

Application of an incomplete factorial design for the formation of an autotrophic biofilm on river bed sediments at a microcosms scale

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Abstract

Purpose River bed sediments are often colonized by microorganisms which can produce large amounts of extracellular polymeric substances (EPS) forming biofilms that may increase the resistance of the bed sediment towards erosion and affect the nutrient and pollutant exchange between water and sediments. The objective of this work was to study the influence of organic carbon, ionic concentration (NaCl) and N/P ratio on the growth of autochthonous biomass and the production of EPS in autotrophic biofilms by measuring the extracellular carbohydrates produced, a specific component of the EPS.

Materials and methods For this purpose, an incomplete 3³ factorial design was carried out to study the influence of the ionic concentration, ranging between 0 and 25 mg/l NaCl; organic carbon concentration, ranging between 0 and 10 mg/l glucose and N/P ratio, ranging between 5 and 20, on the following dependent variables: chlorophyll *a* and

carbohydrate concentration at a microcosms laboratory scale. Heterotrophic density was also included as a third dependent variable.

Results and discussion The ionic and carbon concentrations in the ranged tested were the most influential variables in the production of carbohydrates and chlorophyll *a*. The biofilm formation was stimulated in a medium with a 1:5 ratio for N/P and 10 mg/l of glucose and 25 mg/l of ionic concentration. The extracellular concentration of carbohydrates increased in parallel with the accumulation of chlorophyll *a*, which suggests that the autotrophic biomass may be an important component for the biofilm formation.

Conclusions This work demonstrates that it is possible to develop empirical models that describe the interrelationship between organic carbon, ionic concentration (NaCl) and N/P ratio and the extracellular carbohydrates as well as chlorophyll *a*, contained in the biofilm developed over river bed sediments. It can be concluded that concentrations of 25 mg/l of NaCl and 10 mg/l of carbon improve the biofilm formation from river bed sediments, whereas N/P ratio higher than 5 produced a negative effect in the production of carbohydrates and chlorophyll *a* and consequently in the production of biofilm.

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1 Introduction

The presence of biofilms covering the surfaces of rocks, mineral grains and plant debris is common in aquatic environments (Gerbersdorf et al. 2009; Underwood 2010). Microphytobentic organisms (cyanobacteria, unicellular

algae, particularly diatoms, diatoms and fungi) and bacteria associated in the biofilms produce large amounts of extracellular polymeric substances (EPS), forming adhesive coatings on the individual particles of the sediments, thus increasing the stabilization of the river bed sediments (Gerbersdorf et al. 2007; Sutherland et al. 1998). Also, Ziervogel and Forster (2006), Gerbersdorf et al. (2008a, b) and Flemming (2006) observed that the stability of the sediment was directly correlated with the concentration of certain types of soluble carbohydrates produced by diatoms. Sutherland et al. (1998), Stal (2003) and Tolhurst et al. (2006) observed that biofilms increased the erosion threshold and the sediment resuspension limit, and De Brouwer et al. (2005) found an increase in critical shear stress values in the presence of diatoms, compared with sterile sediment, concluding that there was a biogenic stabilization of intertidal sediments.

The development of biofilms on the surface of the river bed particles may also affect the contaminant mobility, by decreasing sediment resuspension and by modifying the adsorption–desorption behaviour of the contaminants (Stal 2003). The stabilization of the sediment matrix due to the growth of biofilms not only reduces the mobilization of sediments but also of the associated pollutants (León Morales et al. 2006). This fact is particularly important in polluted areas, as those identified in the Anllóns River (NW Spain), with high loadings of arsenic and phosphorus (Devesa-Rey et al. 2008, 2009).

It has been demonstrated that the secretion of EPS by active biomass of algae, cyanobacteria, bacteria and fungi inhabiting river bed sediments is dependent on the type of nutrient supply (Macarelli et al. 2009; Staats et al. 2000). N and P supply are also key components in the cyanobacteria accumulation in sediments (Ladakis et al. 2006). Staats et al. (2000) found that biofilm formation was stimulated when cells of an epipellic diatom were incubated at low N or P concentrations (95.5 μM NH_4Cl and 7.7 μM $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$). Also, Rodrigues et al. (2008) found that a carbon supply can significantly alter the composition of the biofilm formed although the cell density remains relatively unaltered. Carbon supply alters not only the phylogenetic composition or the architecture of the fluvial biofilm but it also favours the development of the heterotrophic population, more than the autotrophic (McArthur et al. 1985; Ylla et al. 2009). The relationship of the N or P concentrations with the carbon supply was also studied by Thompson et al. (2006) for the formulation of a biofilm by *Enterobacter cloacae* Ecl and *Citrobacter freundii* Cf1. These authors found that the highest N/P ratio assayed (N/P=28) may prevent biofilm formation on the sorbent surfaces, by favouring the suspension cells in the planktonic/free-swimming form. Also, low N supplies may alter cell shapes and surfaces roughness of the biofilm formed (Lin and Lay

2004; Thompson et al. 2006). Also, the algal composition may be governed by local rather than larger-scale factors (Pan et al. 1999) such as climate, geology or nutrient levels, and many authors have shown that diatom metrics detect eutrophication effects better than other environmental variables, and they respond most strongly to land-use gradients (Hering et al. 2006; Johnson et al. 2006; Delgado et al. 2010).

