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BIOAUGMENTATION OF ACTIVATED SLUDGE REACTORS TO ENHANCE CHLOROANILINE REMOVAL

BIO-AUGMENTATIE VAN ACTIEF-SLIBREACTOREN VOOR DE VERSNELDE AFBRAAK VAN CHLOORANILINES

door

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NOTATION INDEX

% G + C	percent guanine + cytosine
μ	growth rate
3-CA	3-chloroaniline
Ani	aniline
AOB	ammonia oxidizing bacteria
ARDRA	amplified ribosomal DNA restriction analysis
AS	activated sludge
BOD	biological oxygen demand
bp	base pairs (DNA size indicator)
BSA	bovine serum albumin
C	carbon source
cDNA	copy DNA
CDW	cell dry weight
CFU	colony forming units
COD	chemical oxygen demand
DEPC	diethylpyrocarbonate
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EPS	extracellular polymeric substances
FAME	fatty acid methyl ester
FI	filament index
FISH	fluorescent *in situ* hybridization
g	gravity
GC-MS	gas chromatograph mass spectrometer
gfp	green fluorescent protein
HPLC	high pressure liquid chromatography
HRT	hydraulic retention time
IC	ion chromatography
kb	kilo base pairs (DNA size indicator)
LB	luria broth
LMG	Laboratory of Microbiology Gent (Culture collection)
MDS	multidimensional scaling
MLSS	mixed liquor suspended solids

MMN	mineral medium without nitrogen and carbon
mRNA	messenger RNA
N	nitrogen source
OD	optical density
OUR	oxygen uptake rate
PAGE	poly acryl gel electrophoresis
PC	principal components
PCA	principle component analysis
PCB	polychlorinated biphenyls
PCR	polymerase chain reaction
PFLA	phospholipid fatty acid analyses
ppm	parts per million
rDNA	genes (DNA) coding for rRNA
RFLP	restriction fragment length polymorphism
rfp	red fluorescent protein
rpm	rounds per minute
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase PCR
SCAS	semi continuous activated sludge
SDS	sodium dodecyl sulphate
SRT	sludge retention time
SS	suspended solids
SV_{30}	sludge volume after 30 min. of settlement
SVI	sludge volume index
t_D	doubling time
TGGE	temperature gradient gel electrophoresis
T-RFLP	terminal restriction fragment length polymorphism
UV	ultra violet
VSS	volatile suspended solids

CONTENTS

CHAPTER 1

1. LITERATURE REVIEW

INTRODUCTION

Nowadays, wastewater treatment technology has become indispensable in modern live. However this biotechnological process only exists about 100 years and during this century, the priorities have changed gradually. First, public health was the most important concern and the treatment of domestic wastewater was mainly aimed at the removal of human pathogens. Next, concerns about water quality and eutrophication led to the development of systems to remove nutrients, such as organic carbon, nitrogen and phosphorus. The most recent challenge is the removal of individual organic compounds to low levels, rather than just removing organic matter in general. This has been primarily due to the production and use of the xenobiotic compounds. These xenobiotic compounds are "foreign to the biosphere" and are produced by humankind through the chemical technology.

Over the last decades the use of chemicals in practically every aspect of life has grown very rapidly. Of the 11 million known chemicals, about 100.000 molecules are being produced on an industrial scale and about 1000 to 2000 new chemicals are being introduced each year (Press Release WHO/31, 20 March 1998; www.who.int). Humans can come in contact with many of these chemicals, which may be present as environmental pollutants and contaminants.

In 1874, Zeidler first synthesized DDT (1,1,1-trichloro-2,2-di-[4-chlorophenyl]ethane) and 70 years later the use of chlorinated organic compounds was already integrated in our daily life and its use increased steadily (213). In 1962, Rachel Carson's "Silent Spring" made the public opinion aware of the possible environmental treats resulting from the widespread use and release of these manmade, xenobiotic substances (49). As a result, many organic compounds became environmentally suspect and especially chlorinated compounds, such as DDT, PCB's (polychlorinated biphenyls), dioxins, and pesticides have reached the status of being unacceptable. As the environmental engineers were given the responsibility to protect human health and the aquatic environment only a century ago, their new task now exists in ensuring that these xenobiotic compounds cannot enter the environment in large quantities through treated wastewaters. To accomplish this, environmental engineers and scientists have

made an alliance with micro-organisms. During billion of years, these organisms have been able to adapt to changing environments and thus have developed a battery of enzymes to transform natural as well as xenobiotic compounds.

CONTAMINANTS IN THE ENVIRONMENT: CHLOROANILINES AND THEIR BIODEGRADATION

Properties of 3-chloroaniline

3-chloroaniline is a representative of the substituted anilines. The physical and chemical properties of 3-chloroaniline are presented in Table 1.1, and its chemical structure is shown in Figure 1.1. 3-chloroaniline is prepared by the catalytic hydrogenation of 1-chloro-3-nitrobenzene or by the reduction of 1-chloro-3-nitrobenzene with iron fillings and HCl (1).

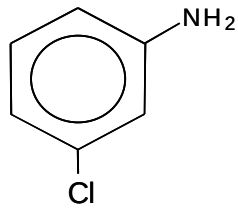

Figure 1.1. Structure of 3-chloroaniline

In recent years, the occurrence of chloroanilines in the environment has become a concern because of their widespread use as intermediates in the production of many herbicides, azo dyes, azo pigments, plastics and pharmaceuticals (158, 202). In addition chloroanilines are formed during (bio)degradation of several pesticides, such as the phenylamide herbicides (129).

Table 1.1. Chemical, physical and biological properties of 3-chloroaniline.

Feature		Reference
CAS number	108-49-9	www.sigmaaldrich.com
Synonyms	1-Amino-3-chlorobenzene	www.itcilo.it
	3-Chlorobenzeneamine	
Molecular formula	C_6H_6ClN	(1)
Molecular mass	127.57	(1)
Melting point	-10.3 °C	(1)
Boiling point	230.5 °C	(1)
Flash point	123 °C	www.itcilo.it
Auto-ignition temperature	> 450 °C	www.itcilo.it
Density (d_4^{22})	1.210 g/cm³	(1)

Table 1.1 continued. chemical, physical and biological properties of 3-chloroaniline.

Solubility		
• in water	6,6 mg/ml (25°C)	(306)
• in organic solvents	soluble	
Refractive index (20 °C)	1,5931	(1)
Octanol/water partition coefficient (log K_{ow})	1.9	(306)
Henry coefficient	$1,5 \times 10^{-6}$ atm.m³/mol	(306)
pK-value	3,9	(241)
Vapor pressure at 20 °C	0,05 mm Hg	(306)
Physical form	colorless to yellow liquid	www.itcilo.it
Odor	sweet	Toxicology Data Management System (TDMS) (157.98.10.135)
Toxicity		
• LD50 rat (oral)	256 mg/kg	Toxicology Data
• LD50 cat (skin)	223 mg/L	Management System
• LDL_0 dog (intravenous)	50 mg/kg	(TDMS) (157.98.10.135)

Chloroanilines can elict an inhibitory action on the respiratory chain, an uncoupling activity or a combination of both (63). Chloroanilines can accumulate in membranes, alter the lipid bilayer structure and thus resulting in disturbance of its functioning (17). Therefore, the chloroanilines are taken up in the European list of priority chemicals (9 of the originally containing 129 chemicals) (2). The water quality objective (WQO), as proposed by the Scientific Advisory Committee on Toxicity and Ecotoxicity of the European Commission, lists 2-, 3- and 4-chloroaniline in WQO class 1, which means that its objective for aquatic ecosystems is 10 µg/L (61). However, quantitative structure-activity relationships (QSAR) and a chemometric approach classified the chloroanilines in the higher toxicity class II, since they show relatively high toxicity values for fish and *Daphnia* in comparison to other class 1 chemicals (315).

Sources of chloroaniline contamination in the environment

Two types of pollution may result from releases of chemicals into the environment (245). In the case of *point source pollution* the concentration of the chemical is usually high and this may occur in landfills, waste dumps and industrial effluents or at sites of accidents associated with transportation and application of chemicals. In *dispersed pollution*, the concentrations are low and result from losses from production sites via volatilization or from agricultural use. Different types of pollution require different strategies of remediation. In case of dispersed chemicals, like in agriculture, their use and prevention can only be regulated by legislation. Technical solutions can be applied for sites with more highly concentrated pollution, such as industrial wastewaters and dumps.

As a result of their use in industry, spills of chloroanilines in the industrial production can result in contaminated soils and groundwater tables. For example at the Bofor-Noble site (Michigan, USA), various owners have operated chemical plants since 1960. Chemicals produced at the site included pesticides, herbicides, benzidine, and other aromatic amines, specifically 3,3'-dichlorobenzidine (DCB). High concentrations of chloroanilines were detected in the groundwater at concentrations of 5,6 ppm (1987) and 3,6 ppm (1988), in the soil surrounding the plant of 160 ppm (1988), in the sludge from the lagoons of 2300 ppm (1988) and in the subsurface soils from the lagoon area of 270 ppm (12) (www.atsdr.cdc.gov/HAC/PHA/bofors-nobel/bof_toc.html). Another example is the Carroll and Dubies site (New York, USA), which contains seven former wastewater lagoons that received waste from septic tanks and industries (primarily two cosmetic manufacturers). On this site, concentration between 4-200 mg/kg (sludge sampling data from the remedial investigation for on-site lagoons), and 510 µg/L (surface water from the on-site lagoons) were detected (13) (www.atsdr.cdc.gov/HAC/PHA/carroll/car_toc.html).

Chloroanilines have also been detected in industrial wastewaters. Lacorte et. al measured concentrations as high as 11,9 mg/L in the effluent of an industrial wastewater (172). Jen et al. even measured a chloroaniline concentration of 230 mg/L in wastewater from a polymer manufacturer (147). Livingston and Willacy (1991) reported that a wastewater from a 3,4-dichloroaniline manufacturing company (Rhone Poulenc Chemicals Ltd. Staveley Works; Derbeyshire, UK) contained aniline (11,6 mg/L), 4-chloroaniline (21,4 mg/L), 2-chloroaniline (1,1 mg/L), 2,5-dichloroaniline (3,1 mg/L), 2,3-dichloroaniline (99 mg/L) and 3,4-dichloroaniline (260 mg/L) (185).

Chloroanilines are also common metabolites resulting from microbial reductive transformation of nitroaromatic compounds, such as some explosives (201), dinitroaniline

herbicides (118) and nitropyrenes (161). Moreover, chloroanilines have been found in environmental waters as a consequence of aerobic transformation of phenylamide herbicides (129). There are three subdivisions of these herbicides, i.e. acylanilides, phenylureas and phenylcarbamates and all groups have a substituted aniline in their molecular structure. Even in the seventies, scientists knew that soil micro-organisms, such as *Pseudomonas striata*, *Fusarium solani*, *Penicillium* sp. and *Pululllaria* sp., are capable to hydrolyse almost all acylanilide herbicides with the formation of the corresponding anilines (129). Also phenylcarbamate herbicides, such a chloropropham, can easily be hydrolyzed by microorganisms (Figure 1.2), such as *Pseudomonas, Agrobacterium, Flavobacterium* and *Achromobacter* (156). The major mechanism of phenylurea detoxification is by *N*-dealkylation or *N*-dealkoxylation (129).

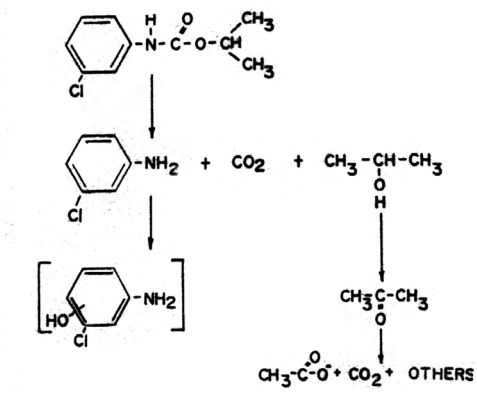

Figure 1.2. Microbial hydrolysis of the potato sprout inhibitor chloropropham into 3-chloroaniline and CO_2 and isopropanol (after Kaufman and Kearny, 1965 (156)**).**

Chloroanilines can have a negative influence on metabolic activities of ecosystems when they are introduced in the environment. If conventional sewage treatment systems are exposed to high loads of these xenobiotics, the effluents of the settling tank exhibit a brown black coloration and contain high concentrations of dissolved organic carbon (175). The bulk of this undecomposed organic matter is due to secondary hydroxylated and polymerized products and cannot be correlated to intact chloroanilines or defined metabolites. Moreover, in soil and aquatic ecosystems these chloroanilines are rather difficult to remove since they can couple to humic substances (25), or undergo oxidative dimerisation, which can yield substituted biphenyls, azobenzenes and triazenes (59).

Microbial degradation of chloroanilines

In four billion years micro-organisms have evolved an extensive range of enzymes and control mechanisms in order to be able to degrade a wide array of naturally occurring aromatic compounds. In contrast, the list of pure, biochemically well characterized cultures able to grow at the expense of haloaromatics is short. Haloaromatic-assimilating strains have been obtained by (i) enrichment from nature, (ii) *in vivo* genetic modification, and (iii) *in vitro* genetic engineering (245). In case of new manmade - xenobiotic - compounds, adaptation of bacteria towards these new molecules is necessary (307).

The limiting step to complete mineralization of haloaromatics seems to be first transformationsteps by the peripheral pathways, which all lead to chlorocatechols, which are then further degraded by a few limited pathways, such as the modified *ortho*-ring cleavage pathway (Figure 1.3). Other central pathways have been recently described as well, such as the *meta* cleavage pathway for chlorocatechols (196) and the *meta* cleavage pathway of a chlorinated protocatechuate intermediate (170, 211).

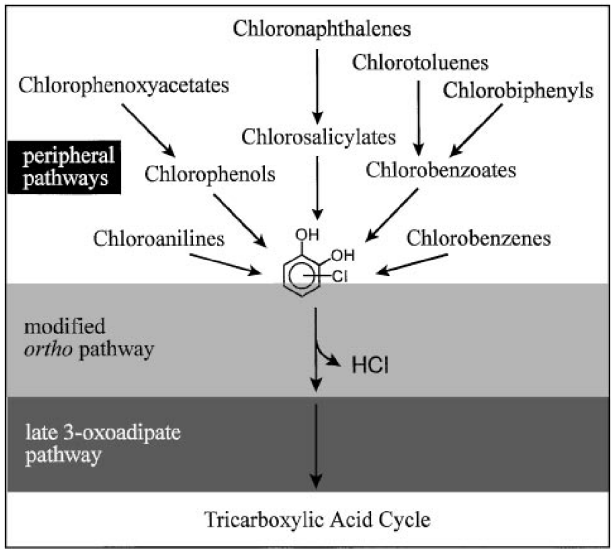

Figure 1.3. Chlorinated aromatics are transformed through peripheral pathways into chlorocatechol, which are further degraded by a few limited central pathways (after Reineke, 1998 (243)).

The major way to remove the non-substituted aniline is through biodegradation (106, 191). A number of aniline degrading strains have already been isolated and described in detail (88, 100, 102, 133, 153, 154, 162, 163, 268, 291). The degradation pathway of aniline starts

with the oxidative deamination, resulting in the formation of catechol, which is then further degraded by an *ortho*-cleavage pathway (15, 189) or a *meta*-cleavage pathway (164). Recently, the different genes of *Pseudomonas putida* UCC22(pTDN1) involved in the transformation of aniline to catechol were sequenced and named as *tdnA1*, *tdnA2*, *tdnB*, *tdnR*, *tdnQ* and *tdnT* (102). Based on sequence similarities with other aniline degradation pathways and on a gene expression study, Fukumori and Saint (102) tentatively concluded that *tdnA1*, *tdnA2*, *tdnB*, and *tdnT* are structural genes and that *tdnR* is a positive regulatory gene. *tdnQ* could be a structural gene, and its gene product TdnQ shows ca. 30% amino acid sequence similarity with glutamine synthetases. Another aniline oxygenase gene was sequenced as *atdA* from *Acinetobacter* sp. strain YAA (100). A number of catabolic plasmids such as pCIT1, pTDN1 and pYA1, containing genes that encode aniline degradation enzymes have been described previously (14, 100, 203, 250).

Whereas aniline is readily degraded by bacteria, the first bacterial isolates capable of degrading halogenated anilines were described in 1982. It was known that the microbial biotransformation, such as methylation or polymerization, can occur (117). Bartha showed that the aliphatic moiety from chloroaniline based herbicides is readily cleaved and metabolized (24). Their aromatic portion, however, was shown to give rise to relative persistent complex residues, such as chlorobenzenes and other polymerized products. Up to 90 % of the chloroanilines released during biodegradation of phenylamide herbicides becomes unextractable by solvents due to binding to the soil organic matter (138). A subsequent step was the isolation of strains that could transform chloroanilines. Walker and Harris (323), who investigated the utilization of aniline by *Pseudomonas* sp., were unable to demonstrate oxidation or co-oxidation of monochloroanilines by this bacterium. A number of years later, cometabolic degradation of chloroanilines by several bacteria was reported. Cells of a *Pseudomonas multivorans* strain, pregrown on aniline, were able to oxidize 2-chloroaniline to 3-chlorocatechol and 3- and 4-chloroaniline to 4-chlorocatechol (241). Aniline grown *Rhodococcus* sp. cells could metabolize 2- and 3-chloroaniline in the presence of glucose with a transient accumulation of 3- and 4-chlorocatchol, respectively (259). Also other organic compounds, such as fructose, acetate, pyruvate, succinate, malate as well as benzoate were found to act as efficient cosubstrate in cometabolic degradation of 3-chloroaniline by resting cells of *Rhodococcus* sp. (146). The same study showed that monochloroanilines, when cometabolized, served as nitrogen sources in experiments with *Rhodococcus* sp. cells growing at the expense of benzoate with either 2- and 3-chloroaniline as the sole source of nitrogen (146).

In 1982, Zeyer and Kearny isolated the first organism that could use chlorinated anilines as sole carbon and nitrogen sources from a chemostat inoculated with soil (339). This organism, a *Pseudomonas* sp. strain G (later re-identified as *Moraxella* sp. G (340)) was grown aerobically with ^{14}C labelled 4-chloroaniline. Of the labeled carbon 64 % was converted into CO_2 and 14 % was associated with the biomass. For the ring substituents, 60 % of the nitrogen and 96 % of the chlorine of 4-chloroaniline accumulated in the medium as ammonium and chloride respectively. Moreover, *Moraxella* sp. G was also able to grow rapidly with aniline and 3-chloroaniline as sole carbon and nitrogen source, while 2-chloroaniline was only degraded slowly. The pathway for the degradation of 4-chloroaniline was investigated by analysis of catabolic intermediates and enzyme activities, and mutants of strain G were isolated which accumulated specific pathway intermediates (340). 4-chloroaniline was converted by a broad substrate specific aniline oxygenase to 4-chlorocatechol, which in turn was degraded via a modified *ortho*-cleavage pathway (Figure 1.4).

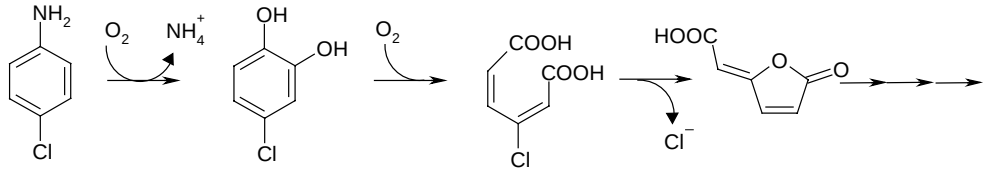

Figure 1.4. 4-chloroaniline degradation pathway by strain G . 4-chloroaniline is oxidatively deaminated to 4-chlorocatechol, which is further tranformed by the modified *ortho*-cleavage pathway. The ring cleavage product 3-chloro-cis,cis-muconic acid is then converted by a lactonizing enzyme to its corresponding lactone. This lactone is further opened and incorporated in the tricarboxylic acid cycle in the bacterial metabolism (after Zeyer et al., 1985 (340)).

In 1979, an approach of natural exchange of metabolic genes was used by Reineke and Knackmuss to construct halobenzoate-utilizing strains (244). By a similar approach, a group of 2-, 3-, and 4-chloroaniline degrading bacteria were obtained by natural gene exchange between an aniline/methylaniline degrading *Pseudomonas* sp. strain JL1 and the chlorocatechol assimilating *Pseudomonas* sp. B13 (175). Strain JL1 can mineralize aniline and methylaniline but it only can cooxidize chloroanilines yielding chlorocatechols as primary metabolites. Strain B13 possesses the genes, necessary for chlorocatechol degradation, the genes of both strains could complement each other.

Hybrid organisms were isolated through cocultivation of the parent strain in a chemostat as well as trough conjugation on solid media in presence of chloroanilines as the selective substrates. Two types of hybrid strains were obtained: hybrids JL2, JL3 and JL4 are organisms originating from *Pseudomonas* sp. B13, while hybrid JL5 resembles the parent *Pseudomonas* sp. strain JL1. All hybrids could utilize 3-chloroaniline as sole source of nitrogen, carbon and energy, while the parent strains could not.

The functional hybrid pathway for chloroanilines in *Pseudomonas* sp. B13 was formed through the acquisition of genes encoding aniline dioxygenation of JL1 (resulting in hybrids JL2, JL3 and JL4), while in the aniline degrading strain JL1, strain B13 donated its genetic information for chlorocatechol assimilation (resulting in strain JL5). Thus, the genetic exchange was feasible in both directions. None of the hybrids could use methylanilines, due to the incompatibility of chloro- and methylsubstituted aromatic compounds as growth substrates. The *meta*-cleavage pathway, used for methylanilines, is often unproductive for chloroanilines so that during the selection, total suppression of this *meta* pathway must occur. Recently, it was revealed that the genes for chlorocatechol degradation (*clc*) from *Pseudomonas* sp, strain B13 are localized on a mobile gene cluster (238). This mobile element employs phage-like integrases. to integrate into specific sites on the chromosome and to excise and transfer to other host bacteria (239, 308).

Table 1.2. Nutritional properties of parent and hybrid strains (after Latorre et al, 1984 (175)).

Substrate	***Pseudomonas* sp.**					
	B13	JL1	JL2	JL3	JL4	JL5
Aniline	-	+	+	+	+	+
2-, 3-, 4-methylaniline	-	+	-	-	-	-
2-chloroaniline	-	-	-	-	+	-
3-chloroaniline	-	-	+	+	+	+
4-chloroaniline	-	-	-	+	+	-

+ = good growth; - = no growth

The next chloroaniline degrading bacterium was *Pseudomonas diminuta* strain INMI KS-7, isolated from a meadow soil, treated over a long period with propanide (284). This strain could use 3- and 4-chloroaniline as sole source of carbon, nitrogen and energy.

However, in contrast to *Moraxella* sp. G (340), *P. diminuta* strain INMI KS-7 grows also on 3,4-dichloroaniline. Interestingly, in contrast to previously described strains, INMI KS-7 cannot use aniline as a substrate.

Of four *Pseudomonas acidovorans* strains (CA26, CA28, CA37 and CA45) that were isolated from soil on their capacity to grow on aniline, 3- and 4-chloroaniline, two of them (CA26 and CA45) showed very low degradation rates (189). During degradation of aniline and 3-chloroaniline by *Pseudomonas acidovorans* CA28, selective induction of two different catechol 1,2-dioxygenases (C12O) was observed (132). C12O I activity was the sole ring-cleaving enzyme detectable in cell-free extracts after growth on aniline, while C12O II was exclusively found after growth on 3-chloroaniline. Also two distinct muconate cycloisomerases are involved in the degradation of aniline and 3-chloroaniline, suggesting that aniline and chloroanilines transformation proceeds via two different *ortho*-cleavage pathways in strain CA28 (131).

During the subsequent years, a set of new chloroaniline degrading strains was isolated. An *Alcaligenes* sp. was isolated with aniline isoenzymes , such as catechol 1,2-dioxygenase I and II and muconate cycloisomerase I and II (286). The type II isoenzymes have a broader substrate specificity and the specific activities are higher towards the chlorinated substrates. Brunsbach and Reineke (1993) used a laboratory selected *Pseudomonas acidovorans* BN3.1 (degrading 2-, 3- and 4-chloroaniline) and a *Pseudomonas* sp. FRB4.5 (degrading 3- and 4-chloroaniline) in soil slurry experiments to enhance 3-chloroaniline degradation (46). Surovtseva et al. isolated four new strains from soils which are able to degrade 3-, 4-chloroaniline, and 3,4-dichloroaniline, i.e. *Aquaspirillum itersonii* 1CA, 10CA, *Aquaspirillum* sp. 2CA and *Paracoccus denitrificans* 3CA (285).

In summary, the major pathway for the aerobic degradation of monochloroanilines has been described (for an overview, see Figure 1.5). In general, the conversion of these compounds proceeds in a way that is similar to that of other chlorinated aromatic compounds: an *ortho* ring cleavage occurs after the formation of chlorocatechols.

An inducible (chloroaniline) dioxygenase not only catalyzes the incorporation of molecular oxygen, but also removes the amino group, yielding a monochlorinated catechol. 3-chlorocatechol can be generated during 2-chloroaniline and 3-chloroaniline metabolism, and 4-chlorocatechol during 4-chloroaniline and 3-chloroaniline metabolism. The chlorocatechols are generally cleaved by a catechol 1,2-dioxygenase (modified *ortho* cleavage pathway) to form chloro-cis-cis-muconic acid, which is further mineralized. In addition to the chlorocatechol pathway describe in Figure 1.5, it must be mentioned that a recent study

showed that an unusual catechol 2,3-dioxygenase can convert 3-chlorocatechol, which enables a *Pseudomonas putida* strain GJ31 to use chloroaromatics for growth (196).

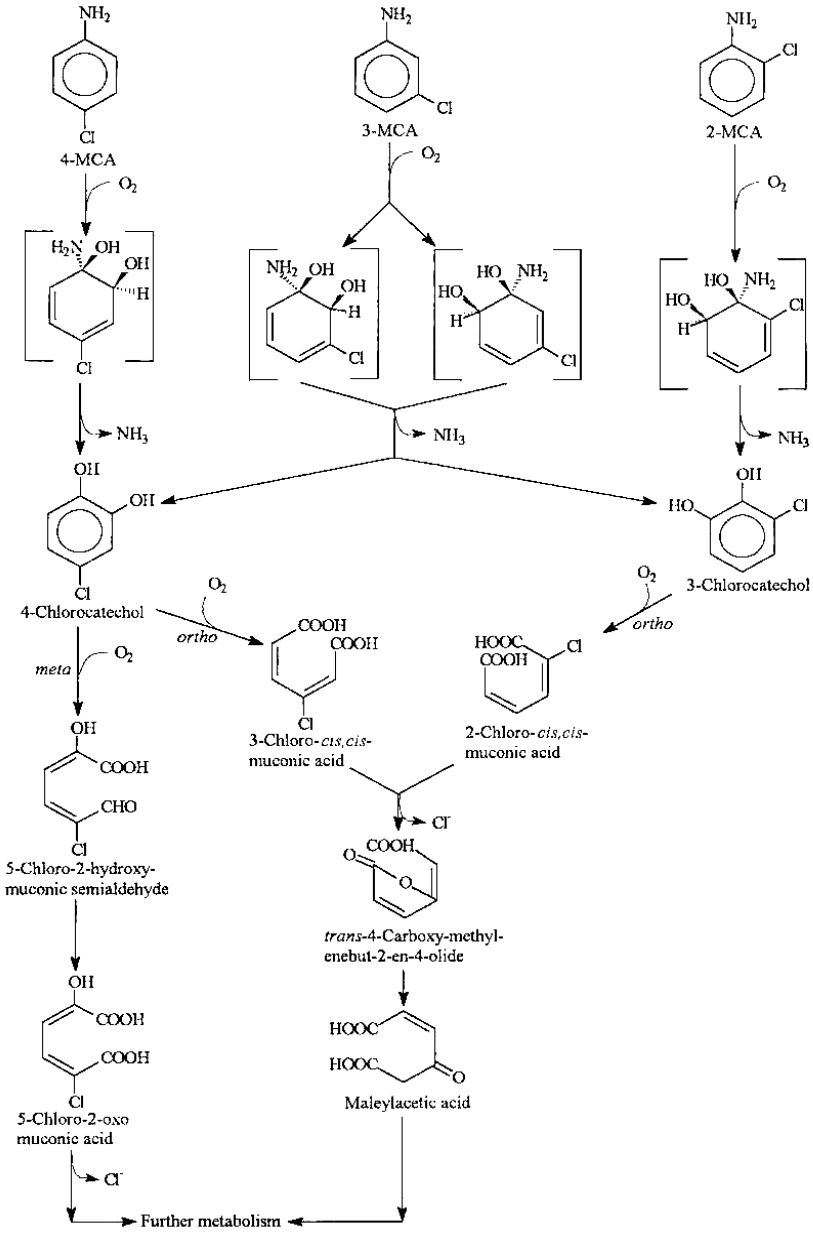

Figure 1.5. The proposed pathways for the bacterial degradation of 2-, 3- and 4-chloroaniline under aerobic conditions (after van Agteren et al., 1998 (306)).

