Pergamon

Wat. Sci. Tech. Vol. 40, No. 4–5, pp. 137–144, 1999

0273–1223/99 $20.00 + 0.00

PII: S0273-1223(99)00495-3

NEW BIOLOGY FOR ADVANCED WASTEWATER TREATMENT

K. Miserez, S. Philips and W. Verstraete

Laboratory of Microbial Ecology and Technology, University of Gent, Coupure L 653, 9000 Gent, Belgium

ABSTRACT

A number of new technologies for the advanced treatment of wastewater have recently been developed. The oxidative cometabolic transformation by methanotrophs and by nitrifiers represent new approaches in relation to organic carbon. The Biological Activated Carbon Oxidative Filters characterized by thin biofilms are also promising in that respect. Moreover, implementing genetically modified organisms with improved catabolic potential in advanced water treatment comes into perspective. For very refractory effluents chemical support techniques, like e.g. strong chemical oxidation, can be lined up with advanced biology. © 1999 IAWQ Published by Elsevier Science Ltd. All rights reserved

KEYWORDS

BACOF; cometabolism; genetically modified organisms; recalcitrant organics; wastewater treatment.

INTRODUCTION

Urban and industrial development is highly dependent on an abundant supply of water. Hydrologists designate water-stressed countries as those with annual supplies of 1000-2000 cubic metres unused water per person. Today, 26 countries over the world fall into the water-scarce category (Postel, 1992). Remarkably, some countries conventionally considered to have ample water supplies such as The Netherlands and Belgium, apparently have rather limited resources of unused water. Of course, the cycling of water on our planet is not only a quantitative problem. Since World War II, human activity has introduced a variety of xenobiotic chemicals into the environment on a very large scale. Every year some 1000 new chemicals are introduced on the market. Many of them have a rather poor biodegradability (Alexander, 1981).

Wastewater reclamation, recycling and reuse by sustainable wastewater treatment serve an important function in water resources management by providing a means to produce quality source water for irrigation, industrial and urban water requirements throughout the world.

This paper focusses on new technologies for the advanced treatment of wastewater, and this in relation to organic carbon, nitrogen, heavy metals and other contaminants.

COMETABOLIC REMOVAL OF RECALCITRANT ORGANICS BY METHANOTROPHS AND NITRIFIERS

Cometabolism is a widespread process in nature, effecting the degradation of many naturally occurring compounds and numerous synthetic man-made compounds. It has been defined as the degradation of a compound by organisms that do not obtain energy or carbon for cell growth from the transformation, and

hence require an alternative source of carbon and energy (Alvarez-Cohen and McCarty, 1991). Cometabolism can bring about a rapid biodegradation of compounds that otherwise would be broken down only very slowly, if at all, in the environment. Also, lower residual contaminant levels may be achieved by cometabolic treatment than could otherwise be attained.

Oxidative cometabolic transformations by methane-utilizing bacteria (methanotrophs) capable of expressing methane monooxygenase (MMO) have been identified as a major mechanism in the degradation of alkanes and different xenobiotics, including halogenated aliphatic compounds and pesticides. Chang and Alvarez-Cohen (1997) developed a two-stage continuous methanotrophic bioreactor for the treatment of wastewater contaminated by chlorinated organic solvents. A bench-scale reactor (with a 4-hour wastewater retention time) was demonstrated to be capable of treating wastewater containing trichloroethylene (4.7 mg/L) and *cis*-1,2-dichloroethylene (4.8 mg/L) to below the maximum contaminant levels (MCLs, 5μg/L each) during a period of at least 31 days.

Cometabolic transformation of commercial linear alkylbenzenesulphonates (LAS) has been studied by using a mixed methanotrophic-heterotrophic culture (termed MM1) and a pure culture of methanotroph type II (strain CSC1) isolated from a mixed culture (Hrsak, 1996). Although the mechanism of LAS transformation by methanotrophs still needs to be elucidated, simultaneous methane oxidation and LAS disappearance was observed. In the experiment with the mixed methanotrophic-heterotrophic culture MM1, LAS was added repeatedly following its depletion, rendering an initial concentration of approximately 12 mg/L. LAS disappeared within 3 days, and with each of the three further additions, in only 1 day (Fig. 1).

