Quality enhancement of chilled fish by including alga *Bifurcaria bifurcata* extract in the icing medium

José M. Miranda[1], Jaime Ortiz[2], Jorge Barros-Velázquez[1], Santiago P. Aubourg[3,*]

[1] Department of Analytical Chemistry, Nutrition and Food Science, School of Veterinary Sciences, University of Santiago de Compostela, Lugo (Spain).

[2] Department of Food Science and Chemical Technology, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago (Chile)

[3] Department of Food Science and Technology, Marine Research Institute (CSIC), Vigo (Spain).

* Corresponding author. Phone: +34986231930; Fax: +34986292762; e-mail: saubourg@iim.csic.es

ABSTRACT

Bifurcaria bifurcata is a widely extended brown macroalga, whose antimicrobial and antioxidant properties have previously been described. In this study, ethanolic extracts of *B. bifurcata* were included in the icing medium employed for the chilled storage of megrim (*Lepidorhombus whiffiagonis*). For it, two different concentrations of this brown macroalga extract (0.67 and 2.50 g lyophilized alga L^{-1} aqueous solution; B-1 and B-2 batches, respectively) were tested for a 14-day storage. The effect of the alga extract was compared with a counterpart batch stored in traditional ice prepared only from water (B-0 batch). Significant ($p<0.05$) inhibitions of microbial activity (aerobes, psychrotrophs, lipolytic bacteria, proteolytic bacteria and Enterobacteriaceae) as well as of pH and trimethylamine formation, were observed as a result of the incorporation of the alga extract in the icing medium, being this effect especially relevant in the B-2 batch. Concerning lipid damage development, a significantly ($p<0.05$) lower formation of free fatty acids (lipid hydrolysis development) and of fluorescent compounds (tertiary lipid oxidation development) in samples corresponding to both alga-including batches could also be observed; this inhibitory effect was more intense in fish belonging to the B-2 batch. The icing medium proposed in this work constitutes a promising strategy in order to apply algae extracts to enhance fish quality retention during the different steps of storage and commercialization of marine species.

Keywords: *Lepidorhombus whiffiagonis*; *Bifurcaria bifurcata*; chilling; microbiological activity; lipid oxidation; quality.

Running Title: Fish preservation with *Bifurcaria bifurcata* extract.

INTRODUCTION

Flake-ice refrigeration has been the most commonly employed method to slow down fish damage. However, deterioration of nutritional value and sensory quality during storage has led important decreases in fish shelf lives, thus provoking important economic losses. With the aim of delaying fish damage during chilled storage, a wide number of preservation strategies have been combined with flake ice. Among them, several chemical and physical treatments and their combination with packaging have been evaluated (Ashie et al. 1996; Oms-Oliu et al. 2010; Senturk and Alpas 2013; Campos et al. 2012). Additionally, recent studies accounted for the incorporation of preservative compounds in the icing medium such as natural low-molecular weight organic acids (Sallam 2007; Sanjuás-Rey et al. 2012), vegetable extracts (namely, thyme, rosemary and oregano) (Oral et al. 2008; Quitral et al. 2009; Özyurt et al. 2012) and ozone (Pastoriza et al. 2008).

Marine algae have traditionally formed part of the Asian diet, especially in countries like Japan, China and Korea, and constitute a relevant source of beneficial nutrients, such as vitamins, trace minerals, lipids, amino acids and dietary fibers (Díaz-Rubio et al. 2009; Paiva et al. 2014). The use of alga in Western countries has been traditionally focused on the extraction of compounds of relevance for pharmaceutical, cosmetics and food industries. Recently, red, green and brown macroalgae have offered the possibility of exploring a wide variety of natural compounds with potential antioxidant (Wang et al. 2010; Halldorsdóttir et al. 2014), antimicrobial (Sandsdalen et al. 2003; Gupta and Abu-Ghannam 2011), anti-inflammatory and anti-tumoral activities (Smit 2004). In this sense, a wide number of preservative metabolites such as polyphenols, terpenes, phlorotannins, steroids, halogenated ketones and alkanes,

fucoxanthin, polyphloroglucinol or bromophenols have been isolated from macroalgae (Fleurence et al. 2012; Peinado et al. 2014).

Among brown macroalgae, *Bifurcaria bifurcata* is a species found in the Atlantic coast of France, Spain and Portugal, also extending to the South and West coasts of England and the West coast of Ireland (Braune 2008; Le Lann et al. 2008). It is usually found in rock pools on the middle and lower shore, particularly on exposed beaches, showing an olive-yellow color and a length up to 50 cm. Previous research on this macroalga accounted for proximate composition analysis (Gómez-Ordóñez et al. 2010) as well as the identification of different kinds of compounds present in it such as diterpenes (Culioli et al. 2001), phenols (Glombitza et al. 1976), sterols (Bouzidi et al. 2008) and polysaccharides (Gómez-Ordóñez and Rupérez 2011). Additionally, the antitumoral and antioxidant activity of this alga has been reported by means of *in vitro* studies (Zubia et al. 2009).

