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Demonstration of the use of *Scenedesmus* and *Carteria* biomass to drive bacterial sulfate reduction by *Desulfovibrio alcoholovorans* isolated from an artificial wetland

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Abstract

A major factor limiting application of bacterial sulfate reduction to removal of sulfate and heavy metals in wetland systems is the requirement to supply carbon and energy to drive the process. Primary production by aquatic plants and algae is a cheap option for driving sustainable bacterial sulfate reduction and most operational systems have relied on plants. The use of harvested, non-growing algal biomass to support bacterial sulfate reduction was investigated. Two genera of green algae, strains N9 and A3, were isolated from treatment cells from the Artificial Wetland Filter at the Ranger uranium mine (Northern Territory, Australia) which successfully removes UO_2^{2+}, Mn^{2+} and nitrate, but little sulfate, from mine waters. These algae were identified as *Carteria* sp. and *Scenedesmus* sp. and were used as the sole carbon and energy source to enrich a sulfate-reducing mixed bacterial culture from the constructed wetland. Bacterial sulfate reduction supported solely by degradation of algal biomass was demonstrated at laboratory scale using both algae. In excess of 300 mg/L, sulfate was reduced in 17 days following an initial period of approximately 8 days during which sulfate levels did not decrease. The amount and rate of reduction was shown to be dependent on the concentration of algal biomass added. *Carteria* algae at low concentration showed reduction earlier; however, yields at higher concentration were affected by unknown inhibition. *Scenedesmus* strain N9 produced a maximum specific yield of 94.3 g of sulfate reduced per gram biomass added compared with 43.5 for *Carteria* strain A3. Sequence analysis of the 16S rRNA gene of members of the bacterial consortium indicated that the sulfate-reducing bacteria (SRB) showed highest homology (98.5%) with *Desulfovibrio alcoholovorans*. A second bacterium, which showed homologies of 91–92% with organisms of the Clostridial assemblage, was also present in the culture and represents a new species, or possibly a new genus.
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Keywords: Bacterial sulfate reduction; Algae; Wetlands; *Desulfovibrio*; Organic carbon

1. Introduction

The use of constructed wetlands to treat mine drainage containing heavy metals and sulfate has been undertaken in the USA and Australia with varying degrees of success (Hammer, 1989; Hedin and Nairn, 1993; Hedin, 1997; Younger, 1997). Whilst the metal and sulfate removal mechanisms were originally thought to be plant mediated, it is now recognised that bacterial sulfate reduction is a key process in effluent treatment (Hedin et al., 1989) although organic carbon is required to provide the energy to drive it (Postgate, 1984; Odom and Singleton, 1993). The Ranger mine was amongst the first in the world to use wetlands to

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treat drainage from uranium mining and although UO_2^{2+} and Mn^{2+} were successfully removed, sulfate levels remained unabated. Factors limiting sulfate reduction were investigated and carbon limitation of bacterial activity was identified as a significant factor in poor sulfate removal efficacy (Jones et al., 1996; Shinners, 1996).

The provision of organic carbon to drive bacterial growth and sulfate reduction in passive water treatment systems is potentially a significant operational cost with attendant technical issues. The treatment of sulfate-containing wastewaters in active, reactor-based systems usually involves provision of low-molecular-weight liquid substrates such as methanol or lactate and is reviewed elsewhere (Lens et al., 1998).

As they are solar-driven, photosynthetic processes producing biomass as feedstock for the bacteria are likely to be a cheap option for passive systems. Although plants are part of the usual wetland design, their tissues contain lignified polymers that are only partly biodegradable. This problem is encountered both in wetlands containing growing plant stock as a carbon source and in wetlands where harvested material of plant origin has been added. The addition of such materials is widely reported and include mushroom compost, peat moss, decomposed wood products, sawdust and straw (Wildeman et al., 1993; Hamilton et al., 1997; Gross et al., 1993). The latter materials are also unsuitable, on ecological grounds, for addition to the constructed wetland at Ranger as it is in the Kakadu World heritage area and the import of non-indigenous plant materials is prohibited.