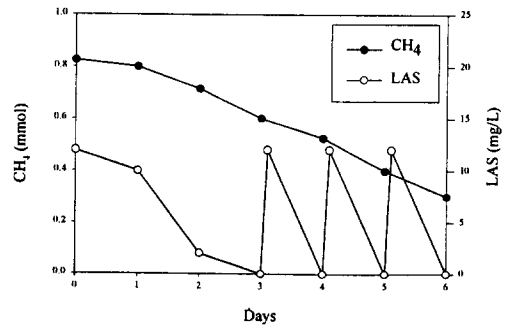

Figure 1. Simultaneous methane oxidation and transformation of commercial LAS using mixed methanotrophic-heterotrophic culture MM1 (after Hrsak, 1996).

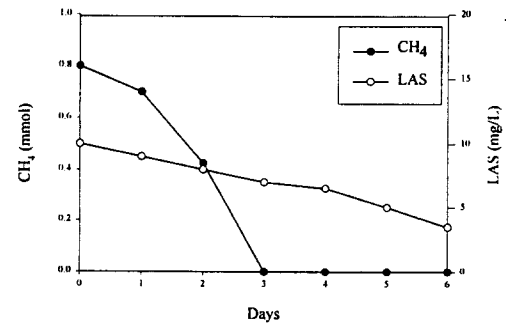

Figure 2. Simultaneous methane oxidation and transformation of commercial LAS using pure culture of type II methanotroph, strain CSC1 (after Hrsak, 1996).

In the experiment with pure culture CSC1, LAS was added to achieve an initial concentration of 10 mg/L. The uptake of natural substrate was faster in this case (Fig. 2). Methane disappeared within 3 days, compared to 9 days with culture MM1. LAS transformation was considerably less rapid. The average

specific transformation rate during a period of 6 days was 0.006 day^{-1}, while the rates obtained with culture MM1 were 0.017 day^{-1} during the 3 day period of the first LAS addition and 0.035-0.036 day^{-1} during each daily subsequent LAS addition. The slower LAS transformation by a pure isolate than by a mixed culture suggests the importance of methanotrophic-heterotrophic interactions in cometabolic transformation of this complex LAS molecule.

Nitrifying activity is associated with the generation of $OH^{\bullet}$ radicals by the ammonia monooxygenase (AMO) (Wood, 1990; Verstraete and Philips, 1998), and with the production of soluble microbial products which can act as co-substrates for heterotrophic microorganisms. The action of the nitrifiers, which can be stimulated by supplying a quite specific substrate, i.e. NH_3, can in this way bring about the initiation of indirect biodegradation of recalcitrant organics.

De Heyder *et al.* (1997) achieved the enhancement of ethene removal in a packed granular activated carbon biobed by specifically stimulating nitrification. The reactor contained an ethene degrading *Mycobacterium E3*. The nitrifying activity was introduced by regular submersion in a nitrifying medium. When combined with a NH_3 dosage, the volumetric removal rate of ethene could be increased to 1.25 kg COD/m^3.d, while the control without activated nitrification showed a volumetric removal rate of 0.72 kg COD/m^3.d (Table 1).

Table 1. Operating conditions and ethene removal rates (Rv), for subsequent submersions in an mineral medium resp. nitrifying activated sludge suspension. The gas loading rate was 3 kg COD/m^3.d (after De Heyder *et al.*, 1997)

Submersion	Estimated Rv (kg COD/m^3.d)
Mineral medium	0.72 to 0.87
Nitrifying suspension	0.97 to 1.25

AMO in cells of *Nitrosomonas europaea* has been reported to oxidize hydrocarbons and aliphatic halogenated hydrocarbons, including the agricultural fumigant 1,2-dibromo-3-chloropropane (Rasche *et al.*, 1990) and industrial pollutants such as trichloroethylene (TCE) (Arciero *et al.*, 1989). Hooper *et al.* (1997) gave an overview of compounds that can be attacked by the ammonium oxygenase. Clearly, the potential of implementing nitrifiers in cometabolism of xenobiotics has not been exhausted yet.

