# Predictive models for bacterial growth in sea bass (*Dicentrarchus labrax*) stored on ice

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## Abstract

The purpose of this paper was to estimate microbial growth through predictive modelling as a key element in determining the quantitative microbiological contamination of sea bass stored on ice and cultivated in different seasons of the year. In the present study, two different statistical models were used to analyse changes in microbial growth in whole, ungutted sea bass (*Dicentrarchus labrax*) stored on ice. The total counts of aerobic mesophilic and psychrotrophic bacteria, *Pseudomonas* sp., *Aeromonas* sp., *Shewanella putrefaciens,* Enterobacteriaceae, sulphide-reducing *Clostridium* and *Photobacterium phosphoreum* were determined in muscle, skin and gills over an 18-day period using traditional methods and evaluating the seasonal effect. The results showed that specific spoilage bacteria (SSB) were dominant in all tissues analysed but were mainly found in the gills. Predictive modelling showed a seasonal effect among the fish analysed. The application of these models can contribute to the improvement of food safety control by improving knowledge of the microorganisms responsible for the spoilage and deterioration of sea bass.


**Keywords**: Sea bass; Microbiology; Statistics; Microorganisms; Predictive modelling.

## INTRODUCTION

Sea bass is a high-quality, delicate and expensive fish. This fish has white flesh, a mild flavour and low fat content and has become very popular in the European aquaculture market (Cakli *et al*., 2006b). The demand for high-quality fresh fish stored on ice in the European market has increased in recent years, but strong competition among producing countries in the Mediterranean area (Spain, Greece, Italy and Turkey) and the

subsequent lowering of market prices have required the differentiation and characterisation of fish produced in aquaculture (Cakli *et al*., 2007).

The Spanish aquaculture market produced 42.675 tons in 2011, a 2.8% (1.213 tons) reduction from 2010 (APROMAR, 2012). The main fish species produced through aquaculture is the sea bream (*Sparus aurata*), at 16.930 tons, 16.8% less than in 2010, followed by the sea bass (*Dicentrarchus labrax*), at 14.367 tons, 15.0% more than in 2010. The Canary Islands are the third most productive sea bass and sea bream producer in Spain, responsible for 24.2% and 19.2% of the total production of these species, respectively, in 2011 (APROMAR, 2012), primarily due to the ideal local marine conditions, including the temperature, salinity, nutrients, currents and the morphology and nature of the sea beds (Pérez-Sánchez & Moreno-Batet, 1991). It is widely known that the physicochemical parameters of the water column are related to bacterial population growth and, as a result, to the microbial load of fish, but no previous study has examined this relationship or determined the exact parameters (oxygen, conductivity and temperature) that affect bacterial populations, nor have such relations been effectively modelled (Iliopoulou-Georgudaki, *et al*., 2009).

The climate of the Canary Islands is subtropical due to the cold Canary Current that crosses the islands from north to south. The water surface temperature in these islands ranges from 18 ºC in the winter to 22-23 ºC in the summer, an important factor to consider when studying the microbiology of farmed fish. However, thus far, no studies have evaluated the effects of the manipulation, distribution and storage on the microbiology of sea bass harvested in the Canary Islands.

Microbial contamination and growth can result in undesirable changes in appearance, texture, flavour and odour that reduce food quality (Sperber & Doyle,

2009). Ice storage has been widely used to prolong shelf life, particularly during transportation both for domestic consumption and export to neighbouring countries (Kostaki *et al*., 2009). Preservation on ice is one of the most efficient ways of retarding spoilage (Özyurt *et al*., 2009). The shelf life of sea bass stored on ice is 6–8 days (Paleologos *et al.*, 2004). Thus, limited shelf life, primarily due to microbial spoilage, is a limiting factor for the distribution and sale of such perishable foods (Kostaki *et al*., 2009). The growing production of this species of fish has increased the importance of maintaining its quality during storage. Fish quality declines due to a complex process involving physical, chemical and microbiological forms of deterioration. Enzymatic and chemical reactions are normally responsible for the initial loss of freshness, whereas microbial activity accounts for obvious spoilage and thereby establishes the product's shelf life (Guillén-Velasco *et al*., 2004; de Koning *et al*., 2004). Many factors can influence the rate of the microbial spoilage of fish, such as the bacterial flora present, storage conditions, handling and temperature (Ward & Baj, 1988), and specific spoilage organisms grow faster than other sea bass microflora (Limbo *et al*., 2009). Some bacterial groups are particularly associated with this spoilage. Fish caught in cold marine waters and stored on ice under aerobic conditions generally spoil because of contamination by *Shewanella putrefaciens*, *Pseudomonas* sp. (Gram & Huss, 1996) and representatives of the family Vibrionaceae (Huss *et al.*, 1995) as well as Enterobacteriaceae, lactic acid bacteria and yeasts (Koutsoumanis & Nychas, 2000). Marine fish from temperate waters stored in a modified atmosphere are spoiled by $CO_2$-resistant *Photobacterium phosphoreum*, whereas Gram-positive bacteria are likely responsible for the spoilage of fish from fresh or tropical waters packed in $CO_2$ (Gram & Huss, 1996).

However, the determination of microbial growth and fish shelf life with traditional microbiological challenge tests is expensive and time-consuming (Bruckner *et al*., 2013). One alternative is the concept of predictive microbiology, which uses mathematical models to predict microbial growth and, thus, to estimate shelf life (McMeekin *et al.*, 1993; Whiting, 1995). Predictive microbiology involves the development of mathematical models to describe the effect of the most important environmental factors on the biology of microorganisms in foods (Ross *et al.*, 2000). The majority of these models are based on experimental data derived from laboratory media, and there is limited information available regarding the assessment of individual species variability in foods (François *et al.*, 2006; Manios *et al.*, 2011). The models have immediate practical application to improve microbial food safety and quality and can also provide quantitative data regarding the microbial ecology of foods (Ross *et al.*, 2000). The implementation of these predictive models contributes to the improved control of food safety and spoilage by quantifying the effect of storage and distribution on microbial proliferation via the Hazard Analysis and Critical Control Points (HACCP) system (Van Impe *et al.*, 2013).

Therefore, the objective of this study was to develop predictive shelf life models that were adequate for use in farmed sea bass harvested in each season and stored on ice based on the growth of eight microorganisms in different tissues.

## MATERIALS AND METHODS

Sea bass with an initial average weight of 430 g (380 g – 590 g) were obtained from an aquaculture farm located in Gran Canaria (Canary Islands, Spain; Atlantic Ocean, 27° 57′ 31″ N, 15° 35′ 33″ W). The fish were cultivated in tanks, and four

samplings were carried out randomly, with the first in winter (January), when the average water temperature for the month was 19.60 ºC (batch 1); the second in spring (April), at 19.30 ºC (batch 2); the third in summer (July), at 22.5 ºC (batch 3); and the fourth in autumn (November), at 21.30 ºC (batch 4). Fourteen fish per batch were sacrificed by immersion in ice water (hypothermia), delivered to the laboratory within 1 h of harvesting and packed in polystyrene boxes with ice.

