Polish Journal of Microbiology
2005, Vol. 54, No 2, 169–173

Biotransformation of Phosphogypsum by Bacteria Isolated from Petroleum-refining Wastewaters

DOROTA WOLICKA, WŁODZIMIERZ KOWALSKI and HANKA BOSZCZYK-MALESZAK*

Institute of Geochemistry, Mineralogy and Petrology, Faculty of Geology, Warsaw Uniwersity
Żwirki i Wigury 93,02-089 Warsaw, Poland
* Department of General Microbiology, Institute of Microbiology, Warsaw Uniwersity
Miecznikowa 1, 02-096 Warsaw, Poland

Received 2 November 2004, received in revised form 2 March 2005, accepted 7 March 2005

Abstract

The biotransformation of phosphogypsum in cultures of sulfate-reducing bacteria (SRB) isolated from crude petroleum-refining wastewaters or purified using activated sludge method was studied. Selection was with the microcosms method on Postgate and minimal medium with different carbon sources, Emerson medium and petroleum-refining wastewaters. Highest hydrogen sulfide production, in excess of 500 mg/L, was observed in culture of microorganisms isolated from purified petroleum-refining wastewaters in Postgate medium with phenol as sole carbon source. 76% phenol reduction with simultaneous biotransformation of 2.7g phosphogypsum/L (1350 mg SO_4/L) was obtained. The results regarding post-culture sediment indicated 66% utilization of phosphogypsum introduced into the culture (5 g/L), which reflects the active biotransformation of phosphogypsum by the community selected from the wastewaters.

K e y w o r d s: sulfate-reducing bacteria, phosphogypsum, petroleum-refining wastewaters, phenol

Introduction

Phosphogypsum is a solid industrial waste that is onerous for the environment and refractory to biodegradation. It is a product that is formed in the chemical industry during the production of phosphoric acid from apatites or phosphorites treated with sulfuric acid. The production of 1 ton of phosphoric acid is accompanied by the formation of about 5 tons of phosphogypsum (Zijlstra, 2001). In Poland enormous mounds of phosphogypsum (about 35 mln tons) are found in Police near Szczecin, in Wiślinka near Gdańsk and in Wizów near Bolesławiec.

Phosphogypsum contains gypsum $CaSO_4 \times 2H_2O$ as well as lesser amounts of bassanite $CaSO_4 \times 0.5\ H_2O$, which together make up 90% of its mass. A mineralogical and geochemical description of phosphogypsums has been presented by Kowalski *et al*. (1990).

SRB are an anaerobic group of microorganisms that use organic compounds as an electron donor and sulfates as an electron acceptor. Sulfates, which comprise about 50% of the mass of phosphogypsum, can be used by sulfate reducing bacteria as a final electron acceptor (Przytocka-Jusiak *et al*., 1995; Deswaef *et al.*, 1996). The preferred carbon sources for these bacteria usually are low molecular weight compounds, such as: organic acids, *e.g.* propionic or formic acids; volatile organic acids, *e.g.* acetic acid, alcohols, *e.g.* ethanol or propanol (Fauque, 1991). Currently, SRB have been shown to use about 125 organic compounds, including certain hydrocarbons (Hansen, 1994).

Sulfate reducing bacteria always accompany the deposits of crude oil and are considered indicator organisms in the search for new beds (Postgate, 1984). The presence of sulfides in crude oil reservoirs was determined even before 1920 and the bacteria responsible for their production were arleady isolated in 1926 (Jenneman, 1999).

Crude oil is a mixture of about one thousand different chemical compounds (Wrenn and Venosa, 1996). The processing of crude oil is carried out at refineries, and the volume of wastewaters formed depends on the quality of the crude oil and extent of processing and is in the 10–18 m^3/ton range. Petroleum-refining

wastewaters contain from 0.3% to 2.0% organic compounds derived from crude oil, *i.e.* hydrocarbons, alcohols, phenols, aldehydes, esters, alcalis, acids and their salts. Phenol is not a characteristic component in crude oil and its presence in petroleum-refining wastewaters stems from the fact that, besides furfural, it is used as a solvent for contaminants and is produced in petrochemical processes.

The aim of this study was to isolate anaerobic communities of microorganisms from petroleum-refining wastewaters (both crude and purified using the activated sludge method) and to determine the effectiveness of the biotransformation of phosphogypsum in cultures of these microorganisms in different selective media.

Experimental

Materials and Methods

Phosphogypsum. The studied phosphogypsum sample was from mounds located in Wizów near Bolesławiec, Lower Silesia.

