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by

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Synopsis

Successful Cr(VI) reduction was achieved over 78 days of continuous operation in a fluidised bed bio reactor (FBBR) and a suspended cell continuously stirred tank reactor (CSTR). All runs were performed at a hydraulic retention time of 30 hours, while the Cr(IV) inlet concentration was varied between 21.4 and 52.1 mg/L. The substrate (glucose) inlet concentration was either 2.5 or 5g/L. Cr(VI) reduction rates ranged between 0.10 and 0.97mg/L.h. No evidence of biofilm growth on the FBBR support particles was found, despite initial attachment investigations suggesting the contrary. However, significant biomass immobilisation was observed on the wall sections of the FBBR column and recycle piping. The FBBR outperformed the CSTR (with no attached biomass) under similar operating conditions with Cr(VI) reduction rates of 0.26 and 0.97mg/L.h compared to 0.18 and 0.41mg/L.h, respectively. Substrate requirements per unit of Cr(VI) reduced were similar for CSTR/FBBR comparative runs indicating similarities between attached biomass and free cells. Results from the FBBR indicate a second order relationship between reduction rate and Cr(VI) concentration under substrate limited conditions, suggesting increases in Cr(VI) reducing enzymes at higher Cr(VI) concentrations. The substrate limited runs were also associated with an increase in reduction yield (milligram Cr(VI) reduced per gram substrate used) as the Cr(VI) concentration increased. Results from both reactors indicate a strong dependency of Cr(VI) reduction rate on substrate concentration. Fourteen distinguishable species from 7 genera were identified as possible Cr(VI) reducers on completion of experimentation. The consortium composition differs from the composition reported by Molokwane *et al*. (2008) who identified 7 species from 2 genera in batch investigations with a different growth medium and higher Cr(VI) exposure concentrations.

Keywords: continuous biological Cr(VI) reduction, CSTR, FBBR, immobilised biomass, free cells.

$^{\Psi}$ Molokwane, Meli & Nkhalambayausi-Chirwa (2008)

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Nomenclature

B_0	Initial biomass concentration	mg/L
C_0	Initial Cr(VI) concentration	mg/L
CFU	Colony forming unit	
$[Cr^{6+}]$	Cr(VI) outlet concentration	mg/L
CRB	Cr(VI) reducing bacteria	
CSTR	Continuous stirred tank reactor	
d_p	Particle diameter	mm
D_0	Percentage Cr(VI) reduction at experiment completion	–
DO	Dissolved oxygen	mg/L
FBBR	Fluidised bed bio reactor	
g	Gravitational constant	m/s^2
GAC	Granular activated carbon	
HRT	Hydraulic retention time	
k	Power law constant	L/mg.h
LB	Luria-Bettani	
MSM	Minimal salt medium	
n	Power of the power law model	Integer
PVC	Poly Vinyl Chloride	
Q	Liquid flow rate	L/h
$r_{Cr(VI)}$	Rate of Cr(VI) reduction	mg/L.h
$r_{glucose}$	Rate of glucose consumption	mg/L.h
rpm	Revolutions per minute	revolution/min
R	Reduction rate	mg_{Cr6+}/L.h
RY	Reduction yield	dimensionless
SEM	Scanning electron microscopy	
SR	Specific reduction rate	mg_{Cr6+}/g_{VSS}.h
V	Volume of reactor	L
VB	Vogel-Bonner	
VSS	Volatile suspended solids	

Greek Letters

τ	Hydraulic retention time	h

1 Introduction

Chromium is used extensively in the chemical, metallic and refractory materials industries (Papp, 2002: 17.3) due to its wide range of possible oxidation states ranging from (-II) to (VI). From these states chromium (III) and (VI) are the most stable and are expected in natural environments like water and soil (James, 2002). South Africa has more than 80% of the world chromium reserves (Papp, 2002) and continual chromium ore production and growing industrial activity result in an increase in Cr(VI) contaminated sites (Papp, 2002: 17.4). These sites pose environmental hazards apart from heavy metal contamination; hexavalent chromium [Cr(VI)] or chromate is known to be toxic, carcinogenic and mutagenic to living organisms (Institute of Medicine, 2001: 197-223). However, Cr(III) is not only nontoxic, but also an essential trace element required by humans (Institute of Medicine, 2001: 197-223). The hazards associated with Cr(VI) are exacerbated by its solubility, mobility and bioavailability (in order of resulting dependence) over Cr(III) compounds (James, 2002). For these reasons scientific research focusing on industrial remediation of contaminated sites, as regulated by government incentives, have received a lot of attention recently (James, 2002).

Various methods exist to remove soluble chromium but if purposeful reduction of Cr(VI) to Cr(III) is neglected, Cr(VI) persists in downstream operations. Traditionally chemical reduction is used, but the process involves strong acid and base reagents which are both costly and hazardous (Molokwane *et al*., 2008). This, in conjunction with ongoing developments in bioreactors, results in increased interest in biological chromate reduction.

Since 1977, when biological Cr(VI) reduction was first reported by Romanenko and Koren'kov, numerous authors have published on biological chromate reduction. A variety of micro-organisms, including bacteria and fungus, have been identified to be able to reduce Cr(VI). Biological reduction is agreed to be enzymatic. Most batch studies have been aimed at optimising physical conditions, establishing the biochemical mechanisms involved and analysing kinetic potential (Caravelli and Zaritzky, 2009). Several published studies on batch as well as continuous biological Cr(VI) reduction processes suggest that optimum conditions are at neutral pH and moderate temperatures (from 25 to 35°C). Reduction is reported under aerobic and anaerobic conditions with micro-organisms utilising a wide variety of organic substrates in conjunction with a minimal salt medium (MSM). Micro-organisms can be employed as free cells or as immobilised cells on a support surface. Complete reduction of Cr(VI) concentrations ranging typically from 20 to 300mg/L was achieved in batch assay experiments which lasted from under ten hours to seven days. Typical Cr(VI) tolerance

concentrations are in the order of 500mg/L beyond which biological activity is ceased. Most continuous Cr(VI) reduction studies were conducted in completely mixed, lab scale reactors with volumes smaller than 5L and relatively large recycle streams. Operating conditions varied with hydraulic retention times between 6 and 50h, substrate load rates from 0.06 to 0.6mg/L.h, Cr(VI) load rates from 0.05 to 4.2mg/L.h and Cr(VI) reduction rates between 0.1 and 4.17mg/L.h.

It is well established that biomass immobilisation improves culture resilience (Stoodley *et al.*, 1999) and allows high specific biomass retention which improves volumetric productivity (Nicolella, Van Loosdrecht and Heijnen, 2000). Biomass immobilisation has been applied in continuous Cr(VI) reduction studies where the presence of biofilm proved to facilitate self-remediation after Cr(VI) overloading (Chirwa and Wang, 1997) and resulted in increased volumetric productivity (Elangovan and Philip, 2009). All biofilm related Cr(VI) reduction studies were performed using stationary support. In studies on other systems, where biofilms are supported on dynamic particles in fluidised bed bio reactors, mass transfer restrictions are reduced with a subsequent increase in productivity. Excessive pressure drop caused by biomass overgrowth is also prevented (Dermou *et al.*, 2005). It is therefore the aim of this work to attempt biomass immobilisation on dynamic support particles in a FBBR for the reduction of Cr(VI) and to evaluate reactor performance. In this investigation the Brits Culture (Molokwane *et al.*, 2008) will be employed in a FBBR as well as a suspended cell CSTR. In addition, the effect of Cr(VI) and glucose concentration on the overall reduction rate will be investigated.

2 Literature

2.1 Chromium, chromium pollution and associated hazards

Chromium is used mainly to harden materials or inhibit corrosion and oxidation - as applied in the manufacturing of stainless steel and nonferrous alloys (Papp, 2002: 17.1). Other important uses are in pigment production, leather processing and tanning, catalysts, metal plating and other surface treatments like wood preservation. The extensive use of chromium in the chemical, metallic and refractory materials industries results from its wide range of possible oxidation states ranging from (-II) to (VI).

Of all the possible oxidation states, Cr(III) and Cr(VI) are the most stable and are expected in natural environments like water and soil (James, 2002). The predominant oxidation state that exists in an environment is relevant; according to the Institute of Medicine (2001: 197-223) Cr(III) is not only nontoxic, but also an essential trace element required by humans. However, hexavalent chromium [Cr(VI)] or chromate is known to be toxic, carcinogenic and mutagenic to living organisms (Institute of Medicine, 2001: 197-223). These hazards are exacerbated by its solubility, mobility and bioavailability (in order of resulting dependence) over Cr(III) compounds (James, 2002). Cr(III) has greater affinity for negatively charged ions found in micro-organisms, plants and animals and negatively charged colloids available in water and soils. Different Cr(III) compounds have different solubility and may be less or more soluble with pH being the main variable. On the other hand, the most common Cr(VI) compounds are soluble freely over a pH ranging from 1-14. Cr(VI) compounds which are insoluble at near-neutral pH ($PbCrO_4$, $BaCrO_4$ and $CaCrO_4$), are soluble at other pH values (James, 2002). Arslan, Beltrame & Tomasi (1987) report that only Cr(VI) can pass through biological membranes implying that Cr(III) is non-bioavailable.

Despite knowledge of the persistence of Cr(VI) in the environment and the dangers associated with exposure, effluent streams from increasing anthropogenic processes and surface run-off water from industrial sites result in increases in Cr(VI) contamination in the environment. In South-Africa the problem is exacerbated by wrongfully decommissioned or abandoned mining operations (Molokwane *et al*., 2008). Further contamination can be prevented and recovery accomplished through remediation by purposeful reduction of Cr(VI) to Cr(III).

2.2 Chromium removal and the importance of Cr(VI) reduction

Removal of highly soluble Cr(VI) is achieved conventionally with chemical reduction and subsequent alkaline precipitation as $Cr(OH)_3$. The method is hazardous because it involves working with strong

acids and bases to change the pH to where reactions should occur. The method is expensive due to the large amounts of chemicals required, often does not remove all Cr(VI) and produces large amounts of chemical sludge that might contain harmful products which require subsequent treatment (James, 2002).

Ion exchange is a more attractive removal method (Ohtake, Fujii & Toda, 1990). However, ion-exchange resins can be expensive. Resins have to be regenerated periodically with hazardous acids/bases which results in down-time and also considerable amounts of a high concentration ion solution with excess acid/base that has to be disposed of (Schutte, 2006: 212-217). Most importantly, ion-exchange cannot reduce Cr(VI) to Cr(III) and subsequent treatment and disposal of regeneration streams, which will contain high concentrations of Cr(VI), have to be considered. Adsorption onto coal, activated carbon, alum, kaolinite and fly ash (Ohtake *et al*. 1990) is another alternative, but the cost of absorbents are high and Cr(VI) is not reduced, requiring subsequent treatment and disposal considerations. Lastly, membrane filtration is a relatively recent technology capable of removing chromium. This method is expensive due to the cost of technology. In addition, periodic backwashing result in downtime (Schutte, 2006: 205) and backwash streams that will contain high concentrations of Cr(VI).

From the above it is clear that many chromium removal methods exist, but if Cr(VI) is not reduced to Cr(III) it is a persistent problem. In the light of this discussion biological reduction is an attractive alternative because (Molokwane *et al*. 2008 and Kathiravan *et al*., 2010):

- Operating conditions are less hazardous with optimum conditions at neutral pH and low temperatures.
- No secondary pollutants are produced requiring less downstream considerations, disposal costs and unit operations that involve additional expense.
- The process is less energy intensive and more cost effective than chemical reduction.
- Operation is sustainable.

