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

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

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Abstract

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

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

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

Nomenclature

EU - European Union

MPN - Most Probable Number

AF - Accumulation Factor

AB - Alfacs Bay

PNC - Poble Nou Channel

CAT - Cefoperazone, Amphotericin B, and Teicoplanin

GM - Geometric Mean

1 Introduction

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

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

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

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

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

2 Experimental procedures

2.1 Location and sampling

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

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

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

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

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

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

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

2.2.2 Detection of *Arcobacter* spp.

Additionally, all the samples were analyzed for the presence of *Arcobacter* species using two methods. The conventional one, described in previous studies (Collado et al., 2008; Levican et al., 2014; Salas-Massó et al., 2016), involved the use of a pre-enrichment in 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 or100 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 ($1.2x10^2$) 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 ºC) than those that were
230 only positive for *E. coli* (18.9 ºC) and those positive for both microbes (19.4 ºC; 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 ($1.2x10^2$±2) was obtained
234 from the shellfish for both microbes, while in the water the values were slightly different
235 i.e. $5.6x10^1$ ± 2.2 for *E. coli* and $1.0x10^2$ ± 3.1 for *Arcobacter* (Table 1). The higher

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

From PNC all the samples of water and shellfish were positive for both bacteria with the MPN method, while with both culture approaches the positive samples for *Arcobacter* ranged between 66.7% and 78.9% (Table 2). The densities of *E. coli* (GM=$6.6x10^4$±5.1) and *Arcobacter* spp. (GM=$5.4x10^5$±7.8) in the shellfish exposed for 72h to the PNC contaminated water were slightly higher than the densities of these bacteria found in water (Table 2). When comparing the *Arcobacter* and the *E. coli* MPN values obtained from both water and shellfish in the PNC, the former had significantly higher MPN than the latter in both matrices (Table 2).

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

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

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

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

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

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

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

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

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

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

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

In general, no correlation between *E. coli* and the *Arcobacter* spp. was observed in the Alfacs Bay samples. However, after a deeper analysis taking into account the different *Arcobacter* species recovered from all the water and shellfish samples with different levels of *E. coli* (Tables S2 and S3) correlations with concrete species were observed. The lack of significant correlation observed between the MPN of *E. coli* and *Arcobacter* in any type of samples from the AB (Table S1) was probably due to the low levels of fecal 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 $4.9x10^3$ 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. buztleri*,
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.

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

Although Alfacs Bay is a good representative of the western Mediterranean shellfish growing areas, its low fecal contamination levels did not provide a wide range of conditions to generate multiple scenarios where the performance of the correlation of *E. coli* and *Arcobacter* spp. could be compared. As a second scenario for the study, the Poble Nou Channel was chosen as the water harbored high levels of fecal pollution (geometric mean of *E. coli* $4.1x10^4$ MPN/100 mL). In this water, the concentration of *Arcobacter* spp. ($4.5x10^5$ MPN/100 mL) was one log higher (p= 0.05) than that of *E. coli*, which agrees with the concentrations described by Collado et al., (2008) in contaminated freshwater that impacted a seawater bathing area ($3.7x10^5$ MPN/100 mL for *Arcobacter* spp. vs. $4.9x10^3$ CFU/100 mL for *E. coli*). This difference in the concentration of both bacteria has also been observed in a recent study that investigated the efficiency of a natural (biological) process of purifying wastewater by storing it in open air lagoons (Fernández-Cassi et al., 2016). The wastewater to be treated showed concentration of *Arcobacter* ($7.51 x10^6$ MPN/100 mL) higher than those of *E. coli* ($7.23 x10^4$ MPN/100 mL) (Fernández-Cassi et al., 2016). Some authors have indicated that the high prevalence of *Arcobacter* spp. found in sewage could be associated to contamination from human 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^+ 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

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

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

5 Conclusions

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

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

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7 Authors and Contributors

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

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9 Tables and Figures

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

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

[a] Geometric mean of the MPN/results obtained from 100 mL of water or 100 g of shellfish.

[b] Enrichment was performed in Arcobacter CAT (Cefoperazone, Amphotericin B, and Teicoplanin) broth.

[c] Enrichment followed by culturing on Blood Agar (BA) after passive filtration.

