Journal of Hazardous Materials 168 (2009) 1346–1358

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

About the performance of *Sphaerotilus natans* to reduce hexavalent chromium in batch and continuous reactors

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ARTICLE INFO

Article history:
Received 24 November 2008
Received in revised form 3 March 2009
Accepted 4 March 2009
Available online 18 March 2009

Keywords:
Biological reduction
Cr(VI)
Batch culture
Continuous culture
Sphaerotilus natans

ABSTRACT

The hexavalent chromium biological reduction constitutes a safe and economical detoxification procedure of wastewaters containing Cr(VI). However, little research has been done to evaluate Cr(VI) tolerance and reduction capacity of microbial cultures under different growth conditions. The aims of this work were (a) to evaluate the capacity of *Sphaerotilus natans* to reduce Cr(VI) to Cr(III) in a continuous system limited in carbon and energy source or in nitrogen source, (b) to evaluate the toxic effect of Cr(VI) on this microorganism, (c) to carry out a complete analysis of Cr(VI) reduction by *S. natans* not only in continuous regime but also in batch system, and (d) to model the obtained results mathematically. *S. natans* exhibited great resistance to Cr(VI) (19–78 mg l^{-1}) and optimal growth in continuous and batch systems using a mineral medium supplemented only with citric acid as organic substrate. In carbon- and energy-limited continuous systems, a maximum percentual decrease in Cr(VI) by 13% was reached for low influent Cr(VI) concentration (4.3–5.32 mgCr(VI) l^{-1}); the efficiency of the process did not notoriously increase as the length of cellular residence time was increased from 4.16 to 50 h. A nitrogen-limited continuous operation with a cellular residence time of 28.5 h resulted in a Cr(VI) decrease of approximately 26–32%. In batch system, a mathematical model allowed to predict the Cr(VI) concentration as a function of time and the ratio between the initial Cr(VI) concentration and that of the biomass. High concentrations of initial Cr(VI) and biomass produced the highest performance of the process of Cr(VI) reduction reached in batch system, aspects which should be considered in detoxification strategies of wastewaters.

1. Introduction

Chromium can be found in the environment, mainly as Cr(III) and Cr(VI) [1], its biological effect being highly dependent on its oxidation state. Trivalent chromium is more stable, less soluble and less toxic than hexavalent chromium [2]. Cr(VI) is an oxidizing agent highly toxic for all life forms [3]. Both wastewater effluent discharge and the inadequate disposal of wastes and sub-products of several industrial activities using Cr(VI) have generated serious environmental pollution [4]. Cr(VI) behaves as an oxyanion (CrO_4^{2-}, $Cr_2O_7^{2-}$) in water solution.

The World Health Organization and the Environmental Protection Agency of the USA have set a maximum limit of Cr(VI) for domestic uses of water of 50 μg l^{-1}.

The conventional physicochemical methods of chromium removal comprise: chemical reduction, ionic interchange and activated carbon adsorption. The use of chemical reducers, followed by alkaline precipitation, generates great amounts of chemical sludges. The ionic interchange and the adsorption are very costly processes [5]. The Cr(VI) biological reduction constitutes an alternative to the conventional methods used in the detoxification of wastewaters. The Cr(VI) biological reduction has been considered a safe, sustainable and economical detoxification process [6].

Most Cr(VI) biological reduction studies have been aimed at establishing the biochemical mechanisms involved in Cr(VI) reduction [7–9], the kinetic analysis [10–12], and the determination of optimal conditions such as pH and temperature [13].

Hexavalent chromium inhibits the nucleic acids synthesis, affecting both the microbial growth rate and the biomass yield [14]. Therefore, the appropriate design of Cr(VI) biological reduction systems not only requires the knowledge of the reduction mechanisms and the factors governing the kinetics of the process but it is also essential to evaluate Cr(VI) toxicity on the microbial cultures used.

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doi:10.1016/j.jhazmat.2009.03.018

Nomenclature

a	coefficient (mgCr(VI) l^{-1})
b	chromium(VI) reduction coefficient (mgCr(VI)$_0$ ($gVSS_0$ $h)^{-1}$)
COD	chemical oxygen demand (mgO_2 l^{-1})
COD_B	chemical oxygen demand corresponding to the biomass (mgO_2 l^{-1})
COD_S	soluble chemical oxygen demand corresponding to organic substrate (mgO_2 l^{-1})
COD_T	total chemical oxygen demand (mgO_2 l^{-1})
$[Cr]_{soluble\,total}$	total soluble chromium concentration (mgCr l^{-1})
$[Cr]_{total}$	total chromium concentration (mgCr l^{-1})
$[Cr(III)]_{soluble}$	soluble Cr(III) concentration (mgCr(III) l^{-1})
[Cr(VI)]	Cr(VI) concentration at different times of batch culture (mgCr(VI) l^{-1})
$[Cr(VI)]_0$	Cr(VI) concentration at the start of batch culture (mgCr(VI) l^{-1})
$[Cr(VI)]_s$	Cr(VI) concentration for steady state condition of continuous culture (mgCr(VI) l^{-1})
$[Cr(VI)]_{E0}$	Cr(VI) concentration at the initial state of the exponential phase of batch culture (mgCr(VI) l^{-1})
$[Cr(VI)]_I$	Cr(VI) concentration in the influent of the bioreactor (mgCr(VI) l^{-1})
$[Cr(VI)]_{soluble}$	soluble Cr(VI) concentration (mgCr(VI) l^{-1})
D	dilution rate (h^{-1})
D_0	ratio between initial Cr(VI) concentration and that of biomass (mgCr(VI)$_0$ ($gVSS_o$)$^{-1}$)
D_S	ratio between influent Cr(VI) concentration and biomass concentration in steady state (mgCr(VI) (gVSS)$^{-1}$)
k	coefficient ((gVSS h)$^{-1}$ l)
k'	apparent chromium(VI) reduction coefficient (h^{-1})
$k_{Cr(VI)}$	half velocity constant (mgCr(VI) l^{-1})
n	coefficient
$q_{Cr(VI)}$	specific Cr(VI) reduction rate (mgCr(VI) (gVSS h)$^{-1}$)
R	maximum Cr(VI) reduction capacity (mgCr(VI) (gVSS)$^{-1}$)
$r_{Cr(VI)}$	Cr(VI) reduction rate (mgCr(VI) (l h)$^{-1}$)
$r_{Cr(VI)max}$	maximum Cr(VI) reduction rate (mgCr(VI) (l h)$^{-1}$)
r_F	substrate feed rate ($mgCOD_S$ (l h)$^{-1}$)
r_S	substrate consumption rate ($mgCOD_S$ (l h)$^{-1}$)
r_X	microbial growth rate (mgVSS (l h)$^{-1}$)
[S]	organic substrate concentration at different time in batch culture ($mgCOD_S$ l^{-1})
$[S]_S$	organic substrate concentration corresponding to steady state condition of continuous culture ($mgCOD_S$ l^{-1})
$[S]_{E0}$	organic substrate concentration at the initial state of the exponential phase of batch culture ($mgCOD_S$ l^{-1})
$[S]_I$	organic substrate concentration in the influent of the bioreactor ($mgCOD_S$ l^{-1})
T	time (h)
VSS	volatile suspended solids
[X]	biomass concentration (mgVSS l^{-1})
$[X]_A$	active biomass concentration with reductase activity (gVSS l^{-1})
$[X]_0$	biomass concentration at the start of batch culture (mgVSS l^{-1})
$[X]_S$	biomass concentration corresponding to steady state condition of continuous culture (mgVSS l^{-1})
$Y_{Cr(VI)/S}$	Cr(VI) reduction yield (mgCr(VI) ($gCOD_S$)$^{-1}$).
$Y_{X/S}$	biomass yield based on consumed organic substrate (mgVSS ($mgCOD_S$)$^{-1}$)
θ_C	cellular residence time (h)
θ_H	hydraulic residence time (h)
μ	specific growth rate (h^{-1})

The toxic effect of Cr(VI) over a pure or mixed microbial culture can be measured through different techniques: inhibition of respiratory activity [15,16], measurement of microbial growth coefficients [17], quantification of kinetic parameters or estimation of the maximum biomass concentration reached at the end of the growth [18].

It has been reported that the specific Cr(VI) reduction rate and the toxic effect of Cr(VI) over the microorganisms depend notoriously on both the physiological state of the culture and the growth conditions [9,18]. However, very few studies evaluate the biological process of Cr(VI) reduction in the different stages of microbial growth or under different culture conditions. In most of these studies, the comparative analysis of Cr(VI) reduction rate and the microbial resistance to this metal along the different stages of growth is generally carried out qualitatively and without considering the microbial growth along the assays [19–22] as well as under zero growth conditions [23].

Most studies of Cr(VI) reduction were performed in batch systems, being scarce those carried out under a continuous regime [24,25]. It should be considered that batch culture generally guarantees surplus supply of all essential nutrients. In continuous systems, a specific nutrient can limit the microbial growth, and it could also affect the performance of the bioreduction process.

In general, in batch and continuous systems, the performance of different organic substrates (such as electron donors) to reduce Cr(VI) to Cr(III) is evaluated [26], without considering the effect of other essential nutrients of the microbial metabolism (such as nitrogen) on the process of Cr(VI) reduction.

In a previous work, Caravelli et al. [16] reported that *Sphaerotilus natans*, a filamentous microorganism present in activated sludge systems, is able to reduce Cr(VI) to Cr(III) using glucose as an energy source in aerobic batch system under stationary growth conditions (zero growth rate). However, the ability of this microorganism to reduce Cr(VI) under different growth conditions has not yet been evaluated.

The aims of this work were (a) to evaluate the capacity of *S. natans* to reduce Cr(VI) to Cr(III) in a continuous system limited in carbon and energy source or nitrogen source, (b) to evaluate the toxic effect of Cr(VI) on this microorganism, (c) to carry out a complete analysis of Cr(VI) reduction by *S. natans* not only in continuous regime but also in batch system, and (d) to model the obtained results mathematically.

2. Materials and methods

2.1. Bacteria and culture conditions

S. natans ATCC #29329 was cultured using monohydrate citric acid as carbon and energy source and ammonium sulphate as nitrogen source. The complete growth medium (M1) was described by Caravelli et al. [16]. The culture medium was sterilized at 121 °C for 45–60 min except vitamin B12, which was sterilized by membrane filtration (0.45 μm Millipore HA) and then added to the sterile

medium. The pH of the culture medium was adjusted to 7.0 with NaOH before autoclaving.