Another factor influencing biofilms formation is the ionic concentration. Kierek and Watnick (2003) found differences in the phylogenetic composition of the biofilm, as well as differences in architecture, when grown in fresh or seawater medium. A 15% NaCl was found to decrease the adhesion of flagellum in a mono-species biofilm formed by *Listeria monocytogenes* (Caly et al. 2009). Instead, Spears et al. (2008) found that the natural multi-species biofilm formed in aquatic ecosystems were more resistant with increasing salinity.

Biofilm characterization may be undertaken by measurements of a pigment marker—chlorophyll *a*—indicative of autotrophic population development. An indirect estimation of such autotrophic growth can be deduced from a specific component of the EPS matrix, the extracellular carbohydrates which may be produced by the algae and the cyanobacteria. The biofilm-forming bacteria can be estimated by determination of the density of the heterotrophic population.

This study explores the application of the response surface methodology in order to predict the optimum production of a fluvial biofilm with varying environmental factors (ionic concentration, N/P ratio and glucose). Since a systematic study of the effects caused by the operational variables on the biofilm production would require a great amount of experimental work, an incomplete, factorial design of experiments was carried out for each environmental factor, to select the best conditions, in which three dependent variables (ionic concentration, N/P ratio and glucose) were assayed at three levels. The study was carried out by taking into consideration the range of natural variations found in the Anllóns River (NW Spain) of the ionic concentration, N/P ratio and glucose which could be found in a natural stream, and the results will enhance our knowledge about the environmental conditions affecting the development of natural biofilms in river bed sediments.

2 Materials and methods

2.1 Development of a fluvial biofilm and environmental variables assayed

The algal inocula were extracted from a river bed sediment sample, taken in the Anllóns River (NW Spain), by means

of an aqueous extraction, with a 1:5 sediment/extractant ratio. Also, bacterial inocula were also extracted in order to evaluate the heterotrophic density. The aqueous solution contained 2% of sodium hexametaphosphate to facilitate sediment dispersion, thus favouring algal and bacterial extraction. Next, 10 ml of this extract was introduced in photobioreactors of 80 ml containing 5 g of sediments as substrata and incubated with algal medium (10.20% Fe, 0.53% Zn, 0.44% Mn, 0.56% Mo, 0.046% Co, 0.049% Cu, 0.162% thiamine, 0.008% biotin, 0.008% cyanocobalamine and 0.390% vitamins) under variable conditions of the main limiting inorganic nutrients (N, P), organic carbon (glucose) and ionic concentration (NaCl), as described in the following section. The analysis of the river water was used to establish the variation range of the independent variables selected. So, their concentrations assayed were ionic concentration (0, 12.5 and 25 mg/l NaCl); carbon (supplemented as glucose; 0, 5 and 10 mg/l), whose lower range was similar to the river water analyzed, although it must be taken into account that glucose is just one component of DOC in rivers; and N/P ratio (5, 12.5 and 20; supplemented as NaNO₃ and NaH₂PO₄, respectively, with a fixed P concentrations of 20 mM and variable N concentrations of 0.4, 0.25 and 0.1 M), whose lower range was similar to the river water analyzed (N/P= 5.25) and the higher might mimic most polluted situations. Table 1 lists the independent and dependent variables considered.

The fluvial biofilm was developed in photobioreactors submitted to 12:12-h light/dark periodicity (Fig. 1). Photobioreactors were maintained with a light intensity of



Fig. 1 Photograph of the aerated photobioreactors employed in this study

170 μmolphoton m⁻²s⁻¹. Lighting was provided by fluorescent lamps (Mazda Fluor Lumiere du Jour C9 TF 65, 85 W), and the light intensity was measured with a radiometer (DHD 2302.0, HERTER). Two glass slides (2× 5 cm) were introduced into each reactor in order to increase the superficial area. CO₂ pulses (10 s) were supplemented between four to eight times per day from 9.00 a.m. to 20.00 p.m. to all the photobioreactors, in order to avoid the development of an excessive alkaline medium (pH>9); however, these pulses were maintained as minimum as possible, in order to simulate a laminar flow. In these conditions, the samples were allowed to stand for 21 days, when the biofilms were chemically characterized.