The present study evaluates the incorporation, to the best of our knowledge for the first time, of *B. bifurcata* extracts in the icing media and their application to the chilled storage of a fish species of commercial relevance. For it, aqueous solutions including ethanolic extracts of lyophilized *B. bifurcata* at two different concentrations were tested as icing media. The effects of the alga extracts on microbial activity inhibition and lipid oxidation stability were monitored in megrim (*Lepidorhombus whiffiagonis*) muscle for up to 14 d of chilled storage. This fish species was chosen due to its abundance in the Northeast Atlantic waters and for its commercial interest in a wide number of European countries such as the United Kingdom, France, Ireland and Spain (FAO 2007).

106 ## MATERIALS AND METHODS

107 ### Preparation of *B. bifurcata* extracts and icing systems

108 The lyophilized alga *B. bifurcata* was provided by Porto-Muiños (Cerceda, A Coruña,
109 Spain). Fifteen g of lyophilized alga were mixed with absolute ethanol (2 x 120 mL),
110 stirred for 30 s and centrifuged at 3500 rpm for 10 min at 4ºC. Then, the supernatant
111 was recovered and diluted to 6 L with distilled water (2.50 g lyophilized alga L^{-1}
112 aqueous solution). This solution was packaged in polyethylene bags, kept frozen at -
113 18ºC and later used as icing medium (B-2 batch). In the same way, 4 g of lyophilized
114 alga were extracted with ethanol as described above in order to provide a more diluted
115 alga-icing medium (0.67 g lyophilized alga L^{-1} aqueous solution; B-1 batch). Finally,
116 traditional ice was prepared from distilled water that was packaged and kept frozen in
117 the same way as the two other ices (B-0, control batch). Before addition to individual
118 fish specimens, the different icing systems were ground to obtain ice flakes.

119 Experimental conditions (contents of lyophilized alga extract in the ice)
120 employed in the present study were based on several preliminary tests carried out at our
121 laboratory in the range of 0.10-5.00 g lyophilized alga L^{-1} aqueous solution. Thus, an
122 increasing presence of alga in the icing medium provided better sensory acceptance
123 (namely, lower putrid odor and taste development). However if a higher concentration
124 than 2.50 was applied, modification of the external odor and color of the whole fish or
125 the flesh odor and flavor would occur as a result of the alga presence in ice (data not
126 shown). In order to avoid such modifications, this concentration (2.50 g lyophilized alga
127 L^{-1} aqueous solution) was considered in the B-2 batch. In order to analyze the effect of
128 the alga presence, a lower concentration of the alga was also tested. Thus, the lowest
129 concentration that led to partial sensory performances during the preliminary trials was
130 also chosen (0.67 g L^{-1}, B-1 batch). According to European Council Regulation (1997)

131 algae are considered food or food ingredients, so their use in the icing medium should
132 not constitute any hazard to health.
133
134 **Fish material, processing and sampling**
135 Fresh megrim (117 specimens) were caught near the Galician Atlantic coast (North-
136 Western Spain) and transported to the laboratory. Throughout this process (10 h), the
137 fish were maintained in ice. The length and weight of the fish specimens ranged from
138 21 to 26 cm and from 105 to 132 g, respectively.
139 Upon arrival to the laboratory, nine individual fish specimens were separated
140 and analyzed as initial fish (day 0). These fish specimens were divided into three
141 different groups (three individuals per group) that were analyzed independently to
142 achieve the statistical analysis (n=3). The remaining fish specimens were divided into
143 three batches (36 individuals in each batch), that were placed in independent boxes and
144 directly surrounded by different kinds of ice (B-0, B-1 and B-2 batches, respectively),
145 prepared as previously described. Ice was added at a 1:1 fish:ice ratio, and all batches
146 were placed inside a refrigerated room (2±1ºC). Boxes that allowed draining of melted
147 ice were used for fish storage. The ice of all batches was renewed when required to
148 maintain the mentioned fish:ice ratio. Fish samples from all of the batches were stored
149 for a 14-day period, being sampled and analyzed on days 4, 7, 11 and 14. At each
150 sampling time, nine specimens were taken from each batch for analysis and divided into
151 three groups (three individuals in each group) that were studied independently (n=3).
152
153 **Microbiological analyses**
154 Samples of 10 g of fish white muscle were taken aseptically from chilled fish
155 specimens, mixed with 90 mL of 0.1% peptone water (Merck, Darmstadt, Germany)

156 and homogenized in sterilized stomacher bags (AES, Combourg, France) as previously
157 described (Ben-Gigirey et al. 1998; Ben-Gigirey et al. 1999). Serial dilutions from the
158 microbial extracts were prepared in 0.1% peptone water.