In the past, the *meta*-cleavage intermediates of chlorinated catechols, i.e. chlorohydroxymuconic semialdehyde, were described as non-productive ("dead end") or toxic ("suicide") metabolites, preventing further degradation (189, 306). Recently, the use of a *meta*-cleavage pathway for the mineralization of 3-chlorocatechol and 4-chlorocatechol as central metabolite of chlorobenzene has been determined (155, 196). Riegert et al. (247) observed a yellow distal *meta*-cleavage product of 3-chlorocatechol with a strongly pH-dependent absorption maximum at 378 nm, and found that this was a chlorohydroxymuconic semialdehyde. Kashabek et al. (155) investigated the oxidation of different types of substituted catechols with the 2,3-dioxygenase of *P. putida* GJ31. Surovtseva et al. mentioned a meta-cleavage of monochloroanilines by *Alcaligenes faecalis*, however it is not clear if this cleavage was metabolic or co-metabolic (287). Minor amounts of *meta*-cleavage products were also found with *P. acidovorans* CA28 and *Moraxella* sp. G (94, 339).

Also micro-organisms other than bacteria can metabolize chloroanilines. In 1985, it was discovered that the white fungus, *Phanerochaeta chrysoporium* ATCC 24725 has the unique capability to mineralize free and lignin-bound chloroaniline (18). A fungal peroxidase preparation was found to react rapidly with chloroanilines, but the main reaction was oligomerization rather than ring opening. More recently, chlorinated anilines and herbicides tightly complexed with native plant cell wall components were found to be mineralized in high yield (197). Also 3,4-dichloroaniline was utilized by *Phanerochaeta chrysoporium* (251). Two other fungi, a *Fusarium* and *Rhizopus*, both could use aniline as sole source of carbon and nitrogen (89). However these two fungi utilized 2- and 3-chloroaniline only as nitrogen sources in the presence of glucose as carbon sources with production of catechol, ammonium and chloride.

WASTEWATER TREATMENT SYSTEMS AND CHLOROAROMATIC COMPOUNDS

An introduction to wastewater treatment

In the middle of the nineteenth century in England, waterborne diseases such as cholera caused epidemics in London, resulting in thousands of victims. Increasing awareness of the role of micro-organisms in diseases led to an enhanced demand for wastewater treatment. As a result, wastewater treatment practice started in the beginning of the twentieth century (28).

Wastewater treatment plants are designed and constructed to treat both domestic and industrial wastes. Nontoxic wastes are contributed mainly by the food industry and by domestic sewage, whereas toxic wastes are contributed by coal processing (phenolics, ammonia, cyanide), petrochemical (oil, petrochemicals, surfactants), pesticide, pharmaceutical and electroplating (toxic metals) industries (171). The objectives of waste treatment are the following:

1. Decrease the organic content of wastewater (i.e. elimination of biochemical or chemical oxygen demand).
2. Removal/reduction of nutrients (N/P) to decrease pollution of receiving surface waters or groundwater.
3. Removal or inactivation of pathogenic micro-organisms and parasites.

The major contaminants found in wastewaters are biodegradable organics, volatile organic compounds, recalcitrant organics, toxic metals, suspended solids, nutrients (carbon, nitrogen, phosphorus,…), microbial pathogens and parasites. Research efforts are now being focused on the fate of toxic substances following passage trough wastewater treatment plants.

Bacteria are the major component of activated sludge. These cells are grouped into in activated sludge flocs, together with inorganic and organic particles. The floc size varies between <1 µm and to 1000 µm (225). In such flocs, different redox potential zones are present (Figure 1.6), from aerobic over anoxic towards anaerobic pockets (336). The oxygen distribution in the activated sludge flocs allows different metabolic processes, such as nitrification and carbon source oxidation (aerobic zone), denitrification (anoxic zone) and sulphate reducers (anaerobic zone).

Exopolymeric substances (EPS) are of fundamental importance for microbial aggregates of cells in biofilms and activated sludge flocs. The floc structure, floc charge, flocculation process, floc settleability and dewatering properties of activated sludge are

determined by EPS (97). The cross-linking EPS and cation bridges lead to the stabilization of the biopolymer network mediating the immobilization of microbial cells. Costerton (2000) (60) defines such microbial multispecies aggregates as multicellular communities, which metabolic potential is greater than the sum of pure strains.

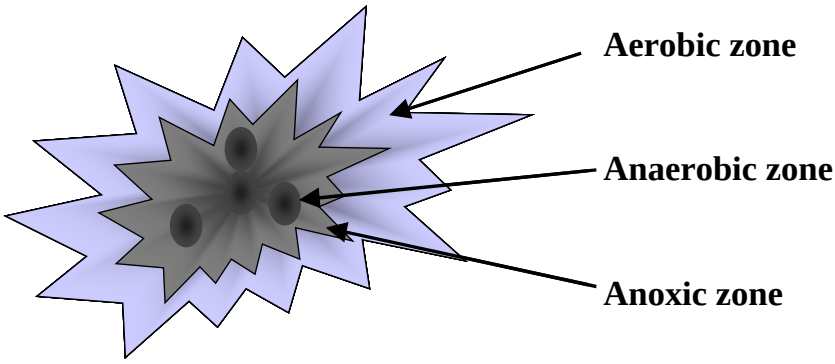

Figure 1.6. Oxygen distribution in an activated sludge floc.

The mixed liquor is transferred from the aeration tank into the settling tank, where the separation of sludge and the treated effluent occurs (Figure 1.7). A portion of the sludge is recycled to the aeration tank and the remaining sludge is wasted. In the settling tank, the separation of the microbial cells in the activated sludge flocs and the purified wastewater occurs.

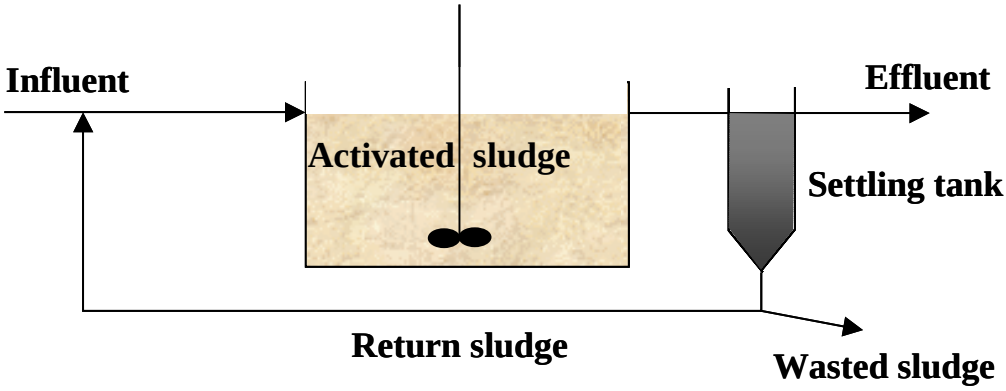

Figure 1.7. Scheme of an activated sludge plant.

However, in a lot of activated sludge samples, poor settling properties are encountered, resulting in a limited solid separation and a decreased effluent quality. The poor settling can be caused by sudden changes in physical parameters (pH, temperature), absence

of nutrients (nitrogen, phosphorus, micronutrients) and the presence of toxicants (inorganic or organic), which can cause partial deflocculation of the activated sludge (28). Filamentous bacteria are commonly observed in activated sludge and contribute to poor settlement of activated sludge flocs in secondary sedimentation tanks, a problem referred to as activated sludge bulking (319). A conventional way to measure the sludge settleability is by determining the sludge volume index (SVI) (115). Mixed liquor drawn from the aeration tank is introduced into a 1 liter graduated cylinder, is allowed to settle for 30 min. and the settled sludge volume is noted. The SVI is calculated as follows:

$$SVI\ (\text{ml/g}) = SV_{30} \times 1000 \times (MLSS)^{-1}$$

where SV_{30} = volume of the settled sludge in the cylinder (ml) and *MLSS* = mixed liquor suspended solids (mg/L).

Microbial populations of activated sludge

Activated sludge is an artificial living ecosystem that is under continuous influence of biotic and abiotic factors.

Table 1.3. Metabolic groups of micro-organisms in activated sludge (after Wanner, 1994 (324)).

Metabolic group	Required carbon form	Energy source	Electron acceptor
Organotrophs	Org.	Oxic oxidation	O_2
Anaerobic fermentators	Org.	Fermentation	Org. carbon
Nitrifiers	Inorg.	Oxic oxidation of NH_4^+	O_2
Denitrifiers	Org.	Anoxic oxidation	NO_3^-
Polyphosphate accumulating bacteria	Org.	Polyphosphate	-/O_2
Sulphide/sulphur oxidizing bacteria	Inorg.	Oxic oxidation	O_2
Sulphate reducing bacteria	Org.	Anaerobic oxidation	SO_4^{2-}

Org. = organic carbon; Inorg. = inorganic carbon.

Activated sludge is cultivated under limiting conditions to reach the desired - low nutrient - effluent concentrations and therefore a constant competition between bacteria is present. The most important metabolic groups of micro-organisms are listed in Table 1.3. Some of the most relevant groups will be discussed further in the text.

Oxic Organotrophic bacteria

The organic matter oxidation happens in the aeration tank of a wastewater treatment plant (Figure 1.7) by heterotrophic micro-organisms. These oxic organotrophic bacteria are fully responsible for complete removal of organic substances from wastewaters. Aeration for only a few hours leads to the transformation of soluble BOD into microbial biomass and CO_2 (28):

$$\text{organic compounds} + O_2 \rightarrow CO_2 + H_2O + NH_4^+ + \text{bacterial mass}$$

Aeration serves two purposes: supplying oxygen to the aerobic bacteria and keeping the activated sludge flocs in constant agitation to provide a constant mixing of the flocs and the incoming wastewater.

The genera *Bacillus, Pseudomonas, Micrococcus, Alcaligenes, Moraxella* and *Flavobacterium* are reported to be able to degrade the complex organic substrates by exo- and endoenzymes (324). Besides these “universalists”, also bacteria specialized in specific substrates can be present in activated sludge, such as *Achromobacter* spp (lipids, alcohols and acids) and *Proteus* spp (proteinaceous substrates). In the group of oxic organotrops, both floc-forming (*Zoogloea*) and filamentous bacteria (often actinomycetes) are found. These Actinomycetes are able to use slow degradable organic substrates (71) and excessive amounts of these bacteria can cause bulking and foaming (192).

Fermentative bacteria

For fermentation, anaerobic conditions are necessary, since these processes only occur in the absence of oxygen and nitrate. Fermentative processes are unlikely to occur in fully aerated low-loaded mixed wastewater treatment plants, but in some cases the conditions are present. For example in plug-flow systems or systems with premixing zones, cores of the aerobic sludge may turn anaerobic (324). Also systems with a high oxygen uptake rate (high loaded systems) can create anaerobic cores.

$$\text{Organic compounds} \rightarrow \text{Ethanol} + \text{2,3-Butanediol} + \text{Succinate}^{2-} + \text{Lactate}^- + \text{Acetate}^- + \text{Formate}^- + CO_2 + H_2 + \text{bacterial mass}$$

Nitrifiers

Nitrification can occur in the aeration tank. Nitrification is the conversion of ammonium into nitrate by microbial conversion (Figure 1.8). Nitrification is the oldest biochemical process used for industrial purposes: the medieval production of saltpeter (potassium nitrate) for gunpowder was based on the microbial conversion of organic nitrogen to nitrates (324). Nowadays, nitrification and subsequent denitrification are used in more peaceful applications to convert nitrogen sources to N_2 gas. Nitrification is carried out by two types of micro-organisms (98):

1. Conversion of NH_4^+ to NO_2^- by autotrophs, such as *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira* and *Nitrosolobus*.
2. Conversion of NO_2^- to NO_3^- by *Nitrobacter*, *Nitrospira* and *Nitrococcus.*

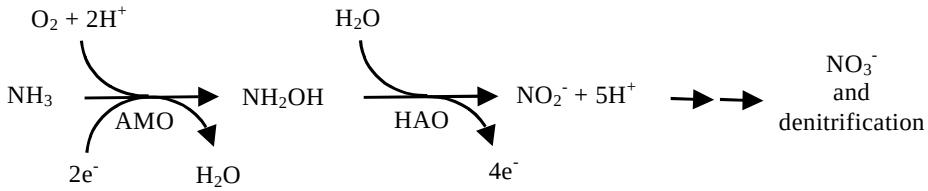

Figure 1.8. First steps in the nitrification, catalyzed by the ammonium monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) (After Keener and Arp, 1993 (159)).

Denitrifiers

Nitrate, originating from the nitrification, can further serve as an electron acceptor (denitrification) and is converted through nitrite to nitric oxide (NO), and nitrous oxide (N_2O) to nitrogen gas, N_2, (98). A large heterogeneous group of micro-organisms involved in denitrification are for example *Achrombacter, Aerobacter, Alcaligenes, Bacillus, Flavobacterium, Micrococcus, Proteus* and *Pseudomonas* (224). This reaction requires anoxic or anaerobic conditions. It has been estimated that 82-97 % of the micro-organisms in the activated sludge from anoxic zones are able to denitrify (104).

Impact of xenobiotic compounds on the functioning of wastewater treatment plants

Xenobiotic substances entering wastewater treatment plants can adversely affect the environment by upsetting the organic and nutrient removal functions of biological treatment processes or by escaping into nature by wash-out, thereby contaminating surface water or

groundwater. The effects of these substances in activated sludge can be (165):

1. lower nutrient (organics and nitrogen) removal
2. decrease in solids separation efficiency
3. modification in sludge settling and compaction properties

The effect of the xenobiotic compounds can be due to inhibition or toxicity (113). According to Grady et al. (113), inhibition occurs when the presence of a chemical reduces the rate of a microbial process, such as growth and substrate utilization. Toxicity can be defined as the presence of a chemical that causes all microbial activity to stop.

The mechanism by which xenobiotics inhibit microbial growth and substrate removal are not well defined. Some of these recalcitrant molecules can have very specific effects on microbial cells, whereas other have a more general or nonspecific effect. Among the specific effects are radical forming reactions, leading to hydroxyl radicals that can bind with macromolecules (113). Also organic acids can react with thiol groups of enzymes, resulting in an altered protein conformation and thus an altered enzyme activity. The active site of an enzyme can also be changed by covalent bounds with the amino acid side chains. Some recalcitrant molecules are analogues of natural occurring molecules and thus can bind irreversibly with the active site of an enzyme. Non-specific inhibition can happen by reacting with general biological components, such as membranes. For example hydrocarbons dissolve in the membrane, disrupting the structural integrity and thus inhibiting normal cellular functioning (270).

The nutrient removal rate of wastewater is reflected by effluent levels of biochemical oxygen demand (BOD) or chemical oxygen demand (COD). Toxic molecules can indeed inhibit the carbon oxidation by aerobic heterotrophic bacteria, but in order to see effects on carbon oxidation the concentration of the pollutant must usually be relatively high (317). A second important nutrient is nitrogen. The autotrophic bacteria responsible for the nitrification (i.e. *Nitrosomonas* and *Nitrobacter*) are generally regarded more sensitive to environmental factors (e.g. temperature, dissolved oxygen, pH) and toxicants than the heterotrophic bacteria (334). In this process, the oxidation of ammonium to nitrite is also more sensitive towards xenobiotic molecules than the oxidation of nitrite to nitrate (134). Thus ammonium oxidation can be considered as the weak link in the nitrification process.

Toxic influents can also cause population shifts in activated sludge towards non flocculating species. In case of pentachlorophenol, only a slight reduction in COD removal

efficiency was observed, however the microbial community turned into dispersed, nonsettleable cells (165). Also bulking can be caused by toxic shocks in wastewater treatment plants (114).

Degradation of chloroaromatic compounds using well defined microbial cultures[*]

Bioaugmentation is the accelerated removal of undesired compounds from contaminated hazardous waste sites or bioreactors by adding a microbial inoculum (311). The inoculum can consist of indigenous or allochtonous wild type or genetically modified organisms. In some cases, catabolic plasmids were used to disseminate catabolic genes among the natural bacterial population. Only a few successes of lab-scale bioaugmentation in activated sludge and other wastewater treating bioreactors have been reported (35, 199, 218, 263, 338) and the use of specialized strains to enhance the removal of pollutants present in wastewater treatment technology is not yet widely applied. In the following overview the successful and unsuccessful cases of bioaugmentation of aerobic bioreactors will be discussed.

In 1989 McClure et al. investigated the survival of a *P. putida* UWC1, which harbored the *in vitro* constructed 3-chlorobenzate degradative non-conjugative IncQ plasmid pD10 (200), inoculated in a labscale activated sludge unit that received 3-chlorobenzate. Despite the long survival of the inoculum and despite the stable maintenance of the plasmid in the sludge, no 3-chlorobenzate removal was observed. Strain UWC1 was reisolated from the activated sludge and some of the isolates showed the ability to transfer plasmid pD10 to another *Pseudomonas* strain. This showed that mobilizing plasmids from the indigenous bacteria were transferred to the introduced UWC1 strain. Plate filter matings showed that indigenous sludge bacteria could act as recipient strains and some of the obtained transconjugants even showed higher 3-chlorobenzate degradation rates then strain UWC1. In a next study, the usefulness of these transconjugants and a new 3-chlorobenzate degrading isolate was evaluated in an new activated sludge experiment (199). Both strains survived for long periods and 3-chlorobenzate degradation was enhanced.

Nüßlein et al. (1992) introduced in a bioreactor that received 3-chlorobenzoate (3CBA) and 4-methylbenzoate (4MBA) contaminated wastewater a genetically modified *Pseudomonas* sp. strain B13FR1(pFRC20P) (218). Strain B13FR1(pFRC20P) contained the not self-transferable plasmid pFRC20P, that simultaneously degrades of 3CBA and 4MBA.

[*] Partially redrafted after **Top, E.M., D. Springael, and N. Boon.** 2002. Mobile genetic elements as tools in bioremediation of polluted soils and waters. FEMS Microbiol. Ecol. Submitted.

Three days after inoculation a drastic decrease in the concentrations of 3CBA and 4MBA (initially 1mM each) in comparison with a non-inoculated reactor. Moreover inoculation protected the indigenous microorganisms against higher concentrations of 3CBA and 4MBA (4 mM). While in filter matings the Tra^- Mob^+ plasmid pFRC20C did not transfer to *Pseudomonas putida* UWC1 due to lack of transfer genes, its spread to sludge bacteria was detected infrequently in the activated sludge, which indicates that mobilizing plasmids present in the sludge must have mobilized pFRC20P. Conclusions on the effect of this low mobilization frequency on the rate of biodegradation of the pollutants could however not be drawn.

Selvartnam et al. (1995) analyzed a phenol receiving sequencing batch reactor bioaugmented with a 3-chlorobezoate-degrading *Pseudomonas putida* ATCC 11172 (262). This strain converts phenol into catechol, which is further metabolized through a *meta* cleavage pathway (263). By means of mRNA expression of the *dmpN* gene, which codes for phenol hydroxylase (converts phenol to catechol), the levels of expression were compared in function of phenol addition and removal. Greatest *dmpN* expression was observed 15 minutes after the maximal phenol concentration was reached and 15 minutes after the start of the aeration. Also decreased phenol concentrations in the reactor corresponded with decreased amounts of *dmpN* mRNA. In the bioaugmented reactor, about 85 % of all phenol was degraded. Two years later, the same authors evaluated the detoxification of phenol in a wastewater system by the introduction of the same catabolic strain, *Pseudomonas putida* ATCC 11172 (263). This time, the bioaugmented reactor maintained a removal between 95 and 100 % during more than 40 days, while in the non-bioaugmented reactor a decreased removal rate was observed.

The involvement of transposons in the adaptation of indigenous bacteria has been shown by Ravatn et al. (240). They showed that the self-transmissible chlorocatechol degradation genes (*clc*) from *Pseudomonas* sp. strain B13 could be transferred at low transfer frequencies in activated sludge microcosms. Springael and coworkers (107) (Springael, D., Ghyoot, W., Nuyts, S., Van Roy, S., and Diels, L. unpublished data) inoculated a *Pseudomonas putida* BN210 strain, which carried the *clc*-element on the chromosome, encoding the genes necessary for 3-chlorobenzoate metabolism, both in a continuous bioreactor and in a sludge system coupled to a membrane separator device, which increases the biomass density in the main reactor unit. The bioaugmented reactors were more resistant towards toxic shocks, as was demonstrated by an improved COD removal. Strain BN120 did not survive well in the reactors and new 3-chlorobenzoate populations developed. The *clc*-

element remained at high levels present in the reactors, indicating transfer of the genetic element to the indigenous bacteria. Similar observations were done by another study of the same group (278), where novel 3-chlorobenzoate degrading populations were formed in a membrane biofilm reactor that treated model wastewater containing 3-chlorobenzoate. *In situ* horizontal transfer of the *clc*-element from the inoculum to indigenous bacteria was observed. The inoculum became undetectable in the reactor and was replaced by several novel 3-chlorobenzoate mineralizing populations, which were more competitive under the defined reactor conditions than the inoculated strain. These are the first studies that showed *in situ* horizontal transfer of a catabolic mobile element in a wastewater treatment reactor and the concomitant removal of the compound for which it codes degradation.

Eichner et al (84) were the first to describe in 1999 a culture-independent approach to determine changes in the indigenous bacterial populations receiving a shock load of chlorinated and methylated phenols. A genetically modified micro-organisms (GMM) was used to bioaugmented the reactor. The GMM, *Pseudomonas* sp. B13 SN45RE, originates from strain B13 and can degrade simultaneously mixtures of chloroaromatics and methylaromatics via a hybrid *ortho*-cleavage pathway without catabolic misrouting of substituted catechols. The non-bioaugmented reactors showed a decrease in oxygen uptake rate (OUR) and the Temperature Gradient Gel Electrophoresis (TGGE) analysis showed that the microbial community collapsed. However the bioaugmented reactor protected the microbial structure by maintaining it highly diversity and the effects on the OUR were minor. The introduction of a specific catabolic pathway encoded by the GMM allowed to protect the activated sludge community from breakdown caused by toxic shock.

Besides the few successes in bioaugmenting reactors, reports of bioaugmentation failures have also been published. These findings are not less important, since future malfunctioned bioaugmentation can be prevented by examining these failures.

Goldstein et al. (110).examined different reasons for possible failure of inoculation in soils, lake water and sewage sludge. A *Pseudomonas* strain, capable of mineralizing 2,4-dichlorophenol and 4-nitrophenol, was isolated from soil. The strain was inoculated in sterile and non-sterile 2,4-dichlorophenol amended sewage. In the non-sterile sewage, the population density declined and 2,4-dichlorophenol was not mineralized. In the sterile sewage, the bacterium grew, however no appreciable degradation of the test compound was observed. The authors suggested different causes that can lead to bioaugmentation failures: (i) the contaminant concentration is too low to support microbial metabolism; (ii) microbial inhibitors are present in the environment; (iii) the growth rate of the degrading organism may

be slower than the rate of removal, for example by predation or wash-out; (iv) the inoculum may use substrates other than the pollutant whose destruction is desired or finally; (v) the organism may physically fail to reach the pollutant.

Tchelet et al. (293) introduced a chlorobenzene degrading *Pseudomonas* sp. strain P51 in a reactor with activated sludge amended and not amended with 1,2,4-trichlorobenzene. The number of inoculated cells dropped 10^7 cells/ml to 10^5 cells/ml in two days and disappeared quickly under the detection limit (10^3-10^4 cells/ml). In addition, no significant trichlorobenzene degradation occurred in the reactors. The same experiment was repeated for a soil system, and in this ecosystem the bioaugmentation was successful. In the sludge microcosm the authors observed two main phenomena: (i) apparently the conditions were not favourable for survival of strain P51, and (ii) the presence of 1,2,4-trichlorobenzene posed no selective advantage for maintenance and growth. The disappearance of the P51 cells was faster than the calculated wash-out from the reactor and the authors concluded that predation by protozoa would be responsible for the decline. Survival and activity thus seem to depend strongly on the type of system into which the strain is introduced.

The poor survival of the inoculated strains would suggest that a regular reinoculation of the specialized strains could increase the success of bioaugmentation. Nevertheless, even massive and repeated inoculation can cause reactor function breakdown. This was observed when an aerobic denitrifying bacterium *Microvirgula aerodenitrificans* was inoculated twice in a nitrifying sequencing batch reactor (41, 42). A first inoculation (70 mg dry mass/L) did not result in an improved nitrogen removal. A second massive inoculation (360 mg dry mass/L) was followed by a nitrification breakdown, while at the same time, nitrification remained stable in a non-bioaugmented reactor. The *Microvirgula aerodenitrificans* almost disappeared within two days and they were found back in digestive vacuoles of protozoa. The second inoculation apparently unbalanced the system and resulted in an overgrowth of protozoa. The increased grazing pressure was fatal for the sensitive autotrophic nitrifying bacteria.

Watanabe et al. (1998) used two different phenol degrading strains, i.e. *Pseudomonas putida* BH and *Comamonas* sp. E6, to treat a phenol contaminated waste stream (328). The population of strain E6 stabilized after 10 days at ca. 10^5 cells/ml, while strain BH declined to 10^4 cells/ml. This difference was due to the slower incorporation of the BH cells into the activated sludge flocs. Approximately 75 % of the E6 cells were incorporated into flocs at day 1, whereas approximately 39 % of the BH population was in the flocs.

To improve the survival of the inoculated bacteria, Watanabe et al. (2000) starved

their inocula prior to addition to the reactors (327). The cells grown on mineral medium with phenol and subsequently starved for two days remained at concentrations that were 50 to 100 times higher than for cells grown in an eutrophic medium, non-starved cells or cells that were starved for seven days. The starved cells showed an increased cell surface hydrophobicity, resulting in a high sludge adhesion capacity. Improved survival by hydrophobic cells, which form clusters within activated sludge flocs, was already demonstrated by McClure et al. (199). Cell surface hydrophobicity of a bacterium is an important factor in its interaction with the environment and may influence the ecological niche it will occupy. Microorganisms can modify their cell surface hydrophobicity, within certain limits, under the influence of environmental parameters, such as stress (40). Bossier et al. (39) inoculated a plasmid-free non-aggregative *Ralstonia eutropha*-like AE815 strain in activated sludge and after reisolation a mutant strain A3 was obtained, showing an autoaggregative behavior. The strain had also acquired an IncP1 plasmid pLME1. A conjugation between strain A3 and another *Ralstonia eutropha* AE176 resulted in AE176 transconjugants with and without aggregation behavior. Therefore, the exact role of the plasmid pLME1 was not clear from this study (39). The acquisition of mobile genetic elements, such as plasmids, thus may lead to increased adherence or adhesion properties and deserves further research.

In conclusion, successful bioaugmentation of bioreactors is still not evident. Future research should aim for a better understanding of the *in situ* physiology of the inoculant cells. This knowledge could then be used to manipulate the cells in different ways to increase the survival and metabolic rates of the inoculated cells. Advanced molecular techniques are now available to obtain this information by monitoring the presence and activity of inoculants both *in situ* or *ex situ*.

MOLECULAR TOOLS TO STUDY MICROBIAL POPULATION DYNAMICS[†]

It has been suggested that between 2 and 3 million bacterial species exist in our biosphere (304). To estimate the diversity of these complex microbial populations, a variety of methods like plating, plain and fluorescence microscopy and recently DNA and RNA analysis have been developed over the century (Figure 1.9).

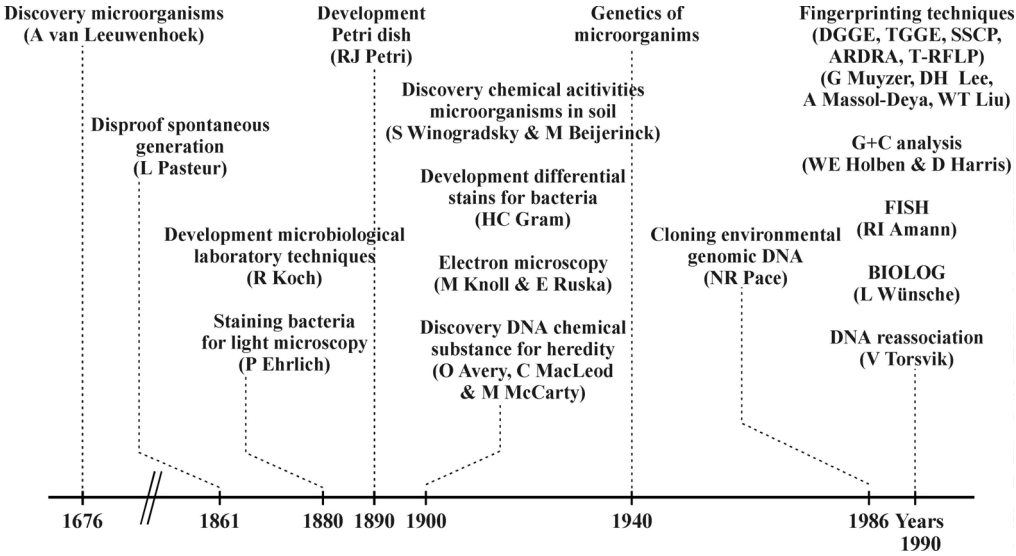

Figure 1.9. Timescale of the most important events in microbiology (after Dejonghe et al., 2001 (75, 229)).

For years, plating (CFU) on different media was the technique to investigate microbial diversity. However, the relative proportion of bacteria growing on agar plates varies from 0.001 to 15 % in aquatic environments. This implies that investigations based on bacterial isolates may include only a minor part of the total bacterial diversity (10). During the last 10 years, different molecular techniques have allowed the investigation of bacterial communities without the need to culture the microorganisms (10, 136) (Figure 1.10).

To examine the active population of a microbial community, molecular techniques have been developed that are based on RNA (mRNA or rRNA). Fluorescent *in situ*

[†] Redrafted after **Dejonghe, W., N. Boon, D. Seghers, E. M. Top, and W. Verstraete.** 2001. Bioaugmentation of soils by increasing microbial richness: missing links. Environ. Microbiol. **3:**649-657.