2.2 Box–Behnken experimental design

Response surface methodology consists of a group of mathematical and statistical techniques based on the fit of empirical models to the experimental data obtained in relation to experimental design (Bezerra et al. 2008). Box–Behnken designs are a class of rotatable or nearly rotatable second-order designs based on three-level, incomplete factorial designs (Box and Behnken 1960). The number of experiments (*N*) required for full Box–Behnken design is given by the formula $N=2k(k-1)+C_0$, where *k* is the number of factors and *C*₀ is the number of central points (Ferreira et al. 2007). The simplest equation describing a linear function is described by the following expression:

$$y = \beta_0 \sum_{i=1}^k \beta_i x_i + \varepsilon \tag{1}$$

where β₀ is the constant factor, β_{*i*} represents the coefficients of the linear parameters, *k* is the number of variables, *x_i* represents the variables and ε is the residual factor associated to the experiments. When the experimental data do not fit to a linear equation because the solution to a

Table 1 Independent and dependent variables employed in this study

Variable	Nomenclature	Definition	Units	Variation range
Independent variables				
Ionic concentration	IC		mg/l	0–25
Carbon	C		mg/l	0–10
N/P ratio	N/P		–	5–20
Dimensionless, coded independent variables				
Dimensionless ionic concentration	<i>x</i> ₁	(IC–12.5)/12.5		(–1, 1)
Dimensionless carbon	<i>x</i> ₂	(C–5)/5		(–1, 1)
Dimensionless N/P	<i>x</i> ₃	(N/P–12.5)/7.5		(–1, 1)
Dependent variables				
Chlorophyll a	<i>y</i> ₁		mg/l	
Carbohydrate	<i>y</i> ₂		mg/l	
Heterotrophic density	<i>y</i> ₃		n° cellsg ⁻¹	

problem is not necessarily the highest or the lowest values of the variables, then it is desirable to include levels in the input variables. In this case, a polynomial response surface must be generated. The Box–Behnken experimental designs were constructed for situations in which it was desirable to fit a second-order model:

$$y = \beta_0 \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \sum_{j \geq i}^k \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

where β_{ij} represents the coefficients of the interaction parameters. These designs include a central point employed to determine the curvature, and the determination of critical or optima conditions are deduced from the above second-order function by including quadratic terms:

$$y = \beta_0 \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{i=1}^k \sum_{j \geq i}^k \beta_{ij} x_i x_j + \varepsilon \quad (3)$$

where β_{ij} represents the coefficients of the quadratic parameters. So, the experimental data allow the development of empirical models describing the interrelationship between operational and experimental variables by equations including linear, interaction and quadratic terms.

In this study, the growing conditions of the fluvial biofilm were optimized by means of an incomplete 3^3 factorial design (Box et al. 1978), in order to study the influence of the ionic concentration (x_1), organic carbon concentration (x_2) and nitrogen/phosphorus ratio (x_3) on chlorophyll *a* (y_1), carbohydrate (y_2) and heterotrophic biomass (y_3) concentrations. So, the quadratic function obtained for the three variables is described as following:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 \quad (4)$$

where y is the dependent variable, β denotes the regression coefficients (calculated from experimental data by multiple regressions using the least-squares method) and x denotes the independent variables. The experimental data were analyzed by the response surface method using the Statistica 7.0 software.

The standardized (coded) dimensionless independent variables employed, with variation limits (−1, 1), were defined as x_1 (coded ionic concentration), x_2 (coded organic carbon source) and x_3 (coded N/P ratio). The correspondence between coded and uncoded variables was established by linear equations deduced from their respective variation limits, according to the following equation (Bezerra et al. 2008):

$$x_i = \left(\frac{z_i - z_i^0}{\Delta z_i} \right) \beta_d \quad (5)$$

where Δz_i is the distance between the real value in the central point and the real value in the superior or inferior level of a variable, β_d is the major coded limit value in the matrix for each variable and z^0 is the real value in the central point. Coded variables are then assigned values of −1, 0 and +1, corresponding to the lowest, central and maximum variation limits for each variable. Thus, the response surface obtained from the coded variables is not influenced by the magnitude of each variable, allowing the combination of factors into a dimensionless scale.

2.3 Extraction and determination of biofilm components: chlorophyll *a*, extracellular carbohydrate and heterotrophic population

Chlorophyll *a* was extracted from a suspension of the bed sediments in the liquid phase with DMSO by following the methodology optimized by Devesa et al. (2007) and determined in a UV–visible spectrophotometer (Varian Cary 100) following the methodology proposed by Wellburn (1994), by applying the following equation:

$$\text{Chla} = 12.47A_{665.1} - 3.62A_{649.1} \quad (6)$$

where Chla represents the concentration of chlorophyll *a* and $A_{665.1}$ and $A_{649.1}$ represent the absorbance of the extracts at 665.1 and 649.1 nm, respectively.