Green and blue-green unicellular algae have long been recognised for their high nutritional value in the context of their use as human (Kay, 1991) or stock feeds (Halama, 1990). Green algae such as *Chlorella* and *Scenedesmus* contain 50–60% by dry weight protein (Becker, 1994; Chowdhury et al., 1995). Carbohydrate and lipid content vary broadly depending on growth conditions and range over 10–52% and 2–40%, respectively (Becker, 1994). The cost of large-scale generation of green algal biomass (*Chlorella* and *Scenedesmus*) was estimated by Chowdhury et al. (1995) at US$1.25 per tonne and is likely to be less than plant-based materials named above. The use of biomass from the blue-green alga *Spirulina* to feed sulfate-reducing bacteria (SRB) in a process treating organic and metal waste effluent has been reported by Rose et al. (1998). The use of unidentified algae to provide a carbon and energy source for denitrifying bacteria involved in the reduction of selenate has also been reported by Lundquist et al. (1994).This paper reports on investigations of the use of *green algae* as a low cost carbon source to promote bacterial sulfate reduction.

2. Materials and methods

2.1. Site description

The Ranger mine is located at Jabiru, 230 km east of Darwin, in northern Australia. The RP1 Artificial Wetland Filter is used to treat ore stockpile runoff and water from the pit and was constructed in two stages (Shinners, 1996). It covers 6 ha and consists of nine cells, varying in size from 2050 to 17,500 m^2, containing a total of 50,000 m^3 of water. The total flow path of the wetland is 1 km and its direction is controlled by concrete spillways of precise height constructed between each cell. The dominant plants in the wetland are *Eleocharis* (spike rush) and *Nymphya* sp. (water lilies).

2.2. Isolation of algae

Two algal cultures, designated A3 and N9, were isolated from the constructed wetland at the Ranger uranium mine. A3 originated from a liquid sample (planktonic) taken from cell 3 of the wetland filter. The sample was inoculated into modified MBL medium (Stein, 1973) and grown at 28 °C in an artificial light cabinet (Laboratory Equipment, Marrickville, NSW) on a 12:12-h light/dark cycle. The culture was spread onto MBL agar plates and colonies were subcultured into the same media until a culture dominated by A3 was obtained. N9 originated from a sample taken in cell 4 of the wetland filter, and was isolated using modified MBL medium as described above. Both algae were identified using standard methods (Greenberg et al., 1992).

2.3. Isolation of sulfate-reducing bacteria (SRB)

Sediment from cell 3 of the Artificial Wetland Filter at the Ranger uranium mine was collected in N_2-sparged sterile containers and transported to the

laboratory with minimal exposure to air. A sulfate-reducing bacterial culture was selectively enriched using a basal medium containing algal cells (*Sceneedesmus* N9) as sole carbon source. Late growth phase algal culture (50 mL) was harvested by filtration under vacuum (8-μm membrane, Millipore, Bedford, MA) then transferred to an anaerobic workstation (Bactron II, Sheldon Manufacturing, Cornelius, OR). All subsequent manipulations were carried out under anaerobic conditions. The cells were dislodged from the filter by forcing a jet of basal medium through a 29-gauge needle attached to a sterile syringe. This medium consisted of the inorganic salts from Postgate's Medium B (Atlas, 1993), with sulfate concentration modified to 200 mg/L. Sterile penicillin vials containing algal-amended salts medium (34 mL) were inoculated with approximately 1 g of sediment, and incubated in the dark at 28 °C. Sulfate reduction was confirmed by blackening of the medium. The resulting sulfate-reducing culture was designated AWF6 and was used as inoculum for the assays described below.

In order to conduct 16S rRNA gene sequence analysis, the dominant SRB was purified by isolation of single colonies on Postgate's Medium B agar containing lactate.

2.3.1. Amplification, cloning, and sequencing

PCR template was prepared by rapid cell lysis in the presence of an ion exchange resin and nonionic detergents. The 16S rRNA genes for the SRB was PCR amplified using the modified consensus primers 27Fl(UFP) and 1494Rc(URP) to the conserved 5′ and 3′ ends of the 16S rRNA gene, respectively. The PCR products were extracted from mineral oil using chloroform/isoamylalcohol (24:1) and purified using the Wizard™ PCR purification system (Promega, Madison, WI) to remove PCR primers, nucleotides and other reaction components. Cloning of PCR products was achieved using the TA Cloning™ system vector, pCR™II (Invitrogen, San Diego, CA). Automated DNA sequencing employed fluorescent nucleotides from the PRISM™ cycle sequencing system and the 373 sequencer (ABI, Foster City, CA). In order to standardise the thermal cycling conditions for DNA amplification and the 12 sequencing reactions, the entire set of primers described were designed to have a theoretical melting temperature of 56 °C. Oligonucleotide primers used for 16S rRNA gene sequencing were synthesised on the Beckman Oligo 1000 DNA synthesis system (Beckman, Fullerton, CA) and purified by reversed-phase chromatography.