Hybridization (FISH), where fluorescent oligonucleotides target rRNA sequences within whole cells (7, 258), is a relative easy technique and is used frequently in wastewater biotechnology and biofilm studies. The advantages of FISH are that the spatial distribution and the quantitative composition of a microbial community can be examined by epifluorescence or confocal laser scanning microscopy. The fluorescence signal emitted by a cell also indicates the physiological state of the cell. The more active a cell, the more ribosomes are present that can serve as a target for the oligonucleotides. Recently, a combination of FISH and microautoradiogaphy was developed to determine *in situ* the identities, activities, and specific substrate uptake profiles of individual bacterial cells within complex microbial communities (177).

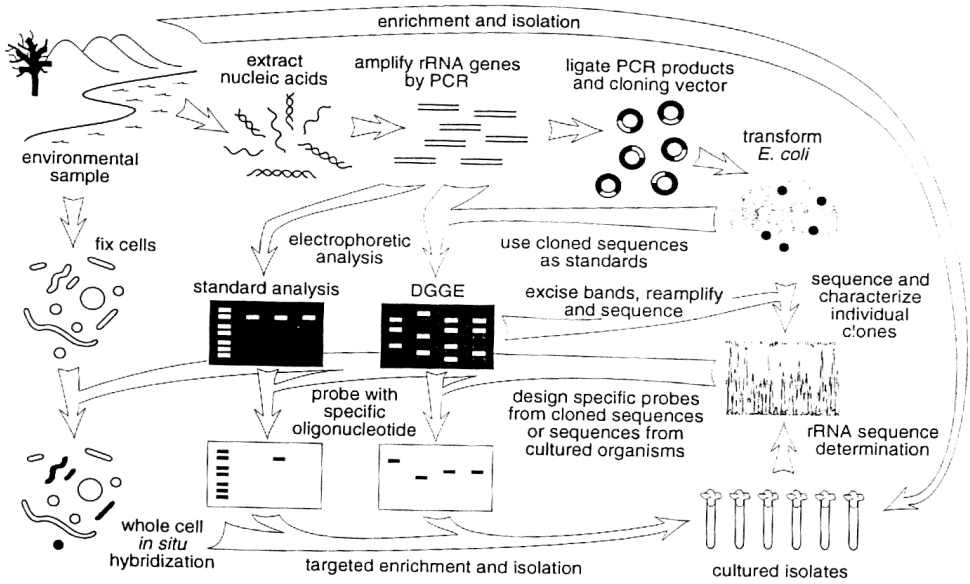

Figure 1.10. Overview of DNA and RNA based molecular methods used in molecular microbial ecology (after Head et al., 1998 (120)).

Another possibility is the extraction of total RNA followed by RT-PCR (reverse transcription PCR) to amplify and study rRNA (92, 168) or mRNA (262). RNA based analysis should give a picture of the most metabolically active, and thus most relevant micro-organisms in the system under those specific test conditions. *In situ* RT-PCR involves amplification of specific nucleic acid sequences (mRNA) inside intact prokaryotic cells followed by color or fluorescence detection of the localized PCR product via bright-field or epifluorescence

microscopy and can be used to study the transcription in active communities (51, 135, 173). A method that is not based on RNA extraction and PCR amplification and examines the active portion of a microbial community is phospholipid fatty acid analyses (PLFA) (143). The total community phospholipid fatty acids are extracted and analyzed by gas chromatography.

DNA based techniques are commonly used to examine the total population of a microbial community. Ribosomal RNA genes are used to obtain phylogenetic relationships between organisms. A first attempt to study the unculturable species of ecosystems was made by cloning random fragments of environmental genomic DNA and then sequencing clones containing rRNA genes (222). Because this technique is very laborious, several researchers started to use Polymerase Chain Reaction (PCR) to selectively amplify rRNA genes from environmental DNA (10). This technique employs different primer sets to amplify the ribosomal genes of all types of organisms (Archaea, Bacteria or Eucarya) present in an environmental sample. The obtained PCR fragments can then be cloned and sequenced (139, 140) or they can be separated and visualized by fingerprinting techniques like Denaturing Gradient Gel Electrophoresis (DGGE). In DGGE and TGGE (Thermal Gradient Gel Electrophoresis) double stranded DNA fragments with the same length but different base-pair sequence, obtained after PCR of rRNA genes, are separated in a denaturant or thermal gradient gel, respectively (208, 209). A similar technique is SSCP (Single Strand Conformation Polymorphism), where PCR amplified single stranded rRNA genes with the same length but different conformational structure are separated in a denaturant gel (176). In both cases a pattern of bands is obtained which provides information about changes in the gross community structure. However, since the microbial diversity of complex ecosystems is so extended and can never be captured in one banding pattern, it may be better to focus on specific subgroups of microorganisms like methylotrophic members of the γ- and α-Proteobacteria (122), actinomyceten (126), α- en β-Proteobacteria (111), ammonia-oxidizing bacteria (167), *Archaea* (221), *Acidobacterium* (34) and fungi (273), rather than on the total rRNA gene pool. Other DNA fingerprinting techniques that are also based on PCR amplification of rRNA genes, but combined with restriction of the amplified fragments are Amplified Ribosomal DNA Restriction Analysis (ARDRA) (310) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) (182). In all the above-mentioned techniques, extraction of RNA instead of DNA, followed by RT-PCR (reverse transcription PCR), can be applied in theory and should give a picture of the most metabolically active.

All the methods mentioned above give only an idea about the dominant ribotypes in an ecosystem. The rRNA based methods only examine a very small part of the total genetic

material of a bacterium and they are not very accurate to assess total microbial diversity. Methods such as base composition profiles expressed as mole percent guanine + cytosine (% G + C) (219) and DNA-reassociation and the so-called "C_0t curves" were a first means to assess the total genetic diversity of bacterial communities (303). In this last technique, the reassociation time is a measure for the genetic diversity in a sample since the more different genomes present in a sample, the slower the DNA reassociation occurs after initial denaturation. Community-level physiological profiles are based on metabolic response patterns of environmental samples, inoculated into a 96 wells BIOLOG® plate (26, 272) and short-term respiration responses of microbial communities are measured by the addition of simple substrates (= *in situ* catabolic potential) (73). These physiological profiles can give an estimation of the functional diversity. More specifically, these methods reflect the organisms directly involved in the mainstream energy flux of the ecosystem. Recently, Rondon et al. (248) combined cloning large fragments of DNA, isolated from soil, into a bacterial artificial chromosome vector for phylogenetic and genomic analysis with screening clones directly for functional gene expression. Using this approach, the phylogenetic and functional information that is present in both culturable and non-culturable micro-organisms can be investigated. The latest advance in molecular technology is the use of nucleic acid microarrays, or DNA chips (30, 52, 271). Oligonucleotides or cDNA are fixed on glass slides and fluorescently labeled DNA, or RNA are hybridized with the probes on the slide, which can be detected by a scanning laser. These chips are currently used for the detection of ribosomal and functional genes and to study expression profiles in bacteria by examining the transcription of RNA. Their use for environmental samples is still limited.

OVERALL CONCLUSIONS

The use of molecular technology in microbial ecology is booming at the moment and techniques, such as PCR, DGGE and FISH have become standard methods. Continuously, new apparatus and techniques are adapted from the medical field to be implemented in the environmental field. In many studies, the new techniques are used in a more descriptive way than in an analytical way. Moreover often the link between the molecular data and the biological processes is still not known. Thousands or even millions of unknown microorganisms, belonging to bacterial lineages of which only a few cultivated members have been described, are still undiscovered (223). As a result, also the metabolic capacities of all these bacteria are unknown and intrinsic biotechnological applications are waiting to be

discovered.

Biotechnology is becoming more and more important in daily life. While in 1996, biotechnology accounted for ca. 18 % of the market share, estimates for 2005 predict that this share will increase to 61 % (48). Pollution control and clean-up by microorganisms is becoming more important. A challenge will be to integrate the fundamental microbial aspects in environmental technology to overcome the present practical problems in the bioaugmentation of environmental samples. Since understanding and steering the behavior of microorganisms in natural systems requires interdisciplinary research, molecular microbiologists, microbial ecologists, and environmental engineers need to cooperate.

GOALS AND OUTLINE OF THE STUDY

Chloroanilines are important precursors in chemical industry and a literature search showed that these types of molecules are found regularly in industrial wastewaters. Since chloroanilines are not removed by the wastewater treatment, these molecules are discharged in the environment. There is obviously a need for more research to enhance chloroaniline degradation in the wastewater treatment systems in order to prevent the release of these compounds in the environment. Literature review indicates that the number of cases of successful bioaugmentation is limited. In addition, the influence of pollutants and bioaugmentation on the microbial communities of activated sludge has not yet been studied thoroughly. This study has the aim to better understand bacterial chloroaniline degradation and to study the potency some bioaugmentation strategies for activated sludge systems.

The specific goals of this work:

- To better understand the diversity of chloroaniline degraders present in the environment, as well as their catabolic genes and the mobile genetic elements they reside on (Chapter 2).
- To develop a bioaugmentation strategy for the continuous removal of 3-CA containing wastewater and to monitor changes in bacterial composition by DGGE analysis. It was hypothesized that a slow release based inoculation of chloroaniline degrading bacteria would be more successful than the inoculation of free suspended bacteria. An alternative bioaugmentation strategy by means of inoculation and transfer of catabolic plasmids may also result in more successful biodegradation than when a strain without mobile chloroaniline degradation genes is inoculated (Chapter 3, 4 and 5).

- To examine the influence of a chloroaniline shock load on the reactor performance, both by chemical and molecular techniques, and evaluate the possible bioprotection of the activated sludge by a chloroaniline degrading strain (Chapter 6).
- To develop an easy and fast nested PCR-DGGE based technique to examine several bacterial groups in activated sludge. This seemed necessary since DGGE analysis with universal bacterial primers typically does not allow detecting changes in populations that are numerically dominant (Chapter 7).

Chapter 1 gives an overview of the literature on chloroanilines in the environment and the biodegradation of chloroanilines. After a short introduction on wastewater treatment plants, an overview of bioaugmentation studies in activated sludge systems is given. Moreover the present molecular techniques which are used in microbial ecology are discussed.

A first part of this research was focused on the diversity of chloroaniline degrading bacteria. Until now all 3-chloroaniline degrading bacteria described in literature were isolated from soil. Since we intended to use a 3-CA metabolizing bacterial strain in an activated sludge bioaugmentation experiment later on, new strains were isolated from activated sludge systems. Before using these strains in bioaugmentation experiments, the strains and their plasmids with genes encoding for chloroaniline degradation, were characterized and identified (Chapter 2).

Activated sludge wastewater treatment is considered as the major technology to minimize the release of pollutants into the aquatic environment. The second objective was to develop a bioaugmentation strategy for the continuous removal of 3-CA containing wastewater and to monitor changes in bacterial composition by DGGE analysis. Three different strategies were assessed. In Chapter 3 the effect of bioaugmentation on the degradation capacity of a SCAS (semi continuous activated sludge) reactor, as well as on the structure of the microbial community was examined by the inoculation of suspended C*omamonas testosteroni* I2*gfp* cells, able to mineralize 3-CA. In Chapter 4 a new slow release approach was tested to obtain a prolonged complete 3-CA removal by the activated sludge reactors. The use of catabolic mobile genetic elements for the bioaugmentation of activated sludge was examined in Chapter 5. The influence of a chloroaniline shock load on the reactor performance was examined in Chapter 6, both by chemical and molecular techniques. The possible bioprotection of the microbial community and of the reactor performance by the inoculation of a specialized strain was examined .

Different bacterial groups each play a particular role in microbial communities. The present techniques do not allow the screening of high numbers of samples for different bacterial groups. Therefore the last objective was the development of an easy and fast nested PCR-DGGE based technique to examine bacterial groups in activated sludge. This is presented in Chapter 7.

In Chapter 8 the obtained results are discussed in the framework of the research objectives. Conclusions are drawn and perspectives for further research are presented.

CHAPTER 2

2. ISOLATION AND CHARACTERIZATION OF 3-CHLOROANILINE DEGRADING STRAINS ‡

ABSTRACT

The diversity has been examined of the plasmids and of the gene *tdnQ*, involved in oxidative deamination of aniline in five bacterial isolates that are able to metabolise both aniline and 3-chloroaniline (3-CA). Two strains have been described and identified previously, i.e., *Delftia acidovorans* CA28 and BN3.1. Strains I2 and B8c were isolated in this study from a wastewater treatment plant and were identified as *Comamonas testosteroni* and *Delftia acidovorans*, respectively. Strain LME1, identified as *Delftia acidovorans*, was isolated from a linuron-treated soil. Both *Delftia* and *Comamonas* belong to the family of the *Comamonadaceae*. All five strains possess a large plasmid of ca. 100 kb, but the plasmids from only 4 strains could be transferred to a recipient strain by selecting on aniline or 3-CA as sole source of carbon and/or nitrogen. Plasmid transfer experiments and Southern hybridization revealed that the plasmid of strain I2 encodes total aniline but not 3-CA degradation, while the plasmids of strains LME1 and B8c were only responsible for the oxidative deamination of aniline. Several transconjugant clones that had received the plasmid from strain CA28 showed different degradative capacities: all transconjugants could use aniline and 3-CA as nitrogen source, while only some of the transconjugants could dechlorinate 3-CA. For all four plasmids, the IS*1071* insertion sequence of Tn*5271* was found to be located on a 1.4 kb restriction fragment, which also hybridized with the *tdnQ* probe. This suggests the involvement of this IS element in the dissemination of aniline degradative genes in the environment. Using specific primers for the *tdnQ* gene, from *Pseudomonas putida* UCC22, the diversity of the PCR amplified fragments in the five strains was examined by denaturing gradient gel electrophoresis (DGGE). With DGGE, three different clusters of the *tdnQ* fragment could be distinguished. Sequencing data showed that the *tdnQ* sequences

‡ Redrafted after **Boon, N., J. Goris, P. De Vos, W. Verstraete and E.M. Top**. 2000. Bioaugmentation of activated sludge by an indigenous 3-chloroaniline degrading *Comamonas testosteroni* strain, I2*gfp*. Appl. Environ. Microbiol. 66:2906-2913. **Boon, N., J. Goris, P. De Vos, W. Verstraete and E.M. Top.** 2001. Genetic diversity among 3-chloroaniline and aniline degrading strains of the *Comamonadaceae*. Appl. Environ. Microbiol. 67:1107-1115.

of I2, LME1, B8c and CA28 were very closely related, while the *tdnQ* fragment of BN3.1 and *P. putida* UCC22 were only about 83 % identical to the other sequences. Northern hybridization revealed that the *tdnQ* gene is only transcribed in the presence of aniline and not when only 3-CA is present.

INTRODUCTION

For many years, anilines and chloroanilines have been among the most important industrially produced amines. They are used widely in the production of polyurethanes, rubber, azo-dyes, drugs, photographic chemicals, varnishes and pesticides (106, 157). As a consequence of this widespread use, they are detected in wastewaters (172, 252). Moreover, chloroanilines have been found in waters as a consequence of the transformation of frequently used acetamide and urea herbicides (172). These toxic and recalcitrant compounds are considered important environmental pollutants (202) and are subjected to legislative control by the 76/464/E.E.C. Directive (2) and the Priority Pollutant List of U.S. Environmental Protection Agency (82).

In aquatic environments, the major way to remove aniline is through biodegradation (106, 191). The first step of the aerobic degradation pathway is oxidative deamination, which results in the formation of catechol, which is then further degraded by an *ortho*-cleavage pathway (15, 189) or a *meta*-cleavage pathway (164). Recently, the different genes of *Pseudomonas putida* UCC22(pTDN1) involved in the transformation of aniline to catechol were sequenced as *tdnA1*, *tdnA2*, *tdnB*, *tdnR*, *tdnQ* and *tdnT* (102) (Figure 2.1); that from *Acinetobacter* sp. strain YAA was sequenced as aniline oxygenase gene *atdA* (100). Based on sequence similarities with other aniline degradation pathways and on a gene expression study, Fukumori and Saint (102) tentatively concluded that *tdnA1*, *tdnA2*, *tdnB*, and *tdnT* are structural genes and that *tdnR* is a positive regulatory gene. *tdnQ* could be a structural gene, and its gene product TdnQ shows ca. 30% amino acid sequence similarity with glutamine synthetases.

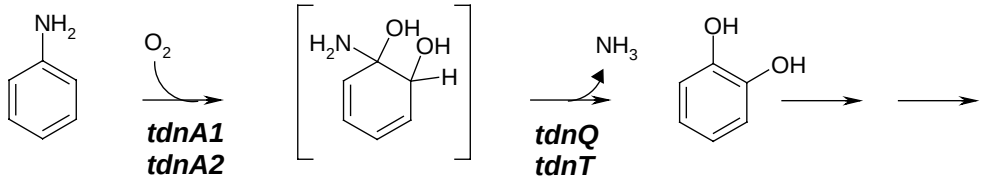

Figure 2.1. Scheme of the genes, involved in the deamination of aniline to catechol.

The hypothetical pathway for aniline conversion is as follows. Both atoms of molecular oxygen are incorporated into the 1 and 2 positions of aniline by the oxygenase (TdnA1 and TdnA2) to form a diol, and subsequently the amino group is transferred to TdnQ

(102). TdnT may further transfer the amino group to an unknown substance or release ammonium. All the *tdn* genes are essential for the conversion of aniline to catechol. A number of catabolic plasmids such as pCIT1, pTDN1 and pYA1, that can degrade aniline have been described previously (14, 100, 203, 250).

In contrast to aniline, which is rapidly metabolized, chloroaniline is more persistent in the environment (137, 288). Therefore, many efforts have been undertaken to isolate bacteria capable of degrading chlorinated anilines. *Moraxella* sp. strain G (339) was the first strain isolated that could use 4-chloroaniline as sole source of carbon, nitrogen and energy. Later, more chloroaniline-metabolizing strains were isolated, such as *Pseudomonas* sp. strain JL2 (175), *Brevundimonas* (previously *Pseudomonas*) *diminuta* INMI KS-7 (284), *Delftia* (previously *Pseudomonas*) *acidovorans* CA28 (189), *D. acidovorans* BN3.1 (46), *Aquaspirillum* sp. strain 2C and *Paracoccus denitrificans* 3CA (285). (For the current taxonomic situation of *B. diminuta* and *D. acidovorans*, see Segers et al. (260) and Wen et al. (332), respectively.) The pathway of monochloroaniline degradation in some of these strains was found to lead directly to a modified *ortho*- or *meta*-cleavage pathway after oxidation of the monochloroaniline to the corresponding chlorocatechol (35, 132, 175, 189, 340). The involvement of plasmids in chloroaniline degradation was not clear from these studies.

In contrast to the situation for the aniline degradation pathway, no specific genes for the transformation of chloroaniline have yet been described. The present study was designed to investigate the genetic diversity of five different aniline- and 3-chloroaniline (3-CA)-degrading strains. We compared the involvement of the plasmids in these strains in the degradation of aniline and 3-CA, as well as the diversity of the *tdnQ* gene, one of the genes involved in oxidative deamination of aniline.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 2.1.

Table 2.1. Bacterial strains and plasmids.

Strain or plasmid	Characteristic(s)[a]	Reference or source
Strains		
Comamonas testosteroni I2	Ani^{NC}, $3\text{-}CA^{NC}$, Rif^{R}, Hg^{R}	This study
Delftia acidovorans LME1	Ani^{NC}, $3\text{-}CA^{NC}$, Rif^{R}	This study
Delftia acidovorans B8c	Ani^{NC}, $3\text{-}CA^{N}$	This study
Delftia acidovorans CA28	Ani^{NC}, $3\text{-}CA^{NC}$, Rif^{R}	(189)
Delftia acidovorans BN3.1	Ani^{NC}, $3\text{-}CA^{NC}$, Rif^{R}	(46)
Ralstonia eutropha JMP228	Rif^{R}	(299)
Ralstonia eutropha JMP228gfp	Rif^{R}, Km^{R}, GFP	This study
E. coli S17-1 λpir		(125)
E. coli S17-1 λpir (pUTgfp)	Amp^{R}, Km^{R}, GFP	This study
Plasmids		
pNB2 (from strain I2)	Ani^{NC}, Hg^{R}	This study
pNB1 (from strain LME1)	Ani^{N}	This study
pNB8c (from strain B8c)	Ani^{N}	This study
pC1-1 (from strain CA28)	Ani^{N}, $3\text{-}CA^{N}$	This study
pC1-2 (from strain CA28)	Ani^{N}, $3\text{-}CA^{N}$	This study
pC1-3 (from strain CA28)	Ani^{N}, $3\text{-}CA^{N}$	This study
pB1 (from strain BN3.1)	-	This study
pUTgfp	Amp^{R}, Km^{R}, expresses GFP	(297)
pTDN1-3112	pUC19 + HindIII (2.59)-EcoRI (4.15) fragment, containing *tdnQ* of *P. putida* UCC22; Amp^{R}	F. Fukumori
pBRH4	Amp^{R}, *IS*1071	(228)

[a] Ani, aniline; GFP, Green Fluorescent Protein; Km, kanamycin; Rif, rifampine; Hg, mercury. Superscript "N" and "C" indicate sole nitrogen source and sole carbon and energy source, respectively.

Strains I2, B8c and LME1 were deposited in the BCCM^{TM}/LMG - Bacterium Collection (Ghent, Belgium) under the numbers LMG 19554, LMG 19553 and LMG 19555 respectively. *Escherichia coli* S17-1 λpir (125) was transformed with plasmid pUTgfp (297) as described by Chung et al. (54). This plasmid contains a mini-Tn*5* transposon with the *nptII* (Km^R) and *gfp* genes, and was used to insert the latter two genes into the chromosome of the rifampin resistant *Ralstonia eutropha* JMP228 (299). This procedure was done by means of biparental mating between *E. coli* S17-1 λpir (pUTgfp) (125, 297) and *R. eutropha* JMP228, with selection on Luria broth (LB) agar plates containing rifampin (100 mg/L) and kanamycin (50 mg/L). The new strain, JMP228*gfp*, is rifampin and kanamycin resistant and shows green fluorescence under UV-light.

Media and culture conditions

The mineral medium MMN (Mineral Medium without Nitrogen and Carbon) is derived from mineral medium MMO (280) by elimination of all nitrogen. The MMN medium contained 1419.6 mg Na_2HPO_4, 1360.9 mg KH_2PO_4, 98.5 mg $MgSO_4$, 5.88 mg $CaCl_2.2H_2O$, 1.16 mg H_3BO_4, 2.78 mg $FeSO_4.7H_2O$, 1.15 mg $ZnSO_4.7H_2O$, 1.69 mg $MnSO_4.H_2O$, 0.38 mg $CuSO_4.5H_2O$, 0.24 mg $CoCl_2.6H_2O$, 0.10 mg MoO_3 and 3.2 mg EDTA in 1 L of distilled water. The liquid mineral medium was supplemented with 200 mg of aniline (Sigma-Aldrich Chemie, Steinheim, Germany) (MMN-A) or 3-CA (Fluka AG Chemische Fabrik, Buchs, Switzerland) (MMN-CA) per liter; for the solidified mineral medium, aniline and 3-CA were each used at a concentration of 500 mg/L. Sodium pyruvate (1000 mg/L of MMN medium) was added as an additional carbon source to MMN-A and MMN-CA, in order to select for bacteria by utilizing aniline or 3-CA as sole source of nitrogen (MMN-AP and MMN-CAP, respectively). LB medium containing 10 g of Bacto Peptone (Difco, Detroit, Mich.), 5 g Bacto Yeast Extract (Difco) and 5 g of NaCl in 1 L of demineralized water was used as a rich medium. These media were solidified with 2 % agar for plate growth.

Kanamycin (50 mg/L), rifampin (100 mg/L) and $HgCl_2$ (10 mg/L) were added when media were cooled down to 50 °C or lower.

Isolation of 3-CA-degrading microorganisms

D. acidovorans CA28 (189) and *D. acidovorans* BN3.1 (46) were isolated previously. In this study, strain B8c was isolated from a wastewater treatment plant of a potato-processing company (Waregem, Belgium); strain I2 was isolated from activated sludge of a domestic wastewater treatment plant (Bourgoyen-Ossemeersen plant, Gent, Belgium); and strain LME1

was obtained from soil that has been treated annually with 3 kg of linuron per ha for at least 10 years (Royal Research Station of Gorsem, Sint-Truiden, Belgium) (see also reference (87)). Erlenmeyer flasks (0.25-liter capacity) containing 100 ml of activated sludge (4 g [dry weight]/L) or soil (5 g in 95 ml of MMN-CA medium) were used to select for 3-CA-degrading microorganisms over a 6-week period by adding 200 mg of 3-CA/liter at the beginning and once a week when less than 5 mg of 3-CA/liter was left in the flasks. The dry-weight determination was performed by incubating a 50-ml sample at 105°C for 24 h and measuring the loss of weight after incubation (115). Subsequently, a 0.5-liter Erlenmeyer flask containing 200 ml of MMN-CA medium (200 mg/liter) was inoculated with 2 ml of the enrichment culture. After 6 days, the second generation of the enrichment culture was transferred to fresh MMN-CA medium (1% inoculum) in a 0.5-liter Erlenmeyer flask. After 6 days of incubation, 100 μl of the culture was spread onto MMN-CA and MMN-CAP agar plates, which were incubated aerobically at 28°C for 1 week. Bacteria that were able to form colonies and that grew in liquid MMN-CA or MMN-CAP medium were regarded as 3-CA-assimilating bacteria.

Cultivation of the isolated microorganisms

Overnight cultures in 5 ml of LB were used as inocula for degradation experiments. After 1 ml of culture was centrifuged for 5 min at 7,000 × *g*, washed, and resuspended in 1 ml of saline (0.85 % NaCl), an inoculum (1% the final volume) was transferred to liquid MMN medium with the previously described concentrations of 3-CA and/or sodium pyruvate. All cultures were incubated aerobically at 28°C in the dark on a shaker (140 rpm).

Identification of the isolates

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of whole-cell proteins was performed as previously described (232). Briefly, cells were harvested from tryptic soy agar plates (BBL) after 48 h of incubation at 37°C. Protein extracts were prepared in a SDS- and beta-mercaptoethanol-containing buffer and separated on a discontinuous SDS-polyacrylamide gel. The gel was then stained with Coomassie blue and scanned with an LKB 2202 Ultroscan laser densitometer (LKB, Bromma, Sweden). The protein extract of *Psychrobacter immobilis* LMG 1125 was used as a standard for normalization. Numerical interpretation of the data was completed with the GelCompar 4.1 software package (Applied Maths, Kortrijk, Belgium).

DNA preparation and determination of the moles percent content guanine plus

cytosine via high-pressure liquid chromatography (HPLC) were done as described by Logan et al. (186); nonmethylated phage lambda DNA (Sigma) was used as the calibration standard. Total genomic DNA-DNA hybridizations were performed by the microplate method of Ezaki et al. (90) using black MaxiSorp (Nunc, Roskilde, Denmark) microplates and a HTS7000 bioassay reader (Perkin-Elmer, Norwalk, Conn.). The hybridization temperature was 55°C.

Methods for plasmid DNA extraction, restriction analysis, and Southern hybridisation

Plasmid DNA was isolated by a modified version (298) of the alkaline extraction procedure for large plasmids (151). Restriction endonuclease digestion was done according to the instructions of the enzyme supplier (Hoffmann-La Roche, Basel, Switzerland). Southern hybridizations were performed at high stringency as described by Top et al. (299). Digested plasmid DNA was separated by electrophoresis on a 0.7 % agarose gel and blotted onto Hybond-N nylon membranes (Amersham International, Buckinghamshire, England). The *tdnQ* probe was prepared by PCR digoxigenin (DIG) labeling mix (Hoffmann-La Roche) according to the instructions of the supplier and using two primers designed in this study (see description of PCR amplification below) and vector pTDN1-3112 (Table 1) as a template. The IS*1071* probe was removed from vector pBRH4 (228) with *HindIII* and subsequently labeled with the DIG DNA random labeling kit. The *korA* probes for the IncP-1α and IncP-β groups were prepared by PCR labeling as previously described using plasmids RP4 (66) and pJP4 (80) as templates respectively.

Plate matings

Biparental matings were performed by using LB agar plates with the donor and the recipient, *R. eutropha* JMP228*gfp*, grown separately overnight in LB. The first selection step for aniline or 3-CA-degrading transconjugants was done with liquid MMN-CA, MMN-CAP, MMN-A and MMN-AP media (5 ml in 20 ml tubes) supplemented with kanamycin (50 µg/ml). After the transconjugants showed growth in the liquid media (as observed by turbidity measurements), they were plated on the corresponding solid MMN media. Green fluorescence under UV light confirmed that potential transconjugant colonies were indeed JMP228*gfp*. Selection for transfer of Hg^R was performed directly on LB agar supplemented with $HgCl_2$ (20 mg/L).

Northern hybridization

C. testosteroni I2 and *D. acidovorans* CA28 were grown overnight at 28°C in LB, LB-

aniline (200 mg/liter), and LB-3-CA (200 mg/liter). Total RNA was extracted as described by Reddy et al. (242). In brief, 10 ml of culture was centrifuged for 10 min. at 12,000 × g and 4°C. The pellet was resuspended in 10 ml protoplasting buffer (15 mM Tris, 0.45 M sucrose, 8 mM EDTA, 0.1 % diethylpyrocarbonate [DEPC] [pH 8.0]), with addition of 80 µl of 50-mg/ml lysozyme and incubated on ice during 15 min. Subsequently, the protoplasts were centrifuged for 5 min. at 5,900 × g, and the pellet was resuspended in 0.5 ml of gram-negative bacterium lysing buffer (10 mM Tris, 10 mM NaCl, 1 mM sodium citrate, 1.5 % SDS, 0.1 % DEPC [pH 8.0]), incubated for 5 min. at 37 °C and chilled on ice. A 250-ml quantity of saturated NaCl (40 g NaCl / 100 ml of H_2O) was added, and the solution was incubated on ice 10 min. and centrifuged at 12000 × g for 10 min. The supernatant was removed to a clean tube, 1 ml ice-cold 100 % ethanol was added, and the RNA was precipitated on dry ice (30 min). Afterwards, the tube was centrifuged at 12000 × g for 15 min., and the pellet was rinsed in 70% ethanol, air dried, and dissolved in 100 µl of DEPC-treated water. Equal amounts of total RNA were loaded on a denaturing agarose gel with formaldehyde, and the gel was Northern blotted onto a Hybond-N nylon membrane (Amersham International, Buckinghamshire, England). Northern hybridization was done as described by Thomas (295).

