A Model Predicting the Microbiological Quality of Aquacultured Sea Bream (*Sparus Aurata)* according to Physicochemical Data: An Application in Western Greece Fish Aquaculture

Joan Iliopoulou-Georgudaki, Chris Theodoropoulos, Danae Venieri, and Maria Lagkadinou

Abstract**—**Monitoring of microbial flora in aquacultured sea bream, in relation to the physicochemical parameters of the rearing seawater, ended to a model describing the influence of the last to the quality of the fisheries. Fishes were sampled during eight months from four aqua farms in Western Greece and analyzed for psychrotrophic, H_2S producing bacteria, *Salmonella sp.*, heterotrophic plate count (PCA), with simultaneous physical evaluation. Temperature, dissolved oxygen, pH, conductivity, TDS, salinity, NO_3^- and NH_4^+ ions were recorded. Temperature, dissolved oxygen and conductivity were correlated, respectively, to PCA, *Pseudomonas sp.* and *Shewanella sp.* counts. These parameters were the inputs of the model, which was driving, as outputs, to the prediction of PCA, *Vibrio sp.*, *Pseudomonas sp.* and *Shewanella sp.* counts, and fish microbiological quality. The present study provides, for the first time, a ready-to-use predictive model of fisheries hygiene, leading to an effective management system for the optimization of aquaculture fisheries quality.

Keywords**—**Microbiological, model, physicochemical, Sea bream

I. Introduction

AQUACULTURE consists one of the world's fastest growing food producing sector, providing high amounts of fish products in order to meet the continuously increasing consumption demands. According to FAO (2005) [1], global fisheries production reached 130.2 million tones in 2001, thus covering the consumption rate which reached 96.3 million tones. In Greece, aquaculture consists one of the most promising sectors, as there exist 269 aquaculture companies, employing almost 10,000 workers and producing 100,000 tones of sea bream and sea bass [2].

This inevitable growth of production emerged the need for simultaneous certification of high fish quality standards, related to physical, chemical and micro-biological data. As a result, the directives 91/67/EEC [3] and 91/493/EEC [4] have set the framework for the production and marketing of high quality fishery products, while several studies and authorities [5], [6], [7] indicated the ideal values of certain sensory, physical, chemical and microbiological characteristics of fish, in order to achieve high standards of quality.

Although it is widely known that physicochemical parameters of the water column are related to bacteria population growth and, as a result, to the microbial load of fishes, no effort has been made in order to examine these relations and locate the exact parameters, which mostly affect bacteria populations, or even model such relations. Scientific research in the field of Predictive Microbiology is mostly related to models concerning bacterial growth along time, in different temperatures, pH or congener parameters, in an effort to predict and extend shelf-life of food products [8]–[12]. Consequently, more attention is given to the post-catch handling and distribution methods of fishery products rather than to the initial quality of the aqua cultured fish immediately after collection from the cages, which has been found to depend on physicochemical and bacteriological properties of the rearing water [13], [14].

In Greece, most aquaculture companies apply a daily monitoring of the main physicochemical characteristics of the rearing water, measuring mainly temperature, pH and dissolved oxygen. As a result, there is a lack of microbiological data and the quality of fish products is presumed, only according to the physicochemical evaluation of the rearing water.

The first goal of this study is to investigate the exact relations between physicochemical parameters of the rearing water and microbial flora of the aqua cultured fish, predicting the microbial quality of the product immediately after collection from the cages and gain a better understanding of the complex interactions between bacteria and the environment. The second goal is to model these relations in order to offer a practical tool for the prediction of microbiological fish quality for everyone concerned to aquaculture settlement, feeding techniques and vaccinations for a final goal as the continuous improvement of the fisheries, which reach to the consumer.