2.2. Operating conditions of the continuous and batch bioreactors

The experiments were carried out using two bioreactors, one in batch mode and the other in continuous form. Both bioreactors (1 l volume) were operated aseptically under the following growth conditions: temperature = 30 °C, air flow rate = 2 l min^{-1}, rotor speed = 600 rpm and dissolved oxygen concentration above 2 $mgO_2\,l^{-1}$. A constant pH 7.0 was maintained throughout the course of the bioreactor operation by automatic addition of 1 M H_2SO_4. At regular intervals samples were taken from the bioreactors to determine organic substrate, biomass and soluble Cr(VI) concentrations.

2.3. Preliminary experiments to analyze the reduction capacity of S. natans over Cr(VI)

Preliminary assays were carried out in batch system to analyze the ability of *S. natans* to reduce Cr(VI) to Cr(III) using citric acid as the unique electron donor. *S. natans* was inoculated in the batch reactor containing 500 ml medium M1 with agitation. Once growth was observed, 250 ml culture was removed from the bioreactor and approximately 250 ml medium M1 was added. A pulse of a stock $K_2Cr_2O_7$ solution (4 gCr(VI) l^{-1}), previously sterilized (121 °C, 15 min), was immediately added producing an initial concentration of 18 mgCr(VI) l^{-1}.

The concentrations of soluble Cr(VI) and total chromium were analyzed as functions of time. Control tests without Cr(VI) were also conducted.

2.4. Experiments to analyze Cr(VI) reduction by S. natans using aerobic continuous culture limited in carbon and energy source or in nitrogen source

Two types of experiments were carried out to analyze the Cr(VI) tolerance and reduction of *S. natans* in an aerobic continuous bioreactor limited in (a) carbon and energy source (CES) and (b) nitrogen source (NS). The bioreactor was inoculated with *S. natans* and was operated without recycling biomass at different dilution rates in order to determine the effect of the physiological state of the microorganism (growth rate) on Cr(VI) reduction. Control tests without Cr(VI) were also conducted for each experiment.

2.4.1. Continuous bioreactor limited in CES

In this case different dilution rates were set (D = 0.02, 0.065, 0.12 and 0.24 h^{-1}) which corresponded to hydraulic and cellular residence times ($\theta_H = \theta_C = 1/D$) of 50.0, 15.38, 8.33 and 4.16 h, respectively. Growth medium M1 was supplemented with different volumes of a stock $K_2Cr_2O_7$ solution (4 gCr(VI) l^{-1}), previously sterilized at 121 °C for 15 min, to obtain Cr(VI) concentrations ranging from 4.3 to 80.1 mgCr(VI) l^{-1} (culture medium M2).

S. natans was inoculated in the bioreactor containing the growth medium M1 without Cr(VI). The bioreactor was operated as a batch until the microbial growth was evident, then under continuous culture regime (D = 0.12 h^{-1}) for approximately 48 h to generate enough biomass. At this time, growth medium M1 was replaced by the culture medium M2 (4.3–80.1 mgCr(VI) l^{-1}) and the bioreactor was maintained under continuous regime for about 10 θ_C, allowing a gradual adaptation of *S. natans* to Cr(VI). In each experiment, a different Cr(VI) concentration of the M2 medium was used; M1 and M2 media have an initial COD_S:N:P ratio of approximately 100:8:5, being COD_S the chemical oxygen demand corresponding to the organic substrate.

At different θ_C, concentrations of organic substrate, biomass and soluble Cr(VI) were determined.

2.4.2. Continuous bioreactor limited in NS

The bioreactor was inoculated and operated similarly to the way it was described in the previous section, according to the initial growth under batch conditions followed by the continuous operation. The growth medium M1 was modified to obtain citric acid and $(NH_4)_2SO_4$ concentrations of 4600 and 721 mg l^{-1}, respectively, giving an initial COD_S:N:P ratio of approximately 100:4:4 (culture medium M1NL) to guarantee excess of carbon and energy source. The bioreactor was operated at D = 0.11 h^{-1} using the culture medium M1NL supplemented with different volumes of the stock $K_2Cr_2O_7$ solution (culture medium M2NL: 4.6–78.7 mgCr(VI) l^{-1}). In each experiment, a different Cr(VI) concentration of the M2NL medium was used.

At different θ_C, concentrations of organic substrate, biomass and soluble Cr(VI) were determined.

2.5. Cr(VI) reduction by S. natans in aerobic batch culture

Cr(VI) tolerance and reduction by *S. natans* was also evaluated in batch culture under different initial Cr(VI) (19–78 mgCr(VI) l^{-1}) and biomass concentrations (170–500 mgVSS l^{-1}). The purpose of these experiments was to determine the favorable experimental conditions to achieve a good performance of the process of Cr(VI) reduction; for this, kinetic and stoichiometric parameters corresponding to both microbial growth and Cr(VI) reduction were determined.

The bioreactor was inoculated and operated in batch mode and then in continuous mode according to what was explained in the Section 2.4.1. After a period of continuous operation of approximately 5 θ_C (M2 medium), different culture volumes (200–500 ml) were removed from the reactor and introduced to the batch reactor containing the same M2 medium in order to obtain initial biomass concentrations ranging from 170 to 500 mgVSS l^{-1}.

The batch bioreactor was operated for 24–32 h. A pulse of a stock citric acid solution (127 gCOD l^{-1}, pH 7.0) was added to the bioreactor in those cases with high initial biomass concentration (400–500 mgVSS l^{-1}). Thus, a comparable initial ratio between organic substrate and biomass (COD_S:VSS) for the different batch experiments was obtained (7.0–12.5 $mgCOD_S$/mgVSS). Experiments were run in duplicates. Control tests without Cr(VI) were also conducted.

At different contact times, concentrations of organic substrate, biomass and soluble Cr(VI) were measured.

2.6. Measurement of the kinetic and stoichiometric parameters of S. natans

The toxic effect of Cr(VI) on *S. natans* was evaluated by the determination of the biomass yield ($Y_{X/S}$, mgVSS ($mgCOD_S$)$^{-1}$). Besides, the process of Cr(VI) reduction was evaluated through the following parameters: Cr(VI) reduction rate ($r_{Cr(VI)}$, mgCr(VI) (l h)$^{-1}$), specific Cr(VI) reduction rate ($q_{Cr(VI)}$, mgCr(VI) (gVSS h)$^{-1}$) and the Cr(VI) reduction yield ($Y_{Cr(VI)/S}$, mgCr(VI) ($gCOD_S$)$^{-1}$).

In the case of the continuous bioreactor operated under steady state conditions, the biomass yield was calculated as follows:

$$Y_{X/S} = \frac{r_X}{r_S} = \frac{D[X]_S}{D([S]_I - [S]_S)} \quad (1)$$

where r_X is the microbial growth rate (mgVSS (l h)$^{-1}$), $[X]_S$ the biomass concentration in steady state (mgVSS l^{-1}), r_S is the substrate consumption rate ($mgCOD_S$ (l h)$^{-1}$), $[S]_I$ is the concentration

of organic substrate in the influent medium of the bioreactor ($mgCOD_S\,l^{-1}$) and $[S]_S$ is the corresponding value to steady state (which is equivalent to that of the effluent of the bioreactor).

In continuous bioreactor (steady state conditions), the Cr(VI) reduction parameters were determined as follows:

$$\text{Cr(VI) reduction rate, } r_{Cr(VI)} = D([Cr(VI)]_I - [Cr(VI)]_S)\,(mgCr(VI)\,(l\,h)^{-1}) \quad (2)$$

where $[Cr(VI)]_I$ is the Cr(VI) concentration of the influent of the bioreactor (culture medium M2 or M2NL) and $[Cr(VI)]_S$ is the steady state value

$$\text{specific Cr(VI) reduction rate, } q_{Cr(VI)} = \frac{r_{Cr(VI)}}{[X]_S}\,(mgCr(VI)\,(gVSS\,h)^{-1}) \quad (3)$$

$$\text{Cr(VI) reduction yield, } Y_{Cr(VI)/S} = \frac{r_{Cr(VI)}}{r_S}\,(mgCr(VI)\,(gCOD_S)^{-1}) \quad (4)$$

which corresponds to the amount of Cr(VI) reduced per unit of organic substrate consumed.

For batch culture, in exponential growth phase, the following parameters were determined: Biomass yield ($Y_{X/S}$, mgVSS $(mgCOD_S)^{-1}$) was determined from the slopes of the graphs: biomass concentration ([X]) vs. consumed organic substrate ($[S]_{E0} - [S]$), where $[S]_{E0}$ is the organic substrate concentration at the initial state of the exponential phase and [S] the value corresponding to different times. Cr(VI) reduction rate ($r_{Cr(VI)}$, mgCr(VI) $(l\,h)^{-1}$) was estimated from the slope of the graphs [Cr(VI)] vs. time. Specific Cr(VI) reduction rate ($q_{Cr(VI)}$, mgCr(VI) $(gVSS\,h)^{-1}$) considered the average concentration of biomass between the initial value and the highest biomass concentration reached in each assay. Cr(VI) reduction yield ($Y_{Cr(VI)/S}$, mgCr(VI) $(gCOD_S)^{-1}$) was determined from the slope of the graphs: total amount of reduced Cr(VI) ($[Cr(VI)]_{E0} - [Cr(VI)]$) vs. consumed organic substrate ($[S]_{E0} - [S]$), being $[Cr(VI)]_{E0}$ the Cr(VI) concentration at the initial state of the exponential phase and [Cr(VI)] the value corresponding to different times.

2.7. Determination of organic substrate, biomass and ammoniacal nitrogen

Organic substrate and biomass in the bioreactors were determined by measuring the chemical oxygen demand (COD) using a commercial kit (Hach Corp., Loveland, USA). The use of COD to determine biomass concentration has been reported by many authors [27–29]. Samples were used to determine total chemical oxygen demand (COD_T), another portion was centrifuged (14,000 rpm, 5 min) and filtered through a 0.45 μm membrane (Millipore HA) to measure soluble chemical oxygen demand that corresponded to organic substrate (COD_S). Biomass concentration (COD_B) was calculated as the difference between COD_T and COD_S and transformed into units of volatile suspended solids (VSS) through a curve given previously [29].