Chemical analysis

Supernatants of bacterial cultures were analyzed by reverse-phase HPLC after the cells were removed by centrifugation (10 min. at 5,000 × g). The HPLC system consisted of a Kontron liquid chromatograph with a DEGASYS DG-1310 system to degas the mobile phase, three Kontron 325 high-pressure pumps, a Kontron MSI 660 injector with a 20 µl loop, a Kontron DAD 495 diode-array detector, and a 450 MT2/DAD software system. An Alltima C_{18} column (250- by 8-mm inner diameter, 5-µm particle size; Alltech, Deerfield, Ill., USA) was used. The mobile phase consisted of CH_3OH-0.1% H_3PO_4 (60:40), the flow rate was 0.75 ml/min, and the UV detector was set to 210 nm. Quantitative determination of aniline and 3-CA were done using an external standard ranging from 1 to 250 mg/liter. The detection limit was ca. 0.5 mg/L.

Supernatants of bacterial cultures were analyzed for chloride content by ion chromatography after centrifugation at 5000 × g for 10 min. and filtering through a 0.45 µm filter. The DX-600 system (Dionex, Wommelgem, Belgium) consisted of a Dionex Pump GP50, a Dionex Autosampler Model AS50 (injection volume is 100 µl), a Dionex ED50 Electrochemical Detector and a PeakNet 6 software system version 6.10. Ionpac AS9-HC

(250 mm × 4 mm ID; 9 µm particle size; Dionex) column and Ionpac CS12-HC (250 mm × 4 mm ID; 8 µm particle size; Dionex) were used for anion separation. The mobile phase consisted of Na_2CO_3 (9 mM) and methanesulfonic acid (20 mM) with a flow rate of 1 ml/min. Quantitative data were obtained by comparing the peak areas of unknown concentrations with the peak areas of standards of known concentrations.

Gas chromatography (GC)-mass spectrometry (MS) analyses were carried out with a model 2700 GC (Varian, Palo Alto, Calif.)-MAT112S (Finnigan, San Jose, Calif.) gas chromatograph-mass spectrometer equipped with a DB-1 capillary column (100% dimethylsiloxane; length, 30 m; internal diameter, 0.53 mm; film thickness, 5 µm). The temperature of the injector was 200°C, and that of the detector was 250°C. The oven temperature was programmed to increase from 40 to 220°C at a rate of 2°C/min. Helium was used as the carrier gas at a flow rate of 3.5 ml/min.

Wavelength scans were recorded on a Kontron Uvikon Spectrophotometer, model 932.

PCR amplification

For pure cultures, the template for PCR amplification was obtained by extracting total genomic DNA by the procedure of Bron and Venema (45). One microliter of genomic DNA solution was used in a PCR. The PCR mixture contained 0.5 µM (each) primer, 100 µM (each) deoxynucleoside triphosphate, 10 µl of 10x Expand High Fidelity PCR buffer and 2 U of Expand High Fidelity DNA polymerase (both from Hoffmann-La Roche, Basel, Switzerland), 400 ng of bovine serum albumin (Hoffmann-La Roche, Basel, Switzerland)/µl, and sterile water (Sigma-Aldrich Chemie, Steinheim, Germany) to a final volume of 50 µl. The *tdnQ* gene was amplified with primers tdnQ1F (5'-TCC-CTG-CCT-GGA-GCC-CGA-AAC-3') and tdnQ1R (5'-TCC-CGC-GCC-GTG-AGT-GAC-TG-3'). The latter were designed in this study on the basis of specific regions of the *tdnQ* sequence (DDBJ-EMBL-Genbank accession number D85415). The length of the expected amplified fragment was 384 bp. A GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3') (208) was attached to the 5' end of the tdnQ1F primer. PCR was performed with a Perkin-Elmer 9600 thermal cycler as follows: 94°C for 5 min, and then 30 cycles of 92°C for 1 min, 53°C for 1 min, and 72°C for 2 min. A final extension was carried out at 72°C for 10 min. For incompatibility group determination, *korA* primers specific for incompatibility group IncP-1 were used, and PCR amplification was performed as described previously (112).

The REP-PCR was done according to Versavolic et al. (313) to distinguish identical

isolates.

DGGE

Denaturing gradient gel electrophoresis (DGGE) based on the protocol of Muyzer et al. (208) was performed with a D Gene System (Bio-Rad, Hercules, Calif., USA). PCR samples were loaded onto 8% (wt/vol) polyacrylamide gels in 1 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA [pH 7.4]). The polyacrylamide gels were made with a denaturing gradient ranging from 50 to 80% (where 100 % denaturant contains 7 M urea and 40% formamide). Electrophoresis was carried out for 5 h at 60°C and 180 V. Then, the gels were stained with SYBR GreenI nucleic acid gel stain (1:10,000 dilution; FMC BioProducts, Rockland, Maine, USA) and photographed (35).

DNA cloning and sequencing

Putative *tdnQ* gene fragments were cloned by using a TOPO TA cloning kit (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's instructions. DNA sequencing was carried out by Eurogentec (Liège, Belgium). Analysis of DNA sequences and homology searches were completed with standard DNA sequencing programs and the BLAST server of the National Center for Biotechnology Information using the BLAST algorithm (5), and using the BLASTN and BLASTX programs for the comparison of a nucleotide query sequence against a nucleotide sequence database and a nucleotide query sequence translated in all reading frames against a protein sequence database, respectively.

Nucleotide sequence accession numbers

Nucleotide sequences for fragments *tdnQ-I2*, *tdnQ-LME1*, *tdnQ-B8c*, *tdnQ-CA28* and *tdnQ-BN3.1* have been deposited in GenBank database under accession numbers, AF315641, AF315640, AF315643, AF315639, and AF315642, respectively.

RESULTS

Isolation and identification of 3-CA-metabolizing bacteria

In order to obtain single strains of 3-CA degrading bacteria from the enrichment cultures, which had been growing with 3-CA as sole carbon and nitrogen source as described above, the culture was plated on MMN medium. The purified colonies were tested in liquid MMN-CA or MMN-CAP medium with 3-CA as sole source of carbon, nitrogen and energy. Strains I2 and B8c, and LME1 were isolated as new 3-CA-metabolizing bacteria from activated sludge (I2 and B8c) and from linuron-treated soil (LME1), respectively. Two of these strains, strain I2 and LME1, and strains CA28, and BN3.1 (Table 2.1) are able to use aniline and 3-CA as sole sources of carbon and nitrogen. When aniline and 3-CA were used as sole carbon sources, all strains could degrade the compounds between 40 and 75 h (Figure 2.2).

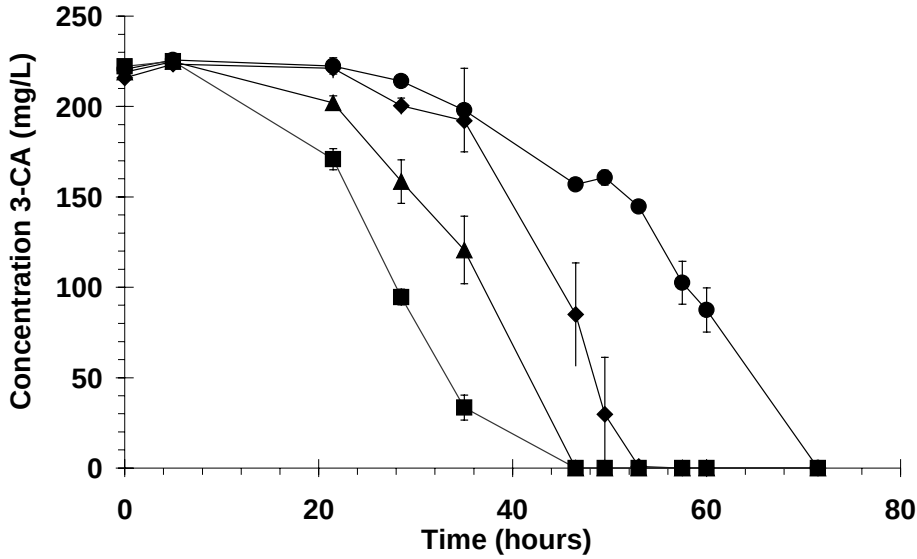

Figure 2.2. Degradation of 3-CA in MMN-CA as sole source of carbon, nitrogen, and energy in *C. testosteroni* I2 (♦), *D. acidovorans* CA28 (▲), LME1 (●) and BN3.1 (■). Data points are averages for duplicate cultures.

On LB agar plates, the strain I2 showed a phenotypic instability, resulting in two types of colonies with different morphology. Further purification of both types of colonies

continued to yield a mixture of the both types.

When aniline and 3-CA were used as sole nitrogen sources and sodium pyruvate was used as an additional carbon source, the degradation of both compounds was already completed between 14 and 24 h. No aromatic intermediates were observed by HPLC analysis. Strain B8c, on the contrary, grew with aniline as a sole carbon source but not with 3-CA (data not shown).

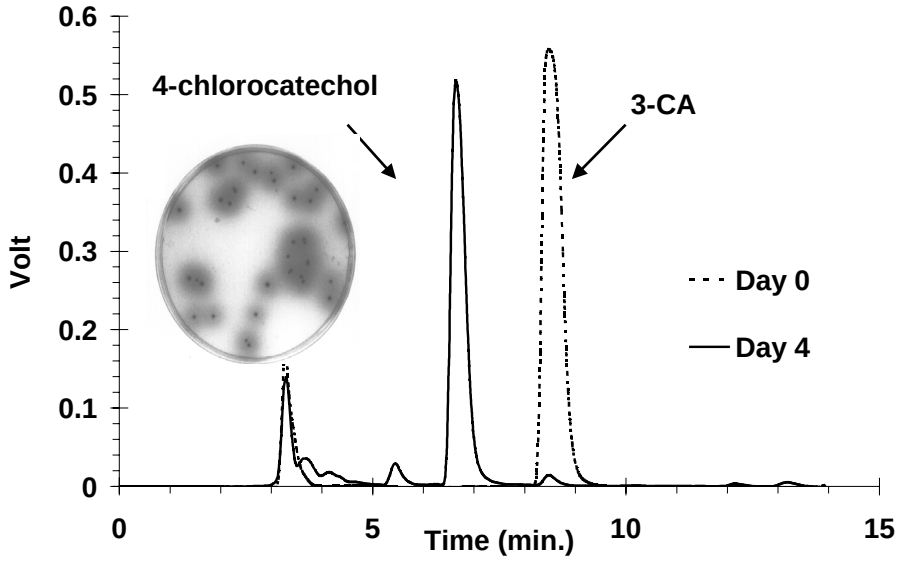

Figure 2.3. HPLC chromatogram for MMN-CAP medium, incubated with *D. acidovorans* B8c at day 0 and at day 4. The inset shows strain B8c grown on MMN-CAP plates. The colonies are surrounded by a brown color.

However, it grew in MMN-CAP medium with 3-CA as a sole N source and formed a brown intermediate (Figure 2.3). This result was corroborated by the detection of an aromatic intermediate by HPLC analysis (Figure 2.3). The mass spectrum of this product, analyzed by GC-MS, was consistent with the structure of 4-chlorocatechol (128). The molecular ion (M) at *m/z* 144 showed the characteristic 3:1 M/M + 2 isotope ratio of a single Cl atom. Major fragment ions had a *m/z* ratios of 126, 98, and 63 (Figure 2.4). The accumulation of 4-chlorocatechol in the culture of strain B8c is consistent with the inability of this strain to use 3-CA as carbon source, and indicates that the strain can only transform 3-CA into 4-chlorocatechol.

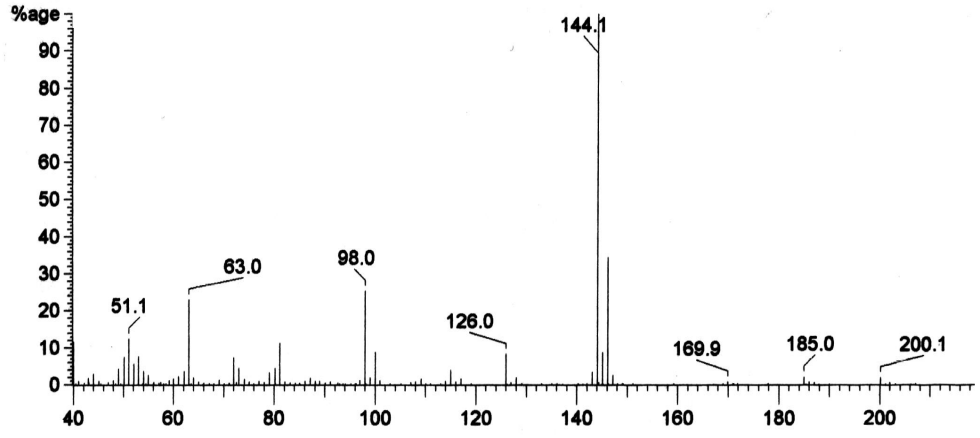

Figure 2.4. GC-MS mass spectrum of the brown intermediate, obtained after incubating MMN-CAP with *D. acidovorans* B8c.

During the growth of I2 in MMN medium (both liquid and solid) with 3-CA as the sole carbon, nitrogen and energy source, a yellow coloration of the medium developed and disappeared again after prolonged incubation. In order to quantify the yellow intermediary product, a wavelength scan of the medium between 200 and 400 nm was performed at different times of incubation of the growth culture (Figure 2.6). During the first 48 hours, no visual changes were observed and no 3-CA was degraded. After 48 h, the culture ended the lag-phase and the medium began to become yellow (Figure 2.5). One day later, the cells were in the logarithmic phase and the intensity of the yellow color was high. Until 77 h, this metabolite accumulated and at the same time the concentration of 3-CA decreased equally. Once the absorption peak at 380 nm had disappeared (82 h) (Figure 2.5 and Figure 2.6), the total 3-CA was metabolized. After 82 hours, an equimolar amount of chloride ions was released during the degradation of 3-CA by strain I2 (data not shown).

The absorption maxima of the yellow intermediate at different pHs were examined. At pH 2, 7 and 12 the maximum absorption of the yellow intermediary product were at 323 nm, 380 nm and 378 nm, respectively.

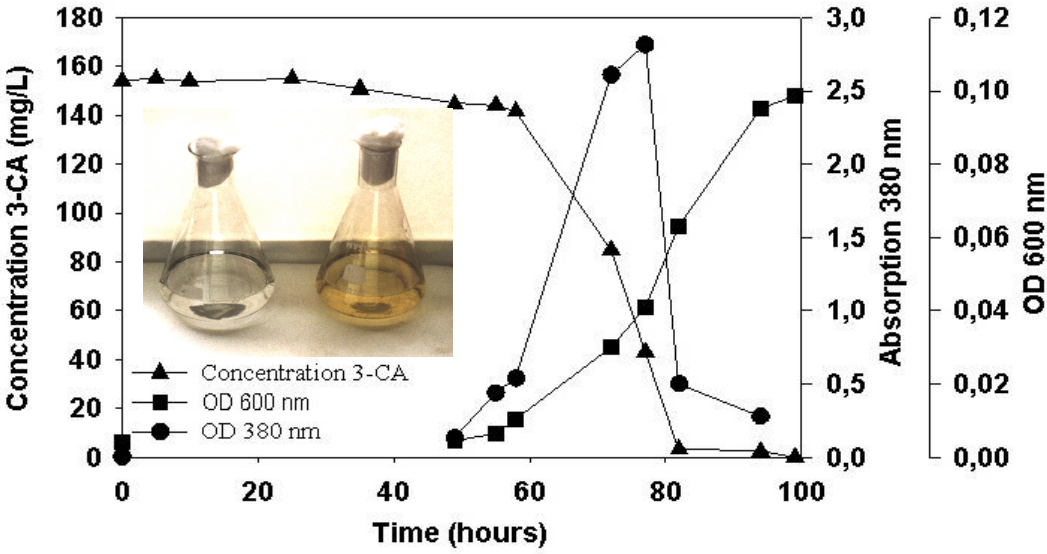

Figure 2.5. Growth of *C. testosteroni* I2 in MMN + 3-CA during 4 days. Degradation of 3-CA (▲) in relation to the cell growth (■)and accumulation of the intermediate (peak at 380 nm) (●) is shown. The inset shows the coloration of the MMN-CA medium after 0 hours (left) and after 80 hours (right is shown).

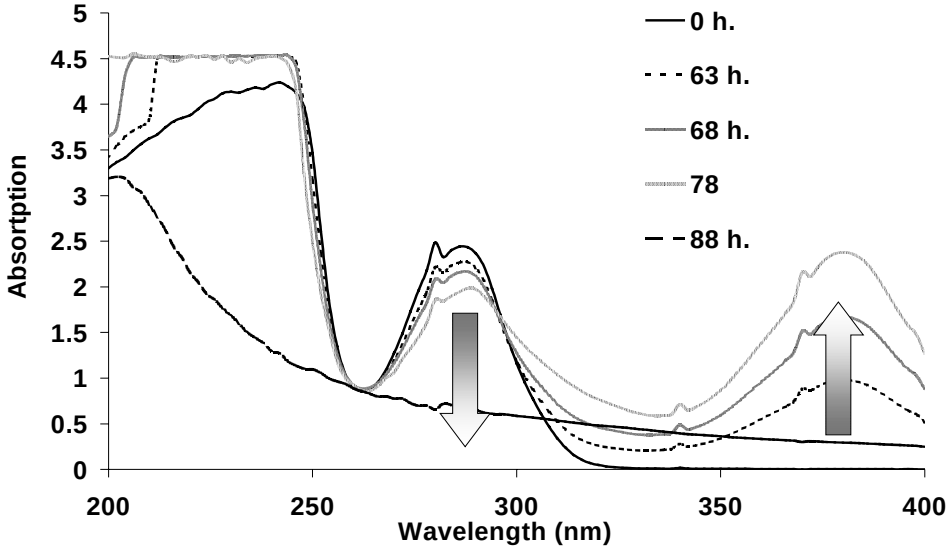

Figure 2.6. Wavelength scans during the growth of *C. testosteroni* I2 in MMN + 3-CA during 4 days. The arrows indicate the changes at a particular wavelength in function of time.

Identification of 3-chloroaniline metabolizing bacteria

Strain I2 had a nucleotide composition of 62 mol % guanosine + cytosine and was identified via FAME analysis (using the commercial MIS-database) as *Comamonas testosteroni*. In DNA-DNA hybridization experiments, the strain showed a DNA reassociation of 76 % with *Comamonas testosteroni* LMG 1800^T (195), and was therefore identified as *Comamonas testosteroni*. Strain B8c has a nucleotide composition of 66.6 mol % G+C and showed 92% DNA reassociation when hybridized with LMG 1226^T(292), the type strain of *Comamonas acidovorans*, which was recently accommodated in the new genus *Delftia* as *Delftia acidovorans* (332). Much lower DNA reassociation values of 28 and 32% were found when strain B8c was hybridized with *Comamonas testosteroni* LMG 1800^T (195) and *Comamonas terrigena* LMG 1253^T (72), respectively. Since strains B8c and LME1 showed identical SDS-PAGE patterns of whole cell proteins, both isolates were unambiguously identified as *Delftia acidovorans*.

Involvement of the plasmids in the degradation

Extraction of plasmids from strains LME1, I2, B8c, CA28, and BN3.1 revealed that they all contained a plasmid with a size of ca. 100 kb, designated pNB1, pNB2, pNB8c, pC1 and pB1, respectively. An *Eco*RI-*Pst*I digest of the plasmids revealed different restriction patterns (Figure 2.7A). All plasmids yielded an amplification product after PCR with the *korA* primers, which are specific for the IncP-1 group of broad-host-range plasmids. Southern hybridization of these *korA* PCR products with RP4 (IncP-1α)- and pJP4 (IncP-1β)-generated probes revealed that plasmids pNB1, pNB2, pNB8c and pC1 hybridized with the IncP-1β-derived probe; pNB8c also hybridized with the IncP-1α-derived probe; and pB1 did not hybridize with either of the two probes, although an amplification product had been obtained (data not shown). Four of the five plasmids clearly belong to the IncP-1 group; three of them appear to be IncP-1β plasmids.

To determine if some of the catabolic genes were plasmid encoded, conjugative transfer of the aniline or 3-CA degradation phenotype from strains I2, LME1, B8c, CA28, and BN3.1 to the recipient strain *R. eutropha* JMP228*gfp* was examined. From these mating experiments different plasmid-encoded functions could be derived, as summarized in Table 2.1 and Table 2.2. All transconjugants, except for that from the mating with *D. acidovorans* BN3.1 used aniline as nitrogen source; however, only JMP228*gfp*(pNB2) degraded aniline completely in MMN-A medium and thus used it as sole carbon source as well.

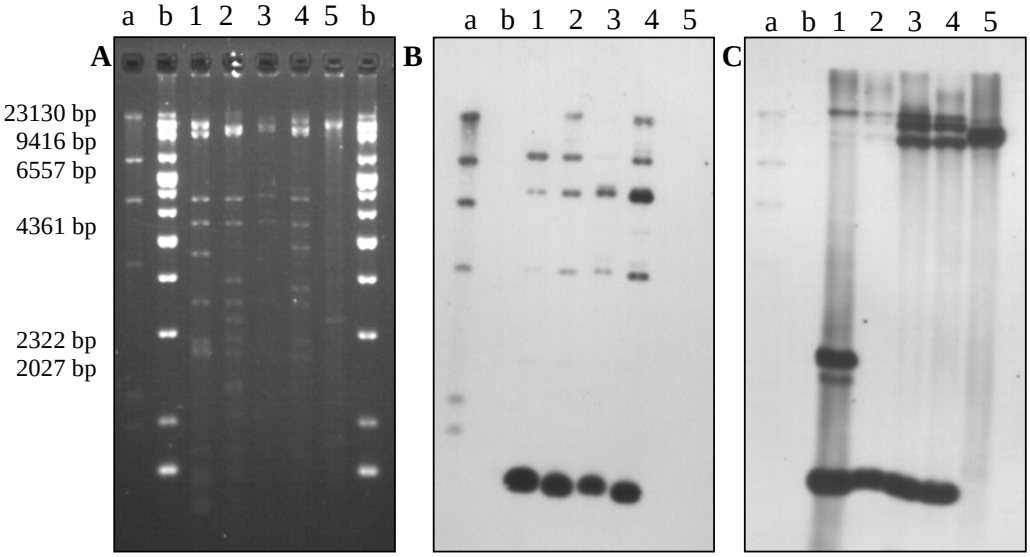

Figure 2.7. Restriction digestion analysis and Southern hybridization. (A) Analysis on a 0.7% agarose gel of *Eco*RI-*Pst*I-digested plasmids. (B and C) Hybridization with *tdnQ* (B) and IS*1071* (C). Lane 1, *C. testosteroni* I2 (plasmid pNB2); lane 2, *D. acidovorans* LME1 (plasmid pNB1); lane 3, *D. acidovorans* B8c (plasmid pB8c); lane 4, *D. acidovorans* CA28 (plasmid pC1); lane 5, *D. acidovorans* BN3.1 (plasmid pB1); lane a, DIG-labeled Marker II (Hoffmann-La Roche); lane b, 1-kb extended marker. The Southern blot in panel C was obtained from a gel different from that shown in panel A and B, but containing the same DNA samples.

Table 2.2. Plasmid encoded properties, determined by conjugation experiments with the different isolates and *R. eutropha* JMP228*gfp*. The composition of the different MMN media are listed in the material and methods section.

Isolate (plasmid)	MMN-CA	MMN-CAP	MMN-A	MMN-AP	Hg
I2 (pNB2)	-	-	+	+	+
LME1 (pNB1)	-	-	-	+	-
B8c (pNB8c)	-	-	-	+	-
CA28 (pC1)	-	+	-	+	-
BN3.1 (pB1)	-	-	-	-	-

+ = growth, - = no growth; Hg: mercury resistant

As determined by HPLC, the other transconjugants, which can use aniline only as nitrogen and not as carbon source, transformed only 50 % of the added aniline in MMN-A medium. This result could have been due to the toxic effect of accumulated intermediates, such as catechol. A brown color, usually caused by polymerization products of catechol, was indeed observed in these cultures. Some of the transconjugants obtained from matings between CA28 and JMP228*gfp* showed different degradation capacities and their respective plasmids were named pC1-1, pC1-2 and pC1-3 (Figure 2.8). Plasmids pC1-1 and pC1-3 contain the genes necessary for the oxidative deamination and dechlorination of 3-CA, while transconjugants with pC1-2 could dechlorinate 3-CA at much lower rates (Figure 2.9). When the three types of transconjugants were grown in MMN-AP, the medium colored brown, probably due to the formation and accumulation of catechol and its polymerization products. This result was not observed when transconjugants JMP228*gfp* (pC1-1) and JMP228*gfp* (pC1-3) were grown in the MMN-CAP.

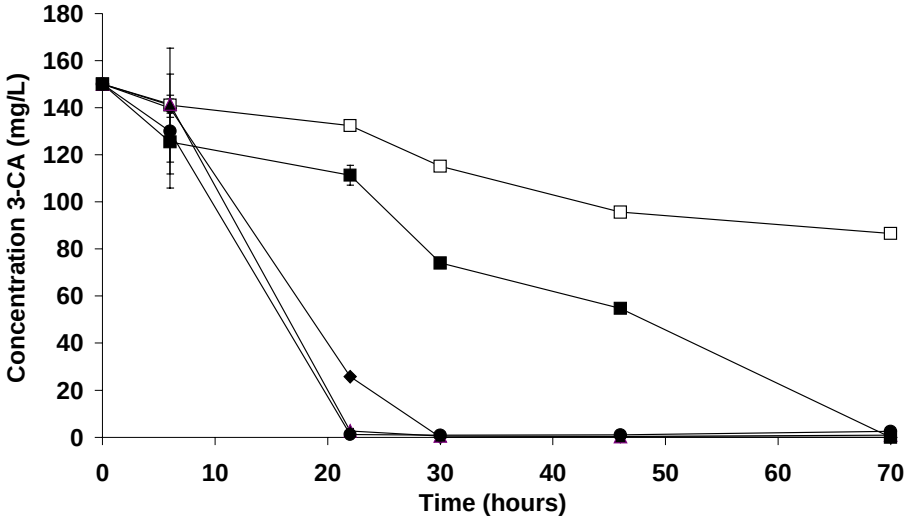

Figure 2.8. Degradation of 3-CA in MMN-CAP as sole source of nitrogen by *D. acidovorans* CA28 (♦); JMP228*gfp* (□); JMP228*gfp* (pC1-1) (▲); JMP228*gfp* (pC1-2) (■) and JMP228*gfp* (pC1-3) (●). Data points are averages of duplicate cultures and error bars represent standard deviations.

Plasmid extraction with *Eco*RI-*Pst*I-digestion and PCR with the *korA*-primers for IncP-1 plasmids revealed the presence of the different plasmids in the JMP228*gfp*

transconjugants. The plasmids in the donors and the respective transconjugants had identical restriction patterns (data not shown). Also no differences in restriction patterns between pC1-1, pC1-2 and pC1-3 were visible. Conjugation between *D. acidovorans* BN3.1 and *R. eutropha* JMP228*gfp* did not yield any transconjugants that were able to metabolize aniline or 3-CA. *C. testosteroni* I2, which was the only mercury resistant strain, could transfer this mercury resistance to JMP228*gfp*, indicating that the resistance gene is also located on plasmid pNB2 (Table 2.1 and Table 2.2).

If the aniline- and/or 3-CA-degradative genes located on the plasmids have enough similarity with some of the cloned and sequenced *tdn* genes of *P. putida* UCC22 involved in the oxidative deamination of aniline (102), we should be able to confirm their localization on the plasmids by hybridization. Therefore, primers were designed and a probe was developed for one of the genes, *tdnQ*. Restriction digestion of plasmids pNB2, pNB1, pNB8c and pC1, showed a clear hybridization signal of a 1.4-kb fragment after Southern hybridization with the *tdnQ*-probe (Figure 2.7B).