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II. Materials and Methods

A. Sample Collection

Samples were collected during eight campaigns from four aquaculture units located in Western Greece (Fig. 1). In every unit, temperature, pH, dissolved oxygen and conductivity were recorded using a permanently laid system of sensors (Global Water Datalogger System), which was placed in a depth of 8m and in a distance of 8m away from the cages, in order to avoid the direct influence of the fish discharches and the food residues. Total dissolved solids and salinity were also recorded using the HORIBA W2010 Portable Multiparameter Meter. Moreover, seawater samples were taken and analyzed for NO3- and NH4+ ions using the HACH DR2800 Spectrophotometer. Sea breams *(Sparus aurata)* were then collected, preserved in 4°C and transferred to the lab for analysis, immediately after the end of the campaign. Each sample consisted of two fishes, thus resulting in a total of 64 sea breams.

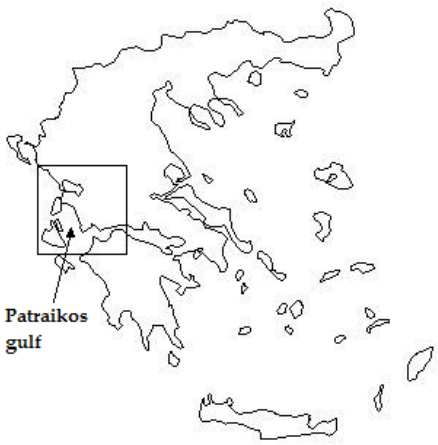

Fig. 1 Location of the study area. The four aquaculture units are located at the Northwest side of Patraikos gulf

B. Sensory Analysis

All fishes were scored from three trained panelists, according to characteristics concerning general appearance, eyes and gills, using the Quality Index Method developed by [15]. Panelists were asked to score surface appearance, flesh firmness, clarity, pupil and shape of the eyes, color and smell of the gills using a 0-1 scale, where 0 represented best quality and higher score indicated lower quality.

C. Microbiological Analysis

Each sample was tested for the presence and enumeration of psychrotrophic bacterial species (*Pseudomonas sp. & Vibrio sp.*), H_2S producing bacteria (*Shewanella putrefaciens*), Enterobacteriaceae (Salmonella sp.) and heterotrophic plate count at 22°C and 37°C according to International Standards Organization (ISO) techniques. Flesh samples were aseptically cut into slices in sterile container. Twenty-five grams of skinless fish flesh were homogenized for 2 min in a stomacher with 225mL of Peptone saline solution (Oxoid).

To detect *Pseudomonas sp*. the surface-plate method was applied on Pseudomonas Agar Base (OXOID) with cetrimide. Plates were incubated at 30°C for 48h. For the isolation of Vibrio sp., the homogenate was firstly incubated at 30°C for 24h and then the surface-plate method was also performed on TCBS agar (Oxoid). Plates were incubated at 30°C for 24h. For the determination of *Shewanella putrefaciens*, Triple Sugar Iron Agar (TSI - Oxoid) was used and plates were incubated at 30°C for 72h. For *Salmonella sp*., enrichment steps were performed. Firstly, the homogenate was incubated at 37°C for 24h, and then portions of 1mL and 100μL were mixed with 10mL of Selenite Cystine broth (Oxoid) and Rappaport – Vassiliadis broth (Oxoid), respectively. Second incubation followed at 37°C for Selenite Cystine broth and 42°C for Rappaport – Vassiliadis broth for 24h. After enrichment steps the surface-plate method was applied on Salmonella – Shigella agar (Oxoid) XLD agar (Oxoid) and all plates were incubated at 37°C for 24h.

For the enumeration of heterotrophic bacteria the pour plate count method was chosen, using 2ml of the homogenate and mixing with melted Plate Count Agar (Oxoid), tempered at 44°C. Two sets of plates were prepared for all samples. One set was incubated aerobically at 37°C for 48h and the other set at 22°C for 72h.