Ammoniacal nitrogen was monitored by the Nessler method (HACH Method No. 8038) and expressed as nitrogen ($mg\,l^{-1}$ NH_3-N).

2.8. Determination of Cr(VI) and total chromium

The residual concentration of Cr(VI) in the culture supernatant was determined by a spectrophotometric method at 540 nm, using diphenylcarbazide [30].

Considering that the tested microorganism could reduce Cr(VI) to Cr(III), and in order to analyze if these Cr species can be adsorbed/precipitated or accumulated by the microorganisms, the following mass balance was carried out at different contact times:

$$[Cr]_{total} = [Cr(VI)]_{soluble} + [Cr(III)]_{soluble} + [Cr(VI)]_{ads} + [Cr(III)]_{ads+precip}$$

ads = adsorbed, precip = precipitated.

The total chromium concentration in the supernatant ($[Cr]_{soluble\,total} = [Cr(VI)]_{soluble} + [Cr(III)]_{soluble}$) was measured using potassium permanganate and ammonium persulfate (to oxidize Cr(III) to Cr(VI)) followed by the reaction with diphenylcarbazide previously described. The Cr(III) concentration present in solution (soluble Cr(III)) was calculated by the mass balance as the difference between total soluble chromium concentration and that of hexavalent chromium. The total Cr(III) concentration (soluble and precipitated fractions) was calculated as the difference between the initial Cr(VI) concentration and the residual Cr(VI) concentration determined at each time.

2.9. Statistical analysis

Analysis of variance was done using Systat software. Linear and non-linear regressions were conducted in the Sigma Plot 9.0 software (Jandel Scientific, Chicago, IL, USA).

3. Results and discussion

3.1. Results obtained from the preliminary experiments

In the batch assays, it was observed that the Cr(VI) concentration decreased in the presence of *S. natans*. The mass balances carried out at different contact times allowed to show that the amount of total chromium present in solution ($[Cr(III)]_{soluble} + [Cr(VI)]_{soluble}$) was equivalent to the initial Cr(VI) concentration, indicating the formation of Cr(III) by biological reduction of Cr(VI) and that the adsorbed and precipitated fractions can be neglected.

Solisio et al. [31] reported that *S. natans* can absorb Cr(III) in aqueous solution, however the experimental conditions were different to those used in the present study. These authors [31] used a synthetic wastewater based on extract of meat and made an acid pre-treatment (pH 3.0–3.5) of the *S. natans* biomass prior to the Cr(III) biosorption assays; besides Cr(III) was not generated by biological activity but dosed in the shaken flasks as $Cr_2(SO_4)_3$. In contrast in our study, the formed Cr(III) remained completely in aqueous phase due probably to the presence of citric acid or cellular organic metabolites that would encourage the formation of soluble organo-Cr(III) complexes preventing any process of metal precipitation (Puzon et al. [32]; Remoundaki et al. [33]).

There is an extensive literature on biological reduction process from Cr(VI) to Cr(III), without simultaneous precipitation and/or adsorption of Cr(III). Opperman and Van Heerden [23] reported that *Thermus scotoductus* reduced Cr(VI) forming soluble Cr(III) end-products and not $Cr(OH)_3$ or Cr_2O_3 precipitates. Similar results were informed by Rege et al. [34] for *Enterobacter cloacae*, Shen and Wang [7] for *Escherichia coli* ATCC 33456, Campos et al. [35] for *Bacillus* strain QC1-2, Nepple et al. [36] for *Rhodobacter sphaeroides* and Megharaj et al. [37] for *Arthrobacter* sp. and *Bacillus* sp., among other studies.

Because neither biosorption nor bioaccumulation of chromium by *S. natans* took place under the experimental conditions of the present work, this microorganism could be able to be reutilized in subsequent reduction processes.

Control tests without microorganisms showed that there is no significant reduction/precipitation of Cr(VI) by abiotic processes. Therefore, it can be demonstrated that in batch system *S. natans* can reduce Cr(VI) to Cr(III) only using citric acid as electron donor.

3.2. Aerobic continuous culture of S. natans limited in carbon and energy source

3.2.1. Cr(VI) tolerance of S. natans

Operating the bioreactor at dilution rates ranging between 0.02 and 0.24 h^{-1} (hydraulic and cellular residence times = 50.0–4.16 h) and using different Cr(VI) concentrations in the influent medium M2 ($Cr(VI)_I$ = 4.3–80.1 mg l^{-1}), the assays in continuous system (limited in carbon and energy source (CES)) allowed to study the effect of *S. natans* physiological state (growth rate) on the tolerance to chromium and Cr(VI) reduction capacity.

The system was considered in steady state condition after a period of operation from 5 to 6 θ_C. In any case, significant changes in the concentrations of biomass, soluble organic substrate and soluble Cr(VI) for prolonged operation times (from 7 to 10 θ_C) were observed ($p > 0.05$).

Once steady state condition was achieved, Cr(VI) tolerance of *S. natans* was assessed by determining the biomass yield $Y_{X/S}$ (mgVSS $(mgCOD_S)^{-1}$) using Eq. (1). For a dilution rate of 0.02 h^{-1}, relatively low values of $Y_{X/S}$ and of $[X]_S$ were obtained for Cr(VI) concentrations ranging between 0 and 76.5 mg l^{-1}, as it can be expected for very low dilution rates (Table 1).

For dilution rates ranging from 0.065 to 0.12 h^{-1}, a gradual increase of $Y_{X/S}$ and consequently of the biomass concentration $[X]_S$ was observed as influent Cr(VI) concentration of the bioreactor increased (Table 1). Similar results were found by Yetiş et al. [17], who reported a significant increase in biomass yield of activated sludge for Cr(VI) concentrations from 1 to 25 mg l^{-1}. In the present work, for a dilution rate higher than 0.12 h^{-1}, values of $Y_{X/S}$ and $[X]_S$ remained relatively stable as influent Cr(VI) concentration of the bioreactor increased (Table 1).

From the results shown in Table 1, it can be concluded that *S. natans* tolerates Cr(VI) concentrations as high as 80 mg l^{-1}, exhibiting optimal growth.

3.2.2. Cr(VI) reduction: kinetic and stoichiometric parameters

When analyzing the effect of dilution rate on Cr(VI) reduction rate, it was observed that for D = 0.02 h^{-1}, Cr(VI) reduction rate ($r_{Cr(VI)}$) determined by Eq. (2) decreased from 0.071 mgCr(VI) $(l\,h)^{-1}$ to values of 0.001 mgCr(VI) $(l\,h)^{-1}$ as the influent Cr(VI) concentration of the bioreactor ($Cr(VI)_I$) increased from 21.8 to 76.5 mg l^{-1} (Fig. 1a). For concentrations higher than 21.8 mgCr(VI) l^{-1}, the amount of Cr(VI) per biomass was high inhibiting *S. natans* reductase activity.

Table 1
Biomass concentration, microbial growth rate and biomass yield for steady state condition of continuous cultures of *S. natans* limited in CES at different dilution rates and influent Cr(VI) concentrations.

D (h^{-1})	$[Cr(VI)]_I$ (mg l^{-1})	$[X]_S$ (gVSS l^{-1})[a]	r_X (gVSS $(l\,h)^{-1}$)	$Y_{X/S}$ (mgVSS $(mgCOD_S)^{-1}$)
0.02	0	0.350	0.007	0.175
	21.8	0.390	0.008	0.191
	39.5	0.335	0.007	0.175
	76.5	0.310	0.006	0.151
0.065	0	0.560	0.036	0.262
	4.3	0.663	0.043	0.299
	19.2	0.684	0.044	0.317
	38.9	0.738	0.048	0.351
	68.7	0.753	0.049	0.356
0.12	0	0.590	0.071	0.279
	4.47	0.634	0.076	0.298
	20.2	0.677	0.081	0.329
	37.9	0.849	0.102	0.391
	76.4	0.944	0.113	0.446
0.24	0	0.703	0.169	0.337
	5.32	0.735	0.176	0.350
	20.6	0.443	0.106	0.443
	38.9	0.567	0.136	0.330
	80.1	0.708	0.170	0.405

[a] Biomass concentration in steady state condition of the bioreactor.

Table 2
Maximum Cr(VI) reduction rate and half velocity constant for steady state condition of continuous cultures of *S. natans* limited in CES at different dilution rates.

D (h^{-1})	$r_{Cr(VI)max}$ (mgCr(VI) $(l\,h)^{-1}$)	$k_{Cr(VI)}$ (mgCr(VI) l^{-1})
0.065	0.200 (0.022)[a]	18.44 (5.92)
0.12	0.344 (0.035)	11.76 (4.28)
0.24	0.607 (0.058)	25.22 (6.31)

[a] Standard deviations between parenthesis.

A different behavior was observed for higher dilution rates (D = 0.065–0.24 h^{-1}); the Cr(VI) reduction rate determined by Eq. (2) followed a saturation kinetics as it can be observed in Fig. 1a. The experimental data of Cr(VI) reduction rate were fitted to the Monod equation as follows:

$$r_{Cr(VI)} = \frac{r_{Cr(VI)max}[Cr(VI)]_I}{k_{Cr(VI)} + [Cr(VI)]_I} \quad (5)$$

where $r_{Cr(VI)max}$ is the maximum Cr(VI) reduction rate (mgCr(VI) $(l\,h)^{-1}$) and $k_{Cr(VI)}$ is the half velocity constant (mgCr(VI) l^{-1}).

The parameters $r_{Cr(VI)max}$ and $k_{Cr(VI)}$ were determined by non-linear regression analysis using the software Sigma Plot 9.0 (Table 2, Fig. 1a). The higher $r_{Cr(VI)max}$ value was determined for a dilution rate of 0.24 h^{-1} ($r_{Cr(VI)max}$ = 0.607 mgCr(VI) $(l\,h)^{-1}$).

Higher values of $r_{Cr(VI)}$ were obtained for increasing D values. This result can be attributed to the fact that as the dilution rate increases (equivalent to the growth rate), the microorganisms gradually increase their general metabolic activity and consequently their capacity to reduce Cr(VI).