The extracellular carbohydrate concentrations were also determined in the suspension of the bed sediments in the liquid phase by using the phenol–sulphuric acid method proposed by Dubois et al. (1956). Standard solutions were prepared with a glucose stock, and the carbohydrate absorbance was measured at 492 nm (UV–visible spectrophotometer, Varian Cary 100).

The heterotrophic population of the biofilm samples was estimated by the most probable number (MPN) method. The biofilm suspensions obtained after the time of incubation were diluted in 10-fold series up to 10^{-7} . Fifty-microlitre aliquots were inoculated in 96-well microtiter plates containing 150 ml/well of liquid yeast extract medium (1.0 g yeast extract, 1.0 g glucose, 0.5 g KNO_3 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g K_2HPO_4 , 0.1 g CaCl_2 , 0.1 g NaCl , 0.01 g FeCl_3 , in 1 l deionized water) plus oligoelements (1.5 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 mg H_3BO_3 , 0.19 mg CoCl_2 , 0.1 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.08 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.036 mg NaMoO_4 , 0.024 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$). The redox dye, tetrazolium violet (2,5-diphenyl-3-(α -naphthyl) tetrazolium chloride (TV), 15 mM), was used to indicate growth and was added to media using the ratio 1:100 (v/v, TV: media; Kidd et al. 2008). Tetrazolium violet serves as an indicator of dehydrogenase activity, forming a deep purple precipitate

upon reduction by electrons flowing through the electron transport system and by superoxide radicals (Kennedy 1994). Four wells were inoculated per each level of dilution, and two replicate plates of each sediment were prepared. After 1, 3 and 8 weeks of incubation at 25°C, the positive wells were recorded and the MPN values were obtained from the appropriate tables. The MPN values obtained were corrected for the initial dilution and volume inoculated and expressed as log₁₀MPN per gram dry sediment (Kidd et al. 2008). The MPN method has been traditionally employed in soil/sediment studies, and it presents the advantage that it measures only living organisms. However, MPN results are estimates of the populations thus having a lower order of precision when compared with other methods.

3 Results and discussion

3.1 Model statistical testing

In this work, an incomplete 3³ factorial design was carried out, in order to elucidate the influence of three environmental factors for the growth and development of biofilms in river bed sediments. Table 1 shows the set of experimental conditions assayed for the independent variables (expressed in terms of coded variables). Moreover, Table 1 also lists the dependent variables considered y_1 , y_2 and y_3 which correspond to chlorophyll *a*, extracellular carbohydrate concentrations and density of cultivable heterotrophs, respectively.

The set of experimental conditions assayed (expressed as coded variables), as well as the experimental data obtained for variables y_1 , y_2 and y_3 , are displayed in Table 2. The sequence for the experimental work was randomly established to limit the influence of systematic errors on the interpretation of results. It can be noted that experiments 1–12 allowed the calculation of the regression coefficients, whereas experiments 13–15 were replicates in the central point of the design to estimate the influence of the experimental error.

The significance of each coefficient was determined by *P* values for the variables y_1 and y_2 , corresponding to chlorophyll *a* and carbohydrates concentrations, respectively (Table 3). The statistical analysis of the heterotrophic population (y_3) did not allow the development of empirical models that describe the interrelationship between operational and experimental variables. Consequently, the coefficients for y_3 were not included in Table 3. Table 4 shows the statistical parameters for y_1 and y_2 (r^2 and *F*) measuring the correlation and the statistical significance of the models, respectively. It can be noted that both models (for y_1 and y_2) showed good

Table 2 Operational conditions considered in this study (expressed in terms of the coded independent variables dimensionless IC x_1 , dimensionless carbon source x_2 and dimensionless N/P ratio x_3) and experimental results achieved for the dependent variables y_1 (chlorophyll *a*), y_2 (carbohydrate) and y_3 (heterotrophic biomass)