Inoculum. The inoculum was autochthonous microflora originally isolated from crude and purified petroleum-refining wastewaters. The inoculum was multiplied using the method of microcrocosms and added to the medium in ratio 1:10. Samples of the studied wastewaters (crude petroleum-refining wastewaters or purified by the activated sludge method) were placed in transparent 100 mL containers and 5 g/L of phosphogypsum was added. The containers were tightly closed and set aside for 6 weeks to allow the selection of anaerobic, sulfidogenic consortia capable of carrying out the biotransformation of phosphogypsum. Incubation was at 30 or 55°C. Four cultures were set up.

Media. The following media were used: modified Postgate medium (Postgate 1984), in which Na_2SO_4 was replaced with phosphogypsum in concentration equivalent to 4.5 g Na_2SO_4/L and minimal medium (1 g/L NH_4Cl). Both media were enriched with sodium lactate 98% (6 mL/L), lactose (9 g/L) casein (6 g/L) or phenol (0.5 g/L); moreover Emerson medium: (K_2HPO_4 – 0.07 g, $MgSO_4$ – 0.5 g, yeast extract – 4.0 g, distilled water – 750 mL, tap water – 250 mL, Tween 80 – 0.3 mL, fuel oil – 5 mL/L) and modified Emerson medium (in which crude oil was replaced with 0.5 g phenol/L); crude petroleum-refining wastewaters or purified using the activated sludge method. All media contained phosphogypsum (5 g/L) as sole electron acceptor for SRB. To all cultures resazurin in concentration 0.001 g/ L was added as an indicator of redox conditions in the medium.

Stationary cultures were maintained in 50 or 300 mL glass vessels tightly closed with rubber stoppers through which a needle, topped by a syringe, was inserted. The cultures were incubated at 30°C or 55°C in the dark.

Determinations. Sulfides in the cultures were determined using the iodometric method, sulfates with the hot barium method, COD by the dichromate method, and the concentration of phenol using a colorometric method involving p-nitroaniline. pH was measured using a pH-meter or with bromothymol indicator and color scale. The reaction of the culture was corrected with 0.1 N HCl or 0.1 N NaOH.

Determinations involving post-culture sediments and fluids were made using the following analytical procedures: 1. IPC emission spectrometry with induced excitation in the medium, 2. X-ray analysis of post-culture sediments using a DRON- 2 X-ray diffractometer

Results and Discussion

After six weeks of incubation, blackening, reflecting the presence of sulfides was observed only for the microcosms incubated at 30°C. Thus obtained mesophilic communities of microorganisms served as an inoculum in further studies. 22 anaerobic stationary cultures were set up. The selective media used were: Postgate, minimal, Emerson and modified Emerson (in which crude oil was replaced with 0.5 g phenol/L) media as well as petroleum-refining wastewaters. The source of carbon was lactate, casein, lactose or phenol. In the course of incubation the concentration of hydrogen sulfide was measured in the cultures, in order to compare SRB activity in the isolated bacterial communities. The results obtained are presented in Fig. 1.

The bacterial communities demonstrating the highest efficiency of biotransformation of phosphogypsum were isolated using Postgate medium with lactate (cultures nos 3 and 14) and phenol (cultures nos 11 and 22) as sole carbon sources, which produced 500, 480, 400 and 396 mg HS-/L, respectively, corresponding to the reduction of 2820, 2707, 2256 and 2233 mg phosphogypsum/L. No growth was observed in minimal medium with lactose (cultures 4 and 15), lactate (culture 17), Emerson medium (culture 9) and modified Emerson medium (culture 18) and in crude petroleum-refining wastewaters (culture 8).

Communities of microorganisms, which produced more than 300 mg HS^-/L, that is reduced more than 846 mg SO_4^{2-}/L, were chosen for further studies.

From among 22 bacterial communities isolated only 6 met the above-mentioned criterion: 3 from crude wastewaters (cultures nos 1, 3, 11) and 3 from wastewaters purified using the activated sludge method (cultures nos 13, 14, 22). These communities were used an inoculum in further studies using Postgate medium with lactate, casein, phenol or lactose. Results for those cultures in which the highest concentration of sulfides over the course of 5 passages was obtained, are presented in Fig. 2.