On the day of slaughter (day 0 of the study), one whole, ungutted fish was analysed, and the other fish were kept in ice in polystyrene boxes with drainage holes. The ice was produced under hygienic conditions in an ice machine (ITV model IQ 135) and replenished when necessary. The fish were kept in a refrigerator with a controlled temperature of 2 ± 1 ºC. Microbiological analyses were performed on days 2, 4, 7, 10, 14 and 18 using a new fish in each analysis. Each sample was analysed in duplicate, and the results are the mean of the two determinations.

Seawater samples were collected over four months, with one sample per week from the principal tank. In every unit, temperature, pH, salinity, total dissolved solids and biological oxygen demand ($BOD_5$) (APHA, 1992) were recorded using a Horiba U 22XD (Kyoto, Japan).

**Sample Preparation and Microbiological Analysis**

Sea bass skin and flesh (25 g) were obtained from the dorsal anterior region of the right side of each fish using the technique described by Slattery (1988). The samples were transferred to a Stomacher bag (Seward Medical, London, UK) containing 225 ml of 0.1% peptone water (Cultimed 413795) with salt (0.85% NaCl w/v) (Drosinos & Nychas, 1996) and homogenised for 60 seconds using a Stomacher Lab Blender 400 at high

speed (Stomacher, IUL Instrument, Barcelona, Spain). From these microbiological extracts, nine decimal dilutions were prepared.

In addition to the skin and flesh, the gills were also analysed and weighed (9.3 g/fish), and the resulting value was multiplied by nine to obtain the millilitres of the first serial dilution (Pascual & Calderón, 2002). The total viable counts (TVC) of mesophilic and psychrotrophic bacteria were determined using Plate Count Agar (PCA Cultimed, 413799) (technique proposed by ISO 17410, 2001, for psychrotrophic bacteria) incubated at 31 ºC for 72 h (Pascual & Calderón, 2002; ISO 4833:2003; Álvarez, *et al.*, 2008; Corbo *et al.*, 2008; Calanche *et al.*, 2013; Genç *et al.*, 2013) and 6.5 ºC for 7-10 days, respectively (Broekaert, 2011). *Pseudomonas* sp. were counted on Pseudomonas F agar (Cultimed, 413796) incubated at 31 ºC for 48 h; cream-coloured, fluorescent or greenish colonies were counted. *Aeromonas* sp. were counted on BD *Yersinia Aeromonas* agar (BD, PA-25405605) after incubation at 31 ºC for 48 h; pale colonies with a rose to red centre that were oxidase positive were counted.

The amount of *Shewanella putrefaciens* ($H_2S$-producing bacteria) was determined on Iron Agar Lyngby (IAL, prepared following instructions and using ingredients provided by OXOID CM964). Iron agar plates were incubated at 20 ºC for 48-72 h, and the black colonies formed by the production of $H_2S$ were counted (Dalgaard, 1995).

Enterobacteriaceae were counted using Violet Red Bile Glucose Agar (VRBG) (Cultimed, 413745). Plates were incubated at 37 °C for 24 h, and these bacteria appeared as large colonies with purple haloes (Pascual & Calderón, 2002).

*Photobacterium phosphoreum* were counted on Iron Agar Lyngby. A subsample of 0.1 ml was spread on a dry surface and incubated at 5 ºC for 14 days. These colonies appeared in the plates as transparent drops of dew (Dalgaard, 1995).

For the sulphide-reducing *Clostridium* (clostridia), spores and vegetative cells were counted on S.P.S. (Cultimed, 414125) and incubated at 46 ºC for 24-48 h. The black colonies observed in the tubes were multiplied by a dilution factor to obtain the number of CFU/g (Pascual & Calderón, 2002).

Counts were performed in duplicate and examined visually for the typical colony types and morphology characteristics associated with each growth medium. The data are reported as colony forming units (log CFU/g). Conventional biochemical tests were carried out to determine the final identification, and the strains were identified according to Barrow and Feltham (1993) (Table 1) and Smith Svanevik and Tore Lunestad (2011).

**Statistical Analysis**

For each bacterial species, $N_{i,j,k,t}$ indicates the count (CFU/g) corresponding to the *i*th fish, *j*th tissue (muscle, gill, skin) and *k*th batch (seasons) on observation day *t*. To determine a pattern of bacterial growth across the observation days, the $\log_{10} N_{i,j,k,t}$ was plotted against the day by tissue (Figure 1) and batch (Figure 2). The plots for mesophilic and psychrotrophic microorganisms*, Pseudomonas* sp., *S. putrefaciens, Aeromonas* sp., and Enterobacteriaceae suggested that the following linear mixed-effects model should be used (Laird & Ware, 1982):

(Model 1) $$\log_{10} N_{i,j,k,t} = \theta + fish_i + \alpha_j + \lambda_k + P_r(t) + e_{i,j,k,t}$$

where $fish_i$ indicates the random effect of the fish ($i = 1....56$), $\alpha_j$ is the fixed tissue effect ($j = muscle, gill, skin$), $\lambda_k$ is the batch effect ($k = 1,2,3,4$), $P_r(t)$ is a polynomial of degree *r* (trend) and $e_{i,j,k,t}$ is the random error. The following formula was used: $\alpha_{muscle} = \lambda_1 = 0$ (muscle and batch 1 were the reference categories). It was assumed that the random effects $fish_i$ were independent random variables distributed according to $N(0, \sigma_f)$ and that the error $e_{i,j,k,t}$ were independent random variables distributed according to $N(0, \sigma_e)$ and independent from the random effects $fish_i$. To determine the optimum degree for the polynomial $P_r(t)$ (trend), we considered the degrees *r* = 1,2,3, and the degree that optimised the Akaike criterion (AIC) was selected. For all the bacteria, the optimum degree obtained was *r* = 2, and thus, $P_2(t) = \beta_1 \cdot t + \beta_2 \cdot t^2$ (the intercept is subsumed in $\theta$). The goodness of fit of the model was evaluated by the coefficient $R^2$, which measures the proportion of the variability corresponding to the fixed effects of the model.

To account for excess zeros for the clostridia and *P. phosphoreum* (44.6% for *Clostridium* and 46.4% for *P. phosphoreum*), the zero-inflated Poisson (ZIP) model was employed as follows (Hall, 2000):

(Model 2)
$$\Pr\left(N_{i,j,k,t} = r \,\middle|\, \mathbf{x}_{j,k,t}\right) = \begin{cases} \pi_t + (1-\pi_t)\exp\left(-\mu_{j,k,t}\right) & ; \quad r = 0 \\ (1-\pi_t)\dfrac{\exp\left(-\mu_{j,k,t}\right)\mu_{j,k,t}^{r}}{r!} & ; \quad r > 0 \end{cases}$$

This model consists of a combination of two distributions to incorporate excess

zeros, where $\ln \mu_{j,k,t} = \theta + \alpha_j + \lambda_k + \beta \cdot t$ and $\pi_t$ is the probability of a measurement being an excess zero. The logistic model $\text{logit}\,\pi_t = \gamma + \eta \cdot t$ (the zeros only depended on day *t*) was considered. Here, the vector $\mathbf{x}_{j,k,t}$ summarises the covariate tissue (*j*), batch (*k*) and day (*t*). Note that the effects of tissue, batch and day are expressed with the same parameters for both models. All of these regression models were fit to our data using the R packages nlme (LME procedure) and PSCL (Zeroinfl procedure).