2.3 Biological Cr(VI) reduction

Biological Cr(VI) reduction was reported first by Romanenko and Koren'kov (1977). When exposed to toxic environments, some bacteria have the ability to protect themselves through oxidation, reduction or methylation of the toxin into less toxic, more volatile or readily precipitating forms

(Chirwa & Wang, 1997; Zhao, Yang & Zhu, 2008). Chromate reduction is agreed to be enzymatic. The enzymes produced for such activity are termed adaptive enzymes and are formed in addition to constitutive enzymes that facilitate normal cell activity (Peavy, Rowe & Tchobanoglous, 1985: 75). Chirwa and Wang (1997) report the following reduction mechanism under aerobic conditions with glucose ($C_6H_{12}O_6$) as electron donor and Cr(VI) fed as K_2CrO_4:

$$C_6H_{12}O_6 + 8CrO_4^{2-} + 34H^+ \rightarrow 8Cr^{3+} + 6HCO_3^- + 20H_2O \quad (1)$$

Molokwane *et al*. (2008) report a general metabolic pathway for Cr(VI) reduction by Cr(VI) reducing bacteria (CRB), as derived from redox half-reactions:

$$CrO_4^{2-} + 8H^+ \xrightarrow{CRB} Cr^{3+} + 4H_2O \quad (2)$$

They report that consumption of large amounts of protons in the reduction reaction aids with subsequent $Cr(OH)_3$ precipitation according to the following reaction:

$$Cr^{3+} + 4H_2O \xrightarrow{neutral\ pH} Cr(OH)_{3\downarrow} + 3H^+ + H_2O \quad (3)$$

Investigations on the metabolic reduction of Cr(VI) are categorised into batch assays and continuous systems and are discussed below.

2.3.1 Cr(VI) reduction with batch assays

Many authors have reported successful biological Cr(VI) reduction, almost exclusively by bacteria as either pure cultures or as a consortium of cultures, under several physical conditions in batch reactors (Ferro Orozco, Contreras & Zaritzky, 2010). Sanghi and Srivastava (2010) report reduction by a fungus (*Coriolus versicolor*) in a continuous column. According to Caravelli and Zaritzky (2009) most batch studies have been aimed at optimising physical conditions, establishing the biochemical mechanisms involved and analysing kinetic potential. Some of the main experimental studies are summarised in table 1 and discussed in the following paragraphs.

Typical cell concentrations in batch reactors are reported to range from 1×10^5 to 1×10^{10}. Reports agree that increased initial cell concentration results in increased initial reduction rates (Llovera *et al*., 1993; Ohtake *et al*., 1990 & Pal an Paul, 2004). Biological reduction rates are affected therefore by parameters that influence biomass concentration. Such parameters include physical conditions (aerobic/anaerobic, temperature and pH), growth medium composition and concentration and lastly the presence and concentration of growth inhibitors – including Cr(VI).

Table 1: Summary of batch studies on bacterial Cr(VI) reduction indicating initial Cr(VI) concentration reduced to completion over time.

Micro-organism [ψ1]	Author(s)	Conditions			Substrate	Toxic exposure concentration [ψ2]	Reduction Concentration [ψ3]		Remarks
		Oxygen	T (°C)	pH			mg/L	h	
Agrobacterium radiobacter	Llovera *et al*. (1993)	Aerobic	10-40	5-8	Resting cells	NA	25.5	(3)	Optimal conditions: pH=7-7.5 & T=25-30°C.
	Llovera *et al*. (1993)	Anaerobic	10-40	5-8	Resting cells	NA	26	(6)	Anaerobic rates were comparable but not tested as extensively. Reduction not complete (45%).
Arthrobacter aurescens	Horton *et al*. (2006)	Aerobic	10	NR [ψ4]	VB broth	100 mg/L	50	(120)	See also their study with *Arthrobacter aurescens* in a consortium lower down.
Arthrobacter sp.	Córdoba, Vargas & Dussan (2008)	Aerobic	30	NR [ψ4]	Glucose-MSM [ψ5]	NA	40	(25.7)	Biofilms in packed bed.
	Córdoba, Vargas & Dussan (2008)	Aerobic	30	NR [ψ4]	Glucose-MSM	NA	30	(27.5)	Biofilms in semi-batch packed bed.
Bacillus sp.	Wang & Xiao (1995)	Aerobic	20-35	5-9	Glucose in VB-broth	NA	25	(100)	Optimal conditions: pH=7.2 & T=30°C. Phenolics and aromatics didn't facilitate reduction at all.
Bacillus sphaericus	Pal & Paul (2004)	Aerobic	15-42	5.7-11	VB-broth & substrates	NA	20	(96)	Optimal conditions: pH=7 & T=30°C. Heavy metal inhibited.
Bacillus subtilis	Garbisu *et al*. (1998)	Aerobic	30	7	Glucose-MSM	50mg/L	25	(30)	No difference in reduction rate by cells inoculated in Cr(VI) vs. cells inoculated under normal conditions.
Desulfovibrio vulgaris	Lovley & Phillips (1994)	Anaerobic, H_2 blanket	30	6.8	Resting cells	NA	2600	(1)	H_2 served as electron donor. No reduction aerobically. Heavy metal inhibited.
Escherichia coli	Shen & Wang (1993)	Aerobic	35	NR [ψ4]	Glucose added to bacto peptone	NA	291	(12)	Reduction near complete (88%). Reduction was inhibited when oxygen was bubbled through the reactor as opposed to air (only 41% reduction after 12h).
	Shen & Wang (1993)	Anaerobic	35	NR [ψ4]	Glucose added to bacto peptone	NA	291	(7)	Anaerobic conditions facilitated by nitrogen bubbling were faster than aerobic conditions.

Bacterium	Author(s)	Conditions			Substrate	Toxic exposure concentration[ψ2]	Reduction Concentration [ψ3]		Remarks
		Oxygen	T (°C)	pH			mg/L	h	
Escherichia coli	Shen & Wang (1994)	Aerobic	10-50	2-8	Bacto-peptone and glucose	NA	30	(43)	Optimal conditions: pH=7 & T=36°C. Phenolic compound and heavy metal inhibited.
	Shen & Wang (1994)	Anaerobic	10-50	2-8	Bacto-peptone and glucose	NA	40	(32)	Optimal conditions: pH=7 & T=36°C. Outperform aerobic conditions.
Enterobacter cloacae	Ohtake, Fujii & Toda (1990)	Anaerobic	30	7	KSC medium, peptone & meat extract	NA	210	(3.5)	No aerobic reduction. Heavy metal inhibited.
Leucobacter sp.	Zhu *et al*. (2008)	Aerobic	15-42	6-12	LB broth	NA	1700	(36)	Optimal conditions: pH=9 & T=30°C.
Pseudomonas	McLean & Beveridge (2001)	Aerobic	20	7	VB broth and glucose	520 mg/L	22	(160)	pH decreased to 5.3 in 170h. Reduction occurred in the presence of As and Cu.
Pseudomonas ambigua	Horitsu *et al*. (1987)	Aerobic	NR [ψ4]	7	Nutrient broth	Tolerant beyond 4000mg/L	150	(36)	Reduction near complete (80%).
Pseudomonas fluorescens	Shen & Wang (1995)	Aerobic	30	7	Glucose and benzoate in VB-broth	NA	25	(96)	Phenolics and aromatics didn't facilitate reduction at all.
Pseudomonas putida	Ishibashi, Cervantes & Silver (1990)	Aerobic	30	7	Tris-hydrochloride and EDTA	NA	1.04	(90)	Reduction only 60%. Outperformed by *Pseudomonas fluorescens and Escherichia coli.*
Sphaerotilus natans	Caravelli, Giannuzzi & Zaritzky (2008)	Aerobic	20	3&7	Glucose-MSM	NA	20	(120)	No biosorption was observed at pH=3.
	Caravelli & Zaritzky (2009)	Aerobic	30	7	Citric acid-MSM	NA	NA[ψ6]		Batch conditions run with limiting substrate concentration; reduction not complete.
Streptomyces griseus	Laxman & More (2002)	Aerobic	28-50	4-8	Broth II medium	NA	50	(72)	Optimal conditions: pH=6-7 & T=28°C.
	Poopal & Laxman (2008)	Aerobic	28	NR [ψ4]	MGYP medium	NA	25	(24)	Immobilisation on polyvinyl alcohol beads (3-5mm).

Bacterium	Author(s)	Conditions			Substrate	Toxic exposure concentration[ψ2]	Reduction Concentration [ψ3]		Remarks
		Oxygen	T (°C)	pH			mg/L	h	
Streptomyces griseus	Poopal & Laxman (2009)	Aerobic	20-60	5-9	Broth II with various carbon sources	NA	59	(30)	Optimal conditions: pH=7 & T=28°C for cell free extract. Enzymes are constitutive. Heavy metal inhibition.
Consortium(NR)	Horton *et al*. (2006)	Aerobic	10	NR [ψ4]	VB-broth	100 mg/L	60	(2160)	Pure culture reduction is much faster than consortium reduction.
Consortium (7 species)	Molokwane *et al*. (2008)	Aerobic	30	NR [ψ4]	LB-broth	400 mg/L	200	(75)	Consortium reduction faster as resulting from isolates contribution.
Consortium (18 species)	Molokwane *et al*. (2008)	Anaerobic	30	NR [ψ4]	LB-broth	300 mg/L	150	(130)	Faster consortium reduction, slower vs. aerobic, lower toxicity tolerance.
Acinetobacter haemolyticus	Ahmad *et al*. (2010)	Aerobic	30-38	6.2-8.4	Liquid pineapple waste	NA	81	(9)	Semi-batch process. Coexistence of 12 species in biofilm .
Consortium(NR)	Dermou *et al*. (2005)	Aerobic	28	7.2-8.9	NaAc-MSM	NA	35	(0.75)	Semi-batch process through a counter current trickle bed with recirculation.

[ψ1] Micro-organism or consortiums (with number of active reducing strains in brackets) can be regarded as bacterial unless stated otherwise.

[ψ2] Concentration where cell metabolic function ceased indicating adverse toxicity; not applicable (NA) when not tested purposefully.

[ψ3] Maximum concentration reduced to completion.

[ψ4] Conditions are not reported.

[ψ5] Minimal salts medium (MSM) discussed below.

[ψ6] Complete reduction was not achieved for any batch assay.

From table 1 it is concluded that most authors report optimum conditions at near neutral pH and moderate temperatures (T=30±5°C). Most studies were done under aerobic conditions. Discrepancies exist between reported studies that compare aerobic versus anaerobic conditions:

- Llovera *et al*. (1993) report comparable rates between resting cells cultivated under aerobic and anaerobic conditions.

- Lovley & Phillips (1994) and Ohtake, Fujii & Toda (1990) report no reduction under aerobic conditions even though reduction did occur under anaerobic conditions.

- Shen & Wang (1993, 1994) report that anaerobic conditions (facilitated by bubbling nitrogen) was faster than aerobic reduction (facilitated by bubbling air); under aerobic conditions when oxygen was bubbled as opposed to air, the reduction rate decreased even more (Shen & Wang, 1993).

- Molokwane *et al*. (2008) report higher reduction rates and toxicity tolerance for their consortium of cultures under aerobic conditions even though a greater number of tolerant strains were present under anaerobic conditions (18) compared to 7 strains under aerobic conditions.

A wide variety of growth media can be used but in essence all media contain the following:

- Macro-nutrients: phosphate as PO_4^{3-}, HPO_4^{2-} or $H_2PO_4^-$; sulphate as SO_4^{2-} and nitrate as NH_4^- or NO_3^-. Macro-nutrients are generally included collectively.

- Micro-nutrients may include: Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, K^+, Na^+, B^-, Cl^-, I^- or Mo^{6+}. They are added as ionic salts with macro nutrients or each other. The salt solution comprising macro and micro nutrients can be termed a minimal salt medium (MSM). MSM's alone cannot sustain microbial activity.

- A substrate is defined as a source of energy and carbon as required above MSM's to support microbial life. A single organic compound can provide both carbon and energy.