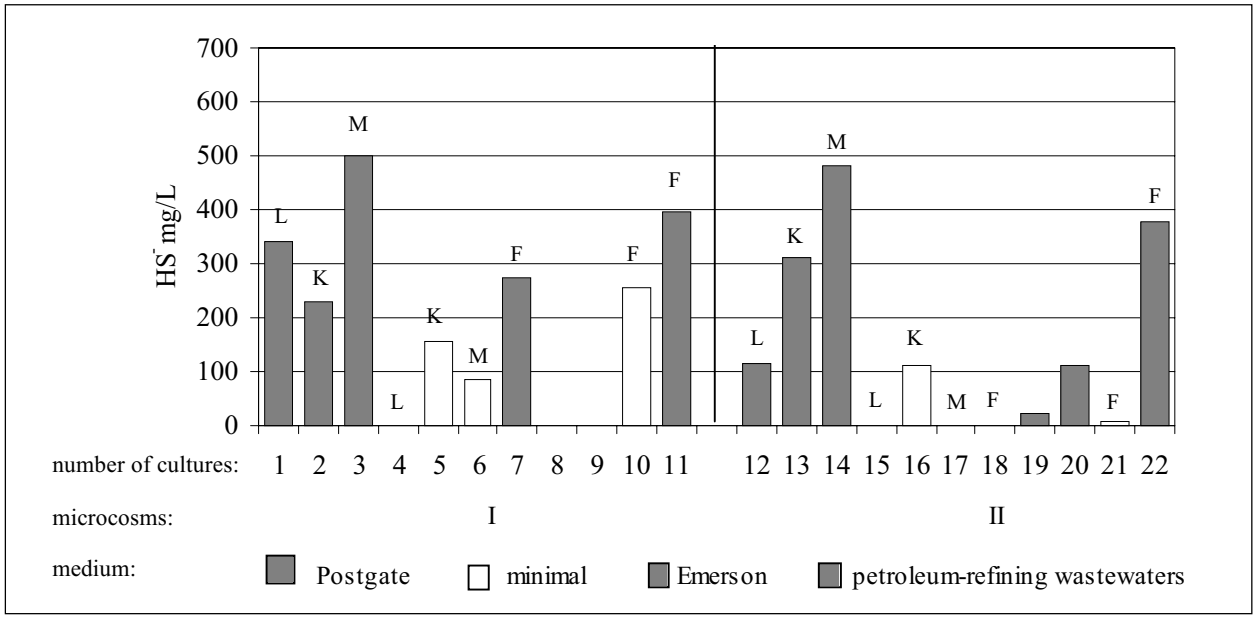

Fig. 1. Activity of SRB isolated from petroleum-refining wastewaters using different media
I – indicates the environment – crude wastewaters, II – purified wastewaters. The letter over the bar indicates the carbon source in the medium: L – lactose, K – casein, M – lactate, F – phenol

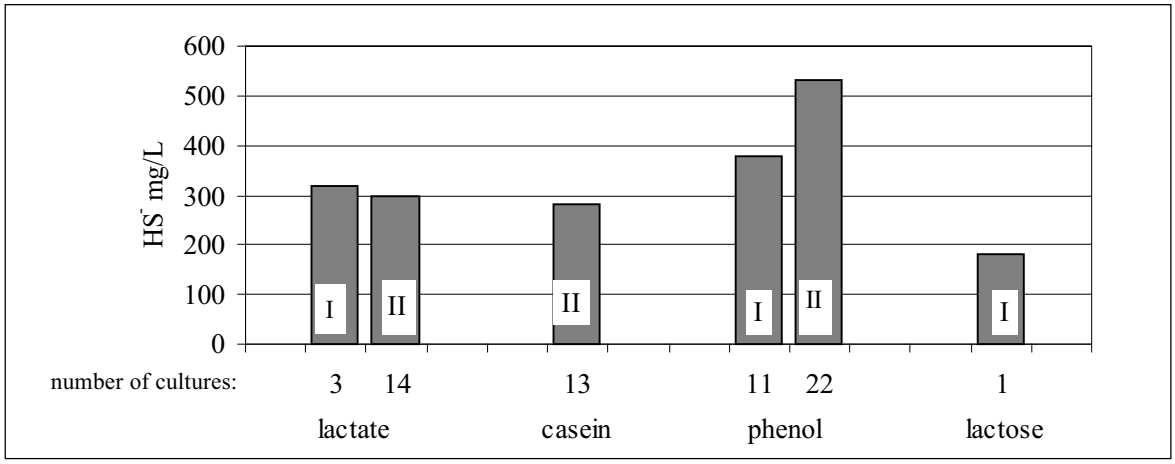

Fig. 2. Maximum SRB activity in various anaerobic communities in five consecutive passages (mg HS^-/L) in Postgate medium with different carbon sources
I – indicates source of isolation – crude petroleum-refining wastewaters, II – purified wastewaters