## RESULTS

### Descriptive Microbiological Analysis

The data on the microbial flora (log CFU/g) of aquacultured, ungutted sea bass stored aerobically on ice at 2 ± 1 ºC are shown by tissue in Tables 2, 3 and 4. The results are expressed as the averages of the four batches sampled.

Total viable counts (TVCs) increased gradually throughout the storage period from day 0, except in the case of *Clostridium* sp. and *P. phosphoreum*, which began to grow from day 7. The TVCs for mesophilic and psychrotrophic bacteria on the initial day (day 0) were 1.89 and 1.07 in muscle, 4.60 and 3.24 log CFU/g in skin and 4.98 and 3.87 log CFU/g in gills, respectively.

Mesophilic and psychrotrophic bacterial counts reached 7 log CFU/g on days 10 and 14 in muscle, days 4 and 7 in skin and days 2 and 4 in gills, respectively. This value is considered to be the maximum acceptable limit for freshwater and marine species as defined by the ICMSF (1986).

252 Furthermore, the mesophilic counts observed in fish in this study after 18 days
253 were higher than the psychrotrophic counts (9.07 and 7.56 log CFU/g in muscle, 11.51
254 and 10.41 log CFU/g in skin and 12.11 and 11.00 log CFU/g in gills, respectively).

255 The initial counts of SSB (specific spoilage bacteria), including *Pseudomonas* sp.,
256 *S. putrefaciens* ($H_2S$-producing bacteria) and *Aeromonas* sp., were 1.65, 1.50 and below
257 the detection threshold (<1 log CFU/g), in muscle; 4.27, 2.46 and 3.28 log CFU/g in skin;
258 and 4.56, 3.18 and 4.17 log CFU/g in gills, respectively.

259 In general, *Pseudomonas* sp. was the dominant population on day 18 of storage,
260 followed by *Aeromonas* sp. and *S. putrefaciens*, with values of 8.64, 7.95 and 8.43 log
261 CFU/g in muscle; 10.88, 8.86 and 8.31 log CFU/g in skin; and 11.21, 10.98 and 8.92 log
262 CFU/g in gills, respectively.

263 The initial Enterobacteriaceae counts were 0.27 log CFU/g in muscle, 2.28 log
264 CFU/g in skin and 2.57 log CFU/g in gills, increasing to 5.42, 6.35 and 6.87 log
265 CFU/g, respectively, after 18 days of ice storage. A low initial population was found in
266 muscle, indicating good hygiene ($<10^2$ CFU/g) in the marine environment where the
267 fish were caught as well as appropriate fishing practices and handling (Kostaki *et al*.,
268 2009).

269 No *Clostridium* sp. or *P. phosphoreum* were detected in all of the tissues analysed
270 at the first sampling. However, these counts increased over 18 days of iced storage to
271 3.33 and 3.80 log CFU/g in muscle, 2.99 and 3.97 log CFU/g in skin and 3.00 and 4.39
272 log CFU/g in gills, respectively. The counts and growth trend of these bacteria were
273 different from the others examined in the present study.

274 Table 7 shows the physicochemical water values, which were constant over the
275 four studied seasons.

## Models for the Analysis of Microbiological Growth

### Model 1

For all bacteria represented by model 1, the optimal polynomial trend obtained was the quadratic with all the $\beta_2$ coefficients negative, which means that the growth exhibited a linear deceleration. Table 5 shows the $R^2$ coefficients, all comparisons among tissues and batches and the $\beta_1$ and $\beta_2$ coefficients corresponding to the quadratic trends.

Figures 1 and 2 show the expected growth values of $\log_{10} N_{i,j,k,t}$ mesophilic aerobic bacteria, psychrotrophic bacteria, *Aeromonas* sp., *Pseudomonas* sp., Enterobacteriaceae and *S. Putrefaciens* plotted against each observation day according to tissue and batch, respectively.

The linear trend for all log counts over time justified the use of this model (1). For these microorganisms, the spoilage levels in gills were higher than in skin and muscle ($P < 0.001$), with muscle the least contaminated tissue.

There was statistical significance between the bacterial counts (except in the case of Enterobacteriaceae) in each of the batches analysed ($\lambda_2 - \lambda_1$, $\lambda_3 - \lambda_1$ and $\lambda_4 - \lambda_1$), with batch 1 (winter) the least contaminated in each case, with different results for each batch depending on the microorganism studied.

### Model 2

Figure 3 shows the counts $N_{i,j,k,t}$ of sulphide-reducing *Clostridium* and *P. phosphoreum* over time according to tissue and per batch. Note that the excess of zeros for both counts suggested the use of model 2. The estimated values $\mu_{j,k,t}$ (expected growth) corresponding to this model and the estimated probabilities of zeros $\pi_t$ are shown in Table 6. The negative coefficients of the parameter $\eta$ indicated that the probability of zeros for sulphide-reducing *Clostridium* and *P*. *phosphoreum* decreased over the observation period.

The plot of these probabilities versus days (Figure 4) showed that the probability of zeros (no counts) decreased over time; from day 10, the probability of spoilage was near 1.

Table 6 shows that the contamination levels for *Clostridium sp.* and *P. phosphoreum* displayed significant differences ($P < 0.001$) among the three tissues sampled and were lower in skin and gills than in muscle.

Significant differences ($P < 0.001$) in the levels of these microorganisms were observed among the four batches studied. These results suggested that the irregular growth observed for these two microorganisms could be dependent on water temperature or other environmental factors.

## DISCUSSION

Previous authors have found similar results in the initial counts for mesophilic and psychrotrophic bacteria in muscle (Cakli *et al*., 2006b; Kostaki *et al*., 2009) or skin in ungutted sea bass stored on ice. However, other authors have reported higher results in

322 the initial TVC values in the muscle of ungutted sea bass (Taliadourou *et al*., 2003; Kilinc
323 *et al.*, 2007), sea bream fillets (Erkan & Ueretener, 2010) and ungutted sea bass
324 (Papadopoulos *et al*., 2003) or the skin of whole sea bream (Cakli *et al*., 2007; Erkan,
325 2007). The differences observed in TVCs in the different studies may be due to the
326 microbiological conditions of fish muscle in ungutted sea bream, which are directly
327 related to the fishing ground, sanitary conditions of the slaughterhouse and
328 environmental factors (Ward and Baj, 1988).

