Biotransformation and Biodegradation of N-Substituted Aromatics in Methanogenic Granular Sludge

Elías Razo Flores

Promotor:	Dr. ir. G. Lettinga bijzonder hoogleraar in de anaërobe zuiveringstechnologie en hergebruik
Co-promotor:	Dr. J.A. Field KNAW onderzoeker aan de Landbouwuniversiteit

Biotransformation and Biodegradation of N-Substituted Aromatics in Methanogenic Granular Sludge

Elías Razo Flores

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
Dr. C.M. Karssen,
in het openbaar te verdedigen
op vrijdag 19 september 1997
des namiddags te half twee in de Aula

This research was carried out at the Department of Environmental Technology, Wageningen Agricultural University, The Netherlands. It was supported by the Consejo Nacional de Ciencia y Tecnología from México. Financial aid from the Instituto Mexicano del Petróleo is gratefully acknowledged.

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Razo Flores, Elías

Biotransformation and Biodegradation of N-Substituted Aromatics in Methanogenic Granular Sludge / Elías Razo Flores. - [S.l. : s.n.]
Thesis Landbouwuniversiteit Wageningen. - With summary in Dutch and abstract in Spanish.
ISBN 90-5485-734-X
Subject headings: anaerobic reactors / azo dyes / biodegradation / nitroaromatics

Propositions

1. Contrary to the statements of Johnson and Young, and Carliell *et al.* nitroaromatics and azo dyes are very toxic and inhibitory to methanogens (Chapters 2 and 6 this dissertation).
   Johnson, L.D., Young, J.C. (1983) Inhibition of anaerobic digestion by organic priority pollutants. *J. Water Pollut. Control Fed.* **55**:1441-1449.
   Carliell, C.M., Barclay, S.J., Naidoo, N., Bucley, C.A., Mulholland, D.A., Senior, E. (1995) Microbial decolourisation of a reactive azo dye under anaerobic conditions. *Water SA* **21**:61-69.

2. Azo dye breakdown products, aromatic amines, can not be regarded as recalcitrant compounds under methanogenic conditions anymore (Chapters 3 and 6 this dissertation).
   Brown, D., Hamburger, B. (1987) The degradation of dyestuffs: Part III - investigations of their ultimate degradability. *Chemosphere* **16**:1539-1553.
   Haug, W., Schmidt, A., Nortermann, B., Hempel, D.C., Stolz, A., Knackmuss, H.J. (1991) Mineralization of the sulfonated azo dye Mordant-Yellow 3 by a 6-aminonaphthalene-2-sulfonate degrading bacterial consortium. *Appl. Environ. Microbiol.* **57**:3144-3149.

3. When Lettinga *et al.* stated that "one of the main drawbacks of anaerobic wastewater treatment is that anaerobic systems are more sensitive to toxic compounds than the aerobic systems", they never imagined the enormous application potential of the UASB reactor.
   Lettinga, G., Hulshoff Pol, L.W., Koster, I.W., Wiegant, W.M., de Zeeuw, W.J., Rinzema, A., Grin, P.C., Roersma, R.E., Hobma, S.W. (1984). High rate anaerobic wastewater treatment using the UASB reactor under a wide range of temperature conditions. *Biotechnology and genetic Engineering Reviews* **2**:253-284.

4. Now it is time not only to clean the environment, but also to design and use "benign" and completely biodegradable chemicals.

5. Dissociated from the human context, development is nothing more than growth without soul and this can be applied to both societies and individuals.
   Los hechos son siempre vacios, huecos. Son recipientes que tomaran la forma de los sentimientos que quieran llenarlos. ***Juan Carlos Onetti***

6. It is not possible to discover new things without having more failures than great successes.

7. Unemployment in Europe is due to problems with the economic model and not the presence of foreigners. History repeats itself?

8. It is surprising that the country which has developed the maximum market economy completely ignores the drug problem.

9. The Wageningen Agricultural University emblem is clearly in descent not in ascent.

10. Now it is possible in México, after the big upset of the state's party, that in politics the gap between the words and the facts can be shortened.

11. Accepting the existence of a problem is the best way of getting started to solve it.

12. Transatlantic nocturnal flights cause so much mental disorder that one can end up buying a tie with an English flag.

13. The Frisian obstinacy is not a fault but an attribute.

14. México City, the feared monster of a thousand heads, is a city with more soul, charm and solidarity than many European capitals.

Propositions belonging to the thesis entitled "**Biotransformation and Biodegradation of N-substituted aromatics in Methanogenic Granular Sludge**".

Elías Razo Flores
Wageningen, 19 September 1997.

A Mario y Margarita

A los Abuelos,
cuyo grato recuerdo siempre me acompaña

Acknowledgments

The dream to come to The Netherlands started almost six years ago during a quite, pleasant and warm weekend in the Mexican high plateau. Now the adventure is finished and I would like to thank those who helped me to accomplish it.

First at all, it was a great pleasure to work with Jim Field, my supervisor and "the boss". Thanks to his excellent and brilliant guidance since the beginning till the end it was possible to arrive to the conclusion of this dissertation. From him I received the most important and very decisive support and I appreciated it a lot.

I express my most sincere gratitude to Gatze Lettinga for allowing me to come to Wageningen to work in his "anaerobic consortia" and for serving as my "promotor". I will never forget his high human warmth and all the endless *avondjes* we had together drinking "very excellent" Belgium beer and trying to solve the world's problems.

Sometimes life puts in our way very nice people, like Brian Donlon, my great Irish friend. I really do not have words to thank his comradeship. We both shared the good and the bad moments of our personal and research lives here in Wageningen. With him I learned to be very passionate with the work and very cool with the personal life. *Go raibh mile maith agat, mo chairde!.*

I also had the opportunity to work, interact and discuss with several students and colleagues, whose hard work and stimulating interest I also would like to acknowledge: Maurice Luijten, Tom Tanghe, Erick-Jan Boots, Marco de Bakker, Antonio Matamala, Anna Svitelskaya, Patrick Smulders, Francesc Prenafeta Boldú and last but not least Nico Tan. Additionally, I would like to express my appreciation to all the members of the Department of Environmental Technology with whom I shared very nice and intense moments. Some of them deserve special thanks like Mario Kato, Lourdinha Florencio, Ching-Shyung Hwu, Miriam van Eekert, Lucas Seghezzo, Robert Kleerebezem, and the Dean of the Colleagues, Mr. Salih Rebac. Of course I should not forget my "guardian angels", Heleen Vos and Ilse Bennehey, who always had a smile and a lot of help for me.

I Consider myself very lucky because I was always surrounded by nice and helpful people; "La Banda del Charro Negro" with Hugo Ramírez, Rebeca Renteria and Armando

García as leaders; The "Spanish Armada" with Francisco Omil, Fran Morales and María Fernández-Polanco as captains of the galleon, and all the other foreigners and Dutch friends who created a sort of "Babel tower" because of the many languages that were spoken. I am indebted to all of them because without all the fun we had together and their support this thesis could have been finished in half of the time!. My special thanks to Reyes Sierra Alvarez for her guidance and friendship and for all the superb suppers she offered. I also thank Dora Lettinga for her kind hospitality.

Despite the distance I always had the support of several friends and colleagues. In fact I can say that the distance shortened the bonds of friendship between us. Special thanks to Mayra, Eugenia, Pilar, Silvia, Luis, Roberto, Victor, Juan and Anne-Marie.

Familia, mi querida familia. El cariño constante y coraje que ustedes me infundieron siempre me ha permitido ver hacia adelante y no rendirme nunca. Consideren este logro como si fuera suyo, que de hecho lo es. Los quiero mucho a todos.

Abstract

Razo Flores, E. (1997) *Biotransformation and Biodegradation of N-substituted Aromatics in Methanogenic Granular Sludge*. Ph.D. Thesis. Wageningen Agricultural University. Wageningen, The Netherlands.

N-substituted aromatic compounds are environmental contaminants associated with the production and use of dyes, explosives, pesticides and pharmaceuticals among others. Nitro- and azo-substituted aromatic compounds with strong electron withdrawing groups are poorly biodegradable in aerobic treatment systems. Therefore anaerobic treatment technologies were considered in this research. The toxicity of these compounds to methanogenic bacteria was studied. Batch toxicity assays indicated that nitroaromatics and azo dyes were highly inhibitory to acetoclastic methanogenic bacteria, with 50% inhibiting concentrations (50% IC) as low as 14 to 538 μM. However, the corresponding aromatic amines were several orders of magnitude less toxic.

The biodegradability of eighteen N-substituted aromatic and six alkylphenol compounds under methanogenic conditions was assessed in batch assays with unadapted and 2-nitrophenol (2NP) adapted granular sludge. Net methane production indicated that all three isomers of aminobenzoate, 2-aminophenol (2AP) and 4-cresol were found to be completely mineralized by the unadapted sludge. All the other compounds tested were not degraded under the experimental conditions employed. The 2NP-adapted granular sludge showed a similar degradation spectrum but also cross acclimatized with other compounds as it was able to also mineralize 4-aminophenol and 5-aminosalicylic acid (5ASA).

The facile reduction of the nitro- and azo-electron withdrawing groups was used as a detoxification strategy in continuous laboratory scale (160 mL) upward-flow anaerobic sludge bed reactors (UASB), supplied with either a mixture of volatile fatty acids (VFA) or glucose and selected nitroaromatic compounds. The nitroaromatics tested included: 2NP, 4-nitrophenol, 2,4-dinitrophenol, 2,4-dinitrotoluene, 4-nitrobenzoic acid (4NBc), 5-nitrosalicylic acid (5NSA) and nitrobenzene. All compounds were efficiently reduced to their corresponding aromatic amines and the primary substrate chemical oxygen demand (COD) was efficiently converted to methane even at influent nitroaromatic concentrations exceeding the 50% IC values by up to 30-fold. After long term reactor operation (several months), aromatic amines were no longer observed to accumulate as products of 4NBc, 2NP and 5NSA elimination. The granular sludge sampled from these reactors were able to fully mineralize 4-aminobenzoate, 2AP and 5ASA to methane when these were offered as the sole carbon and energy source in anaerobic biodegradability assays. These results suggest that 4NBc, 2NP and 5NSA were completely biodegraded in the continuous reactors at nitroaromatic loading rates up to 312, 910 and 553 mg/L-d, respectively.

Continuous UASB reactors were also run with the azo dye Mordant Orange 1 [MO1, 5-(4-nitrophenylazo)salicylic acid] with either no primary substrate, glucose or VFA. Except for the first few weeks, no elimination of azo dye was evident in the column receiving no primary substrates. On the other hand, MO1 was readily cleaved in the reactors (>99%) receiving glucose and VFA at MO1 loading rates up to 295 and 161 mg/L-d, respectively. In these reactors, both 1,4-phenylenediamine (1,4PDA) and 5ASA were detected as products of MO1 cleavage. After 180 days, 5ASA arising from MO1 cleavage could only be detected at trace concentrations in the glucose fed reactor. The sampled sludge was able to rapidly mineralize 5ASA to methane in the anaerobic biodegradability assay. The results suggest that MO1 was cleaved into 1,4PDA and 5ASA; and that 5ASA was fully degraded by the anaerobic consortia; whereas, 1,4PDA persisted. Azodisalicylate (ADS), a pharmaceutical azo dye constructed from two 5ASA units, was completely mineralized in UASB reactors at ADS loading rates up to 225 mg/L-d even in the absence of cosubstrate, indicating that the metabolism of 5ASA could provide the reducing equivalents needed for the azo reduction. Batch experiments confirmed the ADS mineralization.

The results of this research demonstrated that anaerobic treatment is a feasible technology for the treatment of highly toxic nitroaromatics and azo dyes. It was also shown that some nitroaromatic compounds and azo dyes can be completely mineralized and serve serve as a carbon, energy and nitrogen source for anaerobic bacteria, in contrast to the common assumption that they are only biotransformed to mutagenic and carcinogenic aromatic amines.

Resumen

Razo Flores, E. (1997) *Biotransformación y Biodegradación de Compuestos N-aromáticos en Lodo Granular Metanogénico.* Ph.D. Tesis. Universidad Agrícola de Wageningen, Wageningen, Países Bajos.

Los compuestos aromáticos nitrogenados (N-aromáticos) contaminan el ambiente y estan asociados a la producción y uso de colorantes, explosivos, pesticidas y productos farmacéuticos. Los compuestos aromáticos substituidos con grupos nitro o azo son altamente electronegativos, causa por la cual no son fácilmente degradados en los sistemas aerobios convencionales. Por esta razón las tecnologías anaerobias de tratamiento fueron consideradas en esta investigación. La toxicidad de estos compuestos en bacterias metanogénicas fue estudiada en experimentos por lote. Los resultados indicaron que los nitroaromáticos y los colorantes azo inhibieron significativamente la actividad metanogénica de las bacterias acetoclásticas, con concentraciones que causaron una disminución del 50% en dicha actividad (50%IC) en el rango de 14 a 538 μM. Sin embargo, los correspondientes compuestos aminoaromáticos fueron varios ordenes de magnitud menos tóxicos.

La biodegradabilidad de 18 compuestos N-aromáticos y 6 alquilfenoles fue estudiada en experimentos por lote, bajo condiciones metanogénicas y utilizando dos tipos de lodo granular: no adaptado y adaptado a la degradación de 2-nitrofenol (2NF). Los resultados indicaron que los tres isómeros de aminobenzoato, 2-aminofenol (2AF) y 4-cresol fueron completamente mineralizados por el lodo granular no adaptado, mientras que los demás compuestos probados no fueron degradados. Los resultados obtenidos con el lodo granular adaptado mostraron que este lodo degrado, además de los mismos compuestos, el 4-aminofenol y el ácido 5-aminosalicílico (5ASA). Estos resultados indican que es posible adaptar lodo granular para obtener mineralización cruzada de compuestos aromáticos estructuralmente similares.

Es conocido que los grupos nitro o azo son fácilmente reducidos bajo condiciones de anaerobiosis, por lo cual se utilizó la reducción de estos compuestos como una estrategia de detoxificación en reactores anaerobios contínuos (160 mL) de lecho de lodo con flujo ascendente (UASB). Los reactores UASB fueron alimentados con una mezcla de ácidos grasos volátiles (AGV) o glucosa como cosubstratos y diferentes compuestos nitroaromáticos en cada reactor. Los compuestos probados fueron: 2NF, 4-nitrofenol (4NF), 2,4-dinitrofenol (2,4-DNF), 2,4-dinitrotolueno (2,4-DNT), ácido 4-nitrobenzóico (4NBc), ácido 5-nitrosalicílico (5NSA) y nitrobenceno. Todos los nitroaromáticos fueron completamente reducidos a sus correspondientes compuestos aminoaromáticos, mientras que la DQO de los cosubstratos fue convertida eficientemente a metano, incluso a concentraciones de nitroaromáticos que excedian hasta 30 veces el valor de 50%IC. Después de algunos meses de operación se observó que la concentración de los compuestos aminoaromáticos, generados como productos de reducción, disminuía en los reactores UASB que trataban 2NF, 4NBc y 5NSA. Las muestras de lodo granular de estos reactores mineralizaron el ácido 4-aminobenzóico, 2AF y 5ASA en experimentos por lote donde estos compuestos fueron las únicas fuentes de carbono y energía disponibles. Estos resultados demostraron que 2NF, 4NBc y 5NSA fueron mineralizados en los reactores UASB con cargas de nitroaromáticos de hasta de 312, 910 y 553 mg/L-d, respectivamente.

Reactores UASB también fueron operados para estudiar la transformación de compuestos azo con o sin cosubstrato. Excepto en las primeras semanas, la reducción de Mordant Orange 1 (MO1) en el reactor sin cosubstrato fue mínima. Por otra parte, MO1 fue altamente reducido y decolorado (>99%) en los reactores recibiendo AGV y glucosa como cosubstratos, con cargas de MO1 de hasta 161 y 295 mg/L-d, respectivamente. En ambos reactores se detectó 5ASA y 1,4-diaminobenceno (1,4DAB) como productos de ruptura del enlace azo. Después de 180 días de operación se observó que 5ASA era detectado a bajas concentraciones en el reactor con glucosa. El lodo granular de este reactor mineralizó 5ASA en experimentos por lote, mientras que 1,4DAB no fue degradado. De la misma forma, Azodisalicilato (ADS), un compuesto azo formado por dos unidades de 5ASA, fue completamente mineralizado en reactores UASB a cargas de hasta 225 mg/L-d, incluso en ausencia de cosubstrato, indicando que el metabolismo de 5ASA suministro los equivalentes de reducción necesarios para el rompimiento del enlace azo. Los experimentos por lote confirmaron la mineralización de ADS.

Los resultados de esta tesis demuestran que el tratamiento anaerobio es una tecnología viable para el tratamiento de compuestos altamente tóxicos como los nitroaromáticos y los colorantes azo. También se demuestra que algunos nitroaromáticos y colorantes azo son completamente mineralizados y además son utilizados como fuente de carbono, energía y nitrógeno por las bacterias anaerobias, en contraste con lo aceptado generalmente de que sólo son transformados a compuestos mutagénicos y cancerígenos.

Contents

1

General Introduction

1.1 Introduction

Industrialization has resulted in the formation of waste products which are released into the environment in the form of wastewater, gaseous emissions and solid residues leading to environmental pollution and deterioration. Increasing amounts of fuel, industrial chemicals, fertilizers, pesticides, pharmaceuticals, processed food and similar indispensable products will be required for improving the quality of life to mankind. However, the resulting pollution may have a serious impact to the fauna, flora of the ecosystem as well as public health.

The chemical and petrochemical industries represent an important potential source of toxic and recalcitrant priority pollutants. Chemical manufacturing is a multiproduct and multiprocess industry, using a wide range of basic raw materials, it produces several heterogeneous products of diverse nature. Currently, about 100 000 chemicals are produced commercially (113), and aromatic compounds make up about a third of the 91 billion kg of the top 50 chemicals manufactured annually in the United States (6). The environmental impacts of aromatic hydrocarbons and chloroaromatics have received the most research attention. Less studied are the N-substituted aromatic compounds which play an extremely important role in the chemical industry. In fact, some major industries are totally dependent on nitroaromatic feedstocks (46). Table 1 shows the production of nitroaromatics in the U.S. market.

The environmental fate of organic pollutants is known to depend upon a variety of physical and biochemical factors, including chemical structure (aromatic vs. aliphatic, nature of the functional group, etc.), sorption characteristics, volatility, ionic character, solubility and availability of terminal electron acceptors. In anaerobic environments, the predominant electron

acceptor can influence the biological consortium selected, the thermodynamics of a particular reaction and the redox potential. Collectively, these factors can affect the biodegradability, toxicity and kinetics of metabolism for a chemical compound in an anoxic environment.

TABLE 1.1 Production of nitroaromatics in United States in 1980.[a] Adapted from Hartter (46).

Product category	Market size (Mtons)
Polymers[b]	571.5
Rubber chemicals	101.2
Dyes and pigments	21.3
Pharmaceuticals	12.7
Pesticides	68.9
All other	31.8

[a] Include derivatives of benzene, chlorobenzene and toluene.
[b] Mostly polyurethane produced from aniline.

The majority of nitroaromatic compounds found in the environment are released due to anthropogenic activities. However, a few aromatic compounds bearing one nitro group as a substituent are produced as secondary metabolites by microorganisms (118, 120). Nitroaromatics are used in the production of chemicals, dyes, plasticizers, explosives, pharmaceuticals and pesticides among others (40, 44). Consequently, these aromatics appear in the wastes generated by these industries. They are also formed by incomplete combustion of fossil fuels and synthesized photochemically in the atmosphere (37). Nitroaromatics are highly toxic to man and mammals, being easily reduced by enzymes to nitroso and hydroxylamine derivatives (4). These derivatives may lead to the formation of either metahemoglobin, which is unable to bind oxygen, or of nitrosoamines, which are carcinogenic (66). Some nitroaromatics, such as nitropyrene (62), are mutagenic and several nitrophenols have an uncoupling effect on oxidative phosporylation (115). Most nitroaromatics and azo dyes are highly toxic to bacteria and, consequently, may inhibit microbial growth (56, 123). In fact, the toxicity and the poor biodegradability exhibited by these compounds are the main bottlenecks in the application of microbial wastewater treatment processes.

Azo dyes are synthetic organic colorants that exhibit great structural diversity (5), and are used for various purposes in the textile, cosmetic, paper-making and food and pharmaceutical industries (81, 128). The textile and the dyestuff manufacturing industry are the two major sources of released azo dyes (71, 85). Among other factors, these dyes are used because of their resistance to breakdown when exposed to the environment. Consequently, they are often very difficult to biodegrade when present in wastewaters. Azo dyes are regarded as toxic compounds because they were shown to affect microbial activities and microbial population sizes in the sediments and in the water columns of aquatic habitats (26, 56). These colored compounds were also inhibitory to microbial oxidation processes in activated sludge plants (56, 93). In the same way, concentrations of Acid Orange 1 as low as 5 mg/L inhibited all stages of the nitrification process (48).

Biological treatment, either aerobic or anaerobic, is generally the most cost-effective means for the removal of N-substituted aromatics. Evidence is accumulating indicating that microorganisms are able to degrade these compounds (4, 33, 41, 82, 108-110, 122). Under aerobic conditions, aromatic compounds are transformed by monooxygenases and dioxygenases into a few central intermediates. Three intermediates are common to all of the aerobic pathways of metabolism of aromatic compounds: catechol, protocatechuate and gentisate (108). These dihydroxylated compounds are broken down by similar pathways to simple acids and aldehydes which are readily used for cell synthesis and energy (1, 122). Under anaerobic conditions the mechanism of degradation is completely different; the aromatic ring structures are reductively attacked as proposed by Evans and Fuchs (29) and Fuchs *et al.* (35). First, the diverse aromatic compounds are transformed to simplified aromatic intermediates like benzoyl-CoA, resorcinol, phloroglucinol and possibly others through channeling reactions (35, 49). Subsequently, the simplified aromatic intermediates are reductively attacked and cleaved by hydrolysis, and the resulting non-cyclic compounds are transformed by β-oxidation to central metabolites like acetyl-CoA and CO_2. Figure 1.1 shows schematically the anaerobic degradation pathway of aromatic compounds.

Due to the strong electron-withdrawing character of the nitro and azo groups, these compounds are electron deficient. Besides the xenobiotic character of these groups, it is the

electron-withdrawing character and thus the electron deficiency of the aromatic ring (Figure 1.2), which impedes electrophilic attack by oxygenases of aerobic bacteria and fungi (66, 97), being this the reason why polynitroaromatics and azo dyes are not degraded in aerobic wastewater systems (45, 83, 95, 105). The most common biological reaction of nitro and azo groups is reduction. Because the reduction of aromatic nitro and azo groups is such a facile process, reductive transformation of chemicals containing these moieties is often the predominant pathway for their transformation in the environment (18, 45). Consequently, anaerobic reduction can be applied as a first step in the biodegradation of toxic nitro and azo dye compounds.

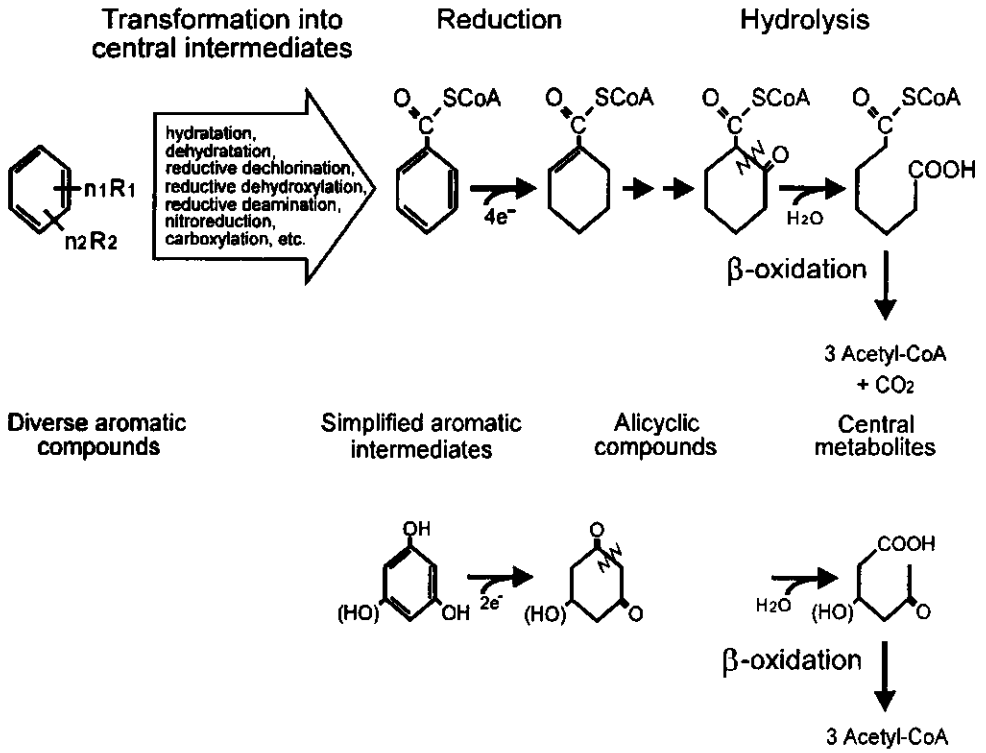


Figure 1.1 Schematic pathway of anaerobic degradation of aromatic compounds: R_1, R_2 = -CH_3, -OH, -COOH, -NH_2, -NO_2, -Cl, etc.; n_1, n_2 = 0-6. Adapted from Holliger and Zehnder (51).

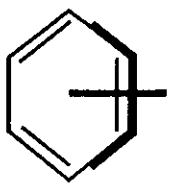


Figure 1.2 Electron-withdrawing groups (EWG) deactivate compounds for electrophilic attack by aerobic microorganisms; R = alkyl or aryl. Adapted from Rieger and Knackmuss (97).

Anaerobic treatment consist of a series of microbiological processes that convert organic compounds to biogas like methane and carbon dioxide. While several types of microorganisms are implicated in aerobic processes, anaerobic processes are driven mostly by bacteria (13). Furthermore, there are synergistic interactions between the various groups of bacteria implicated in anaerobic digestion of wastes. The groups of bacteria catalyzing the reactions taking place during the anaerobic digestion are mainly: fermentative bacteria, hydrogen-producing acetogenic bacteria, hydrogen-consuming acetogenic bacteria, carbon dioxide-reducing methanogens and acetoclastic methanogens (42). The better understanding of the anaerobic microbiological processes allowed the expansion on the application of this technology not only to the treatment of wastewater sludge but to more complex wastewaters.

1.2 Anaerobic Technology; the Upflow Anaerobic Sludge Bed (UASB) Reactor

Anaerobic treatment of wastewater was used in the first half of the century but the predominance of aerobic methods became overwhelming later. However, since the introduction of the "anaerobic high rate systems" in the late 1970's, a breakthrough in the environmental technology came about (10, 43, 111). Now, the upflow anaerobic sludge bed (UASB) reactor, is by far the most widely applied anaerobic treatment system and is extensively used for the treatment of several types of wastewater (55, 72, 73, 76, 101). One of the advantages of the UASB reactor is the ability to retain high biomass concentrations despite the upflow velocity of the wastewater and the production of biogas. Consequently, the reactor can operate at short hydraulic retention time since the sludge retention time is almost

independent of the hydraulic retention time. Successful operation under these conditions requires a highly active biomass with good settling capacities (114, 121). In the UASB reactors, the biomass is retained as aggregates, called granules, formed by self-immobilization of the bacteria which naturally occurs due to the upflow conditions (54, 76, 77). The formation and stability of the granules are essential for successful operation. Retention of active biomass within the system enables good treatment performance at high organic loading rates, and natural turbulence caused by the influent and the biogas production provides good wastewater-biomass contact in the UASB reactor. Higher organic loads can be applied in UASB systems than in aerobic processes (59). Therefore, less reactor volume and space is required while, at the same time, high grade energy can be produced from the biogas (59).

Originally, the UASB reactor was applied for the treatment of wastewaters from the agro and food industries (55), and lately for the municipal wastewaters (43). More recently the UASB reactor has been successfully applied for the treatment of the wastewaters generated by the forestry (74) and the chemical and petrochemical industries (80). The delayed application for the treatment of these kind of wastewaters was due to the *a priori* idea stating that "the anaerobic systems were more sensitive to toxic compounds than the aerobic systems" (13, 75, 98). However, a recent study has demonstrated that aerobic heterotrophs and methanogens have similar sensitivities to toxicants for most classes of chemicals (14). This rather startling conclusion contradicts the standard "wisdom" of the practicing engineer. Experience have demonstrated that even extremely toxic compounds, such as pentachlorophenol, can be successfully treated in UASB reactors (50, 124), indicating that this system is suitable for the treatment of highly toxic compounds.

1.3 Toxicity of N-substituted Aromatics

The presence of toxic chemicals during the anaerobic degradation processes can inhibit the normal sequence of anaerobic biochemical metabolic reactions, thereby causing inefficient treatment and possibly even a complete failure. N-substituted compounds such as nitroaromatics and azo dyes are reported to be toxic to microorganisms (7, 26, 45, 56, 57, 89,

119, 123). Of all the classes of organisms involved in anaerobic degradation, the methanogens are reported to be the slowest growing organisms in the consortium thus a toxic shock can have an important impact. Uberoi and Bhattacharya (119) reported that nitrophenols were more inhibitory to acetate utilization than propionate utilization, and they also noted that high volatile suspended solids concentration produced less severe toxic effects of nitrophenols on methanogenesis. In the same way, Kim *et al.* (60) and Davies-Venn *et al.* (27) reported that chlorophenols and chloroanilines were more toxic to the acetoclastic methanogenesis reactions than to an ethanol-degrading acetogenic reactions. However, in some instances the acetogens, were found to be more sensitive to toxic chemicals than acetoclastic methanogens (84). In the particular case of the methanogens, there are evidences indicating that acetoclastic methanogens are more sensitive to toxic aromatic compounds than hydrogenotrophic methanogens (11, 39, 61). Apparently the methanogens are also more sensitive to the presence of nitroaromatics than the sulphate-reducing bacteria (40).

Nitroaromatics

Table 1.2 shows the anaerobic toxicity effects of several nitroaromatics measured under batch conditions with different kinds of biomass as reported in the literature. As can be seen from Table 1.2, the information about the toxicity exerted by the nitrophenols to the anaerobic biomass is well documented, whereas data for other nitroaromatics is rather scarce. In general, concentrations of nitroaromatics below 100 mg/L exhibited toxic effects which, depending on the compound, ranged from moderate inhibition to complete suppression of microbial activity. With respect to the nitrophenols, their toxicity decreases in the following order: 2,4-dinitrophenol > 4-nitrophenol > 2- or 3-nitrophenol (44, 116). Concentrations of 4-nitrophenol as low as 5 mg/L have been reported to produce severe biogas (methane and carbon dioxide) production inhibition (117) or biogas production suppression (116). Concentrations of 2,4-dinitrophenol of 20 mg/L was reported to irreversibly inhibit biogas production (86). Gorontzy *et al.* (40) reported that nitroaromatics hindered cell growth of several methanogens, sulphate-reducers and *Clostridia*, and additionally, they showed that nitroaromatics caused cell lysis of methanogens. The majority of the toxicity studies reported in Table 1.2 were conducted with anaerobic digester sludge, indicating a lack of data for anaerobic granular sludge. Toxicity

TABLE 1.2 Anaerobic toxicity of nitroaromatic compounds under batch conditions reported in the literature.

Compound	Concentration (mg/L)	Seed	Effect	Reference
nitrobenzene	100	anaerobic digester sludge	>50% reduction in biogas production	58
	12	acetate enrichment culture	50% inhibition acetoclastic methanogenesis	24
	13	acetate enrichment culture	50% inhibition in biogas production	14
pentachloronitrobenzene	26	acetate enrichment culture	50% inhibition in biogas production	14
2-nitrophenol	100	anaerobic digester sludge	>50% reduction in biogas production	58
	100	anaerobic digester sludge	inhibition of methanogenesis	89
	20	acetate enrichment from digested sludge	biogas production inhibition	44
	20	acetate enrichment from digested sludge	reversible biogas inhibition	86
	20	anaerobic digester sludge	severe biogas production inhibition	117
	50	anaerobic sewage sludge	methane production inhibition	16
	100	anaerobic digester sludge	biogas production inhibition	8
	30	acetate enrichment from digested sludge	biogas production inhibition	119
	40	propionate enrichment from digested sludge	biogas production inhibition	119
	70	sediment enrichments	significant inhibition initial methanogenic rates	91
	20	granular sludge from potato-chip industry	50% inhibition acetoclastic methanogenesis	39
	12	acetate enrichment culture	50% inhibition in biogas production	14
3-nitrophenol	7	several methanogenic bacteria	methanogen cell lysis	40
	20	acetate enrichment from digested sludge	biogas production inhibition	44
	10	anaerobic digester sludge	severe biogas production inhibition	117
	50	anaerobic sewage sludge	methane production inhibition	16
	100	anaerobic digester sludge	biogas production inhibition	8
	83	sediment slurry	methane inhibition	90
	70	sediment enrichments	significant inhibition initial methanogenic rates	91

TABLE 1.2 Continuation

	25	granular sludge from potato-chip industry	50% inhibition acetoclastic methanogenesis	39
	18	acetate enrichment culture	50% inhibition in biogas production	14
4-nitrophenol	100	anaerobic digester sludge	>50% reduction in biogas production	58
	100	anaerobic digester sludge	inhibition of methanogenesis	89
	7	several methanogenic bacteria	methanogen cell lysis	40
	>25	halophilic anaerobic eubacteria	inhibition *Haloanaerobium praevalens* growth	94
	5	anaerobic activated biofilm	biogas production suppresion	116
	10	acetate enrichment from digested sludge	biogas production inhibition	44
	20	acetate enrichment from digested sludge	reversible biogas inhibition	86
	5	anaerobic digester sludge	severe biogas production inhibition	117
	50	anaerobic sewage sludge	severe methane production inhibition	16
	100	anaerobic digester sludge	severe biogas production inhibition	8
	72	sediment slurry	methane inhibition	90
	40	acetate enrichment from digested sludge	biogas production inhibition	119
	40	propionate enrichment from digested sludge	biogas production inhibition	119
	100	anaerobic digester sludge	methane production inhibition	53
	70	sediment enrichments	significant inhibition initial methanogenic rates	91
	4	acetate enrichment culture	50% inhibition in biogas production	14
2,4-dinitrophenol	100	anaerobic digester sludge	inhibition of methanogenesis	89
	9	several methanogenic bacteria	methanogen cell lysis	40
	10	halophilic anaerobic eubacteria	inhibition haloanaerobes growth	94
	10	acetate enrichment from digested sludge	biogas production inhibition	44
	20	acetate enrichment from digested sludge	irreversible biogas inhibition	86
	128	anaerobic digester sludge	severe biogas production inhibition	8
	20	acetate enrichment from digested sludge	irreversible biogas inhibition	119
	30	propionate enrichment from digested sludge	irreversible biogas inhibition	119
	0.01	acetate enrichment culture	50% inhibition in biogas production	14
2,5-dinitrophenol	128	anaerobic digester sludge	severe biogas production inhibition	8

TABLE 1.2 Continuation

4-nitroaniline	7	several methanogenic bacteria	methanogen cell lysis	40
2-nitrobenzoate	100	anaerobic digester sludge	biogas production inhibition	8
	60	granular sludge from potato-chip industry	50% inhibition acetoclastic methanogenesis	39
3-nitrobenzoate	100	anaerobic digester sludge	biogas production inhibition	8
	15	granular sludge from potato-chip industry	50% inhibition acetoclastic methanogenesis	39
4-nitrobenzoate	8	several methanogenic bacteria	methanogen cell lysis	40
	100	anaerobic digester sludge	biogas production inhibition	8
2-methyl-4,6-dinitrophenol	100	anaerobic digester sludge	inhibition of methanogenesis	89
2,4-dinitrotoluene	>8	2,4-dinitrotoluene and ethanol adapted biomass	inhibition of its own biotransformation as well as the acetogenesis of ethanol	23
2,6-dinitrotoluene	7.0	acetate enrichment culture	50% inhibition in biogas production	14
1-nitronapthalene	16	acetate enrichment culture	50% inhibition in biogas production	14

studies conducted with anaerobic granular sludge has been reported for few nitroaromatics (39), haloaromatics (39, 107) and alkylphenols (39, 107).

Azo Dyes

Regarding the toxicity of azo dyes to anaerobic microorganisms, there is little information available. Carliell *et al.* (22) demonstrated that concentrations of Reactive Red 141 above 100 mg/L inhibited biogas production in batch experiments using anaerobic biomass. Similarly, Seshadri *et al.* (104) reported that dye concentrations of 15 mg/L of Acid Orange 8 and Acid Orange 10 produced a significant inhibition in dye and chemical oxygen demand (COD) removal in an anaerobic fluidized bed reactor.

Aromatic Amines

The aromatic amines on the other hand are generally regarded as being less toxic compounds for the anaerobic microorganisms compared to nitroaromatic and azo dye compounds. Fedorak *et al.* (30) found no evidence that anilines negatively influenced methanogenesis. Boyd *et al.* (16) and Battersby and Wilson (8) indicated that biogas production inhibition in batch experiments using anaerobic sludge ceased after the nitroaromatics tested were completely reduced. According to these results, it seems that the reductive transformation of nitroaromatics to aromatic amines leads to a detoxification of the compounds.

1.4 Nitroaromatics and Azo Dye Reduction

Nitroaromatics and azo dyes are reduced under anaerobic conditions by different kinds of microorganisms, including the intestinal microflora of several species of mammals (7, 25, 26, 99, 109). The reduction of these compounds proceeds through six-electron (nitro) and four-electron (azo) mechanism as shown in Figure 1.3, resulting in the formation of aromatic amines (18, 40). Reductases (nitro- and azo-) from a variety of sources catalyze the reduction

of the nitro and azo groups. Nitroreductases convert nitro groups to either nitroso derivatives, hydroxylamines or amines by the successive addition of electron pairs donated by cosubstrates. The nitroso derivatives are difficult to detect because they are reactive and unstable (4).

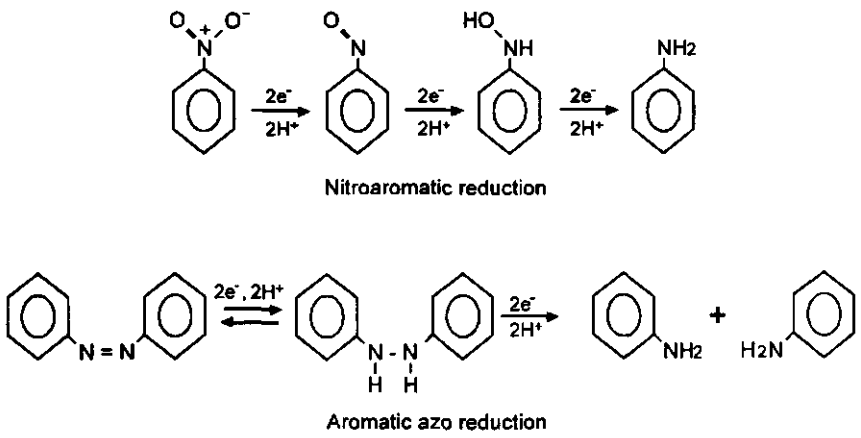


Figure 1.3 Nitroaromatic and azo dye reduction via six- and four-electron mechanism, respectively, under anaerobic conditions.

Specific nitro- and azoreductases have been isolated and characterized (21, 25, 62, 96). Azoreductases have generally been found to be oxygen-sensitive and to require flavins with both NADH and NADPH as active electron donors for optimal activity (25). Gorontzy *et al.* (40) and Chung *et al.* (25) concluded that the microbial reduction of nitro- and azo compounds is an unspecific detoxification reaction mediated by certain enzymes and/or cofactors. Zimmermann *et al.* (125) have shown that certain specific azoreductases of specialized *Pseudomonas* strains are oxygen-insensitive. Reduction of nitroaromatics and azo dyes has also been observed in abiotic systems (70).

1.5 Biodegradation of N-substituted Aromatics

Molecules substituted with electron-withdrawing groups such as nitro-, azo- and chloro-groups are quite resistant to electrophilic attack by oxygenases (65, 97). Consequently,

they generally persist during aerobic wastewater treatment (45, 83, 95, 105). On the other hand, a nucleophilic mechanism of attack is common in anaerobic environments being favorable for the initial reductive attack of these compounds (65, 97). However, electron-donating functional groups, like aromatic amines, are troublesome for the nucleophilic attack of anaerobes (64, 65, 100). The presence of amino electron donating groups is expected to facilitate the electrophilic attack of aromatics. Most of the typical aromatic amine-end products from the anaerobic metabolism of nitro- and azo-aromatic pollutants are eliminated in aerobic biodegradability tests (19, 20, 28, 33). For these reasons, sequencing anaerobic-aerobic biotreatment processes have been proposed for the complete mineralization of electron-withdrawing aromatic compounds such as nitroaromatics and azo dyes as shown in Figure 1.4 (9, 28, 33, 47, 67, 79, 127). However, when a nitroaromatic or azo dye-bearing wastewater is treated anaerobically, the resulting metabolites might well be an unstable aromatic amines which readily are autooxided to colored polymeric products upon exposure to air (63, 69, 83, 88). These autooxidation products are often complex humic compounds that are non-toxic and non-biodegradable (15, 31, 32). Field *et al.* (31, 33) indicated that the intermediary metabolites formed during autooxidation process can increase the methanogenic toxicity when present in wastewater.

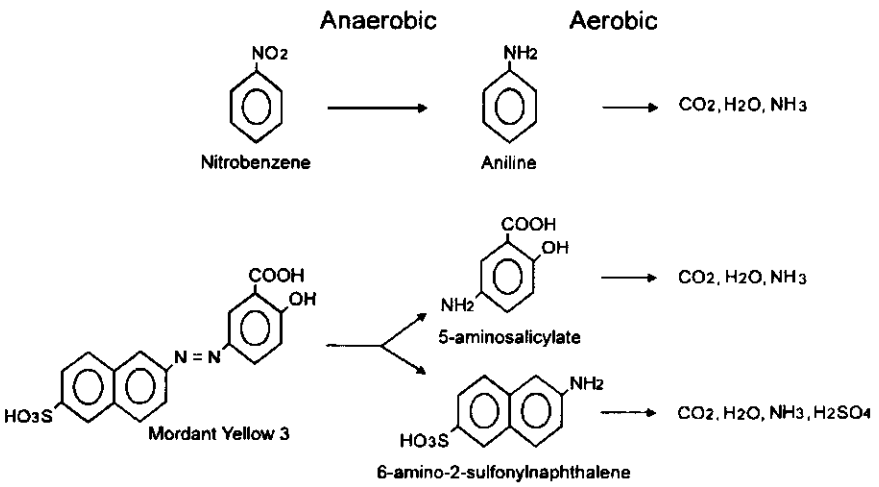


Figure 1.4 Examples illustrating the sequenced anaerobic-aerobic mineralization of nitrobenzene (28) and Mordant Yellow 3 (47).

TABLE 1.3 Anaerobic biodegradability of N-substituted aromatics under methanogenic batch conditions reported in the literature.

Compound	Concentration (mg/L)	Seed	Biodegradation potential[a]	Reference
2-nitrophenol	10	acetate or propionate enrichment culture	+	119
	50	anaerobic sewage sludge	+	16
	20	anaerobic digester sludge	+	89
	70	sediment slurry	+	90
	96	anaerobic digester sludge	+	106
	70	sediment enrichment	±	91
2-aminophenol	72	sediment slurry	+	90
	76	anaerobic digester sludge	+	8
3-nitrophenol	50	anaerobic sewage sludge	+	16
4-nitrophenol	10	acetate or propionate enrichment culture	+	119
	50	anaerobic sewage sludge	+	16
	70	sediment slurry	+	90
	96	anaerobic digester sludge	+	106
	70	sediment enrichment	±	91
4-aminophenol	72	sediment slurry	+	90
	55	sediment enrichment	+	91
2,4-dinitrophenol	10	acetate or propionate enrichment culture	+	119
	20	anaerobic digester sludge	+	89

TABLE 1.3 Continuation

2-aminobenzoate	NI[b]	methanogenic enrichment culture	+	100
	82	municipal digested sludge	+	53
	28	aquifer slurries	+	68
	82	anaerobic digester sludge	+	106
3-nitrobenzoate	NI	freshwater lake sediment	+	53
3-aminobenzoate	20	methanogenic enrichment culture	+	103
	110	freshwater lake sediment	+	53
	23	aquifer slurries	+	68
4-nitrobenzoate	NI	freshwater lake sediment	+	53
4-aminobenzoate	NI	methanogenic enrichment culture	+	100
	82	municipal digested sludge	+	53
	26	aquifer slurries	+	68
	82	anaerobic digester sludge	±	106
	82	anaerobic digester sludge	+	8
Aniline	65	anaerobic digester sludge	-	8
	20	aquifer slurries	-	68
benzamide	20	aquifer slurries	+	68
4-toluamide	30	aquifer slurries	+	68

[a] (+) complete mineralization; (±) partial mineralization; (-) not mineralized.

[b] NI: not indicated.

Nevertheless, there are several reports indicating the complete mineralization of some N-substituted aromatics under anaerobic conditions. Table 1.3 summarizes the batch biodegradability of nitroaromatics and aromatic amines under methanogenic conditions. In general, according to Table 1.3, it appears that all the isomers of the nitrophenols and aminophenols as well as nitrobenzoates and aminobenzoates are mineralized under methanogenic conditions. The mineralization of these monosubstituted aromatics proceeded through reduction of the nitrogroup to the corresponding aromatic amine. Figure 1.5 shows schematically the fate of aromatic amines under anaerobic and anoxic conditions. One of the reactions that has been demonstrated to take place is deamination (Figure 1.5A) from the aromatic ring of 2-aminophenol by an anaerobic methanogenic consortium to produce phenol (116), that was completely mineralized afterwards. Bisaillon *et al.* (12) described a methanogenic consortium that was able to carboxylate and dehydroxylate 2-aminophenol producing 3-aminobenzoate (Figure 1.5B), which accumulated in the culture medium and was not further metabolized.

Other N-substituted aromatics have been reported to be transformed to compounds that are susceptible to be mineralized. Stevens *et al.* (111) reported that an fermentative enrichment culture degrading the herbicide Dinoseb (2-*sec*-butyl-4,6-dinitrophenol) was able to transform Dinoseb to acetate and CO_2. The following nitroaromatics were also transformed by this enrichment culture to the same end products: 4,6-dinitro-*o*-cresol, 3,5-dinitrobenzoate, 2,4-dinitrotoluene and 2,6-dinitrotoluene (111). In the same way, Funk *et al.* (36) reported that under anaerobic conditions 2,4,6-trinitrotoluene was reduced to 2,4,6-triaminotoluene, and a deeper transformation resulted in the accumulation of methylphloroglucinol and *p*-cresol via hydroxylation (Figure 1.5C). Brown and Hamburger (20) indicated that 4,4'-diamino-3,3'-dimethoxybiphenyl was eliminated in anaerobic sludge.