Almost all the communities of microorganisms yielded the highest concentration of sulfides in the third passage (between days 43 and 55 of cultivation). The maximum amount of HS^- found in the cultures studied ranged from 190 in Postgate medium with lactose to 530 mg HS^-/L in Postgate medium with phenol, which corresponds to the reduction of 536 and 1495 mg SO_4/L, respectively. In view of the fact that the degradation of phenol by SRB is not a universal phenomenon, the microorganisms from cultures nos 11 and 22, which produced a maximum of 480 and 530 mg HS^-/L, were passaged. The amount of HS^- obtained on the consecutive days of incubation is presented in Fig. 3.

The maximum concentration of HS^- of 480 mg/L, corresponding to the reduction of 1353 mg SO_4/L, was obtained in culture no. 22 (the microorganisms were isolated from purified petroleum-refining wastewaters). A lower concentration of approx. 310 mg/L was noted for culture no 11 (microorganisms isolated from crude wastewaters). The biotransformation of phosphogypsum is accompanied by 75% COD reduction. The uptake of phenol, related to the reduction of sulfates, calculated on the basis of the stoichiometry of the process, *i.e.* COD /SO_4 = 0.67 (Hao, 1996) was 380 mg/L in culture nos 22 and 245 mg/L in culture no 11. This indicates that SRB utilized 76 and 49% of the phenol, respectively.

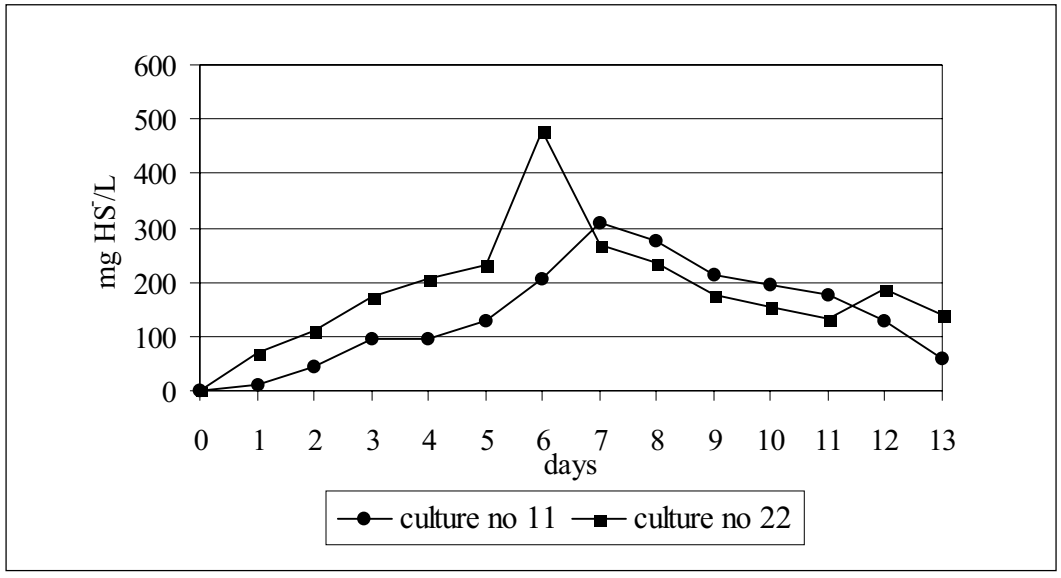

Fig. 3. Amount of HS^- obtained in bacterial cultures in Postgate medium with phenol as sole carbon source