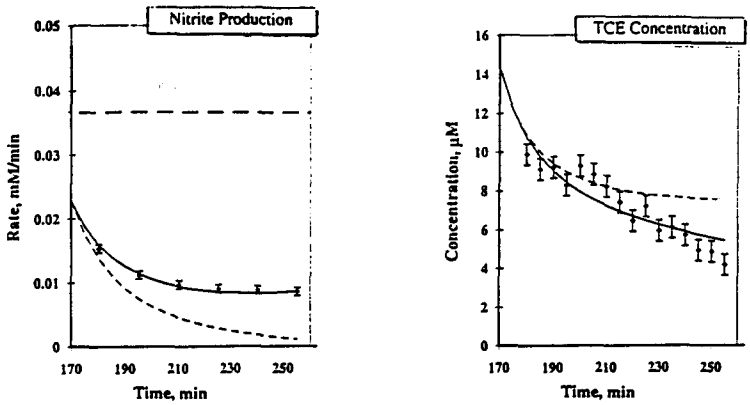

Figure 3. Nitrite production rates and residual TCE concentrations versus time. TCE was injected at t = 170 min. Initial TCE concentration was 14.3 µM (1.9 ppm). Solid lines are best-fit model curves. Heavy dashed line indicates culture activity prior to TCE injection. (after Ely *et al.*, 1995) Light dashed lines indicate hypothetical system behavior in the absence of recovery effects.

If bacteria are to be used effectively in bioremediation strategies, it is important to equally look at the limitations of the biodegradative systems. Indeed, cometabolic transformation of some non-growth substrates can lead to injury and inactivation of bacteria by damaging important cellular constituents (Ely *et*

al., 1997). Cells can normally recover from these cometabolism-associated injuries, even in the presence of the injury-inducing non-growth substrate. For instance, Ely *et al.* (1995) examined trichloroethylene (TCE) degradation by the ammonia-oxidizing bacterium *Nitrosomonas europaea* in a quasi steady-state bioreactor. The injection of TCE in the reactor caused a decrease in ammonia-oxidizing activity, due to the TCE-related inactivation of AMO activity. Yet, the bacteria were capable to initiate recovery provided that ammonia was available, even with TCE present (Fig. 3). However, if cometabolic bacteria are exposed to inactivating, non-growth substrates in the presence of little or no growth substrate, their ability to recover from enzyme inactivation appears often limited. To overcome these difficulties, it has been suggested that bacteria could be grown in one reactor and exposed to the recalcitrant organics in another reactor (Alvarez-Cohen and McCarty, 1991). If bacteria were used once and discarded, substantial quantities of potentially contaminated sludge could be created and biomass utilization would be very inefficient.

An alternative approach would be to provide sustainable treatment by controlling growth and nongrowth substrate concentrations in a way that cells could recover from inhibition and inactivation effects. Cellular growth rates would be expected to decrease as energy is diverted from cell growth to enzyme synthesis. Therefore, cometabolic treatment systems potentially could be designed and operated purposely to stress the cells, causing a lower growth rate and generating less sludge, while concurrently achieving sustainable and acceptably rapid nongrowth substrate degradation (Ely *et al.*, 1997).