All colonies were counted as colony forming units (cfu/g) of the sample. The isolated microorganisms were identified depending on their biochemical characteristics and using standardized identification systems API 20E and API 20NE (BioMérieux, 69280 Marcy-l'Étoile, France).

D. Statistical Analysis

The 1-sample Kolmogorov–Smirnov test was utilized in order to check data for normality. Since some parameters failed to meet the test's criteria, Spearman's rank correlation was used in order to identify correlations between physicochemical and microbiological parameters. For curve fitting between correlated parameters, regression analysis was used and all equations derived, were inputted in the MATLAB software (The MathWorks Inc., Natick, MA, USA). The ANOSIM test [16] of the PRIMER 5 statistical package was also utilized in order to check for significant differences between samples.

III. Results and Discussion

A. Physicochemical Data

Physicochemical data indicate a good chemical quality of the aquaculture's rearing water (table I), except for a certain period from April to June, in which conductivity, total dissolved solids, NO_3^- and NH_4^+ concentrations were higher, although the observed differences were not statistically significant according to the ANOSIM test (R_{max}: 0.301). Water pollution from agricultural and industrial activities from

the adjacent continental land, as well as anthropogenic discharges into Acheloos river, which flows near the sampling area are considered the main factors that contribute to this occasional degradation.

B. Sensory Results

All fishes collected from the aquaculture presented high sensory quality, with bright surface appearance, firm and elastic flesh, transparent and bright clarity, bright black and circular pupil, slightly convex shape of the eye and bright red gill color, indicating the freshness of the aquacultured fishes. The Fish Quality Index was found close to zero, with only minor exceptions (3 individuals), an expected result, taking into account the good physicochemical conditions of the rearing water, during the most months.

C. Microbiological Data

Microbiological results depict more clearly the influence of anthropogenic pressures at the quality of the aquacultured fishes. As it shown in table II, certain microbial populations were found at several samples collected. Total microbial counts were higher for the spring and summer months, in which major pathogens, such as *Vibrio vulnificus, Pseudomonas cepacia, Salmonella sp., Enterobacter cloacae, Pseudomonas aeruginosa* and *Pseudomonas fluorescence* were also found. Agroindustrial pollution transferred through Acheloos river consisted the main degradation factor, which led to increased microbial populations in the aquacultured fishes.

D. Statistical Analysis

Spearman's rank correlation revealed that major influence on microbiological populations of the aquacultured fishes, is provoked by temperature, dissolved oxygen and conductivity. Heterotrophic plate counts (PCA) were positively correlated with water temperature (r: 0.51; $p<0.01$), *Pseudomonas sp.* counts were negatively correlated to dissolved oxygen concentration (r: -0.45; $p<0.05$) and *Shewanella sp.* counts were positively correlated with conductivity (r: 0.52; $p<0.01$). *Vibrio sp.* as well as certain species of the genus *Pseudomonas sp.* occurred only in samples with water temperature higher than 22.9°C.

The regression equation derived for each correlated parameter is graphically represented in Fig. 2, 3 and 4 respectively. For the correlation between water temperature and PCA:

$$y = 0.129x - 0.3433$$

where y is the base 10 logarithm of heterotrophic plate count in cfu/g and x is the temperature (°C). For the correlation between dissolved oxygen and *Pseudomonas sp*. counts:

$$y1 = -0.4709x1 + 4.6629$$

where y1 is the base 10 logarithm of Pseudomonas sp. counts in cfu/g and x1 is the dissolved oxygen concentration in mg/L. For the correlation between conductivity and Shewanella sp. counts:

$$y2 = 0.2565x2 - 13.891$$

where y2 is the base 10 logarithm of *Shewanella sp.* counts in cfu/g and x2 is conductivity in mS/cm.