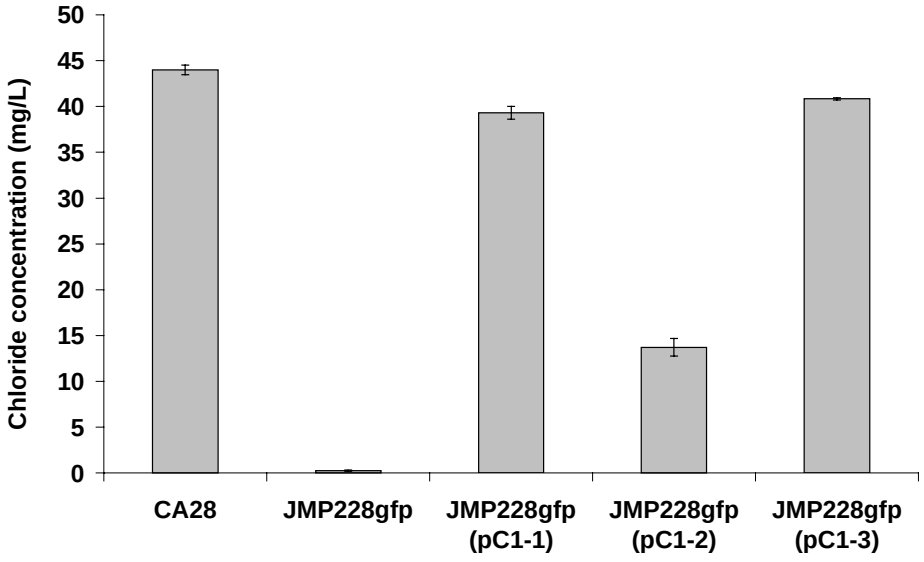

Figure 2.9. Chloride release after 70 hours if incubation of 3-CA in MMN-CAP as sole source of nitrogen by *D. acidovorans* CA28 (♦); JMP228*gfp* (□); JMP228*gfp* (pC1-1) (▲); JMP228*gfp* (pC1-2) (■) and JMP228*gfp* (pC1-3) (●). (A): 3-CA concentration. (B): Data points are averages of duplicate cultures and error bars represent standard deviations.

Only with plasmid pB1 no hybridization signal was obtained. The probe derived from IS*1071* also hybridized with a 1.4-kb fragment (Figure 2.7C). This probe also hybridized to an additional 2.7-kb fragment of plasmid pNB2 as well as to a large fragment (ca. 17 kb) of plasmid pB1. The large fragments of plasmids pNB8c and pC1 are due to incomplete digestion (Figure 2.7C). The data show that *tdnQ*-like genes, very similar to *tdnQ* of *P. putida* UCC22, are located on all four plasmids that could transfer the capacity to deaminate aniline and/or 3-CA by conjugation.

Comparison of partial *tdnQ*-sequences.

To investigate the diversity of the *tdnQ*-like genes in the five strains, PCR amplification with *tdnQ* primers, including one GC clamp, was performed. All the aniline- and 3-CA-metabolizing strains I2, LME1, B8c, CA28, and BN3.1, as well as the positive control (the vector pTDN1-3112) yielded a PCR amplification product of the expected length of 384 bp. An initial comparison of the sequences of the amplified fragments was done via DGGE analysis. A 50% to 80% gradient of denaturing agents resulted in the best separation of the fragments of the different strains. The *tdnQ* fragments were clearly not identical and could be classified in three groups (Figure 2.10).

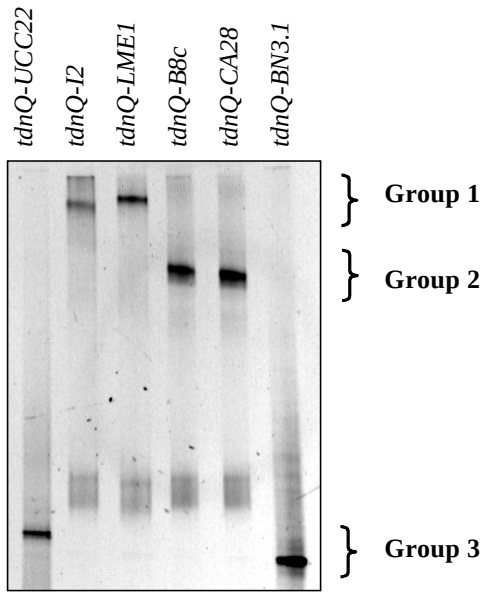

Figure 2.10. DGGE analysis of *tdnQ* fragments of different strains capable of degrading aniline and 3-CA.

The first group, *tdnQ* from *C. testosteroni* I2 (*tdnQ-I2*) and from *D. acidovorans* LME1 (*tdnQ-LME1*), was denatured at rather low denaturant concentrations (upper part in the gel) and a very small difference in migration between the two fragments was observed. The second group, *tdnQ* from *D. acidovorans* B8c (*tdnQ-B8c*) and from *D. acidovorans* CA28 (*tdnQ-CA28)*, was localized at higher concentrations of denaturing agents (middle of the gel) and seemed to migrate at the same rates. The fragments of the original *tdnQ* gene of *P. putida* (*tdnQ-UCC22*) and of *D. acidovorans* BN3.1 (*tdnQ-BN3.1*) both migrated to the bottom of the gel at the highest denaturant concentration and thus formed the third group. While the difference in migration positions between the last two groups of PCR fragments was large, there was only a small difference between the first and the second groups.

Table 2.3. Levels of nucleotide and amino acid sequence identities for the 384-bp amplified portion of the *tdnQ* genes of aniline- and 3-CA-degrading bacteria and for database sequences.

	% Nucleotide or amino acid identity with: [a]						
Strain	*C. testosteroni* I2	*D. acidovorans* LME1	*D. acidovorans* B8c	*D. acidovorans* CA28	*D. acidovorans* BN3.1	*P. putida* UCC22[b]	*Acinetobacter* sp. YAA[b]
C. testosteroni I2	-	95/95	88/88	95/95	88/91	89/91	68/81
D. acidovorans LME1	98	-	91/92	98/98	92/95	91/95	69/82
D. acidovorans B8c	94	95	-	93/93	86/89	86/89	67/81
D. acidovorans CA28	98	98	96	-	92/94	92/97	68/81
D. acidovorans BN3.1	83	84	80	84	-	99/100	70/84
P. putida UCC22[b]	83	83	81	84	99	-	69/84
Acinetobacter sp. YAA[b]	NSS	NSS	NSS	NSS	NSS	NSS	-

[a] Single entries indicate nucleotide identities. Double entries indicate amino acid identities/positives. NSS, no significant similarity was found.

[b] Sequences were obtained from GenBank.

To confirm the sequence differences of these *tdnQ* fragments, all 5 PCR-products (without GC clamp) were cloned and subsequently sequenced. Analysis of the DNA sequences is summarized in Table 2.3. The partial *tdnQ* genes of the five strains were related to the partial *tdnQ* gene of *P. putida* UCC22. While the sequence of the *tdnQ-BN3.1* fragment was nearly identical to that of the *tdnQ-UCC22* fragment, the sequences of the *tdnQ-I2*, *tdnQ-LME1*, *tdnQ-B8c* and *tdnQ-CA28* fragments were only 80-84% similar to that of the *tdnQ-UCC22* fragments. When the partial sequences were translated to amino acid sequence level, all the sequences were found to be highly similar to the amino acid sequence of the TdnQ-protein of *P. putida* UCC22 (102) and less similar to a component of an aniline dioxygenase (glutamine synthetase like protein) of *Acinetobacter* sp. strain YAA (100) (GenBank accession number D86080) (Table 2.3). Only the last 306 bp of the 384 bp *tdnQ* fragment was translated into the partial protein structure of 102 amino acids. The comparison of the DGGE and sequencing results showed that the DGGE approach can be useful for specifically amplifying and analyzing *tdnQ*-like genes from mixed cultures.

Differential expression of *tdnQ*

In order to investigate the role of *tdnQ* in the degradation of 3-CA, strains *C. testosteroni* I2 and *D. acidovorans* CA28 were grown in LB, LB -aniline, and LB-3-CA. No traces of aniline and 3-CA could be detected at the time of cell collection, prior to RNA extraction. This result indicates that at least one or several genes involved in the initial transformation steps had been transcribed. Total RNA was blotted and hybridized with the *tdnQ*-probe (Figure 2.11). With both strains, only the RNA that was extracted from the cells grown with aniline hybridized with the *tdnQ*-probe. These results suggest that under the conditions used here the *tdnQ* gene in these strains is induced by aniline or its metabolites (102) but not by 3-CA or its metabolites. This notion implies that oxidative deamination of 3-CA in these strains may involve different genes than those responsible for aniline degradation.

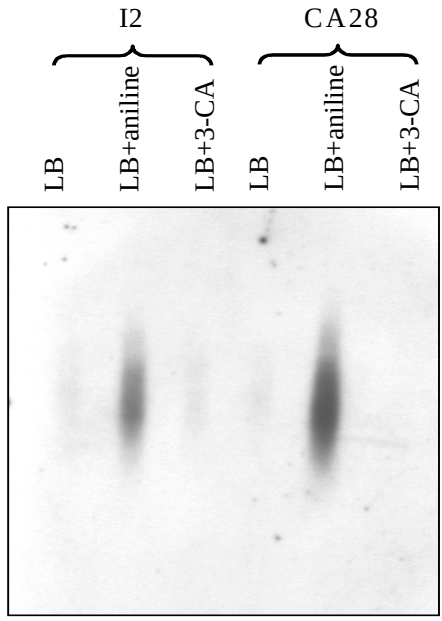

Figure 2.11. Hybridization with *tdnQ* of the total RNA of *C. testosteroni* I2 and *D. acidovorans* CA28, grown in LB, LB-aniline and LB-3-CA.

DISCUSSION

Several bacterial species are known to degrade 3-CA (46, 175, 189, 284, 285, 339). Strains I2, LME1 and B8c, isolated and described in this study, are three new strains of the species *Comamonas testosteroni* and *Delftia acidovorans* that are able to metabolize aniline and 3-CA. *C. testosteroni* I2, *D. acidovorans* CA28 (189), BN3.1 (46) and LME1 show similar metabolic capacities. *D. acidovorans* B8c is not able to use 3-CA as sole carbon source and thus unable to degrade 3-CA completely. This situation leads to accumulation of 4-chlorocatechol in the medium. During the dioxygenation of 3-CA, theoretically two different intermediates may be formed, e.g., 3-chlorocatechol and 4-chlorocatechol (175). The mass spectrometry results in this study, together with data from the literature (189, 259, 340), suggest that *D. acidovorans* B8c degrades 3-CA preferably up to 4-chlorocatechol. *D. acidovorans* LME1 showed no accumulation of chlorinated catechols, probably because of the high level of activity of a chlorocatechol dioxygenase (189).

Strain I2, isolated and described in this study, is to our knowledge the first reported strain of *Comamonas testosteroni* that is able to use 3-chloroaniline as the sole source of carbon and nitrogen. This species, which is often isolated from activated sludge, is resistant to starvation (200) and has been reported to be involved in the degradation of many aromatic products, such as (chloro)phenols (16, 19), p-toluenesulfonic acid (20), polychlorinated biphenyls (23), arylsulfonates (150), 1-(2-chlorobenzoyl)-3-(4-chlorophenyl) urea (267) and chlorinated benzenes (293).

In pure culture, complete degradation was achieved at 3-CA concentration of 200 mg/L and was coupled with quantitative liberation of chloride. During the degradation of 3-CA, a yellow intermediate accumulated temporarily, which was further metabolized, thus indicating total metabolism of the aromatic amines. The chlorinated catechols seem to usually be degraded by a modified *ortho*-cleavage pathway (132, 340). Recently, the use of a functional *meta*-cleavage pathway for the mineralization of 3-chlorocatechol as central metabolite of chlorobenzene has been determined (155, 196). Riegert et al. (247) observed a yellow distal *meta*-cleavage product of 3-chlorocatechol with a strongly pH-dependent absorption maximum at 378 nm, and found that this was a chlorohydroxymuconic semialdehyde. In our study, the λ_{max} values at the different pHs were very similar. This comparison suggests that the degradation of 3-CA by *C. testosteroni* I2 also occurs by means of a distal *meta*-cleavage pathway for chlorocatechol. This is in contrast with the case for most other chloroaniline degraders, which have been shown to degrade 3-CA via modified

ortho-cleavage pathway (132, 340). Only one study, by Surovtseva et al. (287), mentioned a *meta*-cleavage of monochloroanilines by *Alcaligenes faecalis*; however, it is not clear if this cleavage was metabolic or co-metabolic. Recently Walter Reineke (personal communication) determined that our strain I2 is able to use 4-chlorocatechol. This information together with the detection of the yellow chlorohydroxymuconic semialdehyde would indicate that the strain employs a proximal or distal *meta*-cleavage pathway (Figure 2.12).

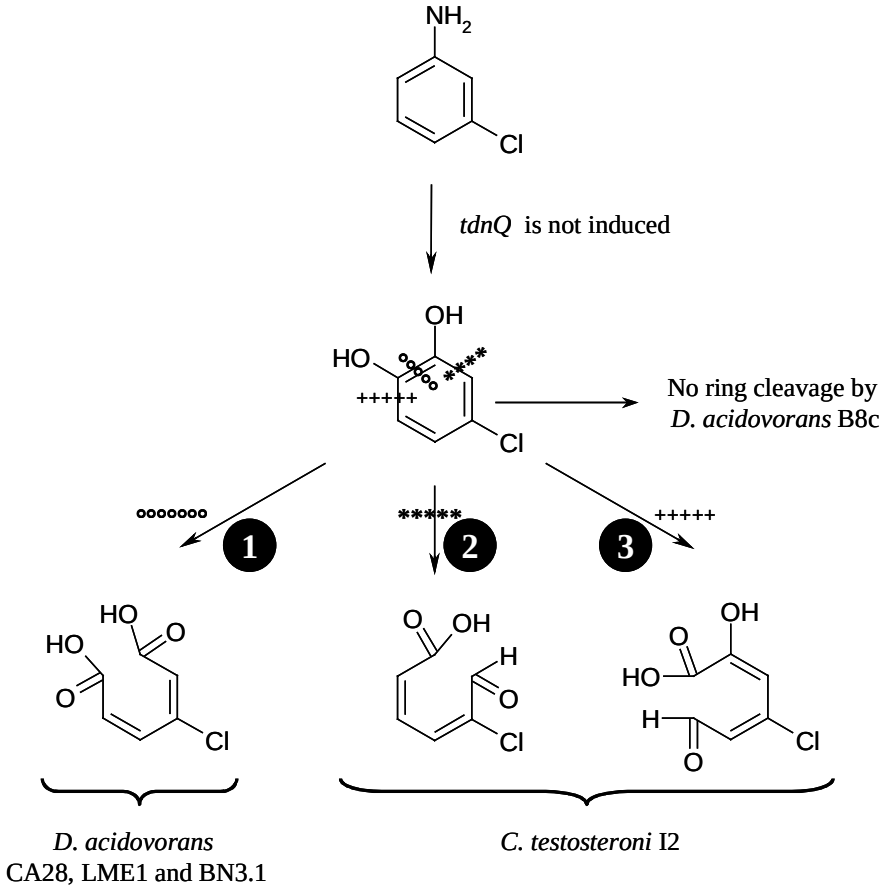

Figure 2.12. Proposed divergent pathways for 3-chloroaniline degradation through 4-chlorocatechol by the five different isolates. 1, 1,2-fission or modified *ortho*-cleavage; 2, 2,3-fission or proximal *meta*-cleavage; 3, 1,6-fission or distal *meta*-cleavage

All the strains that were investigated harbor a large plasmid. Matings between *D. acidovorans* CA28 and the recipient JMP228*gfp* resulted in transconjugants with different

phenotypes. However, no differences in plasmid restriction patterns of the different transconjugant colonies could be shown. The cause of these differences is currently not yet understood. When these transconjugants were grown in MMN-CAP, no formation of chlorocatechol was noticed. However, complete mineralization of 3-CA apparently did not occur, since 3-CA could not be used as carbon source. This result suggests that an aliphatic intermediate accumulated after ring cleavage and dechlorination. This aliphatic intermediate would likely have a six-carbon backbone because release of any carbon should support some growth. It is clear that none of the plasmids codes for complete 3-CA mineralization. To our knowledge, the plasmid pC1 of *D. acidovorans* CA28 is the first and only plasmid characterized so far that codes for partial 3-CA degradation. Plasmid pNB2, on the other hand, was the only plasmid that confers complete degradation of aniline in *R. eutropha* JMP228*gfp*, allowing the strain to use the compound as sole carbon source. This plasmid of *C. testosteroni* I2 is thus a new aniline catabolic plasmid, which also encodes mercury resistance. The observation that 3 plasmids could transfer the ability to use aniline but not 3-CA as sole nitrogen source suggests that the genes carried on these plasmids are insufficient for the oxidative deamination of 3-CA. This suggestion leads to the hypothesis that other, as-yet-unknown chromosomally located genes are required for deamination of 3-CA.

In order to confirm the involvement of the plasmids in aniline and/or 3-CA degradation, hybridization experiments were performed with a *tdnQ* probe. This particular gene was chosen as a representative of the *tdn* genes for several reasons. First, after primers were designed for *tdnQ* and *tdnA*, based on their sequences in the database, the only set that yielded amplification with the 5 strains was the set of *tdnQ* primers. An additional advantage was that the cloned *tdnQ* gene of *P. putida* UCC22 was provided to us by F. Fukumori, allowing us to make a *tdnQ* probe by PCR labeling. The other genes, such as *tdnA2* and *tdnT*, were smaller and did not allow effective primer design. Out of the five plasmids, the four that were able to transfer the ability to use aniline as a nitrogen source also hybridized with the *tdnQ* gene. Only plasmid pB1 of *D. acidovorans* BN3.1, which could not transfer the ability to transform aniline and/or 3-CA, did not yield a hybridization signal. This result suggests that either plasmid pB1 does not carry the catabolic genes, or it carries catabolic genes involved in aniline or 3-CA with lower sequence similarity to *tdnQ* , and it is not conjugative.

All plasmids in this study belong to the IncP-1 incompatibility group, and most of them could be assigned to the IncP-1β subclass by *korA* primers and probes. The incompatibility group and host range of several other catabolic plasmids are still not known. Interestingly, most plasmids involved in degradation of chlorinated aromatics and for which

the incompatibility group has been determined seem to belong to the IncP-1 group (often even IncP-1β), known to contain plasmids with a very broad host range (301). Examples are pJP4 (2,4-dichlorophenoxyacetic acid and 3-chlorobenzoic acid), pAC25 (3-chlorobenzoic acid), pBR60 (3-chlorobenzoic acid) and others (301). In our study, the only plasmid which yielded a *korA* PCR product that did not hybridize with the IncP-1α- or the IncP-1β-derived probe was plasmid pB1 from *D. acidovorans* BN3.1. This result could mean that plasmid pB1 belongs to incompatibility group other than IncP-1, with a more restricted host range. Interestingly pB1 is also the only one of the five plasmids that did not allow conjugative transfer of the 3-CA- or aniline-transforming phenotype and which did not hybridize with the *tdnQ* probe.

Results of recent studies have shown that a variety of catabolic genes and operons are flanked by insertion elements (78). IS*1071* is an insertion sequence that has been found to bracket the class II transposable element Tn*5271*, first described for the 3- and 4-chlorobenzoate-degrading strain *Alcaligenes* sp. strain BR60 (210). Fulthorpe and Wyndham (103) observed that after the introduction of this host strain in lake water and sediment microcosms exposed to 4-chloroaniline, IS*1071* was mobilized into different strains and was found in a plasmid unrelated to the donor, pBRC60. Also, in our study, insertion sequences strongly related to IS*1071* were detected on the plasmids of the aniline- and 3-CA-degrading strains, probably on the same restriction fragment as the *tdnQ* gene. This finding suggests that in our strains, *tdnQ* is flanked by an insertion sequence fragment of the group IS*1071*. Other investigators (102, 231) also identified the *tnpA* transposase sequence, which is related to that of IS*1071*, near the *tdnQ* gene. Furthermore, Fujii et al. (100) found the transposase gene sequence of Tn*1000* on the aniline catabolic plasmid pAS185 of *Acinetobacter* sp. strain YAA. These findings suggest that during bacterial evolution, the genes responsible for aniline degradation have been spread by horizontal transfer aided by transposons, such as Tn*5271*. The additional hybridization signal of a 2.7-kb fragment of plasmid pNB2 with the IS*1071* probe could be related with the plasmid-encoded mercury resistance. This observation corroborates with the findings of Pearson et al. (226), who observed that class II transposase genes are often associated with mercury resistance genes (*mer* genes).

A new approach to study the diversity of functional genes is analysis of PCR products of these genes with DGGE (122, 249). To our knowledge, this is the first study that has used DGGE to examine the diversity of a gene involved in degradation of an aromatic compound. The classification of *tdnQ*-like gene fragments in three groups, based on their rates of migration in the DGGE gel (Figure 2.10), did not correspond entirely with the degree in

sequence similarity between the cloned fragments (Table 2.3). This situation is to be expected, since fragments with different DNA sequences may sometimes end up at the same location in the DGGE gel, while in many other cases, a 1-bp difference can be sufficient to separate two sequences (93). A comparison of DGGE and sequencing data demonstrates, however, that there was sufficient variation at the DNA sequence level to separate the different *tdnQ* like genes in the DGGE gel. This DGGE approach, applied to the total DNA from various environmental habitats, could be especially useful for further investigation of the diversity of *tdnQ*-like genes and other catabolic genes in microbial communities without prior cultivation of the degrading organisms.

Interestingly, different *tdnQ* sequences were found in strains of the same species (*tdnQ-CA28* and *tdnQ-BN3.1*), while almost identical sequences were detected in 2 strains of different genera (*tdnQ-I2* and *tdnQ-LME1* or *tdnQ-BN3.1* and *tdnQ-UCC22*). These results suggest again that horizontal gene transfer has played a role in the evolution of (chloro)aniline-degrading bacteria. None of the obtained *tdnQ* nucleotide sequences was related to the sequence of the aniline dioxygenase gene (glutamine synthetase-like protein) *of Acinetobacter sp.* YAA (100), while there was a good relationship at the level of the amino acid sequence. The *tdnQ*-primers were probably too specific to detect possible genes responsible for oxidative deamination of 3-CA in the strains. Work to identify the latter genes, and their diversity within chloroaniline-degrading bacteria warrants further research.

In the present study and in previous reports (175), the relationship between the degradation of aniline and its chlorinated analogue, 3-CA has been mentioned. The enzymes responsible for *ortho*-ring cleavage of catechol and chlorocatechols are different (132). However, it is not clear if the genes and enzymes responsible for the transformation of aniline and 3-CA into chlorocatechol (oxidative deamination) are also different. Some aniline-degrading bacteria were able to transform 3-CA into chlorocatechol, but these bacteria needed aniline or glucose as a cosubstrate and the cells had to be preincubated with aniline (241, 259). On the one hand, evidence in support of the hypothesis that the oxidative deamination of aniline and its chlorinated analogue is performed by the same enzyme, was given by the work of Latorre et al. (175). The authors obtained 2-chloroaniline-, 3-CA-, and 4-chloroaniline-degrading bacteria by natural gene exchange between an aniline- or toluidine-degrading *Pseudomonas* strain and chlorocatechol assimilating *Pseudomonas* sp. B13. Hybrid organisms were isolated through cocultivation of the parent strains in a chemostat as well as through conjugation on solid media in the presence of chloroanilines as selective substrate. On the other hand, some aniline-degrading bacteria have been reported to be unable to

metabolize or cometabolize monochloroanilines (323), while all 3-CA-degrading bacteria described so far can use aniline as sole carbon source (46, 175, 189, 339). This information suggests the existence of at least two different sets of enzymes, one that can transform only aniline and another that can transform both aniline and 3-CA. In our study, *C. testosteroni* I2 and *D. acidovorans* B8c and LME1 could transfer the genes encoding the oxidative deamination of aniline, while genes encoding the oxidative deamination of 3-CA could not be transferred. These findings, together with the differential transcription of the *tdnQ*-mRNA (Figure 2.11), strongly suggest that two different sets of genes are involved in the oxidative deamination of aniline and 3-CA.

This work has shown that the catabolic plasmids and the *tdnQ* genes involved in oxidative deamination of aniline in five strains of the family *Comamonadaceae* are quite diverse. We described a new plasmid encoding complete aniline degradation and two plasmids that code for the partial oxidative deamination of aniline. Evidence is also given that the plasmid in *D. acidovorans* CA28 is the only one in the five strains that codes for partial 3-CA degradation. The importance of IncP-1 plasmids and insertion sequence elements in the spread of catabolic genes was confirmed. Increasing the understanding of new catabolic plasmids for future studies on the bioaugmentation of polluted environments is warranted.

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CHAPTER 3

3. BIOAUGMENTATION OF ACTIVATED-SLUDGE BY AN INDIGENOUS 3-CHLOROANILINE DEGRADING COMAMONAS TESTOSTERONI *STRAIN, I2*GFP [§]

ABSTRACT

A strain identified as *Comamonas testosteroni* I2 was isolated from activated-sludge and found to be able to mineralize 3-chloroaniline (3-CA). This strain was tested for its ability to clean wastewater containing 3-CA upon inoculation in activated-sludge. To monitor its survival, the strain was chromosomally marked with the *gfp* gene and designated I2*gfp*. After inoculation into a lab-scale semicontinuous activated-sludge (SCAS) system, the inoculated strain maintained itself in the sludge at least 45 days and was present in the sludge flocs. After an initial adaptation period of 6 days, complete degradation of 3-CA was obtained during two weeks, while no degradation at all occurred in the non-inoculated control reactors. Upon further operation of the SCAS system, only 50 % 3-CA removal was observed. Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rRNA genes revealed a dynamic change in the microbial community structure of the activated-sludge. The DGGE patterns of the noninoculated and the inoculated reactors evolved after 7 days to different clusters, which suggests an effect of strain inoculation on the microbial community structure. The results indicate that bioaugmentation, even with a strain originating from that ecosystem and able to effectively grow on a selective substrate, is not permanent and will probably require regular resupplementation.

[§] Redrafted after **Boon, N., J. Goris, P. De Vos, W. Verstraete and E.M. Top**. 2000. Bioaugmentation of activated-sludge by an indigenous 3-chloroaniline degrading *Comamonas testosteroni* strain, I2*gfp*. Appl. Environ. Microbiol. 66:2906-2913.

INTRODUCTION

Bioaugmentation is the accelerated removal of undesired compounds from contaminated hazardous waste sites or bioreactors by using indigenous or allochthonous wild-type or genetically modified organisms (311). These inocula usually are highly efficient in the removal of the xenobiotic targets under laboratory conditions. However, under natural conditions, these laboratory strains have to compete with the established microbial community, resulting in a decrease of the amount of inoculated cells (110). This competition can be controlled by adding a carbon source that the inoculant can degrade (31) or by changing operation parameters (101). Thus far, in water treatment only a few successful cases of small-scale bioaugmentation in activated-sludge by using natural or genetically modified micro-organisms have been described. McClure et al. (199) obtained enhanced but incomplete degradation of 3-chlorobenzoate (3CB) in a laboratory-scale activated-sludge unit after inoculation of activated-sludge-derived bacteria (200), able to mineralize 3CB. Nüßlein et al (218) inoculated a laboratory-scale activated-sludge unit with *Pseudomonas* sp. strain B13 FR1(pFRC20P) and observed a drastic decrease of 3CB and 4CB three days after inoculation, while in the noninoculated reactor respectively 8 and 15 days of adaptation, respectively, were needed. Selvaratnam et al. (262) inoculated a sequencing batch reactor (SBR) with *Pseudomonas putida* ATCC11172 to remove phenol and ca. 85% of the phenol was degraded within 2.5 h. Little is known about the effect of bioaugmentation of activated-sludge reactors on the microbial community. Eichner et al. (84) used Thermal Gradient Gel Electrophoresis (TGGE) to examine the effect of a pollutant shock in the activated-sludge microbial community and observed protection by the inoculation of a genetically modified strain able to avoid formation of toxic intermediates. The addition of specialized strains to activated sludge to enhance the removal of pollutants present in the influent is not yet widely applied, because bioaugmentation is less predictable and controllable than the direct destruction of contaminants, such as incineration.

Aromatic amines, such as chloroanilines, are widely used for the production of dyes, drugs, and herbicides (158) and as a consequence of their application are released in the environment. These compounds are also introduced via the metabolism of phenylamide pesticides (117). These toxic and recalcitrant residues are considered important environmental pollutants (202). Therefore, many efforts were made to isolate bacteria capable of degrading chlorinated anilines. *Moraxella* sp. strain G (339) is the first isolate that was found to be able to use 4-chloroaniline as sole source of carbon, nitrogen and energy. Later, more

chloroaniline-metabolizing strains were isolated, such as *Pseudomonas* sp. strain JL2 (175), *Pseudomonas* (now *Brevundimonas*) *diminuta* INMI KS-7 (284), *Pseudomonas* (now *Delftia*) *acidovorans* CA28 (189), *Pseudomonas* (now *Delftia*) *acidovorans* BN3.1 (46), *Aquaspirillum* sp. strain 2CA and *Paracoccus denitrificans* 3CA (285). The first step of the degradation of chloroanilines is the deamination to chlorocatechols. These chlorinated catechols seem to be usually degraded by a modified *ortho*-cleavage pathway (132, 340), but recently, the use of a *meta*-cleavage pathway for the mineralization of 3-chlorocatechol has been determined (155, 196). In the past, the *meta*-cleavage intermediates of chlorinated catechols, i.e. chlorohydroxymuconic semialdehyde, were described as toxic metabolites, preventing further degradation (189).

The aims of this work was to investigate eventual enhanced 3-CA degradation by the activated-sludge after inoculation with a specialized 3-CA metabolizing strain. In addition, the effect of inoculation on the microbial community structure of the sludge was examined.

MATERIAL AND METHODS

Media and culture conditions

The mineral medium MMN (mineral medium without nitrogen and carbon) is derived from MMO mineral medium (280) by eliminating all nitrogen. The MMN medium contained 1419.6 mg Na_2HPO_4, 1360.9 mg KH_2PO_4, 98.5 mg $MgSO_4$, 5.88 mg $CaCl_2.2H_2O$, 1.16 mg H_3BO_4, 2.78 mg $FeSO_4.7H_2O$, 1.15 mg $ZnSO_4.7H_2O$, 1.69 mg $MnSO_4.H_2O$, 0.38 mg $CuSO_4.5H_2O$, 0.24 mg $CoCl_2.6H_2O$, 0.10 mg MoO_3 and 3.2 mg EDTA in 1 liter of distilled water. The liquid mineral media were supplemented with 150 to 250 mg aniline (Sigma-Aldrich Chemie, Steinheim, Germany) or 3-CA (Fluka AG Chemische Fabrik, Buchs, Switzerland) per liter, while for the solidified media, aniline and 3-CA were supplemented at a concentration of 500 mg/liter. Luria Broth (LB) medium containing 10 g Bacto Peptone (Difco, Detroit, Mich., USA), 5 g Bacto yeast extract (Difco), and 5 g NaCl in 1 liter of distilled water was used as a rich medium. These media were solidified with 2 % agar for plate growth.