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

[e] Species recovered: *A. butzleri*, *A. molluscorum*, and *A. mytili*.

[f] Species recovered: *A. bivalviorum*, *A. butzleri*, *A. cloacae*, *A. ebronensis*, *A. halophilus*, *A. marinus*, *A. molluscorum*, *A. mytili*, *A. skirrowii* and *Arcobacter* sp.

[g] Species recovered: *A. butzleri*, *A. mytili* and *Arcobacter* sp.

[h] Species recovered: *A. bivalviorum*, *A. butzleri*, *A. marinus*, *A. molluscorum*, *A. mytili*, and *Arcobacter* sp.

[i] 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 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.

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 water for 3 days.

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Sample	N	Geometric $\overline{X}$ ± SD[a] **Mean temperature** (ºC)			No (%) positive samples for *Arcobacter* spp. by culture[b]	
		Both	*E. coli*	*Arcobacter*	*Arcobacter* CAT broth + BA[c]	*Arcobacter* CAT-NaCl broth + MA[d]
Water	12	12 (100)	$4.1x10^4 \pm 3.6$	$4.5x10^5 \pm 9.3$[e]	9 (75.0) [f]	8 (66.7) [g]
Shellfish	19	19 (100)	$6.6x10^4 \pm 5.1$	$5.4x10^5 \pm 7.8$[e]	15 (78.9) [h]	15 (78.9) [i]
Total	31	31 (100) **18.3 ºC**	$5.6x10^4 \pm 4.5$	$5.0x10^5 \pm 8.1$	24 (77.4)	23 (74.2)

[a] Geometric mean of the MPN/results obtained from 100 mL of water or 100 g of shellfish

[b] Enrichment was performed in Arcobacter CAT (Cefoperazone, Amphotericin B, and Teicoplanin) broth.

[c] Enrichment followed by culturing on Blood Agar (BA) after passive filtration.

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

[e] 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 samples.

[f] Species recovered: *A. butzleri*, *A. molluscorum*, and *A. mytili*.

[g] Species recovered: *A. bivalviorum*, *A. butzleri*, *A. cloacae*, *A. ebronensis*, *A. halophilus*, *A. marinus*, *A. molluscorum*, *A. mytili*, *A. skirrowii* and *Arcobacter* sp.

[h] Species recovered: *A. aquimarinus*, *A. bivalviorum*, *A. butzleri*, *A. cloacae*, *A. cryaerophilus*, *A. defluvii*, *A. ellisii* and *Arcobacter* sp.

[i] Species recovered: *A. bivalviorum*, *A. butzleri*, *A. cloacae*, *A. cryaerophilus A, halophilus*, *A. marinus*, *A. molluscorum*, *A. mytili*, and *Arcobacter* sp.