THICK VERSUS THIN BIOFILMS

In biological filtration technologies the bacteria are attached to the surface of a carrier material. Various media materials for the growth and capture of the biomass have been used. Recently developed Biological Aerated Filters (BAF) use buoyant synthetic media or plastics as a biofilm support, e.g. polystyrene in the upflow BIOSTYR filter. BAF are continuously aerated, in this way stimulating aerobic biological activity. The biological degradation in combination with the physical retention by filtration allows a high rate of organics- and ammonia-removal as well as the capture of solids. Solids build-up in the filter as a result of both physical and biological processes necessitates periodic backwashing of the bed. BAF contain high concentrations of biomass, ranging from 10 to 15 g/L, enabling them to handle high loading rates. The bacteria form thick biofilms on the carrier material. The substrate has to diffuse from the mass of water to the bacterium. Diffusion is not limiting the uptake of substrate by the cell when diffusion of substrate from the mass of water to the bacterium exceeds that through the bacterial cell wall. This means that a minimum concentration gradient over the biofilm is needed. Typically, the critical cut-off in terms of biokinetics is situated at a substrate concentration of ca. 1 μg C/L (Van der Kooij and Hijnen, 1983).

In a Biological Activated Carbon Oxidative Filter (BACOF), the activated carbon acts as sorbent and as microbial substratum (Liessens *et al.*, 1995). Continuous aeration of the filter enables the growth of a highly performant interactive biomass on the activated carbon. This pronounced biological activity results in an *in situ* biological regeneration of the activated carbon, in this way substantially extending the period between successive reactivations or replacements. Recalcitrant pollutants present at very low concentrations are removed from water by adsorption onto the activated carbon, so that their bioavailability (concentration) increases and their exposure to microorganism breakdown prolonges. In BACOF the biofilm attached to the activated carbon is rather thin, due to the turbulence created by the co-current introduction of air in the filter. Hence, the concentration gradient over the biofilm needed to avoid diffusion limitations is small. In this way BACOF is able to degrade the organic matter present in the water to a very low level.

A BACOF pilot-plant, with an active volume of 4 m^3, was operated at a pharmaceutical and chemical production plant (Beerse, Belgium), treating effluent from a biological wastewater treatment plant (Liessens *et al.*, 1995). Recalcitrant COD in the pharmaceutical effluent was consistently removed with an efficiency of at least 70%, resulting in a final effluent COD concentration below 25 mg/L (Table 2). Micropollutants characterized as toxic towards nitrifying bacteria were removed down to non-toxic levels allowing nitrification of recalcitrant nitrogenous compounds. Moreover, the effluent of the biological wastewater treatment plant was toxic to fish, but the effluent of BACOF showed neither acute nor chronic toxicity to the *Brachydanio rerio* test species.

Table 2. BACOF treatment of effluent of a pharmaceutical production plant at Beerse (after Liessens *et al.*, 1995)

	Influent	Effluent	% Removal
pH	7.8	7.2	-
COD (mg/L)	74.6	20.7	72.3
BOD (mg/L)	11.1	2.0	82.0
KjN (mg/L)	8.9	2.6	70.8
Org N (mg/L)	5.9	2.6	55.9
NH_4^+-N (mg/L)	3.0	0.0	100.0
NO_x^--N (mg/L)	2.3	6.8	-
TSS (mg/L)	36.5	2.6	92.9
NTU	38.6	2.8	92.8
Bio-assay with *Brachydanio rerio* fish			
- acute test			
(% mortality after 96 h)	100	0	-
- chronic test			
(% mortality after 26 d)	100	0	-

NEW GENETICALLY CONSTRUCTED PATHWAYS

The problems with recalcitrant contaminants and the frequent requirement for microbial consortia to ensure complete mineralisation has led to the utilization of molecular techniques in attempts to construct more efficient degradative strains. Natural conjugation has been most commonly employed. The *in vivo* assembly of partial catabolic sequences from different pathways and different organisms is a powerful strategy for the evolution of new complete catabolic pathways. However, genetic engineering can be a powerful technique to help establish effective (non-abortive) degradation pathways and to accelerate the evolution of totally new degradative capabilities (Van Limbergen *et al.*, 1998). It allows the experimenter to precisely select the genes that are essential for the hybrid pathway he intends to construct. By this means, the introduction of superfluous genetic material coding for unproductive or counterproductive enzymes is avoided.