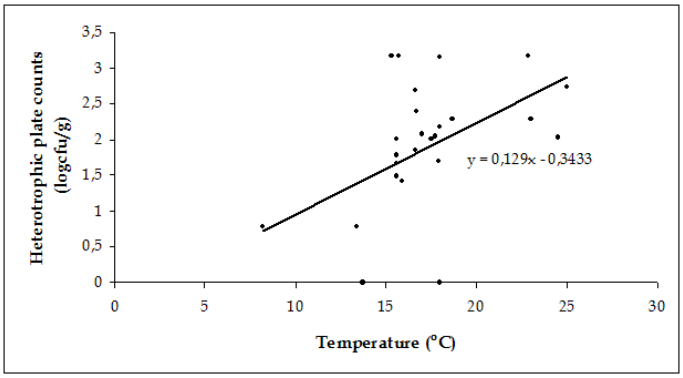

Fig. 2 Graphic representation of the correlation between temperature (°C) and heterotrophic plate counts (log10cfu/g)

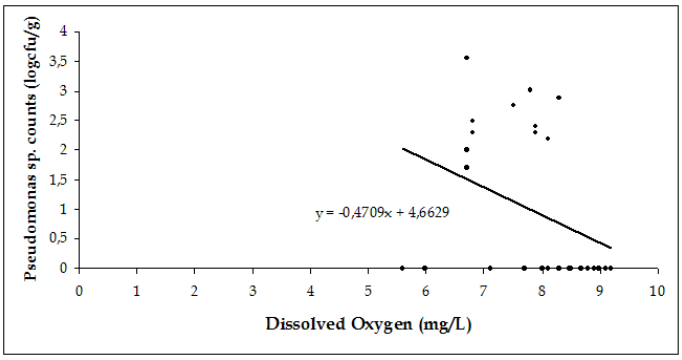

Fig. 3 Graphic representation of the correlation between dissolved oxygen and *Pseudomonas sp.* counts (log10cfu/g)

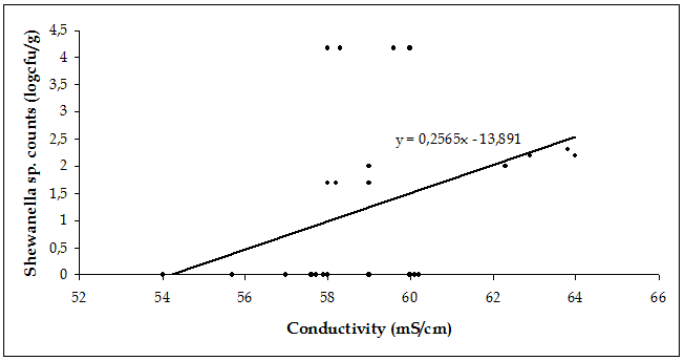

Fig. 4 Graphic representation of the correlation between conductivity and *Shewanella sp.* counts (log10cfu/g)

For the classification of sea bream samples according to microbiological quality (heterotrophic plate count - PCA), boundaries and microbiological values proposed by previous studies had been taken into account. The highest boundary, in which fishes are close to the limit of acceptability, was set at 6log10cfu/g [5], [17], [18] in order for samples to be consumable. The lower value indicated in literature varies

between <1log10cfu/g [19] and 3.5log10cfu/g [20], [21]. Considering the results of the current study, we favored Grigorakis et al. (2003a) [19], as most samples from low physicochemical inputs presented microbiological values below 1.5log10cfu/g.

Five quality classes were formed (table III) in order to express the percentage of divergence from the reference condition (1.5log10cfu/g). No divergence (<1.5 log10cfu/g) was classified as "high", divergence between 1% and 25% was classified as "good", divergence between 25% and 50% was classified as "moderate", divergence between 50% and 75% was classified as "low" and divergence above 75% was classified as "bad".

TABLE III
CLASS BOUNDARIES AS INDICATED BY LITERATURE AND THE RESULTS OF THE CURRENT STUDY (LOG10CFU/G).