159 Total aerobes were investigated by surface inoculation on plate count agar
160 (PCA, Oxoid Ltd., London, UK) after incubation at 30ºC for 48 h. Psychrotrophs were
161 also investigated in PCA, being the incubation carried out at 7-8ºC for 7 days.
162 Enterobacteriaceae were investigated by pour plating using Violet Red Bile Agar
163 (VRBA) (Merck, Darmstadt, Germany) after an incubation period of 24 h at 37±0.5ºC.
164 Microorganisms exhibiting a proteolytic or lipolytic phenotype were determined on
165 casein-agar medium or tributyrine-agar, respectively, after incubation at 30ºC for 48 h,
166 as previously described (Ben-Gigirey et al. 2000).

167 In all cases, microbial counts were transformed into log CFU g^{-1} muscle before
168 undergoing statistical analysis. All of the analyses were conducted in triplicate.

169

170 **Chemical analyses**

171 All solvents and chemical reagents used were of reagent grade (Merck,
172 Darmstadt, Germany). Chemical analyses related to fish quality were carried out on the
173 white muscle of megrim. Total polyphenols content of lyophilized *B. bifurcata* was
174 assessed by means of the Folin-Ciocalteu colorimetric method (Cary 3E UV–Visible
175 spectrophotometer, Varian; Mulgrave, Victoria, Australia) as described previously
176 (Rodríguez-Bernaldo de Quirós et al. 2010). Measurements were made in triplicate.
177 Gallic acid (GA) was used as standard. Results were expressed as mg GA g^{-1}
178 lyophilized alga.

179 The evolution of pH values in megrim muscle along storage time was
180 determined using a 6-mm diameter insertion electrode (Crison, Barcelona, Spain).

181 Trimethylamine-nitrogen (TMA-N) values were determined using the picrate
182 colorimetric (Beckman Coulter, DU 640; London, UK) method, as previously described
183 by Tozawa et al. (1971). This method involved the preparation of a 5% trichloroacetic
184 acid extract of fish muscle (10 g/25 mL). The results are expressed as mg TMA-N kg^{-1}
185 muscle.

186 Lipids were extracted from the fish muscle by the Bligh and Dyer (1959)
187 method, which employs a single-phase solubilization of the lipids using a chloroform-
188 methanol (1:1) mixture. The results were calculated as g lipid kg^{-1} muscle. Free fatty
189 acid (FFA) content was determined in the lipid extract of the fish muscle by the Lowry
190 and Tinsley (1976) method based on complex formation with cupric acetate-pyridine
191 followed by spectrophotometric (715 nm) assessment. Results were expressed as g FFA
192 kg^{-1} lipids and as mg FFA kg^{-1} muscle. Peroxide value (PV) was determined
193 spectrophotometrically (Beckman Coulter, DU 640; London, UK) using the lipid extract
194 via previous peroxide reduction with ferric thiocyanate according to the Chapman and
195 McKay (1949) method. The results were expressed as meq active oxygen kg^{-1} lipids.
196 Thiobarbituric acid index (TBA-i) was determined according to Vyncke (1970). This
197 method is based on the reaction between a trichloracetic acid extract of the fish muscle
198 and thiobarbituric acid. Content of thiobarbituric acid reactive substances (TBARS) was
199 spectrophotometrically measured at 532 nm and calculated from a standard curve using
200 1,1,3,3-tetraethoxy-propane (TEP). Results were expressed as mg malondialdehyde kg^{-1}
201 muscle. Tertiary lipid oxidation compounds resulting from the interaction between
202 oxidized lipids and nucleophilic compounds (namely, protein-like molecules) were
203 measured by fluorescence spectroscopy (Fluorimeter LS 45; Perkin Elmer España; Tres
204 Cantos, Madrid, Spain). In agreement with previous research (Aubourg et al. 2006),
205 fluorescence measurements were carried out at 393/463 nm and 327/415 nm in the

aqueous phase that resulted from the lipid extraction of fish muscle. The relative fluorescence (RF) was calculated as follows: $RF = F/F_{st}$, where F is the fluorescence measured at each excitation/emission wavelength pair and F_{st} is the fluorescence intensity of a quinine sulfate solution (1 μg mL^{-1} in 0.05 M H_2SO_4) at the corresponding wavelength pair. The fluorescence ratio (FR) was calculated as the ratio between the two RF values: $FR = RF_{393/463\ nm}/RF_{327/415\ nm}$.

Statistical analysis

Data obtained from the different microbiological and chemical analyses were subjected to the ANOVA method to explore differences resulting from the effects of both the icing condition and the chilling time; the comparison of means was performed using the least-squares difference (LSD) method. In all cases, analyses were carried out using the PASW Statistics 18 software for Windows (SPSS Inc., Chicago, IL, USA); differences among batches and among chilling times were considered significant for a confidence interval at the 95% level ($p<0.05$) in all cases.

Correlation analysis among parameters (chilling storage time, microbiological values and chemical scores) was also carried out. The results are referred to linear fittings unless indicated.