2.3.2. Phylogenetic analysis

DNA sequences were aligned using the programs Pileus, GCG (Wisconsin Package, 1994) and the multiple sequence alignment tool from Clustal W (Thompson et al., 1994). Manual confirmation of the sequence alignment was performed and checked against both primary and secondary structure considerations of the 16S rRNA molecule. The aligned sequences were applied to genetic distance methods for phylogenetic inference. The program DNADIST utilised the algorithm of Jukes and Cantor (Jukes and Cantor, 1969) to create a pairwise evolutionary distance matrix for input into the neighbour-joining procedure (NEIGHBOR) to construct a dendrogram for illustration of genetic relatedness (Saitou and Nei, 1987). The reliability of each tree node was analysed by statistical resampling of the data by bootstrapping. One thousand bootstrap trees were generated and the consensus tree constructed using the SEQBOOT program (Felsenstein, 1989). For all multiple sequence alignments and phylogenetic inference programs the input order of taxa was randomised. The phylogenetic inference protocols, DNADIST, NEIGHBOR, and SEQBOOT were supplied by the PHYLIP package (version 3.57c) (Felsenstein, 1989). All sequence manipulation and phylogeny programs were made available by the Australian National Genome Information Service (ANGIS, Sydney, Australia).

2.4. Assays

In order to provide algae as a carbon/energy source, a volume of culture (*Carteria* or *Scenedesmus*) was filtered and resuspended in Postgate's Medium C (Atlas, 1993), modified by reducing sulfate to 350 mg/L and omitting carbon sources. All media, except for controls, were inoculated with AWF6 culture. Controls were prepared excluding algae or SRB, and all treatments were incubated in darkness at 28 °C under anaerobic conditions.

For the time course assay, algal culture was harvested by filtration and added to modified Postgate's

Medium C at approximately 2×10^7 A3 algal cells/mL and 5×10^7 N9 algal cells/mL, then inoculated with AWF6 at a final concentration of 1×10^5 cells/mL. Samples were taken at intervals over 25 days, and filtered (0.45 μm) prior to determination of sulfate concentration by ion chromatography. Subsamples were observed microscopically, before filtering, to monitor cell growth.

For the algal concentration assay, algal biomass was added to 30 mL basal medium at different concentrations to determine the effect of algal concentration on sulfate reduction. Sulfate concentrations were measured after 14 and 25 days incubation.

2.5. Sulfate analysis by ion chromatography

Sulfate was analysed using a Dionex 4500 Ion Chromatograph with a Dionex AS4A column eluted with carbonate buffer (1.7 mM Na_2CO_3/1.8 mM $NaHCO_3$) at 2 mL/min. Sulfate was quantified using a suppressed conductivity detector against standards prepared from a NIST Traceable Standard (National Institute of Standards and Technology, Virginia).

3. Results and discussion

3.1. Enrichment and identification of green algae and sulfate-reducing bacteria

Two green algal cultures, designated A3 and N9, were enriched from water samples containing planktonic algae obtained from the constructed wetland at the Ranger uranium mine, Northern Territory, Australia. A3 originated from cell 3 of the wetland filter and was identified by standard methods (Greenberg et al., 1992) as belonging to the genus *Carteria*. Culture N9 originated from cell 4 of the wetland and was identified (Greenberg et al., 1992) as *Scenedesmus* sp. Cell morphologies are shown in Fig. 1a and b. A sulfate-reducing culture, designated AWF6, was enriched using modified Postgate's Medium B (Atlas, 1993) which contained *Scenedesmus* N9 biomass as the sole source of reducing equivalents. This culture was dominated by spirilloid bacteria (Fig. 1c) which displayed corkscrew-like motility. These cells frequently exceeded 5 μm in length. Phylogenetic analysis of 16S rRNA gene sequences (Fig. 2) shows that this bacterium is a *Desulfovibrio* and is most closely related (98.5%) to *Desulfovibrio alcoholovorans* (AFO53751) and an unnamed sulfate-reducing organism, R-SucA1. A second organism in the mixed culture was also sequenced and found to be most closely related to the *Clostridia* assemblage, although at a 91–92% homology, it most likely constitutes a new genus (results not shown).