Cultures were incubated on a rotary shaker under aerobic conditions at 28 °C. Growth was monitored by measuring the turbidity at 600 nm. Two hundred microliters of an overnight-grown LB culture of strain I2, washed twice in saline (0.85 % NaCl), was inoculated in 200 ml of MMN medium with 3-CA (150 mg/L) (0.1 % inoculation) to monitor the transformation of 3-CA.

Marking with *gfp*

Comamonas testosteroni I2 was isolated from a municipal wastewater treatment plant and described previously (35, 36). Strain I2 can mineralize 3-CA completely and is rifampin resistant (100 µg/ml) and on LB-agar plates, the strain I2 showed a phenotypic instability, resulting in two types of colonies with different morphology. The strain *Escherichia coli* S17-1 λpir (pUT-miniTn*5* *gfp*Km) (125, 297) was obtained by transformation (54). The pUT-plasmid, was used for insertion of the *gfp* gene into the chromosome of strain I2. Biparental mating between the donor strain *E. coli* S17-1 λpir (125) and the recipient strain I2 with selection on LB plates with rifampin (100 mg/liter) and kanamycin (50 mg/liter) resulted in I2*gfp* derivatives with the *nptII* and *gfp* genes inserted in the chromosome. This was confirmed by PCR with *gfp*-primers (see below).

SCAS reactors

The experiments were conducted in duplicate with sludge freshly collected from a domestic wastewater treatment plant (Bourgoyen-Ossemeersen, Gent, Belgium). The total count of the sludge was 4.4 10^8 bacteria/ml, determined with the Live/Dead Bacterial Viability Kit (L-13152, Molecular Probes, Eugene) as described by Boulos et al (43). The reactors (2 liters total volume), with an active volume of 1.1 liters, were operated according to the semi continuous activated-sludge (SCAS) procedure at room temperature (ca. 21 °C). The tests were conducted with synthetic influent consisting of skim milk powder (Gloria, Nestlé) dissolved in tap water. The use of skimmed milk powder allowed the use of a constant wastewater that was rich in nutrients, with a chemical oxygen demand (COD)/N/P ratio equal to 100/6/1. The reactors were fed every day after wastage of excess sludge and settling (Figure 3.1).

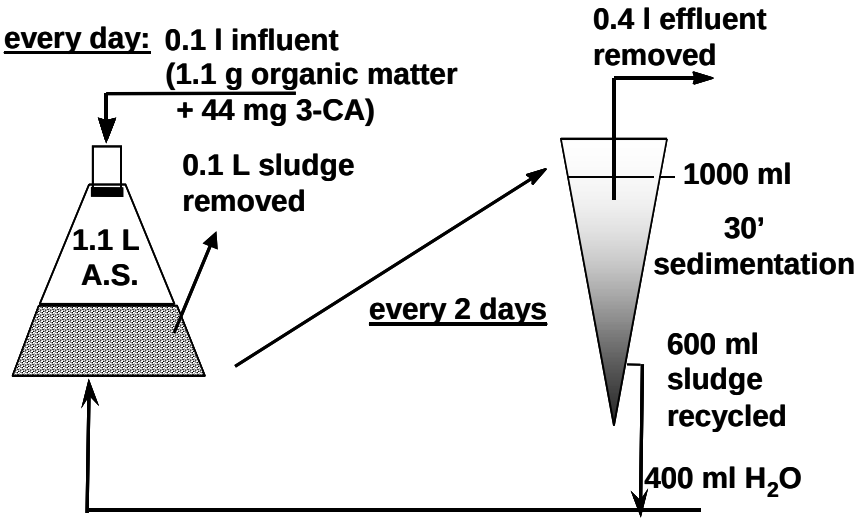

Figure 3.1. Scheme of the operation of a semi continuous activated-sludge (SCAS) system.

The SCAS reactors were operated at a volumetric loading rate of 1 g COD/liter·day, with a hydraulic retention time of 4 days and a sludge retention time of 11 days. All four reactors had a loading rate of 40 mg of 3-CA/liter·day, added as a daily single dose. One liter of the mixed liquor was subjected to a half-hour period of settling in an Imhoff cone to analyze the sludge volume (SV) (115). On days 1, 3 and 5 of each week, the settling was followed by a decantation of 400 ml of the supernatant and the addition of 500 ml of fresh influent. The

wasted sludge was used for analyze as follows: at day 1 of the week, a DNA-extraction of the sludge was performed and pH, oxygen uptake rate (OUR), and concentration of strain I2*gfp* were determined; daily, an HPLC-sample was taken and the suspended solids (SS) and sludge volume index (SVI) were measured (115). Two duplicate reactors were inoculated with *C. testosteroni* I2*gfp* (reactors A), and two duplicate reactors were used as noninoculated control (reactors B). No important differences could be observed between the two duplicate reactors. Hence, unless otherwise indicated, the data reported are averages of both duplicates. The reactors were operated without 3-CA for 12 days to allow the microbial community to adapt to the changed environment and growth conditions. After this period, reactors A were inoculated with *C. testosteroni* I2*gfp* to a final concentration of 3×10^6 cells/ml. The cells were pregrown overnight in LB medium, containing 100 mg 3-CA /L, washed twice with saline, and finally resuspended in saline.

Bacterial counts

Sludge flocs were dispersed by purging a 1-ml sample twenty times through a sterile syringe of 1 ml with a needle (1.2 by 40 mm). LB agar medium, supplemented with rifampin (100 mg/liter) and kanamycin (50 mg/liter) was used to count the I2gfp cells. By using the green fluorescent protein fluorescence, it was possible to detect 10 CFU of strain I2gfp per ml against the background of about 10^3 CFU of total kanamycin- and rifampin-resistant microorganisms per ml.

U.V.-Light microscopy

The sludge samples were analyzed by UV-light microscopy using a Reichert-Jung Polyvar microscope equipped with a Mercury short arc lamp HBO 200 W. Samples were examined with objectives 40x and 100x objectives, using very-low-fluorescence immersion oil.

Respirometric activity measurements

The metabolic activity of the activated sludge in general was expressed as OUR. Therefore, activated-sludge samples (200 ml) were transferred to the vessel and saturated with oxygen by means of bubbling air with a pump. Once oxygen saturation (ca. 8 mg O_2/L) was reached, the aeration was ceased and the oxygen electrode (Oxyguard Probe, Kelma, Niel, Belgium) was placed in such a way that the opening of the vessel was barely closed. Sodium acetate was added to a final concentration of 50 mg/liter. Samples were mixed with a

magnetic stirrer during measurements. The method was further performed as described by Surmacz-Gorska et al. *(283)*, calculating the activity from the constant slope of oxygen concentration over time. The activity measurements resulted in the OUR (grams of O_2 per liter per day).

Analytical methods

The supernatants of cultures were analyzed for 3-CA content by reversed-phase HPLC after centrifugation of the cells at 5,000 × g for 10 min. The HPLC system consisted of a Kontron liquid chromatograph with a DEGASYS DG-1310 system to degas the mobile phase, three Kontron 325 high-pressure pumps, a Kontron MSI 660 injector with a 20-µl loop, a Kontron DAD 495 diode array detector, and a 450 MT2/DAD software system. An Alltima C_{18} reversed-phase column (250 mm × 8 mm [inner diameter], 5-µm particle size; Alltech, Deerfield, Ill., USA) was used. The mobile phase consisted of $CH_3OH/NH_4H_2PO_4$ (0.1 M, pH 3.8)-H_2O (70:25:5), with a flow rate of 0.75 ml/min. The UV-detector was used at 210 nm. Quantitative data for 3-CA were obtained by comparing the peak areas of unknown concentrations with the peak areas of standards of known concentrations.

DNA extraction and purification

Total DNA was extracted from the sludge samples by a method based on the protocols described previously (86, 87). This protocol was modified as follows. Two milliliters of sludge was added to a 14-ml polypropylene round bottom tube (Falcon). To this 3 g of beads (0.10-0.11 mm; B. Braun Biotech International, Melsungen, Germany) and 4 ml of 10 mM Tris/HCl (pH 9) were added. The mixture was beaten three times for 90 s using a bead beater (B. Braun Biotech International) at 2000 rpm. Then, 2 ml of 4 mg of lysozyme per ml in 10 mM Tris-/HCl (pH 9) were added, followed by incubation of the samples for 15 min at 28°C on a rotary shaker. Subsequently, 300 µl of 20% SDS was added and samples were slowly mixed for 5 to 10 min. After this, 1 ml of 8 M ammonium acetate was added. The supernatant was collected after centrifugation at 7,000 × g for 15 min at 4°C. A chloroform-isoamyl alcohol (24:1) purification was done, followed by a centrifugation at 7,000 × g for 15 min at 4°C. The aqueous phase was transferred to a new tube, and 0.8 times volume of isopropanol was added. The precipitation was performed for 1 h at -20°C. Alternatively, 2.5 volumes of ethanol (100 %) were added for an overnight precipitation. The pellet (crude extract) was obtained by centrifugation at 12,000 × g for 25 min and was re-suspended in 250

µl of distilled water. A 100 µl aliquot of the crude extract was further purified using Wizard PCR preps (Promega, Madison, Wis., USA), and the purified DNA was finally recovered in 50 µl of sterile distilled water.

For axenic cultures, the template for PCR-amplification was obtained by suspending a colony in 200 µl sterile distilled water, boiling for 15 min and storing at –20 °C. Two microliters of the lysed cells was used in the PCR reaction.

PCR conditions

Two microliters of the extracted DNA was amplified by PCR with a 9600 thermal cycler (Perkin-Elmer, Norwalk, CT, USA). The PCR mixture used contained 0.5 µM each primer, 200 µM each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 10 µl of Thermophilic DNA Polymerase 10X Reaction Buffer ($MgCl_2$-free), 2.5 U of *Taq* DNA Polymerase (Promega), 400 ng of bovine serum albumin (Boehringer) per µl, and Dnase- and Rnase-free filter-sterilized water (Sigma-Aldrich Chemie, Steinheim, Germany) to a final volume of 100 µl.

The *gfp*-gene was amplified by PCR with a set of primers, based on specific regions of the *gfp*-sequence (Genbank accession number M62653). The set consisted of the primer gfpF (5'-CCA-TGG-CCA-ACA-CTT-GTC-AC-3' [forward]) and gfpR (5'-CTT-TCG-AAA-GGG-CAG-ATT-GT-3' [reverse]).

The 16S rRNA genes from sludge microbial communities were amplified by PCR as suggested by El Fantroussi et al. (87), using the forward primer P63f (5'-CAG-GCC-TAA-CAC-ATG-CAA-GTC-3'-forward) and the reverse primer P518r (5'-ATT-ACC-GCG-GCT-GCT-GG-3'-reverse) (208, 221). A GC-clamp of 40 bp (208, 221) was added to the forward primer. The length of the expected amplified fragment with GC-clamp was 530 bp.

DGGE

Denaturing Gradient Gel Electrophoresis (DGGE) based on the protocol of Muyzer et al. (208) was performed using the D Gene System (Bio-Rad, Hercules, CA, USA). PCR samples were loaded onto 6% (wt/vol) polyacrylamide gels in 1xTAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4). The polyacrylamide gels were made with denaturing gradient ranging from 40 to 60 % (where 100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run for 16 hours at 60°C and 50 V. After the electrophoresis, the gels

were soaked for 5 min in fixation buffer (10% ethanol, 0.5 % acetic acid), and subsequently for 10 min in SYBR GreenI nucleic acid gel stain (1:10,000 dilution; FMC BioProducts, Rockland, Maine, USA). The stained gel was immediately photographed on an UV transillumination table with a video camera module (Vilbert Lourmat, Marne-la vallé, France).

Analysis of DGGE patterns

The statistical comparison of the DGGE patterns on the same gel was done with the GelCompar software 4.1 (Applied Maths, Kortrijk, Belgium). The calculation of the matrix of similarities is based on the Pearson product-moment correlation coefficient. The clustering algorithm of Ward (326) was used to calculate dendrograms.

RESULTS

Survival and activity of *C. testosteroni* I2*gfp* in the SCAS-reactors

In order to monitor the survival of strain I2 in activated sludge, it was chromosomally marked with the *gfp* gene, which was expressed constitutively by a P*psbA*-promotor (297). The insertion of the *gfp* gene in several transconjugants was confirmed by PCR with the *gfp*-specific primers gfpF and gfpR. The *gfp*-marked strain I2*gfp* showed the same degradation characteristics as the original strain in MMN medium (data not shown). Before strain I2*gfp* was inoculated into the sludge reactors (reactors A) and before 3-CA was added, the sludge was adapted for 12 days to the operating SCAS system. At this point (day 0 in Figure 3.2), the daily supplementation with 3-CA started.

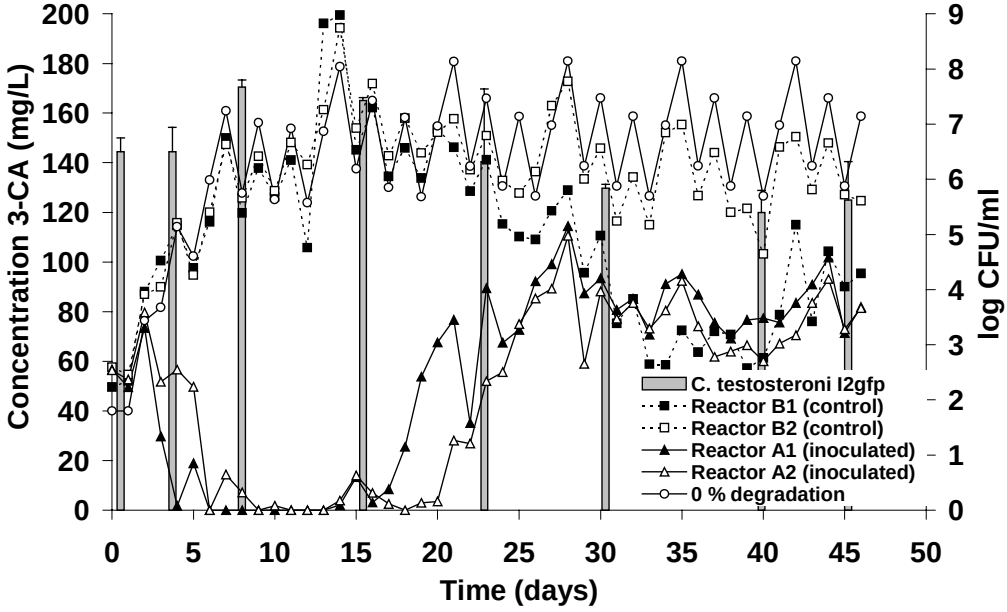

Figure 3.2. Concentration of 3-CA in the inoculated reactors A1 (▲) and A2 (△) and the control reactors B1 (■) and B2 (□), together with a simulation of the 3-CA concentration if no degradation occurred (0 % degradation) (○), and survival *of C. testosteroni* I2*gfp* in reactors A1 and A2 (bars).

During the first three days, no degradation was observed in any of the reactors. In the inoculated reactors A, enhanced degradation was observed from day 4 until day 7, and during the next 12 days complete degradation of 3-CA was achieved. After three weeks, however,

the concentration of 3-CA increased and stabilized at a level corresponding with ca. 50 % degradation. A mass balance calculation of 3-CA in a reactor (with 0 % degradation) showed that the concentration of 3-CA fluctuated weekly, based on the daily addition and the washout. No significant removal of 3-CA occurred in the control reactors B during the first 30 days. However, from day 30, enhanced degradation was observed in reactor B1, comparable to that in the inoculated reactors. The other control duplicate, B2, did not show any enhanced degradation.

The survival and behavior of *C. testosteroni* I2*gfp* were monitored by different methods. The most sensitive way was by plating on LB medium with kanamycin and rifampin (detection limit, 10 CFU/ml). In case of background growth by indigenous bacteria, the inoculated strains could be recognized by green fluorescence protein autofluorescence under a long-wave-length UV lamp. During the first week, the concentration of I2*gfp* cells did not differ much from the initial value, and during week 2 and 3, the concentration of strain I2*gfp* even increased (Figure 3.2). After 30 days, the inoculum size stabilized at ca. 5×10^5 CFU/ml. PCR with *gfp* primers was tested as an alternative technique to monitor the survival in sludge. The detection limit to obtain an amplification signal was ca. 5×10^5 CFU/ml, however, which was higher than for the plating method. In the control reactors no amplification was observed. The distribution in the cell flocs was studied by epifluorescence microscopy. After a few days of inoculation, *C. testosteroni* I2*gfp* cells were visible as green cells under UV-light, although the sludge microbial community had background fluorescence. The inoculated bacteria were not randomly distributed but were observed as clusters within the sludge flocs (Figure 3.3).

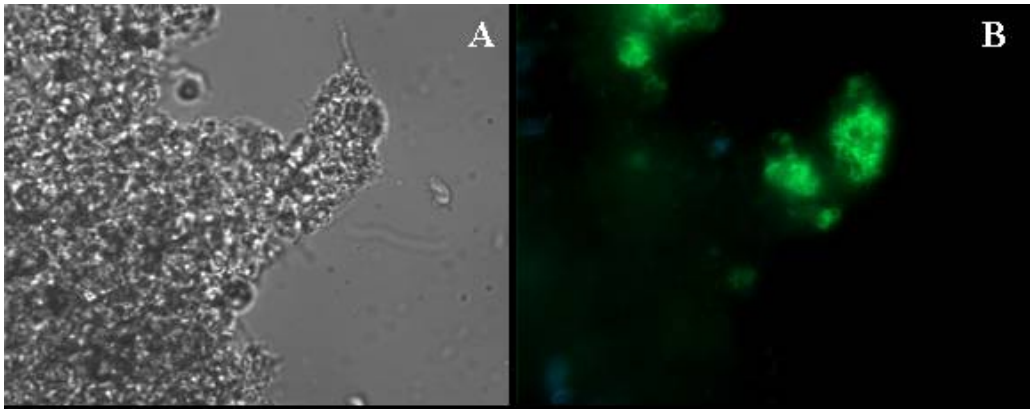

Figure 3.3. Light- (A) and epifluorescence (B) microscopic images of activated-sludge, inoculated with *C. testosteroni* I2*gfp*.

Reactor performance characteristics

At the beginning of the 3-CA supplementation, all reactors showed the same performance parameters (OUR= 74 mg O_2/L.h; SS = 4.2 g/L; SV =250 ml/L; SVI = 60 ml/L; pH = 7.6). The OUR and pH of both reactor sets did not differ much from each other during the experiment (Figure 3.4). The OUR variability of different days of one week was rather high, but the values were in the same range when the same day of different weeks were compared.

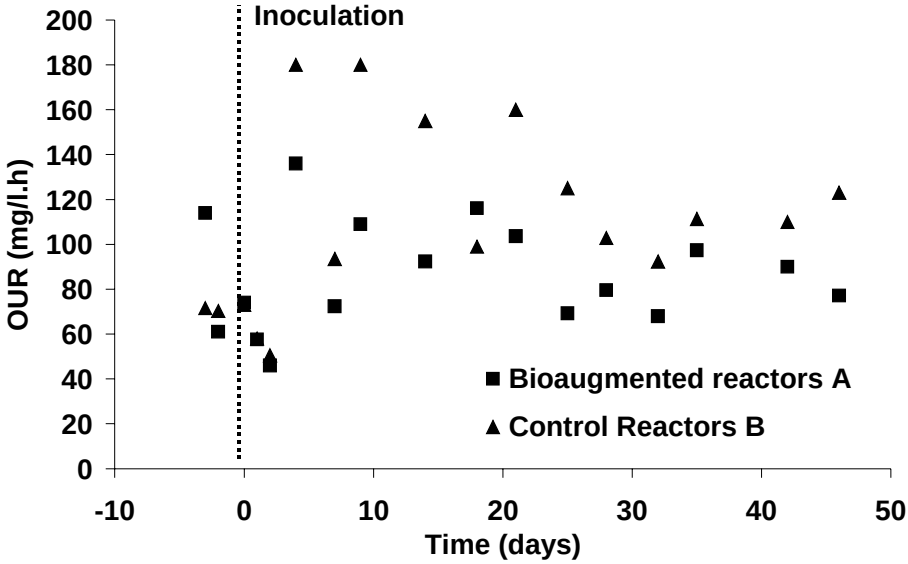

Figure 3.4. Oxygen uptake rates (OUR) of the inoculated reactors A and the control reactors B; the dotted line represents the start of 3-CA supplementation time of inoculation.

After a week the concentration of suspended solids (SS) of the control reactors B decreased about 0.6 g/liter in comparison to that in reactors A. The sludge volume (SV) after 30 minutes of both reactors was significantly different after ca. one week (two-tailed t-test; α = 0.05). During the first week, the SV of reactors A increased from 300 ml to 500 ml, and remained 500 ml for the rest of the experiment. The SV of the control reactors B was stable at 300 ml during the first 40 days and it increased only during the last week. This resulted in a significant difference between the SVI-values of both reactors for the period before 40 days (two-tailed t-test; α = 0.05) (Figure 3.5).

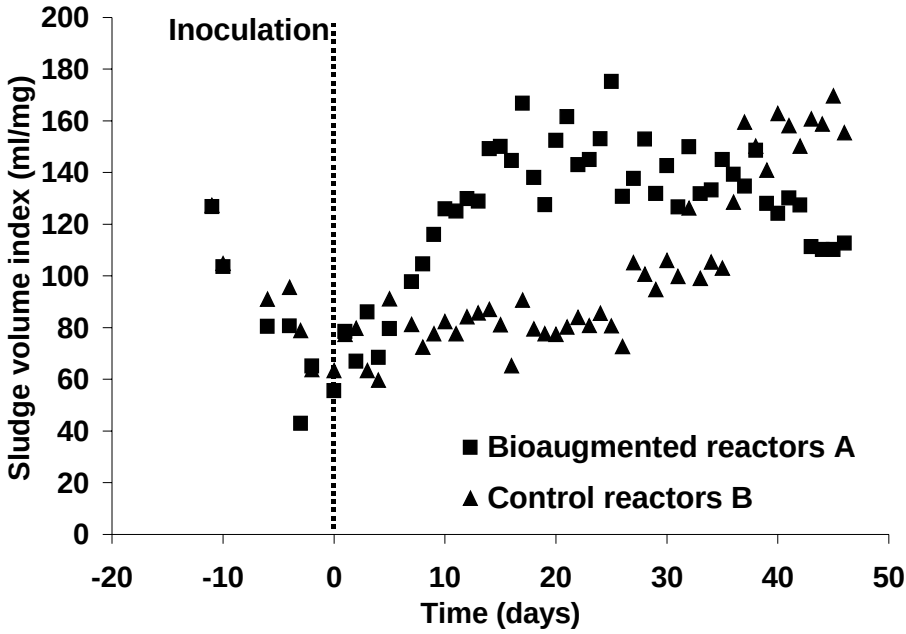

Figure 3.5. Sludge volume index (SVI) of the inoculated reactors A and the control reactors B; the dotted line represents the start of 3-CA supplementation time of inoculation.

DGGE

In order to monitor the changes within the microbial community of the SCAS reactors, the diversity of a 16S rRNA fragment was examined. Each week, a sample was taken from the four reactors and, after DNA extraction and purification, the PCR-amplified product was analyzed on a DGGE-gel (Figure 3.6A and B). The patterns of the four reactors were compared with each other after normalization. To determine the information content of the banding patterns in terms of structural diversity, they were analyzed by clustering (Figure 3.6C). The cluster analysis revealed three major groups. The fingerprints showed several very strong bands, some bands of lower intensity, and an additional number of weak bands, resulting in a smear. Before the adaptation period started (day -12) and at the day of the inoculation and feeding with 3-CA (day 0), the DGGE patterns of both reactors of both times clustered together. After the first week (day 7), each reactor series began to develop a different microbial community, which was clearly separated from that of the other reactor series and from the earlier days of the experiment. On some bands, corresponding with a

bacterial species, the applied treatment and the inoculation had no influence. Some bands became dominant in the reactors, while other bands vanished. The intensity of some fragments seems to be enhanced by the presence of strain I2*gfp*, while other species were disfavored by the inoculation.

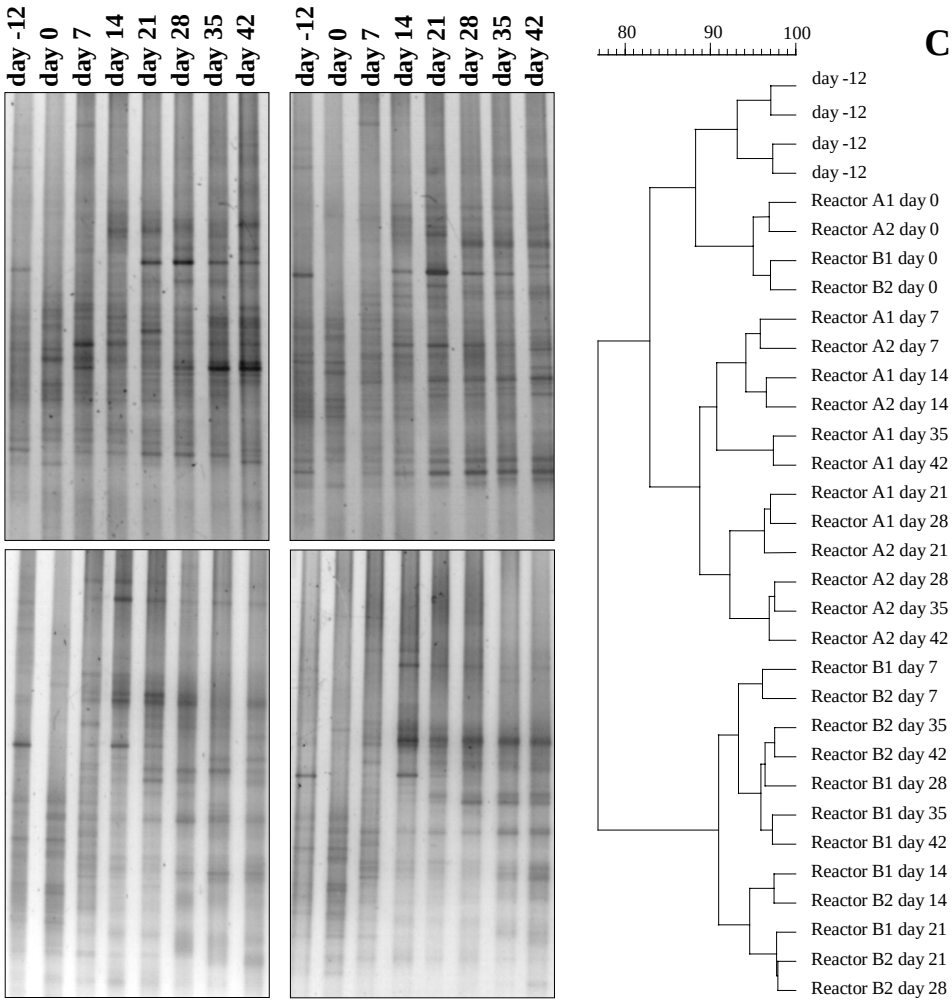

Figure 3.6. Analysis of the DGGE profiles of the different reactors at different times; using partial 16S rRNA gene fragments. (A) DGGE gel of the inoculated reactors A1 and A2; (B) DGGE gel of the control reactors B1 and B2; (C) dendrogram of all reactors, clustered by use of the Ward method (326).

DISCUSSION

In this paper, the 3-CA degrading indigenous activated-sludge bacterium *C. testosteroni* I2 was used to accelerate the removal of 3-CA from wastewater by reinoculating the strain at high density into the same sludge system. The initial sludge microbial community was not able to effectively degrade 3-CA during the first weeks of the experiment, although strain I2 was probably present, since it was isolated from the same activated-sludge plant. Apparently the natural level of the indigenous strain *C. testosteroni* I2 was too low to affect the degradation of 3-CA. To distinguish the inoculated strain from identical or similar indigenous sludge bacteria, the strain was chromosomally marked with the *gfp* gene. The plating method, combined with the *gfp* visualization with UV light, allowed sensitive and reliable monitoring of the survival of the inoculated strain and was preferred over PCR amplification of the *gfp*-gene, which was not as reliable and sensitive. Similar difficulties with PCR-based strain detection were described by Tchelet et al (293), who used specific primers for the 16S rRNA gene and chlorobenzene degradation (*tcb*) genes to monitor an inoculated *Pseudomonas* strain in activated sludge. Their study and ours thus show that plating can still be a reliable method when the strain has natural or inserted (*gfp*-Km) specific phenotypes. It is not known, however, if this culturable fraction (CFU of I2*gfp*) resembles the total viable count of I2*gfp* in the sludge.

