacter* in the shellfish from Alfacs Bay (AB) and Poble Nou Channel (PNC) in relation  
 777 to the sampling months and temperature.