PCA 22^{0}C (log_{10}cfu/g)	PCA 22^{0}C (cfu/g)	Quality Classes
<=1.5	<=31	High
1.51 – 2.62	32 – 415	Good
2.63 – 3.75	416 - 5622	Moderate
3.76 – 4.88	3622 – 75856	Poor
4.89 - 6	75857 - 999999	Bad

E. Built-up of the Model

According to the aforementioned analysis, the model created consists of three inputs (water temperature, dissolved oxygen, conductivity), a calculation code and five outputs (PCA, *Pseudomonas sp.* counts, *Shewanella sp.* counts, occurrence of *Vibrio sp.* and classification of fish into one of the five quality classes). The user is prompted to enter the three physicochemical inputs and after all calculations being executed by the processor, he is informed about the various microbiological properties mentioned (Fig. 5). The computing code of the model is based on the "if-else" and "for" functions of the MATLAB software, which give to the programmer the ability to include or exclude certain physicochemical values, define boundaries and separate different situations.

F. Validation of the Model

Three additional sampling campaigns were conducted in order to check the applicability of the model created. Physicochemical parameters of the rearing water and the observed and predicted microbial flora are presented in table IV. In most cases, the prediction was in accordance to the real data, with an exception, those of heterotrophic plate count at water temperatures close to 24oC, in which the percent relative error was within the –50% to +50% zone [8]. All other values were within the –20% to +20% zone, with only two exceptions (9/2008 - Units 3 and 4 for heterotrophic plate count). Concerning *Pseudomonas sp.* and *Shewanella sp.* counts, predicted and real values presented minor divergence, with almost 90% of the samples being predicted by the model. All samples with water temperature above 22.9^{o}C were found positive in *Vibrio sp.*, in accordance with the prediction.

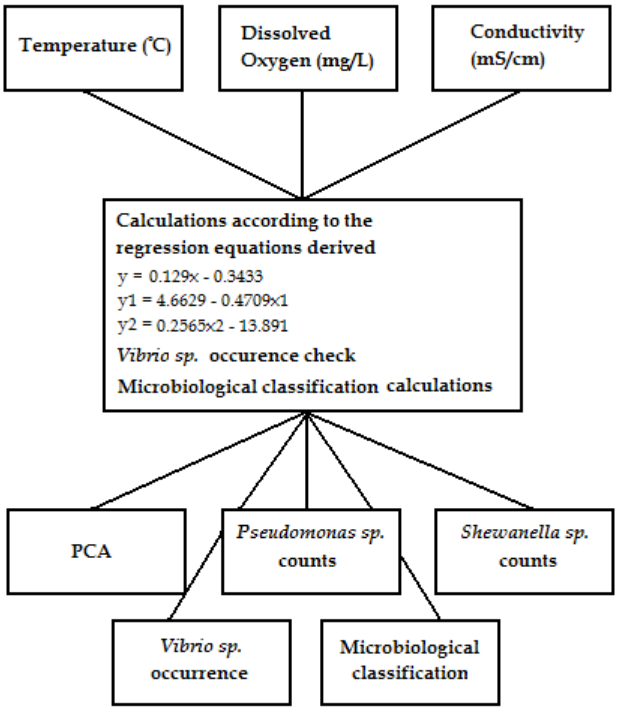

Fig. 5 Graphic representation of the model. It consists of three physicochemical inputs, a calculation code based on the statistical analysis and on the regression equations of raw data and five microbiological outputs.

IV. CONCLUSION

In the present study, the relation between physicochemical parameters of the rearing water and the microbiological quality of aquacultured fish is revealed. Spearman's correlation coefficient, which varied between 0.45 and 0.52 revealed the need for more data in order to increase the precision of the relations between the examined parameters and, consequently, to reinforce the accuracy of the regression equations. Additional future studies would offer more raw data and either retain or modify the equations, increasing the accuracy of the prediction. Anyway, these correlations are qualified in the relative studies [8], [12].