Exp	Independent variables			Dependent variables		
	x_1	x_2	x_3	y_1	y_2	y_3 (log MPN)
1	0	-1	-1	3.4	0.68	3.1
2	0	1	-1	4.5	2.89	4.0
3	0	-1	1	3.4	0.68	5.1
4	0	1	1	12.7	7.16	5.5
5	-1	-1	0	0.8	0.01	5.0
6	-1	1	0	2.0	2.68	3.2
7	1	-1	0	17.3	0.01	0.1
8	1	1	0	36.6	71.04	2.4
9	-1	0	-1	0.7	0.79	5.1
10	-1	0	1	0.1	0.14	4.9
11	1	0	-1	11.7	53.48	4.1
12	1	0	1	1.4	2.01	4.0
13	0	0	0	15.3	3.99	5.1
14	0	0	0	13.7	3.6	6.1
15	0	0	0	14.5	5.24	4.8

y_1 (chlorophyll *a*; milligrams per litre), y_2 (carbohydrate; milligrams per litre) and y_3 (log MPN) (heterotrophic density; number of cells per gram)

statistical parameters for correlation and significance, allowing a close reproduction of experimental data. The results of the *F* test obtained for chlorophyll *a* and carbohydrates suggested the appropriate fit of data to the model, as the factors and the interactions considered in the experimental design were statistically significant ($P < 0.05$) at the 95% confidence level (see Table 4). Additionally, the fit of the model was evaluated through the regression coefficients and the significance level, based on the *F* test

Table 3 Regression coefficients and their statistical significance for the variable y_1 to y_2

Regression coefficients	y_1	P_{y_1}	y_2	P_{y_2}
b_0	14.5	0.0010	3.96	0.0025
b_1	7.92	0.0013	15.37	0.0001
b_{11}	-1.42	0.0758	12.87	0.0002
b_2	3.86	0.0053	10.30	0.0001
b_{22}	1.10	0.1184	1.61	0.0119
b_3	-0.34	0.3551	-5.98	0.0004
b_{33}	-9.60	0.0019	-2.72	0.0042
b_{12}	4.52	0.0077	17.09	0.0001
b_{13}	-2.42	0.0261	-12.71	0.0002
b_{23}	2.05	0.0360	1.07	0.0246

Table 4 Statistical parameters (r^2 and F) and significance level of model for the dependent variable based on the F test

Variable	r^2	F_{exp}	Significance level (based on the F test)
y_1	0.82	2.46	89
y_2	0.85	3.20	94

(see Table 4) which was calculated by taking into account linear, interaction and quadratic coefficients, so from the initial three independent variables were obtained ten parameters that explain the variability of the two dependent variables studied. The model suggests that the 82% ($r^2=0.82$) and 85% ($r^2=0.85$) of the variance for chlorophyll a and extracellular carbohydrates, respectively, can be explained as a function of the ionic concentration, carbon input and N/P ratio. Thus, only a minor percentage of both chlorophyll a and extracellular carbohydrates, varying from 15% to 18%, may be attributed to other factors different from the dependent variables analyzed in this study. Consequently, Eqs. 7 and 8 may serve, respectively, as proxies both for chlorophyll a and extracellular carbohydrate production in the biofilms as a function of the nutrients considered:

$$\begin{aligned} \text{Chla production (mg l}^{-1}\text{)} = & 14.5 + 7.92 \times (\text{IC}) + 3.86 \times \text{C} \\ & - 9.60 \times (\text{N/P})^2 + 4.52 \times (\text{IC}) \\ & \times \text{C} - 2.42 \times \text{C} \times (\text{N/P}) + 2.05 \\ & \times \text{C} \times (\text{N/P}) \end{aligned} \quad (7)$$

$$\begin{aligned} \text{Extracellular carbohydrate production (mg l}^{-1}\text{)} = & 3.96 + 15.37 \times (\text{IC}) + 12.87 \times (\text{IC})^2 + 10.30 \\ & \times \text{C} + 1.61 \times \text{C}^2 - 5.98 \times (\text{N/P}) - 2.72 \\ & \times (\text{N/P})^2 + 17.09 \times (\text{IC}) \times \text{C} - 12.71 \times (\text{IC}) \\ & \times (\text{N/P}) + 1.07 \times \text{C} \times (\text{N/P}) \end{aligned} \quad (8)$$

where IC is the ionic concentration, C is the carbon dose and N/P is the nitrogen/phosphorus ratio.

The above equations were deduced by ignoring those coefficients with an insignificance level at the 95% confidence level ($P>0.05$). So, in the case of chlorophyll a , the linear factor N/P and the interaction factors $(\text{IC})^2$ and C^2 were considered insignificant, whereas all linear, interaction and quadratic terms were significant for the extracellular carbohydrate concentration. From the experimental results and

after the statistical treatment of data, it was observed that the most influential independent variables, in the range tested, were ionic and carbon concentration, whereas the N/P ratio gave the lowest coefficient values (see Table 3). Also, it was noticeable that the statistical coefficients for x_1 and x_2 have positive values, which means that the assayed dependent variables increased with the amount of ionic concentration and carbon tested, whereas the statistical coefficient for x_3 gave a negative value, which means that chlorophyll a and extracellular carbohydrate concentrations decreased with increasing N/P ratios, in the range tested.