3.2. Demonstration of sulfate reduction by bacteria utilising algal feedstock

The suitability of *Carteria* A3 and *Scenedesmus* N9 for use as feedstock to drive bacterial sulfate reduction was investigated at laboratory scale using static cultures containing approximately 350 mg/L sulfate. These organisms occurred naturally in the wetland, and given the favourable growth conditions of tropical temperature and light levels, sufficient biomass could be generated with the addition of suitable nutrients. No significant sulfate reduction was observed in the uninoculated control treatments with sulfate concentration varying $< 5\%$ over the 25-day incubation period. Addition of *Carteria* A3 resulted in sulfate reduction

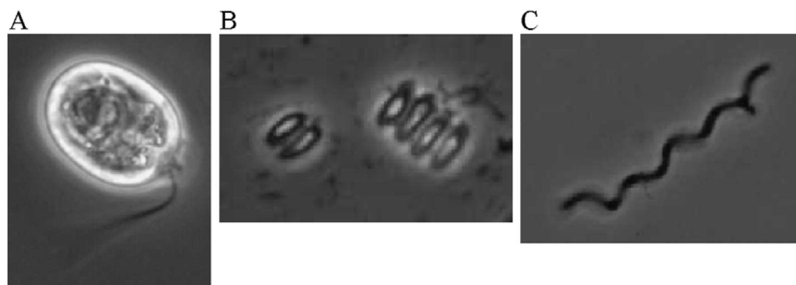

Fig. 1. Phase microscopy images of (A) *Carteria* sp. A3, (B) *Scenedesmus* sp. N9 and (C) *Desulfovibrio* AWF6.

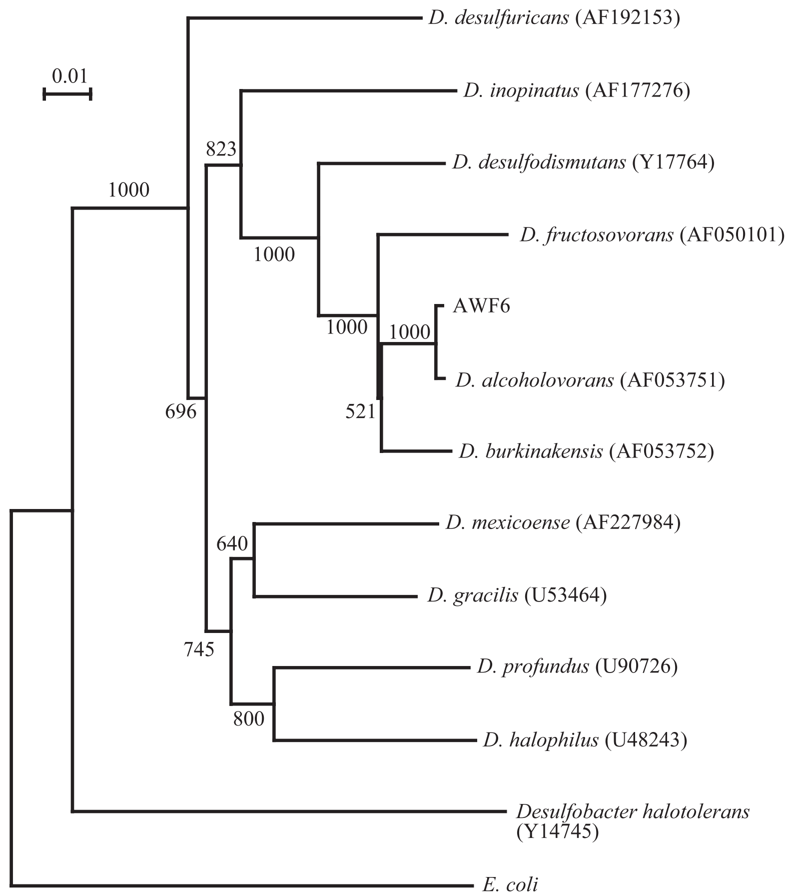

Fig. 2. 16S rRNA phylogenetic tree showing the relationship of AWF6 to other *Desulfovibrio* spp. Bootstrap values are indicated at the branch points.