329 The bacteria in other studies reached counts of 7-9 log CFU/g after 15 days of
330 storage in sea bass muscle (Cakli *et al*., 2006a; Cakli *et al*., 2006b; Kilinc *et al*., 2007;
331 Cakli *et al*., 2007), 7 log CFU/g after 16 days in ungutted sea bass fillets (Papadopoulos
332 *et al*., 2003), 6.7 and 7.6 log CFU/g after 13 and 16 days in sea bream fillets, respectively
333 (Erkan & Ueretener, 2010), and 7 log CFU/g after 11 and 14 days in sea bream muscle
334 with skin, under different culture conditions (López-Caballero *et al*., 2002).

335 Similar results for mesophilic and psychrotrophic bacteria were reported in sea
336 bream skin, with counts of 7.20 and 7.35 log CFU/g, respectively, after 15 days (Cakli *et*
337 *al.*, 2007) and 6.6 and 6.8 log CFU/cm$^2$, respectively, after 13 days of storage (Erkan*,*
338 2007).

339 The slightly higher count of mesophilic microorganisms compared to
340 psychrotrophic microorganisms observed in the three tissues analysed in the present
341 study may have been due to the influence of the water temperature in the breeding tanks,
342 which ranged between 18 and 23 ºC. However, other studies with temperatures ranging
343 between 14 and 27 ºC found significant changes in the bacterial counts and greater
344 growth of psychrotrophic microorganisms (Grigorakis *et al.* 2003) resulting from the
345 greatly reduced intensive thermal shock when the fish were placed on ice (14 to 2 ºC),

resulting in a decreased lag phase and allowing spoilage to proceed more quickly (Ashie *et al.*, 1996; Ward & Bai, 1988). In our study, the microbial load of mesophilic microorganisms on day 0 of ice storage was higher than that of the psychrotrophic microorganisms (0.82 in muscle, 1.36 in skin and 1.11 in gills). This difference was consistently maintained over 18 days of storage and may have been due to an extended lag phase.

Our results are in agreement with the initial counts of *Pseudomonas* sp. for ungutted European hake stored on ice reported by Baixas-Nogueras *et al*. (2009). Other authors described higher initial counts of *Pseudomonas* sp. in muscle, with 3.0 log CFU/g for sea bass (Papadopoulos *et al*., 2003; Paleologos *et al*., 2004) and 3.9 log CFU/g for sea bream (Özden *et al*., 2007), as well as 3.3 log CFU/g in gutted sardine samples (Erkan & Özden, 2008) and 2.88 log CFU/g in horse mackerel (Tzikas *et al*., 2007). Initial *S. putrefaciens* counts accounted for a large proportion of the microflora in the muscle of several species such as sea bass, with values of 2.2 log CFU/g (Paleologos *et al*., 2004); sea bream, with values of 4.4 log CFU/g (Özden *et al*., 2007); sardines, at 3.3 CFU/g (Erkan & Özden, 2008); and sea bream skin, at 3.3 log CFU/g (Erkan, 2007). However, these values differed from those reported in our study, which were more closely aligned with those described by López-Caballero *et al*. (2002), Lougovois *et al*. (2003) and Baixas-Nogueras *et al*. (2009).

The final counts of *Pseudomonas* sp. and *S. putrefaciens* were similar to those reported by other authors in sea bass muscle, at 7-7.2 log CFU/g (*Pseudomonas* sp.) and 6.6 and 7 log CFU/g (*S. putrefaciens*) (Papadopoulos *et al*., 2003; Paleologos *et al*., 2004), and in sea bream, which ranged from 6-7.8 log CFU/g (López-Caballero *et al*., 2002; Lougovois *et al*. 2003; Özden *et al*., 2007); sardines, with values of 4 and 4.9 log

370 CFU/g, respectively, after nine days of storage (Erkan & Özden, 2008); horse mackerel,
371 with values of 6.42 and 5.12 log CFU/g, respectively, after 12 days of storage on ice
372 (Tzikas *et al*., 2007); and sea bream skin, with values of 6.7 log CFU/g after 13 days of
373 storage on ice for $H_2S$-producing bacteria (Erkan, 2007).

374 Similar counts for *Pseudomonas* sp. and *S. putrefaciens* and SSB have been
375 reported in fish from temperate and tropical waters (Gillespie, 1981; Lima dos Santos,
376 1981; Gram & Huss, 1996) and in fresh Mediterranean fish stored aerobically under
377 refrigeration (Koutsoumanis & Nychas, 1999) or on ice (Gennari & Tomaselli, 1988;
378 Gennari *et al*., 1999; Sant'Ana *et al*., 2011). The counts of *S. putrefaciens* reported in the
379 present study were lower than those observed for *Pseudomonas* sp. at the end of the
380 storage period, possibly because *Pseudomonas* sp. and *S. putrefaciens* have specific
381 iron-chelating systems (siderophores), and when these are co-cultured on fish samples,
382 the siderophore-producing *Pseudomonas* sp. inhibits the growth of *S. putrefaciens* (Gram
383 & Dalgaard, 2002; Olafsdóttir *et al*., 2006).

384 In the present study, the initial Enterobacteriaceae counts were lower than
385 those of SSB at the end of the storage period, in agreement with the results reported
386 for different fresh Mediterranean fish at the end of their shelf lives (Gennari &
387 Tomaselli, 1988; Gennari *et al*., 1999; Koutsoumanis & Nychas, 1999; Tejada &
388 Huidobro, 2002). Initial Enterobacteriaceae counts in fresh fish muscle were similar
389 to those reported for ungutted European hake (Baixas-Nogueras *et al*., 2009).
390 However, other authors have described higher initial counts, although the same
391 authors have reported similar values to those described here in different species after
392 a period of storage on ice, such as sea bass, with counts of 2 and 4.2 log CFU/g
393 (initial and final counts, respectively) (Papadopoulos *et al*., 2003); sea bream, with

394 counts of 3.9 and 5.6 log CFU/g (initial and final counts, respectively) (Özden *et al*.,
395 2007); and sardines, with counts of 3.5 and 5.08 log CFU/g (initial and final counts,
396 respectively) (Erkan & Özden, 2008). The contribution of Enterobacteriaceae to the
397 microflora of fish and its spoilage potential must be taken into consideration,
398 especially in the case of polluted water or as a result of a delay in chilling after the
399 catch (Chouliara *et al*., 2004), as well as in the filleting process (Moini *et al*., 2009).
400 Although this group of bacteria can grow at low temperatures, their abundance
401 decreases during storage on ice, possibly because their growth rate is lower than
402 that of other Gram-negative psychrotrophic spoilers, making them poor competitors
403 (Bahmani *et al*., 2011).

404 The counts of *P. phosphoreum* were similar to those found in bogue fish stored
405 aerobically, whereas the contribution of *P. phosphoreum* was very small and relatively
406 unimportant (Koutsoumanis and Nychas, 1999).