The effect of the biomass concentration on the reduction process was also analyzed. Fig. 1b shows that the values of specific Cr(VI) reduction rate ($q_{Cr(VI)}$) determined by Eq. (3) were higher for higher dilution rates. In the case of D = 0.24 h^{-1} and $Cr(VI)_I$ concentrations ranging from 38.9 to 80.1 mg l^{-1}, $q_{Cr(VI)}$ values from 0.6 to 0.7 mgCr(VI) $(gVSS\,h)^{-1}$ were obtained.

In Fig. 1b it can also be observed that $q_{Cr(VI)}$ increases as a function of the influent Cr(VI) concentration (D = 0.065–0.24 h^{-1}). Similar results were reported by Bae et al. [26]; these authors indicated that for *E. coli* growing at a dilution rate of 0.05 h^{-1}, the specific Cr(VI) reduction rate increased from 0.72 to 2.41 mgCr(VI) $(gSS\,h)^{-1}$ as the influent Cr(VI) concentration of the bioreactor increased from 10 to 40 mg l^{-1}.

Plotting $q_{Cr(VI)}$ as a function of D for influent Cr(VI) concentrations ranging from 19.2 to 80.1 mg l^{-1}, the specific Cr(VI) reduction rate increases approximately linearly with a slope of 2.50 (Fig. 2, r^2 = 0.94) indicating that higher growth rates imply higher metabolic activities of *S. natans* and consequently increased Cr(VI) reduction rates. Similar results were obtained by Keyhan et al. [25] for *Pseudomonas putida* growing in chemostat operated at different dilution rates with 0.4 mM K_2CrO_4 (20.8 mgCr(VI) l^{-1}). At low D values, $q_{Cr(VI)}$ is also low due to zero availability of reduction equivalents at low growth rates, according to findings reported by Matin and Gottschal [38].

Cr(VI) reduction yield ($Y_{Cr(VI)/S}$) was not significantly affected ($p > 0.05$) by the dilution rate, thus for each tested $Cr(VI)_I$ concentration, *S. natans* showed the same performance in the use of the energy source (required in the process of Cr(VI) reduction), regardless of the microbial growth rate. However, $Y_{Cr(VI)/S}$ was a function of influent Cr(VI) concentration of the bioreactor.

Fig. 3 shows that Cr(VI) reduction yield $Y_{Cr(VI)/S}$ (Eq. (4)) increases as the influent Cr(VI) concentration of the bioreactor increases. Highest $Y_{Cr(VI)/S}$ values were obtained ranging from 0.9

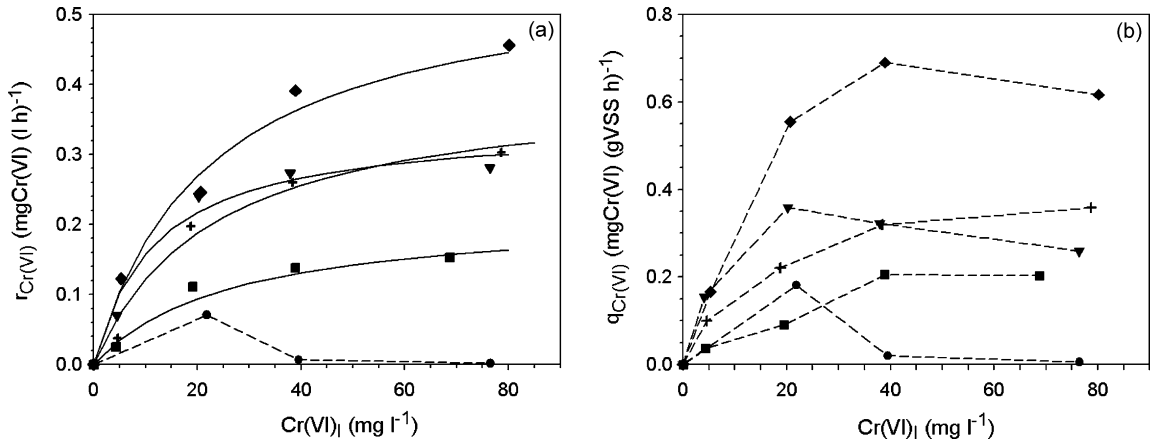

Fig. 1. Cr(VI) reduction for *S. natans* growing in continuous bioreactor at different dilution rates (D, h^{-1}) and influent Cr(VI) concentration (Cr(VI)$_I$): (a) Cr(VI) reduction rate ($r_{Cr(VI)}$); (b) specific Cr(VI) reduction rate ($q_{Cr(VI)}$). Continuous culture limited in CES: D = (●) 0.02, (■) 0.065, (▼) 0.12, (♦) 0.24; continuous culture limited in NS: (+) 0.11. (—) Predictions by Eq. (5).

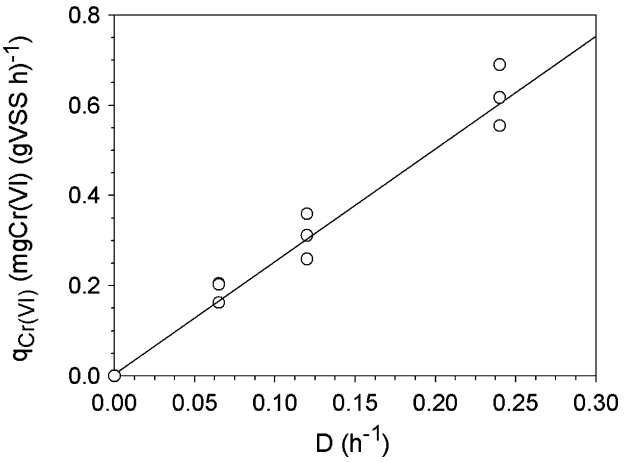

Fig. 2. Specific Cr(VI) reduction rate ($q_{Cr(VI)}$) as a function of the dilution rate (D, h^{-1}) for influent Cr(VI) concentration of the bioreactor ranging from 19.2 and 80.1 mg l^{-1}.

to 1.1 mgCr(VI) (gCOD$_S$)$^{-1}$ for Cr(VI) concentrations from 37.9 to 80.1 mgCr(VI) l^{-1}. A maximum value ($Y_{Cr(VI)/S\,max}$) of 1.370 mgCr(VI) (gCOD$_S$)$^{-1}$ (S.D. 0.162 mgCr(VI) (gCOD$_S$)$^{-1}$) was estimated by fitting a saturation type equation to the experimental data (Fig. 3).

Chirwa and Wang [39] reported similar results for *Bacillus* sp. growing in a fixed-film bioreactor with a hydraulic residence time of 24 h using glucose as energy substrate. Besides, from the analysis of the results reported by Bae et al. [26] about Cr(VI) reduction and glycerol uptake by *E. coli* (rate growth of 0.05 h^{-1}), the parameter $Y_{Cr(VI)/S}$ (mgCr(VI) (g glycerol)$^{-1}$) increased as the influent Cr(VI) concentration of the bioreactor increased.

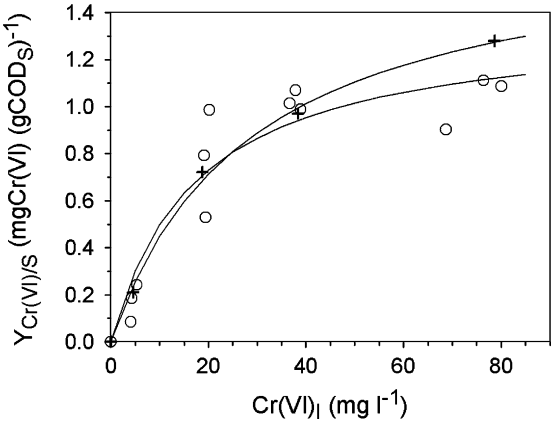

Fig. 3. Cr(VI) reduction yield ($Y_{Cr(VI)/S}$) as a function of the influent Cr(VI) concentration (Cr(VI)$_I$). (○) Continuous culture limited in CES (D = 0.065–0.24 h^{-1}). (+) Continuous culture limited in NS (D = 0.11 h^{-1}). (—) Monod equation.

3.2.3. Maximum Cr(VI) reduction capacity and active biomass concentration of S. natans in continuous system

In continuous system, the maximum Cr(VI) reduction capacity of the biomass (R, mgCr(VI) (gVSS)$^{-1}$) was calculated as follows:

$$R = \frac{r_{Cr(VI)max}}{r_X} \quad (6)$$

where $r_{Cr(VI)max}$ is the maximum Cr(VI) reduction rate (mgCr(VI) (l h)$^{-1}$) and r_X is the microbial growth rate (gVSS (l h)$^{-1}$). R corresponds to the maximum amount of Cr(VI) that can be reduced to Cr(III) per gram of biomass formed.

The maximum Cr(VI) reduction capacity of *S. natans* was determined for the different assays performed under continuous regime at dilution rates ranging from 0.065 to 0.24 h^{-1} and influent Cr(VI) concentrations of the bioreactor from 4.3 to 80.1 mgCr(VI) l^{-1}. For this calculation, r_X and $r_{Cr(VI)max}$ values, shown in Tables 1 and 2, respectively, were utilized.

The R values calculated by Eq. (6) were not significantly different between the tested conditions (p > 0.05, Table 3). An average R value of 4.15 mgCr(VI) (gVSS)$^{-1}$ (S.D. = 0.72 mgCr(VI) (gVSS)$^{-1}$) was determined.

Table 3
Maximum Cr(VI) reduction capacity of the biomass estimated by Eq. (6) for steady state condition of continuous cultures of *S. natans* limited in CES at different dilution rates and influent Cr(VI) concentrations.

D (h^{-1})	[Cr(VI)]$_I$ (mg l^{-1})	[Cr(VI)]$_S$ (mg l^{-1})[a]	R (mgCr(VI) (gVSS)$^{-1}$)
0.065	4.3	3.9	4.65
	19.2	17.4	4.55
	38.9	36.6	4.17
	68.7	66.6	4.08
0.12	4.47	3.9	4.52
	20.2	18.2	4.25
	37.9	35.6	3.37
	76.4	74.0	3.04
0.24	5.32	4.8	3.45
	20.6	19.6	5.72
	38.9	37.2	4.46
	80.1	78.2	3.57

[a] Cr(VI) concentration of the bioreactor under steady state condition.