RESULTS AND DISCUSSION

Microbial evolution in megrim muscle

B. bifurcata ethanolic extracts were preliminarily evaluated in their antimicrobial activity against relevant food-borne spoilage and pathogenic bacteria. Thus, *B. bifurcata* exhibited antimicrobial activity against *Salmonella enterica, Aeromonas hydrophila, Bacillus cereus, Bacillus subtilis, Escherichia coli,*

231 *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas fluorescens, Vibrio*
232 *alginolyticus* and *Vibrio parahaemolyticus* (data not shown). Additionally, high
233 polyphenol content (40.8±8.3 GA g^{-1} lyophilized alga) was determined in ethanolic
234 extracts of *B. bifurcata*.

235 Table 1 shows the evolution of aerobic mesophiles in all three batches.
236 Progressive increases ($p<0.05$) were observed in all batches as storage time progressed
237 (r^2 = 0.91-0.92). The presence of *B. bifurcata* extract in the most concentrated icing
238 medium (B-2 batch) resulted in a significant ($p<0.05$) inhibition of microbial growth as
239 compared with B-1 and B-0 (control) batches. The highest inhibition (1.46 log units)
240 was observed on day 7, and remained at similar levels at advanced storage periods.
241 Unlike other batches, aerobes counts in B-2 batch were always below 6 log units, even
242 at advanced storage periods. B-1 batch exhibited better behavior than B-0 (control)
243 batch (lower mean values), although the differences between both batches were not
244 significant ($p>0.05$).

245 The investigation of psychrotrophs in megrim batches is displayed in Table 1.
246 This bacterial group includes bacteria belonging to *Flavobacterium, Shewanella,*
247 *Acinetobacter, Pseudomonas* and *Moraxella,* among other genera. The evolution of
248 psychrotrophs followed a similar pattern as that observed for aerobes. A progressive
249 increase ($p<0.05$) in microbial counts was observed for all batches as storage time
250 progressed (r^2 = 0.86-0.91). The incorporation of alga extract in the B-2 batch resulted
251 in a significantly ($p<0.05$) better control of psychrotrophs growth as compared with the
252 other batches. These differences were larger on storage days 7 and 11, and reached a
253 maximum of 1.37 log units. As in the case of aerobes, B-1 batch provided a better
254 protection than B-0 (control) batch (lower mean values), although differences between
255 both batches were only significant ($p<0.05$) on day 11. These results are quite in

256 agreement with those observed for aerobes, and clearly indicate a beneficial effect of *B.*
257 *bifurcata* extract in the icing medium in terms of bacterial inhibition in megrim muscle.
258 Table 1 shows the evolution of lipolytic bacteria growth in all three batches. As
259 in the case of the other bacterial groups, a progressive increase ($p<0.05$) was observed
260 in all batches as storage time progressed (r^2 = 0.93-0.94). Significant ($p<0.05$)
261 differences between B-2/B-1 and B-0 (control) batches were observed in the 11-14-day
262 period. These differences reached their maximum (1.11 log units) after 14 d of storage.
263 According to these results, the presence of *B. bifurcata* extracts in the icing media
264 provided an inhibitory effect on the development of lipolytic bacteria in megrim muscle.
265 The investigation of Enterobacteriaceae is presented in Table 2. The results were
266 similar for all batches at all sampling times except for day 14, where the batches
267 containing *B. bifurcata* extract exhibited a significant ($p<0.05$) better behavior than the
268 control batch. The low counts determined for this microbial group confirms the good
269 initial microbial quality of megrim specimens.
270 The study of the evolution of proteolytic microorganisms in all three batches is
271 also presented in Table 2. The active role of proteolytic bacteria in the degradation of
272 fish muscle has been previously informed (Rodríguez et al. 2003). This negative aspect
273 implies that the inhibition of proteolytic bacteria would be of relevance in terms of
274 megrim quality and safety. As for previous bacterial groups, a progressive increase
275 ($p<0.05$) was observed in all batches with storage time (r^2 = 0.88-0.89). The results
276 obtained for proteolytic bacteria indicated that the presence of the alga extract in the
277 icing medium provided significant ($p<0.05$) inhibition towards this microbial group on
278 days 7 (B-2 batch) and 14 (B-1 batch). The bacterial inhibition reached its maximum
279 (0.78 log units) on day 7.

In spite of disposing of a wide variety of information related to the *in vitro* antibacterial activity of brown algae, previous research concerning their practical application to seafood can be considered scarce. In the present study, *B. bifurcata* extracts were included for the first time in the icing media for the chilled storage of a marine fish species. As a result, ethanolic extracts of *B. bifurcata* were found to exert a remarkable antimicrobial effect for all the five microbial groups investigated in this study: aerobes, psychrothrophs, Enterobacteriaceae, proteolytic and lipolytic bacteria. This effect was especially relevant at moderate and advanced storage times and would lead to an extended shelf life in the batches treated with the alga, as compared with the control batch. Other authors have reported the inclusion of natural compounds in the icing system, i.e.: rosemary extract for chilled sardines (*Sardinella aurita*) (Özyurt et al. 2012), and wild-thyme hydrosol extract to chilled Transcaucasian barb (*Capoeta capoeta capoeta*) (Oral et al. 2008).