Sufficient combinations of macro- and micro nutrients are available commercially as ready mixed broths. Luria-Bettani (LB) broth, Vogel-Bonner (VB) broth or peptones are used commonly. These rich media are used routinely as inoculation medium while experimental runs are done using a substrate added MSM (often termed growth medium). Glucose is the most common substrate used in growth media prepared for Cr(VI) reducing batch assays. Pal & Paul (2004) evaluated reduction rates in VB broth supplemented with a variety of substrates (listed in decreasing order of resulting

rate): glucose, yeast extract, glycine, Na-propionate, Na-acetate, Peptone, beef extract and tryptone. Poopal & Laxman (2009) evaluated reduction rates in Broth(II) medium supplemented with 0.2% of glucose, glycerol, acetate, citrate, sucrose, ethanol and tartarate (listed in order of decreasing resultant rate). Reduction performance relies further on substrate concentration. Pal & Paul (2004) report that the percentage reduction (of initial Cr(VI) concentration) increased with increased initial glucose concentration. From their results it is evident that the rate of reduction increased with increased glucose concentration from 0.5 to 4g/L but that an operational optimum exists (estimated at 1g/L) because reduction yield (reported here as $mg_{Cr6+}/g_{glucose}$) decreased with increasing glucose concentration implying counter-efficiency. Sanghi & Srivastava (2010) report rapid increases in reduction rate with increasing glucose concentration from 0g/L to 2g/L. Above this operational optimum concentration reduction rate decreased gradually.

Heavy metals and phenolic compounds may inhibit cellular growth (refer to the remarks column of table 1). These chemicals are reported to be present in industrial waste streams containing Cr(VI) (Chirwa & Wang, 2000; Shen & Wang, 1994 and Elangovan & Philip, 2009). The effect of heavy metals is small below species concentrations of 5mg/L (Elangovan & Philip, 2009). Reported results indicate that Cr(VI) can inhibit cellular growth (refer to the toxic exposure concentration column in table 1). Molokwane *et al*. (2008) report significant decreases in cell concentrations with increasing initial Cr(VI) concentrations. They ascribed the result to irreversible cell inactivation through Cr(VI) poisoning. Correlated to this result, percentage reduction (of initial Cr(VI) concentration) decreased with increasing initial Cr(VI) concentration. Caravelli & Zaritzky (2009) agree with this result. However, even though cellular growth rate decreased with increasing Cr(VI) concentration, cell concentration yields were not affected. A discrepancy exists therefore between the two reports regarding the influence of cell concentration on percentage reduction. Caravelli & Zaritzky (2009) go on to explain that elevated Cr(VI) concentrations did not prevent biomass yield, but rather deactivated cellular functions capable of Cr(VI) reduction, therefore inhibiting percentage reduction. Caravelli *et al*. (2008) report that percentage reduction (D_0) reduced with increasing initial Cr(VI) concentration. Percentage reduction can be predicted as a function of initial Cr(VI) and cell concentration (C_0 and B_0, respectively):

$$D_0 = \frac{C_0}{B_0} \tag{4}$$

They report that percentage reduction was similar for similar values of D_0.

The cultures discussed thus far were inoculated in the absence of Cr(VI). Garbisu *et al*. (1998) report that Cr(VI) exposure during inoculation had no effect on cellular growth rate.

Some authors (as listed in the legend of figure 1 below) report that reduction rates are affected by initial Cr(VI) concentration under the same initial cell concentrations. Figure 1 compares the reported effects of initial Cr(VI) concentration on reduction rate. The figure shows the average initial Cr(VI) reduction rates over the first 20h as calculated by linear approximation as opposed to tangential instantaneous rates. Linearization can be done since most reduction rates remained constant during the first 20 hours of experimentation. Slightly higher instantaneous rates, at time zero, were reported by Shen & Wang (1994) and Poopal & Laxman (2008) but linear approximation is still fair.

Most authors (Caravelli & Zaritzky, 2009; McLean & Beveridge, 2001; Poopal & Laxman, 2009; Shen & Wang, 1994 and Wang & Xiao, 1995) report reduction rates to have a maximum as a function of initial Cr(VI) concentration. Wang & Xiao (1995) ascribe such maxima to toleration thresholds beyond which Cr(VI) toxicity reduces reduction rate. Molokwane *et al*. (2008) report a similar trend but with higher rates and at higher concentration as shown in figure 2. Sanghi & Srivastava (2010) report a similar trend for initial Cr(VI) concentrations ranging from 10 to 80mg/L. Their reported rates are higher than the rates reported by Molokwane *et al*. (2008).

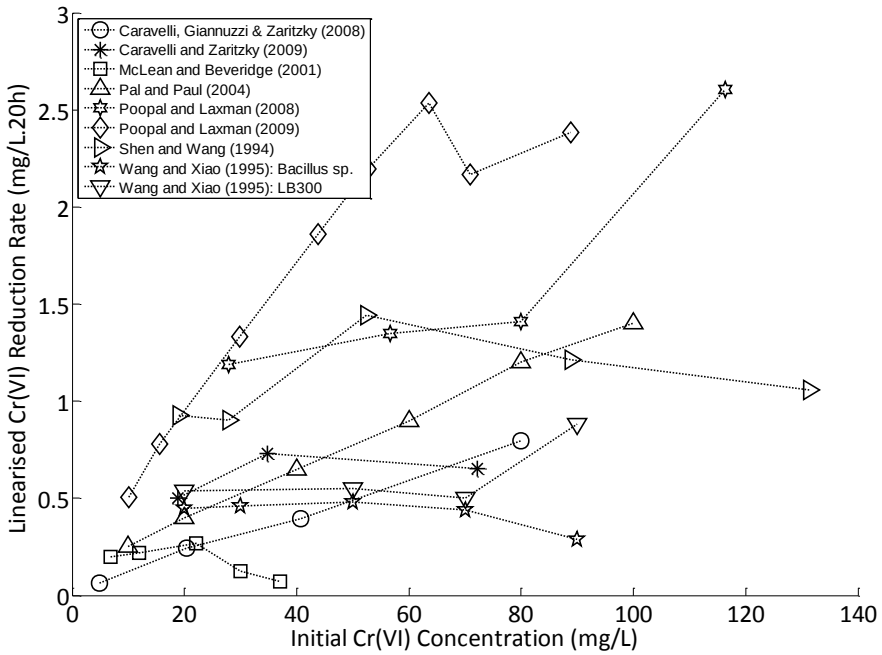


Figure 1: Cr(VI) reduction rate over the first 20h versus initial Cr(VI) concentration.

As shown in figure 1, Caravelli *et al*. (2008) and Pal & Paul (2004) report monotonically increasing trends up to initial concentrations of 80mg/L and 100mg/L, respectively. Poopal & Laxman (2008)

and Wang & Xiao (1995, for *Pseudomonas fluorescens* LB300) report sharp increases in rate beyond certain Cr(VI) concentrations (80 and 70mg/L, respectively). Neither study explains the occurrence.

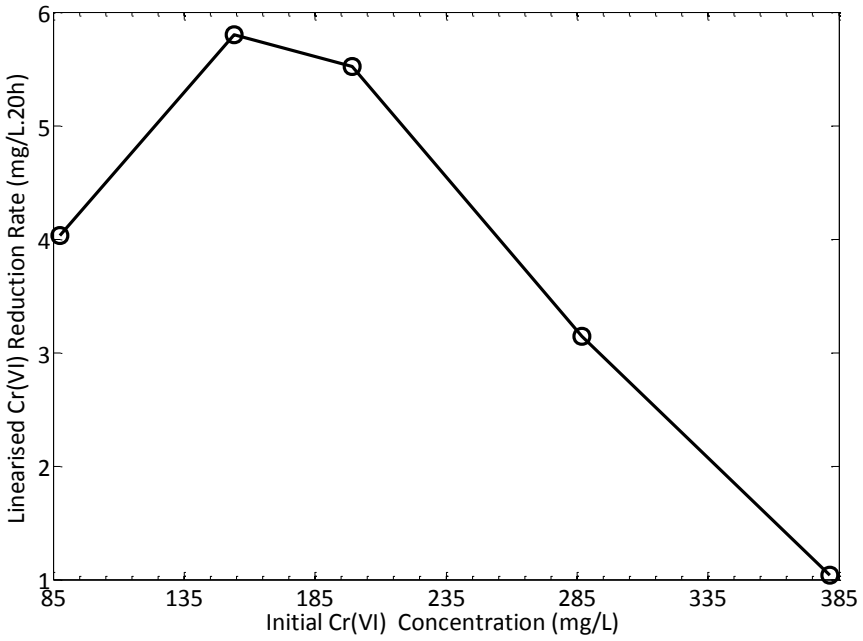


Figure 2: Cr(VI) reduction rate versus initial Cr(VI) concentration (Molokwane *et al.*, 2008).

2.3.2 Cr(VI) reduction with continuous systems

In terms of commercial application continuous operation is likely to be the preferred choice. Continuous systems allow easier handling and simpler operation (Ahmad *et al.*, 2010). Batch systems are complex to describe as resulting from the interdependence between substrate, Cr(VI) and cell concentration ultimately affecting Cr(VI) reduction rate. Cumulative formation of reduction by-products, which may introduce cell toxins into a batch system, will also affect reduction rate. Due to the complexity of the biological reduction system described above, batch kinetics can not be applied directly to continuous systems and separate investigation is required. Studies on continuous reactor systems are less abundant in literature (Caravelli & Zaritzky, 2009). Key continuous studies are reported and summarised in table 2.

Table 2: Summary of continuous Cr(VI) reduction systems

Micro-organism	Author	Reactor [Ψ3]				Conditions [Ψ3]			Substrate [Ψ3]			Cr(VI) [Ψ3]			
		Type	Vol. [Ψ1] (L)	HRT [Ψ2] (h)	Biomass	O_2	T (°C)	pH	Type	Concen-tration (g/L)	Load Rate (g/L.h)	Inlet Conc. (mg/L)	Outlet Conc. (mg/L)	Load Rate (mg/L.h)	Reduction Rate (mg/L.h)
Arthrobacter rhombi	Elangovan & Philip (2009)	CSTR: Internal Circulating	8.2	24-60	Free cells	Yes	NA	7	Molasses-MSM	3	0.0625 – 0.125	18 & 20	0-17	0.33-0.833	0.102
		CSTR: Packed bed	1	24	Biofilm on plastic spirals	Yes	NA	7	Molasses-MSM	3	0.125	18, 20 &36	0-15	0.75-1.5	1.25
		CSTR: Packed bed	1	8 & 24	Biofilm on plastic rings	No	NA	7	Molasses-MSM	3	0.125 – 0.375	18, 20, 36, 60	0-15	0.75-2.5	1.15
Bacillus sp.	Chirwa & Wang (1997)	CSTR	0.0633	6-24	Biofilm on glass beads	Yes	30	NR	Glucose-MSM	2.5	0.104 – 0.417	5-100	0-0.62	0.202 – 4.17	4.17
Desulfomicrobium norvegicum	Battaglia-Brunet *et al*. (2006)	Counter-current trickle bed	200	9-54	Biofilm on volcanic rock	No	15	7-8.5	MSM, CO_2 and H_2	NA [Ψ4]	NA [Ψ4]	4-18	<0.2	0.074-0.99	0.2-2.2
Escherichia coli	Shen & Wang (1995)	PFR	0.245	5.1-10.5	Free cells from CSTR	Yes	35	7	Glucose-MSM	3	0.286-0.588	1.5-25	0-9.1	0.30-3.18	0.30-1.48
Sphaerotilus natans	Caravelli & Zaritzky (2009)	CSTR	1	4.2-50	Free cells	Yes	30	7	Citric acid-MSM	3.48	0.0696-0.835	0-80.1	3.9-78.2	0-19.07	0-0.607
Consortium	Chen & Hao (1997)	CSTR	4	53.3-480	Free cells	No	20& 35	7.9	NaAc-MSM	2	0.0042-0.0375	26	0-5.2	0.0542-0.4878	Maximum of 0.2083
Fungus: *Coriolus versicolor*	Sanghi & Srivastava (2010)	Packed bed	0.118	2.36-5.89	Attached biomass	–	25	7.2 – 3.2	MSM with complex substrate	3.5	0.59-1.48	10-80	0.4-46.4	1.70-33.90	5-18

[Ψ1] Reactor volume is defined as total vessel volume.