3.2 Interaction among dependent variables producing the biofilm

Figures 2 and 3 show the dependence of chlorophyll a and extracellular carbohydrates, respectively, on the ionic and carbon concentration and N/P ratios in the range assayed. The desirability profiles of chlorophyll a and carbohydrates show the levels of the independent variables which produce the most desirable predicted responses of the dependent variables. A visual inspection of the 3D surfaces thus obtained provides information about the optima conditions of the experiment.

Figure 2a shows that the maximum chlorophyll a production is outside the experimental region and was not achieved with the carbon and ionic concentrations tested, which cover the range considered environmentally relevant. Moreover, this fact highlights the importance of these factors in the autotrophic component of the biofilm. Figure 2b, c shows the maximum chlorophyll a production for the combination of N/P and IC or C, respectively. Figure 2c shows a plateau for the N/P ratio in relation to carbon dose, indicating that the N/P variation is the less relevant in the chlorophyll a production.

In relation to the extracellular carbohydrate production, Fig. 3 shows that carbohydrate production may be minimized under low glucose and NaCl concentrations. Again, a plateau is observed in the extracellular carbohydrate production with the NaCl concentrations and the N/P ratios. So, the concentration of chlorophyll a and carbohydrates in the biofilm increased with the concentration of NaCl up to 31.7 and 72.3 mg/l, respectively. Also, the model predicts that the extracellular carbohydrates will achieve the highest concentration using the lowest N/P ratio. So, if the N/P ratio is fixed at the lowest level tested and at the highest ionic and glucose concentration, the model predicts a production of chlorophyll a around 16.3 mg/l and extracellular carbohydrates concentrations about 76.1 mg/l (Table 5).

When comparing the Figs. 2 and 3, it can be observed that those conditions that produce the highest extracellular carbohydrates concentration also give the highest chloro-

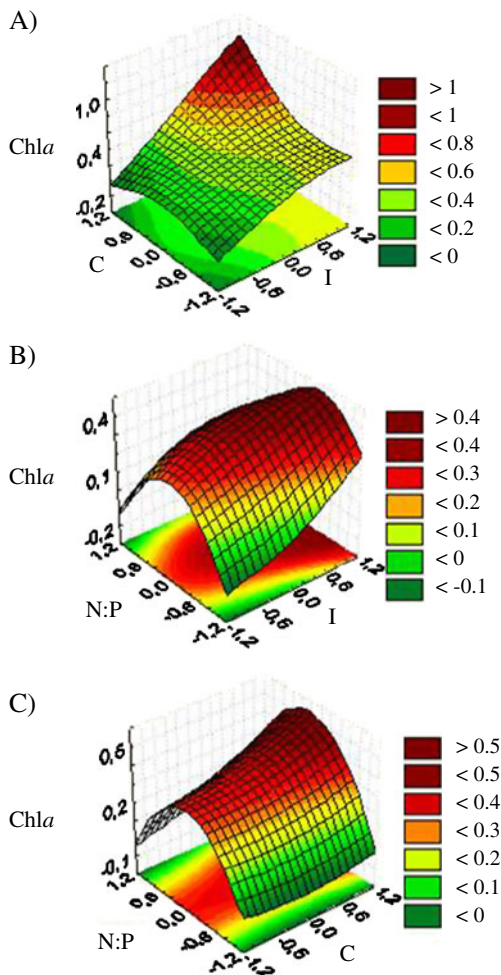


Fig. 2 Desirability profiles obtained by the spline fit method for the evaluation of the dependence of chlorophyll *a* (milligrams per litre) with a concentration of ionic concentration (*I*) and glucose (*C*), **b** N/P ratio and ionic concentration (*I*) and **c** N/P ratio and glucose (*C*) concentration

phyll *a* values, showing a close reproduction between the observed and predicted data (Fig. 4). So, this fact may suggest that the biofilm presents an important autotrophic component.

The heterotrophic organisms may also be an important contributor to the total carbohydrate concentrations of the biofilms. The MPN method gave heterotrophic populations varying between 2.5×10^2 and 1.2×10^6 MPN/g (d.w.), which were highly affected by the carbon concentration of the medium. However, the uncertainty associated to the MPN method did not allow the construction of an empirical model with the variables assayed.