The presence of natural components with antimicrobial activity is widespread in macroalgae, and a wide range of metabolites have been isolated and characterized. In the Order *Fucales* (Class *Phaeophyceae*), natural products of mixed biosynthesis, consisting of terpenes, polyphenols, oligomeric phlorotannins, hydroquinones and halogenated alkanes and alkenes have been identified as key antimicrobial components (Smit 2004; Gupta and Abu-Ghannam 2011; Fleurence et al. 2012). Concerning *B. bifurcata*, Glombitza and Rösener (1974) reported the presence of bifuhalol, a polyhydroxyphenyl ether, on the basis of NMR and IR spectroscopic analysis. In a related Phaeophyceae alga (*Fucus vesiculosus*), Sandsdalen et al. (2003) identified a polyhydroxylated fucophlorethol as responsible for the antimicrobial activity against both Gram-positive and Gram-negative bacteria tested. More recently, García-Soto et al. (2015) showed that *Fucus spiralis* and sorbic acid, when included in a biodegradable

305 film, exerted an inhibitory effect of microbial activity on megrim muscle during chilled
306 storage.
307
308 **Analysis of the chemical changes in megrim muscle**
309 Two chemical parameters (pH and TMA-N), closely related to microbial
310 spoilage, were investigated. With respect to pH, a significant ($p<0.05$) and progressive
311 increase was observed for all batches as storage time progressed (r^2 = 0.77-0.94,
312 quadratic fitting) (Table 3). The increase of pH in fish muscle indicates the formation of
313 ammonia, TMA and other alkaline compounds, mainly derived from microbial action.
314 The incorporation of *B. bifurcata* extracts exerted a better control of the alkalization
315 routes, as compared with the control batch. This effect was significant ($p<0.05$) at
316 advanced storage times (11-14-day period), reaching a maximum inhibition value of
317 0.47 pH units on day 11. pH values showed fair correlation values with the microbial
318 parameters investigated (r^2 = 0.61-0.89), the highest scores being observed with
319 psychrotrophs counts (r^2 = 0.70-0.89).
320 In agreement with these results, previous studies have reported an inhibition of
321 pH increase as a result of using other natural preservative compounds in ice during the
322 chilled storage of marine species. These studies included the use of oregano and
323 rosemary extracts during the chilled storage of Chilean jack mackerel (*Trachurus*
324 *murphyi*) (Quitral et al. 2009), a rosemary extract applied to sardine (*Sardinella aurita*)
325 (Özyurt et al. 2012) and a wild-thyme hydrosol extract employed for the chilled storage
326 of Transcaucasian barb (Oral et al. 2008). Recently, the presence of *Fucus spiralis* and
327 sorbic acid in a biodegradable film led to lower pH values during megrim chilled
328 storage (García-Soto et al. 2015).

329 A marked TMA formation ($p<0.05$) was obtained for all kinds of samples
330 throughout chilled storage (r^2 = 0.90-0.93, quadratic fitting) (Table 3), according to the
331 increasing microbial activity. Values obtained at the end of storage period (115-137 mg
332 kg^{-1}) were in agreement with previous research concerning the refrigerated storage of
333 megrim (Aubourg et al. 2006; Sanjuás-Rey et al. 2012). Results obtained showed that
334 the presence of the alga extract in the ice led to a remarkable inhibitory effect on TMA
335 formation in megrim muscle. Thus, fish specimens corresponding to the B-2 batch
336 showed lower TMA mean values than their counterpart control samples for the 7-14-
337 day period; this difference was significant ($p<0.05$) at days 7 (B-1 batch) and 14 (B-2
338 batch).

339 TMA is one of the most commonly used quality indicators to assess microbial
340 activity in marine species kept under refrigeration conditions. In agreement with the
341 results obtained for the above-mentioned microbial parameters, an inhibitory effect on
342 TMA formation has been obtained as a result of the alga presence in the ice.
343 Accordingly, TMA values showed good correlation values throughout the present study
344 with aerobes (r^2 = 0.73-0.78), psychrotrophs (r^2 = 0.76-0.90), lipolytic bacteria (r^2 =
345 0.75-0.80) and proteolytic bacteria (r^2 = 0.74-0.79) counts as well as with pH value (r^2 =
346 0.88-0.92).