407

408 **Model 1**

409

410 A batch effect was observed, likely due to the different water temperatures in the
411 tanks where the sea bass were cultivated in all seasons. These results agree with those
412 published by Iliopoulou-Georgudaki *et al*. (2009), who observed that temperature,
413 dissolved oxygen and conductivity had a significant influence on microbial populations in
414 aquacultured fish. Other authors (Grigorakis *et al*., 2003, 2004) detected differences
415 between microbial counts in December (water temperature: 14 ºC) and July (water
416 temperature: 25 ºC), showing that summer fish presented higher rates of autolytic activity
417 but lower rates of microbial spoilage.

For the bacteria shown in Figures 1 and 2, the type 1 model is based on the corresponding data represented in Figures 1 and 2, in which the differences in the log counts among the tissues and the batches remain constant throughout the observed days. A polynomial trend is sufficient to explain the microbial kinetics, and the Akaike criterion (AIC) allows for the selection of the optimal degrees of freedom. All $R^2$ coefficients representing the variability due to fixed effects were greater than 95% (except for the outlying integer pair of 93.8%), indicating that the data fit the model well.

## CONCLUSIONS

The present study provides predictive models that can be used as tools for the optimisation of the shelf life of the sea bass throughout the year based on eight species of microorganisms in three different tissues (muscle, skin and gills).

These models revealed significant differences ($P < 0.001$) in the microbial growth between the sampled tissues and batches. The highest contamination was detected in gills, followed by skin and muscle. However, *Clostridium* sp. and *P. phosphoreum* followed a different model than the other studied microorganisms, showing greater contamination in muscle.

Because the measured physicochemical parameters remained relatively constant throughout the year, except for the temperature, temperature may be the main cause of the observed seasonal differences. Further studies on predictive modelling using physicochemical parameters should provide more raw data on microbial evolution in ungutted sea bass.

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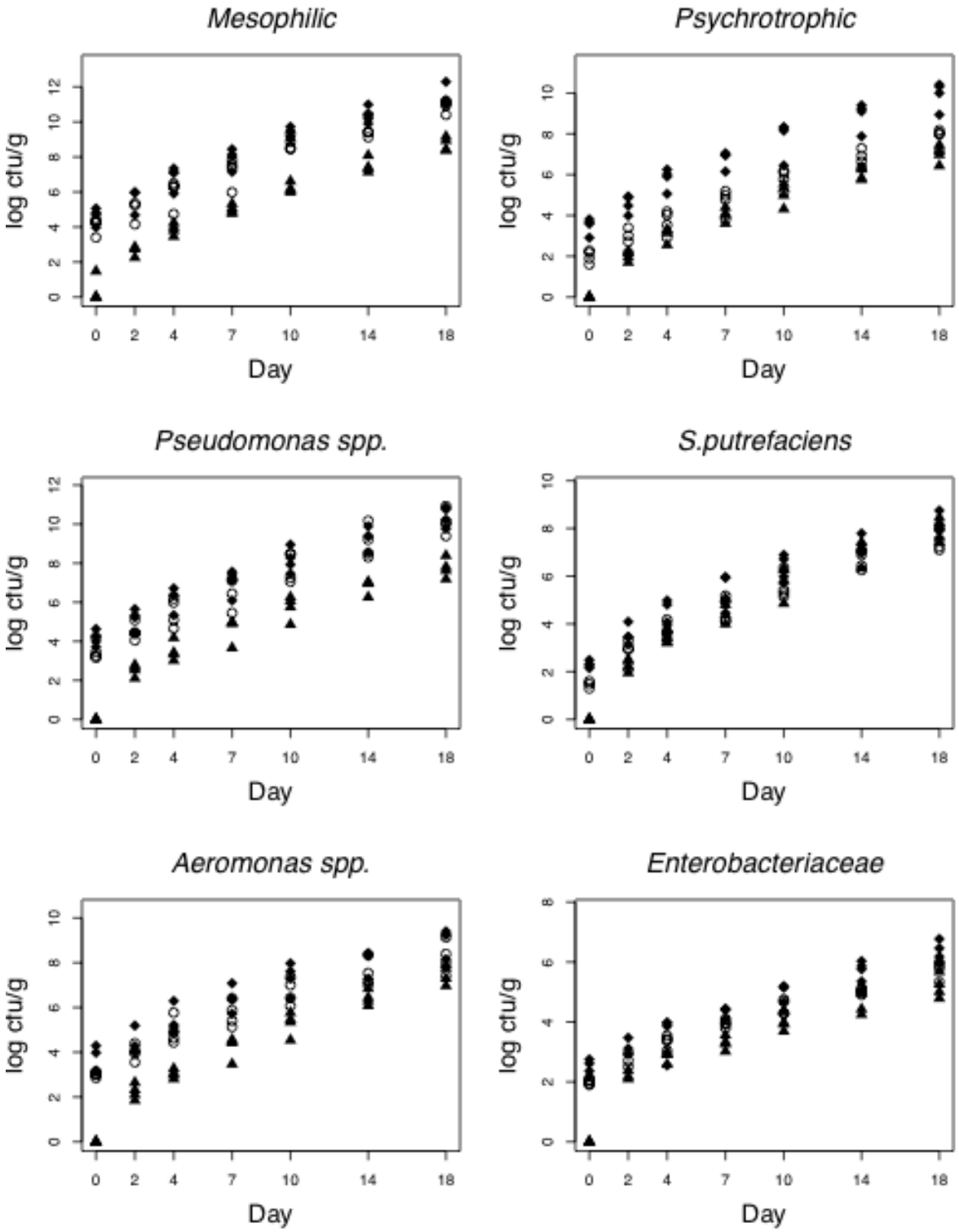
664 Figure 1. Log counts of microorganisms (four batches) over time and according to tissue in sea
665 bass: ▲= Muscle; ◆ = Gill; ○ = Skin.

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669 Figure 2. Log counts of microorganisms (four batches) over time and per batch in sea bass.