As it was demonstrated in Section 3.2.1, Cr(VI) did not negatively affect the biomass yield of *S. natans* growing in continuous system. However, according to Wang and Shen [10], it can be assumed that the active biomass concentration with reductase activity decreases proportionately to the amount of Cr(VI) reduced to Cr(III), because of the toxicity of Cr(VI), as follows:

$$[X]_A = [X]_0 - \frac{[Cr(VI)]_0 - [Cr(VI)]}{R} \quad (7)$$

where $[X]_A$ is the active biomass concentration with reductase activity ($gVSS\,l^{-1}$), $[X]_0$ is the initial biomass concentration ($gVSS\,l^{-1}$), $[Cr(VI)]_0$ is the initial Cr(VI) concentration ($mgCr(VI)\,l^{-1}$).

In steady state for a continuous system, the active biomass concentration was determined by means of Eq. (7), rewritten as follows:

$$[X]_A = [X]_S - \frac{[Cr(VI)]_I - [Cr(VI)]_S}{R} \quad (8)$$

In each condition, $[X]_A$ was estimated using the experimental data ($[Cr(VI)]_I$, $[Cr(VI)]_S$, $[X]_S$) and R values shown in Tables 1 and 3.

For each dilution rate, the active biomass concentration decreased as the influent Cr(VI) concentration of the bioreactor increased. Thus, $[X]_A$ was expressed as follows:

$$[X]_A = [X]_S \frac{a}{[Cr(VI)]_I} \quad (9)$$

where a ($mgCr(VI)\,l^{-1}$) is a coefficient.

$[Cr(VI)]_I/[X]_S$ corresponds to the ratio between influent Cr(VI) concentration and biomass concentration in steady state (D_S, $mgCr(VI)\,(gVSS)^{-1}$). Thus, Eq. (9) was rewritten as

$$[X]_A = \frac{a}{D_S^n} \quad (10)$$

where n is a coefficient.

Eq. (10) was satisfactorily fitted to the $[X_A]$ values as a function of D_S by a non-linear regression (Fig. 4) resulting $a = 1.387\ mgCr(VI)\,l^{-1}$ (S.D. = 0.165 $mgCr(VI)\,l^{-1}$) and $n = 0.478$ (S.D. = 0.044). It must be considered that, even when enough active biomass (with reductase activity) remains in the system at high $Cr(VI)_I$ concentrations, the process of Cr(VI) reduction operates virtually at a maximum rate, i.e. the Cr(VI) reduction rate does not increase significantly as $Cr(VI)_I$ concentration increases as it was indicated in Fig. 1a. These results allow to infer that, in addition to reductase activity, other factors affect the kinetic of Cr(VI) reduction observed.

Besides, it should be considered that under continuous operation limited in CES, the organic substrate concentration in steady state condition ranged from 80 to 200 $mg\,l^{-1}$ (as COD_S), which would imply a low availability of energy and reduction equivalents. These conditions could limit the general metabolic activity and particularly the process of biological reduction of Cr(VI). Previous studies conducted in batch systems using pure cultures and activated sludge have shown that the Cr(VI) reduction is limited when the electron donor substrate is not sufficient [40,41].

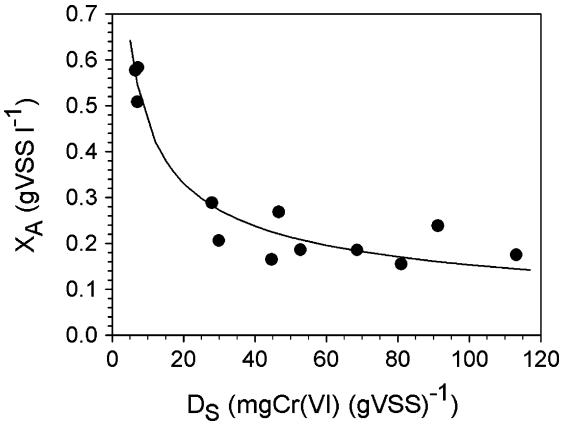

Fig. 4. Active biomass concentration of *S. natans* as a function of the ratio between influent Cr(VI) concentration and biomass concentration in steady state condition of the bioreactor operated under continuous regime limited in CES ($D = 0.065–0.24\ h^{-1}$). (—) Predictions by Eq. (10).

3.3. Aerobic continuous culture of S. natans limited in nitrogen source

Experiments carried out under nitrogen limited conditions (excess of carbon and energy source) using the culture medium M1NL or M2NL with an initial COD_S:N:P ratio of approximately 100:4:4 showed that the organic substrate concentration in the reactor under steady state conditions ranged from 450 to 550 $mg\,l^{-1}$ (as COD_S), while the concentration of ammoniacal nitrogen ranged from 1.5 to 6.5 $mg\,l^{-1}$ N-NH_3. These results confirmed that *S. natans* in the bioreactor was clearly limited by the nitrogen source.

The Cr(VI) reduction rate ($r_{Cr(VI)}$) and the specific Cr(VI) reduction rate ($q_{Cr(VI)}$) were determined by Eqs. (2) and (3), respectively.

Fig. 1(a and b) shows that the curves $r_{Cr(VI)}$ vs. $Cr(VI)_I$ and $q_{Cr(VI)}$ vs. $Cr(VI)_I$ for nitrogen-limited continuous culture are similar to those corresponding to the condition limited in carbon and energy source for a comparable dilution rate ($D = 0.12\ h^{-1}$).

Eq. (5) was fitted to the experimental data of Cr(VI) reduction rate. A maximum Cr(VI) reduction rate of 0.402 $mgCr(VI)\,(l\,h)^{-1}$ (S.D. 0.048 $mgCr(VI)\,(l\,h)^{-1}$) was determined by non-linear regression analysis (Sigma Plot 9.0). A $Y_{Cr(VI)/S\,max}$ value of 1.740 $mgCr(VI)\,(gCOD_S)^{-1}$ (S.D. 0.085 $mgCr(VI)\,(gCOD_S)^{-1}$) was estimated by applying the Monod equation to the $Y_{Cr(VI)/S}$ experimental data (Fig. 3). These values was slightly higher to those estimated for continuous operation limited in CES ($r_{Cr(VI)max} = 0.344\ mgCr(VI)\,(l\,h)^{-1}$, $Y_{Cr(VI)/S\,max} = 1.370\ mgCr(VI)\,(gCOD_S)^{-1}$).

Thus, the process of biological reduction of Cr(VI) was not noticeably affected by the low availability of energy in the citric acid-limited continuous system for the tested dilution rates.

3.4. Performance of continuous systems to reduce Cr(VI) using S. natans

From a technological point of view, it is important to determine the performance of *S. natans* to reduce the influent Cr(VI) concentration of the bioreactor operated under continuous regime using the following expression:

$$Cr(VI)\text{percentual decrease} = 100\frac{[Cr(VI)]_I - [Cr(VI)]_S}{[Cr(VI)_I]} \quad (11)$$

For continuous systems limited in CES and operated at cellular residence times between 4.16 and 15.38 h, the percentual decrease in Cr(VI) decreased linearly as a function of the $Cr(VI)_I$ concentration. A maximum value of approximately 13% was reached for influent Cr(VI) concentration ranging from 4.3 to 5.32 $mgCr(VI)\,l^{-1}$. At a cellular residence time of 50 h, a percentual decrease in Cr(VI) of about 16% was reached for a concentration of 21.8 $mgCr(VI)_I\,l^{-1}$. It must be pointed out that the performance was not markedly improved for this high cellular residence time because probably at low growth rates much of the energy was consumed in cell maintenance more than in other metabolic processes. For higher Cr(VI) concentrations, the performance in Cr(VI) reduction was very low (<1%).

Thus, the percentual decrease in Cr(VI) was independent of the dilution rate for continuous systems limited in CES, i.e. as the cellular residence time increased, the efficiency of the process was not significantly enhanced.

Chirwa and Wang [39] reported that the efficiency of Cr(VI) reduction for *Bacillus* sp. growing in a fixed-film bioreactor was not affected by the hydraulic residence time. On the contrary, DeLeo and Ehrlich [42] reported that in a continuous culture of *Pseudomonas fluorescens* LB300, the percentual decrease in influent Cr(VI) concentration of the bioreactor increased from 28% to 57% as the residence time increased from 11.7 to 38.5 h.

In the present work, nitrogen-limited continuous system operated at a cellular residence time of 9.09 h exhibited a similar performance to that obtained for carbon- and energy-limited continuous system, reaching a maximum removal of 11% for a Cr(VI) concentration of 18.7 mg l^{-1}. However, by increasing the cellular residence time to 28.5 h ($D = 0.035\ h^{-1}$), a significant improvement was achieved for $Cr(VI)_I$ concentrations ranging from 5 to 20 mg l^{-1}. Percentual decreases in Cr(VI) of about 26% and 32% were obtained for concentrations of 19.1 and 5.5 mg$Cr(VI)_I$ l^{-1}, respectively. Moreover, the $Y_{Cr(VI)/S}$ values were significantly higher ($p < 0.01$) to those corresponding to the carbon- and energy-limited continuous culture. For influent Cr(VI) concentrations of 5.5 and 19.1, the values of $Y_{Cr(VI)/S}$ were 0.691 and 2.81 mgCr(VI) $(gCOD_S)^{-1}$, respectively.

It can be assumed that at low growth rates, limiting nutrient (NS) is used more efficiently favoring the general metabolic activities of microorganisms and consequently those linked to the Cr(VI) reduction process.

Moreover, it should be noted that the studies conducted in continuous systems allowed to assess the effect of different factors (growth rate, low availability of carbon and energy or nitrogen, and influent Cr(VI) concentration) on the process of bioreduction for this microorganism.

The obtained results in continuous system were complemented with studies about the feasibility of Cr(VI) reduction by *S. natans* in discontinuous reactors.

3.5. Aerobic batch culture of S. natans

3.5.1. Cr(VI) tolerance of S. natans

Fig. 5 shows biomass, soluble organic substrate and Cr(VI) concentrations as a function of time for batch assays with different initial biomass ($[X]_0 = 170–500$ mgVSS l^{-1}) and hexavalent chromium concentrations ($[Cr(VI)]_0 = 19–78$ mg l^{-1}). The experiments with initial biomass concentration ranging from 170 to 190 mgVSS l^{-1} presented initial COD_S:N:P ratio of approximately 100:8:5 (Fig. 5a–c). An initial ratio of 100:5:3 corresponded to assays with $[X]_0$ ranging from 400 to 500 mgVSS l^{-1} (Fig. 5d–f).