[Ψ2] HRT: Hydraulic retention time defined as liquid retention time through reactor volume.

[Ψ3] Ranges of conditions exist of which the outer limits are reported.

[Ψ4] Not applicable: Acetogenesis process does not require organic substrate.

Most continuous reduction experiments approach completely mixed conditions allowing classification as CSTRs. Where chemostats are not used, fully mixed conditions are achieved with liquid recycle. Once-through systems (with extended retention times) will have significant axial mixing therefore approaching also completely mixed conditions.

It is well established that biomass immobilisation improves culture resilience (Stoodley *et al*., 1999) and allows high specific biomass retention which improves volumetric productivity (Nicolella *et al*., 2000). Elangovan & Philip (2009) did not sterilise their feed and report contamination of the suspended cell culture but not of the attached systems. Chirwa & Wang (1997) report biofilm resilience by observing self-remediation after Cr(VI) overloading. Elangovan & Philip (2009) report drastic increases in volumetric reduction rate for their systems employing biofilm versus free cell operation. Comparison between the reported studies in table 2 supports the notion that attached systems facilitate higher volumetric reduction rates. Biofilms have been reported to facilitate coexistence of 12 bacterial species (Ahmad *et al*., 2010). This trait is important when considering employing a consortium of cultures where possible interactions of inter-species can improve reduction capability considerably (Molokwane *et al*., 2008).

Caravelli & Zaritzky (2009) operated a CSTR under substrate and nitrogen limited conditions in different experimental runs. The authors report Monod behaviour of Cr(VI) reduction rate as a function of Cr(VI) concentration where reduction rate is governed by substrate-enzyme adsorption equilibrium at elevated Cr(VI) concentrations. Similar results are reported by Chirwa & Wang (1997) who operated a CSTR under glucose limited conditions.

3 Experimental

3.1 Cr(VI) reducing bioreactors

The design of the two different reactors (FBBR and CSTR) that were used to conduct Cr(VI) reduction experiments are discussed below. Common operating conditions and further operational parameters applicable to the FBBR are discussed in section 3.1.3.

3.1.1 FBBR design

The FBBR setup can be seen in figure 3.

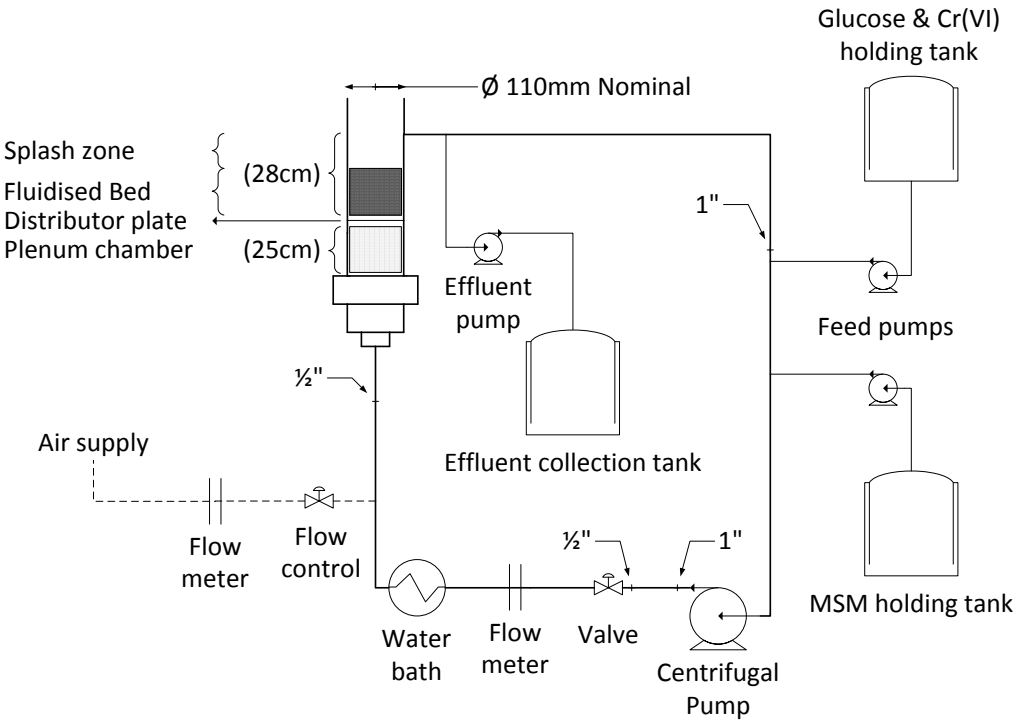


Figure 3: Slurry fluidised bed bioreactor setup.

The reactor was constructed from mostly PVC components and had a working volume of 9.0L. The feed dosing and effluent extraction pumps were Watson-Marlow (520s) peristaltic pumps. Liquid was circulated by a centrifugal pump (0.37kW mechanical seal type). The recycle flow rate was measured (Endress+Hauser Promag 10H) and controlled manually. Temperature was controlled by allowing heat exchange with a temperature controlled water bath through copper tubing (0.6m submerged length). Oxygen was supplied as air; the air delivery point was designed to be just upstream from the plenum chamber to allow gas to be dispersed into the liquid phase. The distributor had radial pitch with 1.8mm holes drilled on the intersecting lines of concentric circles (5mm apart) and diameter lines (6 lines 30° apart). Sand ($1.18<d_p<2$mm) was used as carrier particles. The bulk density (1215g/L), solid density (2430g/L) and voidage (0.5) were determined

experimentally. The reactor was open to the atmosphere at the top where excess air and expected product gas escaped.

3.1.2 CSTR design

The CSTR consisted of a glass beaker (10L) with a 3L working volume placed on a temperature controlled hotplate/magnetic stirrer. The beaker was open to atmosphere with a diameter of 25cm. The beaker content was stirred at 1500rpm. Air was bubbled into the liquid phase via a perforated silicon tube.

3.1.3 Operating conditions and operational parameters

Physical conditions in the reactors were similar. Aerobic growth conditions in the reactors were achieved by providing oxygen as air; the supply stream was not filtered. The dissolved oxygen (DO) concentration and temperature was measured periodically and controlled to be higher than 2mg/L and 30±2˚C, respectively. The growth medium was pH buffering and accordingly pH was maintained at 6.7±0.2.

Both reactors were operated with a constant hydraulic retention time of 30h as calculated by dividing reactor working volume with feed flow rate. The working volume of the FBBR includes the liquid volume in the pipes of the recycle system. Feed flow rates to the FBBR and CSTR were constant at 300mL/h and 100mL/h, respectively. Feed solutions were pumped from two plastic tanks (5L each) which were open to the atmosphere. Load rates (mg_i/L.h with *i*=Cr(VI) or glucose) were varied by varying feed concentrations. Feed-solutions were prepared in 20L batches using non-autoclaved tap water. Cr(VI) was dissolved in the glucose solution to inhibit unintentional growth and was fed separate from the MSM solution which cannot support microbial growth on its own. Potassium chromate (K_2CrO_4) was used as source of Cr(VI). The volumetric flow rates from the feed tanks to the reactor were equal. Effluent was stored in a collection tank and disposed of when required.

The recycle ratio in the FBBR, defined as feed flow rate divided by recycle flow rate, was 5×10^{-5} allowing classification as a fully mixed reactor or CSTR. Liquid recycle flow rate was constant at 10.7L/min corresponding to minimum fluidisation conditions of the fluidised bed (0.0188m/s mean radial velocity). Sand grains (550g) were fluidised, from a static bed height of 4.8cm, to form a plume of solids with the apex approximately 7cm high. The plume resulted from the radial decrease in distributor plate hole-density and allowed solids circulation by facilitating upward solids flow in the centre and downward flow along the sides.

3.2 Bacterial consortium

A dried sludge sample of the Brits Culture, stored at 4˚C, was used as inoculant. The sample was obtained from sand drying beds at the Brits Wastewater Treatment Works in the North-West province of South-Africa (Molokwane *et al*., 2008). The treatment works receives Cr(VI) contaminated water intermittently from nearby chrome foundries as well as a nearby decommissioned sodium dichromate processing facility. Molokwane *et al*. (2008) investigated the Cr(VI) reduction ability of the consortium. Under aerobic conditions the consortium is able to reduce chromate at high concentrations (400mg/L) at higher rates than reported cultures. The consortium is facultative: can be employed under aerobic or anaerobic conditions, but aerobic assays are reported to outperform the anaerobic consortium. Molokwane *et al*. (2008) isolated the consortium cultures using the serial dilution spread plate method and then identified them via phylogenetic characterisation using 16S rRNA partial sequence analysis. Despite better performance, less biodiversity was observed for aerobic cultures (7 identifiable potential Cr(VI) reducing species) compared to anaerobic cultures (18 identifiable species). The Gram-positive *Bacillus* genera predominated consortium composition under aerobic conditions with a small composition of Gram-negative *Microbacterium* sp. Anaerobic cultures also included *Enterococcus*, *Arthrobacter*, *Paenibacillus* and *Oceanobacillus* species. It is possible that the existence of interspecies interactions are necessary for optimum Cr(VI) reduction because individual isolated species did not achieve the same level of Cr(VI) reduction.

3.3 Incubation

Incubation was done by transferring a sample of dried sludge (approximately 2g) to an Erlenmeyer flask (1L) containing 250mL Luria Bertani (LB) broth (Merck biolab series). Broth was autoclaved previously at 121°C for 20min and allowed to cool to room temperature. Incubation was done overnight on a lateral shaker (120rpm) placed in a temperature controlled incubation room (32±2°C). The flask opening was plugged with cotton wool to allow aerobic conditions while restricting unnecessary contamination. Incubated cells were separated from the supernatant through centrifugation at 4°C and 2820*g* (6000rpm, rotor radius 7cm) for 15min. Separated cells were washed through resuspension in approximately 40mL NaCl solution (18.5g/L) followed by centrifugation. Washing was performed twice.

3.4 Growth medium

The growth medium was a D(+) glucose monohydrate (Merck) supplemented MSM. The composition of the MSM can be seen in table 3. The MSM is pH buffering. Control experiments confirmed that the complex growth medium did not reduce chromate. It should be noted that the effective concentrations fed to the reactor are only half of those reported in table 3 as a result of the equal part dilution with the Cr(VI) supplemented substrate feed stream. Molar masses are included because some of the salts used were not anhydrous.