Finally, it is important to mention that the optima conditions of N/P, glucose and ionic concentration obtained in this study may be easily reached in the fluvial ecosystem. The analyses of the Anllóns River waters (data not shown) present a N/P ratio of 5.25, very close to the experimentally optimized in this study (N/P=5). The glucose concentration

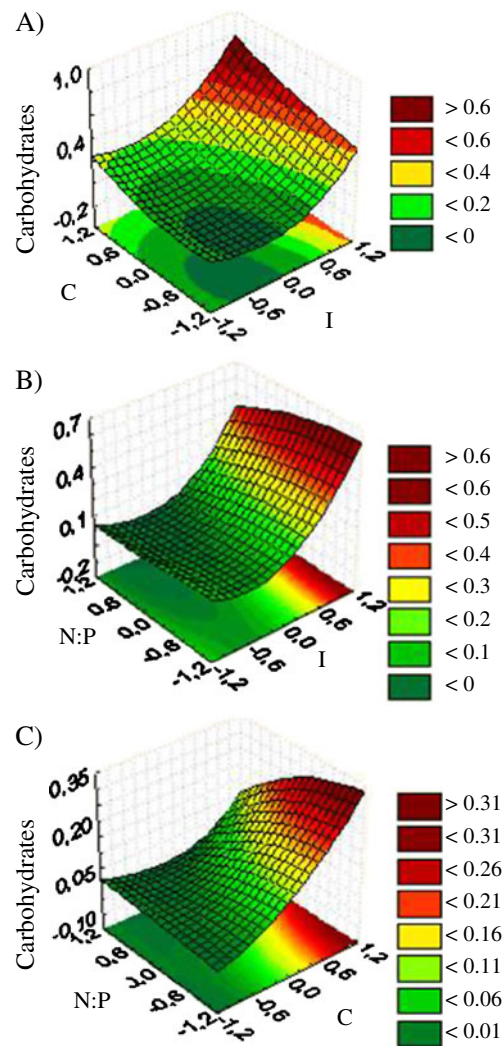


Fig. 3 Desirability profiles obtained by the spline fit method for the evaluation of the dependence of carbohydrates (milligrams per litre) with a concentration of ionic concentration (*I*) and glucose (*C*), **b** N/P ratio and ionic concentration (*I*) and **c** N/P ratio and glucose (*C*) concentration

of 10 mg/l was not reached at the cleanest sites of the Anllóns River, although it was observed at most contaminated sites, so favouring the formation of biofilms. Also, the optima conditions for the biofilm development are achieved under the highest ionic concentrations tested (25 mg/l) observed in the Anllóns watercourse. These results are in concordance with those published by Macarelli et al. (2009). These authors also found that P additions decreased chlorophyll *a* concentration, although there never was a concomitant decrease in the biofilm ash-free dry mass, which includes algal biomass and heterotrophic components such as bacteria and fungi. Moreover, Staats et al. (2000), working with the epipellic diatom *Cylindrotheca closterium*, found that EPS accumulation was stimulated when cells were resuspended in a medium lacking N or P, or with low N or P concentrations (95.5 μ M

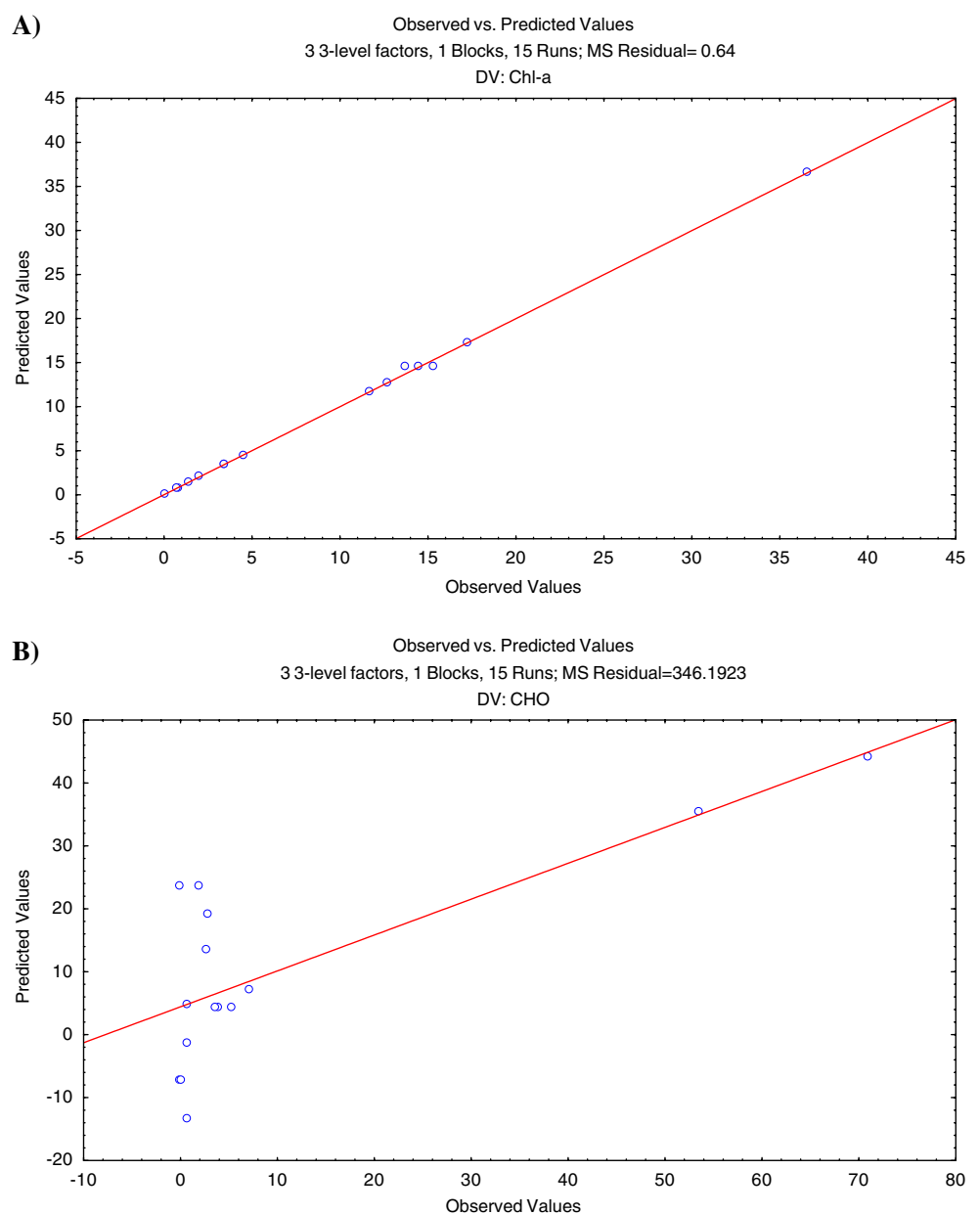
Table 5 Optima conditions predicted by the model for obtaining the maximum concentration of chlorophyll *a* and extracellular carbohydrates