As it was previously indicated in Section 2.5, the initial COD_S:VSS ratio (mg/mg) ranged from 7.0 to 12.5, i.e. the available organic substrate per biomass unit was comparable between the different experiments.

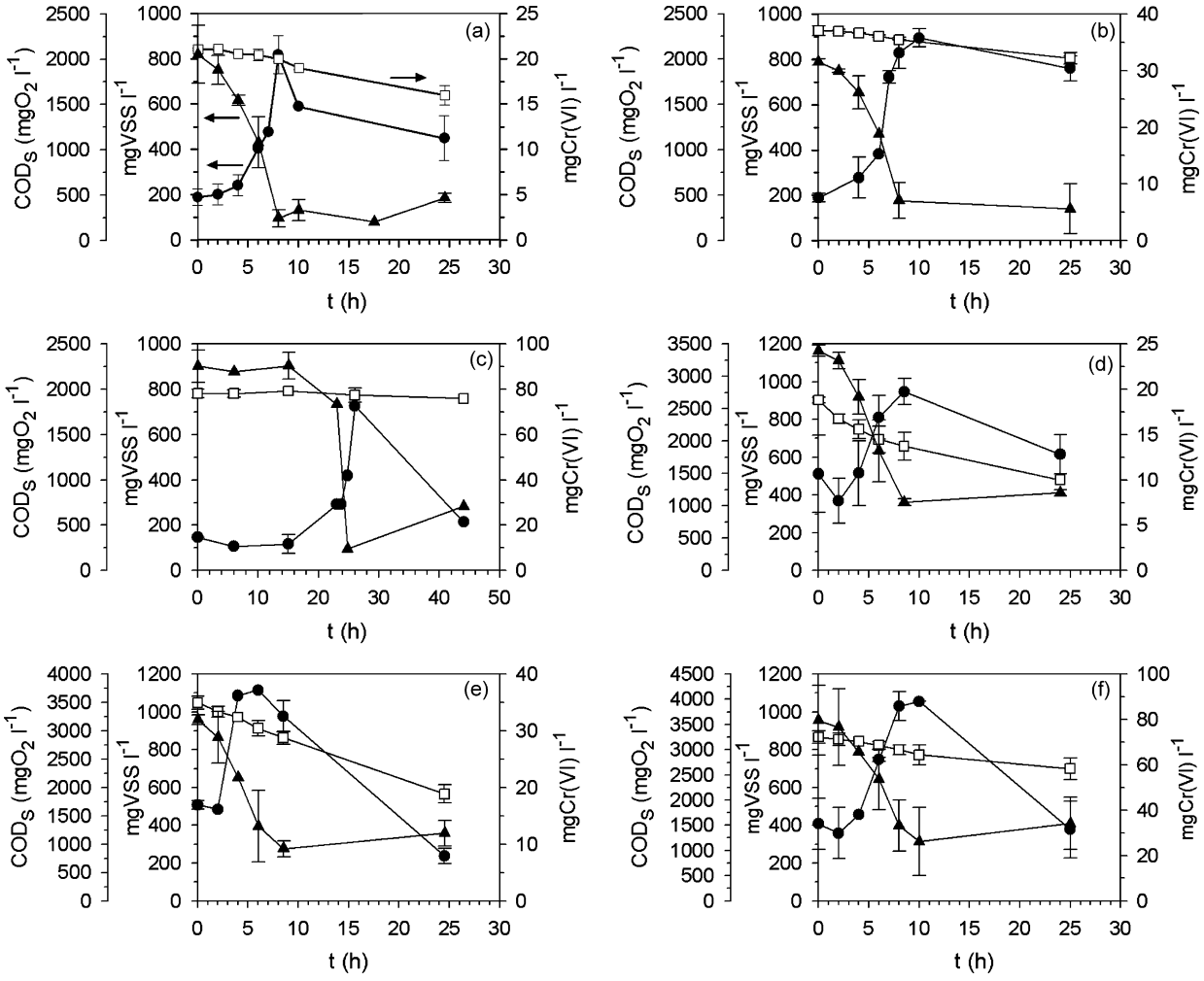

Fig. 5. Growth, organic substrate consumption and Cr(VI) decay for aerobic batch cultures of *S. natans* with different initial Cr(VI) and biomass concentrations ($[X]_0$, mgVSS l^{-1}): (a) 21 mgCr(VI) l^{-1} ($[X]_0 = 185$); (b) 37 mgCr(VI) l^{-1} ($[X]_0 = 190$); (c) 78 mgCr(VI) l^{-1} ($[X]_0 = 170$); (d) 19 mgCr(VI) l^{-1} ($[X]_0 = 500$); (e) 35 mgCr(VI) l^{-1} ($[X]_0 = 500$); (f) 72 mgCr(VI) l^{-1} ($[X]_0 = 400$). (●) Biomass concentration; (▲) organic substrate concentration (COD_S, mgO_2 l^{-1}); (□) soluble Cr(VI) concentration. The standard deviations are indicated by error bars.

Growth curves in the discontinuous reactor were obtained for all experiments (Fig. 5a–f). A lag phase of approximately 2–3 h was observed except for the experiment with the highest initial Cr(VI) concentration (78 mg l^{-1}) and the lowest biomass concentration ($[X]_0$ = 170 mgVSS l^{-1}). In this experiment, the lag phase lasted about 15 h due possibly to the toxic effect of Cr(VI) on the microorganisms (Fig. 5c).

In all experiments, it was observed that after the lag phase, the biomass concentration increased exponentially as the organic substrate was quickly consumed. It must be emphasized that in exponential phase, the growth of *S. natans* was not limited by any nutrient, because they were present in concentrations that exceed all the metabolic requirements.

Cr(VI) tolerance of *S. natans* was evaluated by means of the following parameters of microbial growth: specific growth rate (μ, h^{-1}) and biomass yield ($Y_{X/S}$, mgVSS $(mgCOD_S)^{-1}$).

The specific growth rate of *S. natans* for the different batch assays was determined by a first-order equation as follows:

$$\frac{d[X]}{dt} = \mu[X] \tag{12}$$

where [X] is the biomass concentration (mgVSS l^{-1}), μ is the specific growth rate (h^{-1}) and t is the time (h).

By integrating Eq. (12), between t = 0 (initial state of the batch culture) and t, the following equation was obtained:

$$\mu = \frac{\ln([X]/[X]_0)}{t} \tag{13}$$

where $[X]_0$ is the biomass concentration at the start of batch culture.

The proposed equation satisfactorily fitted the experimental data for the exponential phase of all growth curves shown in Fig. 5. Values of μ are given in Table 4.

Biomass yield ($Y_{X/S}$) was determined in the exponential phase from the slopes of the graphs: biomass concentration ([X]) vs. consumed organic substrate as it as described in Section 2.6. Values of $Y_{X/S}$ are given in Table 4.

Cr(VI) concentrations ranging from 19 to 78 mg l^{-1} did not significantly affect ($p > 0.05$) either the biomass yield or the specific growth rate of *S. natans* for the different conditions assayed (Table 4). These results indicate that *S. natans* can be considered more tolerant to Cr(VI) than other microorganisms reported in literature; the presence of 1 mmolCr(VI) l^{-1} (52 mg l^{-1}) reduced 46% the growth rate of a *Ochrobactrum tritici* strain [21], aerobic cultures of *Shewanellla oneidensis* exposed to Cr(VI) presented growth rates and biomass yield significantly lower than the values corresponding to control tests [18], Cr(VI) concentrations higher than 0.3 mmol l^{-1} reduced the specific growth rate of *T. scotoductus*, while 1 mmolCr(VI) l^{-1} additionally reduced the biomass yield [23]. Ur Rahman et al. [22] reported that the growth rate of a *Pseudomonas* strain decreased gradually as the initial Cr(VI) concentration increased.

Table 4
Growth parameters of *S. natans* obtained from batch assays for different initial Cr(VI) and biomass concentrations.

$[Cr(VI)]_0$ (mg l^{-1})	$[X]_0$ (mgVSS l^{-1})	$Y_{X/S}$ (mgVSS $(mgCOD_S)^{-1}$)	μ (h^{-1})
0	130	0.3406 (0.0831)[a]	0.2545 (0.0148)
19	500	0.2707 (0.0203)	0.2020 (0.0452)
21	185	0.2932 (0.0894)	0.2625 (0.0077)
35	500	0.3031 (0.0548)	0.2110 (0.0198)
37	190	0.4306 (0.0379)	0.2575 (0.0205)
72	400	0.3069 (0.0679)	0.1905 (0.0884)
78	170	0.3823 (0.0671)	0.1582 (0.0414)

[a] Standard deviations in parenthesis.

On the other hand, it has been reported that *Ochrobactrum* could tolerate high concentrations of Cr(VI) (50–200 mg l^{-1}) growing in a complex organic medium such as Luria broth [1].

In the present work, *S. natans* exhibited great resistance to Cr(VI) in the range of 19–78 mg l^{-1} growing in a mineral medium supplemented only with citric acid as organic substrate. This resistance observed in a simple culture medium can be linked to the presence of a sheath around the cells of *S. natans* that would block or reduce the Cr(VI) transport inside the cell, to the formation of complexes between Cr(VI) and citric acid that would reduce the toxicity of the metal and/or it could be due to a detoxification mechanism based on bioreduction from Cr(VI) to Cr(III).

3.5.2. Cr(VI) reduction: kinetic and stoichiometric parameters

In the experiments carried out in the discontinuous reactor, *S. natans* was able to reduce Cr(VI) to Cr(III). Cr(VI) reduction did not ceased once the consumption of CES stopped, because the concentration of soluble Cr(VI) ($[Cr(VI)]_{soluble}$) continued declining until the end of the assays (Fig. 5). Thus, it can be inferred that *S. natans* can also use endogenous reserves as a source of electrons in the process of biological reduction in agreement with results reported by Caravelli et al. [16].