One of the first examples of the improvement of the biodegradative capacity of microbial communities through genetic manipulation was the construction of bacteria degrading chlorinated aromatic compounds (Leisinger *et al.*, 1981). *Alcaligenes eutrophus* and *Pseudomonas putida*, grown on methylarenes (e.g. toluene, cresol, etc.), have a high cometabolic capability for chlorosubstituted analogs and produce the corresponding chlorocathechols. However, they do not grow directly on chlorinated aromatic compounds. These bacteria cleave the ring of the chlorocatechol in the meta-position, which yields a toxic metabolite so that the culture gradually kills itself. A strain of *Pseudomonas sp. B13* able to grow on chlorocatechol has been discovered. This strain uses a non-toxic ortho-cleavage. Following conjugation between *Pseudomonas sp. B13* and *Pseudomonas putida*, transconjugants growing directly on chlorinated aromatic compounds could be isolated. Analysis of these transconjugants demonstrated that they were *Pseudomonas putida* strains that had taken up the genes of *Pseudomonas sp. B13* encoding chlorocatechol mineralisation.

The use of genetically modified strains for bioaugmentation of activated sludge has been reviewed by Van Limbergen *et al.* (1998). A few examples are discussed below. The introduction of the phenol degrading strain, *Pseudomonas putida* ATCC 11172, in an activated sludge reactor was shown to provide stability in phenol degradation (Selvaratnam *et al.*, 1997). *Pseudomonas putida* ATCC 11172 converts phenol to catechol, which is then further metabolized via aerobic meta-cleavage pathway. After inoculation of the strain in an activated sludge reactor, phenol removal increased and was maintained at 95-100%. In the unaugmented reactor, a lower phenol removal efficiency was observed (Fig. 4). Correspondingly, the degradative gene *dmpN* present in *Pseudomonas putida* ATCC 11172 and its expression were detected in the activated sludge unit for over 41 days.

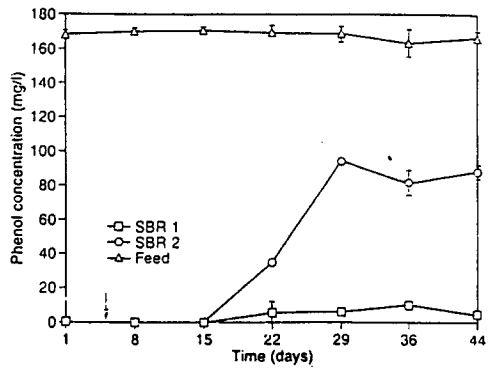

Figure 4. Comparison of phenol removal in bioaugmented SBR 1 and unaugmented control SBR 2. Following establishment of a steady state, $7x10^{11}$ cfu *P. putida* ATCC 11172 were added to SBR 1 on day 4 (arrow). Each datum point is an average of three samples (after Selvaratnam *et al.*, 1997).

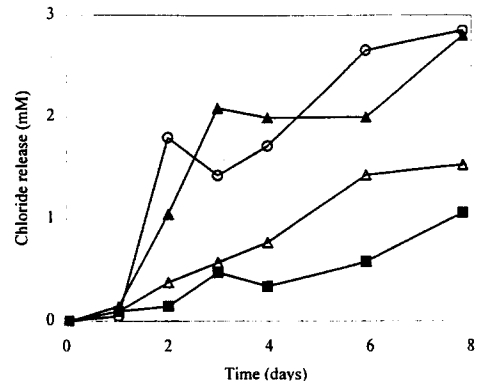

Figure 5. Rates of chloride release from 3CB by strains in batch culture at 15°C. Strains shown are *P. putida* UWC1 (pD10) (-■-) and activated-sludge-derived transconjugant strains *P. putida* ASR5.2 (-○-), *P. putida* ASR2.8 (-▲-)and *P. putida* ASR5.10 (-Δ-) (after McClure *et al.*, 1991)