after an initial lag in activity exceeding 8 days (as observed by stable sulfate concentration), with reduction being complete after 25 days (Fig. 3). A similar trend was observed with treatments amended with *Scenedesmus* N9 with active reduction commencing between days 5 and 8 (Fig. 4). The data suggests that the reduction rate utilising *Scenedesmus* was slower although completion was reached by day 25. Although bacterial cell numbers were difficult to count by phase microscopy, due to their motility and varied morphology, a rapid increase in cell numbers was observed at day 8, corresponding to enhanced sulfate reduction (results not shown). By day 14, *Desulfovibrio* cell density had increased to 4.8×10^6 cell/mL in comparison with 1×10^5 cells/mL at day 0.

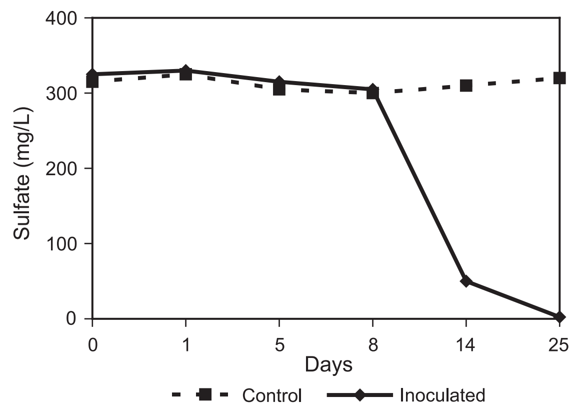

Fig. 3. Bacterial sulfate reduction utilising *Carteria* sp. A3 biomass as carbon and energy source.

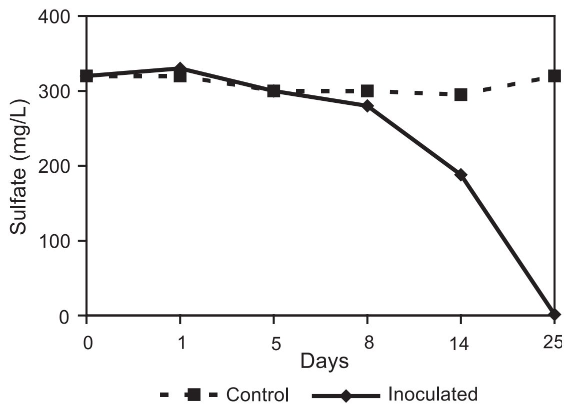

Fig. 4. Bacterial sulfate reduction utilising *Scenedesmus* sp. N9 biomass as carbon and energy source.

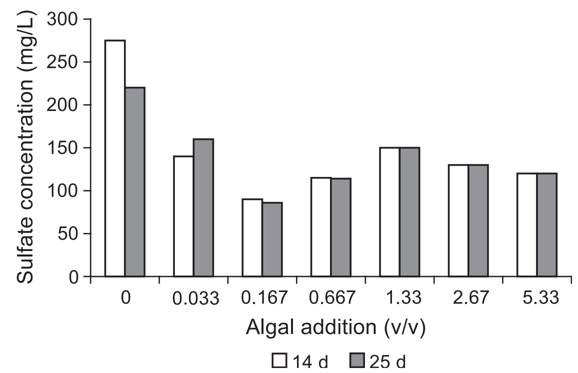

Fig. 6. The effect of *Carteria* sp. A3 biomass concentration on bacterial sulfate reduction.

3.3. Algal concentration vs. sulfate reduction

The effect of algal concentration on bacterial sulfate reduction activity was determined in static culture by addition of harvested algal biomass to SRB medium over a concentration range of 0–4.71 v/v addition of algal culture to experimental volume. Sulfate reduction was essentially complete after 25 days in all treatments receiving *Scenedesmus* N9 (Fig. 5). Faster reduction was observed in treatments receiving at least 1.33 v/v N9 and sulfate concentration approached 0 mg/L after 14 days. In contrast, sulfate concentration only decreased to between 100 and 150 mg/L in media amended with *Carteria* algal culture (Fig. 6). The process was substrate-dependent only at low concentrations (0.033 and 0.167 v/v), above which some unknown limitation prevented further sulfate reduction. Fig. 7 shows the yield of sulfate reduced per mg of algal biomass. Some sulfate reduction was observed in controls without algal amendment. Given the small inoculum size (0.3%), carryover of carbon in the inoculum is unlikely. Residual endogenous activity of sulfate reducers in the inoculum was probably responsible, or it may be due to the small amount of organic acids (ascorbic and thioglycollic acids) used as reductants in the medium. *Carteria* and *Scenedesmus* algal cultures had similar cell concentrations prior to harvesting (8×10^6 and 2×10^7 cells/mL, respectively), but the dry weight of a given volume of *Scenedesmus* was approximately double that of *Carteria*. No algal growth would have been possible under the incubations used, that is, under anaerobic atmosphere, in the