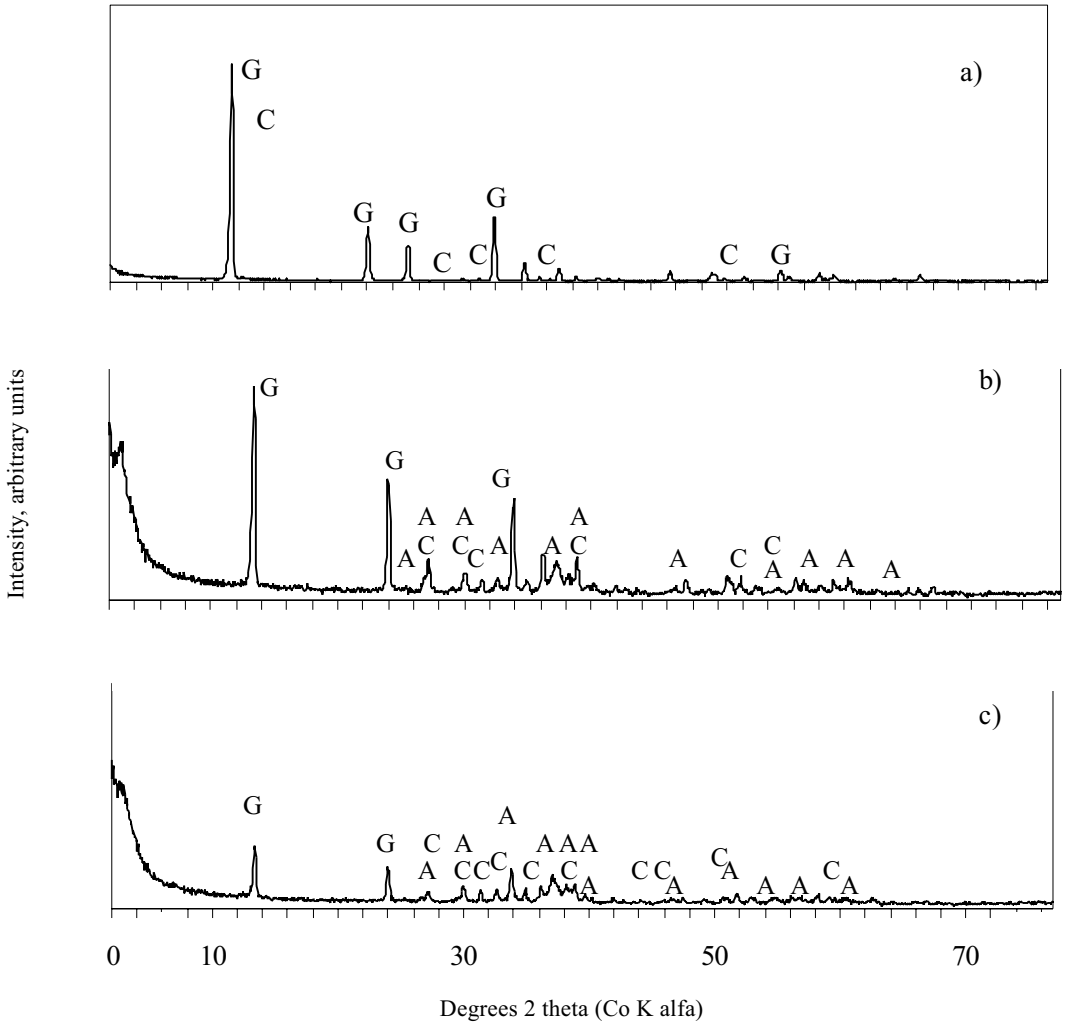

Fig. 4. Diffractograms: a) phosphogypsum, b) post-culture sediment from culture no 22, c) post-culture sediment from culture no 11

The symbols indicate: G – gypsum, C – celestine, A – apatite

The considerable amount of sulfides produced and COD used by the whole bacterial community allowed to calculate, based on the stoichiometry of sulfate reduction (1 mole HS^- from 1 mole SO_4) that the participation of in the utilization of organic compounds was 60% in culture no 22 and 38% in culture no 11. The obtained COD/SO_4 ratio of 1.1 for culture nos 22 and 1.75 for culture no 11 indicates that the conditions created in the cultures favored the selection of SRB (Oude Elferinnck, 1998).

So far only a few strains of desulfurication bacteria capable of degrading phenol have been isolated and described. The anaerobic consortium isolated from swine manure by Boopathy (1997) utilized phenol under conditions favoring the reduction of sulfates in concentration 0.5 mM whereas that isolated by Drzyzga (1993) from marine sediments – in concentration 1 mM. Complete degradation of phenol (2 mM) was described for cultures of the bacterium *Desulfotomaculum gibsoniae* sp. nov. isolated from fresh water sediments (Kuever *et al*., 1999). The first described SRB species able to utilize phenol as a sole carbon source was *Desulfobacterium phenolicum,* isolated from deposits from the North Sea (Bak and Widdel, 1986). This species degraded phenol completely only when its concentration in the medium was not in excess of 2 mM.