Fig. 6a shows that the Cr(VI) reduction rate ($r_{Cr(VI)}$) increases as the initial Cr(VI) concentration increases. For an initial biomass concentration relatively low ($[X]_0$ = 170–190 mgVSS l^{-1}), the Cr(VI) reduction ability reached asymptotic values as the concentration of $Cr(VI)_0$ increased from 21 to 37 mg l^{-1}. For 78 mg$Cr(VI)_0$ l^{-1} ($[X]_0$ = 170 mgVSS l^{-1}) an abrupt decrease of the reduction rate due to the toxic effect of this metal was observed, which also caused a relatively long lag phase but without affecting either the biomass yield or the specific growth rate.

The Cr(VI) reduction rate also depended on the cell density, showing higher values for experiments with higher biomass concentrations ranging from 400 to 500 mgVSS l^{-1} (Fig. 6a). The specific Cr(VI) reduction rates were also higher for high biomass concentrations (Fig. 6b). Particularly, $q_{Cr(VI)}$ values between 0.9 and 1.0 mgCr(VI) $(gVSS\,h)^{-1}$ were obtained for initial biomass concentrations ranging from 400 to 500 mgVSS l^{-1} ($[Cr(VI)]_0$ = 35–72 mg l^{-1}).

In this study, the specific Cr(VI) reduction rates of *S. natans* determined in batch system during the exponential growth phase, were two to four times higher that those reported by Caravelli et al. [16] using *S. natans* cultured in a batch reactor operated under stationary growth conditions with glucose as an energy source. These results permit to conclude that the performance of the batch reactor to reduce Cr(VI) was higher using *S. natans* during growing instead of zero growth conditions.

Saturation kinetics, which describes the process of biological reduction of Cr(VI), has been also reported for other microorganisms [2,23]. The Cr(VI) reduction rates for *S. natans* are similar to those reported in literature for different bacterial strains; however, it is not possible to perform a reliable comparison of Cr(VI) reduction kinetic parameters because the experimental conditions differ considerably between the studies [11].

The literature is controversial regarding the relationship between the specific Cr(VI) reduction rate and the biomass concentration in batch systems. Our results are in agreement with Ur Rahman et al. [22] who reported that the Cr(VI) reduction increased as the initial concentration of inoculum of *Pseudomonas* sp. increased. These authors determined the total amount of reduced Cr(VI) at a certain culture time for different inoculum concentrations (10, 20 and 30%, v/v) obtained from a 18 h-old culture. However, the growth curves and the specific reduction rates were not reported.

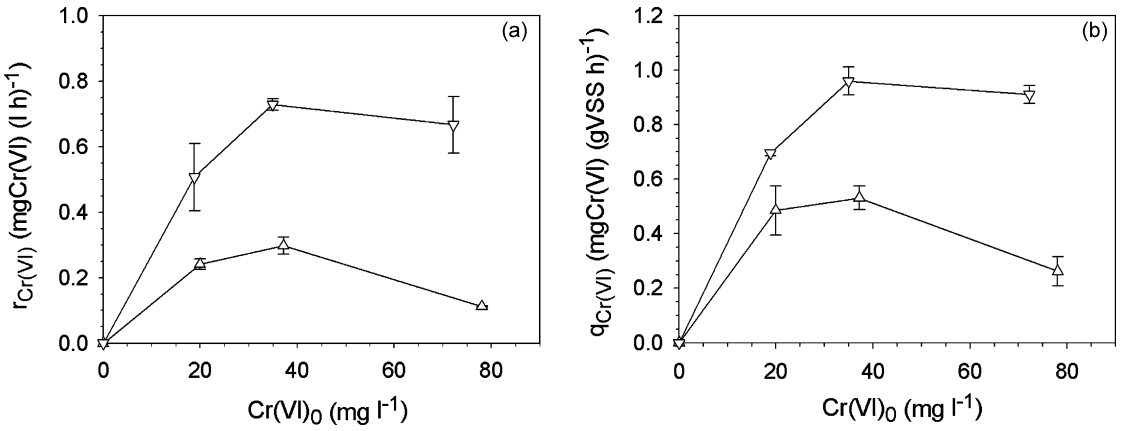

Fig. 6. Cr(VI) reduction for *S. natans* growing in batch bioreactor with different initial Cr(VI) concentration and ranges of biomass concentration (mgVSS l^{-1}): (△) 170–190; (▽) 400–500. (a) Cr(VI) reduction rate ($r_{Cr(VI)}$); (b) specific Cr(VI) reduction rate ($q_{Cr(VI)}$). The standard deviations are indicated by error bars.

3.5.3. Performance of batch systems to reduce Cr(VI) using S. natans

In batch systems, the decrease of the soluble Cr(VI) concentration was determined by means of the following expression:

$$\text{Cr(VI) percentual decrease} = 100\frac{[\text{Cr(VI)}]_0 - [\text{Cr(VI)}]}{[\text{Cr(VI)}]_0} \quad (14)$$

where [Cr(VI)] is the Cr(VI) concentration at time t and $[\text{Cr(VI)}]_0$ is the value corresponding to the start of batch culture.

After 24 h of culture time a decrease in the concentration of soluble Cr(VI) close to 50% was reached, for initial Cr(VI) concentration of 35 mg l^{-1} and initial biomass concentration of 500 mgVSS l^{-1} (Fig. 5e).

3.5.4. Substrate consumption: Cr(VI) reduction yield

Another important aspect to consider is the kinetic of substrate consumption in relation to the biological reduction of Cr(VI). Fig. 7 shows the Cr(VI) reduction yield ($Y_{Cr(VI)/S}$) as a function of the initial Cr(VI) concentration in the range of initial biomass concentration tested (170–190; 400–500 mgVSS l^{-1}). $Y_{Cr(VI)/S}$ values increased as $[\text{Cr(VI)}]_0$ increased with higher values for higher initial concentrations of biomass. A $Y_{Cr(VI)/S}$ value of approximately 2.5 mgCr(VI) (gCOD$_S$)$^{-1}$ was found for initial Cr(VI) and biomass concentrations of 72 mgCr(VI) l^{-1} and 400 mgVSS l^{-1}, respectively.

The parameter $Y_{Cr(VI)/S}$ would determine the approximate amount of organic substrate (citric acid) required in the biological treatment of Cr(VI)-contaminated wastewaters using *S. natans*. Considering that this filamentous bacterium is often found in activated sludge systems [43], the stoichiometric and kinetic parameters calculated in this study allow to estimate the potential contribution of this microorganism in the reduction of Cr(VI).

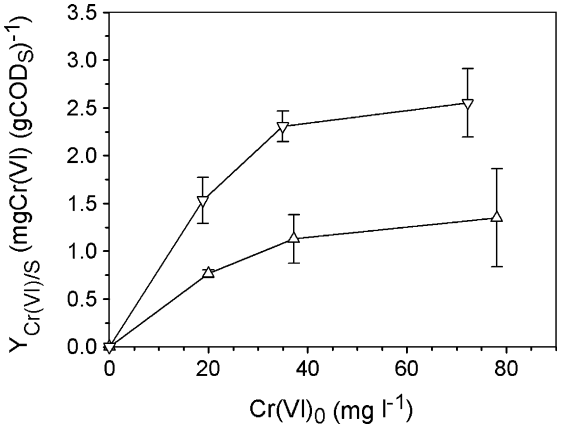

Fig. 7. Cr(VI) reduction yield ($Y_{Cr(VI)/S}$) as a function of the initial Cr(VI) concentration for the assayed ranges of biomass concentration in batch tests (mgVSS l^{-1}): (△) 170–190; (▽) 400–500. The standard deviations are indicated by error bars.

3.5.5. Mathematical model of the process of Cr(VI) reduction

Cr(VI) reduction rate by *S. natans* growing in batch system was modeled by a first-order equation with respect to Cr(VI) as follows:

$$\frac{d[\text{Cr(VI)}]}{dt} = -k'[\text{Cr(VI)}] \quad (15)$$

where k' is the apparent chromium(VI) reduction coefficient (h^{-1}).

By integrating Eq. (15), the following expression was obtained:

$$\ln\frac{[\text{Cr(VI)}]}{[\text{Cr(VI)}]_0} = -k't \quad (16)$$

This equation was satisfactorily fitted by non-linear regression to the experimental data of Cr(VI) as a function of time, for all assayed conditions. Each experimental point was the average of duplicates, measured in two runs carried out under the same $[\text{Cr(VI)}]_0$ and $[X]_0$ (Fig. 8). The kinetic parameter k' is not a constant because it depends on the biomass concentration (Table 5).

In a previous work, Caravelli et al. [16] evaluated the toxic effect of Cr(VI) on the respiratory activity of *S. natans* in a batch system under stationary growth conditions and proposed a mathematical

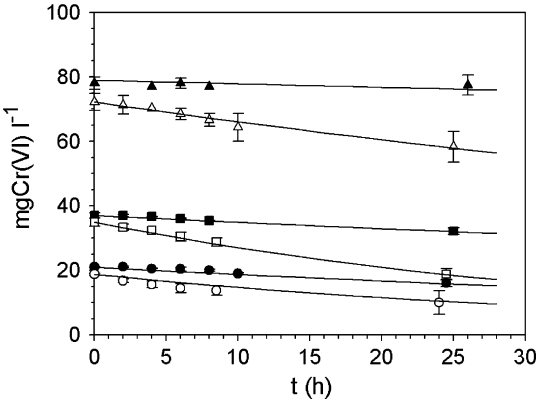

Fig. 8. Experimental data and modeling of Cr(VI) reduction by aerobic batch cultures of *S. natans* with different initial Cr(VI) and biomass concentrations ($[X]_0$, mgVSS l^{-1}): (●) 21 mgCr(VI) l^{-1} ($[X]_0$ = 185); (○) 19 mgCr(VI) l^{-1} ($[X]_0$ = 500); (■) 37 mgCr(VI) l^{-1} ($[X]_0$ = 190); (□) 35 mgCr(VI) l^{-1} ($[X]_0$ = 500); (▲) 78 mgCr(VI) l^{-1} ($[X]_0$ = 170); (△) 72 mgCr(VI) l^{-1} ($[X]_0$ = 400). (—) Predictions by Eq. (16). The standard deviations are indicated by error bars.

Table 5
Apparent chromium (VI) reduction coefficient (k', h^{-1}) for *S. natans* obtained by fitting Eq. (16) to the experimental data.