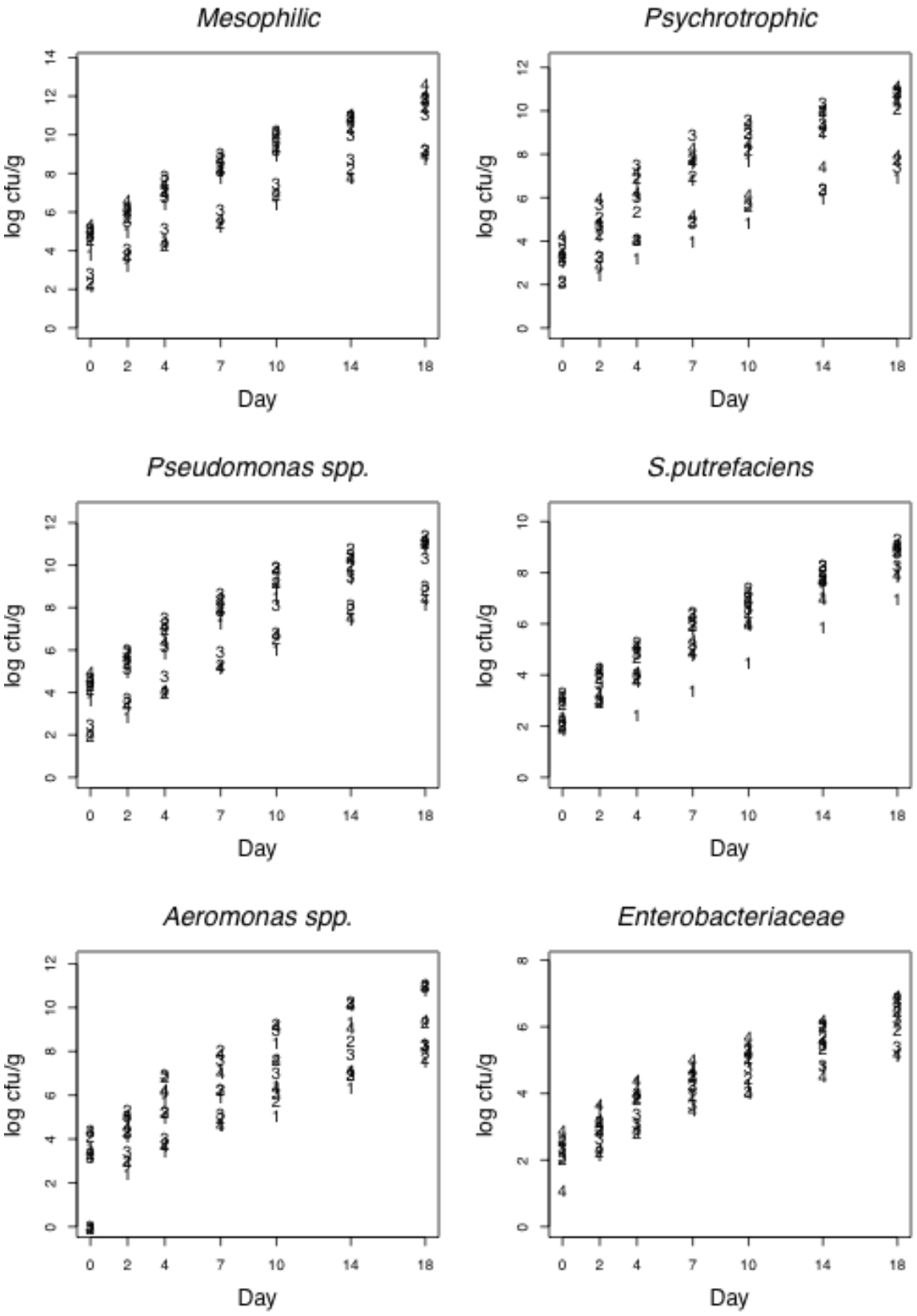


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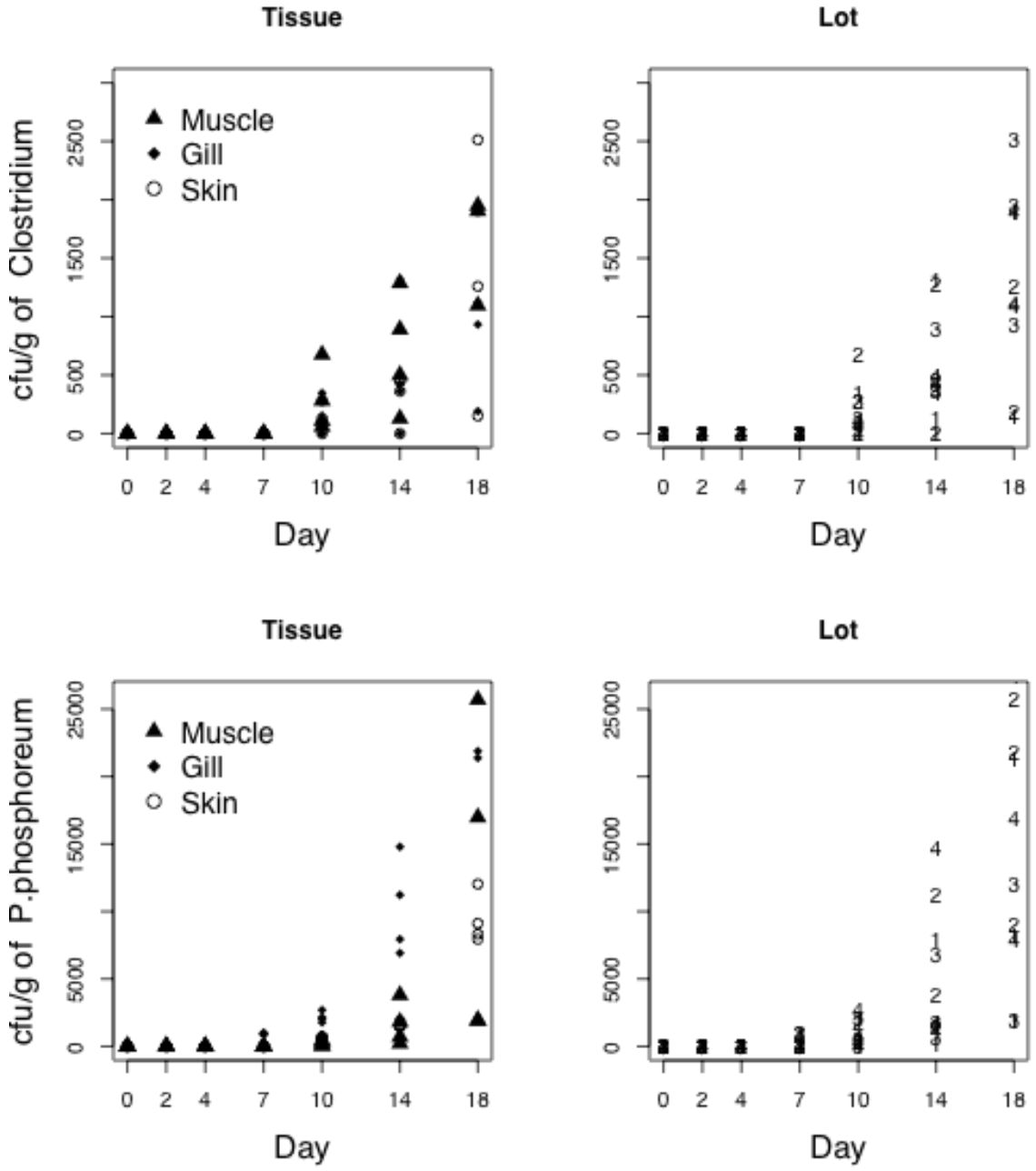
Figure 3. Counts of clostridia and *P. phosphoreum* according to tissue and batch in sea bass. The zero rates were 44.6% for clostridia and 46.4% for *P. phosphoreum*