Reactions leading to the transformation of aromatic amines has been also reported under anoxic conditions. Schnell and Schink (102) and de Alexandra (2) have reported the mineralization of aniline under sulfate-reducing and denitrifying conditions, respectively. The mineralization proceeded via carboxylation of aniline in the *para*-position (Figure 1.5D) to 4-aminobenzoate, which was activated to 4-aminobenzoyl-CoA and further metabolized by

reductive deamination. This latter step has also been demonstrated with 2- and 3-aminobenzoate with strictly anaerobic bacteria (78). In the same way, acetylation of aromatic amines has been reported under nitrate-reducing conditions (Figure 1.5E). Gilcrease and Murphy (38) described the transformation of 2,4-diamino-6-nitrotoluene to 4-acetylamino-2-amino-6-nitrotoluene, whereas Noguera and Freedman (87) reported the transformation of 2,4-diaminotoluene to 2,4-diacetamidetoluene. The two acetylated compounds were not transformed further.

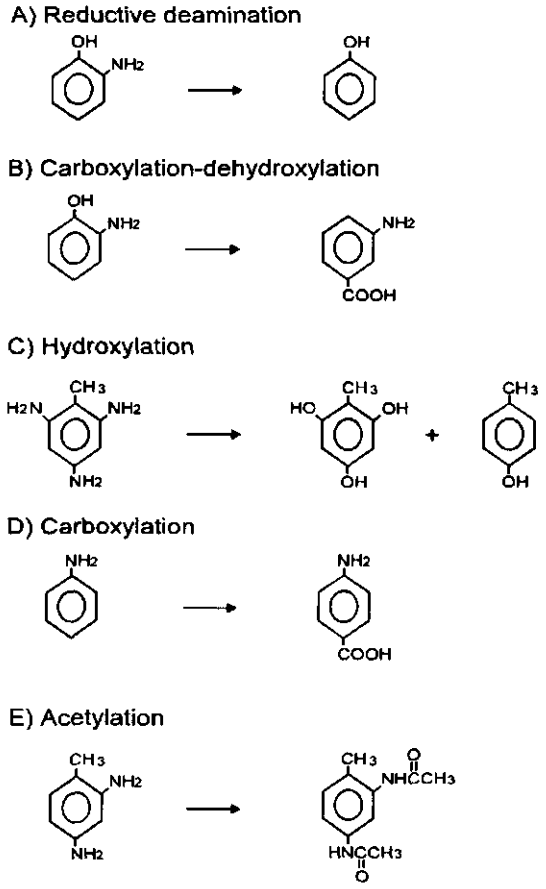


Figure 1.5 Fate of aromatic amines under anaerobic and anoxic conditions; (A) reductive deamination of 2-aminophenol (115), (B) carboxylation-dehydroxylation of 2-aminophenol (12), (C) hydroxylation of triaminotoluene (36), (D) carboxylation of aniline (2, 102), acetylation of 2,4-diaminotoluene (87).

Based on these results, it seems that N-substituted aromatic compounds having carboxy-, hydroxy- and methoxy-substituents are potentially biotransformed and even mineralized under anaerobic conditions. On the other hand, N-substituted aromatics with sulphonic-groups are recalcitrant under methanogenic conditions (68).

The transformation and degradation of azo dyes has been studied for almost 30 years and these compounds have consistently been reported as recalcitrant under aerobic conditions (17, 52, 92, 95, 105). However, during anaerobic conditions the azo dyes were reductively decolorized to the corresponding aromatic amines (18, 20, 22, 34, 85, 104), which are more easily degraded under aerobic conditions (19, 47).

The application of sequenced and simultaneous systems for the mineralization of azo dyes has been very successful, opening a hitherto largely unexploited technology for biological treatment of electron deficient xenobiotics. Haug *et al.* (47) demonstrated the complete mineralization of the azo dye Mordant Yellow 3 under sequenced anaerobic-aerobic conditions. Kudlich *et al.* (67) showed the mineralization of the azo dyes Mordant Yellow 3, Amaranth and Acid Red 1 under simultaneous anaerobic and aerobic conditions using bacterial strains BN6 and 5AS1 immobilized in calcium alginate beads. Other studies have also demonstrated the applicability of this technology for the treatment of different kinds of azo dyes (3, 34). A recent study demonstrating the reduction of a disperse azo dye, *p*-aminoazobenzene, under denitrifying conditions (125) has indicated that it could be possible to reduce azo dyes under redox conditions different to those ones reached under methanogenesis.

1.6 Scope and Structure of the Thesis

N-substituted aromatics, such as nitroaromatics, azo dyes and aromatics amines, are chemical compounds which appear frequently in the wastewaters generated from the chemical and petrochemical industries. All these compounds are of special concern because of their toxic properties against living organisms. Therefore, N-substituted aromatic-bearing wastewaters should be treated before final discharge to receptor aquifers and surface waters. In view of the

scarce information about the role of the toxicity and biodegradability potentials of these aromatic compounds under anaerobic conditions, the aim of this thesis was to study the toxic impact and fate of N-substituted compounds in methanogenic consortia used in anaerobic wastewater treatment systems. The results so obtained could be applied towards developing a general strategy of treatment based on the elucidation of toxic effects, adaptation potential of the microbial ecosystem and process technological aspects. For this purpose, selected N-substituted aromatics were studied under batch and continuous lab-reactor experiments using methanogenic granular sludge consortia as inoculum.

In Chapter 2, the structure-toxicity relationships of N-substituted aromatic compounds to acetoclastic methanogens were examined. In Chapters 3 and 4 the anaerobic biodegradability of N-substituted aromatics and alkylphenols by different samples of granular sludge was evaluated.

In Chapters 5 and 6 continuous experiments were conducted in lab-scale anaerobic reactors treating selected nitroaromatics and azo dyes. N-substituted aromatic mineralization and the role of cosubstrates on the nitro- and azoreduction were also studied. Finally, the results obtained in this research are discussed in Chapter 7.

References

1. Alexander, M. (1977) Introduction to soil microbiology. John Wiley & Sons, New York.
2. Alexandra de, M., O'Connor, O.A., Kosson, D.S. (1994) Metabolism of aniline under different anaerobic electron-accepting and nutritional conditions. *Environ. Toxicol. Chem.* **13**:233-239.
3. An, H., Qian, Y., Gu, X., Tang, W.Z. (1996) Biological treatment of dye wastewaters using an anaerobic-oxic system. *Chemosphere* **33**:2533-2542.
4. Andreoni, V., Baggi, G., Bernasconi, S. (1995) Microbial degradation of nitrogenous xenobiotics of environmental concern. In: Singh, V.P. (Ed.). Biotransformations: microbial degradation of health-risk compounds. Progress in industrial microbiology, Vol. 32, pp 1-35. Elsevier, Amsterdam.
5. Anliker, R. (1979) Ecotoxicology of dyestuffs-a joint effort by industry. *Ecotoxicol. Environ. Saf.* **3**:59-74.
6. Anonymous (1988) Facts and figures for the chemical industry. *Chem. Eng. News*. **66**:34-44.
7. Banat, I.M., Nigam, P., Singh, D., Marchant, R. (1996) Microbial decolorization of textile-dye-containing effluents: a review. *Bioresource Technol.* **58**:217-227.
8. Battersby, N.S. and Wilson, V. (1989) Survey of the anaerobic biodegradation potential of organic chemicals in digesting sludge. *Appl. Environ. Microbiol.* **55**:433-439.

9. Berchtold, S.R., van der Loop, S.L., Suidam, M.T., Maloney, S.W. (1995) Treatment of 2,4-dinitrotoluene using a two-stage system: fluidized-bed anaerobic granular activated carbon reactors and aerobic activated sludge reactors. *Water Environ. Res.* **67**:1081-1091.
10. Berg, van den L., Kennedy, K.J. (1981) Support materials for stationary fixed film reactors for high-rate methanogenic fermentation. *Biotechnol. Lett.* **3**:165-170.
11. Bhattacharya, S.K., Sluder, J.L. Jr., Uberoi, V. (1995) Effects of 4-nitrophenol on H_2 and CO_2 levels in anaerobic propionate systems. *Water Res.* **29**:1249-1255.
12. Bisaillon, J.G., Lepine, F., Beaudet, R., Sylvestre, M. (1993) Potential for carboxylation-dehydroxylation of phenolic compounds by a methanogenic consortium. *Can. J. Microbiol.* **39**:642-648.
13. Bitton, G. (1994) Wastewater microbiology. Wiley-Liss, New York.
14. Blum, D.J.W., Speece, R.E. (1991) A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. *Res. J. Water Pollut. Control Fed.* **63**:198-207.
15. Bollag, J.M., Sjoblad, R.D., Minard, R.D. (1977) Polymerization of phenolic intermediates of pesticides by a fungal enzyme. *Experientia* **33**:1564-1566.
16. Boyd, S.A., Shelton, D.R., Berry, D., Tiedje, J.M. (1983) Anaerobic biodegradation of phenolic compounds in digested sludge. *Appl. Environ. Microbiol.* **46**:50-54.
17. Brown, D., Hitz, H.R., Schefer, L. (1981) The assessment of the possible inhibitory effect of dyestuffs on aerobic wastewater bacteria; experience with a screening test. *Chemosphere* **10**:245-261.
18. Brown, D., Laboureur, P. (1983a) The degradation of dyestuffs: Part I, primary biodegradation under anaerobic conditions. *Chemosphere* **12**:397-404.
19. Brown, D., Laboureur, P. (1983b) The aerobic biodegradbility of primary aromatic amines. *Chemosphere* **12**:405-414.
20. Brown, D., Hamburger, B. (1987) The degradation of dyestuffs: Part III, investigations of their ultimate degradability. *Chemosphere* 16:1539-1553.
21. Bryant, C. and DeLuca, M. (1991) Purification and characterization of an oxygen-insensitive NAD(P)H nitroreductase from *Enterobacter cloacae*. *J. Biol. Chem.* **266**:4119-4125.
22. Carliell, C.M., Barclay, S.J., Naidoo, N., Buckley, C.A., Mulholland, D.A., Senior, E. (1995) Microbial decolourization of a reactive azo dye under anaerobic conditions. *Water SA* **21**:61-69.
23. Cheng, J., Kanjo, Y., Suidan, M., Venosa, A. (1996) Anaerobic biotransformation of 2,4-dinitrotoluene with ethanol as primary substrate: mutual effect of the substrates on their biotransformation. *Wat. Res.* **30**:307-314.
24. Chou, W.L., Speece, R.E., Siddiqi, R.H., McKeon, K. (1978) The effect of petrochemical structure on methane fermentation toxicity. *Prog. Wat. Tech.* **10**:545-558.
25. Chung, K.T., Stevens, S.E., Cerniglia, C.E. (1992) The reduction of azo dyes by the intestinal microflora. *Crit. Rev. Microbiol.* **18**:175-190.
26. Chung, K.T. and Stevens, S.E. (1993) Degradation of azo dyes by environmental microorganisms and helminths. *Environ. Toxicol. Chem.* **12**:2121-2132.
27. Davies-Venn, C., Young, J.C., Tabak, H.H. (1992) Impact of chloroanilines and chlorophenols on the kinetics of acetoclastic methanogenesis. *Environ. Sci. Technol.* **26**:1627-1635.
28. Dickel, O., Haug, W., Knackmuss, H.J. (1993) Biodegradation of nitrobenzene by a sequential anaerobic-aerobic process. *Biodegradation* **4**:187-194.
29. Evans, W.C., Fuchs, G. (1988) Anaerobic degradation of aromatic compounds. *Ann. Rev. Microbiol.* **42**:289-317.
30. Fedorak, P.M., Kindzierski, W.B., Hrudey, S.E. (1990) Effects of anilines and hydantoins on the methanogenic degradation of selected phenols. *Wat. Res.* **24**:921-925.
31. Field, J.A., Kortekaas, S., Lettinga, G. (1989) The tannin theory of methanogenic toxicity. *Biol. Waste* **29**:241-262.

32. Field, J.A., Kortekaas, S., Lettinga, G. (1989) The effect of autooxidation on the methanogenic toxicity and anaerobic biodegradability of pyrogallol. *Biol. Waste* **29**:125-132.
33. Field, J.A., Stams, A.J.M., Kato, M., Schraa, G. (1995) Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consortia. *Antoine van Leeuwenhoek* **67**:47-77.
34. Fitzgerald, S.W., Bishop, P.L. (1995) Two stage anaerobic/aerobic treatment of sulfonated azo dyes. *J. Environ. Sci. Health.* **A30**:1251-1276.
35. Fuchs, G., Mohamed, M.E.S., Altenschmidt, U., Koch, J., Lack, A., Brackmann, R., Lochmeyer, C., Oswald, B. (1994) Biochemistry of anaerobic biodegradation of aromatic compounds. In: Ratledge, C. (Ed.) Biochemistry of microbial degradation, pp 513-553. Kluwer, Dordrecht.
36. Funk, S.B., Roberts, D.J., Crawford, D.L., Crawford, R.L. (1993) Initial-phase optimization for bioremediation of munition compound-contaminated soils. *Appl. Environ. Microbiol.* **59**:2171-2177.
37. Gibson, T.L. (1982) Nitro derivatives of polynuclear aromatic hydrocarbons in airborne and source particular matter. *Atmospheric Environ.* **16**:2037-2040.
38. Gilcrease, P.C., Murphy, V.C. (1995) Bioconversion of 2,4-diamino-6-nitrotoluene to a novel metabolite under anoxic and aerobic conditions. *Appl. Environ. Microbiol.* **61**:4209-4214.
39. Golden, T., Molyneaux, E., Colleran, E. (1994). Toxicity of aromatic chemicals to the bacterial populations involved in anaerobic digestion. In: Proceedings of the 7th International Symposium on Anaerobic Digestion, Poster section pp 19-22. Cape Town, South Africa.
40. Gorontzy, T., Kuver, J., Blotevogel, K.H. (1993) Microbial transformation of nitroaromatic compounds under anaerobic conditions. *J. Gen. Microbiol.* **139**:1331-1336.
41. Gorontzy, T., Drzyga, O., Kahl, M. W., Bruns-Nagel, D., Breitung, J., von Loew, E., Blotevogel, K. H. (1994) Microbial degradation of explosives and related compounds. *Crit. Rev. Microbiol.* **20**:265-284.
42. Gujer, W., Zehnder, A.J.B. (1983) Conversion processes in anaerobic digestion. *Wat. Sci. Technol.* **15**:127-167.
43. Haandel, van A.C., Lettinga, G. (1994) Anaerobic sewage treatment; a practical guide for regions with a hot climate. John Wiley & Sons, Chichester.
44. Haghighi Podeh, M.R., Bhattacharya, S.K., Qu, M. (1995) Effects of nitrophenols on acetate utilizing methanogenic systems. *Wat. Res.* **29**:391-399.
45. Hallas, L.E., Alexander, M. (1983) Microbial transformation of nitroaromatic compounds in sewage effluent. *Appl. Environ. Microbiol.* **45**:1234-1241.
46. Hartter, D.R. (1985) The use and importance of nitroaromatic chemicals in the chemical industry. In: Rickert, D.E. (Ed.). Toxicity of nitroaromatic compounds, pp 1-13. Hemisphere Publishing Corp., Washington.
47. Haug, W., Schmidt, A., Nortermann, B., Hempel, D.C., Stolz, A., Knackmuss, H.J. (1991) Mineralization of the sulfonated azo dye Mordant-Yellow 3 by a 6-aminonaphthalene-2-sulfonate degrading bacterial consortium. *Appl. Environ. Microbiol.* **57**:3144-3149.
48. He, Y., Bishop, P.L. (1994) Effect of Acid Orange 7 on nitrification process. *J. Env. Eng.* **120**:108-121.
49. Heider, J., Fuchs, G. (1997) Microbial anaerobic aromatic metabolism. *Anaerobe* **3**:1-22.
50. Hendriksen, H.V., Larsen, S., Ahring, B. (1992) Influence of a supplemental carbon source on anaerobic dechlorination of pentachlorophenol in granular sludge. *Appl. Environ. Microbiol.* **58**:365-370.
51. Holliger, C., Zehnder, A. (1996) Anaerobic biodegradation of hydrocarbons. *Current Opinion in Biotechnology*. 7:326-330.
52. Horning, R.H. (1976) Characterization and treatment of textile dyeing wastewaters. In: Proceedings National Technical Conference, American Association of Textile Chemists and Colorists, pp 100-104. Research Triangle Park, NC.

53. Horowitz,A., Shelton, D.R., Cornell, C.P., Ticdje, J,M, (1981) Anaerobic degradation of aromatic compounds in sediments and digested sludge. *Dev. Ind. Microbiol.* **23**:435-444.
54. Hulshoff Pol, L.W., de Zeeuw, W.J., Velzeboer, C.T.M., Lettinga, G. (1983) Granulation in UASB reactors. *Wat. Sci. Tech.* **15**:291-304.
55. Hulshoff Pol, L.W., Lettinga, G. (1986) New technologies for anaerobic wastewater treatment. *Wat. Sci. Tech.* **18**:41-53.
56. Hunter, J.V. (1973) The effect of dyes on aerobic systems. In: Dyes and the environment, Vol. 1, Chap. 6. American Dye Manufacturers Institute, New York, NY.
57. Idaka, E., Ogawa, T., Yatome, C., Horitsu, H. (1985) Behavior of activated sludge with dyes. *Bull. Environ. Contam. Toxicol.* **35**:729-734.
58. Johnson, L.D., Young, J.C. (1983) Inhibition of anaerobic digestion by organic priority pollutants. *J. Water Pollut. Control Fed.* **55**:1441-1449.
59. Kato, M. (1994) The anaerobic treatment of low strength soluble wastewaters. Ph.D. thesis, Wageningen Agricultural University. Wageningen, The Netherlands.
60. Kim, I.S., Young, J.C., Tabak, H.H. (1994) Kinetics of acetogenesis and methanogenesis in anaerobic reactions under toxic conditions. *Water. Environ. Res.* **66**:119-132.
61. Kim, I.S., Young, J.C., Tabak, H.H. (1996) Impact of chloroanilines on hydrogenotrophic methanogenesis in ethanol-enriched cultures. *Wat. Res.* **30**:601-612.
62. Kinoushi, T. and Onishi, Y. (1983) Purification and characterization of 1-nitropyrene nitroreductases from *Bacterioides fragilis*. *Appl. Environ. Microbiol.* **46**:596-604.
63. Klivanov, A.M., Alberti, B.N., Morris, E.D., Felshin, L.M. (1980) Enzymatic removal of toxic phenols and anilines from wastewaters. *J. Appl. Biochem.* **2**:414-421.
64. Knackmuss, H.-J. (1992) Potentials and limitations of microbes to degrade xenobiotics. In: DECHEMA preprints soil decontamination using biological processes, pp 3-9. Karlsruhe, Germany.
65. Knackmuss, H.-J. (1996) Basic knowledge and perspectives of bioclimination of xenobiotic compounds. *Journal of Biotechnology* **51**:287-295.
66. Kriek, E. (1979) Aromatic amines and related compounds as carcinogenic hazards to man. In: Emmelot, P., Kriek, E. (Eds.). Environmental carcinogenesis, pp 143-164. Elsevier, Amsterdam.
67. Kudlich, M., Bishop, P.L., Knackmuss, H.-J., Stolz, A. (1996) Simultaneous anaerobic and aerobic degradation of the sulfonated azo dye Mordant Yellow 3 by immobilized cells from a naphthalenesulfonate-degrading mixed culture. *Appl. Microbiol. Biotechnol.* **46**:597-603.
68. Kuhn, E.P., Suflita, J.M. (1989) Anaerobic biodegradation of nitrogen-substituted and sulfonated benzene aquifer contaminants. *Haz. Was. Haz. Mat.* **2**:121-133.
69. Kulla, H.G. (1981) Aerobic bacterial degradation of azo dyes. In: Leisinger, T., Hutter, R., Cook, A.M., Nuesch, J. (Eds.) Microbial degradation of xenobiotic compounds, pp 387-399. Academic Press, London.
70. Larson, R.A., Weber, E.J. (1994) Reaction mechanisms in environmental organic chemistry. Lewis Publishers, Boca Raton.
71. Leisinger, T., Brunner, W. (1986) Poorly degradable sustances. In: Rehm, H.J., Reed, G. (Eds.). Biotechnology, microbial degradation, Vol. 8, pp 497-498. Verlagsgesellschaft, Weinheim, Germany.
72. Lettinga, G. (1995) Anaerobic digestion and wastewater treatment systems. *Antoine van Leeuwenhoek* **67**:3-28.
73. Lettinga, G. (1996) Sustainable integrated biological wastewater treatment. *Wat. Sci. Tech.* **33(3)**:85-98.
74. Lettinga, G., Field, J., Sierra, R., van Lier, J.B., Rintala, J. (1991) Future perspectives for the anaerobic treatment of forestry industry wastewaters. *Wat. Sci. Tech.* **24**:91-102.
75. Lettinga, G., Hulshoff Pol, L.W., Koster, I.W., Wiegant, W.M., de Zeeuw, W.J., Rinzema, A., Grin, P.C. Roersma, R.E., Hobma, S.W. (1984) High-rate anaerobic waste-water treatment using

the UASB reactor under a wide range of temperature conditions. *Biotechnol. Gen. Eng. Rev.* **2**:253-283.

76. Lettinga, G., van Velsen, A.F.M., Hobma, S.W., de Zeeuw, W. Klapwijk, A. (1980) Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. *Biotechnol. Bioeng.* **22**:699-734.
77. Lettinga, G., Vinken, J.N. (1980) Feasibility of the upflow anaerobic sludge blanket (UASB) process for the treatment of low strength wastes. In: Proceedings of the 35th Industrial Waste Conference. Purdue University, West Lafayette, USA.
78. Lochmeyer, C., Koch, J., Fuchs, G. (1992) Anaerobic degradation of 2-aminobenzoic acid (anthranilic acid) via benzoyl-coenzyme A (CoA) and cyclohex-1-enecarboxyl-CoA in a denitrifying bacterium. *J. Bacteriol.* **174**:3621-3628.
79. Loop van der, S.L., Suidan, M.T., Moteleb, M.A., Maloney, S.W. (1995) Two-stage biotransformation of 2,4,6-trinitrotoluene. In: Hinchee, R.E., Anderson, D.B., Hoeppel, R.E. (Eds.). Bioremediation of recalcitrant organics, Bioremediation 3(7), pp 225-230. Battelle Press, Columbus.
80. Macarie, H. (1996) Tratamiento de las aguas residuales de algunas industrias químicas y petroquímicas, un mercado potencial para la digestión anaerobia. In: Kuppusamy, I., Briones, R. (Eds.) Biodegradación de Compuestos Orgánicos Industriales, pp 155-168. Instituto de Ingeniería, UNAM, México D.F. (in Spanish).
81. Maynard, C.W., Jr. (1983) Dye application, manufacture of dye intermediates and dyes. In: Kent, J.A. (Ed.). Riegel's handbook of industrial chemistry, pp 809-861. Van Nostrand Reinhold, New York.
82. Marvin-Sikkema, F. D., de Bont, J. A. M. (1994) Degradation of nitroaromatic compounds by microorganisms. *Appl. Microbiol. Biotechnol.* **42**:499-507.
83. McCormick, N.G., Feeherry, F.E., Levinson, H.H. (1976) Microbial transformation of 2,4,6-trinotrotoluene and other nitroaromatic compounds. *Appl. Environ. Microbiol.* **31**:949-958.
84. McCarty, P.L., Brouseu (1962) Volatile acid digestion at high loading rates. *Inter. J. Air Water Pollut.* **6**:65-70.
85. Meyer, U. (1981) Biodegradation of synthetic organic colorants. *FEMS Symp.* **12**:371-385.
86. Nandipati, R. (1991) Effects and fate of nitrophenols in combined anaerobic-aerobic treatment systems. M.Sc. Thesis, Civil and Environmental Engineering Department, Tulane University, New Orleans, USA.
87. Noguera, D.R., Freedman, D.L. (1996) reduction and acetylation of 2,4-dinitrotoluene by a *Pseudomonas aeruginosa* strain. *Appl. Environ. Microbiol.* **62**:2257-2263.
88. Nortermann, B., Baumgarten, J., Rast, H.G., Knackmuss, H.J. (1986) Bacterial communities degrading amino- and hydroxynapthalene-2-sulfonates. *Appl. Environ. Microbiol.* **52**:1195-1202.
89. O'Connor, O.A., Young, L.Y. (1989) Toxicity and anaerobic biodegradability of substituted phenols under methanogenic conditions. *Environ. Toxicol. Chem.* **8**:853-862.
90. O'Connor, O.A., Young, L.Y. (1993) Effect of nitrogen limitation on the biodegradabilty and toxicity of nitro- and aminophenol isomers to methanogenesis. *Arch. Environ. Contam. Toxicol.* **25**:285-291.
91. O'Connor, O.A., Young, L.Y. (1996) Effects of six different functional groups and their position on the bacterial metabolism of monosubstituted phenols under anaerobic conditions. *Environ. Sci. Technol.* **30**:1419-1428.
92. Ogawa, T. Idaka, E., Hirabayashi, K., Takase, I. (1974) Biological treatments of acid azo dyes by microbial populations in activated sludges. *J. Soc. Fiber Sci. Technol. Jpn.* **30**:T153-159.
93. Ogawa, T., Yamada, Y., Idaka, E. (1978) The respiratory inhibition of activated sludge by dyes. *J. Soc. Fiber Sci. Technol. Jpn.* **34**:T175-180.

94. Oren, A., Gurevich, P., Henis, Y. (1991) Reduction of nitrosubstituted aromatic compounds by the halophilic anaerobic eubacteria *Haloanaerobium praevalens* and *Sporohalobacter marismortui*. *Appl. Environ. Microbiol.* **57**:3367-3370.
95. Pagga, U. Brown, D. (1986) The degradation of dyestuffs. Part II. Behaviour of dyestuffs in aerobic biodegradation test. *Chemosphere* **15**:479-491.
96. Rafii, F., Franklin, W. Heflich, R.H. and Cerniglia, C.E. (1991) reduction of nitroaromatic compounds by anaerobic bacteria isolated from the human gastrointestinal tract. *Appl. Environ. Microbiol.* **57**:962-968.
97. Rieger, P-G., Knackmuss, H-J. (1995) Basic knowledge and perspectives on biodegradation of 2,4,6-trinitrotoluene and related nitroaromatic compounds. In: Spain, J. (Ed.), Biodegradation of nitroaromatic compounds, pp. 1-18. Plenum, New York.
98. Rittmann, B.E., Jackson, D.E., Storck, S.L. (1988) Potential for treatment of hazardous organic chemicals with biological processes. In: Wise, D.L. (Ed.) Biotreatment Systems, Vol. 3, pp 15-64. CRC Press, Boca Raton.
99. Ryan, A.J., Roxon, J.J., Sivayavirojana, A. (1968) Bacterial azo reduction: a metabolic reaction in mammals. *Nature* **219**:854-855.
100. Schink, B. (1988) Fermentative degradation of nitrogenous aliphatic and aromatic compounds. In: Hall, E.R., Hobson, P.N. (Eds.). 5th International Symposium on Anaerobic Digestion, pp 459-464. Pergamon Press, Oxford.
101. Schmidt, J.E., Ahring, B.K. (1996) Granular sludge formation in upflow anaerobic sludge blanket reactors. *Biotechnol. Bioeng.* **49**:229-246.
102. Schnell, S., Schink, B. (1991) Anaerobic aniline degradation via reductive deamination of 4-aminobenzoyl-CoA in *Desulfobacterium anilini*. *Arch. Microbiol.* **155**:183-190.
103. Schnell, S., Schink, B. (1992) Anaerobic degradation of 3-aminobenzoate by a newly isolated sulfate reducer and a methanogenic enrichment culture. *Arch. Microbiol.* **158**:328-334.
104. Seshadri, S. Bishop, P.L. (1994) Anaerobic/aerobic treatment of selected azo dyes in wastewater. *Waste Management* **14**:127-137.
105. Shaul, G.M., Holdsworth, T.J., Dempsey, C.R., Kostal, K.A. (1991) Fate of water soluble azo dyes in the activated sludge process. *Chemosphere* **22**:107-119.
106. Shelton, D.R., Tiedje, J.M. (1984) General method for determining anaerobic biodegradation potential. *Appl. Environ. Microbiol.* **47**:850-857.
107. Sierra, R., Lettinga, G. (1991) The effect of aromatic structure on the inhibition of acetoclastic methanogens in granular sludge. *Appl. Microbiol. Biotechnol.* **34**:544-550.
108. Smith, M.R. (1994) The physiology of aromatic hydrocarbon degrading bacteria. Biochemistry of anaerobic biodegradation of aromatic compounds. In; Ratledge, C. (Ed.) Biochemistry of microbial degradation, pp 347-378. Kluwer, Dordrecht.
109. Spain, J. (1995) Biodegradation of nitroaromatic compounds. *Annu. Rev. Microbiol.* **49**:523-555.
110. Spain, J. (1995) Bacterial degradation of nitroaromatic compounds under aerobic conditions. In: Spain, J. (Ed.) Biodegradation of nitroaromatic compounds, pp 19-36. Plenum Press, New York.
111. Speece, R.E. (1983) Anaerobic biotechnology for industrial wastewater. *Environ. Sci. Technol.* **17**:416-427.
112. Stevens, T.O., Crawford, R.L., Crawford, D.L. (1991) Selection and isolation of bacteria capable of degrading dinoseb (2-sec-butyil-4,6-dinitrophenol). *Biodegradation* **2**:1-13.
113. Swoboda-Colberg, N.G. (1995) Chemical contamination of the environment: sources, types, and fate of synthetic organic chemicals. In: Young, L.Y., Cerniglia, C.E. (Eds.) Microbial transformation and degradation of toxic organic chemicals. Wiley-Liss, New York.
114. Thaveesri, J. Liessens, B., Verstraete, W., Longry, R. (1990) Granular sludge growth under different reactor liquid surface tensions in lab-scale upflow anaerobic sludge blanket reactors treating wastewater from sugar-beet processing. *Appl. Microbiol. Biotechnol.* **43**:1122-1127.

115.Tereda, H. (1981) The interaction of highly active uncouplers with mitochondria. *Biochim. Biophys. Acta* **639**:225-242.

116.Tseng, S.K., Yang, C.J. (1994) The reaction characteristics of wastewater containing nitrophenol, treated using an anaerobic biological fluidized bed. *Wat. Sci. Technol.* **30**:233-240.

117.Tseng, S.K., Wu, Y.F. (1994) The toxicity and biodegradation of nitrophenol in anaerobic digestion. In: Proceedings 7th IUAPPA Regional Conference on Air Pollution and waste Issues, pp 99-109, Vol. IV. Nov. 2-4, Taipei, ROC.

118.Turner, W.B., Aldridge, D.C. (1983) Fungal metabolites II. Academic Press, London.

119.Uberoi, V., Bhattacharya, S.K. (1997) Toxicity and degradability of nitrophenols in anaerobic systems. *Water Environ. Res.* **69**:146-156.

120.Vanulet, J., van Etten, R.L. (1981) Biochemistry and pharmacology of the nitro and nitroso groups. In: Feuer, H. (Ed.) The chemistry of the nitro and nitroso groups. Krieger Publishing Company, New York.

121.Wambeke, van M., Grusenmeyer, S., Verstraete, W., Longry, R. (1990) Sludge bed growth in an UASB reactor treating potato processing wastewater. *Process Biochem.* **25**:181-186.

122.Wilson, L.P., Bouwer, E.J. (1997) Biodegradation of aromatic compounds under mixed oxygen/denitrifyng conditions: a review. *J. Ind. Microbiol. Biotechnol.* **18**:116-130.

123.Won, W.D., di Salvo, L.H., Ng, L. (1976) Toxicity and mutagenicity of 2,4,6-trinitrotoluene and its microbial metabolites. *Appl. Environ. Microbiol.* **31**:576-580.

124.Wu, W.M., Bhatanagar, L., Zeikus, J.G. (1993) Performance of anaerobic granules for degradation of pentachlorophenol. *Appl. Environ. Microbiol.* **59**:389-397.

125.Zimmermann, T., Gasser, F., Kula, H.G. and Leisinger, T. (1984) Comparison of two bacterial azoreductases acquired during adaptation to growth on azo dyes. *Arch. Microbiol.* **138**:37-43.

126.Zissi, U., Lyberatos, G. (1996) Azo dye biodegradation under anoxic conditions. *Wat. Sci. Tech.* **34(5-6)**:495-500.

127.Zitomer, D.H., Speece, R.E. (1993) Sequential environments for enhanced biotransformations of aqueous contaminants. *Environ. Sci. Technol.* **27**:227-244.

128.Zollinger, H. (1987) Color chemistry-syntheses, properties and applications of organic dyes and pigments, pp 92-102, VCH, New York.

2

Toxicity of N-Substituted Aromatics to Acetoclastic Methanogenic Activity in Granular Sludge

Brian A. Donlon
Elías Razo Flores
Jim A. Field
Gatze Lettinga

Summary

N-substituted aromatics are important priority pollutants entering the environment primarily through anthropogenic activities associated with the industrial production of dyes, explosives, pesticides and pharmaceuticals. Anaerobic treatment of wastewaters discharged by these industries could potentially be problematical as a result of the high toxicity of N-substituted aromatics. The objective of this study was to examine the structure-toxicity relationships of N-substituted aromatic compounds to acetoclastic methanogenic bacteria. The toxicity was assayed in serum flasks by measuring methane production rate in granular sludge. Unacclimated cultures were used to minimize the biotransformation of the toxic organic chemicals during the test. The nature and the degree of the aromatic substitution were observed to have a profound effect on the toxicity of the test compound. Nitroaromatic compounds were, on the average over 500-fold more toxic than their corresponding aromatic amines. Considering the facile reduction of nitro groups by anaerobic microorganisms, a dramatic detoxification of nitroaromatics towards methanogens can be expected to occur during anaerobic wastewater treatment. While the toxicity exerted by the N-substituted aromatic compounds was closely correlated with compound apolarity (log P), it was observed that at any given log P, N-substituted phenols had a toxicity that was 2 orders of magnitude higher than that of chlorophenols and alkylphenols. This indicates that toxicity due to the chemical reactivity of nitroaromatics is much more important than partitioning effects in bacterial membranes.

Applied and Environmental Microbiology (1995) 61:3889-3893

2.1 Introduction

N-substituted aromatic compounds, such as nitrobenzenes, nitrophenols, aminophenols and aromatic amines, are widely used in the manufacturing of azo dyes, explosives, pharmaceuticals and pesticides (8, 15). Nitrobenzene is produced annually in the order of 225,000 metric tons and it has been estimated that as much as 9,000 tons of nitrobenzene is discharged annually into natural waters (35). The presence of these aromatic xenobiotics in the environment may create serious public health and environmental problems. Some of these compounds have mutagenic or carcinogenic activity and may bioaccumulate in the food chain (8, 20). Many nitroaromatics have also been shown to be toxic or mutagenic to microorganisms (32, 39). The toxicity has been attributed to the fact that nitrophenols act as uncoupling agents in oxidative phosphorylation. Cell metabolism is affected at concentrations less than 50 mM (32). Aerobic biodegradation of a variety of N-substituted aromatics has been well documented (13, 23, 31) whereas the anaerobic biodegradation and toxicity of these compounds has only recently been addressed (12, 26, 34). Some researchers have reported on their toxicity as part of an overall study surveying the effects of xenobiotic compounds on anaerobic sludge (3, 10). However, the protocols employed in the previous experiments were not fully adequate for N-substituted aromatics (3, 10, 19, 25, 34). In many of the assay procedures, the nitroaromatic test compounds were highly modified by reduction due to inappropriate selection of assay substrates (19, 25). Most authors used anaerobic media containing chemical reducing agents (sulfides) which have been shown to transform several nitroaromatic compounds (11); consequently, the bacteria were exposed only momentarily to the toxic compounds. In some of the previous protocols (25, 26, 34), toxicity was based not on methanogenic production rate but rather on comparison of the methane production of compound-amended cultures with that of controls within a given time period. Such comparisons may underestimate the true toxicity if incubation continues after exhaustion of the assay substrate in the control cultures.

The rationale behind the toxicity assay employed in this study was to minimize test compound biotransformation and to compare the rate of methane production in highly active methanogenic granular sludge. Acetate was used as the assay substrate since it is known to be

a poor electron donor (9) and, thus, would result in minimal nitroaromatic modification. This is the first comprehensive study evaluating a wide range of N-substituted aromatics in a standardized batch toxicity assay. Such knowledge is essential in predicting the impact of these xenobiotics on anaerobic wastewater treatment; thereby, preventing potentially costly upsets of treatment plant operations. A better understanding of the toxicity has made feasible the application of anaerobic treatment technologies to wastewaters containing other aromatic compounds (7, 12, 24, 40).

2.2 Materials and Methods

Biomass

Methanogenic granular sludge from a full-scale upward-flow anaerobic sludge blanket (UASB) reactor treating chemical industry wastewater of Shell Nederland Chemie at Moerdijk, The Netherlands, was used as inoculum. The sludge was elutriated to remove fines and stored at 4 °C before use. The sludge had not previously been acclimated to any of the N-substituted aromatics.

Basal Medium

The basal medium used in the anaerobic toxicity assay contained the following (mg/L): $NaHCO_3$ (5000), NH_4Cl (280), $CaCl_2.2H_2O$ (10), K_2HPO_4 (250), $MgSO_4.7H_2O$ (100), yeast extract (100), H_3BO_3 (0.05), $FeCl_2.4H_2O$ (2), $ZnCl_2$ (0.05), $MnCl_2.4H_2O$ (0.05), $CuCl_2.2H_2O$ (0.03), $(NH_4)_2SeO_3.5H_2O$ (0.05), $AlCl_3.6H_2O$ (2), $NiCl_2.6H_20$ (0.05), $Na_2SeO_3.5H_2O$ (0.1), EDTA (1), resazurin (0.2) and 36% HCl (0.001 mL/L).

Analyses

The methane content in the gas samples was determined by gas chromatography (Packard-Becker, Delft, the Netherlands). The gas chromatograph was equipped with a steel

column (2 m by 2 mm) packed with Poropak Q (80/100 mesh, Millipore Corp., Bedford, Mass.). The temperatures of the column, the injector port and the flame ionization detector were 60, 200 and 220 °C, respectively. The carrier gas was nitrogen at a flow rate of 20 mL/min. Samples for measuring methane content (100 μL) in the headspace were determined with a pressure-gas lock syringe (Pressure-Lok series A-2) which was purchased from Dynatech Precision Sampling Corp., Baton Rouge, LA. An isobaric precise proportion of the known headspace volume could be analyzed. The pH was determined immediately after sampling with a model 511 pH-meter (Knick, Berlin, Germany) and a model N61 double electrode (Scot Gerade, Hofheim, Germany). The UV absorbance was measured with a Spectronic 60 spectrophotometer (Milton Roy/Analytical Products Division, Ostende, Belgium) and a model 100-QS (Hellma Benelux, The Hague, The Netherlands) 1-cm quartz cuvette. Absorption is reported as the absorption of the media containing aromatic compounds minus the absorption of the control medium (which contained no test compounds). All samples were diluted to less than 0.8 absorbance units in 0.2M phosphate buffer (pH 7.0). Nitro group reduction of 2-nitrophenol and 4-nitrophenol was monitored at 370 and 400 nm, respectively. Aromatic ring absorption of 2-nitrophenol/2-aminophenol and 4-nitrophenol/4-aminophenol was monitored at 209 and 225 nm, respectively. All the other analytical determinations were performed as described in *Standard Methods for Examination of Water and Wastewater* (1).

Anaerobic Toxicity Assay

Specific acetoclastic methanogenic activity measurements were performed with 120-mL glass assay bottles sealed with 12-mm thick butyl rubber septa (Rubber B.V., Hilversum, The Netherlands). Granular sludge (2 g of volatile suspended solids per liter) was transferred to vials containing 25 ml of the basal medium and acetate from a neutralized stock solution to yield a final concentration of 39.3 mM (2.5 g of chemical oxygen demand per liter). The maximum specific acetoclastic methanogenic activity of the control sludge was 890 mg methane expressed as chemical oxygen demand per gram of volatile suspend solids per day. Assay bottles were then flushed with 70% N_2-30% CO_2 gas for 5 minutes and incubated overnight at 30 °C. On the following day, vials which were still pink because of the lack of reduction of the redox indicator dye resazurin were discarded. The desired amount of the

toxicant was added to duplicate vials using concentrated stock solutions. However, in the case of poorly soluble compounds, the compound was weighed out and introduced into the vials in solid form. Acidic test compounds were neutralized prior to their addition to the assay medium. Some aromatic amines (e.g., aminophenols), which are prone to autoxidation (7), were prepared fresh with 250 mg/L ascorbic acid to prevent oxidative coupling. Triplicate substrate controls were based on assays where no toxicant was added. Incubations were done in a temperature controlled room at 30 ± 2 °C, in an orbital-motion shaker (Gerhardt, Bonn, Germany) at 70 strokes min^{-1}. After 3 days exposure to the toxicant the acetate concentration was replenished to 15.72 mM (1 g of chemical oxygen demand per liter) to assess the specific methanogenic activity. The headspace was reflushed with 70% N_2-30% CO_2 gas and the assay bottles were reincubated for 1 hr, prior to the determination of the methane production rate. The methane content in the headspace of each assay bottle was determined hourly during the subsequent 6 to 8-h incubation period. The maximum specific methanogenic activity was calculated from the slope of the methane production versus time curve. To determine the degree of inhibition, the methanogenic activities of the control and samples containing inhibitory compounds were determined.

Chemicals

Chemicals were purchased from either Jannsen Chimica (Tilburg, The Netherlands), Merck (Darmstadt, Germany) and Sigma (Bornem, Belgium). All chemicals were of the highest purity available and were not purified further.

2.3 Results

Biotransformation of Test Compounds During Protocol

The nitro-group absorbance maxima of nitrophenols were monitored during the 3-day exposure period of these test compounds with the anaerobic sludge. A small level of nitro-group reduction did occur during the exposure period accounting for 16 and 22% losses of the

nitro-group absorbance maxima of 2-nitrophenol and 4-nitrophenol, respectively. The use of ascorbic acid and the preincubation for the biological removal of dissolved oxygen were found to be sufficient measures in preventing the oxidative coupling of aromatic amines. No formation of visible light absorbance could be detected.

Effect of Aromatic Structure on Methanogenic Inhibition

The inhibitory effects of 29 aromatic compounds on the activity of acetoclastic methanogenic bacteria were evaluated in this study. The inhibition caused by each compound was tested at various levels, from concentrations that were nontoxic to those that were completely inhibitory to acetoclastic methanogenic activity, as seen in a typical experiment with 3-nitrophenol in Figure 2.1. Table 2.1 summarizes the 20%, 50%, and 80% inhibiting concentrations (ICs) of the aromatic compounds evaluated in this study. Those compounds which were not inhibitory at concentrations of 70 mM or less were considered to be nontoxic.

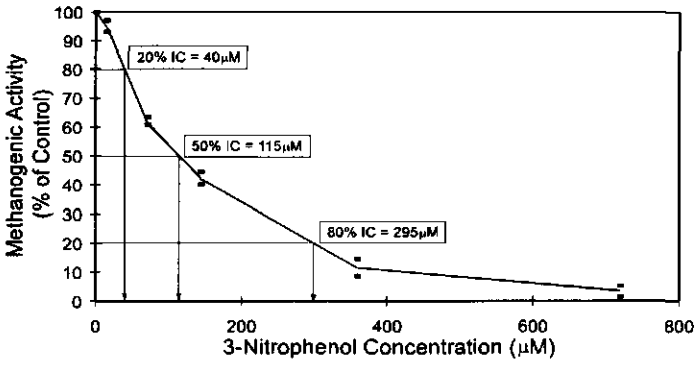


Figure 2.1 Estimation of 20%, 50%, 80% IC values of 3-nitrophenol to acetoclastic methanogens.

The least toxic compounds were benzene, benzoate, phenol and the aromatic amines. Nitrobenzenes, nitrophenols and nitroanilines were among the most toxic compounds. The most toxic compound tested was 2-nitroaniline, having a 50% IC of 14 μM. The results obtained indicate that some general relationships exist between the aromatic structure and their inhibitory effects on methanogenic bacteria. The impact of ring substitution is illustrated in

Figure 2.2. The figure indicates that N-substitutions were more toxic than other ring substituents or benzene itself. The toxicity of the mono-substituted benzenes was observed to increase in the following order: $COOH < H < OH < NH_2 < NO_2$.

TABLE 2.1 The 20%, 50% and 80% IC values observed in this study for various aromatics.

Compound No. and Name	MW	Log P[a]	IC (μM)		
			20%	50%	80%
1. benzene	78	1.95	10580	20500	ND[b]
2. aniline	93	0.9	5000	9670	14100
3. nitrobenzene	123	1.85	41	81	210
4. 2-nitroaniline	138	1.83	7	14	70
5. 3-nitroaniline	138	1.37	7	30	212
6. 2-phenylenediamine	108	0.15	9760	18920	27500
7. 3-phenylenediamine	108	0.03	29500	65700	NT[c]
8. phenol	94	1.46	7140	13830	20510
9. 2-aminophenol	109	0.57	1650	3210	4920
10. 3-aminophenol	109	0.16	9700	18810	27100
11. 4-aminophenol	109	0.104	7330	14220	21100
12. 2,4-diaminophenol	124	NA[d]	146	283	510
13. 2-nitrophenol	139	1.79	46	89	200
14. 3-nitrophenol	139	2.0	40	115	295
15. 4-nitrophenol	139	1.91	31	61	180
16. 2,4-dinitrophenol	184	1.67	22	43	130
17. 2,5-dinitrophenol	184	1.80	6	114	270
18. benzoic acid	122	1.87	34900	NT	NT
19. 2-aminobenzoic acid	137	1.21[e]	31100	67100	NT
20. 3-aminobenzoic acid	137	0.20[e]	NT	NT	NT
21. 4-aminobenzoic acid	137	0.68[e]	NT	NT	NT
22. 2-nitrobenzoic acid	167	1.28[e]	277	538	980
23. 3-nitrobenzoic acid	167	1.83[e]	50	96	140
24. 4-nitrobenzoic acid	167	1.89[e]	30	120	250
25. 2,4-dinitrobenzoic acid	212	NA	226	344	424
26. 5-aminosalicylic acid	153	NA	ND	2800	ND
27. 5-nitrosalicylic acid	183	NA	251	322	377
28. 2,4-diaminotoluene	122	NA	409	1570	ND
29. 2,4-dinitrotoluene	182	2.0	5	27	59

[a] log P values were obtained from the literature (18, 26, 33).
[b] ND; not determined.
[c] NT; not toxic: compounds were considered to be non-toxic if this value was greater than 70 mM.
[d] NA; not available: log P value was not available.
[e] not included in log P correlations because the carboxyl group dissociates at the assay pH of 7.