347 Significantly ($p<0.05$) progressive FFA formation (g kg^{-1} lipids; Table 3) was
348 observed in all kinds of samples throughout chilled storage (r^2 = 0.86-0.93, quadratic
349 fitting). An inhibitory effect derived from the presence of the alga in the icing medium
350 could be concluded on the 11-14-day period. At these times, lower mean FFA values
351 were determined in samples corresponding to B-1 and B-2 batches as compared with
352 control batch. Differences were found to be significant ($p<0.05$) at day 11 (B-2 batch)
353 and at day 14 (B-1 and B-2 batches). Similar conclusions were obtained when FFA

354 content was considered on muscle basis (mg kg^{-1} muscle), this being calculated taking
355 into account the lipid content of fish samples (4.4-5.0 g lipid kg^{-1} muscle, data not
356 shown), On this basis, FFA formation also showed a good correlation value with storage
357 time in all cases (r^2 = 0.85-0.93, quadratic fitting).

358 FFA formation during chilled storage has been reported to be the result of both
359 endogenous enzyme activity and microbial activity (Ashie et al. 1996; Campos et al.
360 2012). Before the end of the microbial lag phase (up to 6-9 days, depending on several
361 factors), FFA formation is mostly a result of endogenous enzyme (namely, lipases and
362 phospholipases) activity. Later on, microbial activity gains importance, so that FFA
363 formation is mainly derived from bacterial catabolic processes. The results obtained in
364 the present study can be explained on the basis of these two mechanisms and periods.
365 During the 0-7-day period, little differences were found so that a definite effect of the
366 presence of *B. bifurcata* in the icing medium could not be concluded. On contrast, later
367 on, when the microbial activity was more intense, the inhibitory effect of the alga was
368 found to be significant. In agreement with this, FFA values determined in the present
369 study showed good correlation values with some indices related to microbial activity,
370 such as psychrotrophs (r^2= 0.78-0.83), pH value (r^2= 0.89-0.94) and TMA-N content
371 (r^2= 0.92-0.93).

372 Peroxide content (Table 4) showed a marked increasing tendency in the 0-11-
373 day period, which was especially relevant in samples corresponding to the B-2 batch.
374 This was followed by a sharp decrease ($p<0.05$) at the end of the chilled storage. Higher
375 mean values were obtained for the B-2 batch when compared with the control batch.
376 Differences were found significant ($p<0.05$) for the 4-11-day period.

377 No significant formation ($p>0.05$) of secondary lipid oxidation compounds (i.e.,
378 TBARS) could be concluded in all batches for the 0-11-day period (Table 4). At the end

379 of the storage time, and in agreement with the peroxide content decrease, a significant
380 ($p<0.05$) formation of TBARS could be depicted in all batches. No significant effect
381 ($p>0.05$) of *B. bifurcata* extract in the icing medium on secondary oxidation compounds
382 could thus be concluded.

383 A low formation ($p>0.05$) of fluorescent compounds was observed in general
384 terms for the 0-7-day period (Table 4). On contrast, a marked increase ($p<0.05$) was
385 determined at day 11, this being in agreement with the important peroxide formation
386 also determined at this time. Finally, FR increased ($p<0.05$) only in megrim specimens
387 corresponding to B-1 batch. Concerning the effect of *B. bifurcata* extracts in the icing
388 media, an inhibitory effect on fluorescent compound formation is concluded in the 11-
389 14-day period for the B-2 batch. In addition, samples corresponding to the B-1 batches
390 also exhibited an inhibitory effect at day 11.

391 Lipid oxidation has been recognized as a complex process where different kinds
392 of molecules are produced, most of them unstable, susceptible to breakdown and to
393 originate low-molecular weight compounds, or to react with other molecules
394 (nucleophilic-type, mostly) present in the fish muscle. As a result of this, determination
395 of each kind of compound cannot always provide an accurate method for the quality
396 loss assessment in fish. In the present study, primary lipid oxidation compounds
397 (peroxides assessment) did not provide a satisfactory correlation with storage time and,
398 accordingly, cannot be considered accurate tools to follow up the lipid oxidation
399 development throughout the whole present experiment. The electrophilic nature of such
400 compounds led them to breakdown or to interact with food constituents possessing
401 nucleophilic functions (Aubourg et al. 2006; Campos et al. 2012). Contrary, TBA-i and
402 FR assessments led to fair correlation values with chilling time (r^2= 0.75-0.89, quadratic
403 fitting, and r^2= 0.78-0.92, respectively). Additionally, FR values showed fair correlation

404 values with aerobes (r^2= 0.82-0.85), lipolytic bacteria (r^2= 0.82-0.89) and proteolytic
405 bacteria (r^2= 0.81-0.86) counts. On contrast, the best correlation scores for TBARS
406 values were obtained with pH (r^2= 0.74-0.86) and TMA-N (r^2= 0.74-0.89).