680 Figure 4. Probabilities of zeros for *Clostridium* and *P. phosphoreum* by observation day in sea bass.

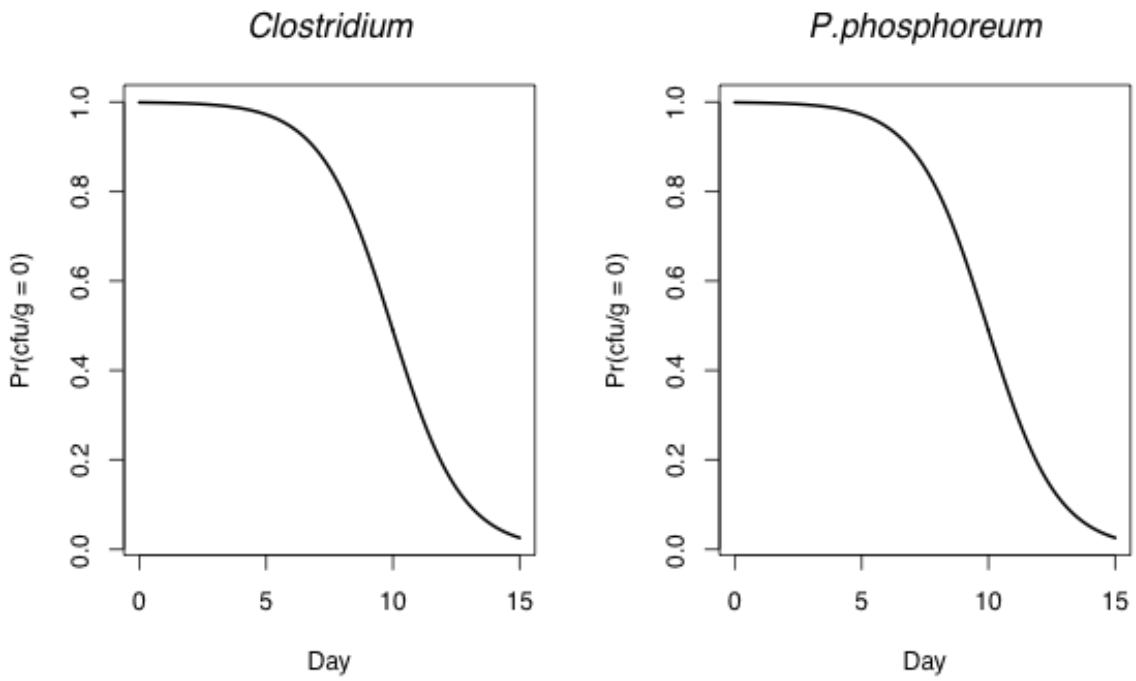



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Table 1. Provisional identification of strains isolated from sea bream stored in ice.

| | Gram reactions | Morph. | Motility | Oxidase | Catalase | HandL | TMAO | $H_2S$ |
|---|---|---|---|---|---|---|---|---|
| *Pseudomonas* sp. | – | r | 15ºC | + | + | Ox | – | – |
| *S. putrefaciens* | – | r | + | + | + | -/Ox | + | + |
| *Aeromonas* sp. | – | r | + | + | + | F | ± | ± |
| *P. phosphoreum* | – | Cb* | ± | – | + | F | ± | – |

Morphology: (cb) coccobacilli, (r) rods, (*) large round cells.
HandL: Oxidative or fermentative metabolism of glucose was performed in the medium of Hugh and Leifson (Hugh and Leifson, 1953).
TMAO: trimethylamine oxide (TMAO) reduction.

753 Table 2. Changes in bacteria count (log CFU/g) in muscle of ungutted sea bass stored in ice.

| | | Days of storage in ice | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Microorganism | 0 | 2 | 4 | 7 | 10 | 14 | 18 |
| I | Mesophilic | 1.89±1.29 | 3.37±0.34 | 4.65±0.38 | 5.64±0.35 | 6.98±0.46 | 8.18±0.42 | 9.07±0.23 |
| | Psychrotrophic | 1.07±1.23 | 3.01±0.39 | 3.88±0.45 | 4.76±0.52 | 5.60±0.53 | 6.56±0.53 | 7.56±0.46 |
| | *Enterobacteriaceae* | 0.27±0.55 | 2.35±0.21 | 2.98±0.14 | 3.77±0.22 | 4.21±0.20 | 4.91±0.36 | 5.42±0.37 |
| | *Aeromonas* sp. | 0.00 | 2.97±0.43 | 3.76±0.24 | 4.87±0.25 | 5.83±0.54 | 6.86±0.35 | 7.95±0.36 |
| | *Pseudomonas* sp. | 1.65±1.12 | 3.40±0.35 | 4.26±0.37 | 5.42±0.36 | 6.59±0.35 | 7.77±0.32 | 8.64±0.41 |
| | *S. putrefaciens* | 1.50±1.01 | 2.23±1.50 | 3.56±0.73 | 4.65±0.83 | 5.91±0.95 | 7.33±0.95 | 8.43±0.97 |
| II | *P. phosphoreum* | <1 | <1 | <1 | <1 | 2.18±0.49 | 3.01±0.54 | 3.80±0.60 |
| | Clostridia | <1 | <1 | <1 | <1 | 2.28±0.46 | 2.72±0.44 | 3.33±0.27 |

754 Mean of four batches (±) standard deviation.
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778 Table 3. Changes in bacteria count (log CFU/g) in skin of ungutted sea bass stored in ice.

| | | Days of storage in ice | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Microorganism | | 0 | 2 | 4 | 7 | 10 | 14 | 18 |
| I | Mesophilic | 4.60±0.54 | 5.66±0.51 | 6.83±0.32 | 8.14±0.26 | 9.37±0.34 | 10.42±0.33 | 11.51±0.43 |
| | Psychrotrophic | 3.24±0.15 | 4.58±0.33 | 5.94±0.38 | 7.38±0.50 | 8.36±0.51 | 9.20±0.20 | 10.41±0.29 |
| | *Enterobacteriaceae* | 2.28±0.25 | 3.08±0.18 | 3.80±0.27 | 4.46±0.21 | 5.05±0.16 | 5.58±0.10 | 6.35±0.20 |
| | *Aeromonas* sp. | 3.28±0.14 | 4.35±0.21 | 5.41±0.54 | 6.40±0.46 | 7.21±0.58 | 8.17±0.83 | 8.86±0.62 |
| | *Pseudomonas* sp. | 4.27±0.42 | 5.33±0.33 | 6.37±0.49 | 7.78±0.34 | 8.79±050 | 9.79±0.43 | 10.88±0.33 |
| | *S. putrefaciens* | 2.46±0.27 | 3.58±0.31 | 4.44±0.43 | 5.54±0.55 | 6.52±0.53 | 7.47±0.53 | 8.31±0.55 |
| II | *P. phosphoreum* | <1 | <1 | <1 | <1 | 2.7±0.10 | 3.20±0.05 | 3.97±0.08 |
| | Clostridia | <1 | <1 | <1 | <1 | 1.63±1.10 | 1.97±1.31 | 2.99±0.55 |