	Independent variables			Dependent variables	
	Ionic concentration (mg/l)	Carbon (mg/l)	N/P (mg/l)	Chlorophyll <i>a</i> (mg/l)	Carbohydrates (mg/l)
Optimum conditions	25	10	5	16.3	76.1

NH_4Cl and $7.7 \mu\text{M NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$). Also, the potential of algal assemblages to form biofilms and the influence of nutrient disposal was studied by Underwood and Paterson (2003), who described the complex microhabitat created by benthic diatoms. A nutrient-rich environment may produce a larger portion of diatom-derived biofilm which, in

addition, provides a greater biodiversity as diatom EPS consists predominantly of carbohydrate-rich polymers (Underwood et al. 2004) increasing the stability of the autotrophic biofilm formed. Thus, as the biofilms formed on the bed sediments have been defined by other authors as ecosystem engineers (Kristensen 2008; Gerbersdorf et al.

Fig. 4 Relation between the observed and the predicted data for the dependent variables assayed chlorophyll *a* (a) and carbohydrates (b), respectively



2009; Schaller et al. 2010), their formation in natural streams may be exploited in bioremediation strategies due to their ability to retain pollutants by creating artificial wetlands where the laminar flow may favour the retention of such pollutants in the biofilms formed on the bed sediments.

4 Conclusions

This study explores the advantages of an incomplete factorial design in order to study the effects of three environmental variables (ionic concentration (NaCl), organic carbon (supplemented as glucose) and N/P ratio) on chlorophyll *a* and extracellular carbohydrate developed from inoculates of river sediments incubated at a laboratory microcosms scale. Heterotrophic density was included as a dependent variable in this study, although it did not render satisfactory results. The results obtained will enhance our knowledge about the environmental conditions affecting the development of natural biofilms in river bed sediments.

This work demonstrates that it is possible to develop empirical models applied to the biofilms developed over river bed sediments that describe the interrelationship between nutrient concentration and the extracellular carbohydrates as well as chlorophyll *a*. The model suggests that the 82% ($r^2=0.82$) and 85% ($r^2=0.85$) of the variance for chlorophyll *a* and extracellular carbohydrates, respectively, can be explained as a function of the ionic concentration, carbon input and N/P ratio. The ionic and carbon concentrations in the ranged tested were the most influential variables in the production of extracellular carbohydrates and chlorophyll *a*. It can be concluded that concentrations of 25 mg/l of ionic concentration—supplemented as NaCl—and 10 mg/l of carbon—supplemented as glucose—improve the biofilm formation from river bed sediments, whereas N/P ratio higher than 5, in the range tested, produced a negative effect in the production of extracellular carbohydrates and chlorophyll *a* and consequently in the development of the biofilm.

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