The type of N-substitution had a profound effect on its toxicity. Figure 2.2 clearly demonstrates that aromatic amines were much less inhibitory than their corresponding nitroaromatic analogues. The nitroaromatics were from 6- to 1350 fold more toxic than their amino-substituted counterparts. Structure-toxicity relationships were also evident for aromatic compounds with more complex substitution patterns. The addition of an ortho carboxyl group to nitrobenzene greatly decreased the toxicity. In strong contrast, the addition of an amino group (i.e., 2-nitroaniline) increased the toxicity towards methanogens.

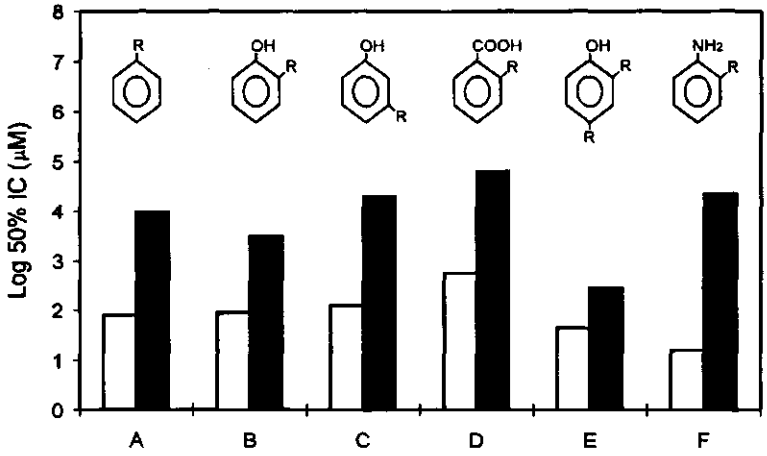


Figure 2.2 Comparison of the toxicity of selected nitroaromatic compounds and their aromatic amine counterparts towards acetoclastic methanogens. Bars: A = nitrobenzene and aniline, B = 2-nitrophenol and 2-aminophenol, C = 3-nitrophenol and 3-aminophenol, D = 2-nitrobenzoic acid and 2-aminobenzoic acid, E = 2,4-dinitrophenol and 2,4-diaminophenol, F = 2-nitroaniline and 2-phenylenediamine. Symbols: □, R = NO_2 group; ■, R = NH_2 group.

Correlation of Toxicity with Compound Hydrophobicity

To determine if the lipophilic character of the aromatics tested could be correlated with their methanogenic toxicity, the logarithm of the 50% IC values of seventeen N-substituted aromatics were plotted against the logarithm of the octanol-water partion coefficient (log P) of the compounds. A significant correlation was obtained ($R^2 = 0.91$, $p < 0.001$) indicating that the partitioning of apolar N-substituted aromatics into lipophilic membranes in bacteria may

have a role in the toxicity. However, certain functional groups might be expected to undergo chemical interactions with proteins, and consequently, enzymes could become inhibited as well. Therefore when comparing compounds that possess different types of substitutions, a perfect correlation with the log P of the compound cannot be expected. A higher correlation could potentially be obtained by comparing compounds in a homologous series.

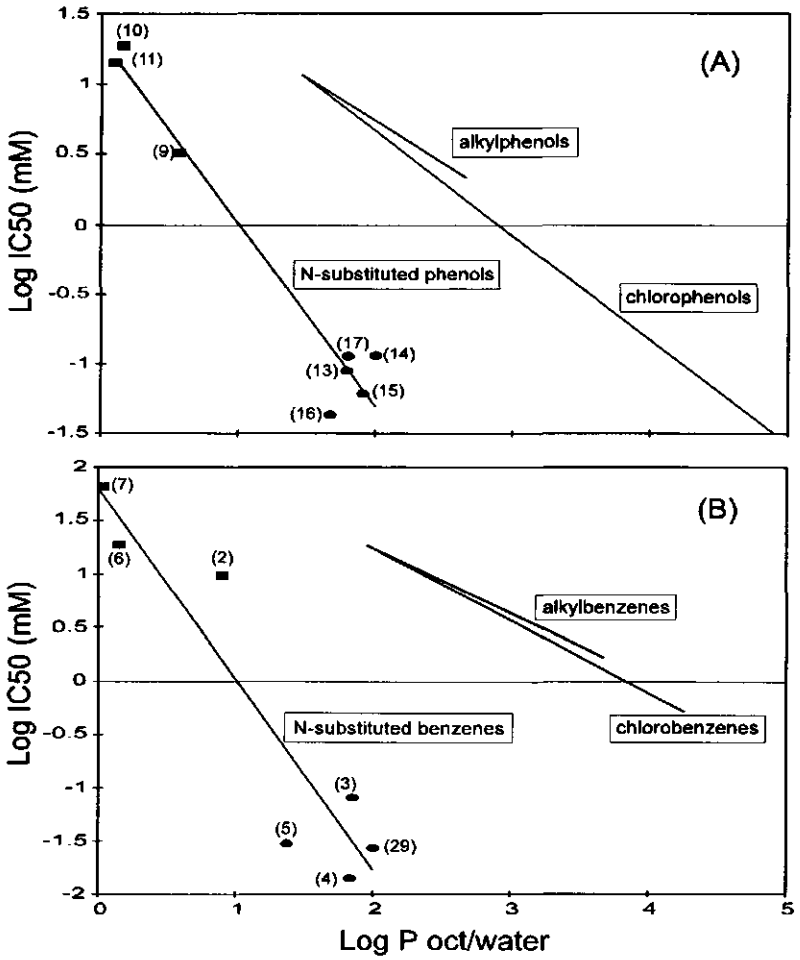


Figure 2.3 (A) Effect of hydrophobicity on the methanogenic toxicity of homologous series of phenols. (B) Effect of hydrophobicity on the methanogenic toxicity of homologous series of benzenes. Compounds are referred to by their compound numbers as reported in Table 1. Alkylphenols (R^2 = 0.989), chlorophenols (R^2 =0.99), alkylbenzenes (R^2 =0.983) and chlorobenzenes (R^2 =0.988) are adapted from a previous study (29).

Figure 2.3 illustrates the correlations determined with the toxicity data of the N-substituted phenols and benzenes, respectively. The methanogenic toxicity of the N-substituted phenols (aminophenols and nitrophenols) were even more highly correlated to the log P data (R^2 = 0.95, $p < 0.001$). However, the correlation with N-substituted benzenes (anilines, nitroanilines, nitrobenzene and dinitrotoluene) was not as high (R^2 = 0.876, $p < 0.01$) because the nitroanilines exerted a higher toxicity than nitrobenzene did with a comparable log P value. In Figure 2.3, regression lines are also plotted from the acetoclastic methanogenic toxicity data of alkyl- and chloro-substituted phenols and benzenes reported by Sierra and Lettinga (29). The measured 50% IC value of phenol and benzene from our study are plotted in the graphs and coincide with their data, indicating that the toxicity results from the two studies are compatible. At any given log P, the alkyl- and chloro-substituents of phenols and benzenes were approximately 2 orders of magnitude less toxic than the N-substituents analogs. This observation clearly indicates a higher chemical reactivity of aromatic nitro- and amino-groups compared with that of alkyl and chloro-groups.

2.4 Discussion

Preventing Test Compound Modification

Nitroaromatic compounds are easily reduced by microorganisms (6, 11, 14) and abiotic reducing agents (11, 16, 33). Even lysed cells of methanogenic bacteria reduce nitroaromatics (11). Consequently, several precautions were taken to minimize nitro-group reduction during the toxicity assay. The practice of adding reducing agents (e.g. sulfide) to chemically remove dissolved oxygen was replaced with a media preincubation step to biologically remove dissolved oxygen.

Anaerobic media were prepared with acetate as the substrate. Results of previous studies concerning with the reductive dehalogenation of chlorinated hydrocarbons indicate that acetate is a poor electron donor (9) compared with other substrates commonly used for methanogenic activity assays. In this study, we were also able to demonstrate that it was not

very effective in reducing nitroaromatics. The reductive biotransformation of nitroaromatics was limited to 25% during the 3-day test-chemical exposure period. This can be viewed as a major improvement over assay substrates that provide interspecies electrons (e.g., H_2), such as ethanol, which was shown to completely convert 500 mg/L of nitrobenzene into aniline in less than 1 day (19, 41). In experiments where ethanol or other good electron donors (e.g. propionate) have been used as an assay substrate, the 50% IC values of nitroaromatics were underestimated by approximately a factor of 10 (25, 41) because of the elimination of nitroaromatics occurring during the assay. Our nitroaromatic toxicity results are only in agreement with literature data in which acetate was used as the sole substrate (3, 5, 38).

Aromatic amines are more persistent to biotransformations in anaerobic environments (7). No losses in aromatic amines could be detected during the 3-day test compound exposure period. Nonetheless, after acclimatization of sludge for longer time periods, anaerobic mineralization of 2- and 4-aminophenol occurs (25, 26). In one study (3), aminophenols were 60-fold more toxic than indicated by our data. A possible explanation for this deviance is the ease by which aminophenols could become partially auto-oxidized during the preparation of the experiment, leading to the formation of more toxic oligomers (7). The auto-oxidation reactions were prevented in our study by preparing stock solutions together with ascorbic acid and adding the stock solution to the culture only after all dissolved oxygen was removed.

Toxicity of N-substituted Aromatics

Nitroaromatics were clearly very toxic compounds to methanogens, with 50% IC values generally ranging from 0.014 to 0.12 mM. Aromatic amines, in contrast, were less inhibitory; the 50% IC values were for the most part between 3.2 and 67 mM. Nitroaromatic compounds were, on the average, 500-fold more toxic than their corresponding aromatic amine analogues, indicating that the facile reduction of nitroaromatics known to occur in anaerobic environments (11, 26, 27, 41) would be responsible for a dramatic detoxification of nitroaromatics towards methanogens. Increasing the number of nitro groups beyond one had little effect in altering the toxicity of nitrobenzenes. On the other hand, the addition of an extra amino group to aminophenol resulted in a more toxic compound, while the addition of an

amino group to aniline resulted in less toxic phenylenediamines. However, the combination of nitro and amino groups, e.g. nitroanilines, was found to be the most toxic substituent pattern, with 50% IC values ranging from 0.014 to 0.030 mM.

Log P-Toxicity Correlations

The hydrophobicity of a compound as indicated by log P is directly related to the partitioning of the compound into bacterial membranes (17, 30). Compounds of greater hydrophobicity are expected to accumulate more efficiently in membranes, causing a greater disturbance to the membrane structure, and consequently, they are responsible for a higher toxicity. The accumulation of apolar pollutants in bacterial membranes causes the membrane to swell and leak, disrupting ion gradients and eventually causing cell lysis (17, 30). Methanogens rely almost entirely on membrane potential (H^+ and Na^+ gradients) to obtain energy during their metabolism (37). When the methanogenic toxicity data of N-aromatics were plotted as a function of the log P, a strong linear fit was obtained, indicating that partitioning into membranes was an important factor contributing to the toxicity of the most toxic N-substituted aromatics. In this study, the compounds ranged from highly polar aromatic amines of low toxicity to apolar nitroaromatics of high toxicity. The decrease in compound toxicity due to the presence of a carboxy group can also be rationalized in terms of compound polarity, since this group (pK_a = 2.16 to 6.94) would be highly dissociated at the assay pH of 7. Consequently, in accordance with a previous report (29), these compounds were not included in the overall correlation.

Many other types of aromatic and phenolic compounds have been reported to be inhibitory to methanogenic bacteria (3, 10, 29). High linear correlations of their methanogenic toxicity to the log P of the compounds has also been observed in the case of alkyl- and chloro-substituted benzenes and phenols (29). However these relatively nonreactive compounds are approximately 100-fold less toxic than N-substituted aromatics with the same log P values. Thus when present at similar concentrations in bacterial membranes, the N-substituted aromatics exert a much higher toxic effect than that which can be accounted for by membrane toxicity alone.

Chemical Reactivity of N-substituents

Nitroaromatics have been reported to be reactive toxicants (3, 18, 22). Nonaromatic nitrogen oxides were reported to inhibit the activity of some component in the methanogenic enzyme complex itself (2). The reactivity of the N-substituents could enable N-aromatics to undergo sorptive and chemical interactions with proteins; thereby, inactivating vital enzymes (4, 18). The toxicity of these compounds to methanogens has been suggested to involve interactions between nitroaromatics or intermediates of the reduction process (nitrosoamines or hydroxylamines) and the unique cell membrane of the methanogens (11). N-substituted aromatics may also interfere with the outcome of a biochemical conversion, such as the uncoupling of phosphorylation reactions (32) or interfering with physiological redox couples. The methanogenic toxicity of N-substituted aromatics was found to be the most pronounced for nitroanilines. The nitroanilines have the highest dipole moment of the compounds tested, making them the most chemically reactive compounds.

In this study, the aminoaromatics were determined to be considerably less toxic than the parent nitroaromatic compound. The ability of anaerobic consortia to remove and detoxify the nitro group in nitroaromatics would make anaerobic processes a useful treatment adjunct and/or alternative to conventional aerobic systems. In particular, anaerobic nitro group reduction may be an important initial step, which when followed by aerobic post-treatment, could result in complete mineralization of such highly nitrated compounds as trinitrotoluene and picric acid, which are highly resistant to aerobic degradation (7).

Acknowledgements

Support from the Human Capital and Mobility programme of the EC is gratefully acknowledged (B.D.). Funding from the Consejo Nacional de Ciencia y Tecnología and the Instituto Mexicano del Petróleo from México is also appreciated (E.R-F.).

References

1. American Public Health Association. (1985) Standard methods for examination of water and wastewater, 16th ed. American Public Health Association, Washington, D.C.
2. Balderston, W.L., Payne, W.J. (1976) Inhibition of methanogens in salt marsh sediments and whole-cell suspensions of methanogenic bacteria by nitrogen oxides. *Appl. Environ. Microbiol.* **32**:264-269.
3. Blum, D.J.W., Speece, R.E. (1991) A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. *Res. J. Water Pollut. Control. Fed.*. **63**:198-207.
4. Bumpus, J.A., Tatarko, M. (1994) Biodegradation of 2,4,6-trinitrotoluene by *Phanerochaete chrysosporium*: Identification of initial degradation products and the discovery of a TNT metabolite that inhibits lignin peroxidases. *Curr. Microbiol.* **28**:185-190.
5. Chou, W.L., Speece, R.E., Siddiqi, R., McKeon, K. (1978) The effect of petrochemical structure on methane formation toxicity. *Prog. Wat. Tech.* **10**:545-558.
6. Dickel, O., Haug, W., Knackmuss, H.J. (1993) Biodegradation of nitrobenzene by a sequential anaerobic-aerobic process. *Biodegradation* **4**:187-194.
7. Field, J.A., Stams, A.J.M., Kato, M., Schraa, G. (1995) Enhanced biodegradation of aromatic pollutants in coculture of anaerobic and aerobic bacterial consortia. *Antonie van Leeuwenhoek* **67**:47-77.
8. Fishbein, L. (1984) Aromatic amines. In: Hutzinger, O. (Ed.) The handbook of environmental chemistry, Volume 3, Part C, pp. 1-40. Springer-Verlag New York.
9. Gibson, S.A., Sewell, G.W. (1992) Stimulation of reductive dechlorination of tetrachloroethene in anaerobic aquifier microcosms by addition of short-chain organic acids or alcohols. *Appl. Environ. Microbiol.* **58**:1392-1393.
10. Golden, T., Molyneaux, E., Colleran, E. (1994) Toxicity of aromatic chemicals to the bacterial populations involved in anaerobic digestion. In: Seventh International Symposium on Anaerobic Digestion, pp. 19-22. Cape Town, South Africa. 1994.
11. Gorontzy, T., Kuver, J., Blotevogel, K.H. (1993) Microbial transformation of nitroaromatic compounds under anaerobic conditions. *J. Gen. Microbiol.* **139**:1331-1336.
12. Haghighi Podeh, M.R., Bhattacharya, S.K., Qu, M. (1995) Effects of nitrophenols on acetate utilising methanogenic systems. *Wat. Res.* **29**:391-400.
13. Haigler, B.E., Spain, J.C. (1991) Biotransformation of nitrobenzene by bacteria containing toluene degradative pathways. *Appl. Environ. Microbiol.* **57**:3156-3162.
14. Hallas L.E., Alexander, M. (1983) Microbial transformation of nitroaromatic compounds in sewage effluent. *Appl. Environ. Microbiol.* **45**:1234-1241.
15. Hartter, D.R. (1985) The use and importance of nitroaromatic chemicals in the chemical industry. In: Rickert, D.E. (Ed.). Toxicity of nitroaromatic compounds, pp. 1-14. Chemical Industry Institute of Toxicology Series. Chemisphere, Washington DC.
16. Heijman, C.G., Holliger, C., Glaus, M.A., Schwarzenbach, R.P., Zeyer, J. (1993) Abiotic reduction of 4-chloronitrobenzene to 4-chloroaniline in a dissimilatory iron-reducing enrichment culture. *Appl. Environ. Microbiol.* **59**:4350-4353.
17. Heipieper, H.J., Weber, F.J., Sikkema, J., Kewelch, H., de Bont., J.A.M. (1994) Mechanisms of resistance of whole cells to toxic organic solvents. *TIBTECH.* **12**:409-415.
18. Jaworska, J.S., Schultz, T.W. (1994) Mechanism-based comparisons of acute toxicities elicited by industrial organic chemicals in procaryotic and eucaryotic systems. *Ecotox. Environ. Saf.* **29**:200-213.
19. Johnson, L.D., Young, J.C. (1983) Inhibition of anaerobic digestion by organic priority pollutants. *J. Water Pollut. Control Fed.* **55**:1441-1450.

20. Kriek, E. (1979) Aromatic amines and related compounds as carcinogenic hazards to man. In: Emmelot, P., Kriek, E. (Eds.), Environmental carcinogenesis, pp. 143-164. Elsevier, Amsterdam.
21. Leo, A., Hansch, C., Elkins, D. (1971) Partition coefficients and their uses. *Chem. Rev.* **71**:525-616.
22. Manahan, S. E. (1993) In: Fundamentals of environmental chemistry. Lewis Publishers, Inc. Chelsea, Michigan.
23. Marvin-Sikkema, F.D., de Bont, J.A.M. (1994) Degradation of nitroaromatic compounds by microorganisms. *Appl. Microbiol. Biotechnol.* **42**:499-507.
24. Narayanan, B., Suidan, M.T., Gelderloos, A.B., Brenner, R.C. (1993) Treatment of semivolatile compounds in high strength wastes by expanded-bed GAC reactor. *Water Res.* **27**:171-180.
25. O'Connor, O.A., Young, L.Y. (1989) Toxicity and anaerobic biodegradability of substituted phenols under methanogenic conditions. *Environ. Toxicol. Chem.* **8**:853-862.
26. O'Connor, O.A., Young, L.Y. (1993) Effect of nitrogen limitation on the biodegradability and toxicity of nitro- and aminophenol isomers to methanogenesis. *Arch. Environ. Contam. Toxicol.* **25**:285-291.
27. Oren, A., Gurevich, P., Henis, Y. (1991) Reduction of nitrosubstituted aromatic compounds by the halophilic anaerobic eubacteria *Haloanaerobium praevalens* and *Sporohalobacter marismortui*. *Appl. Environ. Microbiol.* **57**:3367-3370.
28. Schwarzenbach, R.P., Stierli, R., Folsom, B.R., Zeyer, J. (1988) Compound properties relevant for assessing the environmental partitioning of nitrophenols. *Environ. Sci. Technol.* **22**:83-92.
29. Sierra R., Lettinga, G. (1991) The effect of aromatic structure on the inhibition of acetoclastic methanogens in granular sludge. *Appl. Microbiol. Biotechnol.* **34**:544-550.
30. Sikkema, J., de Bont, J.A.M., Poolman, B. (1994) Interactions of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.* **269**:8022-8028.
31. Spain, J.C., van Veld, P.A., Monti, C.A., Pritchard, P.H., Cripe, C.R. (1984) Comparison of *p*-nitrophenol biodegradation in field and laboratory test systems. *Appl. Environ. Microbiol.* **48**:944-950.
32. Tereda, H. (1981) The interaction of highly active uncouplers with mitochondria. *Biochim. Biophys. Acta* **639**:225-242.
33. Tratnyek, P.G., Malcady, D.L. (1989) Abiotic reduction of nitroaromatic pesticides in anaerobic laboratory systems. *J. Agric. Food Chem.* **37**:248-254.
34. Tseng, S.K., Yang, C.J. (1994) The reaction characteristics of treatment of wastewater containing nitrophenol by anaerobic biological fluidized bed. In: Seventh International Symposium on Anaerobic Digestion, pp. 297-305. Cape Town, South Africa.
35. U.S. Environmental Protection Agency (1978) Nitrobenzene. Initial report of the TSCA Interagency Testing Committee to the administrator. EPA 560-10-78/001. U.S. Environmental Protection Agency, Washington, D.C.
36. Verscheuren, K. (1983) In: Handbook of environmental data on organic chemicals, 2nd edition, Van Nostrand Reinhold Company, New York.
37. Vogels, G.D., Keltjens, J.T., van der Drift, C. (1988) Biochemistry of methane production. In: A. J. B. Zehnder (Ed.). Biology of anaerobic microorganisms, pp. 707-770. John Wiley & Sons, New York.
38. Wang, Y.T., Gabbard, H.D., Pai. P.C. (1991) Inhibition of acetate methanogenesis by phenols. *J. Envir. Engrg.* **117**:487-500.
39. Won, W.D., di Salvo, L.H., Ng. L. (1976) Toxicity and mutagenicity of 2,4,6-trinitrotoluene and its microbial metabolites. *Appl. Environ. Microbiol.* **31**:576-580.
40. Wu, W.M., Bhatanagar, L., Zeikus, J.G. (1993) Performance of anaerobic granules for degradation of pentachlorophenol. *Appl. Environ. Microbiol.* **59**:389-397.
41. Young, J.C., Tabak, H.H. (1993) Multilevel protocol for assessing the fate and effect of toxic organic chemicals in anaerobic treatment processes. *Wat. Environ. Res.* **65**:34-45.

3

Biodegradability of N-substituted Aromatics and Alkylphenols under Methanogenic Conditions using Granular Sludge

Elías Razo Flores
Brian A. Donlon
Jim A. Field
Gatze Lettinga

Summary

The biodegradability of seventeen N-substituted aromatic and six alkylphenol compounds were evaluated under methanogenic conditions. Biodegradation was assessed in batch assays inoculated with unacclimated and predigested anaerobic granular sludge at 30°C under agitated conditions over a 150 day period. The compounds were supplied at sub-toxic concentrations in the assays in order to prevent inhibition to the methanogens. The biodegradability test was performed by the measurement of the methane composition in the headspace of the serum flasks. The methanogenic consortia completely mineralized 2-, 3-aminobenzoate, 2-aminophenol and 4-cresol; whereas, 4-aminobenzoate was only partially degraded. The other N-substituted compounds and the alkylphenols tested were not biodegradable under the experimental conditions employed. An additional biodegradability assay was conducted with sludge from an upward-flow anaerobic sludge bed reactor adapted to the degradation of 2-nitrophenol. This sludge mineralized 2-aminophenol without any lag phase while the unadapted sludge required 110 days of acclimatization. The three aminobenzoate isomers were fully mineralized by the adapted sludge after similar lag periods observed in the unadapted sludge. The 2-nitrophenol adapted sludge cross-acclimatized to the mineralization of 5-aminosalicylate and 4-aminophenol. This constitutes the first report demonstrating the anaerobic mineralization of 5-aminosalicylate, which indicates that at least some azo dye cleavage products can be degraded in methanogenic consortia.

*Water Science and Technology (1996) **33(3)**:47-57*

3.1 Introduction

N-substituted aromatics and alkylphenols are usually found in the wastewaters generated in the chemical and petrochemical industries such as in the production of dyes, explosives, pesticides, pharmaceuticals and petrochemicals (3, 14, 31). These aromatic compounds are toxic and highly mutagenic and/or carcinogenic (18) and are generally considered to be difficult for anaerobic degradation (4, 10).

Biological treatment, either aerobic or anaerobic, is generally the most cost-effective means of removing the bulk of the pollutants in a high-strength organic wastewater. While aerobic treatment has been studied extensively and used to treat aromatic-bearing wastewater (6, 22, 26), anaerobic treatment has received comparatively little attention. One of the major problems confronting biological treatment of this kind of wastewater is inhibition to microorganisms due to the high toxicity of the aromatic compounds. Generally the toxicity of the aromatic compounds increases with greater apolarity (7, 27). The N-substituted aromatic compounds cause 50% inhibition (50% IC) to methanogenic bacteria at concentrations ranging from 2 to 2100 mg/L (7, 11), whereas the 50% IC of the alkylphenolic compounds ranged from 250 to 4000 mg/L (4, 11, 27).

The possibility of aromatic compound mineralization under anaerobic conditions would determine the applicability of anaerobic wastewater treatment technology to the effluents of the chemical and petrochemical industry. The purpose of this study was to evaluate the anaerobic mineralization of 23 common N-substituted aromatic and alkylphenol pollutants in a standard bioassay utilizing predigested anaerobic granular sludge. Due to problems of high toxicity in previous anaerobic biodegradability assays (2, 19), the compounds were tested at subtoxic concentrations. Acclimatization of anaerobic sludge to aromatic compounds has been shown to result in a significant enhancement in the mineralization of other structurally similar compounds (16, 32). Consequently, selected compounds were also evaluated in granular anaerobic sludge that was adapted to the continuous degradation of 2-nitrophenol.

3.2 Materials and Methods

Analyses

The methane content in the headspace of the serum flasks was determined by gas chromatography. A 100 μL gas sample was injected using a pressure-lock gas syringe (Dynatech Precision Sampling Corp., LA, USA) to a gas chromatograph, equipped with a molecular sieve 5A (mesh 60-80) column. The temperature of the column, the injection port and the flame ionization detector were 60, 200 and 220°C respectively. The carrier gas was nitrogen at a flow rate of 14.3 mL/min. The pH was determined with a Knick 511 pH-meter and a Scot Gerade N61 double electrode. Total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to *Standard Methods for Examination of Water and Wastewater* (1).

Biomass

The methanogenic granular sludge used was obtained from a full-scale upward-flow anaerobic sludge bed reactor (UASB) treating a petrochemical wastewater containing benzoate and acetate as primary substrates (Shell Nederland Chemie B.V., Moerdijk, The Netherlands). The sludge was elutriated to remove the fines and predigested at 30°C during a 30 days period in order to deplete all endogenous substrate in the sludge. The rationale behind using predigested granular sludge is to minimize the background methane production. The sludge was not previously acclimated to any of the test chemicals, except for benzoate. The sludge contained 10.5% TSS and 8.5% VSS. The maximum specific acetoclastic methanogenic activity of the sludge, as determined in standard batch activity tests (7) was 0.89 g COD-CH_4/g VSS-d at 30 °C. Adapted sludge was used for the cross acclimatization experiment, this sludge was taken from a UASB reactor which was mineralizing 2-nitrophenol (8). The sludge contained 11.2% TSS and 10.4% VSS and its maximum specific acetoclastic activity was 0.6 g COD-CH_4/gVSS-d.

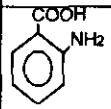
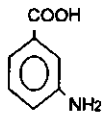
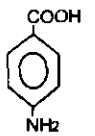
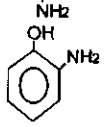
Anaerobic Biodegradability Assay

The batch anaerobic biodegradability assays were conducted in 120 mL glass serum flasks. The basal medium used in the bioassays was as described previously by Sierra and Lettinga (27), with the exception of $NaHCO_3$ supplied at 5 g/L. Predigested granular sludge (1 g VSS/L) was transferred to serum flasks containing 24 mL of the basal medium and acetate from a neutralized stock to yield a final concentration of 50 mg of chemical oxygen demand (COD)/L. The serum flasks were sealed with 12 mm thick butyl rubber stoppers (Rubber B.V., Hilversum, The Netherlands) and flushed with 70% N_2-30% CO_2 gas for 5 minutes and incubated overnight at 30°C to allow for biological consumption of residual O_2.

On the following day, serum flasks which were still pink due to the redox indicator dye resazurin were discarded. The desired amounts of the target compounds (Table 3.1) were then added to triplicate serum flasks using concentrated stock solutions. The compounds were supplied at sub-toxic concentrations in the assay medium in order to prevent inhibition to the methanogens. The concentrations employed were based on non-inhibitory values of alkylphenols and N-substituted aromatics as determined by Blum *et al.* (4), Golden *et al.* (11) and Donlon *et al.* (7). When toxicity data was not available a concentration of 100 mg/L was used because this is the minimal concentration that should be employed in order to minimize interferences of the background methane production. Acidic test compounds were neutralized prior to their addition to the assay medium.

Some aromatic amines (e.g. aminophenols) are prone to autoxidation and were prepared fresh with 250 mg/L ascorbic acid to prevent premature coloration. The ascorbic acid-COD concentration in the assay bottle was less than 24 mg/L and the sludge blank received a dose with the same level of ascorbic acid. The serum flasks were incubated with shaking (50 rpm) in a temperature controlled room at 30°C over a 150 day period. Sludge blanks, to correct for background gas production from the sludge, were based on assays where no test compounds were provided. All biodegradability data are the average of triplicate run experiments, except the sludge blanks which utilized six serum flasks to assess the methane production.

TABLE 3.1 Anaerobic biodegradability of N-substituted aromatic and alkylphenol compounds using granular sludge.

Compound[a]	Structure	Concentration[b] (mg/L)	Methane % TMP	S.D.[c] (% TMP)	Lag Phase (days)	Degradation potential[d]
2-aminobenzoate		300	92.0	5.0	25	+
3-aminobenzoate		300	112.5	5.4	75	+
4-aminobenzoate		300	41.9	10.1	35	+/-
2-aminophenol		200	91.6	10.3	110	+
4-cresol		250	78.0	0.4	18	+

[a] The following compounds were tested and were not found to be mineralized after 150 days (mg/L): 3-aminophenol (1000), 4-aminophenol (1000), aniline (200), 1,2-diaminobenzene (200), 1,3-diaminobenzene (200), 2-nitroaniline (100), 4-aminosalicylate (120), 5-aminosalicylate (120), 2-amino-4-chlorophenol (100), 4-aminobenzenesulfonate (200), 2-amino-4-nitrophenol (100), 4-amino-2-nitrophenol (100), 3,4-diaminobenzoate (100), 2-cresol (250), 3-ethylphenol (250), 2,5-xylenol (100), 3,4-xylenol (100), 4-methylcatechol (100).

[b] Concentration of the test compound in the bioassay.

[c] S.D.= standard deviation (as % TMP) of the methane production from the compound.

[d] Biodegradation potential: (+) completely degradable, (+/-) partially degradable.

The methane composition in the headspace of each serum flask was monitored periodically during the assays. The serum flasks were shaken vigorously before gas measurements were taken. Methane production was calculated from the volume of the headspace and the methane composition in the gas. Net methane production was calculated by subtracting background methane production in the controls from that in the test vials. The corrected methane production (M) was expressed as a percentage of the theoretical methane

production (TMP) expected from the test chemical mineralization based on the Buswell equation (30). All Chemicals were purchased from Acros Chimica (Geel, Belgium), Merck (Darmstadt, Germany) and Sigma (Bornem, Belgium) and were used without further purification.

3.3 Results

Evaluation of the Anaerobic Biodegradability Assay

Benzoate and phenol were used as reference aromatic compounds to assess the accuracy of the bioassay method used. The concentrations of benzoate and phenol used were 250 mg/L. The benzoate was completely degraded in 20 days and the phenol in 45 days (Figure 3.1). The ultimate conversion of the substrate COD to methane was equal to 85.5% ± 1.82 and 82.8% ± 2.32 for benzoate and phenol respectively. Reproducibility of the methane production among replicate sludge blank serum flasks was satisfactory, with standard deviations accounting for less than 2% of the mean.

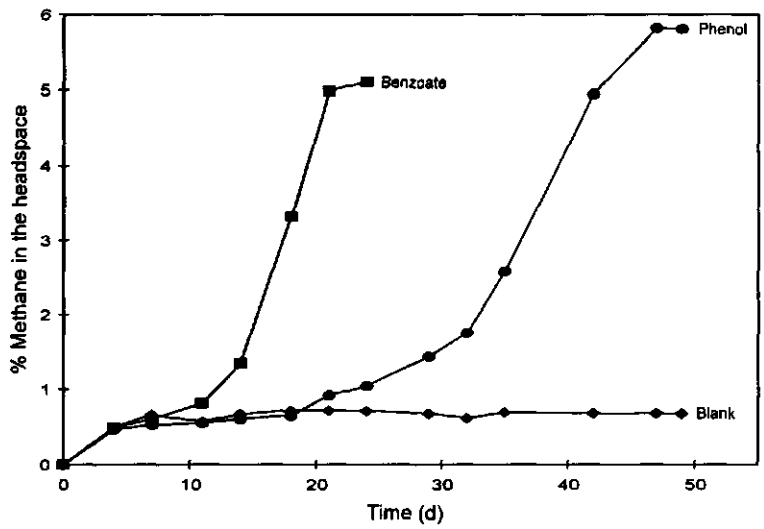


Figure 3.1 Biodegradability of the reference aromatic compounds: benzoate and phenol.

Anaerobic Biodegradability of N-substituted Aromatics and Alkylphenols

The biodegradability of seventeen N-substituted aromatic and six alkylphenol compounds were evaluated under methanogenic conditions with unadapted sludge. Of the N-substituted aromatics, only 2-aminobenzoate, 3-aminobenzoate and 2-aminophenol were fully mineralized (Table 3.1). The lag phase prior to the onset of mineralization ranged from 25 to 110 days. Also, 4-aminobenzoate was partially mineralized by 42% (Table 3.1). The other isomers of aminophenol, all N-substituted benzenes, salicylates and benzenesulfonate were not degraded after 150 days. Two compounds, 2-amino-4-nitrophenol and 4-amino-2-nitrophenol tested at 100 mg/L were found to be toxic and consequently their biodegradation could not be evaluated. Figure 2.2 illustrates the time course of methane production with 2-aminobenzoate, 4-aminobenzoate and 4-aminobenzenesulfonate as sole substrates representing biodegradable, partially biodegradable and recalcitrant compounds, respectively. Of the alkylphenols tested, only 4-cresol was mineralized after an 18 day lag phase (Table 3.1). The other alkylphenols, 2-cresol, 3-ethylphenol, xylenols and 4-methylcatechol were not even slightly mineralized after 150 days.

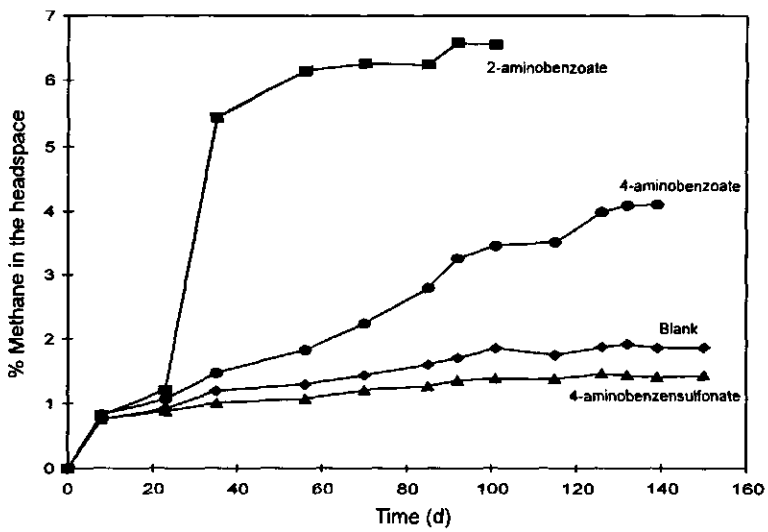
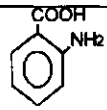
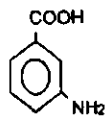
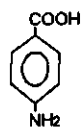
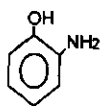
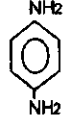
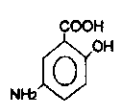
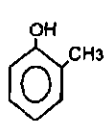


Figure 3.2 Biodegradability of N-substituted aromatics by the unadapted sludge.

TABLE 3.2 Anaerobic biodegradability of N-substituted aromatics and alkylphenols using 2-nitrophenol adapted granular sludge.

Compound	Structure	Concentration[a] (mg/L)	Methane % TMP	S.D.[b] (% TMP)	Lag Phase (days)	Degradation potential[c]
2-aminobenzoate		300	95.7	5.2	25	+
3-aminobenzoate		300	75.1	1.9	50	+
4-aminobenzoate		300	76.2	0.4	50	+
2-aminophenol		200	83.1	3.8	<5	+
4-aminophenol		1000	45.5[d]	5.8	70	+
aniline		200	1.0	4.5	>150	-
5-amino salicylate		120	95.6	5.4	65	+
2-cresol		250	-5.8	1.2	>150	-

[a] Concentration of the test compound in the bioassay
[b] S.D.= standard deviation (as % TMP) of the methane production from the compound.
[c] Biodegradation potential:(+) completely degradable, (+/-) partially degradable, (-) non degradable.
[d] Incubated over a 220 day period.

Cross-Acclimatization Studies

In order to evaluate the cross-acclimation properties of a granular sludge adapted to 2-nitrophenol, an additional biodegradability experiment was set up. Seven N-substituted aromatics and one alkylphenol were tested (Table 3.2). 2-aminophenol was immediately mineralized without any obvious lag phase while 110 days were required to initiate degradation in the unadapted sludge. The three isomers of aminobenzoate were mineralized by the adapted sludge after lag phases similar to those observed in the unadapted sludge. However, 5-aminosalicylate, which was recalcitrant with the unadapted sludge, was mineralized by the adapted sludge after a 65 day lag period suggesting that cross-acclimatization to this compound occurred. 4-aminophenol was only partially mineralized whereas 2-cresol and aniline were not mineralized by the adapted sludge. Figure 3.3 shows the time course of methane production with 2-aminophenol, 4-aminobenzoate and 5-aminosalicylate.

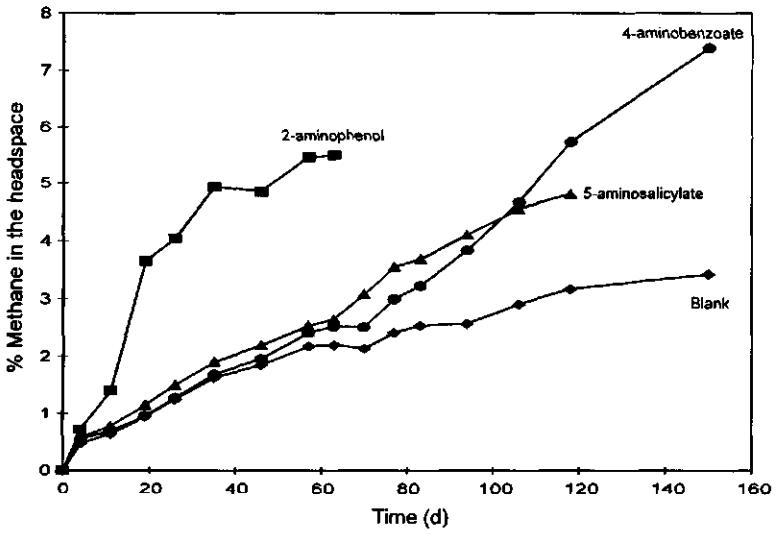


Figure 3.3 Biodegradability of N-substituted aromatics by the 2-nitrophenol adapted sludge.

3.4 Discussion

Biodegradability Studies with Unadapted Sludge

The biodegradability method used in this study has some advantages compared to the conventional methods (2, 21, 25), namely: interferences due to high background methane production is minimized using low concentrations of predigested granular sludge and the utilized concentrations of the test chemicals are based on non-toxic values to prevent inhibition of the methanogens. According to the degradation results of the reference compounds, benzoate and phenol, this method was found to be reliable for assaying the anaerobic biodegradation of aromatic compounds.

In the literature, biodegradability studies of the 3 isomers of mono-aminobenzoate have indicated that 2- and 4-aminobenzoate are mineralized by methanogenic consortia (2, 17, 23 25), but there are conflicting reports on the biodegradability of 3-aminobenzoate (2, 17, 24). In our studies, 2-aminobenzoate was completely mineralized, whereas 4-aminobenzoate was partially mineralized. In the case of 3-aminobenzoate, we found that this compound is completely mineralized, confirming the findings of Schnell & Schink (24). According to Schnell & Schink (24), 2- and 4-aminobenzoate are compounds that exist in nature. However, 3-aminobenzoate is a xenobiotic compound produced mainly for synthesis of azo dyes.

Of the aminophenols tested only 2-aminophenol was mineralized, 3- and 4-aminophenol were left unmetabolized by the unadapted sludge. Previous reports in the literature suggest that 2-aminophenol can be completely mineralized under methanogenic conditions (2, 20), while 3-aminophenol is recalcitrant (2, 20) and 4-aminophenol is sometimes mineralized (20) and other times is left unmetabolized (2). None of the other N-substituted aromatic compounds tested were found to be mineralized and there is also no evidence for the degradation of such compounds in methanogenic consortia. Based on the results of the nitroaromatics tested, we can conclude that it is not feasible to study the biodegradability of such highly toxic compounds in batch assays.

Six alkylphenolic compounds were tested and only 4-cresol was found to be degraded. The mineralization of 4-cresol has been demonstrated by several authors (4, 9, 32). Fedorak & Hrudey (9) reported that none of the isomers of xylenol were degraded nor was 2-cresol, which is the most anaerobically recalcitrant alkylphenol. Blum *el al.* (4) reported a partial degradation (based on gas production) of 3-ethylphenol (19%) and 4-methylcatechol (36%), using phenol-acclimated culture for the later compound. However, the low mineralization levels do not substantiate their biodegradability. Our results confirm the recalcitrance of the alkylphenols assayed.

Biodegradability Studies with Adapted Sludge

The results of this study using sludge adapted to the continuous degradation of 2-nitrophenol in laboratory columns showed that cross-acclimatization of N-substituted aromatics did occur: 2-aminophenol, 4-aminobenzoate and 5-aminosalicylate were completely mineralized, whereas 4-aminophenol was partially mineralized. Degradation of 2-aminophenol was also obtained with unadapted sludge, but in the case of adapted sludge the lag phase of 2-aminophenol was reduced from 110 days to less than 5 days. It is known that nitroaromatics are easily reduced by microorganisms to aminoaromatics (12, 13), and it was shown that 2-aminophenol is an intermediate of 2-nitrophenol degradation (8). Consequently, it was not surprising that the lag phase of the 2-aminophenol was reduced to such a short period. 4-aminobenzoate was completely degraded using acclimated sludge, whereas the other two isomers of aminobenzoate showed the same behavior in both sludges.

Previously it was shown that cells of *Klebsiella pneumoniae* were able to decarboxylate 5-aminosalicylate to 4-aminophenol when incubated under anaerobic conditions (28). However, this study constitutes the first report of 5-aminosalicylate mineralization in the absence of molecular oxygen by anaerobic bacteria. The degradation of 5-aminosalicylate under aerobic conditions, on the other hand, is well documented (15, 29)

The anaerobic biodegradability of 5-aminosalicylate has important implications for the environment since this compound is an important industrial precursor of many of the

commonly used azo dyes. Likewise 5-aminosalicylate is a degradation product of anaerobic reductive azo dye cleavage (15). Previously it was assumed that azo cleavage products would be recalcitrant to anaerobic degradation (5, 15) and subsequent aerobic mineralization would be required. However this study shows that certain azo cleavage products such as 3-aminobenzoate and 5-aminosalicylate are in fact fully biodegradable in methanogenic consortia.

Acknowledgments

This research was supported by the Consejo Nacional de Ciencia y Tecnología from México. Financial aid from the Instituto Mexicano del Petróleo from México is gratefully acknowledged (E.R.F.). Support from the Human Capital and Mobility programme of the EC is also appreciated (B.D).

References

1. American Public Health Association (1985) Standard methods for examination of water and wastewater, 16th. ed. American Public Health Association, Washington, DC
2. Battersby, N.S., Wilson, V. (1989) Survey of the anaerobic biodegradation potential of organic chemicals in digesting sludge. *Appl. Environ. Microbiol.* **55**:433-439.
3. Beychok, M.R. (1967) Aqueous Wastes from Petroleum and Petrochemical Plants. John Wiley & Sons, London.
4. Blum, D. J. W., Hergenroeder, R., Parkin, J.F., Speece R.E. (1986) Anaerobic treatment of coal conversion wastewater constituents: biodegradability and toxicity. *J. Water Pollut. Control Fed.* **58**:122-131.
5. Brown, D., Hamburger, B. (1987) The degradation of dyestuffs: investigations of their ultimate degradability. *Chemosphere* **16**:1539-1553.
6. Deshpande, S.D., Chakrabarti, T., Subrahmanyam, P.V.R., Sundaresan, B.B. (1985) Biological treatability of *m*-aminophenol plant wastewater containing structural isomers of benzene with different substituents. *Wat. Res.* **19**:293-298.
7. Donlon, B., Razo-Flores, E., Field, J.A., Lettinga, G. (1995a) The toxicity of N-substituted aromatics to acetoclastic methanogenic activity in granular sludge. *Appl. Environ. Microbiol.* **61**:3889-3893.
8. Donlon, B., Razo-Flores, E., Hwu, C.S., Field, J., Lettinga, G. (1995b) Toxicity and biodegradability of selected N-substituted phenols under anaerobic conditions. In: Hinchee, R., Brockman, F.J., Vogel, C.M. (Eds.). Microbial Processes for Bioremediation; Bioremediation 3(8), pp. 251-257. Battelle Press, Columbus.
9. Fedorak, P.H., Hrudey, S.E. (1984) The effects of phenol and some alkyl phenolics on batch anaerobic methanogenesis. *Wat. Res.* **18**:361-367.