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

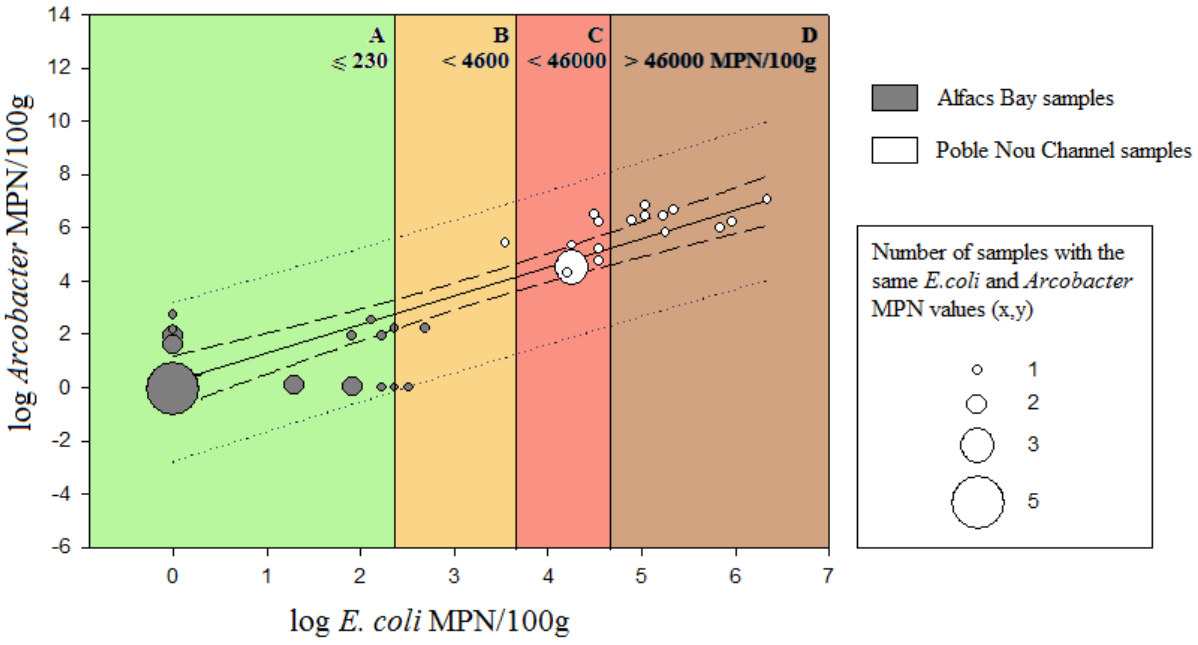

Figure 2. Linear regression showing the correlation between the MPN concentration of *E. coli* and *Arcobacter* sp. in the AB (grey) and in the PNC (white) for 100 mL of water (rho= 0,791, P= 0.000). The different colors indicate the standards of the four categories (A, B, C and D) established by the 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
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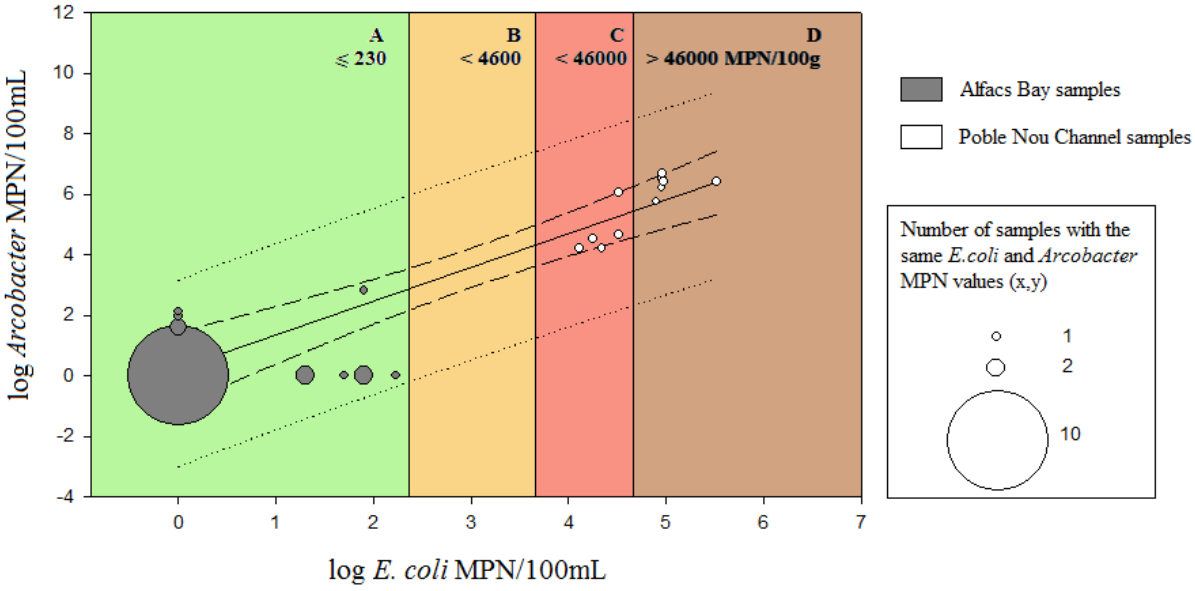
773 interval.