In the light of these data it can be said that the use of phenol by the described SRB communities is not surprising but its use by cultures in concentrations far higher than described in the available literature is worth of particular attention. In our studies phenol was used in the medium at a concentration of 500 mg/L (5.3 mM), and utilized in the amount of 4 mM (culture no 22) and 2.6 mM (culture no 11).

At the end of incubation in culture no. 22 a 66% (3.3 g/L) drop in mass of the phosphogypsum added was observed and in culture no 11 – about 76% (3.8 g/L). Phase changes of the sediment compared to phosphogypsum were observed for the sediments from two cultures nos 11 and 22. The results of the diffractrometric studies on phosphogypsum and post-culture sediment are presented in Fig. 4.

The post-culture sediment was composed of phosphogypsum residues; gypsum and copestone. A considerable content of apatite was also found. The diffractometric results for the sediments correlate well with data obtained using chemical methods and point to the occurrence of the biotransformation of phosphogypsum.

The obtained results indicate that the community of bacteria isolated from refinery wastewaters contained SRB capable of the biotransformation of phosphogypsum in cultures in medium containing phenol as sole carbon source. In the post-culture sediment phase changes of the phosphogypsum were determined indicating the appearance of apatite, which reflects the biotransformation of the former by SRB.

Literature

Bak F. and F. Widdel. 1986. Anaerobic degradation of phenol and phenol derivatives by *Desulfobacterium phenolicum. Arch. Microbiol.* **54**: 177–180.

Boopathy R. 1997. Anaerobic phenol degradation by microorganisms of swine manure. *Curr. Microbiology* **35**: 64–67.

Deswaef S., T. Salmon, S. Hiligsmann, X. Taillieu, N. Milande, Pn. Thonart and M. Cruine. 1996. Treatment of gypsum waste in a two stage anaerobic reactor. *Water Sci. Tech.* **34**: 367–374.

Drzyzga O., J. Kuever and K.H. Blotevogel. 1993. Comlete oxidation of benzoate and 4-hydroxybenzoate by a new sulfate-reducing bacterium resembling *Desulfoarculus*. *Arch. Microbiol.* **159**: 109–113.

Fauque G., J. Legall and L.L. Barton. 1991. Sulfate-reducing and sulfur reducing bacteria. Variations in autotrophic life. J.M.I. Shively and L.L. Barton (eds), Academic Press Ltd.

Hansen T.A. 1994. Metabolisms of sulfate-reducing procaryotes. *Antonie van Leevenhoek* **66**: 165–185.

Hao O. J., J.M., Chen, L. Huang and R.L. Buglass. 1996. Sulfate-reducing bacteria. *Crit. Rev. Environm. Sci. Technol*. **26**: 155–187.

Jenneman G.E. and D. Gevertz. 1999. Identification, characterization and application of sulfide-oxidizing bacteria in oil fields In: Microbial Biosystems: New Frontiers. Proceedings of the 8th International Symposium on Microbial Ecology, C.R. Bell, M. Brylinsky and P. Johnson-Green (eds), Atlantic Canada Society for Microbial Ecology, Halifax, Canada.

Kowalski W., J. Parafiniuk and M. Stępisiewicz. 1990. Mineralogy and geochemistry of phosphogypsum from a dump of Chemical Works "Wizów" (in Polish). *Archiwum Mineralogiczne* **45**: 115–134.

Kuever J., F.A. Rainey and H. Hippe. 1999. Description of *Desulfotomaculum* sp.Groll as *Desulfotomaculum gibsoniae* sp. nov. *Int. J. Syst. Bacteriol.* **49**: 1801–1808.

Oude Elferinck S.J.W.H., W.J.C. Vorstman, A. Sopjes and A.J.M. Stams. 1998. *Desulforhabdus amnigenus* gen. sp. nov., a sulfate reducers isolated from anaerobic granular sludge. *Arch. Microbiol.* **164**: 119–124.

Postgate J.R. 1984.The sulphate reducing bacteria. Cambridge University Press.

Przytocka-Jusiak M., W. Kowalski, M. Rzeczycka, M. Błaszczyk and R. Mycielski. 1995. Products of microbial transformation of phosphogypsum in anaerobic thermophilic cultures (in Polish). *Biotechnologia* **29**: 102–112.

Wrenn B.A. and A.D. Venosa. 1996. Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most-probable-number procedure. *Can. J. Microbiol.* **42**: 252– 258.

Zijlstra J.J.P. 2001. Geochemical engineering of phosphogipsum tailings. p. 15–17. In: Raport of Geochem. Reasearch BV, HA DeBilt, The Netherlands.