10. Field, J.A., Stams, A.J.M., Kato, M., Schraa, G. (1995) Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and anaerobic bacterial consortia. *Antonie van Leeuwenhoek* **67**:47-77.
11. Golden, T., Molyneaux, E., Colleran, E. (1994) Toxicity of aromatic chemicals to the bacterial populations involved in anaerobic digestion. In: Proceedings of the Seventh International Symposium on Anaerobic Digestion, pp. 19-22. Cape Town, South Africa.
12. Gorontzy, T., Kuver, J., Blotevogel, K.H. (1993) Microbial transformation of nitroaromatic compounds under anaerobic conditions. *J. Gen. Microbiol.* **139**:1331-1336.
13. Hallas, L.E., Alexander, M. (1983) Microbial transformation of nitroaromatic compounds in sewage effluent. *Appl. Environ. Microbiol.* **45**:1234-1241.
14. Hartter, D.R. (1985) The use and importance of nitroaromatic chemicals in the chemical industry. In: Rickert, D.E. (Ed.). Toxicity of Nitroaromatic Compounds, pp. 1-13. Hemisphere Publishing Corp., Washington D.C.
15. Haug, W., Schmidt, A., Nörtemann, B., Hempel, D.C., Stolz, A., Knackmuss, H.-J. (1991) Mineralization of the sulfonated azo dye mordant yellow 3 by a 6-aminonaphtalene-2-sulfonate-degrading bacterial consortium. *Appl. Environ. Microbiol.* **57**:3144-3149.
16. Healy, J.B., Young, L.Y. (1979) Anaerobic biodegradation of eleven aromatic compounds to methane. *Appl. Environ. Microbiol.* **38**:84-89.
17. Horowitz, A., Shelton, D.R., Cornell, C.P., Tiedje, J.M. (1981) Anaerobic degradation of aromatic compounds in sediments and digested sludge. *Dev. Ind. Microbiol.* **23**:435-444.
18. Kriek, E. (1979) Aromatic amines and related compounds as carcinogenic hazards to man. In: Emmelot P., Kriek E. (Eds.). Environmental Carcinogenesis, pp. 143-164. Elsevier, Amsterdam.
19. O'Connor, O.A., Young, L.Y. (1989) Toxicity and anaerobic biodegradability of substituted phenols under methanogenic conditions. *Environ. Toxicol. Chem.* **8**:853-862.
20. O'Connor, O.A., Young, L.Y. (1993) Effect of nitrogen limitation on the biodegradability and toxicity of nitro- and aminophenol isomers to methanogenesis. *Arch. Environ. Contam. Toxicol.* **25**:285-291.
21. Owen, W.F., Stuckey, D.C., Healy, J.B. Jr., Young, L.Y., McCarty, P.L. (1979) Bioassay for monitoring biochemical methane potential and anaerobic toxicity. *Wat. Res.* **13**:485-492.
22. Pandey, R.A., Parhad, N.M., Kumaran, P. (1991) Biological treatment of low temperature carbonization wastewater by activated sludge process-a case study. *Wat. Res.* **25**:1555-1564.
23. Schink, B. (1988) Fermentative degradation of nitrogenous aliphatic and aromatic compounds. In: Hall, E.R., Hobson P.N. (Eds.). Proceedings of the 5th International Symposium on Anaerobic Digestion held in Bologna, Italy, 22-26 May, pp. 459-464. Pergamon Press, Oxford.
24. Schnell, S., Schink, B. (1992) Anaerobic degradation of 3-aminobenzoate by a newly isolated sulfate reducer and a methanogenic enrichment culture. *Arch. Microbiol.* **158**:328-334.
25. Shelton, D.R., Tiedje, J. M. (1984) General method for determining anaerobic biodegradation potential. *Appl. Environ. Microbiol.* **47**:850-857.
26. Siegrist, T.W. (1982) Chemicals and allied products. *J. Water Pollut. Control Fed.* **54**:737-7749.
27. Sierra, R., Lettinga, G. (1991) The effect of aromatic structure on the inhibition of acetoclastic methanogens in granular sludge. *Appl. Microbiol. Biotechnol.* **34**:544-550.
28. Sivolodskii, E.P., Rovnov, N.V., Petrov, L.N. (1994) Mechanism of specific chromogenic reaction of *Klebsiella* spp. during growth on nutrient medium with 5-aminosalicylic acid. *Microbiology* **63**:272-274.
29. Stolz, A., Nörtemann, B., Knackmuss, H.-J. (1992) Bacterial metabolism of 5-aminosalicylic acid: Initial ring cleavage. *Biochem. J.* **282**:675-680.
30. Tarvin, D., Buswell, A.M. (1934) The methane fermentation of organic acids and carbohydrates. *J. Amer. Chem. Soc.* **56**:1751-1755.

31. Vernick, A.S., Lanik, P.D., Langer, B.C., Hrudey, S.E. (1984) The management of wastewater from the petroleum refining industry. In: Barnes, D., Forster, C., Hrudey S.E. (Eds.). Surveys in industrial wastewater treatment, Vol. 2, pp. 1-64. Pitman, London.
32. Young, L.Y., Rivera, M.D. (1985) Methanogenic degradation of four phenolic compounds. *Wat. Res.* **19**:1325-1332.

4

The Effect of Granular Sludge Source on the Anaerobic Biodegradability of Aromatic Compounds

Elías Razo Flores
Anna Svitelskaya
Brian A. Donlon
Jim A. Field
Gatze Lettinga

Summary

The ability of bacterial consortia of five different granular sludge sources to anaerobically biodegrade aromatic compounds was evaluated. The biodegradability of phenol, 4-cresol, 2-aminobenzoate (2ABc) and 5-aminosalicylate (5ASA) was determined by measuring compound conversion to methane in batch serum bottles at 30 °C under agitated conditions over a period of at least 100 days. Phenol and 4-cresol were completely mineralized by all the granular sludges tested. This observation indicates a universal capacity of granular sludge to degrade phenol and 4-cresol; which would be expected since these compounds are intermediates during the anaerobic degradation of the commonly occurring amino acid tyrosine. On the other hand, 5ASA and 2ABc were degraded only by one or two granular sludges. Previous acclimation to an N-substituted aromatic was a prerequisite for 5ASA degradation.

Bioresource Technology (1996) 56:215-220

4.1 Introduction

Over the past 20 years anaerobic treatment has gained a solid position in the biological wastewater treatment field. Anaerobic treatment has been successfully applied to the treatment of municipal (15) and food industry wastewater (14, 21, 23). More recently, anaerobic treatment has been utilized for the treatment of aromatic-bearing wastewaters from the chemical and petrochemical industries (4, 24) and from the forestry industry (22). The successful application can be attributed to the high retention of active bacterial aggregates inside the high-rate reactors, such as granular sludge in the upflow anaerobic sludge bed (UASB), and fixed films in the attached-film processes. Granular sludge forms naturally from the tendency of the anaerobic bacteria to attach to one another under the upflow conditions prevailing in the UASB reactors (20). It is well known that the structural characteristics of bacterial aggregates and the high biomass retention protect and improve the tolerance of anaerobic bacteria to toxic compounds, and also allow the bacteria to adapt to inhibitory compounds (3, 19, 11).

Lately, the tremendous degradation potential of anaerobic granular sludge with respect to aromatic compounds has been shown (6, 7, 9, 25). However, there are some reports in the literature which show significant differences in the response of different seed cultures with respect to their ability to degrade compounds and their acclimation rates as well (3, 16, 18). These observations emphasize the importance of the seed sludge source used during the start-up of UASB reactors treating aromatic compounds. Consequently, granular sludges from different sources were assayed to evaluate their biodegradative capacities with respect to the onset of the degradation, the degradation rate and the degradation potential. Four compounds previously reported to be anaerobically biodegraded and that are frequently present in the wastewaters of the chemical and petrochemical industries were selected for this study, namely, 2-aminobenzoate (2), phenol (16), 4-cresol (5) and 5-aminosalicylate (25).

4.2 Materials and Methods

Biomass

The methanogenic granular sludges used in this study were obtained from 5 different UASB reactors treating effluents from: Aviko (potato industry), Borculo (whey industry), Gist-Brocades (yeast/antibiotics industry), 2-nitrophenol (2NP) adapted sludge (2-nitrophenol/VFA) and from Shell Nederland Chemie (wet oxidized petrochemical industry effluent); fresh sample (SNC) or a 2 year old sample stored at 4 °C (SNC-2). It should be pointed out that 2NP sludge was originally cultivated from SNC after 200 days of adaptation to 2-nitrophenol in a laboratory-scale UASB reactor. The sludges were elutriated to remove the fines and predigested at 30°C during 30 days period in order to deplete most of the endogenous substrates in the sludge.

Analyses

The methane content in the gas samples was determined by gas chromatography (Packard-Becker, Delft, the Netherlands). The gas chromatograph was equipped with a steel column (2 m by 2 mm) packed with Poropak Q (80/100 mesh, Millipore Corp., Bedford, Mass.). The temperatures of the column, the injector port and the flame ionization detector was 60, 200 and 220 °C, respectively. The carrier gas was nitrogen at a flow rate of 20 mL/min. Samples for measuring methane content (100μL) in the headspace were determined using a pressure-lock gas syringe (Pressure-Lok series A-2, Dynatech Precision Sampling Corp., LA, USA). An isobaric precise proportion of the known headspace volume could be analyzed. The pH was determined immediately after sampling with a Knick 511 pH-meter (Berlin, Germany) and a Scot Gerade N61 double electrode (Hofheim, Germany). Total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to *Standard Methods for Examination of Water and Wastewater* (1).

Basal Medium

The basal medium used contained (mg/L): $NaHCO_3$ (5000), NH_4Cl (280), $CaCl_2.2H_2O$ (10), K_2HPO_4 (250), $MgSO_4.7H_2O$ (100), yeast extract (100), H_3BO_3 (0.05), $FeCl_2.4H_2O$ (2), $ZnCl_2$ (0.05), $MnCl_2.4H_2O$ (0.05), $CuCl_2.2H_2O$ (0.03), $(NH_4)_2SeO_3.5H_2O$ (0.05), $AlCl_3.6H_2O$ (2), $NiCl_2.6H_2O$ (0.05), $Na_2SeO_3.5H_2O$ (0.1), EDTA (1), rezazurin (0.2) and 36% HCl (0.001 mL/L).

Anaerobic Activity Assay

Maximum specific acetoclastic methanogenic activity measurements were performed according to the method described by Donlon *et. al.* (8) in 120 mL glass serum flasks sealed with 12 mm thick butyl rubber septa. Predigested granular sludge (2 g VSS/L) was transferred to serum flasks containing 25 mL of basal medium and acetate from a neutralized stock solution to yield a final concentration of 2.5 g of chemical oxygen demand (COD)/L. Assay serum flasks were then flushed with 70% N_2-30% CO_2 for 5 minutes and incubated at 30°C. On the following day bottles which were still pink due to the redox indicator dye resazurin were discarded. After 3 days incubation, the acetate concentration was replenished to 1 g COD/L in order to assay the specific methanogenic activity. The headspace was reflushed with 70% N_2-30% CO_2 gas and the assay bottles were reincubated for 1 hr, prior to the determination of the methane production rate. The methane content in the headspace of each assay bottle was determined hourly during the subsequent 6-8 hour incubation period. The maximum specific methanogenic activity was calculated from the slope of the cumulative methane production (mL) versus time curve (d).

Anaerobic Biodegradability Assay

The batch anaerobic biodegradability assays were conducted according to the method described by Razo-Flores *et. al.* (25) in 120 mL glass serum flasks. Predigested granular sludge

(1 g VSS/L) was transferred to serum flasks containing 24 mL of the basal medium and acetate from a neutralized stock to yield a final concentration of 50 mg COD/L. The serum flasks were sealed with 12 mm thick butyl rubber stoppers and flushed with 70% N_2-30% CO_2 gas for 5 minutes and incubated overnight at 30°C to allow for biological consumption of residual O_2.

On the following day, the desired amounts of the target compounds were then added to triplicate serum flasks using concentrated stock solutions. The compounds were supplied at sub-toxic concentrations in the assay medium in order to prevent inhibition to the methanogens, with the following concentrations in the serum flasks (mg/L): 2-aminobenzoate (2ABc), 300; phenol, 250; 4-cresol, 250 and 5-aminosalicylate (5ASA), 120. The serum flasks were incubated with shaking (50 rpm) in a temperature controlled room at 30°C over at least a 100 day period. Sludge blanks, to correct for background gas production from the sludge, were based on assays where no test compounds were provided. All biodegradability data are the average of triplicate run experiments. The methane composition in the headspace of each serum flask was monitored periodically during the assays. The serum flasks were shaken vigorously before gas measurements were taken. Methane production was calculated from the volume of the headspace and the methane composition in the gas. Net cumulative methane production was calculated by subtracting background methane production in the controls from that in the test vials. The net cumulative methane production was expressed as a percentage of the theoretical methane production (TMP) expected from the test chemical mineralization based on the Buswell equation (26). The degradation rate of the compounds was calculated from the slope of the net cumulative methane production (mL) versus time curve (d) by converting the measured mg COD-CH_4 to mg of compound of the corresponding aromatic amine. A conversion factor of 0.388 mL CH_4/mg COD (1 atm and 30°C) was also used for these calculations.

Chemicals

Chemicals were purchased from Acros Chimica (Geel, Belgium). All chemicals were of the highest purity and were used without further purification.

4.3 Results

Five different sources of granular sludge were assayed. In the case of the Shell sludge, we tested a fresh sample (SNC) and 2-year old sample stored at 4 °C (SNC-2) in order to measure the effect of cold storage on the activity and biodegradability potential of the granular sludge. Table 1 shows the sources and characteristics of the granular sludges used. The sludges contained a TSS and VSS percentage in the range of 9.6-12.8% and 7.5-10.2% respectively. The specific acetoclastic methanogenic activity of the sludges was relatively high and was in the range of 0.5 - 1.14 g CH_4-COD/g VSS-d. The acetoclastic activity of the SNC-2 sludge was 54% lower than that of the SNC sludge after 2 years of cold storage. Nonetheless the remaining activity was quite high.

The four compounds selected for this study: 2ABc, phenol, 4-cresol and 5ASA, were tested for anaerobic biodegradability by the different sludges (Table 2). The background methane production of the sludge blanks accounted for 2.7 - 8.1 mg CH_4-COD. Phenol and 4-cresol were completely mineralized by all sludges. The lag phase was minor for the Aviko and Borculo sludges. SNC and 2NP sludges were capable of degrading 2ABc after 3 weeks. In the case of 5ASA, only 2NP sludge was able to mineralize the compound after a 65 day adaptation period. The TMP of the compounds that were mineralized was generally >70%. SNC-2 lost the ability to degrade 2ABc; whereas, 2NP sludge kept this ability despite the fact that it had been adapted to another N-substituted aromatic (2-nitrophenol). The degradation rates of the compounds that were mineralized were fairly similar with all the sludges. However, the lag phase before the onset of the degradation were quite different from compound to compound and from sludge to sludge. Figure 1 shows the net cumulative methane production of the 4-cresol degradation with Aviko and Gist-brocades sludges.

The effect of biomass concentration on the phenol (250 mg/L) degradation was also studied. Three concentrations of SNC granular sludge were assayed: 1, 3 and 10 g VSS/L. Figure 2 shows the time course of the net cumulative methane production. Figure 2 clearly illustrates that methane production rate is quite similar for all sludge concentrations tested. On the other hand, while the standard deviation (SD) of the TMP was rather good for 1 and

TABLE 4.1- Sources and characteristics of the anaerobic granular sludges used in this study.

Source	Aviko	Borculo	Gist-Brocades	SNC	SNC-2[a]	2NP[b]
Wastewater type	potato	whey	yeast/antibiotic	w.o.p.[c]	w.o.p.	2-nitrophenol/VFA
Reactor type	UASB	UASB	UFB[d]	UASB	UASB	UASB
Reactor volume	1700 m^3	590 m^3	400 m^3	1280 m^3	1280 m^3	0.16 L
Total suspended solids (w/w)	9.6 %[e]	12.7 %[e]	11.8 %	11 %	10.6 %	12.8 %
Volatile suspended solids (w/w)	7.5 %[e]	9.1 %[e]	10.2 %	9 %	8.4 %	9.5 %
Acetoclastic activity (g CH_4-COD/g VSS-d)	0.52	0.57	0.5	1.14	0.53	0.63

[a] SNC sludge storaged 2 year at 4 °C.
[b] SNC sludge adapted to 2-nitrophenol for 200 days.
[c] w.o.p.: wet oxidized petrochemical.
[d] Upflow fluidized bed.
[e] Values taken from Hwu *et. al.* (19)

TABLE 4.2 Anaerobic biodegradability of aromatic compounds using different sources of granular sludge.

Compound	Phenol	4-Cresol	2ABc	5ASA
Sludge		Aviko		
Lag phase (d)	13	11	>100	>100
Degradation rate (mg/g VSS-d)	10	11.3	0	0
Methane (% TMP)	86 ± 4	93 ± 3	-3 ± 1	-16 ± 2
Sludge		Borculo		
Lag phase (d)	11	14	>100	>100
Degradation rate (mg/g VSS-d)	7.5	14.1	0	0
Methane (% TMP)	92 ± 2	102 ± 1	-5 ± 0.5	-1 ± 13
Sludge		Gist Brocades		
Lag phase (d)	47	27	>100	>100
Degradation rate (mg/g VSS-d)	9	11.3	0	0
Methane (% TMP)	70 ± 20	100[a]	4 ± 8	5 ± 2
Sludge		SNC		
Lag phase (d)	26	18	23	>100
Degradation rate (mg/g VSS-d)	11.5	12.1	8.1	0
Methane (% TMP)	93 ± 0.4	78 ± 0.4	101 ± 14	6 ± 3
Sludge		SNC-2		
Lag phase (d)	32	19	>100	>100
Degradation rate (mg/g VSS-d)	8.2	9.8	0	0
Methane (% TMP)	77 ± 5	102 ± 3	9 ± 2	0[a]
Sludge		2NP		
Lag phase (d)	40	ND[b]	25	65
Degradation rate (mg/g VSS-d)	9.1	ND	11.7	1.43
Methane (% TMP)	98 ± 2	ND	96 ± 5	96 ± 5

[a] no standard deviation data, n = 2

[b] ND: not determined

3 g VSS/L, this was not the case for the 10 g VSS/L where the SD accounted for 50% of the TMP. The background methane production corresponded to 37.9, 84.2 and 212.8% of TMP expected from phenol for 1, 3 and 10 g VSS/L respectively. The relatively high background methane production from 10 g VSS/L would explain the high standard deviation.

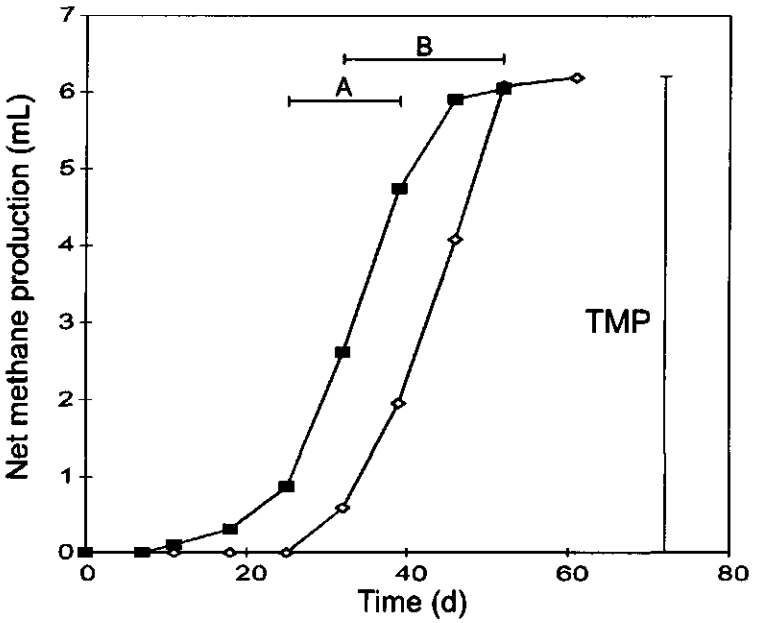


Figure 4.1 Time course of the net cumulative methane production of the 250 mg/L 4-cresol biodegradation; (■) Aviko sludge, (◊) Gist-Brocades sludge. The vertical line shows the theoretical methane production (TMP) that should be achieve if the 4-cresol were completely mineralized to methane (6.01 mL). The horizontal lines indicates the time period used to calculate the slope of the net cumulative methane production: (A) Aviko sludge, (B) Gist-Brocades sludge.

4.5 Discussion

The 5 different granular sludge sources tested in this study varied with respect to their ability to anaerobically degrade compounds and the time period required for the onset of degradation. The compounds were selected based on their proven anaerobic degradability in methanogenic consortia (2, 5, 16, 25) and were tested at sub-toxic concentrations to avoid

methanogenic inhibition. The inhibitory concentration of the compounds tested that causes a 50% decrease in the methanogenic activity is (g/L): 1.3, 1, 9.18 and 0.43 for phenol, 4-cresol, 2ABc and 5ASA respectively (3, 8, 9, 25). Phenol and 4-cresol were mineralized by all sludges at similar degradation rates, with an average of 9.2 and 11.7 mg/g VSS-d, respectively. The ubiquitous capacity for anaerobic phenol and 4-cresol degradation is not surprising since these compounds are intermediates during the anaerobic degradation of flavonoids and tyrosine (12, 13). The common occurring amino acid tyrosine would be expected in many different types of wastewater from the food industry or to result from the degradation of proteins in bacterial biomass. The fact that phenol and 4-cresol were readily degraded by the Aviko sludge after very short lag phases corresponds to the occurrence of tyrosine in potato starch wastewater (12).

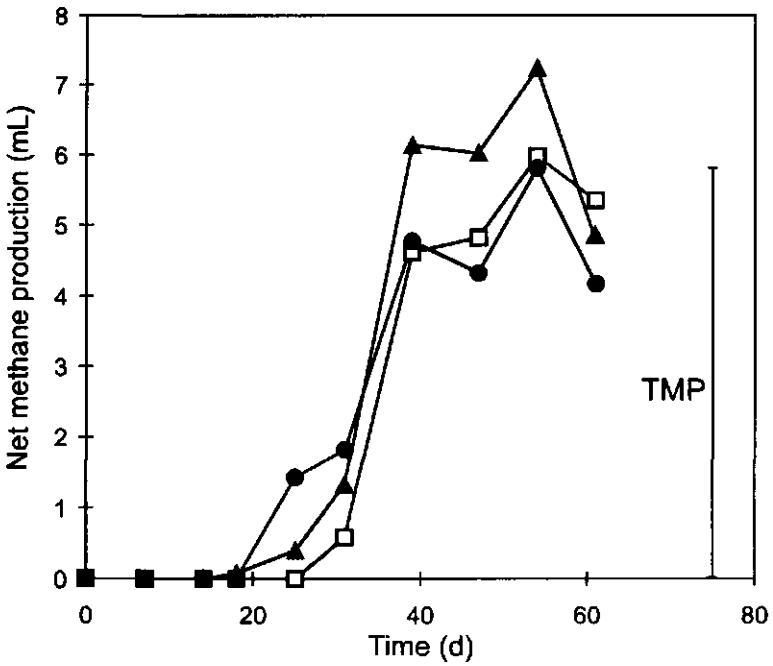


Figure 4.2 Net cumulative methane production of the 250 mg/L phenol biodegradation using 3 different concentrations of SNC granular sludge under stirred conditions (50 rpm). The vertical line shows the theoretical methane production (TMP) that should be achieve if the phenol were completely mineralized to methane (5.77 mL). (□) 1 g VSS/L; %TMP = 92.7 ± 0.4, (●) 3 gVSS/L, %TMP = 71.9 ± 4.7, (▲) 10 g VSS/L; %TMP = 83.1 ± 44.

2ABc was degraded by SNC and 2NP sludges, but not by SNC-2 and the other sludges. Based on these results it is clear that the 2 years of cold storage of the SNC sludge had a detrimental effect on its ability to degrade 2ABc. Probably, the microorganisms responsible for 2ABc degradation have special maintenance energy requirements and they are apparently not involved in the phenol and 4-cresol breakdown. 2NP adapted sludge, kept the ability to degrade 2ABc after 200 days exposure to 2-nitrophenol and VFA. 2NP sludge was also able to degrade 5ASA. This finding confirms the results of Healy & Young (17) and Young & Rivera (27), that is possible to cross-acclimate sludge for the mineralization of other structurally similar compounds using aromatic-acclimated sludge. Our study indicates that previous adaptation to an N-substituted aromatic is a prerequisite for 5ASA mineralization.

Based on these results, it is clear that one of the bottlenecks of aromatic degradation is the adaptation. Once the microorganisms are adapted, the degradation proceeds at similar rates, independently of the origin of the sludge. According also to the results of this study, it seems that the granular sludge, independently of the source, has a universal capacity to degrade phenol and 4-cresol. In contrast, only some granular sludge sources are able to degrade N-substituted aromatics like 2ABc and 5ASA.

Acknowledgments

This research was supported by the Consejo Nacional de Ciencia y Tecnología from México. Financial aid from the Instituto Mexicano del Petróleo from México is gratefully acknowledged (E.R.F.). Support from the Human Capital and Mobility programme of the European Community is also appreciated (B.D.).

References

1. American Public Health Association (1985) Standard methods for examination of water and wastewater. 16th. ed. American Public Health Association, Washington, D.C.
2. Battersby, N.S., Wilson, V. (1989) Survey of the anaerobic biodegradation potential of organic chemicals in digesting sludge. *Appl. Environ. Microbiol.* 55:433-439.

3. Blum, J.W., Hergenroeder, R., Parkin, G.F., Speece, R.E. (1986) Anaerobic treatment of coal conversion wastewater constituents: biodegradability and toxicity. *J. Water Pollut. Control Fed.* **58**:122-231.
4. Borghans, A.J.M.L., van Driel, A. (1988) Application of the biothane UASB reactor to a chemical waste water, containing phenol and formaldehyde. In: Tilche, A. Rozzi, A. (eds.). 5th. International Symposium on Anaerobic Digestion, pp. 627-630. Monduzzi Editore, Bologna.
5. Boyd, S.A., Shelton, D.R., Berry, D., Tiedje, J.M. (1983). Anaerobic biodegradation of phenolic compounds in digested sludge. *Appl. Environ. Microbiol.* **46**:50-54.
6. Christiansen, N., Ahring, B.A. (1994) Introduction of bioremediation ability into granular sludge. *Wat. Sci. Technol.* **29**:189-193.
7. Donlon, B., Razo-Flores, E., Hwu, C.-S., Field, J., Lettinga, G. (1995) Toxicity and biodegradability of selected N-substituted phenols under anaerobic conditions. In: Hinchee, R.E., Brockman, F.J., Vogel, C.M. (eds.). Microbial processes for bioremediation, Bioremediation 3(8), pp..251-257. Batelle Press, Columbus.
8. Donlon, B., Razo-Flores, E., Field, J. A., Lettinga, G. (1995) Toxicity of N-substituted aromatics to acetoclastic methanogenic activity in granular sludge. *Appl. Environ. Microbiol.* **61**:3889-3893.
9. Donlon, B., Razo-Flores, E., Luijten, M., Swarts, H., Lettinga, G., Field, J. (1997) Detoxification and partial mineralization of the azo dye Mordant Orange 1 in a continuous upflow anaerobic sludge blanket reactor. *Appl. Microbiol. Biotechnol.* **47**;83-90.
10. Dwyer, D., Krumme, M.L., Boyd, S.A., Tiedje, J.M. (1986) Kinetics of phenol biodegradation by an immobilized methanogenic consortium. *Appl. Environ. Microbiol.* **52**:345-351.
11. Fedorak, P.M., Hrudey, S.E. (1986) Anaerobic treatment of phenolic coal conversion wastewaters in semicontinuous cultures. *Wat. Res.* **20**:113-122.
12. Field, J., Lettinga, G., Geurts, M. (1987) The methanogenic toxicity and anaerobic degradability of potato starch wastewater phenolic amino acids. *Biological Wastes* **21**:37-54.
13. Field, J.A., Lettinga, G. (1992) Biodegradation of tannins. In: Sigel, H. (ed.). Metal ions in biological systems, Vol. 28, Degradation of environmental pollutants by microorganisms and their metalloenzymes, pp. 61-97. Marcel Dekker Inc., New York.
14. Frankin, R.J., Koevoets, W.A.A., van Gils, W.M.A., van der Pas, A. (1992) Application of the biobed upflow fluidized bed process for anaerobic wastewater treatment. *Wat. Sci. Technol.* **25**:373-382.
15. Haandel van, A.C., Lettinga, G. (1994) Anaerobic sewage treatment; a practical guide for regions with a hot climate. John Wiley & Sons, Chischester, U.K.
16. Healy J.B., Young, L.Y. (1978) Catechol and phenol degradation by a methanogenic population of bacteria. *Appl. Environ. Microbiol.* **35**:216-218.
17. Healy, J.B., Young, L.Y. (1979) Anaerobic biodegradation of eleven aromatic compounds to methane. *Appl. Environ. Microbiol.* **38**:84-89.
18. Horowitz, A., Shelton, D.R., Cornell, C.P., Tiedje, J.M. (1981) Anaerobic degradation of aromatic compounds in sediments and digested sludge. *Dev. Ind. Microbiol.* **23**:435-444.
19. Hwu, C.-S., Donlon, B., Lettinga, G. (1996) Comparative toxicity of long-chain fatty acid to anaerobic sludges from various origins. *Wat. Sci. Technol.* **34(5-6)**:351-358.
20. Lettinga, G., Velsen, A.F.M. van, Hobma, S.W., de Zeeuw, W., Klapwijk, A. (1980) Use of the upflow sludge blanket (USB) reactor concept for biological treatment, especially for anaerobic treatment. *Biotechnol. Bioeng.* **22**:699-734.
21. Lettinga, G., Hulshoff Pol, L.W., Koster, I.W., Wiegant, W.W., de Zeeuw, W.J., Rinzema, A. Grin, P.C., Roersma, R.E., Hobma, S.W. (1984) High-rate anaerobic waste-water treatment using the UASB reactor. *Biotechnol. Genetic Eng. Rev.* **2**:253-284.
22. Lettinga, G., Field, J.A., Sierra, R., van Lier, J.B., Rintala, J. (1991) Future perspectives for the anaerobic treatment of forestry industry wastewaters. *Wat. Sci. Technol.* **24**:91-102.

23. Lettinga, G., van Haandel, A.C. (1993) Anaerobic digestion for energy production and environmental protection. In: Johansson, T.B., Kelly, H., Reddy, A.K.N, Williams R.H. (eds.). Renewable Energy; sources for fuels and electricity, pp. 817-839. Island Press, Washington D.C.
24. Macarie, H. , Noyola, A., Guyot, J.P. (1992) Anaerobic treatment of a petrochemical wastewater from a terephtalic acid plant. *Wat. Sci. Tech.* **25**:223-235.
25. Razo-Flores, E., Donlon, B., Field, J., Lettinga, G. (1996) Biodegradability of N-substituted aromatics and alkylphenols under methanogenic conditions using granular sludge. *Wat. Sci. Technol.* **33(3)**:47-57.
26. Tarvin, D., Buswell, A.M. (1934) The methane fermentation of organic acids and carbohydrates. *J. Amer. Chem. Soc.* **56**:1751-1755.
27. Young, L.Y., Rivera, M.D. (1985) Methanogenic degradation of four phenolic compounds. *Wat. Res.* **19**:1325-1332.

5

Continuous Detoxification, Transformation and Degradation of Selected Nitroaromatics in Upflow Anaerobic Sludge Blanket (UASB) Reactors

Elías Razo Flores
Brian A. Donlon
Antonio Matamala
Patrick Smulders
Gatze Lettinga
Jim A. Field

Summary

The anaerobic transformation and degradation of selected nitroaromatic compounds by granular sludge was investigated in laboratory-scale upflow anaerobic sludge blanket (UASB) reactors continuously fed with either a volatile fatty acid (VFA) mixture or glucose as a cosubstrate. During the start-up, subtoxic concentrations of 2-nitrophenol (2NP), 4-nitrophenol (4NP), 2,4-dinitrophenol (2,4DNP), 5-nitrosalicylate (5NSA), 4-nitrobenzoate (4NBc), 2,4-dinitrotoluene (2,4DNT), or nitrobenzene (NB) were utilized. The nitroaromatic concentrations were gradually increased during reactor operation and the efficiency of nitro-group reduction was higher than 98%. Reactors treating 4NP, 2,4DNP, and NB readily converted the nitroaromatics to their corresponding aromatic amine; whereas 2NP, 5NSA and 4NBc were mineralized via intermediate formation of their corresponding aromatic amines. 2,4DNT was completely reduced, and the corresponding aromatic amine was partially transformed to an unidentified metabolite. These conversions led to a dramatic detoxification of the nitroaromatics since the reactors were able to treat these compounds at concentrations which were over 30 times higher than the 50% inhibitory concentration to methanogenic activity. Cosubstrate-COD removal efficiencies greater than 87% were achieved, except for 2,4DNP (75%), at loading rates up to 13.3 g COD/L-d even at volumetric loading of nitroaromatics up to 910 mg/L-d.

The sludges sampled from selected reactors at the end of the continuous experiments were assayed for their specific nitroaromatic reducing activity in the presence of different primary substrates. Reduction rates of 44.6, 26.0 and 11.3 mg/g VSS-d were observed for 2NP, 4NP and 5NSA, respectively when utilizing the VFA mixture as primary substrate. Hydrogen, an interspecies reduced compound, and substrates that provide interspecies reducing equivalents; such as butyrate, propionate and ethanol stimulated nitroaromatic reduction; while direct substrates of methanogens, acetate and methanol did not. Anaerobic batch biodegradability assays with the 2NP, 5NSA and 4NBc adapted sludges could readily mineralize the corresponding aromatic amines, (2-aminophenol, 5-aminosalicylate or 4-aminobenzoate) to methane at rates of 14.5, 13.2 and 6.8 mg/g VSS-d, respectively. These activities were sufficient to account for the complete mineralization of aromatic amines formed from the reduction of 2NP, 5NSA and 4NBc in the UASB reactors. The results of this study indicate that UASB reactors can be applied to rapidly detoxify nitroaromatics and certain nitroaromatic compounds are even mineralized.

Biotechnology and Bioengineering (1996) 51:439-449
Biotechnology Progress (1997) submitted for publication

absorbance was detected at 230 nm. Determination of the anaerobic intermediates of aromatic amines degradation were also performed by gas chromatography as has been previously described (15). Authentic standards tested included benzoic acid, carboxycyclohexane, cyclohexanone and phenol. Aromatic amines were also determined colorimetrically after reacting with 4-dimethylaminobenzaldehyde-HCl (Ehrlich Reagent) according to the method described by Oren et al. (42). The pH was determined immediately after sampling with a model 511 pH-meter (Knick, Berlin, Germany) and a model N61 double electrode (Scot Gerade, Hofheim, Germany). All the other analytical determinations were performed as described in *Standard Methods for Examination of Water and Wastewater* (2).

Laboratory UASB Reactor Experiments

The continuous reactor experiments were performed in seven separate glass UASB reactors (0.145 m of length and 0.039 m of internal diameter) with liquid volumes of 160 mL placed in a temperature controlled room at 30 ± 2°C (Figure 5.1). Reactor 1 (R1), Reactor 2 (R2), and Reactor 3 (R3) were inoculated with 20 g of volatile suspended solids (VSS) per liter of Shell Nederland Chemie anaerobic granular sludge. Reactor 4 (R4), Reactor 5 (R5), and Reactor 6 (R6) were inoculated with 20 g VSS/L of an sludge mixture: Shell Nederland Chemie and 2NP-adapted anaerobic granular sludge in a 2:1 ratio. Reactor 7 (R7) was started immediately after the experiment in R4 was finished by shifting the nitroaromatic compound. The reactors were started-up (except R7) with partially neutralized (pH=6.0) VFA mixture (acetate:propionate:butyrate, 23:34:41 on a COD basis) at a concentration of 4 g COD/L for 15 days. Thereafter, the reactors received sub-toxic concentrations of the nitroaromatic compounds in addition to the VFA substrate: R1, 8 mg/L 2NP; R2, 12 mg/L 4NP; R3, 5 mg/L 2,4DNP; R4, 60 mg/L 5NSA; R5, 20 mg/L 4NBc; and R6, 5 mg/L 2,4DNT. Sub-toxic concentrations were defined as those causing a 50% inhibition of acetoclastic methanogens (IC50 values) and were determined previously (11). R7 received 50 mg/L NB in addition to 1 g COD/L of glucose as cosubstrate. The methane production was measured with 10 liter Mariotte flasks filled with a 3% (w/v) NaOH solution to scrub out the carbon dioxide from the biogas

Anaerobic Activity Assays

The specific acetoclastic methanogenic activity test was performed in 120 mL serum vials sealed with butyl rubber septa and aluminum caps. Measurements were performed at 30°C ± 2°C as outlined previously (11). All results are reported in g CH_4-COD/g VSS-d, as the mean value of triplicate values.

Determination of Nitroaromatic Reduction Rate

The nitroaromatic-reducing activity of the sludge sampled from the UASB reactors was also determined. The vials were set-up similarly to the biodegradation technique outlined previously (47) and were incubated overnight at 30°C with shaking. Then the nitroaromatic test compounds (2NP, 4NP or 5NSA) were introduced into the vials at concentrations of 100 mg/L. Reduction of the nitroaromatics to their corresponding aromatic amines was monitored by measuring the loss in the nitro-group absorbance. The effect of electron donors on the nitroaromatic reducing activity was determined by adding the VFA mixture used as in the column experiments (500 mg COD/L), acetate (500 mg COD/L), methanol (432 mg COD/L), and ethanol (432 mg COD/L). The use of hydrogen as primary substrate was tested by pressurizing the vials with a gas mixture of H_2:CO_2 (80:20, vol:vol) to 0.5 bar. The approximate headspace:liquid volume ratio in these vials was 4:1. Sludge that was autoclaved for one hour served as the control. All results are reported as the mean value of triplicate incubated experiments.

Biodegradation of Aromatic Amines

The biodegradation assay was conducted for the granular sludge sampled from all the columns except R3 and R7 as outlined previously (47). The aromatic amines, 2AP, 4AP, 5NSA, 4NBc and 2,4DAT were tested at a concentration of 200, 200, 300, 300, and 130 mg/L in the vials containing sludge samples withdrawn from the reactors treating 2NP, 4NP, 5NSA, 4NBc, and 2,4DNT, respectively. The methane produced was monitored by periodic measurement of the headspace gas content using gas chromatography. Methane production

due to the mineralization of aromatic amines was calculated by subtracting background methane production in the sludge blank controls from that in the vials with test compound. The corrected methane production was expressed as a percentage of the theoretical methane production (TMP) expected from the test chemical mineralization according to the Buswell equation (51). The specific mineralization rate of the aromatic amines was calculated by converting the measured mg COD-CH_4 to mg of compound of the corresponding aromatic amine. Parallel experiments where the sludge received the specific methanogenic inhibitor 2-bromoethanesulfonate (BESA, 50mM) were also established in order to identify intermediates of 2AP and 5ASA degradation. All results are reported as the mean value of triplicate incubated experiments.

Chemicals

Chemicals were purchased from either Jannsen Chimica (Tilburg, The Netherlands), Merck (Darmstadt, Germany) and Sigma (Bornem, Belgium). All chemicals were of the highest purity available and were not purified further. The purity of all standards was 95% or greater.

5.3 Results

Anaerobic Treatment of Nitroaromatics in UASB Reactors

Seven laboratory scale UASB reactors were operated in order to investigate the continuous anaerobic treatment of nitroaromatics. All the reactors (except R7) were initially started up after a 15 day adaptation period to VFA. After this adaptation time period the reactors were initially fed with sub-toxic concentrations of the nitroaromatics. Either VFA or glucose were used as a cosubstrate to provide the electrons for the reduction of nitroaromatics. The concentration of nitroaromatic in the influent was increased periodically after at least 10 hydraulic retention times (HRT, equal to 8 h, unless otherwise indicated) and when greater than 75% removal of nitroaromatics and cosubstrates was obtained. The rationale behind this

approach is that the sludge is expected to withstand higher concentrations of the incoming nitroaromatics by reducing the nitro-group to the less toxic amino-group. The operational parameters and treatment efficiencies obtained during the continuous operation of the seven UASB reactors treating 2NP (R1), 4NP (R2), 2,4DNP (R3), 5NSA (R4), 4NBc (R5), 2,4DNT (R6), and NB (R7) in the final period of the reactor operation are summarized in Table 5.1.

The time course of the treatment performance of R1 treating 2NP is shown in Figure 5.2. The reduction of 2NP was highly efficient throughout the entire operation of the reactor, even after increasing the 2NP concentration up to 300 mg/L period (Days 180-194). 2NP concentrations higher than 350 mg/L exhibited toxicity for the biomass (downward dotted arrow in Figure 5.2A). However, the reactor quickly recovered when the influent concentration was lowered to 200 mg/L. By the end of the experiment up to 300 mg/L 2NP in the influent could be tolerated. From day 15 to day 60, 2NP was converted to 2AP in stoichiometric quantities. After day 60, less than 20% of the nitrophenol removed was recovered as the corresponding aromatic amine (Figure 5.2C). This indicates that the 2AP formed was being further transformed as was evident from the decrease in UV_{209} which is indicative of aromatic compounds. The reduction of the nitro group to the less toxic amino substituent ensured that a detoxification of the influent was obtained and there was greater than 90% removal of VFA. In this reactor, the measured methane production accounted for 101% of the VFA consumed (Table 5.1).

The time course of the treatment performance of R2 treating 4NP is shown in Figure 5.3. In this reactor a highly efficient reduction of 4NP to the corresponding aromatic amine (>99%), 4AP, was also observed (Figure 5.3C). This reduction was responsible for the detoxification of the influent even after increasing the 4NP concentration up to 260 mg/L. Nitroaromatic concentrations higher than 260 mg/L were toxic as indicated by the downward dotted arrow in Figure 5.3A. As soon as the concentration was lowered again, the reactor recovered. Unlike R1, the aminophenol was always recovered in near stoichiometric yields; there was 94% 4AP recovery as determined by HPLC in this reactor. This indicates that no further conversion of the aminophenol was observed although the reactor was in operation for

TABLE 5.1 The operational parameters and treatment efficiency during continuous operation of the UASB reactors (160 ml) treating 2-nitrophenol (2NP), 4-nitrophenol (4NP), 2,4-dinitrophenol (2,4DNP), 5-nitrosalicylic acid (5NSA), 4-nitrobenzoic acid (4NBC), 2,4-dinitrotoluene (2,4DNT), and nitrobenzene (NB) together with cosubstrates during the final period of reactors operation.

	2NP	4NP	2,4DNP	5NSA	4NBC	2,4DNT	NB
Final period reactor operation (d)	180 to 194	180 to 194	180 to 194	200 to 253	300 to 356	162 to 202	1-100
Operational parameters							
Cosubstrate influent (g COD/L)	4.18[a]	3.76[a]	3.81[a]	3.6[a]	3.6[b]	4.0[a]	1.0[b]
Cosubstrate loading rate (g COD/L-d)	12.7	11.4	11.5	13.3	9	12.8	0.9
Nitroaromatic conc. influent (mg/L)	300	260	30	150	125	120	50
Nitroaromatic loading rate (mg/L-d)	910	790	91	553	312	384	43
Hydraulic retention time (h)	8	8	8	6.5	9.6	7.5	28
Efficiency							
Cosubstrate removal (%)	99.3 ± 0.4	99.1 ± 0.4	75.1 ± 6.2	99.5 ± 0.5	96.8 ± 1.4	98.5 ± 1.8	86.6 ± 4.3
Cosubstrate conversion to CH_4 (%COD_{in})	101.3 ± 8.2	88.5 ± 10.6	68.9 ± 8.7	94 ± 21.5	101.1 ± 10	97.6 ± 15.2	89.4 ± 16.3
Nitroaromatic elimination (%)[c]	98.7 ± 0.2	99.2 ± 0.3	68.2 ± 3.1[d]	>99.2	>99.2	>99.9	>99.9
Aromatic amines molar yield (%)[c]	1.83 ± 0.9	93.7 ± 4.2	103 ± 6.6	2.8 ± 2.8	15.5 ± 3.9	52.4 ± 5.6	91.5 ± 1.3

[a] Volatile fatty acids (VFA) mixture as a cosubstrate.
[b] Glucose as a cosubstrate.
[c] Determined by HPLC.
[d] Measured by loss in absorbance of the nitro-group as determined by UV-VIS spectroscopy (see Materials and Methods).

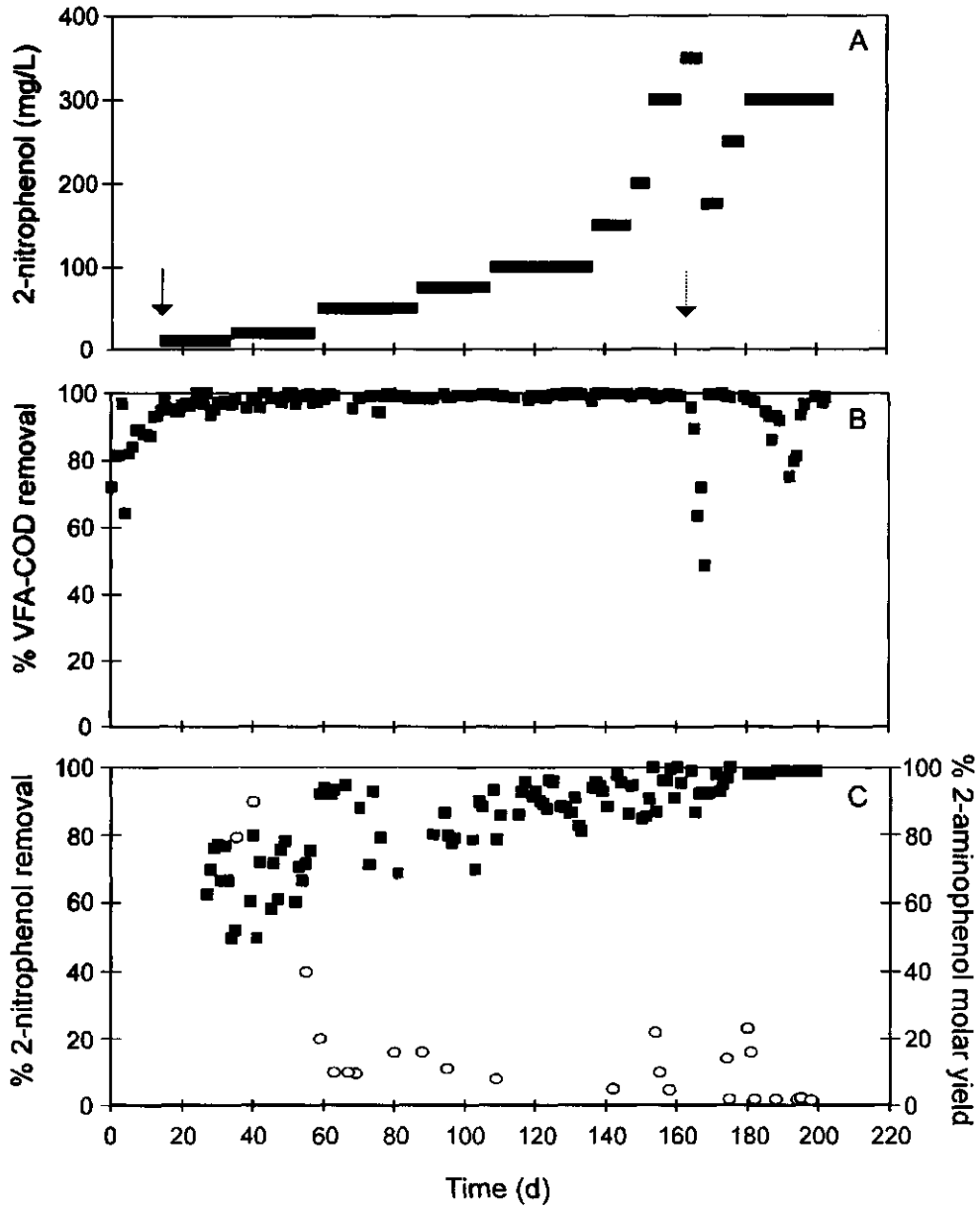


Figure 5.2 Operational efficiency during the continuous anaerobic treatment of 2-nitrophenol: (A) 2-nitrophenol concentration in the influent; (B) %VFA removal; (c) % 2-nitrophenol removal as determined by loss in UV at 370nm (■), recovery as a percentage (molar terms) of 2-nitrophenol eliminated (o). The concentration of 2-aminophenol was determined colorimetrically after reacting with 4-dimethylaminobenzaldehyde-HCl (Ehrlich Reagent) according to the method described by Oren et al. (42). Solid arrow indicates the introduction of 2-nitrophenol in the influent. Dotted arrow indicates nitroaromatic toxicity.