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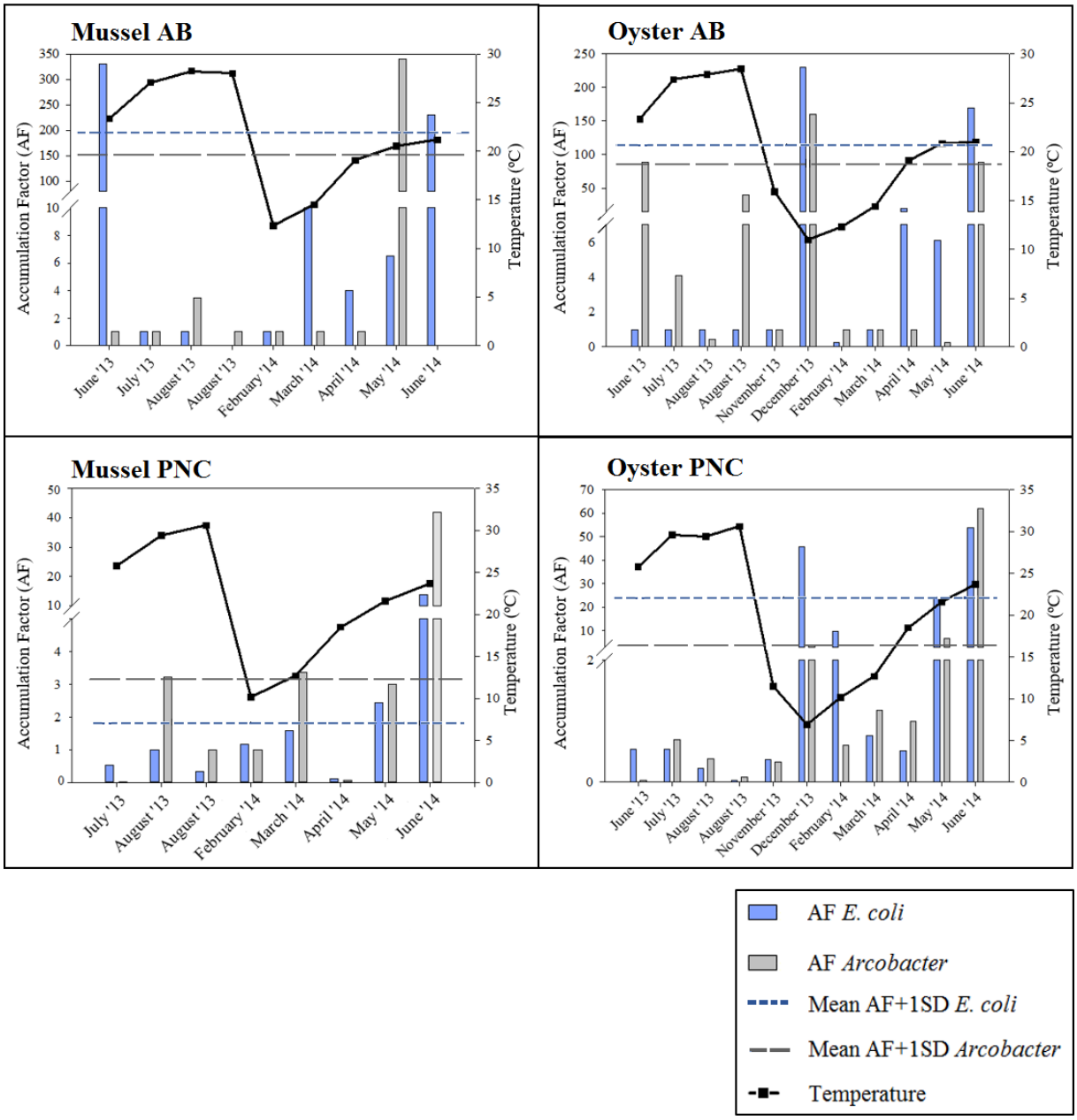
775 **Figure 3.** Accumulation factor (AF; GM MPN shellfish/ GM MPN water) of *E. coli* and
776 *Arcobacter* in the shellfish from Alfacs Bay (AB) and Poble Nou Channel (PNC) in relation
777 to the sampling months and temperature.


Mussel AB
Oyster AB
Mussel PNC
Oyster PNC
Accumulation Factor (AF)
Temperature (ºC)
June '13
July '13
August '13
August '13
February '14
March '14
April '14
May '14
June '14
November '13
December '13
AF E. coli
AF Arcobacter
Mean AF+1SD E. coli
Mean AF+1SD Arcobacter
Temperature

778