779 Mean of four batches (±) standard deviation.
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803 Table 4. Changes in bacteria count (log CFU/g) in gills of ungutted sea bass stored in ice.
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| | | Days of storage in ice | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Microorganism | | 0 | 2 | 4 | 7 | 10 | 14 | 18 |
| I | Mesophilic | 4.98±0.40 | 6.27±0.31 | 7.53±0.35 | 8.75±0.33 | 9.96±0.38 | 11.02±0.03 | 12.11±0.39 |
| | Psychrotrophic | 3.87±0.35 | 5.42±0.55 | 7.08±0.35 | 8.15±0.57 | 9.10±0.57 | 10.10±0.21 | 11.00±0.14 |
| | *Enterobacteriaceae* | 2.57±0.24 | 3.36±0.31 | 4.17±0.25 | 4.74±0.23 | 5.44±0.17 | 6.09±0.08 | 6.87±0.08 |
| | *Aeromonas sp.* | 4.17±0.35 | 5.12±0.16 | 6.45±0.55 | 7.70±0.36 | 9.00±0.39 | 10.00±0.44 | 10.98±0.12 |
| | *Pseudomonas* sp. | 4.56±0.40 | 5.77±0.31 | 7.13±0.41 | 8.31±0.48 | 9.67±0.49 | 10.38±0.45 | 11.21±0.22 |
| | *S. putrefaciens* | 3.18±0.14 | 4.19±0.1 | 5.14±0.15 | 6.23±0.30 | 7.07±0.36 | 7.93±0.48 | 8.92±0.46 |
| II | *P. phosphoreum* | <1 | <1 | 0.54±1.08 | 2.97±0.03 | 3.33±0.07 | 3.99±0.15 | 4.39±0.06 |
| | Clostridia | <1 | <1 | <1 | <1 | 1.14±1.33 | 2.08±1.41 | 3.00±0.57 |

805 Mean of four batches (±) standard deviation.
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828 Table 5. Mixed model for microbiological growth in sea bass for model (1).

| | Mesophilic | Psychrotrophic | *Pseudomonas* sp. | *S. putrefaciens* | *Aeromonas* sp. | *Entero-bacteriaceae* |
|---|---|---|---|---|---|---|
| $R^2$ | 0.989 | 0.975 | 0.988 | 0.971 | 0.968 | 0.973 |
| $\alpha_{gill} - \alpha_{muscle}$ | 2.87 (0.07)** | 3.10 (0.11)** | 2.70 (0.08)** | 1.16 (0.10)** | 3.03 (0.13)** | 1.18 (0.06)** |
| $\alpha_{skin} - \alpha_{muscle}$ | 2.28 (0.07)** | 2.30 (0.11)** | 2.15 (0.08)** | 0.54 (0.10)** | 1.64 (0.13)** | 0.80 (0.06)** |
| $\alpha_{gill} - \alpha_{skin}$ | 0.58 (0.07)** | 0.80 (0.11)** | 0.55 (0.08)** | 0.62 (0.10)** | 1.39 (0.13)** | 0.38 (0.06)** |
| $\lambda_2 - \lambda_1$ | 0.42 (0.10)** | 0.37 (0.13)* | 0.59 (0.09)** | 0.97 (0.12)** | 0.58 (0.15)** | 0.02 (0.09) |
| $\lambda_3 - \lambda_1$ | 0.52 (0.10)** | 0.71 (0.13)** | 0.58 (0.09)** | 0.85 (0.12)** | 0.51 (0.15)** | -0.12 (0.09) |
| $\lambda_4 - \lambda_1$ | 0.54 (0.10)** | 0.71 (0.13)** | 0.53 (0.09)** | 0.56 (0.12)** | 0.70 (0.15)** | 0.06 (0.09) |
| $\lambda_2 - \lambda_3$ | -0.10 (0.10) | -0.34 (0.13) | 0.00 (0.10) | 0.12 (0.12) | 0.07 (0.15) | 0.14 (0.09) |
| $\lambda_2 - \lambda_4$ | -0.12 (0.10) | -0.33 (0.13) | 0.05 (0.10) | 0.41 (0.12) | -0.12 (0.15) | -0.04 (0.09) |
| $\lambda_3 - \lambda_4$ | -0.02 (0.10) | 0.006 (0.13) | 0.05 (0.10) | 0.29 (0.12) | -0.19 (0.15) | -0.18 (0.09) |
| $\beta_1$ | 0.58 (0.02)** | 0.61 (0.03)** | 0.59 (0.02)** | 0.47 (0.03)** | 0.61 (0.03) | 0.35 (0.02)** |
| $\beta_2$ | -0.01 (0.001)** | -0.01 (0.002)** | -0.01 (0.001)** | -0.008 (.001)** | -0.01 (0.002)** | -0.007 (.001)** |

829 (*) $P < 0.05$; (**) $P < 0.001$; all p-values correspond to multiple linear comparison.
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Table 6. Zero-inflated models with its Poisson and logistic parts for *Clostridium* and *P. phosphoreum* in sea bass stored in ice.

| | **Poisson part** | | | **Logistic part** | |
|---|---|---|---|---|---|
| | *Clostridium* | *P. phosphoreum* | | Clostridium | *P. phosphoreum* |
| $\beta$ (slope) | 0.215 (0.003)** | 0.323 (0.001)** | Intercept | 7.141 (1.798)** | 7.903 (2.384)** |
| $\alpha_{gill} - \alpha_{muscle}$ | -0.493 (0.018)** | 1.014 (0.005)** | Day | -0.7187 (0.182)** | -1.090 (0.327)** |
| $\alpha_{skin} - \alpha_{muscle}$ | -0.246 (0.016)** | -0.156 (0.006)** | | | |
| $\alpha_{gill} - \alpha_{skin}$ | -0.247 (0.018)** | 1.170 (0.006)** | | | |
| $\lambda_2 - \lambda_1$ | 0.170 (0.023)** | 0.536 (0.006)** | | | |
| $\lambda_3 - \lambda_1$ | 0.332 (0.020)** | 0.208 (0.006)** | | | |
| $\lambda_4 - \lambda_1$ | 0.063 (0.021)* | 0.512 (0.006)** | | | |
| $\lambda_2 - \lambda_3$ | -0.162 (0.020)** | 0.328 (0.006)** | | | |
| $\lambda_2 - \lambda_4$ | 0.108 (0.021)** | 0.023 (0.006)** | | | |
| $\lambda_3 - \lambda_4$ | 0.269 (0.018)** | -0.304 (0.006)** | | | |

(*) P < 0.05; (**) P < 0.001; all p-values correspond to multiple linear comparison

868 Table 7. Mean values of seawater parameters in the principal tank.

| **Parameters** | **January** | **April** | **June** | **November** |
|---|---|---|---|---|
| Temperature (ºC) | 18.2 | 19.50 | 22.4 | 21.8 |
| pH | 7.9 | 8.1 | 8.1 | 7.7 |
| Salinity (g/l) | 33 | 35.6 | 32.2 | 33.6 |
| $BOD_5$ (mg/l) | <5 | 5.3 | <5 | <5 |

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