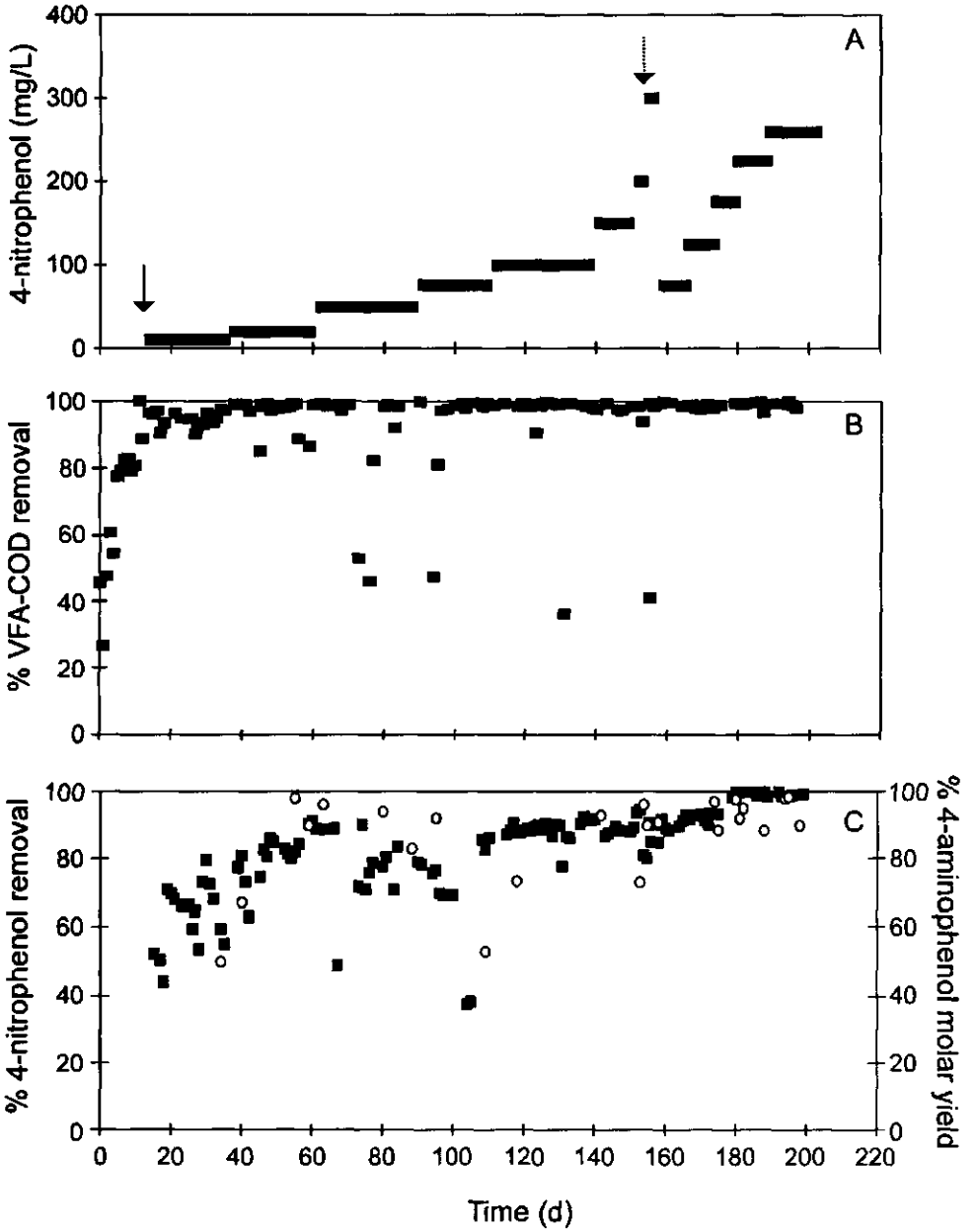


Figure 5.3 Operational efficiency during the continuous anaerobic treatment of 4-nitrophenol. (A) 4-nitrophenol concentration in the influent; (B) %VFA-COD removal; (C) % 4-nitrophenol removal as determined by loss in UV at 400nm (■), 4-aminophenol recovery as a percentage (molar terms) of 4-nitrophenol eliminated (o). For the other symbols see legend of Figure 5.2.

approximately 200 days. High VFA removal efficiencies were obtained over the entire period and the measured methane accounted for 89% of the VFA consumed (Table 5.1).

Figure 5.4 shows UV-VIS scans of influent and effluent samples from the reactors treating 2NP and 4NP. When comparing these scans to similar scans made for nitrophenol and aminophenol standards it can be concluded that 4NP was converted to 4AP. This was confirmed with the HPLC (Table 5.1). On the other hand, it appears that 2NP was not only reduced to 2AP but it was degraded further since only a trace of the 2AP spectrum could be detected in the effluent. The effluent UV-VIS absorbance (Figure 5.4B) was similar to that obtained in the effluent of a reactor treating VFA only. Analysis of the reactor performance by UV-VIS determination demonstrated that greater than 94% nitro-group removal and 87% removal of the aromatic structure of 2AP was obtained in R1. There was greater than 99% nitro-group removal in R2; whereas, there was 84% recovery of the aromatic structure of 4NP.

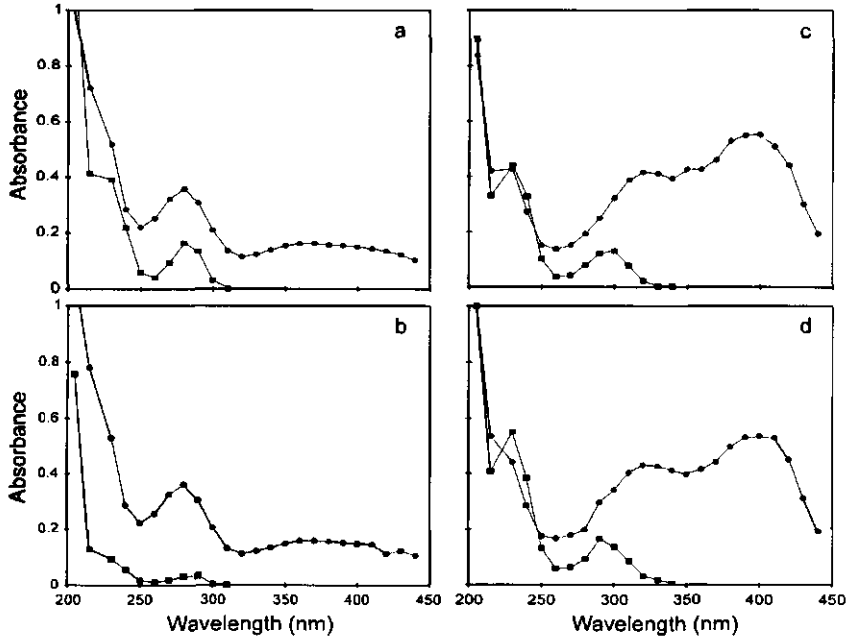


Figure 5.4 UV-VIS scans of (a) authentic 2-nitrophenol (•) and 2-aminophenol (■) standards, (b) influent (•) and effluent (■) samples from reactor treating 2-nitrophenol, (c) authentic 4-nitrophenol (•) and 4-aminophenol (■) standards, (d) influent (•) and effluent (■) samples from reactor treating 4-nitrophenol. All samples were diluted to give a concentration of 72μM in 0.2M phosphate buffer pH 7.

R3 treating 2,4DNP gave high nitro-group reduction efficiencies up to concentrations of 25 mg/L 2,4DNP in the influent for the first 116 days of operation. Highly reliable VFA (84%) removal was also obtained in this period. In order to study the effects of nitroaromatic feed interruptions upon reactor performance, on day 117 to 124, the dinitrophenol was omitted from the influent. After one week operation, 2,4DNP was reintroduced into the reactor influent (25 mg/L). This caused a major perturbance in the reactor performance and resulted in lower VFA removal rates (approx. 43%). The influent nitrophenol and VFA concentrations were then reduced to 5 mg/L and 0.5 g COD/L, respectively, and the reactor became more stable. Afterwards, nitrophenolic and organic loading rates were gradually increased. At the end of the reactor operation, an apparent steady-state was reached with nitro-group and VFA removal rates of 68 and 75%, respectively. Stoichiometric production of 2,4DAP from the elimination of the parent nitroaromatic compound in this reactor was confirmed by HPLC analysis (Table 5.1). The methane production accounted for 69% of the VFA consumed.

5NSA was reduced at high efficiencies during the whole experimental period in R4 (Figure 5.5 and Table 5.1). 5ASA was recovered in stoichiometric amounts during the first 90 days of operation. However after day 100, less than 20% of the 5NSA removed was recovered as the corresponding aromatic amine (Figure 5.5C), indicating that 5ASA was transformed further. The system demonstrated to be sensitive to pH changes; on day 70 and 116 the pH dropped from 7.3 to 6.7 and 6.1, respectively, producing a sharp decrease in both the VFA removal and nitro reduction (upward dotted arrows in Figure 5.5A). Consequently, the 5NSA concentration in the influent was lowered to protect the biomass against toxic effects. After the VFA removal and the nitro-reduction stabilized, the 5NSA concentration in the influent was again increased to a maximum of 150 mg/L. A further increase of 5NSA in the influent to 200 mg/L produced a temporary accumulation of 5ASA in the effluent between days 170 and 220. The VFA removal was quite high over the course of the experimental period, and the methane production accounted for 94% of the VFA consumed during the final period of reactor operation.

The time course of the treatment performance of R5 treating 4NBc is shown in Figure 5.6. R5 was operated for 356 day period. VFA mixture (4 g COD/L) was used as cosubstrate

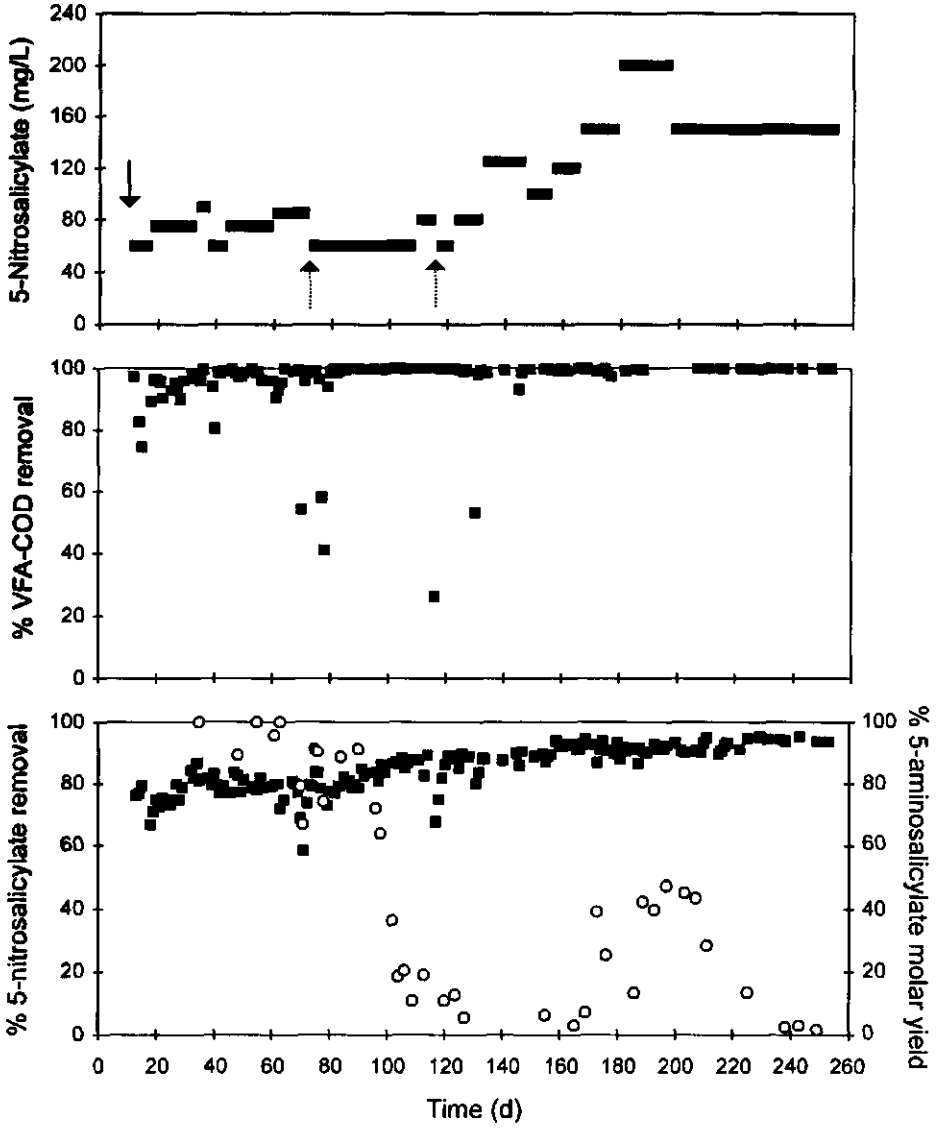


Figure 5.5 Operational efficiency during the continuous anaerobic treatment of 5-nitrosalicylate. (A) 5-nitrosalicylate concentration in the influent; (B) %VFA-COD removal; (c) % 5-nitrosalicylate removal as determined by loss in UV at 280nm (■), 5-aminosalicylate recovery as a percentage (molar terms) of 5-nitrosalicylate eliminated (o). Solid arrow indicates the introduction of 5-nitrosalicylate in the influent. Up dotted arrow indicates pH drop. Down dotted arrow indicates nitroaromatic toxicity.

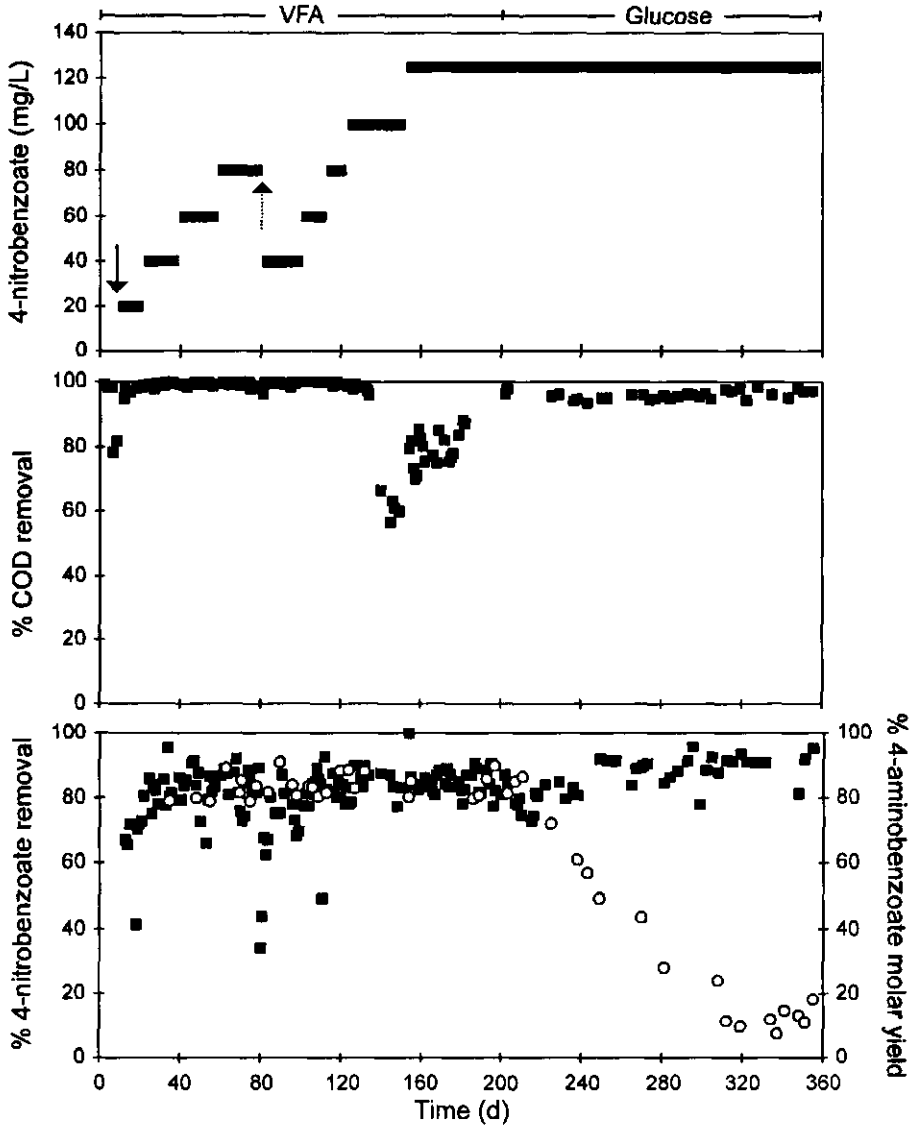


Figure 5.6 Operational efficiency during the continuous anaerobic treatment of 4-nitrobenzoate. (A) 4-nitrobenzoate concentration in the influent; (B) %COD removal (glucose-COD); (c) % 4-nitrobenzoate removal as determined by loss in UV at 320nm (■), 4-aminobenzoate recovery as a percentage (molar terms) of 4-nitrobenzoate removed (o). For the other symbols see legend of Figure 5.5.

from day 0 to day 200, whereas glucose (4 g COD/L) was used from day 201 and onwards. During the VFA period, 4NBc was reduced at high efficiencies (> 85% as measured by UV-VIS), and 4ABc was recovered in stoichiometric amounts, indicating that the aromatic amine produced was not degraded. On day 80 there was a pH drop (upward dotted arrow in Figure 5.6A) and 4NBc concentration in the influent was lowered to protect the biomass against toxic effects. Afterwards the 4NBc concentration was again increased gradually up to 120 mg/L. After the cosubstrate was shifted to glucose, the 4NBc reduction was still highly efficient. On day 225, the recovery of 4ABc steadily started to decrease to less than 15% (molar yield) by the end of reactor operation (Table 5.1), indicating that a population of 4ABc degraders had developed. The COD removal (VFA or glucose) was greater than 95% during almost the entire period operation. In this reactor the measured methane production accounted for 101% of the glucose-COD consumed.

R6 treating 2,4DNT gave high nitro-group reduction efficiencies over the course of the experimental period (>90% as measured by UV-VIS), even at 2,4DNT concentrations of 120 mg/L. It was not possible to apply higher 2,4DNT concentrations due to its limited aqueous solubility. 2,4DAT was recovered in stoichiometric amounts until day 125. Thereafter, the aromatic amine accounted for only 52% molar yield of the 2,4DNT removed, indicating its further transformation. VFA removal efficiency was quite high over the course of the experiment (98.5%). Methane production accounted for 97% of the VFA consumed in this reactor.

R7 treating NB was started in R4 immediately after the corresponding experiment was finished by shifting the nitroaromatic compound from 4NBc to NB. The concentration of both NB and glucose was kept constant for the entire period of reactor operation (100 days). The reduction of NB was highly efficient (>99.9%) with the concomitant production of AN. There was 92% AN recovery (molar yield) as determined by HPLC in this reactor, indicating no further conversion of the aromatic amine produced. Glucose-COD removal efficiency was greater than 87% and the methane production accounted for 89% of the glucose-COD removed.

At the end of the continuous experiments in R1, R2 and R3, the biomass concentrations and the maximal methanogenic activity of the sludge was measured (Table 5.2). The specific methanogenic activity of the sludge from the column fed 4NP was the highest. There was only a slight decrease in maximal methanogenic activity in all reactors compared to the initial methanogenic activity. The quantity of sludge in the reactors treating 4NP and 2,4DNP were similar to the starting inoculum concentration (20 g VSS/L). However, there was a doubling of biomass in the reactor treating 2NP (R1) after 200 days of continuous operation which is indicative of growth and retention of granular sludge.

TABLE 5.2 The methanogenic activity and biomass concentrations in reactors R1, R2, and R3 before and after 200 day operation.

Granular sludge	Activity (g CH_4-COD/g VSS-d)	Sludge concentration (g VSS/L)
Inoculated in all three reactors	0.891	20.0
R1 (2NP) reactor	0.633	48.7
R2 (4NP) reactor	0.877	16.7
R3 (2,4DNT) reactor	0.513	21.5

Effects of Primary Substrate on Nitroaromatic Reduction

At the end of the reactor operation, sludge was withdrawn from the lab-scale reactors (R1, R2 and R4) to study the extent of chemical and biological nitro-group reduction (Table 5.3). A small amount of chemical nitroaromatic reduction to aromatic amine was obtained using autoclaved granular sludge. However, the rate was doubled when living sludge was used without any primary substrate, indicating a biological enhancement of the nitroaromatic reduction rate with endogenous substrate. The VFA mixture, acetate, ethanol, methanol, and hydrogen were tested as a source of additional reducing equivalents for enhancing reductive transformation of nitroaromatics. Acetate and methanol did not improve either the yield of

aromatic amines or the rate of nitroaromatic reduction beyond that obtained with endogenous substrate (sludge control) sample. However, the rate of nitro- group reduction and the percentage aromatic amines recovery was enhanced when hydrogen and other primary substrates such as ethanol and VFA mixture were used.

TABLE 5.3 Rates of nitro-group reduction and molar yield of aromatic amines by granular sludge sampled from the continuous experiments at the final period of reactor operation (R1, R2, and R4) in the presence of various primary substrates.

Substrate	Nitroaromatic reduction rate (mg/gVSS-d)	Molar yield aromatic amines[a] (%)
2-nitrophenol		
None and sludge autoclaved	9.92	12.8
None	18.8	54
Acetate	20.0	55
VFA mixture[b]	44.6	102.8
4-nitrophenol		
None	10.6	19.9
Acetate	10.5	29.5
VFA mixture	26.0	96.6
Ethanol	16.8	NM[c]
Hydrogen	36.2	NM
Methanol	11.0	NM
5-nitrosalicylic acid		
None and sludge autoclaved	3.06	NM
VFA mixture	11.32	99.8

[a] Molar yields determined for 2-aminophenol, 4-aminophenol, and 5-aminosalycilic acid after 69, 102, and 147 h, respectively.

[b] VFA mixture, which was partially neutralized (pH = 6), contained acetate:propionate:butyrate in a ratio of 23:34:41 on a COD basis.

[c] NM; not measured.

As shown in Figure 5.7, 2NP was degraded in the presence of VFA mixture (500 mg COD/L) substrate without a lag period together with a concomitant release of 2AP as the biotransformation product. This in turn was further degraded as can be seen from the decreases in the UV_{209} absorbance indicating aromatic structure and by a loss in the colorimetrically determined aromatic amine concentration.

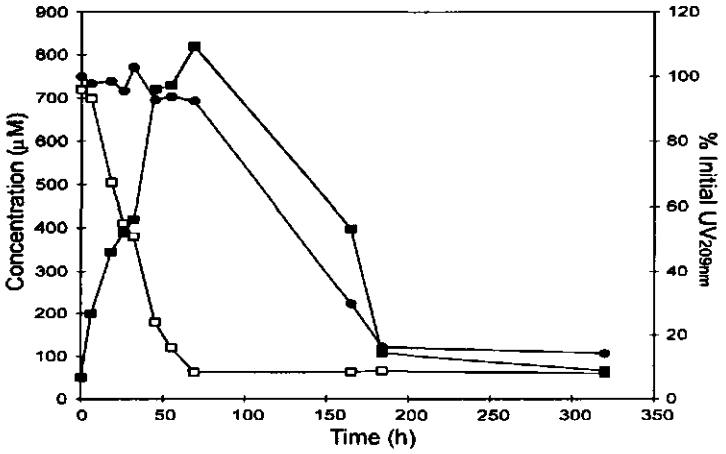


Figure 5.7 Profile of 2-nitrophenol biodegradation (□) and temporal 2-aminophenol accumulation (■) using sludge recovered from Reactor 1 (treating 2-NP) at the end of the continuous experiments. Nitrophenol concentration was determined by measuring the absorbance at 370nm by spectroscopy. Aromatic ring cleavage was determined by measuring the absorbance at 209nm (•) as the extinction coefficients of 2-nitrophenol/2-aminophenol prepared in phosphate buffer (0.2M, pH 7) overlap at this wavelength. Confirmation of 2-aminophenol concentrations was also determined colorimetrically.

Mineralization of N-substituted Aromatics by Sludge from the UASB Reactors

Background methane production can interfere in the determination of the biodegradability of highly toxic compounds tested at low concentrations in anaerobic bioassays based on methane production potential (47, 49). Consequently, aromatic amines instead of nitroaromatics were used to study the mineralization potential of the sludges sampled from the continuous reactors (except R3 and R7). The rationale behind our approach is that it was easier and more accurate to examine the mineralization of the daughter aromatic amines which

could be tested at higher concentrations than their toxic parent nitroaromatic compounds. Table 5.4 indicates that the methane obtained from sludges receiving 2AP, 5ASA or 4ABc as a sole substrate was higher than 75% of the TMP, suggesting their complete anaerobic mineralization. 4AP and 2,4DAT amended sludges produced low %TMP after long incubation periods, indicating the recalcitrance of these compounds.

TABLE 5.4 Anaerobic mineralization of aromatic amines to methane by sludge sampled from the reactors treating the corresponding nitroaromatic compounds.

	R1	R2	R4	R5	R6
Aromatic amine tested	2AP	4AP	5ASA	4ABc	2,4DAT
Sampling day	194	194	175	175	175
Concentration assayed (mg/L)	500	500	300	300	130
Methane[a] (%TMP)	98.7 ± 6.1	22.8 ± 3.4	74.9 ± 0.8	82 ± 2.9	4.9 ± 5.1
Lag phase (day)	<3	60	<5	60	>170
Max. mineralization rate (mg/g VSS-d)	14.5	0.76	13.2	6.8	~0

[a] Methane production was corrected for the values in the sludge blank controls, which were used for comparison to the theoretical methane production (TMP) of the test compound.

Parallel experiments where conducted with the specific methanogenic inhibitor, BESA, with the sludges that were mineralizing 2AP and 5ASA in order to determine intermediate product formation. BESA effectively blocked the methane production from the aromatic amines. The aromatic amines instead were converted to mainly acetate in both cases, which equaled approximately 75% of the COD of the aromatic amines added after correcting for the acetate levels in the sludge blank controls (results not shown). However, we did not detect phenol, benzoic acid, carboxycyclohexane, cyclohexanone nor any other aromatic intermediates in uninhibited or BESA inhibited cultures.

5.4 Discussion

Anaerobic Treatment of Nitroaromatics in UASB Reactors

Previously, we reported that nitroaromatic compounds were several orders of magnitude more toxic than their reduced aromatic amine products (11). Since it was previously reported that anaerobic bacteria readily reduce nitroaromatics (7, 23, 41), considerable detoxification would be expected to result from the treatment of the highly toxic nitroaromatic compounds in anaerobic continuous reactors.

The results of this study confirm that extensive detoxification can be obtained. Laboratory scale UASB reactors treating the nitroaromatics were able to convert cosubstrate-COD (either VFA or glucose) to methane at organic loading rates up to 13.3 g COD/L-d, with greater than 87% cosubstrate-COD removal efficiency (except R3), even though the influent concentration of the nitroaromatics was 30-fold higher than the 50% inhibiting concentration to acetoclastic methanogenesis. The specific methanogenic activity of the sludges from the reactors treating the nitrophenols indicated no significant decrease in the performance of the VFA-degrading methanogenic consortia. Stoichiometric reduction of 4NP, 2,4DNP and NB to 4AP, 2,4DAP, and AN were obtained in R2, R3, and R7; whereas, degradation of 2NP, 5NSA and 4NBc to nonaromatic products was observed in R1, R4, and R5 via intermediate formation of the corresponding aromatic amines respectively. In the case of R6, 2,4DNT was completely reduced, but only 52% of the compound was recovered as 2,4DAT, suggesting partial transformation to an unidentified aromatic compound.

In this study, very high volumetric loading rates of 2NP and 4NP (910 and 790 mg/L-d, respectively) were treated in laboratory scale UASB reactors. The maximum 2NP sludge loading rate applied in the continuous reactor experiment (18.7 mg/gVSS-d) was approximately the half of the specific rate obtained in batch assays with the VFA mixture (44.6 mg/g VSS-d) as primary substrate. This indicates that the reactor was underloaded with respect to its nitrophenol removal potential. Greater than 98% removal of nitrophenol were obtained in both reactors. These removal rates are much higher than that obtained in a lab-scale

digestor (0.36 mg/L-d with 82% removal of 4NP) reported by Haghighi Podeh et al. (26). After more than 600 days of acclimatization to a mixture of hydroxylated aromatics, Giot *et al.* (21) reported that the maximum loading rate tolerated by the adapted biomass was 30 mg 2NP/L-d in a fixed-film digester. However, Tseng and Lin (54) reported COD and nitrophenol removal efficiencies of greater than 90% in the treatment of a synthetic wastewater (glucose, and beef extract) being loaded with 900 mg mononitrophenols/L-d in an anaerobic biological fluidized bed reactor system. Continuous reactor studies for the treatment of 2,4DNP, 5NSA, and 4NBc are not reported in the literature. 2,4DNT removal of more than 99.9% has been reported by Berchtold et al. (4), using an fluidized-bed anaerobic granular activated carbon (GAC) reactor operated for almost 600 days with ethanol as cosubstrate. However, the contribution of adsorption onto the GAC in the overall removal of 2,4DNT is not described. The 2,4DNT loading rate applied in R6 of our study was the double of that applied in the GAC reactor (183.3 mg/L-d) of the cited study.

Detoxification based on the facile reduction of highly toxic polychlorinated aromatic compounds to less chlorinated products of lower toxicity was suggested by Sierra and Lettinga (50). The successful operation of continuous anaerobic reactors fed with the highly toxic compound, pentachlorophenol, reliant upon this reductive detoxification have been cited in the literature (39, 60). Wu et al. (60) reported high removal efficiencies of pentachlorophenol at loading rates of up to 96 mg/L-d in laboratory-scale UASB reactor fed with a mixture of VFA and methanol as cosubstrate. The dechlorination of pentachlorophenol was compared in two reactor systems (UASB and fixed-film) and a better performance and higher process stability was observed in the UASB system (39).

The effective treatment regimens cited here for nitro and polychlorinated aromatics rely on the easy reduction of these compounds to products of lower toxicity (11, 22, 50). Furthermore, immobilizing anaerobic bacteria and maintaining high concentrations of biomass in the reactor are factors which are known to improve the tolerance to toxic substances by anaerobic treatment systems (13, 43); methanogens inside granules are protected from exposure to toxic compound. Biogas production together with an efficient influent distribution system ensured a completely mixed hydraulic regime in our UASB reactors thereby preventing

localized high concentrations of the toxic parent compounds. Previous studies have demonstrated that adequate hydraulic mixing is brought about through high biogas production in anaerobic reactors (24, 38).

Effects of Primary Substrate on Nitroaromatics Reduction

Methanol and acetate did not support significant nitroaromatic reducing activity during the time course of the batch experiments performed on the sludge sampled from the reactors. The rate of nitro group reduction was similar to that determined for the unamended sludge control. Neither would be expected to produce large amounts of interspecies hydrogen in mesophilic granular sludge consortia (18, 20, 45). When granular sludge was incubated with methanol in the presence or absence of the specific eubacterial inhibitor, vancomycin, it was shown that this substrate is almost exclusively utilized directly by methanogens (18). There are some reports, however, that these compounds can produce some hydrogen under thermophilic conditions or if methanogens are inhibited (9, 61). However, our experimental results did show that the addition of H_2 or organic compounds which more readily provide interspecies hydrogen (propionate, butyrate, ethanol) under mesophilic conditions significantly enhanced the rate of nitroaromatic reduction and yield of amino aromatics. These findings are in agreement with previous studies that investigated the role of various electron donors on the reduction of chlorinated aromatics (20) and aromatic aldehydes (53) by methanogenic consortia.

When comparing the reduction rate of nitroaromatics using the VFA mixture, it was observed that the nitro-group reduction in the *ortho*-position proceeded two and four times faster with respect to the *meta*- and *para*-position, respectively. This results indicates that the position of the nitro-group in relation to the other susbstituents in the aromatic ring plays a key role in the rate of nitro-group reduction. Preuss and Rieger (46) indicated that the reduction rate of nitro compounds in general is determined by the chemical properties of the whole molecule and is therefore influenced by other ring substituents. Hudlicky (30) reported a preferential reduction of nitro group in the *ortho*-position with resonance electron-donating groups (e.g., $-NH_2$ or -OH) in the 1-position for both chemical and enzymatic reduction.

McCormick et al. (37) stated that the rate of reduction of nitro compounds by enzyme preparations from *Veillonella alkalescens* increases with increasing electron withdrawing power of the groups at the *para*-position in the following order: $-NH_2$<-OH<-H<$-CH_3$<-COOH<$-NO_2$. The same authors stated that biologically mediated reduction mainly occurred at the *ortho*- rather than at the *para*-position in the case of nitroanilines and nitrophenols. On the other hand, the rates of nitroreduction increases as more nitro groups are placed on the aromatic ring (37).

Mineralization of Aminoaromatics by Sludge from Reactors

The anaerobic sludges sampled from the reactors were incubated with the non toxic concentrations of aromatic amines corresponding to the parent nitroaromatic compound in the reactor feed. It was observed that 2AP, 5ASA, and 4ABc were completely mineralized to methane, while 4AP was only partially (22%) mineralized, and 2,4DAT was not mineralized at all. The original sludge inoculum was also able to mineralize 2AP albeit after a lag phase of 120-day (47). The long lag phase prior to 4ABc mineralization was due to the time of sampling; the sludge was sampled from the reactor on day 175 when it was not yet fully adapted. 4ABc degradation was first evident in R5 after day 225. In the batch experiments assaying the conversion of 2AP to methane, a specific mineralization rate of 14.5 mg/g VSS-d was observed. This rate was similar to the 2NP sludge loading rate used in the continuous column experiment, which when expressed in terms of 2AP equivalents amounted to 14.4 mg/g VSS-d. Any further increase in the nitrophenol loading rate would have resulted in incomplete 2AP removal in this reactor.

Previous reports in the literature suggest that 2AP can be completely mineralized, by municipal digested sludge and sediment samples, under methanogenic conditions (3, 40, 41); whereas, 4AP is sometimes mineralized (40, 41) and other times is left unmetabolized (3). 4ABc is also reported to be degraded in methanogenic consortia (3, 47). The mineralization of 5ASA was reported previously using 2NP-adapted granular sludge after a lag phase of 65 days (47). 2,4DAT was not mineralized in batch assays but the compound was removed in part,

indicating that 2,4DAT was partially transformed to a non-identified and non-degradable metabolite in R6.

Although few studies have addressed the biodegradability pathways of 2AP under methanogenic conditions, some have shown that transformation reactions occur via deamination to phenol (54) or by carboxylation-dehydrogenation to 3-aminobenzoate (5). However, these compounds were not detected as intermediates in our consortia even in experiments where the sludge received the specific methanogenic inhibitor BESA in order to block methanogenesis. Neither was it possible to detect aromatic intermediates during 5ASA degradation. The only compound detected in these experiments was acetate in relatively high yields. Consequently, the results of our study indicate that the degradation of 2NP, 5ASA and 4NBc proceed via nitrogroup reduction to aromatic amines, which are subsequently mineralized to methane and carbon dioxide via homoacetogenic fermentation. In a similar fashion, Funk et al. (19) reported that during the anaerobic degradation of 2,4,6-trinitrotoluene acetate accumulated as the end product by strictly anaerobic soil microflora, which was formed via the intermediates methylphloroglucinol and *p*-cresol.

The results of this study indicate that UASB reactors can be applied to rapidly detoxify nitroaromatics and that certain nitroaromatics are even mineralized in the methanogenic consortia. However, several aromatic amines accumulated in the continuous reactors. These can be mineralized by a subsequent aerobic treatment step. Sequenced anaerobic-aerobic systems have been successfully applied for the treatment of aromatic compounds (16, 62). NB and 2,4DNT were completely mineralized using this approach (4, 10). Alternatively, anaerobic degradation can be obtained using electron acceptors aside from CO_2. Mineralization of AN under denitrifying and sulfate reducing conditions is reported in the literature (1, 48). In both cases carboxylation of AN in the *para*-position producing 4ABc was the initial step for further degradation. The 4ABc-adapted granular sludge from R7 was unable to carboxylate AN, confirming the methanogenic recalcitrance of AN (1).

Acknowledgments

Funding from the Consejo Nacional de Ciencia y Tecnología and the Instituto Mexicano del Petróleo from México is appreciated (by E.R-F.). Support from the Human Capital and Mobility programme of the EC is gratefully acknowledged (by B.D.).

References

1. Alexandra de, M., O'Connor, O.A., Kosson, D.S. (1993) Metabolism of aniline under different anaerobic electron-accepting and nutritional conditions. *Environ. Toxicol. Chem.* **13**:233-239.
2. American Public Health Association. (1985) Standard methods for examination of water and wastewater, 16th ed. American Public Health Association, Washington, D.C.
3. Battersby, N. S., Wilson, V. (1989) Survey of the anaerobic biodegradation potential of organic chemicals in digesting sludge. *Appl. Environ. Microbiol.* **55**:433-439.
4. Berchtold, S.R., van der Loop, S.L., Suidan, M.T., Maloney, S.W. (1995) Treatment of 2,4-dinitrotoluene using a two-stage system: Fluidized-bed anaerobic granular activated carbon reactors and aerobic activated sludge reactors. *Water Environ. Res.* **67**:1081-1091.
5. Bisaillon, J. G., Lepine, F., Beaudet, R., Sylvestre, M. (1993) Potential for carboxylation-dehydroxylation of phenolic compounds by a methanogenic consortium. *Can. J. Microbiol.* **39**:642-648.
6. Blum, D. J. W., Speece, R. E. (1991) A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. *Res. J. Water Pollut. Control. Fed.* **63**:198-207.
7. Boopathy., R. (1994) Transformation of nitroaromatic compounds by a methanogenic bacterium, Methanococus sp. (strain B). *Arch. Microbiol.* **162**:167-172.
8. Borghans, A.J.M.L., van Driel, A. (1988) Application of the biothane UASB reactor to a chemical wastewater, containing phenol and formaldehyde. In: Tilche, A., Rozzi, A. (eds.). 5th. International Symposium on Anaerobic Digestion, pp 627-630. Monduzzi Editore, Bologna.
9. Cord-Ruswisch, R., Seitz, H. J., Conrad, R. (1988) The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. *Arch. Microbiol.* **149**:350-357.
10. Dickel, O., Haug, W., Knackmuss, H. J. (1993) Biodegradation of nitrobenzene by a sequential anaerobic-aerobic process. *Biodegradation* **4**:187-194.
11. Donlon, B., Razo-Flores, E., Field, J., Lettinga, G. (1995) Toxicity of N-substituted aromatics to acetoclastic methanogenic activity in granular sludge. *Appl. Environ. Microbiol.* **61**:3889-3893.
12. Dorlgan, J., Hushon, J. (1976) Air pollution assessment of nitrobenzene. Technical report MITRE-7228. The Mitre Corp., MacLean, Va.
13. Dwyer, D., Krumme, M.L., Boyd, S.A., Tiedje, J.M. (1986) Kinetics of phenol biodegradation by an immobilized methanogenic consortium. *Appl. Environ. Microbiol.* **52**:345-351.
14. Eckenfelder, W. W. (1989) Industrial Water Pollution Control. McGraw-Hill, New York.
15. Field, J. A., Lettinga, G., Geurts, M. (1987) The methanogenic toxicity and anaerobic degradability of potato starch wastewater phenolic amino acids. *Biological Wastes* **21**:37-54.
16. Field, J. A., Stams, A. J. M., Kato, M., Schraa, G. (1995) Enhanced biodegradation of aromatic pollutants in coculture of anaerobic and aerobic bacterial consortia. *Antonie van Leeuwenhoek* **67**:47-77.

17. Fishbein, L. (1984) Aromatic amines. In: Hutzinger, O.(ed.). The handbook of environmental chemistry, Volume 3 Part C, pp 1-40. Springer-Verlag, New York.
18. Florencio, L., Field, J. A., Lettinga, G. (1994) The importance of cobalt for individual trophic groups in an anaerobic methanol degrading consortium. *Appl. Environ. Microbiol.* **60**:227-234.
19. Funk, S., Roberts, D. J., Crawford, D. L., Crawford, R. L. (1993) Initial-phase optimization for bioremediation of munition compound-contaminated soils. *Appl. Environ. Microbiol.* **59**:2171-2177.
20. Gibson, S.A., Sewell, G. W. (1992) Stimulation of reductive dechlorination of tetrachloroethene in anaerobic aquifer microcosms by addition of short-chain organic acids or alcohols. *Appl. Environ. Microbiol.* **58**:1392-1393.
21. Giot, R., Van Damme, F., Naveau, H. P., Nyns, E. J. (1995) Acclimation of a methanogenic consortium to a mixture of hydroxylated aromatic compounds. In: Hinchee, R. (ed.). Third International In-Situ and On-Site Bioreclamation Symposium. April, San Diego, USA.
22. Gorontzy, T., Kuver, J., Blotevogel, K. H. (1993) Microbial transformation of nitroaromatic compounds under anaerobic conditions. *J. Gen. Microbiol.* **139**:1331-1336.
23. Gorontzy, T., Drzyga, O., Kahl, M. W., Bruns-Nagel, D., Breitung, J., von Loew, E., Blotevogel, K. H. (1994) Microbial degradation of explosives and related compounds. *Crit. Rev. Microbiol.* **20**:265-284.
24. Grobicki, A., Stuckey, D.C. (1992) Hydrodynamic characteristics of the anaerobic baffled reactor. *Wat. Res.* **26**:371-378.
25. Grosjean, D. (1985) Reactions of *o*-cresol and nitrocresol with NOx in sunlight and with ozone-nitrogen dioxide mixtures in the dark. *Environ. Sci. Technol.* **19**:968-974.
26. Haghighi Podeh, M. R., Bhattacharya, S. K., Qu, M. (1995) Effects of nitrophenols on acetate utilising methanogenic systems. *Wat. Res.* **29**:391-400.
27. Hartter, D.R. (1985) The use and importance of nitroaromatic chemicals in the chemical industry. In: Rickert, D.E. (ed.). Toxicity of nitroaromatic compounds. Chemical Industry Institute of Toxicology Series, pp 1-14. Chemisphere, Washington DC.
28. Heitkamp, M. A., Camel, V., Reuter, T. J., Adams, W.J. (1990) Biodegradation of *p*-nitrophenol in an aqueous waste stream by immobilised bacteria. *Appl. Environ. Microbiol.* **56**:2967-2973.
29. Hirzel, S. (1993) 2-nitrophenol, 4-nitrophenol, BUA Report 75. Wissenschaftliche Verlagsgesellschaft , Stuttgart, Germany.
30. Hudlicky, M. (1984) Reductions in organic chemistry. p. 74. Halsted Press, New York.
31. Hwang, P.-C., Cheng, S.-S. (1991) The influence of glucose supplement on the degradation of catechol. *Wat. Sci. Technol.* **23**:1201-1209.
32. Hwang, P.-C., Cheng, S.-S. (1991) Treatment of *p*-cresol with a recirculating UASB reactor using the concept of kinetic control. *Wat. Sci. Technol.* **24**:133-140.
33. Janeczko, A., Oleszkiewicz, J. A. (1993) Response of acclimated and un-acclimated activated sludge to 2-nitrophenol. *Environ. Technol.* **14**:351-358.
34. Keith, L. H., Telliard, W. A. (1979) Priority pollutants. I - a perspective view. *Environ. Sci. Technol.* **13**:416-423.
35. Kriek, E. (1979) Aromatic amines and related compounds as carcinogenic hazards to man. In: Emmelot, P., Kriek, E. (eds.). Environmental carcinogenesis, pp 143-164. Elsevier, Amsterdam.
36. Lettinga, G., van Velsen, A. F. M., Hobma, S. W., de Zeeuw, W., Klapwijk, A. (1980) Use of the upflow sludge blanket (USB) reactor concept for biological treatment, especially for anaerobic treatment. *Biotechnol. Bioeng.* **22**:699-734.
37. McCormick, N.G., Feeherry, F.E., Levinson, H.S. (1976) Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. *Appl. Environ. Microbiol.* **31**:949-958.
38. Meer van der, R.R. (1979) Anaerobic treatment of wastewater containing fatty acids in upflow reactors. Ph.D. Thesis, Delft University of Technology.