Nevertheless, a user friendly and practical tool for aquaculturers is created for the first time, giving them the ability for a rapid assessment of the microbiological fish quality, based on the physicochemical parameters of the rearing water, avoiding the time-consuming microbiological analyses. Plus, the application of this model can lead to an effective management system, which will optimize the quality of aquaculture fisheries.

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TABLE I

PHYSICOCHEMICAL PROPERTIES OF THE AQUACULTURE'S REARING WATER. DO: DISSOLVED OXYGEN, O%: OXYGEN SATURATION, COND: CONDUCTIVITY, TDS: TOTAL DISSOLVED SOLIDS

Date	Units	Temperature (°C)	pH	DO (mg/L)	O%	Cond (mS/cm)	TDS (mg/L)	Sal (psu)	NO_3^-	NH_4^+
9/2007	1	24.5	8.1	5.98	70	57.7	37	38	0.3	0.04
	2	23	8.1	7.5	92.80	55.7	35.5	36.8	0.4	0.1
	3	22.9	8.1	6.7	83	57.9	37.1	38.6	0.4	0
	4	25	8.13	7.8	100	57	36.5	37.9	0.3	0
10/2007	1	18	8.1	6	72	57.2	36	38.2	0.3	0.03
	2	17.7	8.14	7	90	55.4	35.5	36.9	0.3	0
	3	17.5	8.1	6.5	82.5	57	36.7	38.4	0.4	0
	4	17.5	8.2	7.4	96	56.8	36	38	0.3	0
1/2008	1	19	8.23	6.8	69	59	37	38.3	0.3	0.05
	2	15.6	8.27	6.7	68.30	58.2	36.3	38.2	0.3	0.02
	3	15.9	8.29	7.9	78	58	36	38.2	0.4	0.02
	4	15.9	8.2	8.1	82	57	36	38.3	0.3	0.02
2/2008	1	13.8	8.2	7.7	74	60	37	38.4	0.5	0.06
	2	13.73	8.24	5.6	78	58	36	38.2	0.2	0
	3	8.24	8.26	8.1	74	59.6	36.6	38.2	0.3	0.02
	4	13.4	8.2	8.3	80	58.3	35.9	38.2	0.2	0.02
3/2008	1	15.6	8.2	8.9	93	59	38	38	0.7	0.01
	2	15.3	8.16	8.67	90	58	36	38.2	0.2	0.01
	3	15.7	8.2	9.1	93.4	57.6	36.5	38.1	0.5	0.005
	4	15.6	8.1	9.2	94.6	54	35.9	38.4	0.5	0.005
4/2008	1	16.7	8.02	8.99	96	62.3	37	38	0.3	0.04
	2	16.6	8.12	8.3	89	64	38	38.1	0.3	0.04
	3	17	8.16	8.46	95.5	62.9	38	38.2	0.5	0.05
	4	16.6	8.13	8.79	96.6	63.8	38	38	0.5	0.03
5/2008	1	17.9	7.98	7.1	83	60	36	38.6	0.5	0.08
	2	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-
	4	18	8.1	8.1	84	59	36	38.4	0.4	0.03
6/2008	1	18	8.13	8	88	60.1	36	38.3	0.7	0.01
	2	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-
	4	18.7	8.15	8.5	98	60.2	36	38.2	0.9	0.03

TABLE II
MICROBIOLOGICAL PROPERTIES OF THE AQUACULTURED FISHES. PCA 22oC: HETEROTROPHIC PLATE COUNT INCUBATED AT 22°C, PCA 37°C: HETEROTROPHIC PLATE COUNT INCUBATED AT 37°C