McClure *et al.* (1991) clearly showed that the transfer of genes into indigenous bacteria can improve biodegradation. A 3-chlorobenzoate (3CB) degradative non-conjugative plasmid pD10 was constructed by cloning the *SstI*-C fragment of the 2,4-dichlorophenoxyacetic acid plasmid pJP4 in the broad host range vector pKT231. *Pseudomonas putida* UWC1 (pD10) was inoculated in the activated sludge unit. Despite the long survival of this genetically modified bacterium in the sludge, no breakdown of the 3CB was observed. Autochthonous bacteria, which had taken up the plasmid bound catabolic genes from the introduced strain, were isolated from the activated sludge. In contrast to the initially introduced strain *Pseudomonas putida* UWC1 (pD10), the isolated transconjugants could enhance the biodegradation after reinoculation in the activated sludge unit (Fig. 5).

INTEGRATED BIOLOGICAL AND CHEMICAL OXIDATION OF RECALCITRANT ORGANICS

In order to efficiently treat very refractory effluents, certainly in cases of high concentrations of toxic or inhibitory compounds, biological methods require support techniques. Waste streams from the use and production of insecticides and herbicides have been treated successfully by an integrated biological and chemical oxidation (Haverhoek *et al.*, 1997). The treatment comprised an aerobic fluidized bed and an aerobic trickling filter, removing the major part of the contaminants, followed by two chemical oxidation reactors. In the first oxidation reactor ozone is introduced under pressure of around 15 bar. After pressure

reduction to about 5 bar hydrogen peroxide is added into a second oxidation reactor with a heterogeneous catalyst. Radical reaction mechanisms oxidize the refractory compounds not totally, but only to such an extent that they are more amenable to biological attack. After chemical oxidation the stream is recirculated to the biological reactors, where biological conversion of the now better degradable compounds occurs. The residual ozone dosing is utilized in the aerobic reactors, thereby making optimum use of energy input.

The results showed that it is possible to break down pesticides with this integrated biological and chemical oxidation. Chemical oxidation is required in order to enhance biodegradability, while the consumption of oxidation agents is drastically reduced by the biological activity. Residual pesticide levels to below 0.1 ppb for individual and 0.5 ppb for total concentrations can be reached.

CONCLUSIONS

The new approaches in relation to the removal of organic matter from water are designed to result in a higher effluent quality. In a world more and more threatened by water scarcity, this opens perspectives for the reuse of wastewater treatment plant effluent. Yet, a few questions remain to be answered. There is a growing concern for micropollutants with the undesirable capability of having estrogenic activity on various higher forms of life. It has to be investigated whether those micropollutants can be removed biologically down to the levels safe for the currently feared endocrine disrupting effects (Tanghe *et al.*, 1998 ; 1999). Secondly, it is possible that the public will not accept the large-scale use of genetically modified organisms.

ACKNOWLEDGEMENTS

K.M. thanks Aquafin and Severn Trent for the research grant. S.P. wishes to thank the Fund for Scientific Research – Flanders for providing a Ph-D Scholarship.

REFERENCES

Alexander, M. (1981). Biodegradation of chemicals of environmental concern. *Science,* **211**, 132-138.

Alvarez-Cohen, L. and McCarty, P.L. (1991). Two-stage dispersed growth treatment of halogenated aliphatic compounds by cometabolism. *Environ. Sci. Technol.*, **25** (8), 1387-1393.

Arciero, D., Vannelli, T., Logan, M. and Hooper, A.B. (1989). Degradation of trichloroethylene by the ammonia-oxidizing bacterium *Nitrosomonas europaea. Biochem. Biophys. Res. Commun.*, **159**, 640-643.

Chang, H.L. and Alvarez-Cohen, L. (1997). Two-stage methanotrophic bioreactor for the treatment of chlorinated organic wastewater. *Water research,* **31** (8), 2026-2036.