Table 3: Make-up of the MSM used in this study

Chemical name	Chemical formula	MM (g/mol)	Mass (g)	Chemical name	Chemical formula	MM (g/mol)	Mass (g)
Ammonium Chloride	NH_4Cl	53.49	1.07	Copper Chloride	$CuCl_2$	170.48	34.1×10^{-6}
Sodium Phosphate	Na_2HPO_4	141.96	8.52	Sodium Bromide	NaBr	102.90	10.3×10^{-6}
Potassium di-hydrogen Phosphate	KH_2PO_4	136.09	5.44	Sodium Molybdate	Na_2MoO_4	241.95	12.1×10^{-6}
Sodium Sulphate	Na_2SO_4	142.02	0.227	Manganese Chloride	$MnCl_2$	197.84	19.8×10^{-6}
Magnesium Sulphate	$MgSO_4$	246.48	48.2×10^{-3}	Potassium Iodide	KI	166.0	16.6×10^{-6}
Calcium Chloride	$CaCl_2$	110.98	7.35×10^{-3}	Boric Acid	H_3BO_3	61.83	12.3×10^{-6}
Iron Sulphate	$FeSO_4$	278.02	6.95×10^{-3}	Cobalt Chloride	$CoCl_2$	237.93	23.8×10^{-6}
Zinc Chloride	$ZnCl_2$	136.30	13.6×10^{-6}	Nickel Chloride	$NiCl_2$	128.69	23.8×10^{-6}

3.5 Preliminary attachment investigation

Possible attachment to five different support media was tested in batch assays. The support media investigated were:

- Smooth glass beads 3mm.
- Sanded glass beads 3mm. Sanding was done on a sieve shaker (Fritsch: laborgerätebau set at amplitude 5 for 6 hours) with sanding paper (80 grain). Sanding paper was glued to the bottom of a sieve pan and pressure was applied on the beads by a 2.5kg weight with sanding paper glued to the bottom.
- River sand was sieved on a sieve shaker (Fritsch: laborgerätebau set at amplitude 4 until no more fines were sieved out) to obtain particle size $1.18<d_p<2$mm.
- Aquarium sand sieved in the same way as above.
- Granular activated carbon (GAC) with a particle diameter similar to that of the sand samples.

A sample of each support material (35g) was washed with tap water then sterilised with ethanol (99% purity) for 10min followed by rinsing with distilled water. The samples were transferred to five Erlenmeyer flasks (250mL capacity) which were sterilised with ethanol. One hundred millilitres of growth medium containing incubated cells was added to each flask. Liquid volumes had uniform cell concentrations and were enough to submerge the solid phases. The flask openings were plugged with cotton wool. Flasks were swirled at least once a day over the duration of cultivation. After two weeks of cultivation, the supernatant was decanted from each flask without losing any support particles. Support particle samples were then washed serially to remove suspended cells from the assays. Each sample wash washed twice with 100mL solution followed by four washes with 50mL of previously autoclaved NaCl solution. After each wash the supernatant was discarded without losing any support particles. Suspended cells were removed. Each sample was submerged again in previously autoclaved growth medium (96mL) supplemented with approximately 4mL Cr(VI) stock solution (1000mg/L) to yield a final liquid volume of 100mL at 40mg_{Cr6+}/L. Specific reduction rates (mg_{Cr6+}/h.35g_{media}) were compared over one week. Control experiments were conducted with no inoculated bacteria. No changes in Cr(VI) concentration were observed in the control experiments, except for the GAC assay which indicated Cr(VI) absorption. Cr(VI) reduction was observed with the washed and re-used river sand, aquarium sand, sanded glass and smooth glass beads, with the highest specific reduction rates observed with river sand and the lowest rates with smooth glass beads. This was assumed to be a positive indication of biomass immobilisation. Sieved river sand was subsequently used as support medium in the FBBR.

3.6 FBBR inoculation with biofilm on river sand

Biomass immobilisation on sand particles was achieved in two 1L Erlenmeyer flasks each containing incubated cells, 250mL LB-broth and 275g ethanol-sterilised river sand. The flask openings were plugged with cotton wool. The flasks were placed on a lateral shaker (110rpm) situated in a temperature controlled incubation room (32±2°C) and cultivated for ten days. After this time the supernatant was discarded without losing any sand particles. The solids in each of the flasks were re-submerged in equal volumes of growth medium (250mL) supplemented with 2.025mL stock Cr(VI) solution to have a final Cr(VI) concentration of 10mg/L. The assays were allowed to reduce all chromate before the inoculated sand particles were transferred to the FBBR.

3.7 Analytical methods

3.7.1 Sampling

Two 1.5mL samples were collected from the effluent stream into Eppendorf-type centrifuge tubes at various intervals of operation. The samples were centrifuged at 6000rpm, 2000g (Hermle GmbH Z

100 M minicentrifuge) for 15min to remove the cells as pellets at the bottom of the tubes. The cell free supernatant used for analytical procedures was extracted from the centrifuge tubes with a pipette without resuspending the separated cells.

3.7.2 Suspended biomass

The combined mass of two sample tubes was weighed on an electronic balance accurate to 10^{-4} gram (Ohaus Adventurer AR2140) before sampling. After all the required supernatant was extracted from the tubes, the remaining supernatant was discarded carefully not to lose separated cells. The remaining cell pellets were allowed to dry in the tubes for approximately 24h. The tubes were weighed and the mass recorded; the initial mass was subtracted from the resulting mass and the answer multiplied with a factor to yield a dry cell mass concentration in units of mg_{dry_cells}/L.

3.7.3 Viable cell concentration

Living cell concentration was determined using the serial dilution spread plate method. One millilitre of suspended cell solution was diluted serially into 9mL NaCl solution (18.5g/L) contained in ten test tubes. Test tubes were sterilised just before use with 99% purity ethanol and then rinsed with distilled water. One millilitre of suspended cell solution was transferred from test tubes 8, 9 and 10 to three Petri dishes with agar-medium. The agar-medium was a mixture of Plate Count Agar and LB Agar (Merck) dissolved collectively into tap water at half the recommended concentration each; 11.5g/L and 22.5g/L were dissolved, respectively. The agar-medium was autoclaved at 121˚C for 20min before use. The suspended cell solutions were spread onto the agar-medium, then the Petri dishes were turned upside-down and were incubated overnight in a temperature controlled oven at 30±3˚C. The cell colonies on each plate were counted and the geometric mean between the three plates is reported as colony forming units (CFU) per millilitre of sampled solution.

The procedure was performed once for every steady state condition.

3.7.4 Cr(VI) measurement

Chromate concentration was determined through absorbance measured by a UV/visible spectrophotometer (Pharmacia Biotech Ultrospec 1000) at 540nm through a 10mm light path. Samples of cell free supernatant (0.2mL) were acidified with 0.4mL sulphuric acid solution (2M), then diluted with 9.2mL water followed by discoloration to purple with 0.2mL of 1,5-diphenyl carbazide (DPC) solution. DPC solution was prepared by dissolving 0.5g DPC into 100mL chromatography grade acetone; the solution was stored in a brown glass bottle covered with tin foil.

Calibration was done using a water filled cuvette as reference cell. The absorbance of standard Cr(VI) solutions with concentrations below 20mg/L were measured – the upper limit of the linear range of the calibration curve. Cell free supernatant samples were diluted to Cr(VI) concentrations below 20mg/L. Four repeat Cr(VI) measurements were done from each sample and their geometric mean is reported as the chromate concentration of that sample. If dilution was necessary the geometric mean of the concentration was multiplied with the dilution ratio.

3.7.5 Total chromium measurement

Total chromium was measured using a flame atomic absorption spectrophotometer (AAS) (PerkinElmer AAnalyst 400) at a wavelength of 359.9nm. Calibration was done using standard chromium solutions below 5mg/L; samples were diluted to within this range.

3.7.6 Glucose

The glucose concentration was determined according to the Somogyi-Nelson method described in detail by Whitaker (2001: 579-580).

3.7.7 Scanning electron microscopy

Support particles were prepared for electron microscopy by:

- Fixing the biomass with 2.5% glutaraldehyde dissolved in 0.075M phosphate buffer (pH=7.4-7.6) for 30min.
- Rinsing 3 times for 5min each time with phosphate buffer.
- Fixing with 0.25% aqueous osmium tetroxide 3 times for 5min each time (in a fume hood).
- Rinsing 3 times with distilled water (in the fume hood).
- Dehydrating with 20, 50, 70, 90 and 99% ethanol for five minutes at a time.
- Drying twice for 15min at a time with hexamethyldisilazane.
- Evaporating hexamethyldisilazane from the particles under atmospheric conditions for approximately 30min.
- Attaching particles to carbon tape which in turn was fixed to an aluminium support.
- Covering in gold under argon plasma.

A control sand sample of dry sand, that was sieved and sterilised, was prepared according to the last two steps listed above.

Scanning electron microscopy was done only once at the end of the last run in the FBBR. A sample of sand (approximately 5g in total) was removed from more than one location in the FBBR to represent a randomly selected sample.

3.7.8 Culture isolation and identification

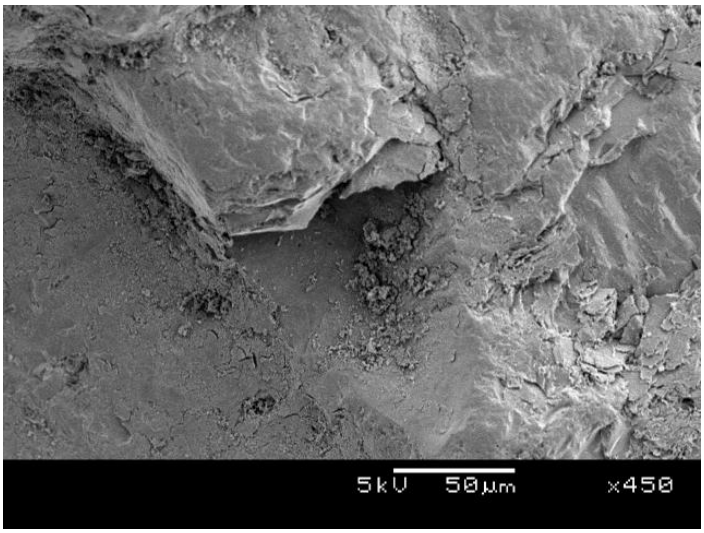
Isolation and identification of the consortium were done using the serial dilution spread plate method and 16S rRNA partial sequence analysis, respectively. Blast test matches were done to determine a best blast match for each isolate. Further classification was done by categorising isolates in genus trees.

Culture isolation & identification was done only once at the end of all experimental runs.

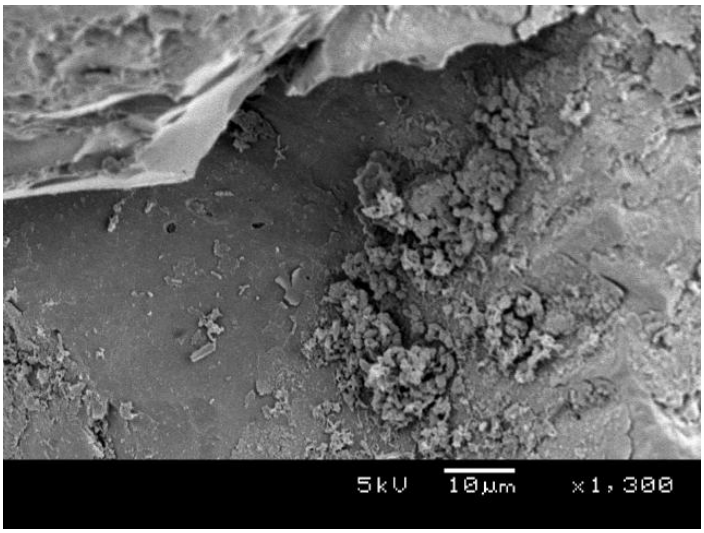
4 Results and Discussions

4.1 Biomass characteristics in the FBBR

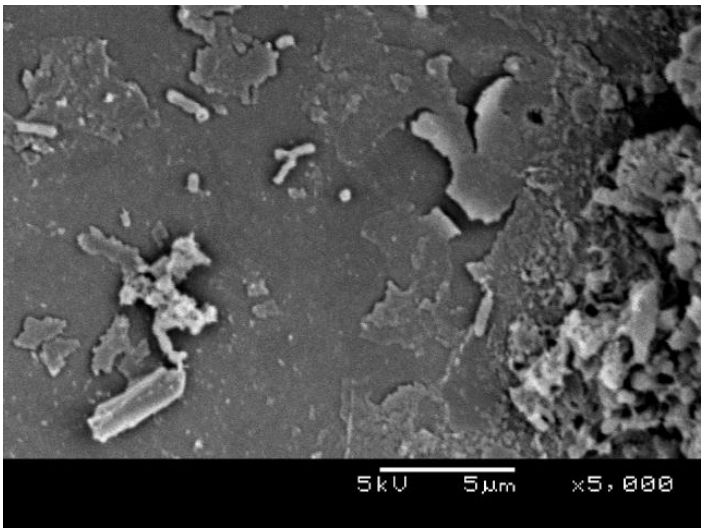
No evidence of biofilms was found on the sand particles, even in the surface crevasses where attrition effects are negligible. Only a few scattered cells were observed in these crevasses (as can be seen in figure 4c). However extensive biofilm were observed on all wall surfaces of the FBBR setup (column wall and recycle pipes) except for the column section in contact with the fluidised sand bed. This suggests that the shear effect of sand particle collisions (on each other and on the wall) outweighs flow induced shear with regards to biofilm formation of the specific consortium.