39. Nicholson, D. K., Woods, S.L., Istok, J. D., Peek, D. C. (1992) Reductive dechlorination of chlorophenols by a pentachlorophenol-acclimated methanogenic consortium. *Appl. Environ. Microbiol.* **58**:2280-2286.
40. O'Connor, O. A., Young, L. Y. (1989) Toxicity and anaerobic biodegradability of substituted phenols under methanogenic conditions. *Environ. Toxicol. Chem.* **8**:853-862.
41. O'Connor, O. A., Young, L. Y. (1993) Effect of nitrogen limitation on the biodegradability and toxicity of nitro- and aminophenol isomers to methanogenesis. *Arch. Environ. Contam. Toxicol.* **25**:285-291.
42. Oren, A., Gurevich, P., Henis, Y. (1991) Reduction of nitrosubstituted aromatic compounds by the halophilic anaerobic eubacteria *Haloanaerobium praevalens* and *Sporohalobacter marismortui*. *Appl. Environ. Microbiol.* **57**:3367-3370.
43. Parkin, G.F., Speece, R.E. (1983) Attached versus suspended growth anaerobic reactor: response to toxic substances. *Wat. Sci. Technol.* **15**:261-289.
44. Patil, S.S., Shinde, V. M. (1988) Biodegradation studies of aniline and nitrobenzene in aniline plant wastewater by gas chromatography. *Water Res.* **22**:1160-1165. 1988.
45. Phelps, T. J., Conrad, R. Zeikus, J. G. (1985) Sulfate-dependent interspecies H_2 transfer between *Methanosarcina barkeri* and *Desulfovibrio vulgaris* during coculture metabolism of acetate or methanol. *Appl. Environ. Microbiol.* **50**:589-594.
46. Preuss, A., Rieger, P.-G. (1995) Anaerobic transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. In: Spain, J.C. (ed.). Biodegradation of nitroaromatic compounds, pp 69-85. Plenum Press, New York.
47. Razo-Flores, E., Donlon, B., Field, J., Lettinga, G. (1996) Biodegradability of N-substituted aromatics and alkylphenols under methanogenic conditions using granular sludge. *Wat. Sci. Technol.* **33(3)**:47-57.
48. Schnell, S., Schink, B. (1991) Anaerobic aniline degradation via reductive deamination of 4-aminobenzoyl-CoA in *Desulfobacterium anilini*. *Arch. Microbiol.* **155**:183-190.
49. Sierra, R. (1990) The role of natural wood constituents on the anaerobic treatability of forest industry wastewater. Ph.D. thesis. Wageningen Agricultural University, The Netherlands.
50. Sierra, R., Lettinga, G. (1991) The effect of aromatic structure on the inhibition of acetoclastic methanogens in granular sludge. *Appl. Microbiol. Biotechnol.* **34**:544-550.
51. Tarvin, D., Buswell, A.M. (1934) The methane fermentation of organic acids and carbohydrates. *J. Amer. Chem. Soc.* **56**:1751-1755.
52. Thaller, V., Turner, J. L. (1972) Natural acetylenes. Part XXXV. Polyacetylenic acid and benzoid metabolites from cultures of the fungus *Lepista diemii* Singer. *J. Chem. Soc. Perkins Trans.* 2032-2034.
53. Todini, O., Hulshoff Pol, L. (1992) Anaerobic degradation of benzaldehyde in methanogenic granular sludge: the influence of additional substrates. *Appl. Microbiol. Biotechnol.* **38**:417-420.
54. Tseng, S. K., Lin, M. R. (1994) Treatment of organic wastewater by anaerobic biological fluidized bed reactor. *Wat. Sci. Technol.* **29(12)**:157-166.
55. Tseng, S.K., Hwu, C.S., Chang, W.Z. (1996) Anaerobic degradation of *o*- and *m*-nitrophenols in plug flow biofilters. *Environ. Technol.* **17**:1225-1233.
56. US-EPA (1978) Nitrobenzene. Initial report of the TSCA Interagency Testing Committe to the Administrator. EPA 560-10-78/001. U.S. Environmental Protection Agency, Washington D.C.
57. US-EPA (1980) Ambient water quality for nitrophenols. US Environmental Protection Agency Report EPA 440/5-80-063.
58. US-EPA (1986) Health and environmental effects profile of nitrotoluenes (*o*, *m* and *p*). Report EPA/600/x-86/143. Environmental Criteria and Assessment Office, Cincinnati, Ohio.
59. Wakabayashi, T., Igarashi, H., Yamamoto, Y. (1983) Degradation of EPN in soils under laboratory conditions. *J. Pesticide Sci.* **8**:155-165.

60. Wu, W. M., Bhatnagar, L.,Zeikus, J. G. (1993) Performance of anaerobic granules for degradation of pentachlorophenol. *Appl. Environ. Microbiol.* **59**:389-397.
61. Zinder, S.H., Koch, M. (1984). Non-acetoclastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. *Arch. Microbiol.* **138**:263-272.
62. Zitomer, D.H., Speece, R. E. (1993) Sequential environments for enhanced biotransformation of aqueous contaminants. *Environ. Sci. Technol.* **27**:227-244.

6

Biotransformation and Biodegradation of Azo Dyes by Anaerobic Granular Sludge Bed Reactors

Elías Razo Flores
Brian A. Donlon
Maurice Luijten
Gatze Lettinga
Jim A. Field

Summary

Biological treatment of wastewaters discharged by the textile industry could potentially be problematical due to the high toxicity of the commonly-used azo dye compounds. In batch toxicity assays, azo dye compounds were found to be many fold more toxic than their cleavage products aromatic amines towards methanogenic activity in anaerobic granular sludge. Considering the ability of anaerobic microorganisms to reduce and decolorise azo compounds, detoxification of azo dyes towards methanogens can be expected to occur during anaerobic wastewater treatment. In order to test this hypothesis, continuous upflow anaerobic sludge blanket reactors were run with the azo dye Mordant Orange 1 (MO1) with either no cosubstrate, glucose or a VFA mixture. Except for the first few weeks, no elimination of MO1 was evident in the reactor receiving no cosubstrate. On the other hand, MO1 was readily cleaved in the reactors receiving glucose and VFA at MO1 loading rates up to 295 and 161 mg/L-d, respectively. The azo dye was decolorised and reductively cleaved (>99%) to less toxic aromatic amines (1,4-phenylenediamine (1,4PDA) and 5-aminosalicylic acid (5ASA)). In the reactor receiving glucose as cosubstrate, 5ASA could only be detected at trace levels in the effluent after day 189 of operation. Batch biodegradability assays with the sludge sampled from this reactor confirmed the mineralization of 5ASA to methane, whereas 1,4PDA persisted.

A pharmaceutical azo dye, azodisalicylate (ADS), constructed from two 5ASA molecules was also evaluated in the glucose fed reactor after the MO1 experiment was finished. The reactor was operated for an additional 340 days period with and without cosubstrate. During this period, ADS was completely decolorised and mineralized to CH_4 at dye loading rates up to 225 mg/L-d. The anaerobic metabolism of 5ASA was shown to provide the electrons required for the initial reductive cleavage of the azo group under continuous experiments. Batch biodegradability assays with ADS as the only carbon and energy source confirmed the dye mineralization to CH_4 and NH_3, indicating that ADS can be anaerobically degraded as a sole substrate. These results indicate that some azo dyes are in fact biodegradable in anaerobic environments in contrast to the common assumption that they are only biotransformed to mutagenic and carcinogenic aromatic amines.

Applied Microbiology and Biotechnology (1997) 47:83-90
Environmental Science and Technology (1997) 31:2098-2103

6.1 Introduction

Azo dyes are one of the oldest industrially synthesized organic compounds. The azo dye production in the United States alone in 1985 amounted to over 100 million kg which was used as colouring agents, by textile, printing, drug and pharmaceutical industries (34). Generally, azo dyes contain between one and three azo linkages (-N=N-) linking phenyl and/or naphthyl rings that are usually substituted with some combination of functional groups including: amino, chloro, hydroxy, methyl, nitro and sulphonate groups. Approximately 10,000 dyes are currently manufactured (41) and it is estimated that at least 15% of these are released into the environment (35). They occur in industrial effluent, groundwater, contaminated soils and sediments. The presence of very small amounts of dyes in water (less than 1 mg/L) is highly visible and aesthetically unacceptable. These compounds are of concern because some of the dyes, dye precursors or their biotransformation products, such as the aromatic amines, have been shown to be carcinogenic (17, 20). Azo dyes are intentionally designed to be recalcitrant under typical product service conditions and, it is this property allied with their toxicity to microorganisms that makes biological treatment difficult (16, 24).

Azo dyes are resistant to aerobic degradation by bacteria. The strong electron withdrawing character of the azo group stabilizes these aromatic pollutants against conversions by oxygenases (15, 18, 26). Pagga and Brown (26) and Shaul et al. (31) tested the degradation of more than 100 dyes in aerobic activated sludge systems and found that only a few of them were actually biodegraded. On the other hand, anaerobic treatment systems may have promising applications for the removal of azo dye compounds since it is widely reported that the azo dyes are gratuitously reduced and decolorised by anaerobic sludges, anaerobic sediments and anaerobic bacterial enrichment cultures (4, 5, 9, 15, 37).

The anaerobic cleavage products of azo dye compounds (aromatic amines) are more easily degraded under aerobic conditions (6, 15). The aromatic amines are generally not metabolized further under anaerobic conditions, particularly those ones used to construct azo dyes (5, 9). Nonetheless, some aromatic amines such as the three isomers of aminobenzoate, 2-aminophenol and 4-aminophenol were shown to be mineralized in methanogenic consortia (22,

23, 28). Recently, biodegradation of 5-aminosalicylic acid (5ASA) has been demonstrated after long adaptation periods (28).

Toxic compounds can be tolerated by methanogens in continuous upflow anaerobic sludge bed (UASB) reactors if they are degraded or undergo biotransformations to less toxic products. Toxic chloro-, nitro- and azo-substitutions of aromatics are subject to reductive biotransformations in anaerobic environments (12). Previously, we reported that nitroaromatic compounds were on the average 500-fold more toxic than their corresponding aromatic amine analogues (10). The reduction of the nitro-substituents during treatment in UASB reactors was shown to be responsible for a dramatic detoxification of several nitrophenols towards methanogens (11).

A similar strategy towards detoxification was tested in this study for azo dyes. Firstly, batch toxicity studies were conducted in order to compare the methanogenic toxicity of selected model azo dye compounds with that of the corresponding aromatic amines. Secondly, the detoxification and degradation of the azo dyes, Mordant Orange 1 (MO1) and Azodisalicylate (ADS), in continuous laboratory-scale UASB reactors were evaluated, as well as the final fate of aromatic amines.

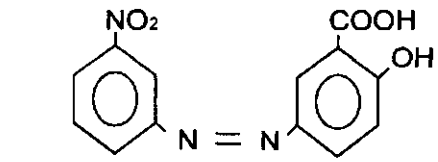
6.2 Materials and Methods

Anaerobic Granular Sludge and Basal Medium

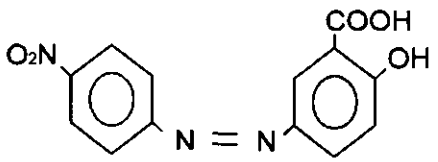
Methanogenic granular sludge from a full-scale UASB reactor treating wet oxidized petrochemical industry effluent of Shell Nederland Chemie (Moerdijk, The Netherlands) was used as inoculum (except when indicated otherwise). The sludge was washed to remove fines and stored at 4°C before use. The sludge has not previously been exposed to any of the compounds being tested. The basal medium used in the experiments was as described previously (11). The micro-nutrients were supplemented to the media for the continuous column experiments at a 10-fold lower concentration.

Anaerobic Toxicity Assay

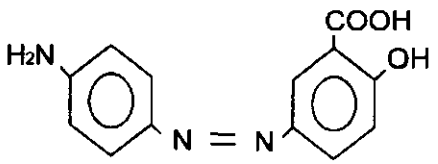
Methanogenic toxicity assays were performed in 120 ml glass serum vials, containing 25 ml of basal medium as described previously (10). The maximum specific acetoclastic methanogenic activity of the control sludge was 420 mg of methane expressed as chemical oxygen demand (COD) per gram of volatile suspended solids (VSS) per day. The exact toxic concentrations of the azo dyes were calculated taking into account the purity of the compound tested. Figure 6.1 shows the aromatic structure of the azo dyes used in the toxicity study.



Mordant Yellow 1



Mordant Orange 1



Mordant Yellow 12

Figure 6.1 Azo dye compounds used in the toxicity study.

Anaerobic Continuous Reactors

Mordant Orange 1 (MO1) Reactors. The continuous experiments were performed in three separate glass UASB reactors with liquid volumes of 160 ml placed in a temperature controlled room at 30 ± 2°C. All reactors were inoculated with 20 g of VSS/L of anaerobic granular

sludge. To start the experiment the granular sludge was initially fed with the medium containing partially neutralized (pH=6.0) volatile fatty acids (VFA) mixture (acetate:propionate:butyrate, 23:34:41 on COD basis) at a concentration of 4 g COD/L during the start-up. After 1 month of operation, the three reactors received a sub-toxic concentration of the azo dye (3 mg/L of MO1). This is referred to as the first day of the experiment, Day 0 in the figures. Reactor 1 (R1) received no cosubstrate (the control), the VFA feed was no longer supplied. Reactor 2 (R2) received glucose (1.3 g COD/L) and Reactor 3 (R3) was fed the VFA mixture (1.5 g COD/L), in addition to the azo dye in both reactors. The methane production was measured with 10 liter Mariotte flasks filled with a 3% (w/v) NaOH solution to scrub out the carbon dioxide from the biogas.

Azodisalicylate (ADS) Reactor. The 160 ml glass UASB reactor 4 (R4) was operated at 30 ± 2°C, and was inoculated with 20 g VSS/L of MO1-adapted granular sludge (withdrawn from R2 immediately after the corresponding experiment was finished on day 217). The reactor was started-up with glucose (1.5 g COD/L) and ADS. The concentration of ADS was increased periodically after at least 10 hydraulic retention times (HRT). Methane production was measured in the same way as MO1-reactors.

Biodegradability Studies

Aromatic Amine Biodegradability Assay. The biodegradation assay of the aromatic amines from MO1-breakdown was conducted for the granular sludge sampled from the reactors (R2, R3) using a protocol outlined previously (28). The sludges were sampled from the reactors at days 0, 166 and 203. The sludges (1 g VSS/L) were incubated at 30°C under N_2/CO_2 (70%/30%) atmosphere in serum bottles (120 ml) containing 28 ml of basal medium. Serum bottles were incubated overnight and only those bottles in which anaerobic conditions prevailed as indicated by the redox indicator resazurin were used for the assay. The concentration of the target aromatic amines tested was 250 mg/L for the 5ASA and 1,4-phenylenediamine (1,4PDA). Sludge blank controls received equal additions of basal medium without the aromatic amines. The serum bottles were incubated for a 150 day period under on an orbital shaker (Gerhardt, Bonn, Germany) at 50 strokes/min. Methane was measured by

sampling the head space of the serum bottles using a pressure-lock (Dynatech Precision Sampling Corp., Baton Rouge, USA) gas-tight syringe (injection volume = 0.1 mL). Methane values reported were corrected by subtracting the values from those in the sludge blanks. The corrected methane production was expressed as a percentage of the theoretical methane production expected from the test aromatic amines mineralization based on the Buswell equation (33). All results are reported as the mean value of triplicate serum vials.

ADS Biodegradability Assay. The same biodegradation assay protocol outlined above was used here as well. ADS-adapted sludge (0.7 g VSS/L) withdrawn from R4 on day 130 of operation was incubated at 30°C under N_2/CO_2 atmosphere in serum bottles (120 mL) containing 28 ml of basal medium. In this particular case, NH_4^+-N nutrient concentration was reduced to 22 mg/L and the yeast extract was omitted from the basal medium. ADS was added as a sole source of substrate from a concentrated stock solution to give a final concentration of 200 mg ADS/L. The serum bottles were incubated for a 50 day period under on an orbital shaker at 50 strokes/min. Sludge blanks, to correct for background NH_4^+-N and methane production from the sludge, were based on assays where no ADS was provided. Sludge that was autoclaved at 121°C for 1 h received ADS, and was used to measure abiotic reduction and/or adsorption of the azo dye. The ADS reduction, the temporal accumulation of 5ASA and the NH_4^+-N released were measured periodically by sampling the liquid phase. Methane was sampled in the head space of the serum bottles using a pressure-lock gas-tight syringe (injection volume = 0.1 mL). The net values of NH_4^+-N released and methane produced were obtained by subtracting the values in the compound amended sludge with those in the sludge blank controls. The corrections for methane production were the same as described in the previous section. All the values reported are the means of triplicate or quadruplicate incubations.

To elucidate intermediates during the metabolism of ADS, a parallel experiment was done. Incubations were made as described before, with the difference that 300 mg/L of the ADS breakdown product, 5ASA, was added to the serum bottles instead of ADS. The methanogenesis was blocked using 50 mM of the methanogenic inhibitor 2-bromoethanesulfonate (BESA), allowing the accumulation of intermediates, which were

determined by gas chromatography (GC), and high-performance liquid chromatography (HPLC). Sludge blank controls were also used in these determinations.

Analyses

Methane and volatile fatty acids (VFA) were determined by gas chromatography as described previously (32). The pH was determined immediately after sampling to avoid any change due to the CO_2 evolution, using a pH-meter 511 (Knick, Berlin, Germany) and a model N61 double electrode (Scot Gerade, Hofheim, Germany). All the other analytical determinations were performed as described in *Standard Methods for Examination of Water and Wastewater* (2).

The MO1, 4-nitroaniline (4NA), ADS and 5ASA concentrations were determined with an HPLC. The HPLC determinations were conducted on an Spectra-Physics SP8810 HPLC (Thermo Separation Products, Breda, The Netherlands). MO1 and 4NA were detected with the following method: a C18 (125 X 3 mm, particle size 5μm) reverse-phase column (Chromosphere 18, Chrompack, Bergen op Zoom, The Netherlands) was used to separate individual compounds which were detected using a Kratos Superflow 773 UV detector (Separations, H. I. Ambacht, The Netherlands). Absorbance was detected at 280 nm. The solvent phase was methanol and 1% acetic acid (40:60, vol:vol). The solvent flow rate was 0.5 mL/min, and the column temperature was 20°C. ADS and 5ASA were detected using a C_{18} (250 X 3mm, particle size 5 μm) reverse-phase column to separate individual compounds which were detected using a Kratos Superflow 773 UV-VIS detector The solvent phases were 98% methanol and 0.5% acetic acid in demineralized water (adjusted to pH 4.5 with NaOH). The column was run with methanol/acetic acid solution in a ratio 2/98 for 5 min, then the ratio was changed to 80/20 over the next 2.5 min and sustained for 7.5 min. The solvent flow rate was 0.3 mL/min, and the column temperature was 20°C. The retention times of 5ASA and ADS were 5 and 15.5 min, and were detected at 330 and 300 nm, respectively.

The aromatic amine mixture of 1,4PDA and 5ASA arising from the effluent of MO1-reactors could not be determined by the HPLC method (similar retention times). Consequently,

these compounds were analyzed by GC-MS as follows: After centrifugation of the samples the supernatant was removed and the pH was adjusted to 2 with 4 M sulfuric acid followed by extraction three times with freshly distilled ethyl acetate. The combined organic layers were washed with demineralized water and evaporated till dryness under reduced pressure at ambient temperature. In order to make 5ASA amenable to GC-MS analysis, a derivatization step was performed using the method described by Brook and Chan (3). For this purpose, the residue was redissolved in 5 ml of dry methanol and 0.5 ml of thechlorotrimethylsilane was added to give the methyl ester to 5ASA (5ASA-ME). The reaction mixture was left overnight under a nitrogen atmosphere. The internal standard, 4-Bromoanisole, was used at a concentration of 0.55 mM. All the samples were analyzed on an HP5970B quadrupole mass spectrometer coupled to an HP5890 gas chromatograph (Hewlett Packard, Palo Alto, USA) equipped with a fused silica capillary column (DB17, 30 m X 0.25 mm i.d., film thickness 0.25 μm, J&W Scientific, USA). Helium was used as the carrier gas at a flow rate of 1.1 ml/min. Injector temperature was 220 °C. Temperature programme: 70-250 °C at 7 °C per min and, thereafter, held for 20 min. The injection volume was 10 μl; split ratio 1:100. EI-MS were obtained at 70eV, and the quantification was based on the total ion current which gave an indication of the relative concentration of the compound in the extract. The identification of the aromatic amines was achieved by comparison of retention times and mass spectra to data of respective standard compounds.

MO1 and ADS were also measured spectrophotometrically by UV-VIS absorbance with a Spectronic 60 spectrophotometer (Milton Roy/Analytical Products Division, Ostende, Belgium) using a model 100-QS (Hellma Benelux, The Hague, The Netherlands) 1 cm quartz cuvette. Absorption is reported as the absorption of the medium containing N-aromatic compounds minus the absorption of the control media (which contained no test compounds). All samples were diluted to less than 1 absorbance unit in 0.2 M phosphate buffer (pH 7.0). Azo dye reduction was monitored at absorbance maxima of 373 and 380 nm for MO1 and ADS, respectively. There was less than 5% interference from the azo dye cleavage products (5ASA and 1,4PDA) at these wavelengths. The aromatic amines were also determined colorimetrically at 440nm after reacting with 4-dimethylaminobenzaldehyde-HCl (Ehrlich Reagent) according to the method described by Oren et al. (25). The expected absorbance

units per mmol of MO1 removed (*total aromatic amines*, TAA) was determined to be 3.055 by using an equimolar mixture of 5ASA and 1,4PDA as a standard. The % of TAA recovery was expressed as: 100 X (absorbance/3.055).

NH_4^+-N was measured spectrophotometrically based on the modified Berthelot reaction with an Skalar autoanalyzer (Skalar Analytical B.V., Breda, The Netherlands) according to Dutch standards (21).

Chemicals

Chemicals were purchased from either Jansen Chimica (Tilburg, The Netherlands), Merck (Darmstadt, Germany) or Sigma (Bornem, Belgium). All the chemicals were of the highest purity commercially available. None of the chemicals were purified further. ADS (under the commercial name "Olsalazine") was kindly supplied by Pharmacia AB (Uppsala, Sweden). ADS was 99.5% pure.

6.3 Results

Effect of N-substituted Aromatic Structure on Methanogenic Inhibition

The inhibitory effects of 8 N-substituted aromatic compounds on the activity of acetoclastic methanogenic bacteria were evaluated in this study. The inhibition caused by each compound was tested at varying levels covering non-toxic to completely inhibitory concentrations of the compounds. Table 6.1 summarizes the 50% inhibitory concentrations (IC) of the aromatic compounds tested and outlines the relationship between the azo dye and the reduced cleavage products. The type of N-substitution had a profound effect on their toxicity. The least toxic compounds were the aromatic amines: 1,3-phenylenediamine (1,3PDA), 1,4PDA and 5ASA. The azo compounds and the nitroanilines arising from incomplete reduction of azo dyes were more toxic. The most toxic compound tested was MO1 having a 50% IC of 0.014 mM.

TABLE 6.1 Toxicity of selected azo dye compounds and their suspected cleavage products to acetoclastic methanogens.

Compound name	mW[a]	50% IC[b] (mM)	Theoretical Reductive Biotransformation Products
Mordant Orange 1 (MO1)	287	0.04	5ASA, 4NA, 1,4PDA
Mordant Yellow 1 (MY1)	287	0.063	5ASA, 3NA, 1,3PDA
Mordant Yellow 12 (MY12)	279	0.25	5ASA, 1,4PDA
3-Nitroaniline (3NA)	138	0.03	1,3PDA
4-Nitroaniline (4NA)	138	0.017	1,4PDA
1,3-Phenylenediamine (1,3PDA)	108	65.7	N.A.[c]
1,4-Phenylenediamine (1,4PDA)	108	30	N.A.
5-Aminosalicylic acid (5ASA)	153	2.9	N.A.

[a] mW: molecular weight.
[b] 50% IC: compound concentration that caused 50% inhibition of the methanogenic activity.
[c] N.A.: not applicable.

Continuous Anaerobic Treatment of Azo Dyes

MO1 Reactors. Three laboratory scale UASB reactors were operated in order to investigate the continuous anaerobic treatment of MO1. The reactors (R1, R2, and R3) were initially started up after one month adaptation period to VFA. After this adaptation time period the reactors were initially fed with sub-toxic concentrations of MO1 (3 mg/L). The concentration of MO1 in the influent was increased periodically after at least 10 HRT (the HRT was approximately 0.32 d) and when greater than 75% removal of MO1 and the cosubstrate COD had been obtained. The rationale behind this approach is that the sludge can withstand higher concentrations of the incoming dye by cleaving the azo bond to form less toxic aromatic amines. The reactors differed from each other in the selection of the cosubstrate; R1 (the control) received no cosubstrate, whereas R2 and R3 received glucose and VFA mixture, respectively. Glucose and the VFA mixture were chosen as model wastewater substrates deemed necessary for providing the electrons for the reduction of the azo compounds.

R1 was able to decolorise and remove the azo dye for 20 days with the production of aromatic amines. The yield of aromatic amines was based on TAA. The %TAA recovered were fairly constant at 98.2% of MO1 removal. Azo dye removal exceeded 60% at concentrations up to 12 mg/L for 20 days. The concentration of MO1 was increased stepwise to 75 mg/L for an additional 30 days. During this time period there was a major decrease in the azo dye removal to less than 10% with a parallel decrease in TAA production (results not shown). In addition, 4NA which previously had not been detected in the reactor effluent was observed in the HPLC analyses (data not shown). Due to the fact that MO1 was not being reduced completely, the concentration of both MO1 and 4NA increased. Finally, complete failure of the reactor occurred as evidenced by the lack of azo dye reduction. It is likely that the endogenous substrate present in the sludge was initially contributing the reducing equivalents to reduce the azo bond. The endogenous substrate was probably exhausted after 50 days.

The operational parameters and treatment efficiency during the continuous operation of R2 and R3 at the end of the reactor operation are listed in Table 6.2. The operational conditions and treatment performance of R2 and R3 are shown in Figure 6.2 and 6.3, respectively. From day 75 up to day 166, MO1 was greatly decolorised and reduced (at least by 90%) in R2. The %TAA recovered were fairly constant yielding approximately 98% of the TAA expected, indicating a stoichiometric yield of the aromatic amines (Figure 6.2A). The reduction of the parent azo compound to the less toxic daughter aromatic amines ensured that a detoxification of the influent occurred as was evidenced by the glucose degradation (86.6% COD removal) and methane production (79.8% of the incoming cosubstrate COD). By use of HPLC and GC-MS, 4NA was not detected as a cleavage product that accumulated. From day 189 onwards, the %TAA recovered decreased, indicating that part of the amines produced by the reduction were converted further (Figure 6.2A). The maximum MO1 loading rate applied in this period was 295 mg/L-d with a MO1 dye removal efficiency of >99%. Effluent samples collected in this period were subjected to derivatization conditions and were analyzed with a GC-MS. The GC-MS spectra confirmed the presence of 1,4PDA while only trace levels of 5ASA-ME were detected (Figure 6.4A). These results indicated biological removal of the 5ASA.

TABLE 6.2 The operational parameters and treatment efficiency during the continuous operation of the UASB reactors treating Mordant Orange 1 at the final period of reactor operation (Days 174-217).

Operational parameters	Reactor 2	Reactor 3
Influent cosubstrate (g COD/L)[a]	1.42	0.91
Cosubstrate loading rate (g COD/L-d)	4.16	2.88
Conc. influent Mordant Orange 1 (mg/L)	100	50
MO1 loading rate (mg/L-d)	295	161
HRT (d)	0.34	0.31
Efficiency		
COD removal (%)[b]	86.6 ± 3.9	81.8 ± 5.5
Methane (% CODin)[c]	79.8 ± 11.1	58.3 ± 20
VFA effluent as % of CODin	1.2 ± 0.5	2.6 ± 2.1
Azo dye removal (%)		
Absorbance[d]	95.1 ± 1.6	91.8 ± 1.3
HPLC	>99	>99

[a] g COD of cosubstrate: reactor 2, glucose; reactor 3, VFA mixture.

[b] removal of cosubstrate in terms of COD elimination.

[c] % conversion of influent cosubstrate COD to methane.

[d] Azo dye absorbance removal indicates loss in azo group absorbance at 373nm.

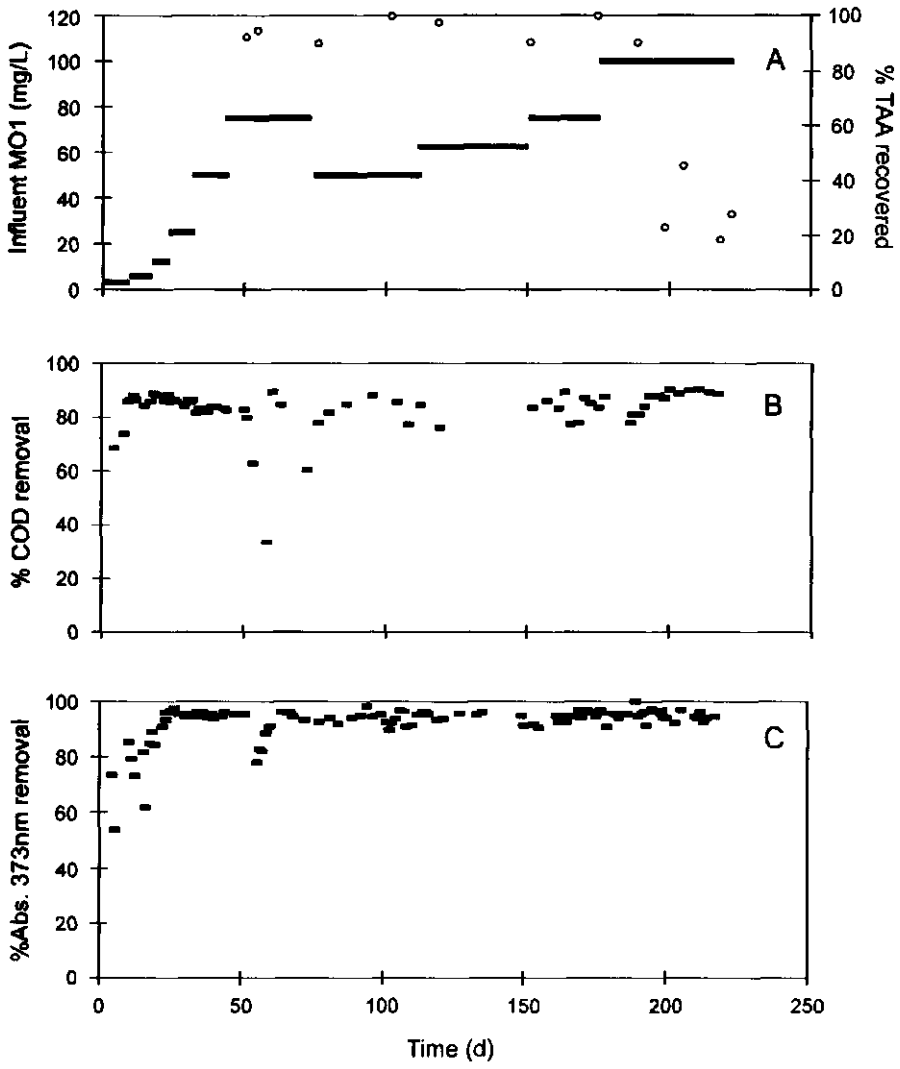


Figure 6.2 Operational parameters and removal efficiencies during the continuous anaerobic treatment of MO1 when glucose was employed as cosubstrate (R2): (A) MO1 (—) concentration in the influent, (o) TAA produced; (B) % removal of glucose in terms of COD; (C) % MO1 removal as determined by loss in UV absorbance at 373nm.

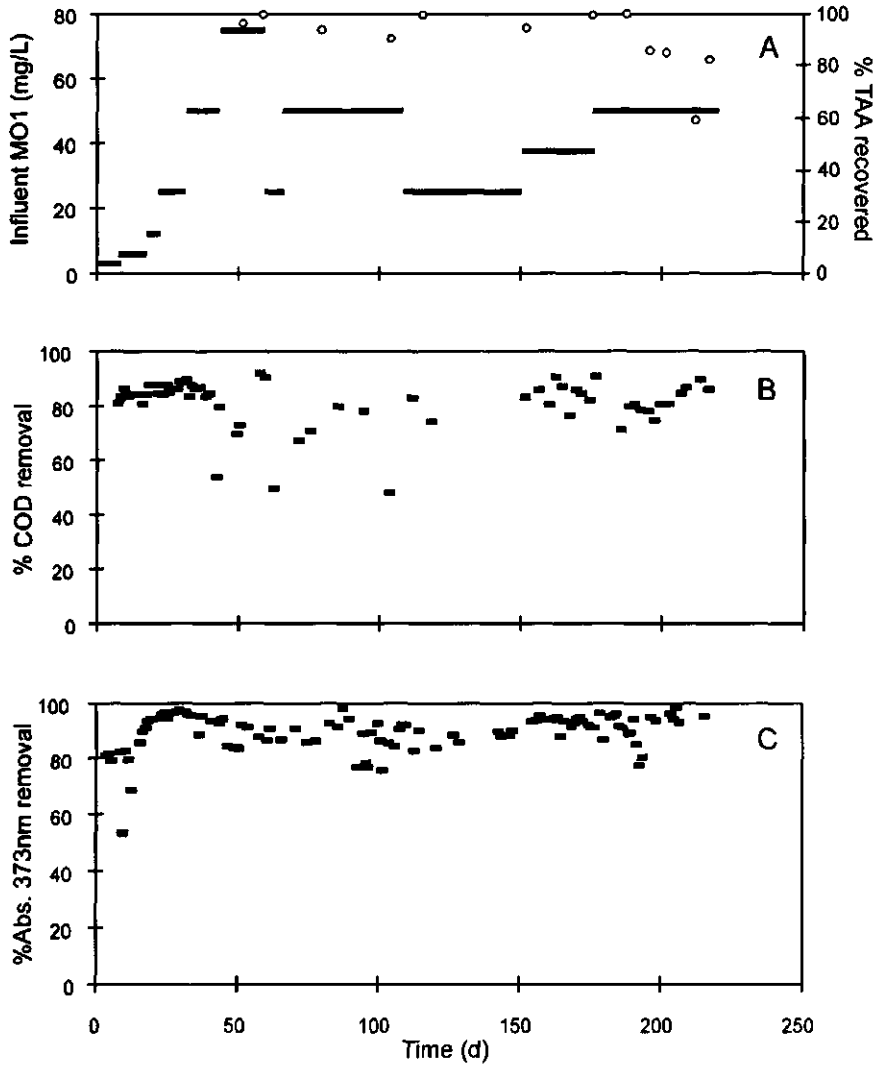


Figure 6.3 Operational parameters and removal efficiencies during the continuous anaerobic treatment of MO1 when VFA was employed as cosubstrate (R3): (A) MO1 (—) concentration in the influent, (o) TAA produced; (B) % VFA removal in terms of COD; (C) % MO1 removal as determined by loss in UV absorbance at 373nm.

R3 gave high VFA removal efficiencies (81.8% on a COD basis) and methane production (58.3% of the incoming cosubstrate COD) over the course of the experimental period (Table 6.2). The decolorisation and reduction of MO1 to 5ASA and 1,4PDA was attributed to the high MO1 removal efficiencies (91%), even after increasing MO1 concentrations up to 50 mg/L (Figure 6.3). Again it was observed that 4NA could not be detected as a cleavage product by use of the HPLC and GC-MS methods. The %TAA recovered was approximately equivalent to that expected from a stoichiometric recovery of aromatic amines during the whole period (Figure 6.3A). The GC-MS spectra data confirmed the presence of 1,4PDA and 5ASA-ME (Figure 6.4B). 5ASA-ME was present at much higher concentration than in R2. Consequently, no further conversion of the aromatic amines was observed in R3 although the reactor was in operation for 217 days. The maximum MO1 loading rate applied was 161 mg/L-d with an MO1 efficiency removal of >99%.

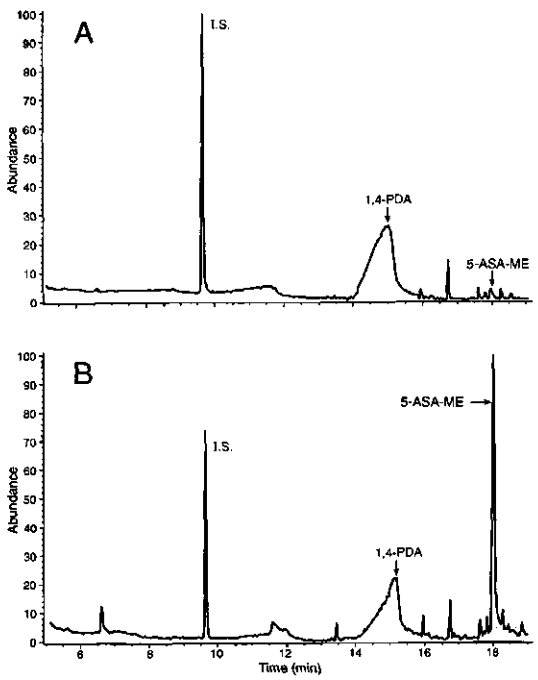


Figure 6.4 Gas chromatogram of metabolites detected during continuous anaerobic treatment of MO1 in (A) the reactor containing the glucose-degrading granular sludge, (B) the reactor containing the VFA-degrading granular sludge. 1,4-phenylenediamine (1,4PDA), 5-aminosalicylic acid-methylester (5ASA-ME). The peak observed at 9.5 minutes is 4-bromoanisole which was used as the internal standard (I.S.).

ADS Reactor. The 160 ml UASB reactor was operated under several operational conditions for a 340 days period to study the anaerobic transformation and mineralization of ADS. This reactor (R4) was started up with granular sludge from R2 after 200 days of operation and was fed with ADS and glucose. Glucose was used initially as a cosubstrate to provide the reducing equivalents for the reduction and cleavage of the azo chromophore, later the reactor was operated without glucose (from day 206 onwards). The applied influent values of ADS (ADS_{in}), ADS loading rate (ADS-LR), cosubstrate concentration (CoS_{in}), cosubstrate organic loading rate (CoS-LR) and HRT, as well as the assessed treatment efficiencies in the various periods of the experiment are listed in Table 6.3 for R4. The daily azo dye treatment performance is also shown in Figure 6.5.

In periods 1 and 2, the ADS was partially removed to an extent of more than 60%; whereas, the ADS breakdown product, 5ASA, was recovered at low concentrations in the effluent (Figure 6.5). During periods 3 and 4, the ADS was greatly decolorised and removed (at least by 95%) and the corresponding aromatic amine was recovered by less than 2% (molar yield of influent ADS). This indicates that the 5ASA released from azo dye cleavage was being metabolized. The cosubstrate removal was also quite high during all these periods accounting for more than 86% of the influent COD (Table 6.3). After the cosubstrate was excluded (period 5), ADS continued to be decolorised and reduced by more than 60%. On day 236 the upflow velocity was increased in the reactor from 0.02 to 0.2 m/h by recirculating the effluent. Nonetheless, the increase in upflow velocity did not produce any improvement in the ADS reduction as can be observed in Figure 6.5. 5ASA was still detected at very low concentration levels in the effluent (<1% molar yield of the influent ADS). In period 6, the HRT was doubled from 8 to 16 h and there was a slight increase in the ADS reduction to 70%. In this period, the sulfates and nitrogen nutrient sources were removed from the basal medium to avoid any possible competition for electron acceptors and forcing the consortia to utilize ADS as an N-source. A further increase of the HRT to 26 h in period 7 resulted in a great increase in the ADS reduction to 89%. These results indicated the dye could be anaerobically degraded as a sole substrate in the continuous bioreactor for an extended period of time

TABLE 6.3 Operational conditions and treatment efficiency of the continuous UASB reactor (R4) treating ADS with glucose or no cosubstrate at 30°C.

Parameter[a]	Experimental periods[b]						
	1	2	3	4	5[c]	6	7
Operational							
ADS_{in} (mg/L)	25	50	50	75	75	75	75
CoS_{in} (g COD/L)	1.5	1.5	3	3	0	0	0
CoS-LR (g COD/L-d)	4.5	4.5	9	9	NA[d]	NA	NA
ADS-LR (mg/L-d)	75	150	150	225	225	114	69
HRT (h)	8	8	8	8	8	16	26
Efficiency (%)							
ADS removal[e]	69.4 ± 6.6	63.5 ± 4.5	96.7 ± 1.5	98.8 ± 0.8	63.4 ± 9.1	69.1 ± 2.8	88.9 ± 1.8
5ASA recovered[e]	13.4 ± 3.6	11.5 ± 2.0	1.0 ± 1.2	0.8 ± 1.1	0.12 ± 0.6	<0.06	<0.06
CoS removal (%COD)	89.0 ± 2.9	88.7 ± 0.2	93.3 ± 1.7	94.8 ± 0.9	NA	NA	NA
CH_4 (%CoS_{in}-COD)[f]	74.6 ± 15.8	70.5 ± 3.6	96.7 ± 12.6	100.9 ± 8.0	NA	NA	NA

[a] Azodisalicylate concentration influent (ADS_{in}), cosubstrate influent concentration (CoS_{in}), cosubstrate volumetric loading rate (Co-LR), ADS volumetric loading rate (ADS-LR), and hydraulic retention time (HRT).

[b] Periods: 1 (day 0-25), 2 (day 26-40), 3 (day 41-136), 4 (day 137-205), 5 (day 206-257), 6 (day 258-307), 7 (day 308-340).

[c] On day 236 (period 5) the upflow velocity in the bioreactor was increased from 0.02 to 0.2 m/h through recirculation of the effluent. This upflow velocity was applied until the end of the experiment.

[d] NA: not applicable.

[e] ADS removal and 5ASA recovered were determined by HPLC, except for periods 1 and 2 when the determinations were done by spectrophotometry and colorimetry; respectively (see materials and methods). 5ASA recovered is expresed as molar yield of ADS removed.

[f] Yield of methane in COD as a % of CoS_{in}-COD.

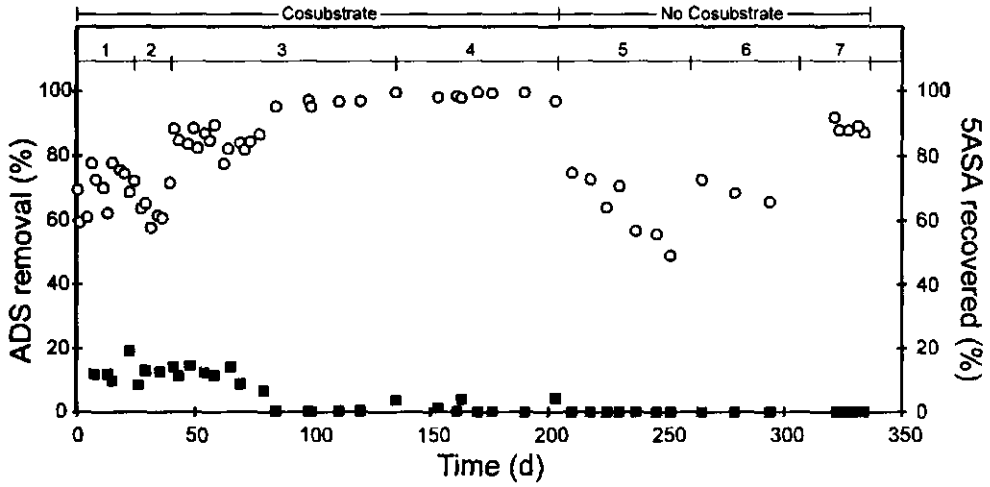


Figure 6.5 Treatment efficiency of ADS degradation with glucose (periods 1 to 4) or no cosubstrate (periods 5 to 7) in UASB reactor (R4); %ADS removal (O), %5ASA recovered (as a molar yield of ADS in influent) (■). Numbers in the top of the figure indicates the experimental periods (see Table 3).

Biodegradability Studies

MO 1 Cleavage Products. In order to confirm the mineralization of the aromatic amines arising from MO1-breakdown, the sludges sampled from R2 and R3 at various time intervals of the continuous experiments were assayed for the conversion of 5ASA and 1,4PDA to methane in batch assay experiments. There was no conversion of 1,4PDA to methane in any of these samples. On the other hand, complete mineralization of 5ASA to methane in batch assays from sludge sampled from R2 on days 166 and 203 was observed, with a maximum degradation rate of 35.2 mg/g VSS-d (Table 6.4). The conversion of the 5ASA-COD to methane-COD was more than 90% of the theoretical methane production expected for both sludges. In contrast, the lag phase of 5ASA degradation was reduced from 21 to <5 days for the sludges sampled on days 166 and 203, respectively. This fact indicated that sludge adaptation due to an increase in 5ASA degrading microorganisms had occurred. The seed sludge and the sludge sampled from R3 were unable to convert 5ASA to methane.

TABLE 6.4 Anaerobic biodegradability of 5-aminosalicylic acid (250 mg/L) by sludges sampled from the reactors at various time intervals.

Parameter	Seed sludge	Reactor 2		Reactor 3		Reactor 4
	day 0[a]	day 166	day 203	day 166	day 203	day 130[b]
Lag period (d)	>150	21	<5	>150	>150	<5
Degradation rate (mg/g VSS-d)[c]	0.15	27.5	35.2	0.15	0	41.9
Methane (%TMP)[d]	6 ± 3	93 ± 2.7	119 ± 6	4.7 ± 0.8	0	76 ± 1

[a] Day 0 refers to the initial inoculum sludge.
[b] This reactor was started up with granular sludge from R2 at the end of the respective experiment on day 217.
[c] The degradation rate was calculated by converting the measured mg CH_4-COD to mg of the compound.
[d] Conversion of the test compound COD to CH_4-COD. %TMP refers to the theoretical methane production.

Biodegradability Assay of ADS. In order to confirm that ADS could be mineralized into methane and NH_3, ADS-adapted granular sludge withdrawn from R4 was incubated in anaerobic serum bottles with and without 200 mg/L of ADS as a sole source of substrate. During the course of the 50 day incubation period, ADS concentration steadily decreased at an average rate of 8.3 mg/g VSS-d (Figure 6.6A). No significant decrease in the ADS concentration was observed in the autoclaved sludge after more than 50 days, indicating that the dye removal in the living sludge was biologically mediated. The elimination of ADS was associated with a net increase in methane production and ammonium concentration beyond that observed in the sludge blanks (Figure 6.6B). The net methane production and ammonium released at the end of the 50-day incubation period accounted for 78% COD and 75% of the nitrogen contained in the ADS, respectively. These results indicate that ADS was extensively mineralized by the adapted methanogenic consortium. During ADS mineralization, 5ASA was detected as a transient degradation intermediate (Figure 6.6A), demonstrating that reductive cleavage of the azo dye was the first step in the degradation. By day 20, this intermediate was

no longer detectable suggesting that it was metabolized as was confirmed in a separate experiment by the conversion of exogenously added 5ASA to methane in the same sludge, with a maximum degradation of 41.9 mg/g VSS-d (Table 6.4).