Successful bioaugmentation mainly depends on the behavior of the inoculated strain in the environment where it is introduced. Therefore, a first criterion is good survival and retention of the strain in the system. The growth rate of the organism may be slower than washout (328) and the rate of predation, for example, by protozoa (110). In our experiment, an equilibrium seemed to be reached between washout, predation, and growth rate after three weeks with an inoculum cell densities of ca. 5×10^5 CFU of strain I2*gfp* per ml. The origin and the type of inoculated strain also can play an important role in the survival of the strain. Tchelet et al. (293) used *Pseudomonas* sp. P51, originally isolated from sediments, for a bioaugmentation experiment in a soil column and sewage sludge. The survival and activity of strain P51 in the soil column were successful, but the strain was not able to maintain itself in the sludge reactors and thus no degradation was observed. McClure et al. (199) showed that a sludge isolate, AS2, was able to reach a stable level after inoculation, in contrast to other inocula tested. Strain AS2 had a characteristic flocculation, which may have been an important factor in the survival. *C. testosteroni* I2*gfp*, used in this study, also maintained a stable population in the activated-sludge system. The original strain I2, also isolated from a

sludge environment, tends to form clusters within the sludge flocs. This observation, together with the unstable colony morphology on agar plates, suggests the formation of exopolysaccharide production, which was described by Bossier and Verstraete (40). The authors describe *C. testosteroni* A20, which expresses a phenotypic shift between mucoid-colony-forming (MCF) cells and non-MCF cells under different conditions. When the strains were cultured under unfavorable conditions, the cells shifted to the hydrophobic non-MCF-form and dense flocs were formed, providing cellular protection. Under favorable conditions, MCF cells were formed and resulted in loose associations. The possibility that strain I2*gfp* changes phenotype under unfavorable conditions may be an important factor in the maintenance of a stable population in the SCAS-reactor.

The second criterion for successful bioaugmentation is the activity of the inoculum. In our experiment, after an initial adaptation period of 6 days, complete degradation of 3-CA was obtained during two weeks, while no degradation at all occurred in the noninoculated control reactor. Upon further operation of the SCAS system, 50 % 3-CA removal was observed. The lower 3-CA removal of the inoculated reactors can be due to the declining population density of *C. testosteroni* I2*gfp* or due to a decreased metabolic activity of the I2*gfp* cells. Although McClure et al. (199) could establish a stable population of the introduced strain in the sludge environment, no enhanced degradation of chlorobenzoate was observed. Different authors proposed that the inability of the inoculated strains to degrade the xenobiotics may have been due to the availability of alternative substrates (31, 110, 199, 200, 256, 290). However *C. testosteroni* I2*gfp* received daily only 40 mg 3-CA/liter together with 1 g of COD per liter (diluted milk powder) and performed its specific activity within 2 weeks. Two preliminary SCAS experiments with the same strain I2*gfp* showed a very similar positive effect on 3-CA degradation during at least two weeks (data not shown). This observation was corroborated by the findings that when the pure culture was grown in LB medium supplemented with 100 mg 3-CA/liter, 3-CA was not detectable after one day (data not shown). The degradation of 3-CA by strain I2*gfp* therefore is not repressed by additional nutrients. Compared with the calculated 0 % degradation curve, no degradation of 3-CA was observed in either noninoculated control reactor B within 4 weeks. However, during the fifth week, enhanced degradation was observed in one of the reactors, probably due to the enrichment of indigenous bacteria with degradative capacities. It has been reported that in some cases indigenous bacteria become capable of removing xenobiotics after a long exposure time, either by metabolism or co-metabolism (200, 218, 309, 330). However, in our parallel SCAS reactors, the differences in degradation rates between the inoculated and

control reactor were striking and stable over a prolonged time period, suggesting that the bioaugmentation was effective and not ephemeral.

The microbial community structure of the SCAS-reactors was monitored by DGGE of 16S rRNA genes. The changes of the patterns over time suggest that the structure of the microbial communities was not static but rather dynamic. After 7 days, the microbial communities in both series of reactors evolved into separate clusters. The inoculated strain could not be seen in the DGGE patterns, most probably because its proportion of the total bacterial cell count was too small and a DGGE pattern only reveals the numerically dominant populations. Eichner et al. (84) investigated the bioprotection of activated sludge from pollutant shocks by the related method TGGE (Temperature Gradient Gel Electrophoresis). Those authors observed subtle shifts in community structure during adaptation to laboratory conditions. In their tests, the microbiota of the non-inoculated control reactor collapsed after the shock load of xenobiotics, resulting in both a lower OUR and a decrease in bands in the TGGE-pattern. In our studies, the diversity of bands in the pattern of the control reactor did not visibly decrease, and no drastic changes in the reactor performance were observed during the experiment. This was confirmed by the OUR- measurements, where only small differences could be observed with the inoculated reactor. In contrast to shock load, applied by Eichner et al. (84), the continuous supplementation of low concentrations of 3-CA in our experiment gave the sludge time to adapt. Remarkably, the DGGE-technique combined with clustering analysis revealed subtle responses to the inoculation of strain I2*gfp*. Indeed some species seemed to be enriched after the inoculation, while others tended to be less abundant.

This work indicates that bioaugmentation of activated-sludge systems for specific trace organics, such as 3-CA, can be achieved successfully. Moreover, this work corroborates what is often experienced in the use of activated sludge systems, i.e., that inoculation with a specific strain generally has only a transient effect. The fact that even an indigenous strain is only temporarily effective in activated-sludge communities substantiates the experience that biological supplements for such systems have to be added on a regular basis in order to assure continuous treatment efficacy. Further research will be performed to try to prolong the period of efficient degradation by the inoculum.

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CHAPTER 4

4. BIOAUGMENTING BIOREACTORS FOR THE CONTINUOUS REMOVAL OF 3-CHLOROANILINE BY A SLOW RELEASE APPROACH[**]

ABSTRACT

The survival of inoculated catabolic strains in bioreactors is critical to obtain successful biodegradation. Achieving this in industrial wastewater reactors is technically challenging. We evaluated a strategy to obtain complete and stable bioaugmentation of activated sludge, used to treat a 3-chloroaniline (3-CA) contaminated wastewater in a lab-scale Semi-Continuous Activated Sludge (SCAS) system. A 3-CA metabolizing bacterium, *Comamonas testosteroni* strain I2, was mixed with molten agar and encapsulated in 4 mm diameter open ended silicon tubes of 3 cm long. The tubes containing the immobilized bacteria represented about 1 % of the volume of the mixed liquor. The bioaugmentation activity of a reactor containing the immobilized cells was compared with a reactor with suspended I2*gfp* cells. From day 25-30 after inoculation, the reactor with only suspended cells failed to completely degrade 3-CA, due to a decrease in cell concentration and metabolic activity. In the reactors with immobilized cells however, 3-CA continued to be removed. A mass balance indicated that ca. 10 % of the degradation activity was due to the immobilized cells. Slow release of the growing embedded cells from the agar into the activated sludge medium resulted in a higher number of active 3-CA degrading I2 cells and was responsible for ca. 90 % of the degradation. Our results demonstrate that this simple immobilization procedure was effective to maintain a 3-CA degrading population within the activated sludge community.

[**] Redrafted after **Boon, N., L. De Gelder, H. Lievens, S.D. Siciliano, E.M. Top and W. Verstraete.** 2002. Bioaugmenting bioreactors for the continuous removal of chloroaniline by a slow release approch. Environ. Sci. Technol. Submitted.

INTRODUCTION

Xenobiotics transiently present in sewage persist for extended periods of time and disrupt reactor function. Because of slow microbial adaptation and growth, there is seldom sufficient metabolic capacity to protect reactors from these toxicants that only rarely appear. The removal of these chemicals is often enhanced by inoculating specialized xenobiotic degrading bacteria (311). These laboratory-cultured inocula, which can transform the xenobiotica very efficiently in pure cultures, are however usually of little effectiveness once they are inoculated into an established microbial community (110). It is hypothesized that bioaugmentation failures arise because, (i) the contaminant concentration is too low to support microbial metabolism; (ii) microbial inhibitors are present in the environment; (iii) the growth rate of the degrading organism may be slower than the rate of cell removal, for example by predation or wash-out; (iv) the inoculum may use substrates other than the pollutant whose destruction is desired, or finally; (v) the organism may physically fail to reach the pollutant (110).

As a result of these practical problems, few successful cases of lab-scale activated sludge bioaugmentation have been described (35, 199, 218, 263, 327, 338), and most of these report only partial success. These studies are limited to the inoculation of suspended cells into the mixed liquor. After the introduction, cell densities decline (35, 200, 327, 338) and there is relatively minor influence on reactor functions (200, 328). To increase the success rate of bioaugmentation strategies, investigators use carrier materials such as alginate, agarose, polyurethane (282), Lentikats (81) or hollow fibers (188) to provide a temporary protective environment to the inoculum (312). However, there is no clear understanding of the mechanism by which these encapsulation processes protect bacteria and there is no information available on how these protective measures influence the kinetics of biodegradation processes in activated sludge.

3-chloroaniline (3-CA) is a chemical used in industry during the production of polyurethanes, rubber, azo-dyes, drugs, photographic chemicals, varnishes and pesticides (106, 157). Chloroanilines are often transiently detected in wastewaters (172, 252) at concentrations ranging from 12 mg/L (172) to 230 mg/L (147), and they can cause reactor failure (38). Recently, we reported successful degradation of 3-chloroaniline (3-CA) in activated sludge by inoculating the strain *Comamonas testosteroni* I2*gfp* (35). During 14 days, the 3-CA was completely degraded, after which the degradation rate declined. This resulted in accumulation of 3-CA in the reactor and only 50 % 3-CA removal was achieved after these

two weeks. The decreased 3-CA removal rate correlated with declining *C. testosteroni* I2*gfp* concentrations in the reactor. Regular re-inoculation of the strain seemed to be the logical solution to prevent this problem. However, repeated inoculations can themselves disrupt reactor function and thus, can cause the very problem the inoculums are designed to prevent (41). Faced with the same difficulties encountered by other investigators, we evaluated a novel encapsulation method, i.e. silicone tubes (5.4 mm outer diameter, 4.0 mm inner diameter, 3 cm in length) as a means to protect the degradative activity. These tubes were open at their ends, so that release of cells from inside the tubes would occur. We estimated the 3-CA removal rate to elucidate how encapsulation alters biodegradation kinetics in Semi Continuous Activated Sludge (SCAS) reactors. To differentiate between encapsulated and suspended bacterial cells in the sludge liquor, we used two marker genes that encode two different fluorescent proteins, wild-type GFP and DsRed (RFP) (152, 296). This enabled us to count the inoculants as fluorescent colonies after plating but also allowed *in situ* monitoring of the inoculants by epifluorescence microscopy.

MATERIAL AND METHODS

Chemicals

3-chloroaniline (3-CA) is obtained by Fluka AG (Chemische Fabrik, Buchs, Switzerland; 99% pure). The octanol water partition coefficient is 1.89 (log $K_{O/W}$) and the Henry coefficient is 1.5×10^{-6} atm.m³/mol (306). The antibiotics rifampin and kanamycine were obtained from Ducheva (Haarlem, The Netherlands).

Bacterial strains

The chromosomally *gfp*-marked strain *Comamonas testosteroni* I2*gfp* originates from strain *C. testosteroni* I2, isolated from a municipal wastewater treatment plant (35, 36). Strain I2*gfp* mineralizes 3-CA completely and is rifampin resistant (100 µg/ml). Due to the marking with pUT-miniTn*5 gfp*Km by means of the pUTgfp delivery vector (70), the strain is also kanamycin resistant (50 µg/ml) and fluoresces green (excitation at 396/476 nm, emission at 508 nm) under UV-light (TLD 18W/08, Philips, The Netherlands).

The genes for *dsRed* fluorochrome (Red Fluorescent Protein or RFP) and kanamycin resistance were inserted into the chromosome of *Comamonas testosteroni* I2 by using the *rrnB*P1-RBSII-*dsRed*-T0-T1 cassette, which was located on a mini-Tn*5* cassette and inserted in the chromosed by means of the pUT delivery plasmid (296). The delivery plasmid was mobilized from *E. coli* CC118 pir to the recipients using the helper strain *E. coli* HB101(RK600) as previously described (11). The obtained RFP marked I2*rfp* cells were selected on LB plates supplemented with kanamycin (50 µg/ml) and rifampin (100 µg/ml). RFP fluorescence (excitation at 558 nm, emission at 583 nm) was visualized by a Dark Reader illuminator (Clare Chemical Research, Denver, USA) and by epifluorescence microscopy.

Strain I2, I2*gfp* and I2*rfp* are deposited in the BCCM^{TM}/LMG - Bacteria Collection (Ghent, Belgium) under the numbers LMG 19554, LMG 21409 and LMG 21402, respectively.

Semi-Continuous Activated Sludge (SCAS) reactors

The experiments were conducted in duplicate with sludge freshly collected from a domestic wastewater treatment plant (Bourgoyen-Ossemeersen, Ghent, Belgium), according to a slightly modified SCAS (Semi-Continuous Activated Sludge) procedure as described previously (35). In brief, the reactors (2 L plastic Erlenmeyer flasks) had an active volume of

1.2 L. The tests were conducted with synthetic influent (skimmed milk powder (Gloria, Nestlé); COD/N/P ratio equal to 100/6/1) at room temperature (ca. 21 °C). The reactors were fed every other day after wastage of excess sludge and settling. The SCAS reactors were operated at a volumetric loading rate of 1 g COD/L.d, with a hydraulic retention time (HRT) of 4 days, a sludge retention time (SRT) of 12 days and 4 g/L suspended solids (SS). The volatile suspended solids (VSS) consisted out of 77 % of the SS. All three reactors received a loading rate of 37 mg 3-CA/L.d, added as a single dose every other day. Every two days, one liter of the mixed liquor was subjected to a half-hour period of settling in an Imhoff cone to analyze the sludge volume (SV) (115). The other 200 ml of mixed liquor were used for analysis and discharged. The settling was followed by a decantation of 400 ml of the supernatant, and the addition of 600 ml of fresh influent. The wasted sludge was used for analyses. Unless otherwise indicated, the data reported are averages of both duplicates. During the start-up, the reactors were operated without 3-CA for at least 8 days to allow the microbial community to adapt to the changed environment and growth conditions.

Bacterial counts

At different time intervals the densities of strain I2*gfp* or I2*rfp* were determined. Sludge flocs were dispersed by purging a 1 ml sample 20 times through a sterile 1 ml syringe with a sterile needle (1.2 × 40 mm). LB agar medium, supplemented with rifampin (100 mg/L) and kanamycin (50 mg/L) was used to count I2*gfp*.

Cell dry weight (CDW) of *C. testosteroni* was determined as follows (n = 2). Glass tubes were dried overnight at 105 °C and the weight was measured. An overnight grown culture of 100 ml in LB broth was concentrated 10 times by centrifugation (5000 × g for 15 min.), resuspended in saline and plated to determined the amount of CFU/ml. The remaining suspension was transferred to the glass tubes and was dried for 24 hours at 105 °C. After cooling, the glass tubes were weighed again. After correction for the saline in the medium, the cell dry weight (CDW) of the I2 cells was determine to be 1.14 g CDW/10^{12} cells.

Inoculum preparation

The inocula were grown overnight at 28 °C in 5 ml LB medium (1L contains 5 g NaCl, 10 g trypton and 5 g yeast extract) containing 100 mg 3-CA/L. Subsequently, these 5 ml cultures were used to inoculate 500 ml LB medium + 3-CA (100 mg/L). After shaking the cultures overnight at 28 °C, they were washed twice with saline (0.85% NaCl) and

resuspended finally in 50 ml of saline.

The I2*gfp* or I2*rfp* containing silicon tubes (product code 990.0040.007, bore size 4 mm, wall size 0.7 mm, Wadson-Marlow, Cornwall, England) were prepared as follows: an overnight grown culture of I2*gfp* or I2*rfp* cells was centrifuged and resuspended in saline and mixed with liquid LB agar (for one liter: 5 g NaCl, 10 g peptone, 5 g yeast extract, 40 g agar) at 40°C (ratio 1:1). Subsequently, while the mixture was still fluid, it was injected into the sterile silicone tubes. When the mixture was solidified, the tubes were cut into pieces of 3 cm long and placed in the reactors (100 cm total length, 12.7 g or 12.5 ml agar per reactor); the silicone pieces remained open at both ends.

First experimental run

In the first experimental run, the use of a carrier system, i.e. agar in silicone tubes for enhanced 3-CA degradation was assessed for a long time period. One reactor was used as non-inoculated control (A1). Duplicate reactors (n=2) were inoculated with either *C. testosteroni* I2*gfp* in suspension (B1), or with immobilized as well as suspended *C. testosteroni* I2*gfp* (C1). The final concentration of *C. testosteroni* I2*gfp* was 9.0×10^7 and 1.2×10^7 cells/ml mixed liquor in reactors B1 and C1 respectively. The final concentration of I2*gfp* cells in the agar mixture was ca. 5.0×10^8 CFU/ml agar mixture corresponding with 6.3×10^7 CFU/cm tube. The absolute total number of cells added via the silicon tubes was 5.2×10^6 CFU/ml mixed liquor.

Second experimental run

In the second experimental run, we used an RFP marked variant of I2 as immobilized cells (I2*rfp*) to differentiate them from the cells that were inoculated as suspended cells (I2*gfp*). Additionally, an experiment was set up to evaluate the bioaugmentation potential of immobilized I2*rfp* cells alone. Duplicate reactors (n=2) were inoculated with either *C. testosteroni* I2*gfp* in suspension (B2), with *C. testosteroni* I2*gfp* in suspension as well as immobilized in silicone tubes (C2), or with *C. testosteroni* I2*rfp* immobilized in silicone tubes (D2). Reactors B2 and C2 were inoculated with *C. testosteroni* I2*gfp* to a final concentration of 4.8×10^8 and 1.4×10^8 cells/ml respectively. The final concentration of I2*rfp* cells in the agar mixture in reactors C2 and D2 was ca. 1.3×10^9 CFU/ml agar mixture corresponding to 1.6×10^8 CFU/cm silicone tube. The absolute total number of I2*rfp* cells added via the silicon tubes was 1.4×10^7 CFU/ml mixed liquor or 18.5 mg CDW_{I2rfp}/L mixed liquor.

The rate of I2*rfp* cell release was determined every six days as follows: one tube was transferred from the SCAS reactor, to 100 ml physiological solution (0.85% NaCl) for 1 hour (shaking at 140 rpm). Subsequently, the amount of I2*rfp* cells was determined by plating on LB agar supplemented with 50 µg/ml kanamycin and 100 µg/ml rifampin. Theoretically, it is possible that the 3-CA absorbed on this tube was released in this side-test. Thus the tube when returned to the reactor was calculated to potentially have released 0.1 mg 3-CA (see further).

Absorption of 3-CA through silicone-tubes

For the absorption test, 60 ml of sterile H_2O, containing 200 mg/L 3-CA, was incubated with different amounts of autoclaved silicone tubes filled with agar suspension (the inoculum was replaced by sterile saline), resulting in final concentrations of 0 to 170 g tubes per liter. The 3-CA concentration in the water was determined daily by HPLC.

Diffusion of 3-CA and chloride ions through silicone-tubes.

The diffusion of 3-CA was measured by bringing one ml stock solution of 3-CA (10 mg 3-CA/ml methanol; methanol was used because of the low solubility of 3-CA in water) in an empty autoclaved silicone tube, placed in a 100 ml sterile bottle filled with 50 ml sterile H2O, resulting in a final concentration of 200 mg/L. The ends of the tube were placed out of the flask, so that the stock solution was not in direct contact with the water. A control for the diffusion test had the same set-up, but 1 ml of the stock solution was directly added in the water. In case of total diffusion of 3-CA through the tubes, the 3-CA concentration in the diffusion experiment should be the same as the control test.

An identical set up was used to test if the chloride ions, released during the mineralization of 3-CA, could diffuse back into the medium. One ml of a 25 mM NaCl solution was injected inside the silicone tube and placed in 100 ml of distilled water. In function of time, the chloride content was determined. In case of total diffusion, the chloride concentration should increase to 8.9 mg of Cl^-/ml.

Degradation of 3-CA by suspended cells

The degradation rate of suspended I2 cells was estimated by incubating I2 cells in a MMN mineral medium containing 75 or 100 mg 3-CA per liter. The different media were inoculated with 1.9×10^7 I2*gfp* cells per ml and every 30 minutes a HPLC sample was taken. The degradation rate was calculated from the slope of the removal curve.

Degradation of 3-CA by cells contained within the silicone tubes

Three different set ups were examined in duplicate: one with I2*gfp* cells (1.3×10^9 CFU/ml), one with *Escherichia coli* cells (3.5×10^9 CFU/ml) and one with sterile saline. A long tube was partially filled with different agar mixtures for a tube length of 8 cm and the filled part was submerged in 100 ml water containing 250 mg 3-CA/L (same ratio tube length/mixed liquor as in the SCAS experiments). The ends of the tube were placed outside the flask, so that the cells could not be in direct contact with the 3-CA in the water (Figure 4.4). The 3-CA and chloride concentration in the water was determined daily by HPLC and IC, respectively, and possible contamination of the water by the cells in the tubes was determined by plating the water on LB agar to count *E. coli* and on LB agar with 50 µg/ml kanamycin and 100 µg/ml rifampin to count the I2*gfp* cells. The flasks were incubated at 28°C and shaken at 140 rpm.

Analytical methods

The effluent was analysed for 3-CA content by reversed phase HPLC after centrifugation at 5000 × g for 10 min. The Summit HPLC system (Dionex, Wommelgem, Belgium) consisted of a Dionex Pump Series P580, a Dionex Autosampler Model ASI-100 (injection volume is 20 µl), a STH585 Column Oven (at 28°C), a Dionex UV/VIS Detector UVD 340S and a Chromeleon software system version 6.10. A Hypersil Green Env column (150 mm × 8 mm ID; 5 µm particle size; Alltech, Deerfield, IL, USA) was used. The mobile phase consisted of CH_3OH/ 0.1% H_3PO_4 (ratio 70/30), with a flow rate of 0.8 ml/min. The UV-detector was used at 210 nm.

Chloride concentrations were determined by ion chromatography (IC) after centrifugation of the samples at 5000 × g for 10 min. and filtering through a 0.45 µm filter. The DX-600 system (Dionex, Wommelgem, Belgium) consisted of a Dionex Pump GP50, a Dionex Autosampler Model AS50 (injection volume is 100 µl), a Dionex ED50 Electrochemical Detector and a PeakNet 6 software system version 6.10. Ionpac AS9-HC (250 mm × 4 mm ID; 9 µm particle size; Dionex) column and Ionpac CS12-HC (250 mm × 4 mm ID; 8 µm particle size; Dionex) were used for anion separation. The mobile phase consisted of Na_2CO_3 (9 mM) and methanesulfonic acid (20 mM) with a flow rate of 1 ml/min. Quantitative data were obtained by comparing the peak areas of unknown concentrations with the peak areas of standards of known concentrations.

Microscopy

The GFP and RFP marked cells were observed by standard epifluorescence microscopy on a Zeiss Axioskop II microscope (Carl Zeiss, Jena, Germany). The microscope was equipped with a Peltier cooled single chip digital color CCD camera (Hamamatsu Orca IIIm, Hamamatsu, Massy Cedex, France) connected to a PC to obtain digital images.

RESULTS

First bioaugmentation experiment

Encapsulation in silicone tubes increased the period of complete 3-CA removal by I2*gfp*-cells (Figure 4.1). Starting from day 2 complete removal of 3-CA was obtained in all inoculated reactors with a minimal removal rate of 37 mg 3-CA/L mixed liquor·d.

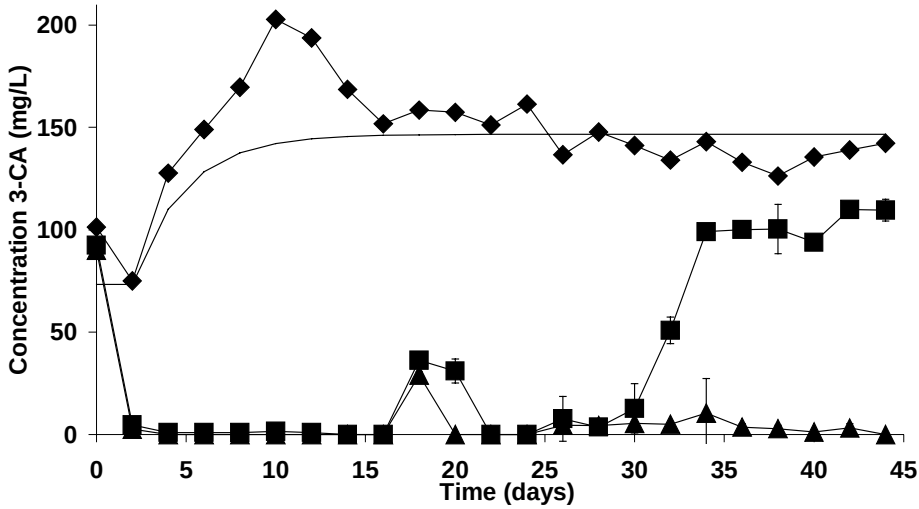

Figure 4.1. Concentration of 3-CA in the control reactor A1 (♦); the reactors B1 (I2*gfp*) (■), and the reactors C1 (I2*gfp* and immobilized I2*gfp*) (▲). The solid line represents the theoretical 3-CA concentration at the imposed loading rate of 37 mg 3-CA/L mixed liquor•d. Values represent the mean ± error bars (*n* = 2); in some cases, the error bars were too small to be visible.

Due to technical problems on day 17, anoxic conditions were created for 12 hours and a small amount of 3-CA accumulated on day 18. Encapsulated cells recovered from this anoxic shock more quickly than suspended cells (in 2 days compared to the 4 days). By day 30, the 3-CA biodegradation rate in reactors without encapsulated cells decreased to 30% (12 mg 3-CA/L mixed liquor·d) of that of the encapsulated cells. The reduced biodegradation rate was accompanied by a 100 fold decrease in culturable I2*gfp* cells from their initial value of 10^7 CFU/ml mixed liquor to 10^5 CFU/ml mixed liquor (data not shown). In contrast, the reactor with the encapsulated cells maintained its biodegradation rate of 37 mg 3-CA/L mixed liquor·d for the duration of the experiment (44 days in total) and culturable I2*gfp* cells

remained quite stable. No significant removal of 3-CA was observed in the control reactor A1 during the first 25 days and only a small amount of 3-CA was removed from day 25 until the end of the experiment with a biodegradation rate of 2,3 mg 3-CA/L mixed liquor·d.

Diffusion and sorption experiments

The ability of 3-CA to diffuse through the walls of the silicon tubes was examined by adding a concentrated 3-CA solution inside the tube and by monitoring its concentration in the water outside the tubes as a function of time. In case of total diffusion, the concentration outside the tubes should have been 196 mg/L. 3-chloroaniline freely diffused through silicone tubes within 24 hours with 93% (170 ± 13 mg 3-CA/L) of the expected 3CA found in the reactor liquor by 24 hours. From day two on, the 3-CA concentrations stabilized at 107% of the level expected.

To estimate the amount of 3-CA that could be absorbed on the silicon tubes in reactors C1, the amount of 3-CA that disappeared from the solution in a separate absorption experiment was determined. Silicone tubes filled with agar (0, 33, 67, 100, 133 and 167 g/L) were incubated in 200 mg/L 3-CA containing water until maximal absorption occurred (Figure 4.2).

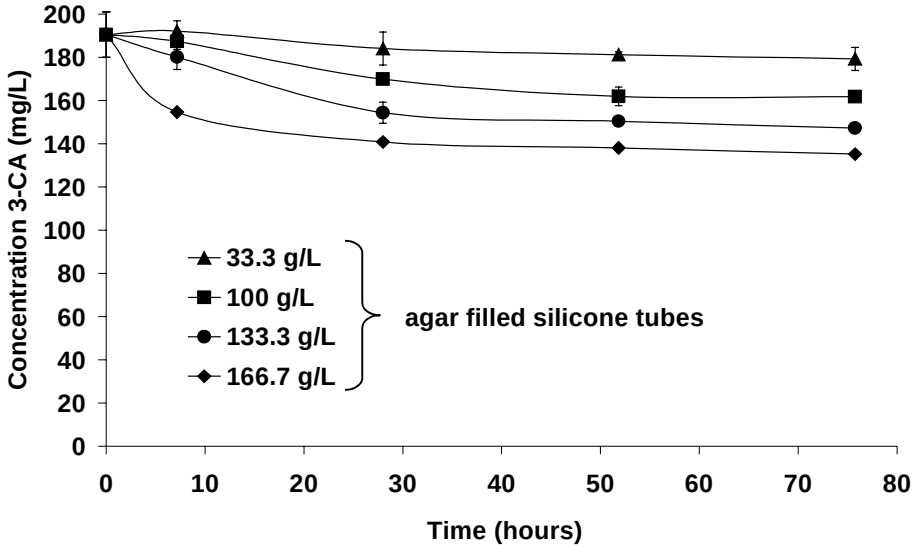

Figure 4.2. Absorption of 3-CA by increasing amounts of agar containing silicone tubes. Values represent the mean ± error bars (*n* = 2); in some cases, the error bars were too small to be visible.

Sorption by agar filled silicone tubes was minimal with tubes absorbing ca. 0.27 mg 3-CA/g within a 75 hour period (linear sorption, see Figure 4.3). This corresponds to 3.3 mg 3-CA absorbing to tubes in the first reactor experiment.