$[Cr(VI)]_0$ (mg l^{-1})	$[X]_0$ (mgVSS l^{-1})	D_0 (mgCr(VI)$_0$ (gVSS$_0$)$^{-1}$)	k' (h^{-1})
19	500	38	0.0333 (0.0099)[a]
21	185	113	0.0112 (0.0009)
35	500	70	0.0253 (0.0044)
37	190	195	0.0061 (0.0006)
72	400	180	0.0089 (0.0021)
78	170	459	0.0012 (0.0003)

[a] Standard deviation in parenthesis.

model that describes the Cr(VI) reduction considering the initial Cr(VI) concentration and the active biomass concentration.

In the present work, as it was previously demonstrated in Section 3.5.1, Cr(VI) did not affect significantly either the biomass yield or the growth rate. Considering these results, the following equation was proposed to consider the effect of active biomass concentration $[X]_A$ on the Cr(VI) reduction rate:

$$\frac{d[Cr(VI)]}{dt} = -k[Cr(VI)][X]_A \quad (17)$$

A simplified expression was proposed to describe the relationship between the active biomass concentration and D_0 = ratio between initial Cr(VI) concentration and initial biomass concentration $[Cr(VI)]_0/[X]_0$ ((mgCr(VI)$_0$ (gVSS$_0$)$^{-1}$)

$$[X]_A = \frac{a}{D_0^n} \quad (18)$$

where n is a coefficient to be determined.

By replacing Eq. (18) in Eq. (17) the following was obtained:

$$\frac{d[Cr(VI)]}{dt} = -k[Cr(VI)]\frac{a}{D_0^n} \quad (19)$$

Comparing Eq. (19) with Eq. (15) results:

$$k' = k\frac{a}{D_0^n} = \frac{b}{D_0^n} \quad (20)$$

being b the chromium (VI) reduction coefficient (mgCr(VI)$_0$ (gVSS$_0$ h)$^{-1}$).

Different values of k' were obtained for all the tested batch conditions (Table 5). Besides k' values were plotted as a function of D_0 (Fig. 9) according to Eq. (20). Non-linear regression allowed to determine the parameters b (0.965 mgCr(VI)$_0$ (gVSS$_0$ h)$^{-1}$, S.D. 0.542 mgCr(VI)$_0$ (gVSS$_0$ h)$^{-1}$) and n (0.910, S.D. 0.139).

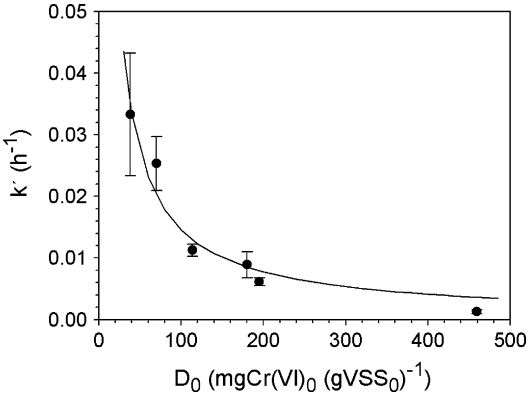

Fig. 9. Apparent chromium(VI) reduction coefficient (k', h^{-1}) as a function of the ratio between initial Cr(VI) concentration and initial biomass concentration (D_0, mgCr(VI)$_0$ (gVSS$_0$)$^{-1}$) for batch culture of *S. natans*. (●) Experimental data. (—) Predictions by Eq. (20).

By combining Eq. (20) with Eq. (19) and integrating:

$$\ln\frac{[Cr(VI)]}{[Cr(VI)]_0} = -\frac{b}{D_0^n}t \quad (21)$$

Eq. (21) allows to estimate Cr(VI) concentration as a function of time and D_0 (Fig. 10). For initial conditions of 20 mgCr(VI) l^{-1} and 40 mgCr(VI)$_0$ (gVSS$_0$)$^{-1}$, a decrease of 60% in the Cr(VI) concentration would be achieved after 27.2 h of culture time.

The proposed model considers the initial Cr(VI) concentration and its effect on the metabolic activity of the biomass, specifically on its reductase activity.

3.6. Analysis of the S. natans performance in batch and continuous systems

S. natans showed good tolerance to Cr(VI) in batch and continuous systems exhibiting optimal growth for the different tested conditions. For dilution rates of 0.065 and 0.12 h^{-1} a gradual increase of the biomass yield, and consequently an increase in the biomass concentration reached at steady state, were observed as the influent Cr(VI) concentration increased from 0 to 76.4 mg l^{-1}.

In batch systems, concentrations ranging from 19 to 78 mgCr(VI) l^{-1} did not significantly affect either the biomass yield or specific growth rate for the ranges of biomass assayed.

The highest specific Cr(VI) reduction rates $q_{Cr(VI)}$ were obtained in batch assays. For initial Cr(VI) concentrations ranging from 35 to 72 mgCr(VI) l^{-1} and biomass concentrations from 400 to 500 mgVSS l^{-1}, $q_{Cr(VI)}$ values between 0.9 and 1.0 mgCr(VI) (gVSS h)$^{-1}$ were observed. These were slightly higher to the highest values reached in continuous culture ($q_{Cr(VI)}$ = 0.6–0.7 mgCr(VI) (gVSS h)$^{-1}$) at a dilution rate D = 0.24 h^{-1} and a similar range of Cr(VI) concentration (38.9–80.1 mgCr(VI)$_l$ l^{-1}). It must be emphasized that in batch systems the parameters of Cr(VI) reduction were determined under non-limited growth conditions.

Unfortunately, there are few studies regarding the biological reduction of Cr(VI) in batch and continuous systems. The analysis of the results reported by Bhide et al. [24] shows that a *Pseudomonas mendocina* strain growing under continuous regime at dilution rates between 0.11 and 0.16 h^{-1} exhibited Cr(VI) reduction rates two to three times greater than those calculated in batch system. However, the biomass concentration was not indicated in any case and the results reported for batch system corresponded to global Cr(VI) reduction rates calculated virtually over the entire duration of the experiment.

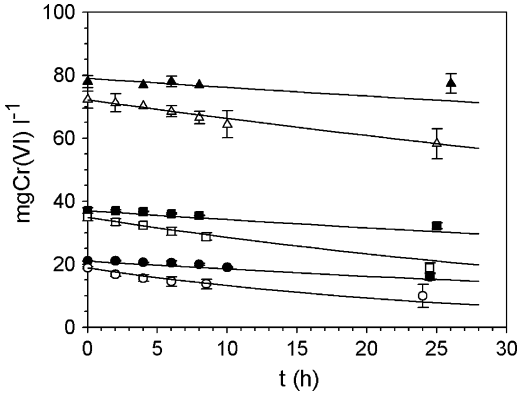

Fig. 10. Experimental data and modeling of Cr(VI) reduction by aerobic batch cultures of *S. natans* with different initial Cr(VI) and biomass concentrations ($[X]_0$, mgVSS l^{-1}): (●) 21 mgCr(VI) l^{-1} ($[X]_0$ = 185); (○) 19 mgCr(VI) l^{-1} ($[X]_0$ = 500); (■) 37 mgCr(VI) l^{-1} ($[X]_0$ = 190); (□) 35 mgCr(VI) l^{-1} ($[X]_0$ = 500); (▲) 78 mgCr(VI) l^{-1} ($[X]_0$ = 170); (△) 72 mgCr(VI) l^{-1} ($[X]_0$ = 400). (—) Predictions by Eq. (21). The standard deviations are indicated by error bars.

In batch system, a decrease in the concentration of soluble Cr(VI) close to 50% was reached after 24 h of culture time, for initial Cr(VI) and biomass concentrations of 35 mg l^{-1} and 500 mgVSS l^{-1}, respectively. In nitrogen-limited continuous culture operated at a cellular residence time of 28.5 h, percentual decreases in $Cr(VI)_l$ of about 26 and 32% were obtained for influent Cr(VI) concentrations of 19.1 and 5.5 mgCr(VI) l^{-1}, respectively.

In continuous system, Eq. (10) allowed to estimate the active biomass concentration as a function of influent Cr(VI) concentration of the bioreactor and biomass concentration. The same equation was included in the mathematical model used to describe the process of Cr(VI) reduction in batch system (Eq. (19)). Thus, this model considered the initial Cr(VI) concentration and its effect on the biological activity of the biomass, specifically on its reductase activity. The proposed model allowed to predict Cr(VI) concentration as a function of both time and ratio between initial Cr(VI) concentration and that of the biomass for batch culture.

4. Conclusions

- *S. natans* exhibited optimal growth in batch and continuous systems exposed to Cr(VI) concentrations as high as 80 mg l^{-1} showing a significant Cr(VI) tolerance and the ability to reduce Cr(VI) to Cr(III) using only citric acid as electron donor.
- In carbon- and energy-limited continuous system, the specific Cr(VI) reduction rate increased as the Cr(VI) concentration of the influent of the bioreactor ($Cr(VI)_l$) increased from 4.3 to 80.1 mg l^{-1}; however, the percentual decrease in Cr(VI) raised linearly as $Cr(VI)_l$ concentration decreased. The efficiency of the process did not significantly increase prolonging the cellular residence time up to 50 h.
- Considering that at low growth rates much of the energy is consumed in cell maintenance, a continuous system with an excess of energy source was studied. A nitrogen-limited continuous culture operated at a low dilution rate ($D = 0.035\ h^{-1}$), equivalent to a cellular residence time of 28.5 h, achieved a higher percentual decrease of Cr(VI) (26–32%) for influent Cr(VI) concentrations ranging between 5.5 and 19.1 mgCr(VI) l^{-1}.
- In batch system, a decrease of soluble Cr(VI) concentration close to 50% was obtained after 24 h of culture time. In this system, high initial Cr(VI) and *S. natans* biomass concentrations allowed to achieve the highest performance of the Cr(VI) reduction process in terms of both, rate and efficient use of energy source. These are important aspects which should be considered in detoxification strategies of wastewaters.
- In batch systems, a mathematical model allowed to predict the Cr(VI) concentration as a function of time and the ratio between initial Cr(VI) concentration and that of the biomass. This model considers the initial Cr(VI) concentration and its effect on the biological activity of the *S. natans* biomass, specifically on its reductase activity.

Acknowledgements

The authors gratefully acknowledge the financial support given by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de La Plata, and Agencia Nacional de Promoción Científica y Tecnológica, Argentina.

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