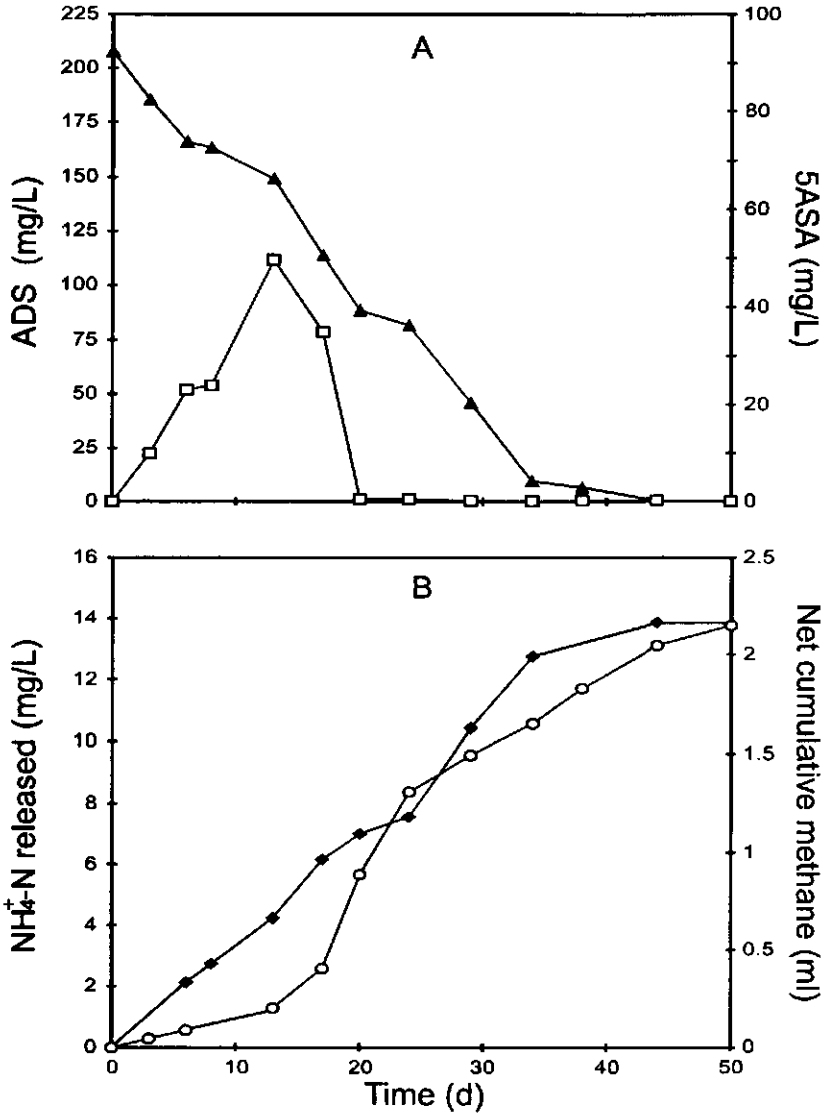


Figure 6.6 The time course of ADS mineralization in anaerobic batch assays using ADS-adapted granular sludge withdrawn from bioreactor R2. (A) Reduction of ADS (▲), and transient accumulation of the intermediate 5ASA (□); (B) Net methane production (◆), and net NH_4^+-N released (○) due to the ADS mineralization.

During the degradation of 5ASA, it is possible that electrons are used to reduce the azo dye. This hypothesis was confirmed by adding exogenous 5ASA (300 mg/L) to the sludge incubated with ADS (200 mg/L). The rate of azo dye reduction was enhanced by 65% with the addition of exogenous 5ASA (results not shown). To elucidate intermediates during the metabolism of 5ASA, a specific methanogenic inhibitor, BESA was added to sludge incubated with 5ASA. BESA effectively blocked the methane production from 5ASA. Several VFA were identified in the BESA amended cultures after 50 days of incubation accounting for up to 60% of the COD contained in ADS. Acetate was the major VFA formed, responsible for 79% of the VFA pool.

6.4 Discussion

Toxicity of N-substituted Aromatic Compounds

In our study, the azo dyes and incomplete reduction products of azo dyes, the nitroanilines, were clearly very toxic compounds to methanogens, with 50% IC values ranging from 0.014 to 0.25 mM. Of the three azo dyes tested, Mordant Yellow 12 (MY12) was the least toxic probably because this dye does not contain a nitro-functional group in its structure. However, since MY12 did cause inhibition at 0.25mM, it can be concluded that the azo group itself has a toxic effect on the methanogens. The toxic concentrations found here were in the same range of those reported by Seshadri et al. (30), who studied the inhibitory effects of some azo dyes on COD and dye removals in anaerobic fluidized bed reactors. They found that Acid Orange 10 and Acid Orange 8 dye concentrations of 0.037 and 0.051 mM, caused a significant inhibition in dye and COD removal, respectively.

Previously, we have shown that aromatic nitro-substituents are responsible for severe methanogenic toxicity. Aromatic amines in contrast were less inhibitory compounds with 50% IC values between 2.9 to 65.7 mM (10). In a similar fashion, here we observe that the toxicity of azo compounds was very much greater than their aromatic amine cleavage products. MO1 is over 200-fold and 2140-fold more toxic than 5ASA and 1,4PDA, respectively. These results

indicate that the reduction of azo dyes known to occur in anaerobic environments (4, 5, 37) would be responsible for their dramatic detoxification towards methanogens.

Azo Dye Reduction in Continuous UASB Reactors

The application of high-rate reactors, such as the UASB reactors, have proven to be capable of treating various wastewaters bearing toxic aromatic compounds with a high degree of efficiency and stability (11, 19, 39). It is well known that azo dyes are easily decolorised and reduced under anaerobic conditions. The stoichiometric reduction of an azo bond yielding aromatic amines requires four reducing equivalents, which are typically supplied by an electron donor. Consequently, it was of interest to examine the cosubstrate requirement for the azo group reduction. Both glucose and the VFA mixture supported azo dye reduction for the entire duration of the continuous experiments. In the reactor to which no cosubstrate was fed (R1), some azo dye reduction occurred in the initial period of operation when low MO1 loading rates were applied. The reducing equivalents for this conversion were presumably due to endogenous substrates in the anaerobic sludge. Eventually the endogenous substrate would be expected to become exhausted which might explain why R1 performance slowly dropped in time, failing completely by day 50. The presence of 4NA as an incomplete reduction end product in R1, prior to its failure, is analogous to the recent paper by Cheng et al. (8), where they reported an incomplete reduction of 2,4-dinitrotoluene to nitro-aminotoluenes by an anaerobic culture in the absence of cosubstrate.

Highly toxic concentrations of MO1 up to 100 mg/L could be tolerated in the continuous UASB reactors fed with cosubstrate, exceeding the 50%IC of MO1 to methanogenic bacteria by 25-fold in R2. Nonetheless, the methanogenic consortia appeared to be healthy since the cosubstrate was degraded at moderate organic loading rates with a high conversion efficiency to methane. Decolorisation and detoxification of MO1 was clearly taking place in the UASB reactors. The reducing equivalents produced during cosubstrate degradation were used to support the complete reduction of MO1 to less toxic breakdown products. In the case of R4, this reactor could also handle high ADS loading rates with a high reduction efficiency (up to 99%).

Many reports indicate that electron donating cosubstrates are required for azo reduction by bacteria (7, 15, 38). It has been postulated that the presence of cosubstrates enhances the reduction rate of the azo compounds by increasing the rate of formation of reduced enzyme cofactors which are able to fortuitously and nonspecifically reduce the azo dyes. Gingell and Walker (14) found that soluble flavins play an important role in azo dye reduction in *Streptococcus faecalis*. Reduced flavins, acting as two electron donors, rapidly reduced Red 2G nonenzymatically, and the reduced flavins can act as electron shuttles from NADPH-dependent flavoproteins to the acceptor azo compound. Methanogenic and acetogenic bacteria in the granular sludge contain unique reduced enzyme cofactors; such as F_{430} and vitamin B_{12}, that could also potentially chemically reduce azo bonds, similar to what has been found for the reductive dechlorination of the chlorinated aliphatics (36). It is, therefore, not surprising that azo reduction rates are sensitive to the amount of available fermentation substrate in an anaerobic system, since catabolism of these substrates is ultimately responsible for the production of reduced enzyme cofactors.

It is also likely that cosubstrates could act as donors of reducing equivalents (e.g., via NAD(P)H) to specific azoreductases. Roxon et al. (29) reported that both NADH and NAD(P)H are active electron donors for the reduction of tartrazine in whole-cell suspensions of *Proteus vulgaris*. In the same way, Zimmermann et al. (40) have also shown that certain specific oxygen-insensitive azoreductases of *Pseudomonas sp.* have NAD(P)H dependency to catalyze the reductive cleavage of the azo group of carboxy-Orange I and carboxy-Orange II under aerobic conditions.

The results from this study showed that UASB reactors could handle high azo dye loading rates up to 295 mg/L-d for MO1 and 225 mg/L-d for ADS. These loading rates are a big improvement over those obtained in previous studies with anaerobic reactors. Seshadri et al. (30) used an anaerobic fluidized bed reactor for the treatment of selected azo dyes. The authors applied azo dye loading rates of 36.4 mg Acid Orange 8/L-d. Fitzgerald and Bishop (13) applied azo dye loading rates of 7.7 mg Acid Orange 10/L-d to an anaerobic fixed fluidized bed reactor.

Mineralization of Aromatic Amines in Continuous UASB Reactors

The GC-MS results combined with the chemical assay for TAA and the anaerobic batch biodegradability assays indicated that 5ASA was being mineralized in R2. In the batch experiments assaying the conversion of 5ASA to methane by sludge sampled from R2, a specific mineralization rate of 35 mg/g VSS-d was observed. This rate was much greater than the maximum MO1 sludge loading rate used in the continuous column experiment, which when expressed in terms of 5ASA equivalents amounted to 5 mg/g VSS-d. Consequently we concluded that the removal of 5ASA cleaved from MO1 in R2 was due to its anaerobic degradation. In the case of the other MO1 breakdown product, there was no or very minor conversion of 1,4PDA to methane in all of these sludge samples. The proposed pathway for the partial mineralization of MO1 in R2 can be depicted schematically as shown in Figure 6.7.

The elimination of 1,4PDA in aerobic sludge has been previously documented (1, 27). Consequently, an aerobic post treatment step would be required for the complete mineralization of the azo dye MO1. Coupled anaerobic-aerobic systems have proven to be successful in achieving the complete biodegradation of azo dyes. In such systems, azo dyes are reduced anaerobically, followed by subsequent aerobic degradation of the aromatic amines produced (12, 15).

The extent of ADS reduction was rather high in R4. Based on this fact, a high recovery of the ADS breakdown product, 5ASA, was expected. However, the recovery of 5ASA was very low by the end of period 4 and onwards (Table 6.3), when accounted for less than 1% (molar yield of the influent ADS) of the expected value, indicating that this aromatic amine was being mineralized or being degraded to another product. On day 206 (period 5) the cosubstrate was no longer added to R4. If 5ASA was being mineralized, we hypothesized that the reducing equivalents needed for the azo bond reduction could be generated. The hypothesis was confirmed since 60% to 89% ADS removal was obtained without any cosubstrate. ADS was used as a sole substrate for more than 100 days. According to this results, it is likely that the cosubstrate was only necessary to establish an active methanogenic consortium during the

adaptation to the azo dye, and that cosubstrate supplementation was no longer essential once 5ASA-degrading bacteria developed in the consortium.

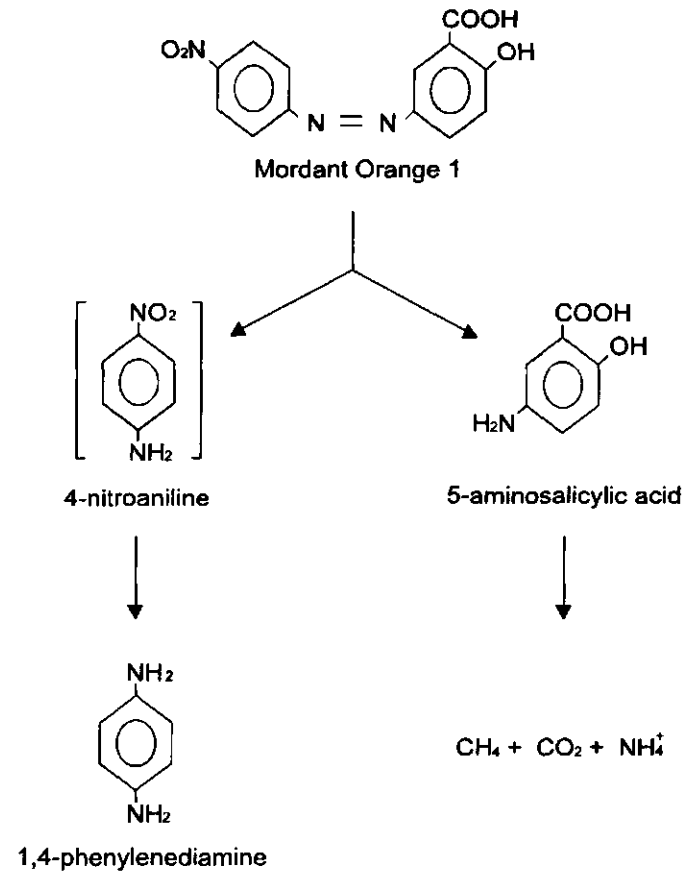


Figure 6.7 Proposed scheme detailing the anaerobic breakdown of the azo dye MO1 by granular sludge present in the reactor receiving glucose as cosubstrate.

Anaerobic Biodegradation of ADS in Batch Experiments

In order to confirm that ADS was being mineralized, the anaerobic sludge withdrawn for R4 was incubated with ADS as a sole source of substrate. ADS was largely mineralized to methane and ammonium with transient accumulation of 5ASA as a degradation intermediate. The concentration of ADS in the dye-amended autoclaved sludge remained constant for more

than 50 days, indicating that the degradation of ADS was biologically mediated. The specific ADS degradation rate under batch conditions (8.3 mg/g VSS-d) was high enough to account for ADS mineralization in continuos reactors; the ADS removal rate at the end of period 7 in R4 (VSS concentration was 21.83 g/l) was 3.15 mg/g VSS-d.

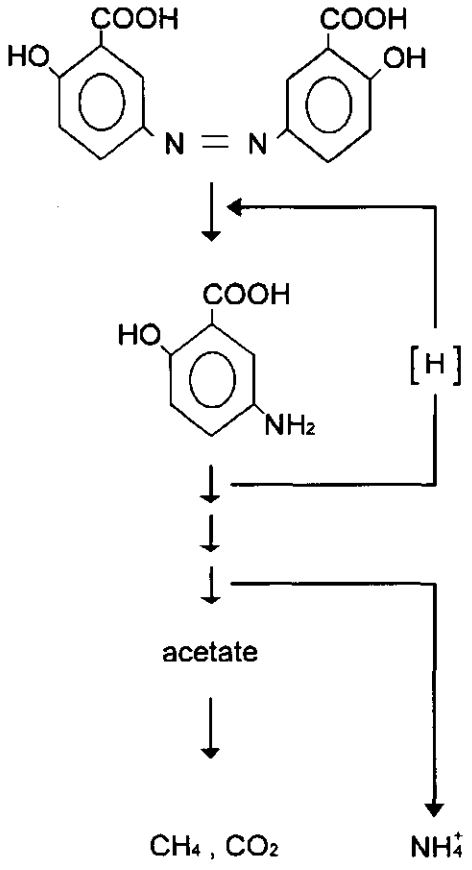


Figure 6.8 Biodegradation pathway proposed for the complete mineralization of ADS under anaerobic conditions.

The electrons required for the reductive cleavage of azo dyes by anaerobic microorganisms is known to be derived from cosubstrates. While no exogenous cosubstrates were added in the batch biodegradation assays described here, background levels of endogenous substrates in the sludge inoculum were most likely used to prime azo dye

reduction. Thereafter, the metabolism of 5ASA released from the azo dye cleavage could provide cosubstrates supporting continued reduction of the dye. This hypothesis was confirmed by adding exogenous 5ASA to the sludge incubated with ADS, resulting in an enhancement of the azo dye reduction rate.

5ASA was incubated with the methanogenic consortia in the presence of the specific methanogenic inhibitor BESA. Acetate was identified as the major intermediate formed, indicating that the degradation of 5ASA occurs via acetogenic fermentation. The results taken as a whole indicate a biodegradation pathway going from ADS, 5ASA, acetate to methane as shown in Figure 6.8.

This constitutes the first report indicating that an azo dye compound is completely decolorised and biodegraded in the absence of oxygen. Previously, it was assumed that azo cleavage products would be recalcitrant to anaerobic degradation, and that subsequent aerobic mineralization would be required (4, 12, 15, 30). However, here we demonstrate that an azo dye constructed from anaerobically metabolizable aromatic amines is completely biodegraded supplying itself with electrons to support reductive azo bond cleavage.

Acknowledgments

This research was supported by the Consejo Nacional de Ciencia y Tecnologia from México. Support from the Human Capital and Mobility programme of the EC is gratefully acknowledged (B.D.).

References

1. Alexander, M., Lustigman B.K. (1966) Effect of microbial degradation of substituted benzenes. *J. Agr. Food Chem.* **14**:411-413.
2. American Public Health Association (1985) Standard methods for examination of water and wastewater, 16th ed. American Public Health Association, Washington, DC.
3. Brook, M.A., Chan, T.H. (1983) A simple procedure for the esterification of carboxylic acids. *Synthesis* 201-203.
4. Brown, D., Hamburger, B. (1987) The degradation of dyestuffs: Part III. investigations of their ultimate degradability. *Chemosphere* **16**:1539-1553.

5. Brown, D., Laboureur, P. (1983) The degradation of dyestuffs: Part I, primary biodegradation under anaerobic conditions. *Chemosphere* **12**:397-404.
6. Brown, D., Laboureur, P. (1983) The aerobic biodegradability of primary aromatic amines. *Chemosphere* **12**:405-414.
7. Carliell, C.M., Barclay, S.J., Naidoo, N., Bucley, C.A., Mulholland, D.A., Senior, E. (1995) Microbial decolourisation of a reactive azo dye under anaerobic conditions. *Water SA* **21**:61-69.
8. Cheng, J., Kanjo, Y., Suidan, M.T., Venosa, A.D. (1996) Anaerobic biotransformation of 2,4-dinitrotoluene with ethanol as primary substrate: mutual effect of the substrates on their biotransformation. *Wat. Res.* **30**:307-314.
9. Chung, K.T., Stevens, S.E., Cerniglia, C.E. (1992) The reduction of azo dyes by the intestinal microflora. *Crit. Rev. Microbiol.* **18**:175-190.
10. Donlon, B., Razo-Flores, E., Field, J., Lettinga, G. (1995) Toxicity of N-substituted aromatics to acetoclastic methanogenic activity in granular sludge. *Appl. Environ. Microbiol.* **61**:3889-3893.
11. Donlon, B., Razo-Flores, E., Lettinga, G., Field, J. (1996) Continuous detoxification, transformation, and degradation of nitrophenols in upflow anaerobic sludge blanket (UASB) reactors. *Biotechnol. Bioeng.* **51**:439-449.
12. Field, J.A., Stams, A.J.M., Kato, M., Schraa, G. (1995) Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consortia. *Antoine van Leeuwenhoek* **67**:47-77.
13. Fitzgerald, S.W., Bishop, P.L. (1995) Two stage anaerobic/aerobic treatment of sulfonated azo dyes. *J. Environ. Sci. Health* **A30(6)**:1251-1276.
14. Gingell, R., Walker, R. (1971) Mechanisms of azo reduction by *Streptococcus faecalis*. II. The role of soluble flavins. *Xenobiotica* **1**:231-239.
15. Haug, W., Schmidt, A., Nörtermann, B., Hempel, D.C., Stolz, A., Knackmuss, H.J. (1991) Mineralization of the sulfonated azo dye mordant yellow 3 by a 6-aminonaphtalene-2-sulfonate-degrading bacterial consortium. *Appl. Environ. Microbiol.* **57**:3144-3149.
16. Idaka, E., Ogawa, T., Yatome, C., Horitsu, H. (1985) Behavior of activated sludge with dyes. *Bull. Environ. Contam. Toxicol.* **35**:729-734.
17. Kriek, E. (1979) Aromatic amines and related compounds as carcinogenic hazards to man. In: Emmelot, P., Kriek, E. (eds). Environmental carcinogenesis, pp 143-164. Elsevier, Amsterdam.
18. Kulla, H.G., Klausener, F., Meyer, U., Ludeke, B., Leisinger, T.H. (1983) Interference of aromatic sulfo groups in the microbial degradation of the azo dyes Orange I and Orange II. *Arch. Microbiol.* **135**:1-7.
19. Lettinga, G., Field, J.A., Sierra, R., van Lier, J.B., Rintala, J. (1991) Future perspectives for the anaerobic treatment of forestry industry wastewaters. *Wat. Sci. Technol.* **24**:91-102.
20. Longstaff, E. (1983) An assessment and categorization of the animal carcinogenicity data on selected dyestuffs and an extrapolation of those data on the relative carcinogenic risk to man. *Dyes and Pigments* **4**:243-304.
21. NEN 6646 (1992) Photometric determination of ammonium content with a continuous flow-throug system, NNI, Delt, The Netherlands.
22. O'Connor, O.A., Young, L.Y. (1989) Toxicity and anaerobic biodegradability of substituted phenols under methanogenic conditions. *Environ. Toxicol. Chem.* **8**:853-862.
23. O'Connor, O.A., Young, L.Y. (1993) Effect of nitrogen limitation on the biodegradability and toxicity of nitro and aminophenol isomers to methanogenesis. *Arch. Environ. Contam. Toxicol.* **25**:285-291.
24. Ogawa, T., Yamada, Y., Idaka, E. (1978) The respiratory inhibition of activated sludge by dyes. *J. Soc. Fiber Sci. Technol. Jpn.* **34**:T175-180.
25. Oren, A., Gurevich, P., Henis, Y. (1991) Reduction of nitrosubstituted aromatic compounds by the halophilic anaerobic eubacteria *Haloanaerobium praevalens* and *Sporohalobacter marismortui*. *Appl. Environ. Microbiol.* **57**:3367-3370.

26. Pagga, U., Brown, D. (1986) The degradation of dyestuffs: Part II, behaviour of dyestuffs in aerobic biodegradation test. *Chemosphere* **15**:479-491.
27. Pitter, P. (1976) Determination of biological degradability of organic substances. *Wat. Res.* **10**: 231-235.
28. Razo-Flores, E., Donlon, B., Field, J., Lettinga, G. (1996) Biodegradability of N-substituted aromatics and alkylphenols under methanogenic conditions using granular sludge. *Wat. Sci. Technol.* **33(3)**:47-57.
29. Roxon, J.J., Ryan, A.J., Wright, S.E. (1967) Enzymic reduction of tartrazine by *Proteus vulgaris* from rats. *Food Cosmet. Toxicol.* **5**:645-656.
30. Seshadri, S., Bishop, P.L., Agha, A.M. (1994) Anaerobic/aerobic treatment of selected azo dyes in wastwater. *Waste Management* **14**:127-137.
31. Shaul, G.M., Holsdworth, T.J., Dempsey, C.R., Kostal, K.A. (1991) Fate of water soluble azo dyes in the activated sludge process. *Chemosphere* **22**:107-119.
32. Sierra, R., Lettinga, G. (1991) The effect of aromatic structure on the inhibition of acetoclastic methanogens in granular sludge. *Appl. Microbiol. Biotechnol.* **34**:544-550.
33. Tarwin, D., Buswell, A.M. (1934) The methane fermentation of organic acids and carbohydrates. *J. Amer. Chem. Soc.* **56**:1751-1755.
34. United States International Trade Commission (1986) Synthetic Organic Chemicals. USITC Publication 1892, Washington, DC
35. Vaidya, A.A., Datye, K.V. (1982) Environmental pollution during chemical processing of synthetic fibers. *Colourage* **14**:3-10.
36. Wackett LP, Schanke CA (1992) Mechanism of reductive dehalogenation by transition metal cofactors found in anaerobic bacteria. In: Sigel H, Sigel, A. (eds). Metal ions in biological systems, vol 28, pp 329-356. Marcel Dekker, New York.
37. Weber, E.J., Wolfe, N.L. (1987) Kinetic studies of the reduction of aromatic azo compounds in anaerobic sediment/water systems. *Environ. Toxicol. Chem.* **6**:911-919.
38. Wuhrmann, K., Mechsner, K., Kappeler, K. (1980) Investigation on rate-determining factors in the microbial reduction of azo dyes. *European J. Appl. Microbiol. Biotechnol.* **9**:325-338.
39. Wu, W.M., Bhatanagar, L., Zeikus, J.G. (1993) Performance of anaerobic granules for degradation of pentachlorophenol. *Appl. Environ. Microbiol.* **59**:389-397.
40. Zimmermann, T., Gasser, F., Kulla, H.G., Leisinger, T. (1984) Comparison of two bacterial azoreductases acquired during adaptation to growth on azo dyes. *Arch. Microbiol.* **138**:37-43.
41. Zollinger H. (1987) Color chemistry-syntheses, properties and applications of organic dyes and pigments, pp 92-102. VCH Publishers, New York.

7

Biotransformation and Biodegradation of N-Substituted Aromatics in Methanogenic Granular Sludge: Discussion and Conclusions

Elías Razo Flores
Brian A. Donlon
Gatze Lettinga
Jim A. Field

Summary

N-substituted aromatic compounds are environmental contaminants associated with the production and use of dyes, explosives, pesticides and pharmaceuticals. In this chapter the results of this dissertation on the potential of anaerobic granular sludge from anaerobic treatment systems towards the detoxification, transformation, and mineralization of nitroaromatic and azo compounds is reviewed and discussed. Nitroaromatics and azo dyes with strong electron withdrawing groups are highly inhibitory to acetoclastic methanogenic bacteria. However, nitro and azo substituted aromatics are readily reductively detoxified in methanogenic consortia to their respective aromatic amines which are several orders of magnitude less toxic. This reductive detoxification has allowed the successful operation of anaerobic reactors for the treatment of highly toxic nitroaromatic and azo dye compounds. In the course of the experiments it was discovered that some aromatic amines were mineralized in the absence of oxygen in methanogenic consortia. These results indicate that hydroxy and carboxy substituted aromatic amines can be completely mineralized and serve as a carbon and energy source for anaerobic bacteria.

Adapted from FEMS Microbiology Reviews (1997) ***20(3-4)****:525-538*

7.1 Introduction

N-substituted aromatics, such as nitroaromatics, azo dyes and aromatic amines, are important priority pollutants entering the environment primarily through anthropogenic activities associated with the industrial production of dyes, explosives, pesticides and pharmaceuticals (15, 32). The presence of these aromatic xenobiotics in the environment may create serious public health and environmental problems. Some of these compounds have mutagenic or carcinogenic activity and may bioaccumulate in the food chain (10, 22).

Many nitroaromatics have been shown to be toxic or mutagenic to microorganisms (26, 28). The toxicity of these compounds to microorganism together with their recalcitrant nature can prove to be problematic for their effective biological treatment. It has been suggested that aromatics with multiple nitro substituents and azo dyes are resistant to electrophilic attack by oxygenases (14, 16). However, these compounds are readily reduced by anaerobic consortia to aromatic amines (4, 11). In many cases, the aromatic amines are not metabolized further under anaerobic conditions (4, 9). However, the reduction of nitro- and azo-groups increases the susceptibility of the aromatic molecule for aerobic degradation (5, 8). Consequently, sequenced degradation of nitroaromatics and azo dyes has been proposed (8, 9, 16).

Here we summarize the results of studies conducted in this dissertation, evaluating the toxicity and biodegradability of a wide range of N-substituted aromatics in standardized anaerobic batch assays using granular sludge. Biodegradability studies of N-substituted aromatics were also conducted in continuous UASB reactors.

7.2 Toxicity of N-substituted Aromatics

Nitroaromatics

The knowledge of the toxicity of N-aromatic compounds is essential in predicting the impact of these priority pollutants on anaerobic wastewater treatment systems. Of all the

classes of organisms involved in anaerobic degradation reactions, the methanogens are reported to be the most sensitive to inhibition (30). Consequently, acetoclastic methanogenic toxicity for selected N-substituted aromatics was studied. The results of Chapter 2 indicate that nitroaromatics were on the average over 500-fold more toxic than their aromatic amine analogous, highlighting the detoxification process that occurs by the anaerobic reduction of nitroaromatics. Some general relationships between the aromatic structure and their inhibitory effects on methanogenic bacteria were also observed. N-substitutions were more toxic than other ring substituents or benzene itself. The toxicity of the mono-substituted benzenes was observed to increase in the following order: COOH < H < OH < NH_2 < NO_2. Nitrosubstituted phenols and benzenes were at least 2 orders of magnitude more toxic than their alkyl- and chloro-substituted analogous of equivalent hydrophobicity. This observation clearly indicates a higher chemical reactivity of the nitro-groups compared with that of alkyl and chloro groups. Thus, when present at similar concentrations in bacterial membranes, the nitrosubstituted aromatics exert a much higher toxic effect than that which can be accounted for by membrane toxicity alone. Nitroaromatics have been reported to be reactive toxicants (2). The reactivity of the nitrosubstituents could enable these compounds to undergo chemical interactions with proteins; thereby inactivating vital enzymes (19). The toxicity of these compounds to methanogens has been suggested to involve interactions between nitroaromatics or intermediates of the reduction process (nitrosoamines or hydroxylamines) and the unique cell membrane of the methanogens (12). N-substituted aromatics may also interfere with the outcome of a biochemical conversion, such as the uncoupling of phosphorylation reactions or interfering with physiological redox couples (28).

Azo Dyes

Azo dyes are synthetic chemicals used for dyeing. Consequently, colour is the first contaminant to be recognized in the wastewaters of the industries using these compounds. Azo dyes are intentionally designed to be recalcitrant under typical product service conditions and, it is this property allied with their toxicity to microorganisms that makes biological treatment difficult (18). The results of Chapter 6 indicate that azo dyes were highly toxic against the

acetoclastic methanogens. The azo dyes tested and the nitroanilines were much more toxic than their respective aromatic amine biotransformation products, including Mordant Yellow 12 (MY12) that does not have any nitro substituent in its structure, indicating that the azo group itself has a toxic effect on the methanogens. The 50% inhibitory concentration (IC) value of 0.25 mM for MY12, is comparable to the toxicity of some nitroaromatic compounds.

7.3 Reduction of Nitroaromatics and Azo Dyes

Nitroaromatics and azo dyes are easily reduced under anaerobic conditions to aromatic amines (6, 7, 27). However, cosubstrates are required as an electron source for the reduction. Enhancement of nitro- and azo-reduction through addition of cosubstrates was demonstrated in Chapters 5 and 6. As shown in Table 7.1, it was observed that hydrogen, an interspecies-reduced compound, and substrates that provide interspecies-reducing equivalents such as butyrate, propionate, and ethanol stimulated nitro-reduction; whereas, acetate and methanol which are directly used by the methanogens as substrates did not. Similarly, it was found that glucose and volatile fatty acid (VFA) mixture enhanced the reduction of the azo dyes Mordant Orange 1 (MO1) and Azodisalicylate (ADS). Results in Chapter 6 show that 5-aminosalicylic acid (5ASA), which is an ADS-breakdown product, enhanced the reduction of the parent dye, indicating that even aromatic amines could be a good electron donors once an aromatic amine degrading population of bacteria had developed in the consortium.

7.4 Continuous Detoxification of Nitro and Azo Compounds in UASB Reactors

The application of high-rate reactors, such as the UASB reactor, have proven to be capable of treating various wastewaters bearing toxic aromatic compounds with a high degree of efficiency and stability (3, 29). The facile reduction of nitro- and azo- electron withdrawing groups was used as a detoxification strategy in continuous laboratory scale UASB reactors, treating selected nitroaromatic and azo dye compounds fed with either glucose or VFA

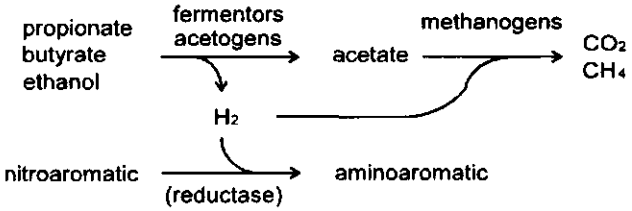
mixture as cosubstrates. All the nitroaromatics tested in Chapter 5 were converted to aromatic amines in stoichiometric quantities. The reduction of the nitro groups to less toxic amino substituents ensured that a detoxification of the influent was obtained. The reactors treating the mononitroaromatics were able to convert the cosubstrate to methane at organic loading rates in excess of 13.2 g COD/L-d, with greater than 96% cosubstrate removal efficiency, even though, the influent concentration of the mononitroaromatics was up to 30-fold higher than the 50% IC to acetoclastic methanogenesis. In the case of the dinitroaromatics, the cosubstrate removal efficiency was high for 2,4-dinitrotoluene (2,4DNT), but lower for the 2,4-dinitrophenol (2,4DNP), possibly due to an acute toxicity of the latter compound. Results in Chapter 5 indicated that very high mononitroaromatic loading rates (up to 910 mg/L-d) were treated in the UASB reactors, with greater than 98% removal of the nitroaromatics.

TABLE 7.1 Effect type of cosubstrate on rate nitroaromatic reduction.

- Endogenous substrates in sludge support limited reduction of nitroaromatics.

- Rate reduction is not stimulated by using acetate nor methanol.

- Rate reduction is stimulated by using propionate, butyrate, ethanol or hydrogen.



In a similar way, the anaerobic transformation of the azo dye MO1 in continuous UASB reactors using granular sludge was studied in Chapter 6. MO1 was readily cleaved in

the reactors receiving glucose and VFA at MO1 loading rates up to 295 and 161 mg/L-d, respectively. These concentrations exceeded the 50%IC of the MO1 to methanogenic bacteria by 25 and 13 fold in the glucose and VFA reactors, respectively. In these reactors, both 1,4-phenylenediamine (1,4PDA) and 5ASA were detected as products of MO1 cleavage.

The effective regimes applied for the treatment of highly toxic nitro and azo aromatics rely mainly on three key factors as shown in Figure 7.1: reductive anaerobic detoxification to products of lower toxicity as demonstrated in Chapters 2 and 6; good hydraulic and gas mixing conditions minimizing biological dead-space and preventing localized high concentrations of the toxic nitroaromatic or azo dye compounds (13, 24); and protection of the methanogens inside the granules (presence of less toxic aromatic amines due to the reducing conditions) from toxic compounds of the bulk of the reactor (nitroaromatic or azo dye compounds).

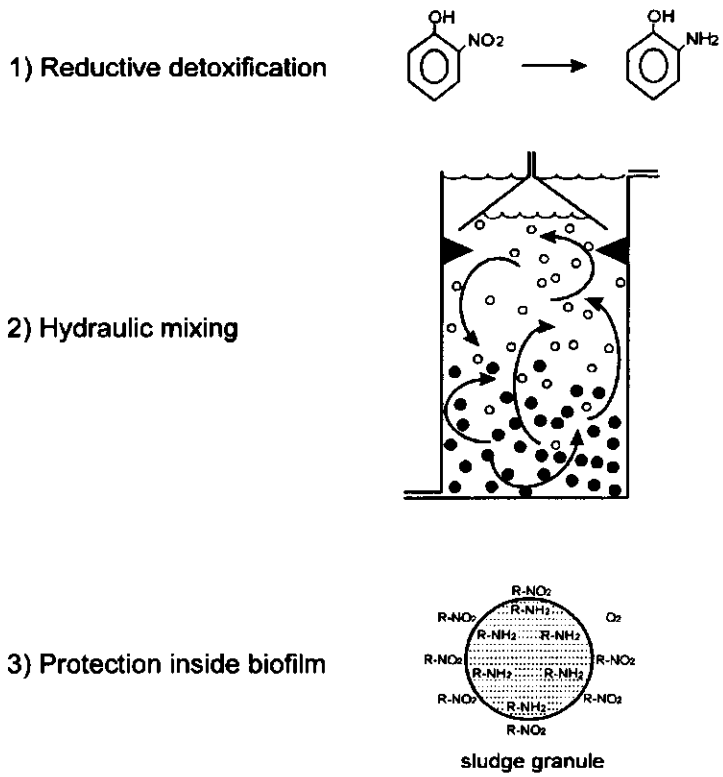


Figure 7.1 The three factors contributing to detoxification of nitroaromatics in upflow anaerobic sludge bed reactors.

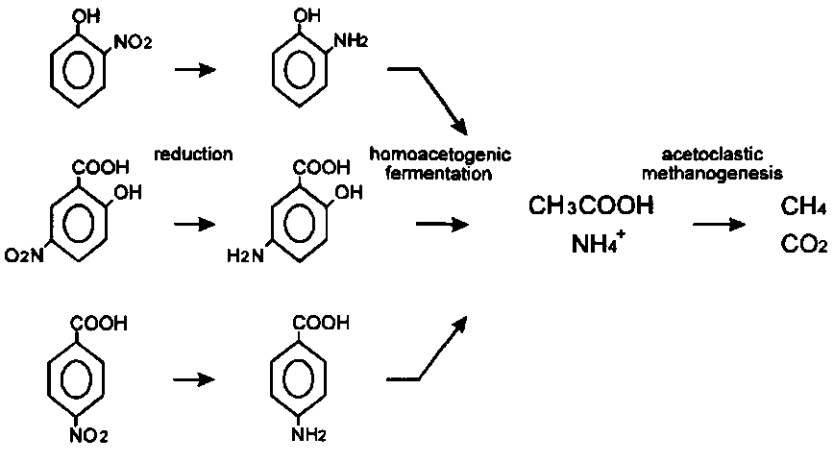
7.5 Anaerobic Biodegradation of Aromatic Amines

The electron donating amino groups formed from the reduction of nitro and azo groups are expected to pose a serious problem for further reductive biotransformations by anaerobes (9). In fact, aniline, the simplest aromatic amine has been found to be recalcitrant in methanogenic consortia (1, 25). However, there is growing evidence that anaerobic microorganisms and consortia can mineralize many aromatic amines. Aromatic amines with carboxy, hydroxy and methoxy substitutions are potentially mineralizable in methanogenic consortia. Results in Chapter 3 show that four out of seventeen aromatic amines were mineralized using unadapted granular sludge, namely: the three isomers of aminobenzoate and 2-aminophenol (2AP). However, 5ASA and 4-aminophenol (4AP), which were recalcitrant with the unadapted sludge, were mineralized by the 2-nitrophenol (2NP) adapted granular sludge after a 65 and 70 day lag period. Additionally, the anaerobic mineralization of 5ASA was observed for the first time.

The results of the study using 2NP-adapted granular sludge, and the parallel ones in Chapter 4 evaluating the ability of bacterial consortia from five different granular sludge sources to anaerobically biodegrade aromatic compounds, showed that cross-acclimatization of N-substituted aromatics occur. This finding confirms the possibility to cross-acclimatize sludge for the mineralization of other structurally similar compounds using aromatic-adapted sludge (17, 31). Based on these results, it is clear that one of the bottlenecks of aromatic degradation is the adaptation process. Once the sludge is adapted, the degradation proceeds at similar rates, independent of the origin of the sludge.

Microbial communities in sediments could use alternative electron acceptors such as NO_3^-, $SO_4^=$, Mn(IV) and Fe(III) (20, 21, 23). Aniline, the simplest aromatic amine, was not degraded under methanogenic conditions but was under denitrifying and sulfate reducing conditions (1, 25), highlighting the enormous potential of microbial communities in anaerobic environments to degrade aromatic amines when these alternative electron acceptors are available.

A) Complete mineralization: 2-nitrophenol, 5-nitrosalicylate and 4-nitrobenzoate



B) Biotransformation: 4-nitrophenol, 2,4-dinitrophenol, 2,4-dinitrotoluene and nitrobenzene

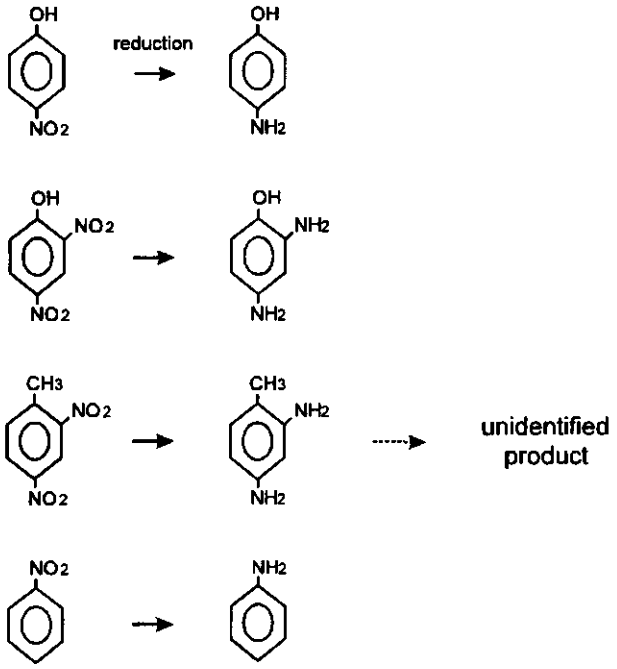


Figure 7.2 Final fate of nitroaromatics in the UASB reactors.

7.6 Complete Biodegradation of Nitro and Azo Aromatics

Results from the continuous UASB reactors experiments in Chapters 5 and 6 suggest that some nitroaromatics and azo dyes are fully biodegradable in anaerobic environments. After long term reactor operation, aromatic amines were no longer observed to accumulate as products of 2NP, 4-nitrobenzoic acid (4NBc) and 5-nitrosalicylic acid (5NSA) elimination, suggesting that they were completely biodegraded in the continuous reactors. The granular sludge sampled from these reactors were able to fully mineralize 2AP, 4-aminobenzoate (4ABc) and 5ASA via homoacetogenic fermentation when these were offered as the sole carbon and energy source in anaerobic biodegradability assays. On the other hand, 4AP and 2,4-diaminophenol accumulated as biotransformation products from 4-nitrophenol (4NP) and 2,4DNP, and were not eliminated further in the continuous reactors nor were they mineralized by the sampled sludge in batch assays. However, in the case of 2,4DNT, only 52% of the compound was recovered as 2,4-diaminotoluene (2,4DAT), indicating that the other 48% was biotransformed. Lack of mineralization indicated that 2,4DAT was transformed to a non-identified and non-degradable dead-end metabolite. These results are in agreement with those obtained in the batch biodegradability assays in Chapter 3. The final fate of nitroaromatics in the UASB reactors is depicted in Figure 7.2.

In the case of azo dyes in Chapter 6, it was found that MO1 was partially mineralized. After 180 days of operation, 5ASA arising from MO1 cleavage could only be detected at trace concentrations in the glucose fed reactor. 5ASA was completely mineralized in the batch anaerobic biodegradability assay with the sampled reactor sludge, but the other cleavage product, 1,4PDA was not mineralized. These findings indicate that 5ASA was fully degraded by the anaerobic consortia; whereas, 1,4PDA persisted.

The degradation of a pharmaceutical azo dye, ADS, constructed from two 5ASA molecules, was also studied in Chapter 6 under batch and continuous conditions using 5ASA-adapted sludge. ADS was highly mineralized in continuous reactors even when cosubstrate was not present in the basal medium. Batch experiments with ADS as the sole carbon an energy source confirmed the dye mineralization to methane and ammonium, and it was also

shown that the metabolism of 5ASA released from ADS cleavage could provide electrons supporting continued reduction of the dye. According to this result, it is likely that the cosubstrate was only necessary to establish an active methanogenic consortium during the adaptation to the azo dye, and that cosubstrate supplementation was no longer essential once 5ASA-degrading bacteria developed in the consortium. According to the results obtained in Chapter 6, complete mineralization of azo dyes under methanogenic and/or anoxic conditions could be obtained as proposed in Figure 7.3.

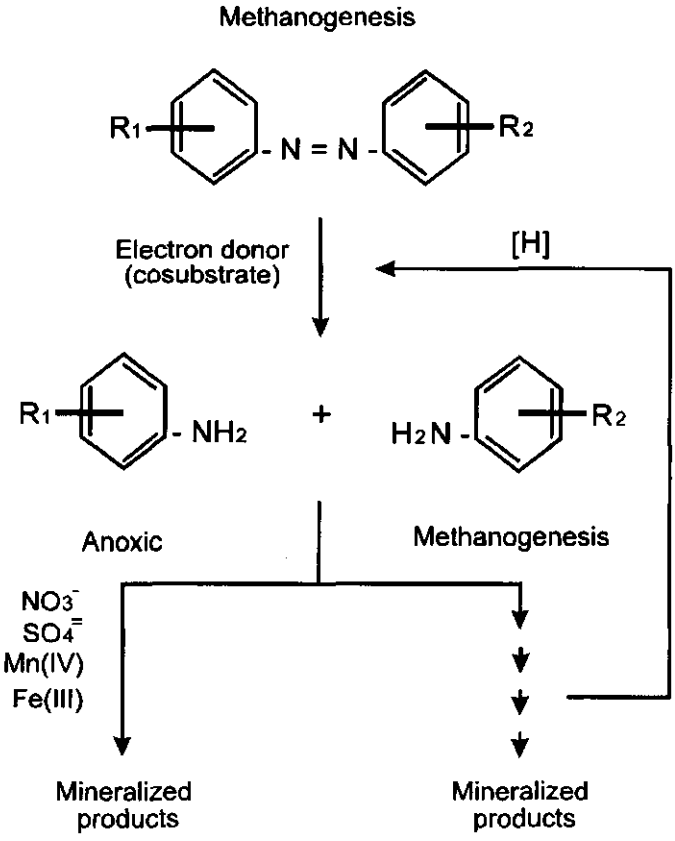


Figure 7.3 Biodegradation of azo dyes under methanogenic and/or anoxic conditions. R_1 and/or R_2 = -COOH, -SO_3H, -NO_2, -NH_2, -OH, -CH_3, -OCH_3, -H and/or any other substituent. If R_1 and/or R_2 = -COOH, -OH, -OCH_3 the azo dyes can be completely mineralized under methanogenic conditions; with all the other substituents the aromatic amines could potentially be degraded by the anoxic pathways with the alternative electron acceptors.

7.7 Conclusions and Recommendations

The anaerobic biodegradation of N-aromatic compounds was still doubted twenty years ago when very little knowledge was known about its microbiological potential. However, nowadays anaerobic biodegradation of N-aromatic compounds is a promising alternative to aerobic degradation processes. The results shown here indicate that the anaerobic reductive transformation of nitroaromatic and azo compounds leads to a detoxification of these substances. We have also shown that some nitroaromatic compounds and azo dyes can be completely mineralized and serve as a carbon and energy source for anaerobic bacteria, in contrast to the common assumption that they are only biotransformed to mutagenic and carcinogenic aromatic amines. Therefore, we conclude that it should be possible for industry to design nitro and azo aromatic compounds that are fully biodegradable in anaerobic environments.

More research should be conducted in order to gain insight in both microbiological (the know) and technological aspects (the how) of N-aromatic biodegradation. Below are a few suggestions for the continuation of this research:

- fate of aromatic amines and reduction of nitroaromatics and azo dyes under alternative electron acceptors environments such as NO_3^-, $SO_4^=$, Mn(IV), Fe(III) and/or microaerobic conditions.
- studies to identify the microbial degradation pathway(s) and the microorganism(s) responsible for the degradation. The role of the microbial adaptation should be clarified.
- toxicity and biodegradability studies with more complex azo dyes (e.g., sulphonated and/or naphtholic) and their breakdown products.
- comparison studies evaluating the performance of different kind of high rate anaerobic reactors treating nitroaromatics and azo dyes. The use of "real" wastewater instead synthetic wastewater is strongly advised.