(a)



(b)



(c)

Figure 4: SEM photographs of a crevice at different magnifications showing some scattered micro-organisms but no biofilm.

4.2 Continuous, aerobic Cr(VI) reduction

The results of the continuously operated reactors are summarised in table 4 and illustrated on figure 5. Successful Cr(VI) reduction was achieved over 78 consecutive days of continuous operation. The different regimes marked on figure 5a coincide with a change in inlet concentration and/or reactor type as listed in table 4. The Cr(VI) load rate used in run I was chosen as to compare with the initial load rate reported by Chirwa and Wang (1997). Subsequent Cr(VI) inlet concentrations were changed by 15ppm to prevent sudden Cr(VI) over-exposure. Runs V and VI were done at Cr(VI) load rates comparable to runs IV and II, respectively. Table 4 reports the steady state operating conditions and geometric means of steady state results. Steady states were assumed when glucose and Cr(VI) concentrations remained constant for at least three hydraulic retention times (as indicated on figure 5b).

Throughout experimentation total chromium was accounted for with 96% accuracy as measured by AAS and reported in Table 4. The result indicates that chromium was not absorbed into the biofilm. The difference between the inlet and outlet Cr(VI) concentrations were assumed to be Cr(III). This assumption is fair because experimental conditions concur with natural conditions, with specific regard to pH which remained constant at 6.7±0.2, where Cr(VI) and Cr(III) are expected (James, 2002).

The results for total suspended biomass concentration are of the same order of magnitude (as listed in Table 4 below). Similarly, viable cell count results indicated that the concentration of living cells were of the same order (10^8CFU/mL) for all experimental runs (results not listed). The similarity indicates a constant fraction of living cells constituting total suspended biomass concentration for all steady state operating conditions.

After only six weeks of operation the reactor system was robust to the extent where it could recover from a non-routine maintenance operation. During run IV (day 44) the mechanical seal of the recycle pump failed and the pump had to be replaced by the backup pump. Within four days the reactor recovered to an operational state similar to conditions before the failure (refer to figure 5). On day 50 the backup pump also broke down. A suitable replacement could not be procured within the time limits of the experimental schedule. Reduction experiments were continued therefore in the CSTR (runs V & VI) by transferring 3L of liquid from the FBBR to the CSTR. Unfortunately due to the FBBR breakdown planned repeat experiments could not be performed.

Table 4: Steady state values for inlet and outlet concentrations as well as calculated rates for all continuous runs.

		Cr(VI)				Glucose						
Run	Reactor type [Ψ1]	Inlet conc. [Ψ2] (mg/L)	Outlet conc. (mg/L)	Load rate [Ψ3] (mg/L.h)	Reduc-tion rate (mg/L.h)	Inlet conc. [Ψ2] (mg/L)	Outlet conc. (mg/L)	Load rate [Ψ3] (mg/L.h)	Consump-tion rate (mg/L.h)	Cell conc. (g/L)	Total Cr conc. (mg/L)	Reduction yield (mg_{Cr6+}/g_{glu})
I	FBBR	21.4	18.5	0.72	0.10	2.5	0.0	84.4	84.4	0.67	20.89	1.16
II	FBBR	34.4	26.9	1.16	0.26	2.5	0.0	84.4	84.4	0.63	33.78	3.02
III	FBBR	52.1	39.2	1.76	0.44	2.5	0.0	84.4	84.4	0.70	50.14	5.18
IV	FBBR	51.7	23.0	1.75	0.97	5.0	2.5	168.9	84.4	–	49.83	11.46
V	CSTR	53.2	41.1	1.79	0.41	5.0	3.8	168.3	40.4	0.77	52.38	10.08
VI	CSTR	32.4	27.1	1.09	0.18	2.5	0.9	84.2	52.5	0.57	31.47	3.40

Ψ1 Hydraulic retention time was constant at 30h for both reactors. Volumes were 9L for the FBBR and 3L for the CSTR.

Ψ2 Feed concentrations listed as the effective reactor inlet concentration, that is half the concentration in the feed tank as a result of equal part dilution.

Ψ3 Values calculated based on total volumetric flow rates to the reactor (FBBR: 300mL/h and CSTR: 100mL/h).

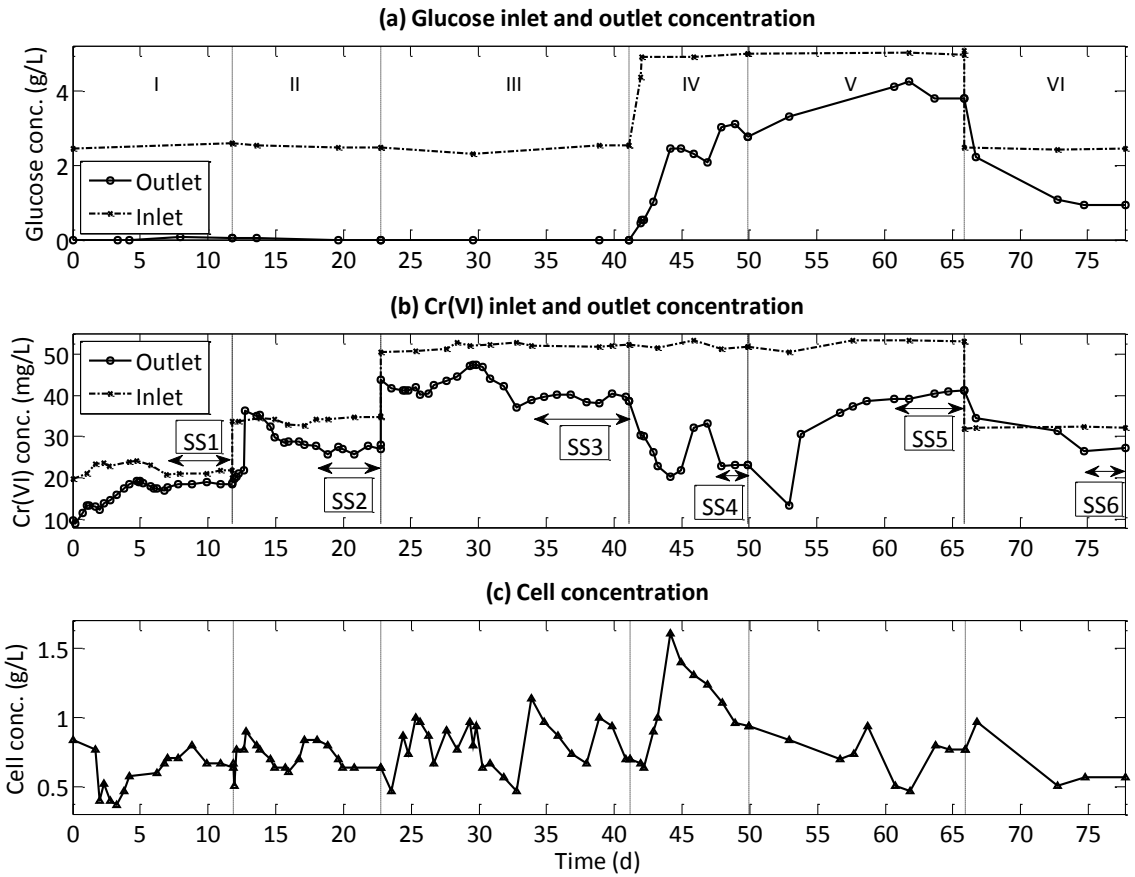


Figure 5: Continuous reactor experiments: reactor inlet and outlet concentrations vs. time; steady states (SS) are indicated on figure (b).

Significant data scatter prevents unambiguous interpretation of the system's dynamic behaviour for kinetic study. Steady state results could not be described by a single reduction rate expression with regard to cell, substrate and Cr(VI) concentration. This might be attributed to insufficient quantification of the amount of biofilm in the recycle system of the FBBR. The inconsistencies might also be linked to changes in consortium composition under different operating conditions. Repeat experiments at different stages in the lifetime of the consortium would have served as a test for this hypothesis, but was not possible due to the failure of the FBBR system.

The contribution of biofilm to the overall Cr(VI) volumetric reduction rate is clear when comparing the results of the FBBR to the CSTR. Comparison under similar operating conditions and suspended cell concentrations (runs II and IV compared to runs VI and V, respectively) showed higher reduction rates achieved by the FBBR (0.26 and 0.97mg/L.h) compared to the CSTR (0.18 and 0.41mg/L.h). This is in agreement with the findings of Elangovan & Philip (2009) who reported higher rates for systems with biofilm than for a system with free cells.

Since substrate requirement is part of the cost objective of a Cr(VI) reducing system, it is worthwhile to compare the glucose requirement per unit Cr(VI) reduced, of the FBBR and CSTR. Biofilms might be expected to have higher substrate requirements than their suspended counterparts due to additional metabolic activities like EPS production. For this comparison reduction yield is defined as milligram Cr(VI) reduced per gram substrate consumed as calculated with (Caravelli & Zaritzky, 2009):

$$RY = \frac{r_{Cr(VI)}}{r_{glucose}} \tag{5}$$

where $r_{Cr(VI)}$ is the Cr(VI) reduction rate (mg/L.h) and $r_{glucose}$ the glucose consumption rate (g/L.h). Calculated values for reduction yield are listed in table 4. Similar substrate requirements for comparative runs (II & IV and VI & V) of the FBBR and CSTR indicate similarities between suspended and attached cells.

Steady state results for the first three runs (under glucose limited conditions) indicate a highly non-linear relationship between Cr(VI) concentration and reduction rate. Conventional bio-kinetic rate forms, like the Monod equation, could not fit the data. The rate can be predicted by a second order linear relationship as indicated in figure 6. This result contradicts the work by Caravelli & Zaritzky (2009) and Chirwa & Wang (1997) where Monod kinetics were able to predict reduction rates achieved by pure culture organisms under glucose limited conditions. The result implies that substrate-enzyme adsorption equilibrium cannot account for reduction rate changes. The severe

changes in reduction rates are linked likely to increases in Cr(VI) reducing enzymes (despite similar cell concentrations). The hypothesis was not tested by analysing enzyme concentration, with for instance comparative reduction experiments by enzymes obtained from lysed cells in a French pressure cell (Garbisu *et al*., 1998), because the result was unexpected. Enzyme increases might have been facilitated by a different species distribution in the consortium at higher Cr(VI) concentrations, where organisms with higher reduction efficiencies were more abundant. Reduction yield showed analogous rapid increases with Cr(VI) concentration under glucose limited conditions.