407 As photosynthetic organisms, algae are exposed to a combination of light and
408 high oxygen concentration. The lack of structural damage in their organs has led to
409 consider that their protection against oxidation would arise from their natural content on
410 antioxidant substances (Frankel and Meyer 2000). The inhibitory effect of ethanolic
411 extracts of *B. bifurcata* on the formation of tertiary lipid oxidation compounds (namely,
412 FR value) can be explained on the basis of the high level of polyphenol compounds
413 (40.8±8.3 GA g^{-1} lyophilized alga) and previous related research. Thus, Glombitza et al.
414 (1976) isolated different polyhydroxyphenyls und phenylethers from *B. bifurcata* and
415 established their structure by NMR and IR spectroscopy. Later on, other authors
416 reported the antioxidant effect of aqueous methanolic extracts (Connan et al. 2007; Le
417 Lann et al. 2008) and of dichloromethane/methanol extracts (Zubia et al. 2009) of alga
418 *B. bifurcata* on the basis of different *in vitro* assays (DPPH, reducing activity and beta-
419 carotene methods). In such studies, a marked correlation between phenol content (0.96-
420 4.00% dry weight) and antioxidant activity was proved. In a related alga, *F. spiralis*, a
421 preliminary identification of active compounds was carried out by means of quadrupole
422 time-of-flight mass spectrometry (Q-TOF-MS) (Tierney et al. 2013). The results
423 supported the assumption that pholorotannins were present and were probably involved
424 in the antioxidant activity. More recently, Ortiz et al. (2014) showed that the inclusion
425 of different kinds of algae (*Durvillaea antarctica, Ulva lactuca*, *Pyropia columbina*,
426 *Macrocystis piryfera and Gracilaria chilensis*) in the covering medium during salmon
427 canning led to a remarkable rancidity stabilization. Recently, García-Soto et al. (2015)

showed that *F. spiralis* and sorbic acid, when included in a biodegradable film for megrim chilled storage, led to a significant inhibitory effect on lipid oxidation.

CONCLUSIONS

The presence of *B. bifurcata* extracts in the icing media employed for the chilled storage of megrim led to a significant ($p<0.05$) inhibition of microbial activity (aerobes, psychrotrophs, lipolytic bacteria, proteolytic bacteria and Enterobacteriaceae), as well as of pH, trimethylamine formation and lipid damage mechanisms (formation of free fatty acids and tertiary oxidation compounds). These inhibitory effects proved to be more intense in fish specimens corresponding to B-2 batch. According to the strong inhibitory effect on the microbial activity, this effect is considered the main reason that would explain an extended shelf life in the batches treated with the alga, as compared with the control batch.

Most studies on the preservative (namely, antioxidant and antimicrobial) activity of algae have been performed *in vitro*; however, the practical data derived from studies with commercial foods are still scarce. In this sense, the icing medium proposed in this work may constitute a promising strategy in order to open the way to the application of natural algae extracts for fish storage, to enhance the retention of quality during storage and commercialization.

449 **ACKNOWLEDGMENTS**

450 The authors thank Mr. Marcos Trigo and Ms. Monserrat López for their excellent
451 technical assistance. Porto-Muiños (Cerceda, A Coruña, Spain) is greatly acknowledged
452 for kindly providing the lyophilized alga. This work was supported by the Consejo
453 Superior de Investigaciones Científicas (CSIC; Spain) through the project PIE
454 201370E001.

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TABLE 1

Microbiological count assessment (aerobic mesophiles, psychrotrophs and lipolytics; log CFU g^{-1})* in chilled megrim muscle stored under different icing conditions**

Chilling time (days)	Aerobic mesophiles			Psychrotrophs			Lipolytics		
	B-0	**B-1**	**B-2**	**B-0**	**B-1**	**B-2**	**B-0**	**B-1**	**B-2**
0		3.04 (0.13)			4.62 (0.44)			2.00 (0.00)	
4	3.82 a (0.59)	3.66 a (0.68)	3.15 a (0.48)	5.06 a (0.74)	4.85 a (0.61)	4.23 a (0.41)	3.73 a (0.44)	3.63 a (0.22)	3.28 a (0.35)
7	5.51 b (0.33)	4.86 ab (0.65)	4.09 a (0.76)	6.34 b (0.67)	5.46 b (0.17)	4.97 a (0.21)	4.31 b (0.49)	4.17 b (0.32)	3.39 a (2.1)
11	6.31 b (0.54)	6.14 b (0.08)	4.92 a (0.89)	7.26 b (0.20)	5.89 a (0.29)	5.95 a (3.0)	5.93 b (0.15)	4.87 a (0.14)	4.89 a (0.19)
14	6.38 b (0.33)	6.04 b (0.14)	5.15 a (0.44)	7.25 a (0.43)	6.98 a (0.12)	6.46 a (0.37)	6.35 b (0.27)	5.45 a (0.29)	5.24 a (0.39)

* Mean values of three replicates (n = 3); standard deviations are indicated in brackets. For each parameter and for each chilling time, mean values followed by different letters (a, b) indicate significant ($p<0.05$) differences as a result of the icing condition.

** Icing conditions: B-0 (Control; no seaweed extract presence in ice), B-1 (ice prepared from a 0.67 g L^{-1} aqueous solution of *B. bifurcata* extract) and B-2 (ice prepared from a 2.50 g L^{-1} aqueous solution of *B. bifurcata* extract).