References

1. Alexandra de, M., O'Connor, O.A., Kosson, D.S. (1994) Metabolism of aniline under different anaerobic electron-accepting and nutritional conditions. *Environ. Toxicol. Chem.* **13**:233-239.
2. Blum, D.J.W., Speece, R.E. (1991) A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. *Res. J. Water Pollut. Control Fed.* **63**:198-207.
3. Borghans, A.J.M.L., van Driel, A. (1988) Application of the Biothane UASB reactor to a chemical wastewater containing phenol and formaldehyde. In: Tilche, A., Rozzi, A. (Eds.). 5th. International Symposium on Anaerobic Digestion, pp. 627-630. Monduzzi Editore, Bologna.
4. Brown, D., Laboureur, P. (1983) The degradation of dyestuffs: Part I primary biodegradation under aerobic conditions. *Chemosphere* **12**:397-404.
5. Brown, D., Laboureur, P. (1983) The aerobic biodegradability of primary aromatic amines. *Chemosphere* **12**:405-414.
6. Chung, K.T., Stevens, S.E., Cerniglia, C.E. (1992) The reduction of azo dyes by the intestinal microflora. *Crit. Rev. Microbiol.* **18**:175-190.
7. Chung, K.T. and Stevens, S.E. (1993) Degradation of azo dyes by environmental microorganisms and helminths. *Environ. Toxicol. Chem.* **12**:2121-2132.
8. Dickel, O., Haug, W., Knackmuss, H.J. (1993) Biodegradation of nitrobenzene by a sequential anaerobic-aerobic process. *Biodegradation* **4**:187-194.
9. Field, J.A., Stams, A.J.M., Kato, M., Schraa, G. (1995) Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consortia. *Antoine van Leeuwenhoek* **67**:47-77.
10. Fishbein, L. (1984) Aromatic amines. In: Hutziger, O. (Ed.). The handbook of environmental chemistry, Vol. 3, pp. 1-40, Springer, New York.
11. Gorontzy, T., Drzyga, O., Kahl, M.W., Bruns-Nagel, D., Breitung, J., von Loew, E., Blotevogel, K.H. (1994) Microbial degradation of explosives and related compounds. *Crit. Rev. Microbiol.* **20**:265-284.
12. Gorontzy, T., Kuver, J., Blotevogel, K.H. (1993) Microbial transformation of nitroaromatic compounds under anaerobic conditions. *J. Gen. Microbiol.* **139**:1331-1336.
13. Grobicki, A., Stuckey, D.C. (1992) Hydrodynamic characteristics of the anaerobic baffled reactor. *Wat. Res.* **26**:371-378.
14. Hallas, L.E., Alexander, M. (1983) Microbial transformation of nitroaromatic compounds in sewage effluent. *Appl. Environ. Microbiol.* **45**:1234-1241.
15. Hartter, D.R. (1985) The use and importance of nitroaromatic chemicals in the chemical industry. In: Rickert, D.E. (Ed.). Toxicity of nitroaromatic nompounds; Chemical Industry Institute of Toxicology series, pp. 1-14. Chemosphere, Washington DC.
16. Haug, W., Schmidt, A., Nortermann, B., Hempel, D.C., Stolz, A., Knackmuss, H.J. (1991) Mineralization of the sulfonated azo dye Mordant Yellow 3 by a 6-aminonaphtalene-2-sulfonate-degrading bacterial consortium. *Appl. Environ. Microbiol.* **57**:3144-3149.
17. Healy, J.B., Young, L.Y. (1979) Anaerobic biodegradation of eleven aromatic compounds to methane. *Appl. Environ. Microbiol.* **38**:84-89.
18. Idaka, E., Ogawa, T., Yatome, C., Horitsu, H. (1985) Behavior of activated sludge with dyes. *Bull. Environ. Contam. Toxicol.* **35**:729-734.
19. Jaworska, J.S., Schultz, T.W. (1994) Mechanism-based comparisons of acute toxicities elicited by industrial organic chemicals in prokaryotic and eukaryotic systems. *Ecotoxicol. Environ. Saf.* **29**:200-213.

20. Kazumi, J., Haggblom, M.M., Young, L.Y. (1995) Diversity of anaerobic microbial processes in chlorobenzoate degradation: nitrate, iron, sulfate and carbonate as electron acceptors. *Appl. Microbiol. Biotechnol.* **43**:929-936.
21. Langenhoff, A. (1997) Biotransformation of toluene, benzene and naphthalene under anaerobic conditions. Ph.D. Thesis, Wageningen Agricultural University, The Netherlands.
22. Longstaff, E. (1983) An assesment and categorization of the animal carcinogenicity data on selected dyestuffs and an extrapolation of those data on the relative carcinogenic risk to man. *Dyes and Pigments* **4**:243-304.
23. Lovley, D.R., Woodward, J.C., Chapelle, F.H. (1994) Stimulated anoxic biodegradation of aromatic hydrocarbons using Fe (III) ligands. *Nature* **370**:128-131.
24. van der Meer, R.R. (1979) Anaerobic treatment of wastewater containing fatty acids in upflow reactors. Ph.D. Thesis, Delft University of Technology, The Netherlands.
25. Schnell, S., Bak, F., Pfennig, N. (1989) Anaerobic degradation of aniline and dihydroxybenzenes by newly isolated sulfate-reducing bacteria and description of *Desulfobacterium anilini*. *Arch. Microbiol.* **152**:556-563.
26. Shea, P.J., Weber, J.B., Overcash, M.R. (1983) Biological activities of 2,4-dinitrophenol in plant-soil systems. *Residue Reviews* **87**:2-41.
27. Spain, J.C. (1995) Biodegradation of nitroaromatic compounds. *Annu. Rev. Microbiol.* **49**:523-555.
28. Tereda, H. (1981) The interaction of highly active uncouplers with mitochondria. *Biochimica et Biophysica Acta* **639**:225-242.
29. Wu, W.M., Bhatnagar, L., Zeikus, J.G. (1993) Performance of anaerobic granules for degradation of pentachlorophenol. *Appl. Environ. Microbiol.* **59**:389-397.
30. Yang, J., Speece, R.E. (1986) Effects of engineering controls on methane fermentation. *J. Water Pollut. Control Fed.* **57**:1134-1140.
31. Young, L.Y., Rivera, M.D. (1985) Methanogenic degradation of four phenolic compounds. *Wat. Res.* **19**:1325-1332.
32. Zollinger, H. (1987) Color chemistry-syntheses, properties and applications of organic dyes and pigments, pp. 92-102. VCH Publishers, New York.

7

Biotransformatie en Biodegradatie van N-gesubstitueerde Aromaten in Methanogeen Korrelslib: Discussie en Conclusies

Elías Razo Flores
Brian A. Donlon
Gatze Lettinga
Jim A. Field

Samenvatting

N-gesubstitueerde aromatische verbindingen zijn milieuverontreinigende stoffen, waarvan de aanwezigheid in het milieu voortkomt uit de productie en het gebruik van kleurstoffen, explosieven, pesticiden en farmaceutische verbindingen. In dit hoofdstuk wordt een overzicht gegeven van de resultaten in dit proefschrift aangaande de toepassing van anaëroob korrelslib bij de detoxificatie, transformatie en mineralisatie van nitroaromaten en azoverbindingen. Tevens worden de resultaten bediscussieerd. Nitroaromaten en azokleurstoffen hebben sterk elektronegatieve groepen, die zeer remmend werken op acetoclastische methanogene bacteriën. Nitro- en azogesubstitueerde aromaten worden echter gemakkelijk reductief gedetoxificeerd in methanogene consortia, hetgeen leidt tot de vorming van hun aromatische amine-analogen, die aanzienlijk minder toxisch zijn. Deze reductieve detoxificatie maakt een succesvolle behandeling van zeer toxische nitroaromaten en azokleurstoffen in anaërobe reactoren mogelijk. In de loop van het onderzoek werd duidelijk dat sommige aromatische amines in afwezigheid van zuurstof worden gemineraliseerd in methanogene consortia. Dit geeft aan dat hydroxy- en carboxygesubstitueerde aromatische amines bij volledige mineralisatie kunnen dienen als koolstof- en energiebron voor anaërobe bacteriën.

*Bewerkt naar FEMS Microbiology Reviews (1997) **20(3-4)**:525-538*

7.1 Inleiding

N-gesubstitueerde aromaten, zoals nitroaromaten, azokleurstoffen en aromatische amines, zijn belangrijke verontreinigende verbindingen, die vooral in het milieu terecht komen door antropogene activiteiten zoals de industriële productie van kleurstoffen, explosieven, pesticiden en farmaceutische verbindingen (15, 32). Hun aanwezigheid in het milieu zou ernstige problemen kunnen veroorzaken voor de volksgezondheid en het milieu. Sommige van deze verbindingen hebben mutagene of carcinogene activiteit en kunnen bioaccumuleren in de voedselketen (10, 22).

Veel nitroaromaten zijn toxisch of mutageen voor micro-organismen (26, 28). Dit, tezamen met het feit dat ze moeilijk worden afgebroken, zou problematisch kunnen zijn voor een effectieve biologische behandeling van afvalstromen, die nitroaromaten bevatten. Er is gesuggereerd dat aromaten met meerdere nitro-substituenten en azokleurstoffen niet kunnen worden afgebroken via een electrofiele aanval door oxygenases (14, 16). Echter, dit soort verbindingen wordt door anaërobe consortia wel gemakkelijk gereduceerd tot aromatische amines (4, 11). Deze aromatische amines worden vaak niet verder gemetaboliseerd onder anaërobe omstandigheden (4, 9), maar ze kunnen door de reductie van de nitro- en azogroepen in veel gevallen wel gemakkelijker worden omgezet via aërobe afbraak (5, 8). Daarom wordt een sequentiële anaërobe-aërobe afbraak van nitroaromaten en azokleurstoffen voorgesteld (8, 9, 16).

In dit hoofdstuk vatten wij de resultaten van de verschillende studies die zijn uitgevoerd in het kader van dit proefschrift samen. In het beschreven onderzoek is de toxiciteit en biologische afbreekbaarheid van een breed scala aan N-gesubstitueerde aromaten in gestandaardiseerde anaërobe batchtesten met anaëroob korrelslib geëvalueerd. Tevens zijn biodegradatie-studies uitgevoerd in continu bedreven UASB-reactoren.

7.2 Toxiciteit van N-gesubstitueerde Aromaten

Nitroaromaten

Kennis van de toxiciteit van N-aromatische verbindingen is van grote waarde bij het voorspellen van hun invloed op anaërobe waterzuiveringssystemen. Van alle verschillende klassen van micro-organismen die betrokken zijn bij anaërobe afbraak, zijn methanogene bacteriën het meest gevoelig voor remming door toxische stoffen (30). Daarom is de toxiciteit van enkele N-gesubstitueerde aromaten onderzocht. De resultaten in Hoofdstuk 2 laten zien dat nitroaromaten gemiddeld 500 maal meer toxisch zijn dan hun respectievelijke aromatische amine-analogen. Dit geeft duidelijk het belang aan van de detoxificering die plaatsvindt bij de anaërobe reductie van nitroaromaten. Er zijn enkele algemene verbanden waargenomen tussen de aromatische structuur en de remmende effecten op methanogene bacteriën. N-substituties zijn toxischer dan andere ringsubstituenten of benzeen zelf. De toxiciteit van monogesubstitueerde benzenen werd groter in de volgorde: $COOH < H < OH < NH_2 < NO_2$. Nitrogesubstitueerde fenolen en benzenen waren minstens twee maal zo toxisch als de alkyl- en chloorgesubstitueerde analogen met vergelijkbare hydrofobiciteit. Deze observatie wijst duidelijk op een hogere chemische reactiviteit van de nitrogroepen vergeleken met alkyl- of chloorgroepen. Ergo, bij aanwezigheid in vergelijkbare concentraties in bacteriële membranen oefenen nitrogesubstitueerde aromaten een veel groter toxisch effect uit dan dat wat kan worden toegekend aan de membraantoxiciteit alleen. Uit literatuurgegevens blijkt dat nitroaromaten reactieve toxicanten zijn (2). Door de reactiviteit van de nitrosubstituenten zouden chemische interacties met eiwitten mogelijk zijn, die inactivatie van vitale enzymen tot gevolg hebben (19). Daarnaast wordt verondersteld dat de toxiciteit van deze verbindingen voor methanogene bacteriën wordt veroorzaakt door interacties tussen nitroaromaten of intermediairen in het reductieproces (nitrosamines of hydroxylamines) met het unieke celmembraan van deze bacteriën (12). N-gesubstitueerde aromaten zouden ook het resultaat van biochemische omzettingen kunnen beïnvloeden, bijvoorbeeld via het ontkoppelen van fosforyleringsreacties of beïnvloeding van fysiologische redoxkoppels (28).

Azokleurstoffen

Azokleurstoffen zijn synthetische chemicaliën, die worden gebruikt in verfstoffen. Als gevolg daarvan is "kleur" de eerste vervuilende eigenschap in het afvalwater van de industrieën die dit soort stoffen gebruiken. De chemische structuur van azokleurstoffen maakt dat ze onafbreekbaar zijn onder de typische omstandigheden waarbij ze worden toegepast. Het is deze eigenschap, samen met de toxiciteit voor micro-organismen, die de biologische behandeling van afvalstromen met azokleurstoffen moeilijk maakt (18). De resultaten in Hoofdstuk 6 geven aan dat azokleurstoffen zeer toxisch zijn voor acetoclastische methanogene bacteriën. De onderzochte azokleurstoffen en nitroanilines waren veel toxischer dan hun respectievelijke aromatische amine-analogen, die ontstaan bij biotransformatie. Dit was ook het geval voor Mordant Yellow 12 (MY12), dat geen nitrogroepen aan de aromatische structuur gesubstitueerd heeft. Dit laatste wijst erop dat de azogroep zelf de methanogene bacteriën remt. De concentratie van 0.25 mM MY12, waarbij 50% remming van de methanogenese (50% IC) optreedt is vergelijkbaar met de toxiciteit van sommige nitroaromaten.

7.3 Reductie van Nitroaromaten en Azokleurstoffen

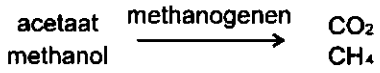
Onder anaërobe condities worden nitroaromaten en azokleurstoffen gemakkelijk tot aromatische amines gereduceerd (6, 7, 27). De toevoeging van een cosubstraat als elektronendonor is echter een vereiste. Verbetering van de nitro- en azoreductie door de toevoeging van cosubstraten is beschreven in de Hoofdstukken 5 en 6. Er is, zoals weergegeven in Tabel 7.1, waargenomen dat waterstof, waarmee elektronen tussen de bacteriën kunnen worden "doorgegeven" (via "interspecies hydrogen transfer") en (co)substraten, zoals butyraat, propionaat en ethanol, die reductie-equivalenten zoals waterstof opleveren, nitroreductie stimuleren. Substraten zoals methanol en acetaat, die direct door methanogenen kunnen worden gebruikt, bevorderen de omzetting niet. Analoog aan deze resultaten werd gevonden dat glucose en een mengsel van vluchtige vetzuren (VFA) de reductie van de azokleurstoffen Mordant Orange 1 (MO1) en Azodisalicylaat (ADS) stimuleren. De resultaten uit Hoofdstuk 6 laten tevens zien dat 5-aminosalicylzuur (5ASA), een

afbraakprodukt van ADS, de reductie van ADS bevordert. Dit geeft aan dat, na ontwikkeling van een aromatische amine-afbrekende populatie in het consortium, zelfs aminoaromaten als elektronendonor kunnen dienen.

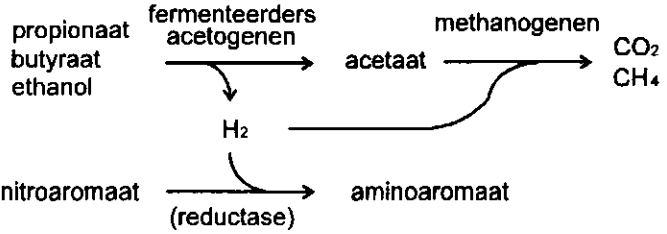
TABEL 7.1 Het effect van het type cosubstraat op de snelheid van nitroaromatische reductie.

- Endogene substraten in het slib leiden tot len beperkte reductie van de nitroaromaten.

- De reductiesnelheid wordt niet hoger bij gebruik van acetaat of methanol.



- De reductiesnelheid wordt hoger bij gebruik van propionaat, butyraat of waterstof.



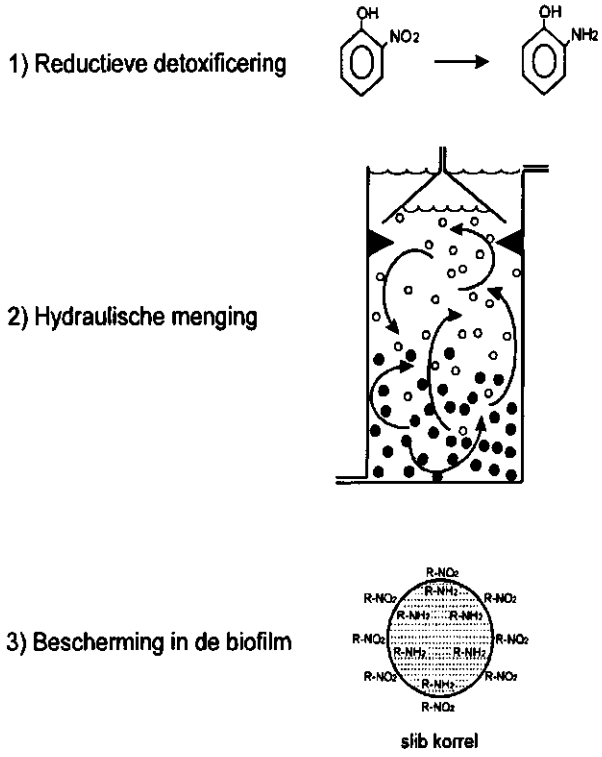
7.4 Continue Detoxificatie van Nitro- en Azoverbindingen in UASB reactoren.

Afvalwater dat toxische aromatische verbindingen bevat, kan efficiënt en stabiel behandeld worden in hoog-belaste reactoren, zoals de UASB-reactor (3, 29). De eenvoudige reductie van elektronenzuigende nitro- en azogroepen, is gebruikt als detoxificeringsstrategie in continu bedreven UASB-reactoren op laboratoriumschaal. Hierin werden geselecteerde nitroaromaten en azokleurstoffen afgebroken met glucose of een VFA-mengsel als cosubstraat.

Al de nitroaromaten, die werden onderzocht in de experimenten in Hoofdstuk 5, werden in stoichiometrische hoeveelheden omgezet naar aromatische amines. De reductie van de nitrogroepen naar minder toxische aminosubstituenten zorgde voor een detoxificering van het influent. De reactoren met mononitroaromaten in het influent waren in staat het cosubstraat volledig om te zetten in methaan bij organische belastingen hoger dan 13.2 g COD/L-d en met een verwijderingsefficiëntie voor het cosubstraat van meer dan 96%. Dit ondanks een nitroaromaatconcentratie in het influent die 30 maal hoger was dan de 50% IC voor acetoclastische methanogenen. Bij de behandeling van dinitroaromaten was de verwijderingsefficiëntie van het cosubstraat hoog in aanwezigheid van 2,4-dinitrotolueen (2,4DNT) maar, waarschijnlijk door acute toxiciteit, lager voor 2,4-dinitrofenol (2,4-DNP). De resultaten in Hoofdstuk 5 laten eveneens zien dat zeer hoge mononitroaromaat-belastingen (tot 910 mg/L-d) in UASB-reactoren kunnen worden behandeld met meer dan 98% verwijdering van de nitroaromaten.

Op vergelijkbare wijze is de anaërobe omzetting van de azokleurstof MO1 onderzocht in continu bedreven UASB-reactoren met korrelslib (Hoofdstuk 6). MO1 werd in aanwezigheid van glucose of VFA als cosubstraat gemakkelijk gesplitst in 1,4-diphenyleendiamine (1,4PDA) en 5ASA bij MO1-belastingen van respectievelijk 295 en 161 mg/L-d. De MO1-concentraties in het influent van de met glucose of VFA gevoede reactoren waren respectievelijk 25 en 13 maal groter dan de 50% IC van MO1 voor methanogene bacteriën.

De strategie die succesvol is toegepast bij de behandeling van de zeer toxische nitro- en azoaromaten berust voornamelijk op de drie sleutelfactoren die zijn weergegeven in Figuur 7.1: reductieve omzetting naar minder toxische producten (Hoofdstuk 2 en 6); goede hydraulische omstandigheden en gasmenging, zodat de hoeveelheid biologische "dode ruimte" wordt geminimaliseerd en wordt voorkomen dat er lokaal hoge concentraties van de toxische nitroaromaten en azokleurstoffen ontstaan (13, 24); en bescherming van de methanogenen in de korrels tegen de toxische verbindingen in de bulkvloeistof van de reactor (nitroaromatische of azokleurstof-verbindingen) door de aanwezigheid van minder toxische aromatische amines, die zijn ontstaan door de reducerende omstandigheden.



Figuur 7.1 De drie factoren die bijdragen aan detoxificatie van nitroaromaten in UASB-reactoren.

7.5 Anaërobe Biodegradatie van Aromatische Amines

Er wordt verwacht dat de elektronenstuwende aminogroepen die worden gevormd bij de reductie van nitro- en azogroepen moeilijk verder kunnen worden omgezet via reductieve biotransformatie door anaërobe bacteriën (9). Er is inderdaad voor aniline, het meest simpele aromatische amine, gevonden dat het niet kan worden afgebroken door methanogene consortia (1, 25). Echter, de hoeveelheid bewijs dat de omzetting van veel aromatische amines door anaërobe micro-organismen en consortia wel degelijk mogelijk is neemt toe. Aromatische amines met carboxy-, hydroxy- en methoxy-substituties zijn in aanleg mineraliseerbaar door methanogene consortia. De resultaten in Hoofdstuk 3 laten zien dat vier van de zeventien onderzochte aromatische amines werden gemineraliseerd door ongeadapteerd korrelslib,

namelijk: de drie isomeren van aminobenzoaat en 2-aminofenol (2AP). 5ASA en 4-aminofenol (4AP), die beiden niet werden afgebroken in aanwezigheid van ongeadapteerd slib, werden door aan 2-nitrofenol (2NP) geadapteerd slib na een lagfase van 65 en 70 dagen wel gemineraliseerd. Bovendien werd voor de eerste maal de anaërobe mineralisatie van 5ASA waargenomen.

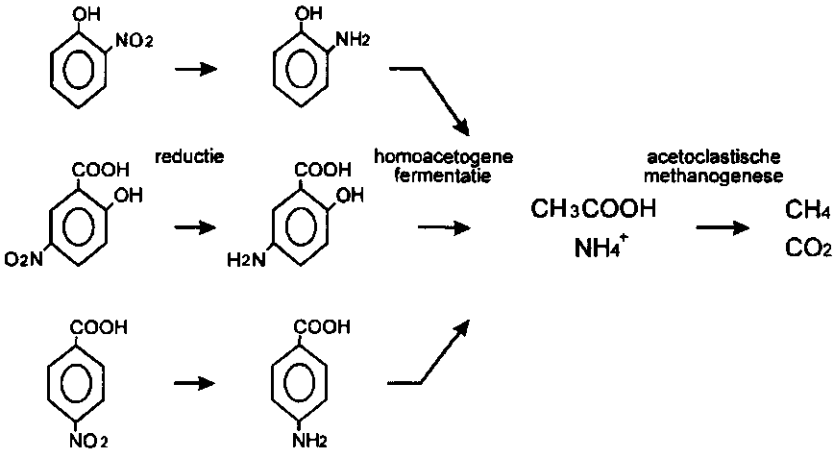
"Cross-acclimatisering" van N-gesubstitueerde aromaten is mogelijk. Dit werd aangetoond in het onderzoek met aan 2-NP geadapteerd korrelslib en in de studie waarin het vermogen van korrelslib om aromatische verbindingen af te breken is onderzocht met slib afkomstig van vijf verschillende bronnen (Hoofdstuk 4). Deze bevindingen ondersteunen de mogelijkheid om slib te "cross-acclimatiseren" zodat het verbindingen af kan breken die structureel analoog zijn aan de aromatische verbindingen waaraan het slib is geadapteerd (17, 31). Op grond van deze resultaten, is het duidelijk geworden dat het adaptatieproces een van de knelpunten in de afbraak van aromatische verbindingen is. Zodra het slib is geadapteerd, vindt het afbraakproces met vergelijkbare snelheden plaats, onafhankelijk van de oorsprong van het slib.

Microbiële gemeenschappen in sedimenten kunnen alternatieve elektronen-acceptoren zoals NO_3^-, $SO_4^=$, Mn(IV) en Fe(III) gebruiken (20, 21, 23). Aniline, het meest simpele aromatische amine, werd niet afgebroken onder methanogene omstandigheden, maar wel onder denitrificerende en sulfaatreducerende omstandigheden (1, 25). Dit geeft het enorme potentieel aan voor de afbraak van aromatische amines door microbiële consortia in anaërobe milieus waar alternatieve elektronenacceptoren aanwezig zijn.

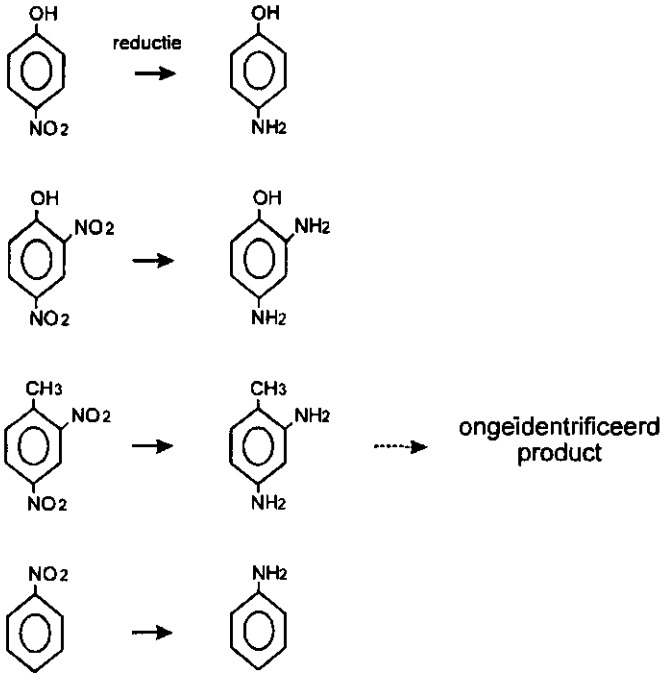
7.6 Complete Biodegradatie van Nitro- en Azoaromaten

Uit de resultaten met de continu bedreven UASB-reactoren (Hoofdstukken 5 en 6) kan worden afgeleid dat sommige nitroaromaten en azokleurstoffen volledig biodegradeerbaar zijn in anaërobe milieus. Na langdurig bedrijven van de reactoren werd bij de afbraak van 2NP, 4-nitrobenzoëzuur (4NBc) en 5-nitrosalicylzuur (5NSA) niet langer accumulatie van aromatische

A) Complete mineralisatie: 2-nitrofenol, 5-nitrosalicylaat en 4-nitrobenzoaat



B) Biotransformatie: 4-nitrofenol, 2,4-dinitrofenol, 2,4-dinitrotolueen en nitrobenzeen



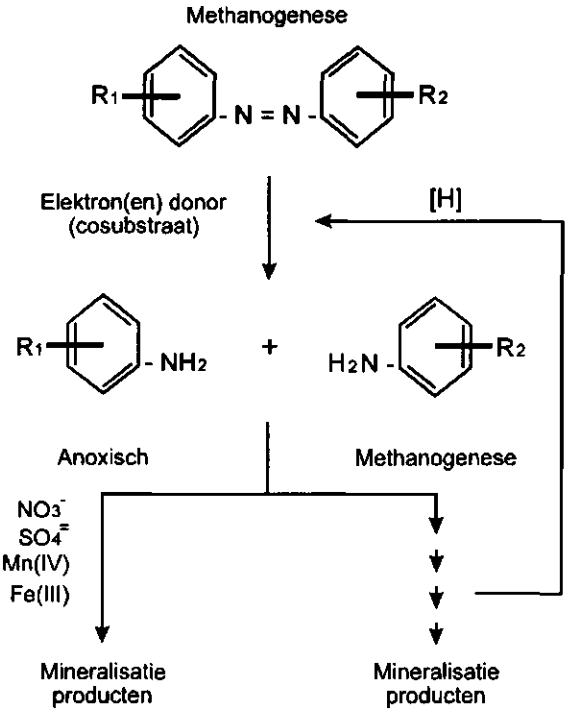
Figuur 7.2 Lot en gedrag van nitroaromaten in UASB-reactoren.

amines waargenomen. Dit wijst op een complete biologische afbraak. Korrelslib afkomstig uit deze reactoren was in staat in anaërobe biodegradatiestesten 2AP, 4-aminobenzoaat (4ABc) en 5ASA via homoacetogene fermentatie volledig te mineraliseren, wanneer deze stoffen werden aangeboden als enige C- en energiebron. Anderzijds vond er wel accumulatie van 4AP en 2,4-diaminofenol plaats bij de afbraak van respectievelijk 4-nitrofenol (4NP) en 2,4DNP. Ook in batchtesten met slib uit deze reactoren werden 4AP en 2,4-diaminofenol niet verder gemineraliseerd. Van 2,4DNT werd slechts 52% van de verbinding "teruggevonden" als 2,4-diamino-tolueen (2,4DAT). Dit duidt erop dat de resterende 48% biologisch is omgezet. Het uitblijven van mineralisatie wijst op de omzetting van 2,4DAT naar een ongeïdentificeerd en niet afbreekbaar metaboliet ("dead-end-metabolite"). Deze resultaten zijn in overeenstemming met die verkregen in de batch afbreekbaarheidstesten in Hoofdstuk 3. Het lot en gedrag van nitroaromaten in UASB-reactoren is weergegeven in Figuur 7.2.

In het geval van azokleurstoffen (Hoofdstuk 6) werd gevonden dat MO1 gedeeltelijk werd gemineraliseerd. Na 180 dagen werd 5ASA, afkomstig van MO1-splitsing, slechts in zeer lage concentraties waargenomen in de met glucose gevoede reactor. 5ASA werd volledig gemineraliseerd in de anaërobe biodegradatietesten met slib afkomstig uit deze reactor. Het andere splitsingsproduct, 1,4PDA, werd echter niet gemineraliseerd. Deze resultaten wijzen op de volledige afbraak van 5ASA door anaërobe consortia, terwijl 1,4PDA niet wordt afgebroken.

De afbraak van de farmaceutische azokleurstof ADS die is opgebouwd uit twee 5ASA-moleculen werd onderzocht in batch- en continutesten met aan 5ASA-geadapteerd slib (Hoofdstuk 6). ADS werd in hoge mate gemineraliseerd in continu bedreven reactoren, zelfs wanneer er geen cosubstraat in het basale medium aanwezig was. Batchexperimenten met ADS als enige koolstof- en energiebron bevestigden de mineralisatie naar methaan en ammonium en er werd eveneens aangetoond dat het metabolisme van 5ASA (product van de ADS-splitsing) voldoende elektronen produceert om continue reductie van de kleurstof te bewerkstelligen. Uit deze resultaten volgt dat de toevoeging van cosubstraat waarschijnlijk alleen noodzakelijk is om een actief methanogeen consortium te creëren tijdens de adaptatie van het slib in de reactor aan de azokleurstof. Supplementatie met het cosubstraat was niet langer essentieel nadat een

5ASA-afbrekende bacteriepopulatie zich had ontwikkeld. Volgens de resultaten verkregen met de experimenten beschreven in Hoofdstuk 6, is complete mineralisatie van azokleurstoffen in methanogene en/of anoxische omstandigheden mogelijk bij de condities zoals voorgesteld in Figuur 7.3.



Figuur 7.3 Biodegradatie van azokleurstoffen in methanogene en/of anoxische omstandigheden. R_1 en/of R_2 = -COOH, -SO_3H, -NO_2, -OH, -CH_3, -OCH_3, -H en/of een andere substituent. Als R_1 en/of R_2 = -COOH, -OH, -OCH_3 kunnen de azokleurstoffen volledig worden gemineraliseerd in methanogene omstandigheden; met alle andere substituenten kunnen de aromatische amines mogelijk worden afgebroken via anoxische routes met alternatieve elektronacceptoren.

7.7 Conclusies en Aanbevelingen

Aan de anaërobe biodegradeerbaarheid van N-aromatische verbindingen werd twintig jaar geleden nog getwijfeld, toen zeer weinig bekend was omtrent de mogelijkheden van microbiële afbraak in afwezigheid van zuurstof. Tegenwoordig is anaërobe biodegradatie van

N-gesubstitueerde aromaten echter een veelbelovend alternatief voor aërobe processen. De resultaten die hier worden beschreven wijzen op detoxificatie van nitroaromaten en azokleurstoffen via anaërobe reductieve transformatie. Wij hebben eveneens aangetoond dat sommige nitroaromatische verbindingen en azokleurstoffen volledig kunnen worden gemineraliseerd en kunnen dienen als koolstof- en energiebron voor anaërobe bacteriën, dit in tegenstelling tot de algemene aanname dat ze biologisch worden omgezet naar mutagene en carcinogene aromatische amines. Daarom concluderen wij dat het voor "de industrie" mogelijk moet zijn om nitro- en azoverbindingen te ontwerpen die volledig biologisch afbreekbaar zijn in anaërobe milieus.

Verder onderzoek zou moeten worden uitgevoerd om meer inzicht te verkrijgen in zowel microbiologische ("the know") als technologische aspecten ("the how") van de biologische afbreekbaarheid van N-gesubstitueerde aromaten. Hierna volgen enige suggesties voor de continuering van dit onderzoek.

- het lot van aromatische amines en de reductie van nitroaromaten en azokleurstoffen in milieus met alternatieve electronacceptoren, zoals NO_3^-, $SO_4^=$, Mn(IV), Fe(III) en/of in microaërobe omstandigheden.
- studies om de microbiële afbraakroute(s) en de micro-organismen die verantwoordelijk zijn voor de afbraak te identificeren. De rol van de microbiële adaptatie zou moeten worden opgehelderd.
- onderzoek naar de toxiciteit en biologische afbreekbaarheid van meer complexe azokleurstoffen (bijv. gesulfoneerde en/of naftaleenachtige) en hun afbraakproducten.
- vergelijkend onderzoek waarin de zuiveringsresultaten van verschillende hoog-belaste anaërobe reactoren met nitroaromaten en azokleurstoffen worden geëvalueerd. Het gebruik van "echt" afvalwater in plaats van synthetisch afvalwater wordt sterk aanbevolen.

Referenties

1. Alexandra de, M., O'Connor, O.A., Kosson, D.S. (1994) Metabolism of aniline under different anaerobic electron-accepting and nutritional conditions. *Environ. Toxicol. Chem.* **13**:233-239.
2. Blum, D.J.W., Speece, R.E. (1991) A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. *Res. J. Water Pollut. Control Fed.* **63**:198-207.

3. Borghans, A.J.M.L., van Driel, A. (1988) Application of the Biothane UASB reactor to a chemical wastewater containing phenol and formaldehyde. In: Tilche, A., Rozzi, A. (Eds.) 5th. International Symposium on Anaerobic Digestion, pp. 627-630. Monduzzi Editore, Bologna.
4. Brown, D., Laboureur, P. (1983) The degradation of dyestuffs: Part I primary biodegradation under aerobic conditions. *Chemosphere* **12**:397-404.
5. Brown, D., Laboureur, P. (1983) The aerobic biodegradability of primary aromatic amines. *Chemosphere* **12**:405-414.
6. Chung, K.T., Stevens, S.E., Cerniglia, C.E. (1992) The reduction of azo dyes by the intestinal microflora. *Crit. Rev. Microbiol.* **18**:175-190.
7. Chung, K.T. and Stevens, S.E. (1993) Degradation of azo dyes by environmental microorganisms and helminths. *Environ. Toxicol. Chem.* **12**:2121-2132.
8. Dickel, O., Haug, W., Knackmuss, H.J. (1993) Biodegradation of nitrobenzene by a sequential anaerobic-aerobic process. *Biodegradation* **4**:187-194.
9. Field, J.A., Stams, A.J.M., Kato, M., Schraa, G. (1995) Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consortia. *Antoine van Leeuwenhoek* **67**:47-77.
10. Fishbein, L. (1984) Aromatic amines. In: Hutziger, O. (Ed.) The handbook of environmental chemistry, Vol. 3, pp. 1-40, Springer, New York.
11. Gorontzy, T., Drzyga, O., Kahl, M.W., Bruns-Nagel, D., Breitung, J., von Loew, E., Blotevogel, K.H. (1994) Microbial degradation of explosives and related compounds. *Crit. Rev. Microbiol.* **20**:265-284.
12. Gorontzy, T., Kuver, J., Blotevogel, K.H. (1993) Microbial transformation of nitroaromatic compounds under anaerobic conditions. *J. Gen. Microbiol.* **139**:1331-1336.
13. Grobicki, A., Stuckey, D.C. (1992) Hydrodynamic characteristics of the anaerobic baffled reactor. *Wat. Res.* **26**:371-378.
14. Hallas, L.E., Alexander, M. (1983) Microbial transformation of nitroaromatic compounds in sewage effluent. *Appl. Environ. Microbiol.* **45**:1234-1241.
15. Hartter, D.R. (1985) The use and importance of nitroaromatic chemicals in the chemical industry. In: Rickert, D.E. (Ed.), Toxicity of nitroaromatic nompounds; Chemical Industry Institute of Toxicology series, pp. 1-14. Chemosphere, Washington DC.
16. Haug, W., Schmidt, A., Nortermann, B., Hempel, D.C., Stolz, A., Knackmuss, H.J. (1991) Mineralization of the sulfonated azo dye Mordant Yellow 3 by a 6-aminonaphtalene-2-sulfonate-degrading bacterial consortium. *Appl. Environ. Microbiol.* **57**:3144-3149.
17. Healy, J.B., Young, L.Y. (1979) Anaerobic biodegradation of eleven aromatic compounds to methane. *Appl. Environ. Microbiol.* **38**:84-89.
18. Idaka, E., Ogawa, T., Yatome, C., Horitsu, H. (1985) Behavior of activated sludge with dyes. *Bull. Environ. Contam. Toxicol.* **35**:729-734.
19. Jaworska, J.S., Schultz, T.W. (1994) Mechanism-based comparisons of acute toxicities elicited by industrial organic chemicals in prokaryotic and eukaryotic systems. *Ecotoxicol. Environ. Saf.* **29**:200-213.
20. Kazumi, J., Haggblom, M.M., Young, L.Y. (1995) Diversity of anaerobic microbial processes in chlorobenzoate degradation: nitrate, iron, sulfate and carbonate as electron acceptors. *Appl. Microbiol. Biotechnol.* **43**:929-936.
21. Langenhoff, A. (1997) Biotransformation of toluene, benzene and naphthalene under anaerobic conditions. Ph.D. Thesis, Wageningen Agricultural University, The Netherlands.
22. Longstaff, E. (1983) An assesment and categorization of the animal carcinogenicity data on selected dyestuffs and an extrapolation of those data on the relative carcinogenic risk to man. *Dyes and Pigments* **4**:243-304.
23. Lovley, D.R., Woodward, J.C., Chapelle, F.H. (1994) Stimulated anoxic biodegradation of aromatic hydrocarbons using Fe (III) ligands. *Nature* **370**:128-131.

24. van der Meer, R.R. (1979) Anaerobic treatment of wastewater containing fatty acids in upflow reactors. Ph.D. Thesis, Delft University of Technology, The Netherlands.
25. Schnell, S., Bak, F., Pfennig, N. (1989) Anaerobic degradation of aniline and dihydroxybenzenes by newly isolated sulfate-reducing bacteria and description of *Desulfobacterium anilini*. *Arch. Microbiol.* **152**:556-563.
26. Shea, P.J., Weber, J.B., Overcash, M.R. (1983) Biological activities of 2,4-dinitrophenol in plant-soil systems. *Residue Reviews* **87**:2-41.
27. Spain, J.C. (1995) Biodegradation of nitroaromatic compounds. *Annu. Rev. Microbiol.* **49**:523-555.
28. Tereda, H. (1981) The interaction of highly active uncouplers with mitochondria. *Biochimica et Biophysica Acta* **639**:225-242.
29. Wu, W.M., Bhatnagar, L., Zeikus, J.G. (1993) Performance of anaerobic granules for degradation of pentachlorophenol. *Appl. Environ. Microbiol.* **59**:389-397.
30. Yang, J., Speece, R.E. (1986) Effects of engineering controls on methane fermentation. *J. Water Pollut. Control Fed.* **57**:1134-1140.
31. Young, L.Y., Rivera, M.D. (1985) Methanogenic degradation of four phenolic compounds. *Wat. Res.* **19**:1325-1332.
32. Zollinger, H. (1987) Color chemistry-syntheses, properties and applications of organic dyes and pigments, pp. 92-102. VCH Publishers, New York.

Curriculum vitae

The author of this dissertation, Elías Razo Flores, was born on September 3rd, 1962 in Puebla City, México. He received his high school diploma in 1979 from the Universidad Autónoma de Puebla. From the same University, he was granted his bachelor of science degree in 1984 from the Department of Chemical Engineering. His master of science degree with *Magna cum lauda* was granted in 1991 from the Universidad Nacional Autónoma de México (UACPyP-CCH) at México City. The topic of the master thesis was on the scale-up of a fermentation process for the production of bioinsecticides. From 1984 till 1988 he worked as process engineer in the Department of Process Engineering (Volkswagen de México S.A. de C.V) at Puebla City. Since 1991 he is a full time researcher of the Department of Environmental Protection at the Instituto Mexicano del Petróleo, México City. In March, 1994, he started his Ph.D. studies at the Department of Environmental Technology at the Landbouwuniversiteit Wageningen, The Netherlands. In November, 1997 he reassumes his research position in México City. The address will be:

E-mail: Erazo@www.imp.mx

Instituto Mexicano del Petróleo,
Subdirección de Protección Ambiental,
Eje Central Lázaro Cárdenas 152,
07730, México D.F.,
México.

List of Publications

Razo-Flores, E. (1992). Application of anaerobic biotechnology for wastewater treatment. *Biotecnología*, **2**:85-96. In Spanish.

Rodriguez, M., **Razo-Flores, E.**, Villafaña, J., Urquijo, E., de la Torre, M. (1993) Development of a high productivity process for the production of bioinsecticides by *Bacillus thuringiensis*. In: Salama, H.S., Morris, O.N., Rached, E. (eds.). The biopesticide *Bacillus thuringiensis* and its applications in developing countries, pp 137-147. AL-AHRAM Commercial Press, Cairo.

Rivera, R., Rodriguez, G., **Razo-Flores, E.**, Bremauntz, P. (1993). Some aspects about the anaerobic digestion process. Part I: microbiology. *Revista Latino Americana de Microbiología*, **35**:451-458. In Spanish with abstract in English.

Rodriguez, R., Rivera, R., **Razo-Flores, E.**, Bremauntz, P. (1993). Some aspects about the anaerobic digestion process. Part II: Biochemistry. *Revista Latino Americana de Microbiología*, **35**:459-468. In Spanish with abstract in English.

Razo-Flores, E., Rodriguez, R., Rivera, R., Bremauntz, P. (1993). Some aspects about the anaerobic digestion process. Part III: UASB reactor. *Revista Latino Americana de Microbiología*, **35**:469-475. In Spanish with abstract in English.

Villafaña, J., **Razo-Flores, E.**, De la Torre, M. (1993). Development of a *Bacillus thuringiensis* fermentation process for the production of bioinsecticides. *Revista Internacional de Contaminación Ambiental*. **9**:84-90. In Spanish.

Razo-Flores, E., Rivera, R., Toledo, N., Arias, I., Garcia, D., Bremauntz, P. (1994). Factibility studies for the application of an UASB reactor for the treatment of waste sludge. *Revista del Instituto Mexicano del Petróleo*. **26**:31-37. In Spanish.

Donlon, B., **Razo-Flores, E.**, Field, J., Lettinga, G. (1995). Toxicity of N-substituted aromatics to acetoclastic methanogenic activity in granular sludge. *Applied Environmental Microbiology*, **61**:3889-3893.

Donlon, B., **Razo-Flores, E.**, Hwu, C.-S., Field, J., Lettinga, G. (1995). Toxicity and biodegradability of selected N-substituted phenols under anaerobic conditions. In: Hinchee, R., Brockman, F., Vogel, C. (eds.). Microbial Processes for Bioremediation; Bioremediation 3(8), pp 251-257. Battelle Press, Columbus.

Razo-Flores, E., Donlon, B., Field, J., Lettinga, G. (1996). Biodegradability of N-substituted aromatics and alkylphenols under methanogenic conditions using granular sludge. *Water Science & Technology*, **33(3)**:47-57.

Razo-Flores, E., Svitelskaya, A., Donlon, B., Field, J., Lettinga, G. (1996). The effect of granular sludge source on the anaerobic biodegradability of aromatic compounds. *Bioresource Technology*, **56:**215-220.

Donlon, B., **Razo-Flores, E.**, Lettinga, G., Field, J. (1996). Continuous detoxification, transformation, and degradation of nitrophenols in upflow anaerobic sludge blanket (UASB) reactors. *Biotechnology and Bioengineering*, **51:**439-449.

Donlon, B., **Razo-Flores, E.**, Luijten, M., Swarts, H., Lettinga, G., Field, J. (1997). Detoxification and partial mineralization of the azo dye Mordant Orange 1 in a continuous anaerobic sludge-blanket reactor. *Applied Microbiology and Biotechnology*, **47:**83-90.

Razo-Flores, E., Luijten, M., Donlon, B., Lettinga, G., Field, J. (1997). Complete biodegradation of the azo dye Azodisalicylate under anaerobic conditions. *Environmental Science & Technology*, **31:**2098-2103.

Razo-Flores, E., Donlon, B., Lettinga, G., Field, J. (1997). Biotransformation and biodegradation of N-substituted aromatics in methanogenic granular sludge. *FEMS Microbiological Reviews*, **20(3-4):**525-538.

Razo-Flores, E., Pérez, G.F., de la Torre, M. (1997). Scale-up of Bacillus thuringiensis fermentation based on oxygen transfer rate. *Journal of Fermentation and Bioengineering*, **31(6)**, in press.

Razo-Flores, E., Luijten, M., Donlon, B., Lettinga, G., Field, J. (1997). Biodegradation of selected azo dyes under methanogenic conditions. *Water Science & Technology*, in press.

Razo-Flores, E., Matamala, A., Lettinga, G., Field, J. (1997). Biotransformation and biodegradation of nitroaromatics under anaerobic conditions. *Biotechnology Progress*, submitted.

Razo-Flores, E., Smulders, P., Prenafeta, B.F., Lettinga, G., Field, J. (1997) Anaerobic treatment of anthranilic acid in an expanded granular sludge bed (EGSB) reactor at low concentrations. In preparation.