Date	Units	PCA 22°C	PCA 37°C	*Pseudomonas* sp. counts	Vibrio sp.	*Shewanella* sp. counts	*Salmonella* sp.	Identified bacteria
9/2007	1	105	145	0	+	0	-	*Pseudomonas fluorescence & P. putida* *Vibrio vulnificus & Vb. alginolyticus* *Sphingomonas paucimobilis*
	2	195	1500	550	+	0	-	
	3	1500	1500	3500	+	0	-	
	4	535	110	1000	+	0	-	
10/2007	1	150	200	200	-	100	-	-
	2	110	150	50	-	50	-	-
	3	100	235	200	-	50	-	-
	4	100	60	0	-	0	-	-
1/2008	1	60	340	300	-	50	-	*Pseudomonas aeruginosa, Ochrobactrum antrhopi*
	2	30	300	100	-	50	-	-
	3	25	250	250	-	0	-	-
	4	25	75	0	-	0	-	-
2/2008	1	0	0	0	-	15000	-	-
	2	0	0	0	-	15000	-	-
	3	5	5	150	-	15000	-	-
	4	5	0	750	+	15000	-	*Vibrio vulnificus*
3/2008	1	100	100	0	-	0	-	*Pseudomonas cepacia*
	2	1500	1500	0	-	0	+	*Pseudomonas cepacia & Salmonella sp.*
	3	1500	1500	0	-	0	-	*Pseudomonas cepacia*
	4	45	140	0	-	0	-	*Pseudomonas cepacia*
4/2008	1	240	1500	0	-	100	-	*Citrobacter freundii, Providencia rettgeri*
	2	70	1500	0	-	150	-	*Pseudomonas pseudomallei, Enterobacter cloacae*
	3	120	1500	0	-	150	-	*Pseudomonas aeruginosa*
	4	480	1500	0	-	200	-	*Serratia liquefaciens, Enterobacter cloacae*
5/2008	1	50	60	0	-	0	-	-
	2	-	-	-	-	0	-	-
	3	-	-	-	-	0	-	-
	4	0	0	0	-	0	-	*Pseudomonas cepacia, Enterobacter cloacae*
6/2008	1	1415	1335	0	-	0	-	*Enterobacter cloacae, Photobacterium damselae*
	2	-	-	-	-	0	-	-
	3	-	-	-	-	0	-	-
	4	195	10	0	-	0	-	*Pseudomonas fluorescence*

TABLE IV
VALIDATION OF THE MODEL FROM THREE ADDITIONAL SAMPLING CAMPAIGNS SHOWING THE RELATION BETWEEN OBSERVED AND PREDICTED PARAMETERS

Date	Units	Physicochemical data Temperature (°C)	Observed Microbiological PCA (cfu/g)	Predicted Microbiological PCA (cfu/g)
7/2008	1	20	160	171
	2	20.7	200	211
	3	20.5	180	199
	4	20.7	250	211
8/2008	1	24	350	564
	2	23.9	400	548
	3	24.3	410	617
	4	24	300	564
9/2008	1	19	120	127
	2	18.9	140	123
	3	18.6	150	112
	4	18.7	140	116
		Dissolved Oxygen (mg/L)	Pseudomonas sp. counts (cfu/g)	Pseudomonas sp. counts (cfu/g)
7/2008	1	6.4	50	0-50
	2	7.1	0	0-50
	3	6.7	0	0-50
	4	6.5	0	0-50
8/2008	1	7.2	0	0-50
	2	7.3	50	0-50
	3	5.8	100	50-100
	4	7	0	0-50
9/2008	1	6.9	50	0-50
	2	7.1	0	0-50
	3	6.4	50	50-100
	4	7.1	50	0-50
		Conductivity (mS/cm)	Shewanella sp. counts (cfu/g)	Shewanella sp. counts (cfu/g)
7/2008	1	59	0	0-50
	2	58.5	0	0-50
	3	58.7	50	0-50
	4	58	0	0-50
8/2008	1	57	0	0-50
	2	56.6	0	0-50
	3	57.4	50	0-50
	4	55	0	0
9/2008	1	56.4	0	0
	2	55	0	0
	3	57	50	0-50
	4	55.6	100	0-50