TABLE 2

Microbiological count assessment (Enterobacteriaceae and proteolytics; log CFU g^{-1})* in chilled megrim muscle stored under different icing conditions**

Chilling time (days)	**Enterobacteriaceae**			**Proteolytics**		
	B-0	**B-1**	**B-2**	**B-0**	**B-1**	**B-2**
0		< 1.00			3.01 (0.19)	
4	< 1.00 a	1.10 a (0.70)	< 1.00 a	2.95 a (0.43)	3.03 a (0.13)	2.77 a (0.21)
7	< 1.00 a	1.10 a (0.17)	< 1.00 a	4.71 b (0.36)	4.27 ab (0.25)	3.93 a (0.24)
11	1.49 a (0.61)	1.53 a (0.47)	< 1.00 a	5.98 a (0.29)	5.46 a (0.17)	5.30 a (0.78)
14	1.81 b (0.48)	1.10 a (0.17)	< 1.00 a	5.87 b (0.23)	5.28 a (0.19)	5.34 ab (0.29)

* Mean values of three replicates (n = 3); standard deviations are indicated in brackets. For each parameter and for each chilling time, mean values followed by different letters (a, b) indicate significant ($p<0.05$) differences as a result of the icing condition.

** Abbreviations of icing conditions as expressed in Table 1.

TABLE 3

Assessment of pH, trimethylamine-nitrogen (TMA-N) and free fatty acid (FFA) values* in chilled megrim muscle stored under different icing conditions**

Chilling time (days)	pH			TMA-N (mg kg^{-1} muscle)			FFA (g kg^{-1} lipids)		
	B-0	B-1	B-2	B-0	B-1	B-2	B-0	B-1	B-2
0		6.53 (0.06)			0.7 (0.1)			16.1 (4.1)	
4	6.51 a (0.15)	6.39 a (0.10)	6.48 a (0.01)	0.9 a (0.5)	0.6 a (0.2)	1.1 a (0.3)	13.4 a (1.9)	14.0 a (1.8)	14.1 a (1.2)
7	6.73 a (0.14)	6.55 a (0.06)	6.58 a (0.04)	9.9 b (3.7)	2.9 a (1.5)	7.3 ab (2.5)	17.1 ab (3.2)	13.3 a (1.3)	19.1 b (2.1)
11	6.97 c (0.09)	6.79 b (0.05)	6.51 a (0.05)	64.7 b (19.9)	72.6 b (21.4)	35.4 a (3.0)	45.9 b (12.3)	34.5 b (4.8)	20.2 a (6.7)
14	7.29 b (0.13)	7.05 ab (0.12)	6.94 a (0.10)	136.5 a (26.7)	133.7 a (5.3)	115.9 a (13.7)	62.3 b (3.3)	38.7 a (9.0)	49.3 a (9.6)

* Mean values of three replicates (n = 3); standard deviations are indicated in brackets. For each parameter and for each chilling time, mean values followed by different letters (a, b, c) indicate significant ($p<0.05$) differences as a result of the icing condition.

** Abbreviations of icing conditions as expressed in Table 1.

TABLE 4

Assessment of lipid oxidation development* in chilled megrim muscle stored under different icing conditions**

Chilling time (days)	**Peroxide value (meq active oxygen kg^{-1} lipids)**			**Thiobarbituric acid index (mg malondialdehyde kg^{-1} muscle)**			**Fluorescence ratio**		
	B-0	**B-1**	**B-2**	**B-0**	**B-1**	**B-2**	**B-0**	**B-1**	**B-2**
0		0.28 (0.01)			0.06 (0.03)			1.66 (0.42)	
4	0.80 a (0.55)	1.24 ab (0.88)	4.22 b (2.04)	0.13 a (0.03)	0.09 a (0.03)	0.09 a (0.03)	2.19 a (0.41)	2.53 a (0.27)	2.17 a (0.25)
7	1.63 a (0.42)	1.52 a (0.57)	6.21 b (2.69)	0.15 a (0.07)	0.12 a (0.05)	0.12 a (0.07)	2.38 a (0.60)	2.60 a (0.31)	2.64 a (0.56)
11	3.10 a (0.66)	4.40 a (1.28)	15.91 b (2.34)	0.11 a (0.03)	0.13 a (0.01)	0.12 a (0.02)	5.38 b (0.21)	3.47 a (0.22)	3.81 a (0.56)
14	2.27 a (0.38)	2.10 a (0.55)	4.65 a (2.03)	0.24 a (0.01)	0.32 a (0.05)	0.21 a (0.04)	4.61 b (0.70)	4.52 b (0.27)	2.89 a (0.75)

* Mean values of three replicates (n = 3); standard deviations are indicated in brackets. For each parameter and for each chilling time, mean values followed by different letters (a, b) indicate significant ($p<0.05$) differences as a result of the icing condition.

** Abbreviations of icing conditions as expressed in Table 1.