De Heyder, B., Vanelst, T., Vanlangenhove, H. and Verstraete, W. (1997). Enhancement of ethene removal from waste gas by stimulating nitrification. *Biodegradation,* **8**, 21-30.

Ely, R.L., Williamson, K.J., Guenther, R.B., Hyman, P.R. and Arp, D.J. (1995). A cometabolic kinetics model incorporating enzyme inhibition, inactivation and recovery. *Biotechnol. Bioeng.*, **46**, 218-245.

Ely, R.L., Williamson, K.J., Hyman, M.R. and Arp, D.J. (1997). Cometabolism of chlorinated solvents by nitrifying bacteria : kinetics, substrate interactions, toxicity effects and bacterial response. *Biotechnol. Bioeng.*, **54**, 520-534.

Haverhoek, S., Koning, J., van Staveren, N.A. and de Ruyter, M.A. (1997).Advanced treatment of effluents from an agrochemical formulation plant. *Water Sci. Technol.*, **35**(10), 155-163.

Hooper, A.B., Vanelli, T., Bermann, D.J. & Arciero, D.M. (1997). Enzymology of the oxidation of ammonia to nitrite by bacteria. *Antonie van Leeuwenhoek*, **71**, 59-67.

Hrsak, D. (1996). Cometabolic transformation of linear alkylbenzenesulphonates by methanotrophs. *Water research,* **30** (12), 3092-3098.

Leisinger *et al.* (1981). Microbial degradation of xenobiotics and recalcitrant compounds. FEMS symposium n° 12.

Liessens, J., Pipyn, P., Mingneau, C., Vanrolleghem, P., Verstraete, W., Van Der Zanden, J. and Weytjens, D. (1995). Personal communication. Biotim, Th. Van Rijswijckplaats 7, 2000 Antwerpen. Janssen Pharmaceutica, Turnhoutsesteenweg 30, 2340 Beerse.

McClure, N.C., Fry, J.C. and Weightman, A.J. (1991). Survival and catabolic activity of natural and genetically engineered bacteria in a laboratory-scale activated sludge unit. *Appl. Environ. Microbiol.*, **57**, 366-373.

Postel, S. (1992). *The last oasis : Facing water scarcity*. W.W. Norton & Company, New York.

Rasche, M.E., Hyman, M.R. and Arp, D.J. (1990). Biodegradation of halogenated hydrocarbon fumigants by nitrifying bacteria. *Appl. Environ. Microbiol.*, **56**, 2568-2571.

Selvaratnam, C., Schoedel, B.A., McFarland, B.L. and Kulpa, C.F. (1997). Application of the polymerase chain reaction (PCR) and the reverse transcriptase/PCR for determining the fate of phenol-degrading *Pseudomonas putida* ATCC 11172 in a bioaugmented sequencing batch reactor. *Appl. Microbiol. Biotechnol.*, **47**, 236-240.

Tanghe, T., Devriese, G. and Verstraete, W. (1998). Nonylphenol degradation in lab scale activated sludge units is temperature dependent *Water Reasearch*, **32**, 2889-2896.

Tanghe, T., Devriese, G. and Verstraete, W. (1999). Nonylphenol and estrogenic activity in aquatic environmental samples. *Journal of environmental Quality*, **28**, 702-709.

Van der Kooij, D. and Hijnen, W.A.M. (1983). Verwijdering van organische stoffen door micro-organismen bij filtratieprocessen. *H_2O*, **13**, 306-311.

Van Limbergen, H., Top, E.M. and Verstraete, W. (1998). Bioaugmentation in activated sludge : current features and future perspectives. *Appl. Microbiol. Biotechnol.*, **50**(1), 16-23.

Verstraete, W. and Philips, S. (1998). Nitrification-denitrification processes and technologies in new contexts. *Environmental pollution*, **102**(1), 717-726.

Wood, P.M. (1990). Autotrophic and heterotrophic mechanisms for ammonia oxidation. *Soil use and management*, **6**(2), 78-79.