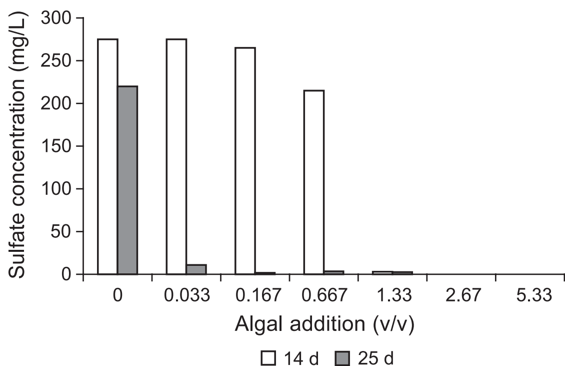

Fig. 5. The effect of *Scenedesmus* sp. N9 biomass concentration on bacterial sulfate reduction.

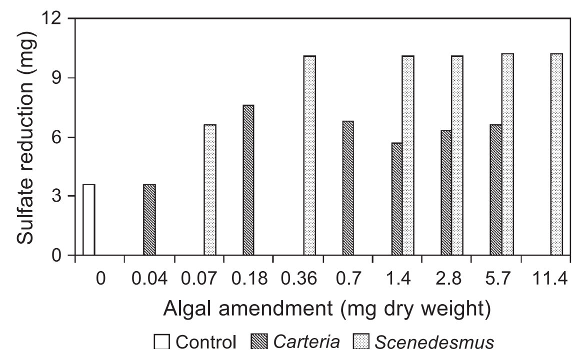

Fig. 7. Specific yield of sulfate reduced utilising *Carteria* sp. A3 and *Scenedesmus* sp. N9 biomass.

dark, in the absence of added organic carbon. Each treatment contained 10 mg sulfate which was completely reduced by 0.36 mg *Scenedesmus* algal biomass (dry weight). The maximum sulfate reduction achieved by *Carteria* amended medium in 25 days was approximately 7 mg sulfate and required 0.175 mg dry weight algal biomass. Only 0.07 mg of *Scenedesmus* biomass was required to reduce the equivalent mass of sulfate. *Scenedesmus* strain N9 produced a maximum specific yield of sulfate reduced per mg biomass added of 94.3 compared with 43.5 for *Carteria* strain A3.

The role of the second member of the bacterial mixed culture, which reports somewhat distantly with *Clostridium* sp. upon phylogenetic analysis, is not known but may be crucial in the degradation of algal cell biomass and production of low molecular weight metabolites, principally organic acid and alcohol and hydrogen (Smith, 1993), which sulfate-reducing bacteria require as reducing equivalents to reduce sulfate. Sulfate reducers do not degrade natural polymers such as proteins, lipids or starch (Hansen, 1993) which constitute up to 80% of algal cell contents as described earlier (Becker, 1994). The determination of the chemical content of the algal biomass produced under defined growth conditions and the role of the *Clostridium* like organisms in metabolising such products bears further investigation.

4. Conclusions

The main conclusions of this paper are summarised as follows:

(i) Reduction of sulfate by a *Desulfovibrio* culture grown on algal biomass as carbon/energy source was demonstrated at bench scale using both *Scenedesmus* and *Carteria* algal cultures.
(ii) The amount and rate of sulfate reduction was dependent on algal concentration.
(iii) *Carteria* algae at low concentration showed reduction earlier, however yields at higher concentration were affected by unknown inhibition.
(iv) *Scenedesmus* produced a higher specific yield of sulfate reduced per mg algal biomass. The highest yield observed was 94.3 g of sulfate reduced per g of algal biomass added.

This papers clearly demonstrates that bacterial sulfate reduction supported by the addition of *Scenedesmus* biomass is technically feasible and has potential as a "natural" system for treatment of mine waters in ecologically sensitive areas in the tropics.

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