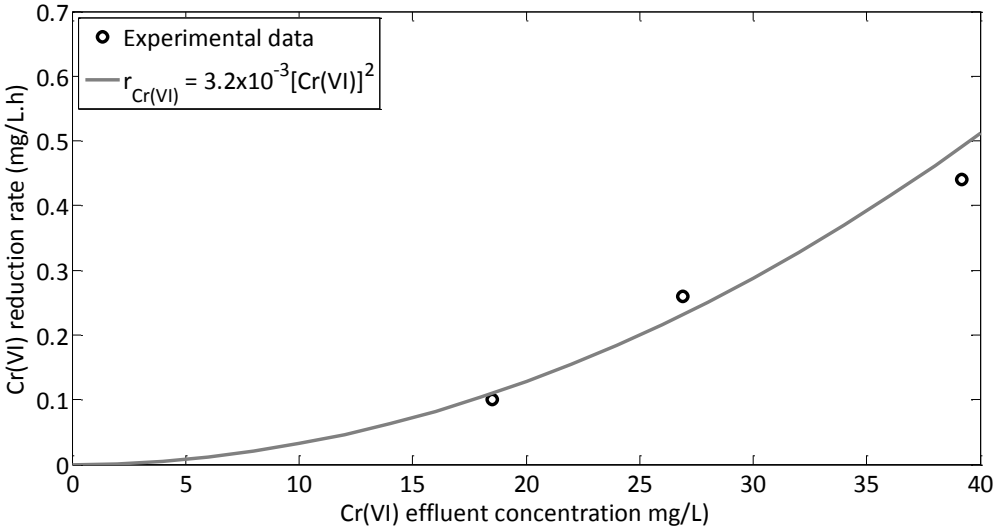


Figure 6: Rate prediction as a function of Cr(VI) outlet concentration by a second order power law model.

For both reactors the Cr(VI) reduction rate showed a strong dependency on the glucose concentration. This is evident when runs III and VI are compared to runs IV and V. This result is in agreement with the results of Pal & Paul (2004) and Caravelli & Zaritzky (2009). The effect of substrate concentration can also be observed when comparing run III to run V, where similar reduction rates at similar Cr(VI) concentrations were achieved, despite run III having attached biomass.

4.3 Bacterial consortium analysis: 16S rRNA partial sequence analysis

Results indicated that 14 possible Cr(VI) reducing microbial species were present in the reactor upon completion of experiments. Best blast match species are summarised in table 5. The isolate numbers are indicated on the genus trees in Appendix A.

Table 5: Bacterial consortium analysis results indicating best matches and reported Cr(VI) reducing micro-organisms of the same genus.

Isolate(s)	Best blast match	Genus reported for Cr(VI) reduction
1 & 5	*Acinetobacter baumannii*	*Acinetobacter haemolyticus* (Ahmad *et al*., 2010)
6, 12 & 15	*Acinetobacter beijerinckii*	
3	*Bacillus pumilus*	*Bacillus* (Molokwane *et al*., 2008)
8 & 10	*Cellulomonas flavigena*	
13	*Microbacterium deminutum*	*Microbacterium* sp. (Molokwane *et al*., 2008)
2	*Planomicrobium koreense*	
4 & 9	*Staphylococcus caprae*	
11 & 14	*Stenotrophomonas maltophilia*	

The consortium composition obtained in this study differs from the composition reported by Molokwane *et al*. (2008) who identified 7 species from 2 genera under aerobic conditions. Concurrently, both *Bacilli* and *Microbacteria* were present in this investigation. The dissimilarities might be attributed to the different growth medium and elevated Cr(VI) exposure concentrations employed in the batch assays of Molokwane *et al*. (2008).

5 Conclusions

Successful Cr(VI) reduction was achieved over 78 days of continuous operation in a FBBR and a CSTR. It was planned initially to perform all the experimental runs in the FBBR, but due to unforeseen system failure a CSTR, without any biofilm, was used for the last 28 days of the investigation. Due to the FBBR breakdown, no repeat runs (at different consortium ages) could be performed.

Initial attachment investigations suggested biomass attachment as biofilm on river sand particles and therefore river sand was used as solid support medium in the FBBR. After 50 days of continuous operation no evidence of biofilm growth on these particles was found. However, significant biomass immobilisation was observed on the wall sections of the FBBR column and recycle piping.

The volumetric Cr(VI) reduction rates achieved in the FBBR was higher than that in the CSTR (with no attached biomass) under similar operating conditions, proving the enhanced contribution of biomass as biofilm. Substrate requirements per unit of Cr(VI) reduced were similar between the CSTR and the FBBR indicating similarities between attached biomass and free cells. Results from the FBBR indicate a second order relationship between reduction rate and Cr(VI) concentration under substrate limited conditions. Reduction yield showed analogous rapid increases with Cr(VI) concentration for the glucose limited runs. Results from both reactors indicate a strong dependency of Cr(VI) reduction rate on substrate concentration.

Fourteen distinguishable species from 7 genera were identified as possible Cr(VI) reducers on completion of experimentation. The consortium composition differs from the composition reported by Molokwane *et al*. (2008) who identified 7 species from 2 genera in batch investigations with a different growth medium and higher Cr(VI) exposure concentrations.

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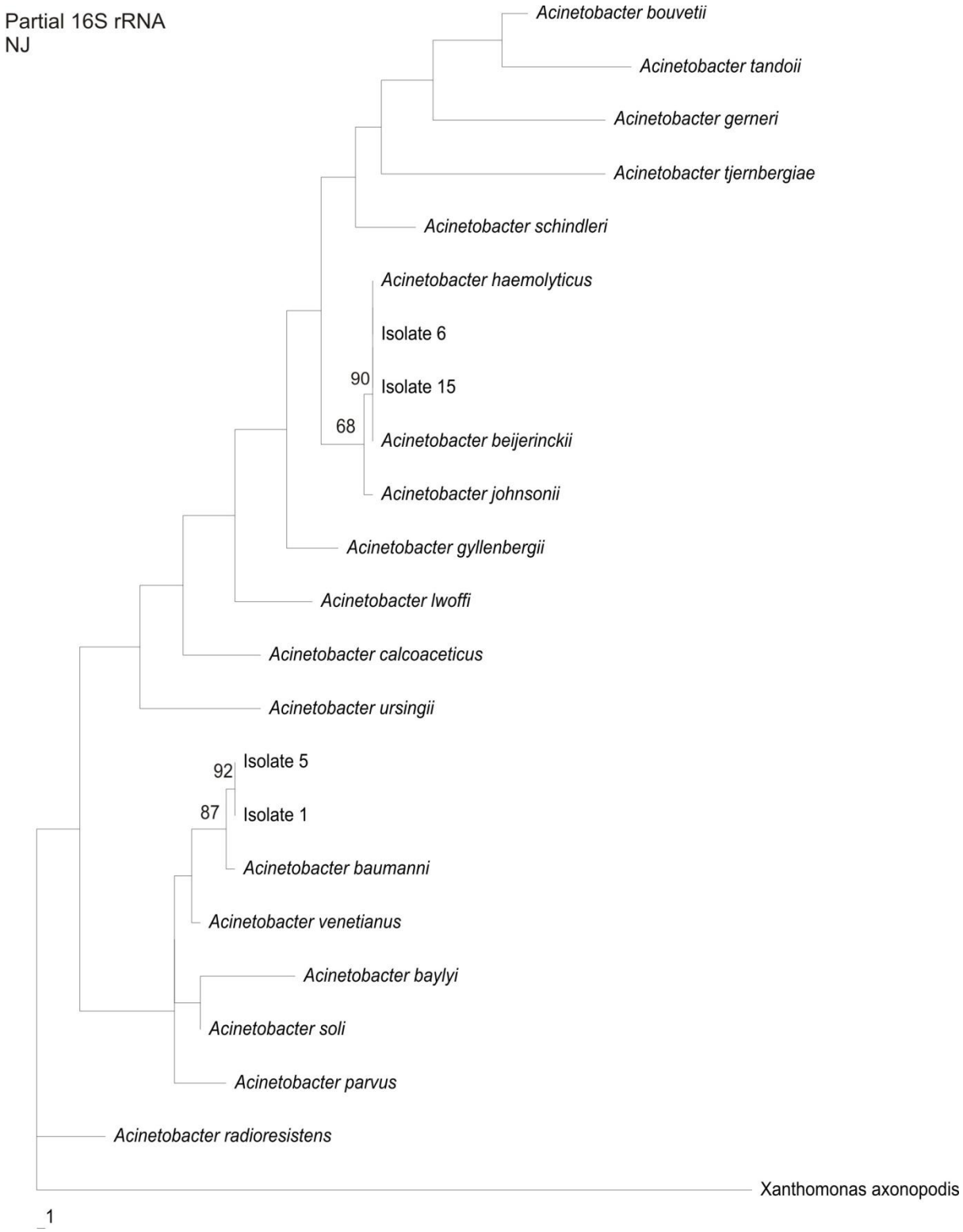
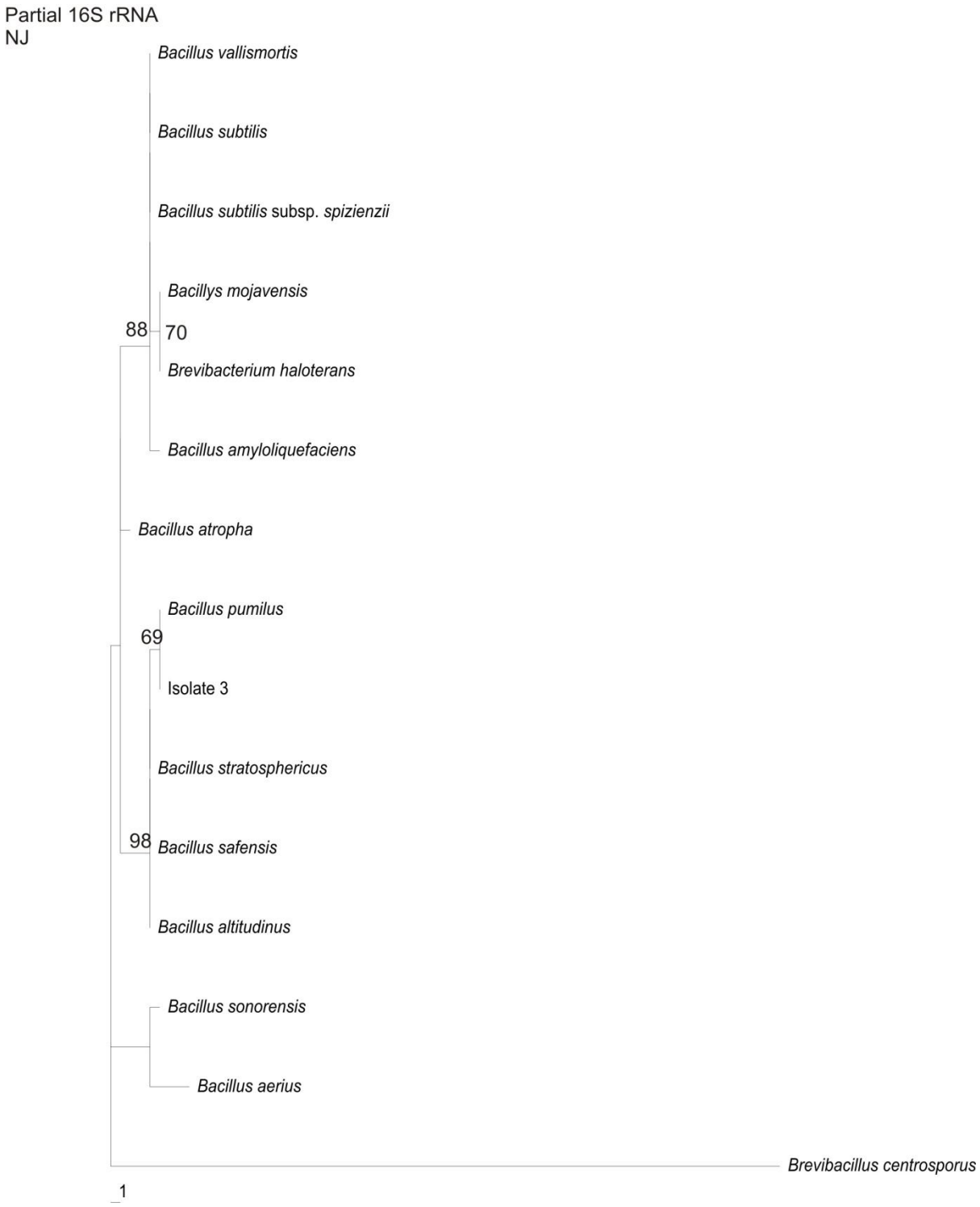
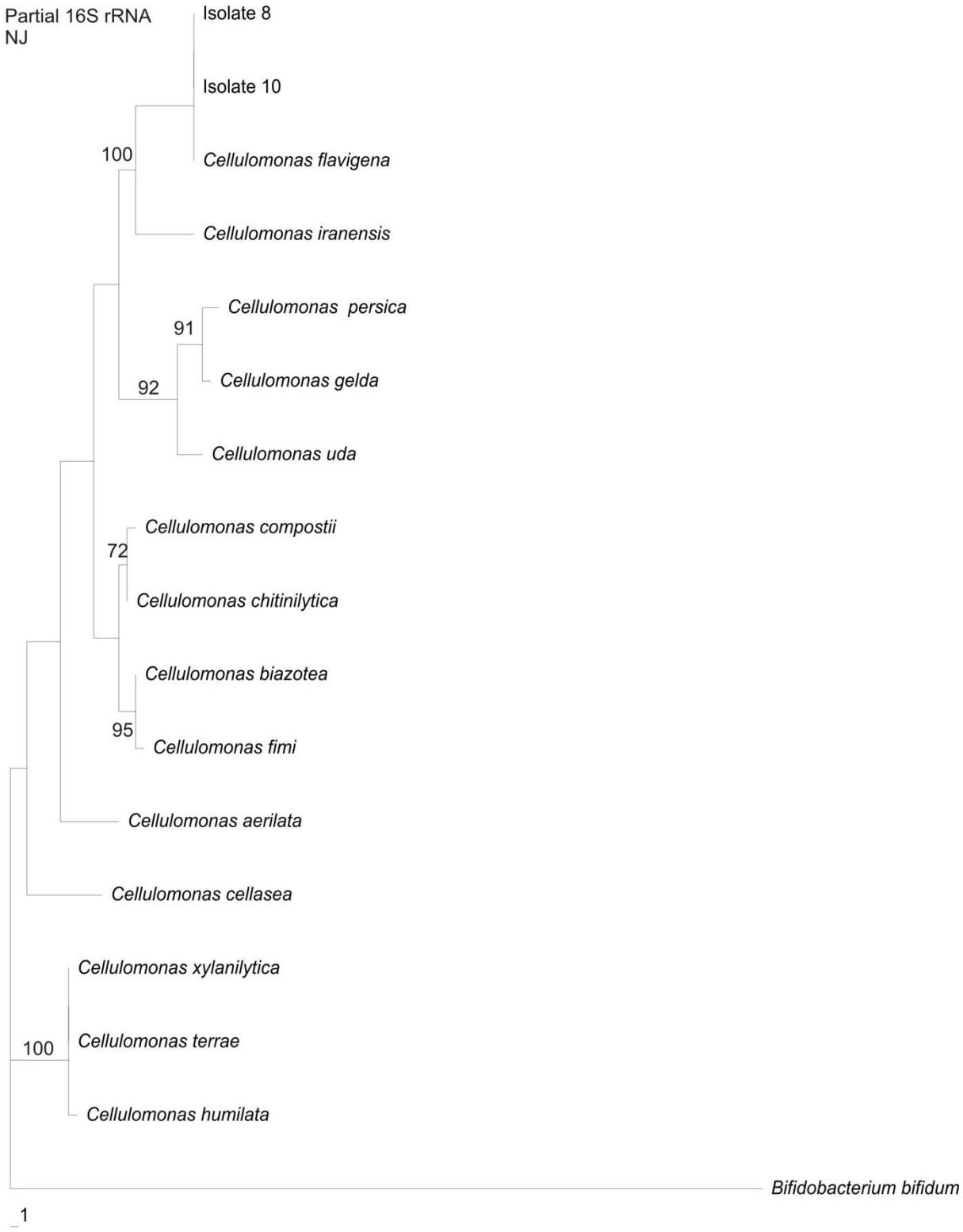
Appendix A: Genus trees (*Acinetobacter* Tree)



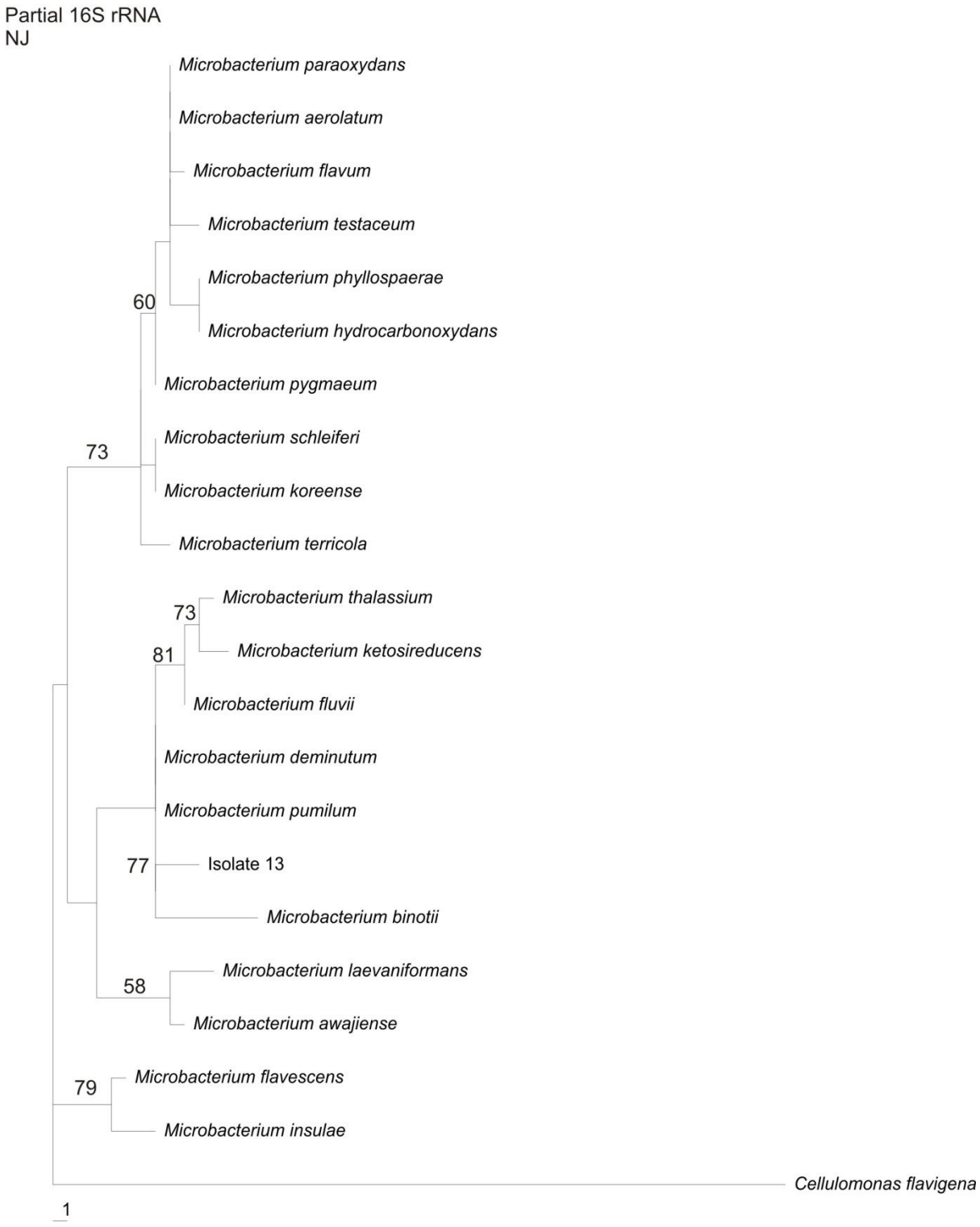
Bacillus Tree



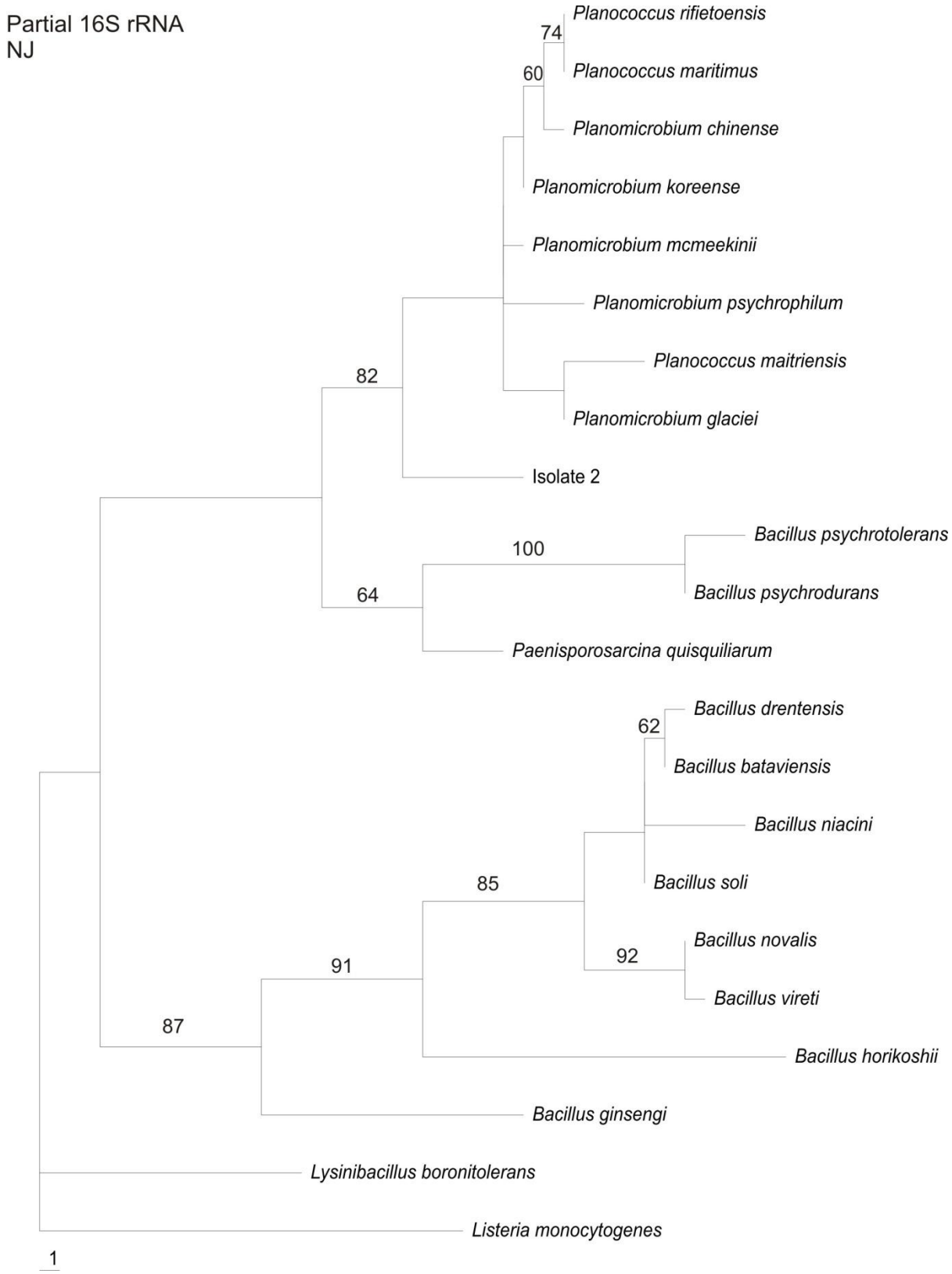
Cellulomonas Tree



Microbacterium Tree



Planomicrobium Tree



Staphylococcus Tree

Stenotrophomonas Tree