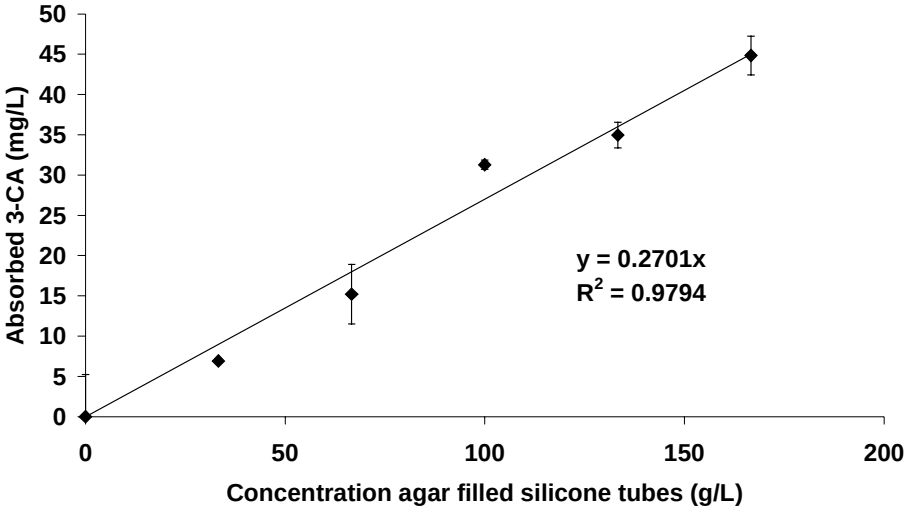

Figure 4.3. Linear regression of the absorption of 3-CA in function of increasing amounts of agar containing silicone tubes after 75 hours. Values represent the mean ± error bars (*n* = 2); in some cases, the error bars were too small to be visible.

The removal rate of free suspended cells was estimated by incubating pregrown cells in mineral medium. The average degradation rate was 64.6 mg 3-CA/L·h. This makes a specific removal rate for I2 of 71.9 mg 3-CA/mg CDW_{I2gfp}·d. This degradation is rate is rather high, but comparable with metabolic rates of other micro-organisms, such as yeast (341).Cells contained within the silicone tubes were able to degrade the 3-CA present in the liquid phase (Figure 4.4). The linear degradation rate of encapsulated I2*gfp*cells was 77 mg 3-CA/L·d. This corresponded with a specific removal rate of 0.53 mg 3-CA/mg CDW_{I2gfp}·d [(0.1 L × 77 mg 3-CA/L·d)/(1.3×10^{10} cells × 1.14 mg $CDW_{I2gfp}/10^9$ cells)]. This makes that the degradation rate was 136 times higher of the free suspended cells than of the encapsulated cells. No I2*gfp* was detected in the medium by plate counts or optical density. Encapsulated *E. coli* and sterile agar displayed no 3-CA degradation and minor absorption. It should be noted that in this experiment the cells as well as 3-CA metabolites were contained in the silicone tubing. No increased chloride concentrations were measured in the water, indicating that the chloride

released during the 3-CA mineralization remained inside the tubes doubling the chloride concentration to 9.7 mg Cl^-/ml or 277 mM.

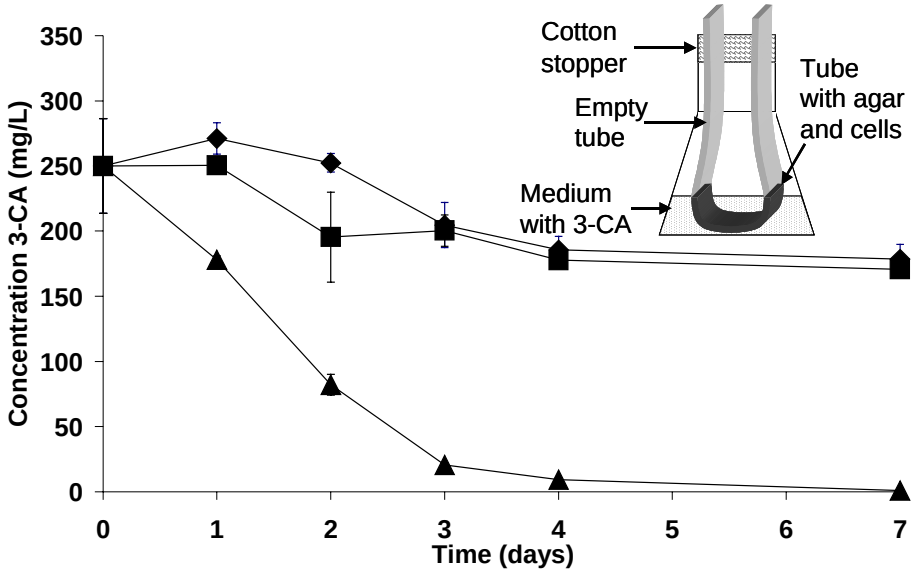

Figure 4.4. Concentration of 3-CA in the diffusion mediated degradation experiment: control without cells (♦); agar embedded I2*gfp* cells (▲); agar embedded *E. coli* cells (■). Values represent the mean ± error bars (*n* = 2); in some cases, the standard deviations were too small to be visible. A representation of the experimental set up is shown besides the graph.

Second bioaugmentation experiment

In a second SCAS experiment, we differentiated between encapsulated and released cells by marking strain I2 with two different genes. Cells marked with the green fluorescent protein (I2*gfp*) were freely suspended and cells marked with the red fluorescent protein (I2*rfp*) were encapsulated. Strain I2 marked with the *DsRed* gene showed the same 3-CA degradation characteristics as the wild type strain (data not shown).

Similar to the first run, complete removal of 3-CA was obtained by day 2 in all inoculated reactors (Figure 4.5). From day 26 on, 3-CA degradation decreased to only 24 mg/L mixed liquor·d in the reactors inoculated with suspended I2, compared to a rate of at least 37 mg/L mixed liquor·d for the reactors with encapsulated cells for the duration of the experiment. Reactors containing encapsulated and suspended cells degraded 3-CA at a lower rate for a four day period from day 20 until day 24 but reactors containing only encapsulated

cells continuously removed all 3-CA throughout the experiment.

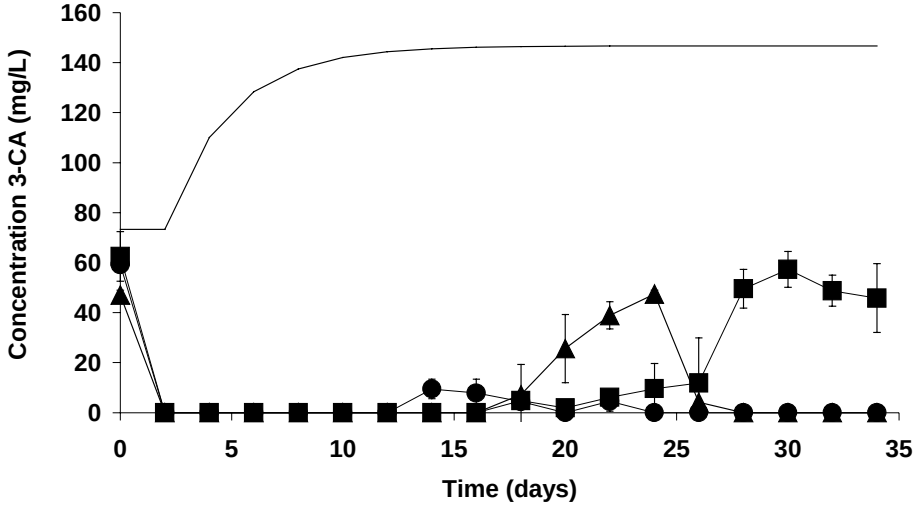

Figure 4.5. Effects of I2*gfp* and I2*rfp* cells in the mixed liquor on the amount of 3-CA in the effluent. Concentration of 3-CA (in the reactors B2 (suspended I2*gfp*) (■), the reactors C2 (suspended I2*gfp* and immobilized I2*rfp*) (▲) and the reactors D2 (immobilized I2*rfp*) (●). The solid line represents the theoretical 3-CA concentration at the imposed loading rate of 37 mg 3-CA/L mixed liquor·d. Values represent the mean ± error bars (*n* = 2); in some cases, the error bars were too small to be visible.

Suspended *C. testosteroni* I2*gfp* inoculated in the reactors B2 remained quite constant at ca. 10^7 CFU/ml mixed liquor throughout the study period despite the loss of 3-CA biodegradation activity (Figure 4.6). In contrast, I2*gfp* in the presence of encapsulated I2*rfp* in reactors C2 only remained at 10^7-10^8 CFU/ml mixed liquor until day 15 when the number of cells declined drastically to 4.0×10^3 CFU/ml mixed liquor. The encapsulated I2*rfp* cells of reactors D2 remained quite stable at 10^7 CFU/ml mixed liquor except for a brief period between day 15 and 25. This corresponds to the observed lapse in 3-CA biodegradation activity. By transferring one tube out of the reactors to saline for one hour and plating the amount of released cells, it was observed that the silicone tubes released ca. 10^6 cells/h·silicone tube via their open end (Figure 4.7). These measurements were repeated at regular intervals and throughout the entire period of the SCAS tests this rate of release of cells essentially unchanged (average release rate over was log 6.0 ± 0.3 CFU/h.tube).

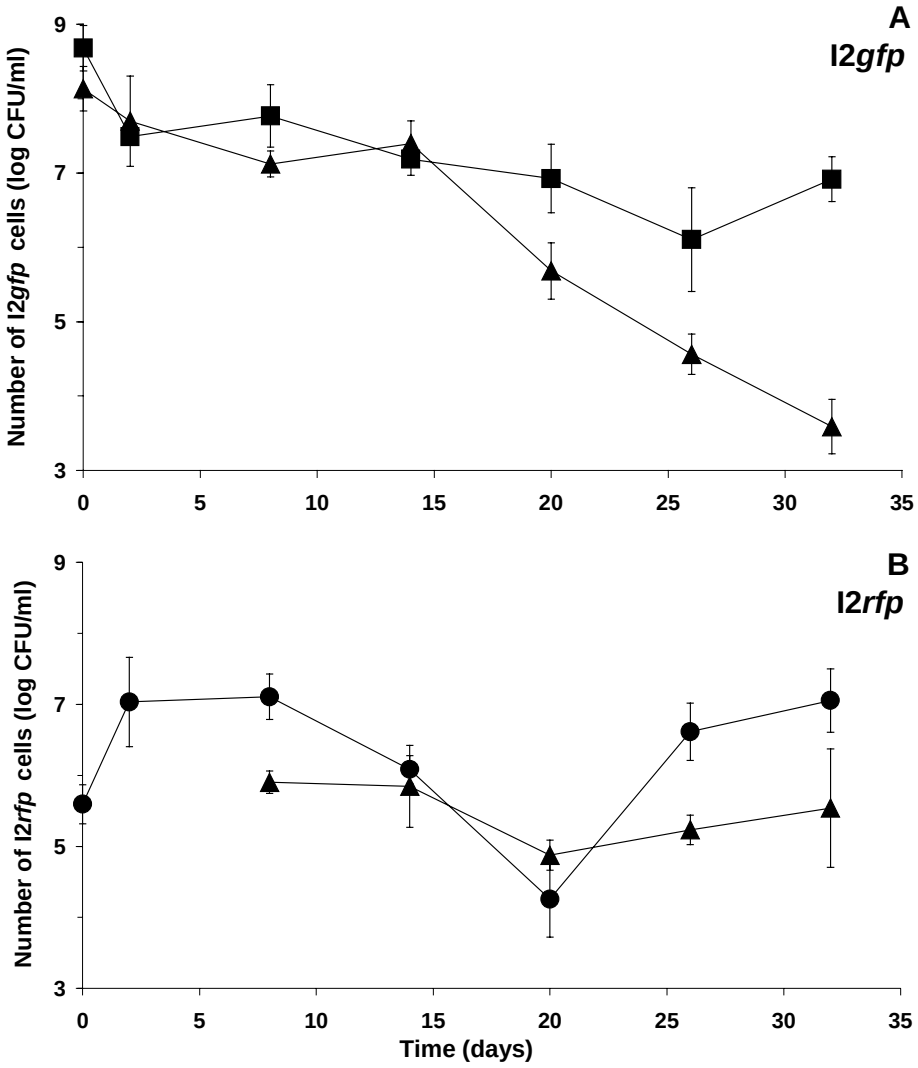

Figure 4.6. Number of I2*gfp* (A) and I2*rfp* (B) cells in the reactors B2 (suspended I2*gfp*) (■), the reactors C2 (suspended I2*gfp* and immobilized I2*rfp*) (▲) and the reactors D2 (immobilized I2*rfp*) (●). The solid line represents the theoretical 3-CA concentration at the imposed loading rate of 37 mg 3-CA/L mixed liquor·d. Values represent the mean ± error bars (*n* = 2).

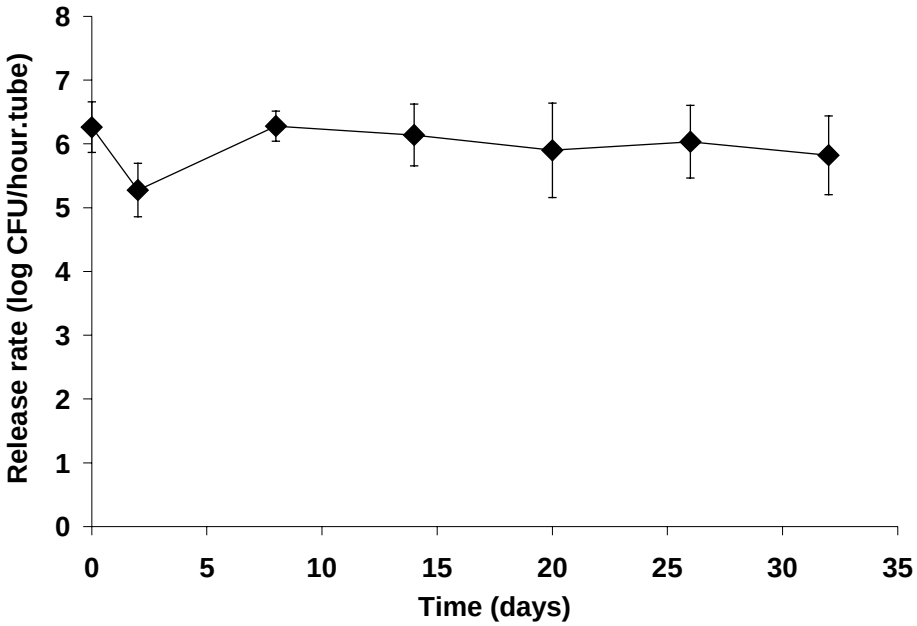

Figure 4.7. Estimates of the rate of release of I2*rfp* cells from the agar containing silicone tubes into the mixed liquor, as determined by transferring one tube from the reactors into saline and shaking it for one hour, followed by enumerating the number of released cells by plate counts.

Bacterial activity estimates

Based on the data from the second experimental run, bacterial kinetics were calculated and the relative proportion of each mode of action estimated. The calculations of the specific activity of cells present in the sludge for some time (aged cells) are based on reactors B2 (with only free suspended cells) and on the period where 3-CA accumulation occurred. During this period (reference day 32) the removal rate was for the suspended cells 2.5 mg 3-CA/mg CDW_{I2gfp}·d [(24 mg 3-CA/L·d) / (8.3×10^9 cells/L mixed liquor × 1.14 mg $CDW_{I2gfp}/10^9$ cells)] (Figure 3). This was not sufficient to remove the daily dosed 37 mg 3-CA/L, so accumulation occurred.

In the reactors D2 with only the immobilized I2*rfp* cells, four different modes of 3-CA removal are possible: absorption, diffusion with degradation by the immobilized cells inside the tubes, degradation by the freshly released cells in the mixed liquor and by the aged cells already present in the mixed liquor. The absorption effect is minimal since only 3.3 mg 3-CA was able to bind to the tubes the first day and once the tubes are saturated they will no longer

absorb 3-CA. Theoretically, it is possible that the 3-CA absorbed on the tube was released in the side-test for the determination of the release rate of I2*rfp* cells from one tube. In theory, the tube when returned to the reactor, was calculated to potentially release a negligible 0.1 mg 3-CA.

The second effect of the tubes on the degradation rate is the 3-CA degradation by immobilized cells inside the tubes. This estimated biodegradation rate potential, mediated by diffusion through the silicone walls, was minor, since the encapsulated cells in the batch experiment had the potential to degrade 9.8 mg/L mixed liquor·d [(0.53 mg 3-CA/mg CDW_{I2rfp}·d) × (inside the tubes 18.5 mg CDW_{I2rfp}/100 cm tube in 1.2 L of mixed liquor)].

A third fraction of the degradation can be considered to be due to the ongoing released I2*rfp* cells in the mixed liquor. Based on the 3-CA degradation rates of freshly released (within 24 h), active I2*rfp* cells in mineral medium (71.9 mg 3-CA/ mg CDW_{I2gfp}·d; see batch tests), the 3-CA removal rate of the freshly 6.6×10^8 released cells/L mixed liquor·d [24 h × (10^6 I2*rfp* cells per tube) × 33 tubes in 1.2 L of mixed liquor] in reactor D2 was approximated at maximum 54.1 mg 3-CA/L mixed liquor·d [(71.9 mg 3-CA/ mg CDW_{I2gfp}·d) × (6.6×10^8 freshly released I2*rfp* cells/L mixed liquor) × (1.14 mg $CDW_{I2rfp}/10^9$ I2*rfp* cells)]. The 12.9 mg CDW_{I2rfp} aged cells/L mixed liquor [(1.13×10^{10} aged I2*rfp* cells/L mixed liquor) × (1.14 mg $CDW_{I2rfp}/10^9$ I2*rfp* cells)] already present in the activated sludge (from earlier release) also counted for 33.0 mg 3-CA/L mixed liquor·d degradation [(12.9 mg CDW_{I2rfp}/L mixed liquor) × (2.5 mg 3-CA/mg CDW_{I2gfp}·day)]. The 3-CA degradation rate of the aged suspended cells from reactor was assumed to be the same as for the aged suspended cells from reactor B2.

In conclusion, four different factors played a role in the 3-CA removal of reactors (Figure 4.8). The prolonged 3-CA degradative activity in reactor D may have been caused by a combination of minor adsorption on the silicone tubes (0.1%), and degradation activities by three different types of I2*rfp* cells: immobilized cells in the agar (estimated to be responsible for only 10% of the 3-CA removal); freshly released active cells (56% of the degradation capacity), and aged cells present in the sludge (34% of the degradation capacity).

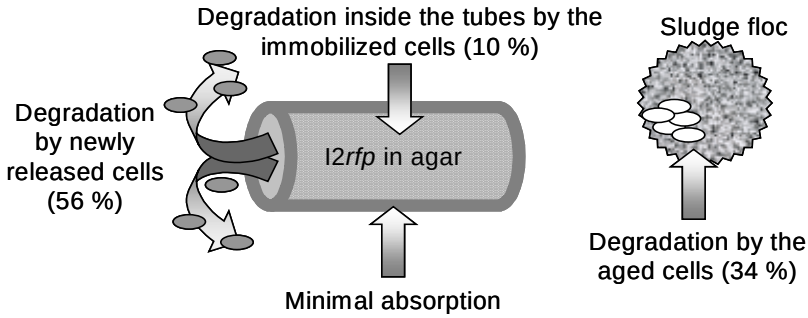

Figure 4.8. Schematic representation of the second bioaugmentation experiment. The modes of action and respective removal rates of 3-CA by agar immobilized *C. testosteroni* I2 cells in silicone tubes are schematized. The value represented between brackets is the estimated relative contribution of each mode of action to the total 3-CA removal in the reactor experiment.

I2*rfp* cell growth and release from the silicone tubes

During the whole experiment, about 40 mg CDW_{I2rfp} were released [(10^6 cells/h·tube) × (33 tubes) × (24 h) × (44 d) × (1.14 mg $CDW_{I2rfp}/10^9$ I2*rfp* cells)]. Initially, only 18.5 mg CDW_{I2rfp} was immobilized in the tubes [(1.3×10^6 cells/h·tube) × (33 tubes) × (24 h) × (45 d) × (1.14 mg $CDW_{I2rfp}/10^9$ I2*rfp* cells)]. Thus, growth of the I2*rfp* cells inside the tubes occurred and they were released into the mixed liquor. The I2*rfp* cell growth rate μ and doubling time t_D were determined by:

$$\mu = \ln(X_t/X_0)/t \qquad \textbf{Equation 1}$$

$$t_D = \ln 2/\mu \qquad \textbf{Equation 2}$$

with X_t = total biomass released and present in the tubes after 44 days (18.5 + 40 mg CDW_{I2rfp}), assuming that the cell mass inside the tubes did not change over time ; X_0 = biomass present in the tubes at day 0 (18.5 CDW_{I2rfp}); t = duration of the experiment (44 days). The overall growth rate (0.026/day) and doubling time (26.5 days) were rather low.

The substrate necessary for growth was obtained from three sources. The first substrate source was the LB medium, which was mixed with the bacteria and inserted in the silicone tubes. One liter of LB medium contains as substrates 10 g peptone/L and 5 g yeast extract/L. This means that in the tubes 94 mg of substrate was present [(6.25 ml LB) × (15 mg

substrate/L)]. A second source of substrate provision was obtained by the addition of 3-CA. During the whole experiment, 1936 mg 3-CA [(44 mg/L·d) × (44 d)] was added to the reactors, of which 6 %, or 119 mg was degraded by the immobilized cells (see previous). The third substrate source could be the milk powder, however it was not possible to determine how much substrate the immobilized cells received from this source. This means that for a yield factor of 40 %, sufficient substrates were present to release 40 mg CDW_{I2rfp} during the experiment. In conclusion the cells immobilized in the silicone tube were growing slowly and released into the mixed liquor. Substrates necessary for growth support was sufficiently provided by the LB medium, present in the agar, and by the 3-CA metabolism.

DISCUSSION

Inoculation of a catabolic strain by immobilizing it in agar and surrounding it by open ended silicone tubes significantly prolonged the bioaugmentation capacity of the strain in the SCAS system exposed to realistic 3-CA concentrations. The increase in bioaugmentation capacity was primarily due to degradation of 3-CA by the released suspended cells (56 %), followed by the aged suspended cells (34 %) and only a minor part by the encapsulated cells (10 %).

The slow release concept is to our knowledge a new bioaugmentation strategy for bioreactors. In several studies where the use of carrier materials was examined, positive effects on survival and activity of inoculated strains have been reported (312). Loh et al. (188) used hollow fiber membranes to immobilize phenol degrading *Pseudomonas putida* cells to treat high concentrations of phenol (1 g/L). Encapsulation of pentachlorophenol-degrading bacteria in polymeric material had no negative effect on the metabolic activity of the immobilized cells (282). Our work identifies an additional mechanism by which encapsulation improves biodegradation, namely continuous but relatively low level release of 'non-starved' cells. In our set-up in reactors D2, cell release could account directly or indirectly for 90% of the degradation occurring in a bioaugmented reactor, due to the aged cells in the sludge flocs (34 %), but also due to the freshly released cells (56 %). The release of free suspended cells may become of critical importance for trace toxicants that need to be removed from industrial waste water streams because, typically, biodegradation rates at low concentrations are better in suspended compared to fixed cultures (149). Thus, a slow release bioaugmentation set up is at least as effective as the systems with complete immobilization or massive inoculation of specialized cells.

There was a significant difference between the capability of newly released or inoculated I2 cells to degrade 3-CA and aged I2*gfp* cells. While newly released cells continuously degraded 3-CA, the biodegradative activity of the original inoculum decreased substantially in time. In our previous work, lower degradation activity was also observed for I2*gfp* cells continuously exposed to 3-CA (35), resulting in an accumulation of 3-CA in the effluent. Bossier and Verstraete described a *C. testosteroni* strain, A20, that expressed a phenotypic shift when the strains were cultured under unfavorable conditions (40). The cells altered to the hydrophobic form and dense flocs were formed, providing cellular protection. The I2 cells also showed a similar phenotypic instability and formed flocs in the activated sludge, once they were inoculated (35). We postulate that this phenotypic shift is detrimental

to 3-CA degradation capacity. In a similar vein, starvation induced flocculation by *Ralstonia eutopha,* increased hydrophobicity and a complete loss of phenol-oxygenation activity (327). Thus, the continuous provision of new suspended I2*rfp* cells from the tubes could result in a higher metabolic rate, since these cells are not yet starved and flocculated by the harsh environmental conditions.

The use of regular reinoculation of metabolic active cells can be an option to maintain a high biodegradative capability. Repeated massive inoculation can however give rise to the disappearance of critical bacterial groups, such as nitrifiers due to the overgrowth of protozoa (41), so that the reactor is no longer achieving it's desired function. Over a 44 day period about 3.5×10^{10} I2*rfp* cells were released into the mixed liquor with no detrimental effect on reactor function, as was measured by analysis of chemical oxygen demand in the effluent (data not shown). The substrates, provided by the LB medium in the agar and by the 3-CA diffused into the silicone tubes were sufficient to sustain growth of the immobilized cells, which were subsequently released. These slowly released I2*rfp* cells in reactors C2 survived better (3.5×10^5 CFU/ml mixed liquor at day 32) compared to the suspended I2*gfp* cells (3.9×10^3 CFU/ml mixed liquor at day 32). Thus, I2*rfp* cells embedded in silicone tubes grew and cells slowly diffused out of the silicone tubes into the mixed liquor where they rapidly degraded 3-CA.

In most activated sludge bioaugmentation experiments, inoculation with a specific strain generally has no or only a transient effect. This study has demonstrated that encapsulation of the inoculum improves biodegradation of 3-CA for an extended period of time in (industrial) wastewaters. The slow release of specialized, metabolically active cells was essential to maintain prolonged bioaugmentation, in contrast to their 'aged' counterparts, which lost biodegradation activity after 30 days in the reactor.

Acknowledgments

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CHAPTER 5

5. PLASMID MEDIATED BIOAUGMENTATION OF ACTIVATED SLUDGE CONTAMINATED WITH 3-CHLOROANILINE

ABSTRACT

Specific degrading bacteria inoculated in bioreactors used for wastewater treatment usually do not maintain their high cell numbers, or the metabolic activity declines in function of time, and as a result the success of bioaugmentation is often limited. A possible alternative to overcome this problem is to deliver the catabolic genes to the indigenous bacteria. However, plasmid mediated bioaugmentation has not yet been achieved in activated sludge bioreactors. We examined the introduction of the catabolic plasmid pC1-3, involved in deamination and dechlorination of 3-chloroaniline (3-CA), into activated sludge that received a daily 3-CA dose. The introduction of the plasmid donor did not result in sustained bioaugmentation. In contrast, co-introduction of the donor of pC1-3 and the 3-CA metabolizing strain *Comamonas testosteroni* I2*gfp* resulted in a significant prolongation of complete removal of 3-CA from the reactors. Inoculation of strain I2gfp alone could not support this prolonged 3-CA degradation after a few weeks. Since plasmid pC1-3 lacked suitable selection markers the possible formation of transconjugants could not been shown in the coinoculated reactor. Before a next inoculation experiment was set up, plasmid pC1-3 was marked with a mini-Tn*5* transposon, containing genes encoding for the green fluorescent protein (GFP) and kanamycin resistance. The *gfp* marked plasmid, pC1::gfp, was subsequently used in batch experiments to show that plasmid transfer towards the indigenous bacteria occurred. Transfer frequencies between 4×10^{-6} and 1×10^{-5} were detected. We postulate two possible reasons to explain the success of co-inoculation: (i) transfer of plasmid pC1-3 occurred and allowed the indigenous sludge bacteria to degrade 3-CA or (ii) an as yet unknown synergistic effect between the two co-inoculated strains caused a better degradation than when both strains were inoculated separately. In conclusion bioaugmentation of bioreactors can be improved by the co-inoculation of the metabolic strain I2*gfp* and a catabolic plasmid bearing donor strain.

INTRODUCTION

During the last two decades, many research groups focused on the isolation and characterization of bacterial strains, able to degrade recalcitrant man-made chemicals. In many of these bacterial strains, catabolic genes are encoded on plasmids and they can often be transferred to other species (for an overview, see reference (301)). Horizontal gene transfer is indeed a major mechanism for microorganisms to acquire new metabolic traits in new combinations. As a consequence, horizontal gene transfer can have important implications for public health by the spread of antibiotic resistance (69) and genetically modified genes (281). However horizontal gene transfer can also be beneficial for society when it involves degradative genes. Nevertheless, its use in biotechnological processes, such as bioremediation, is still limited, despite the numerous reports of new catabolic plasmids.

The bioaugmentation of bioreactors is still not an established procedure and failures are common (41, 110, 293). Poor survival due to slow growth rates, wash-out or predation, and poor metabolic activity, due to too low contaminant concentration, microbial inhibitors and preferential substrate uptake, are some of the problems that can occur (110). Therefore, alternative bioaugmentation strategies, such as cell immobilization (188), inoculum starvation (327) and slow release (33) can be a possible solution to overcome the poor survival of inocula. An alternative approach may be the use of mobile genetic elements to establish new metabolic traits in indigenous microbial populations, without the requirement of inoculum survival. In soils, the use of catabolic plasmids was evaluated to enhance biodegradation of herbicides, such as 2,4-dichlorophenoxyacetic acid. Although the introduced strains survived poorly, plasmid transfer occurred and enhanced degradation was observed (77, 300, 302). Very few studies have evaluated the introduction of degradative bacteria with mobile catabolic genes into activated sludge or different wastewater treatment reactor systems in order to enhance biodegradation of recalcitrant organic compounds. In most of these studies the exact role of the indigenous transconjugants in accelerated degradation was never investigated in detail (199, 200, 218).

Substituted anilines are used widely as a precursor for the synthesis of chemicals, such as rubber, azo-dyes, drugs, photographic chemicals, varnishes and pesticides (106, 157). As a consequence, these chemicals are often detected in industrial wastewaters (147, 172, 184). The removal of these chemicals is in most cases very low, and more and more chloroanilines have been found in waters (331) and are considered as important environmental pollutants (202). Therefore they are subjected to legislative control by Europe (76/464/E.E.C. Directive,

see reference (2)) and the United States of America (Priority Pollutant List of U.S.E.P.A., see reference (82)).

In our previous work, successful degradation of 3-chloroaniline (3-CA) in activated sludge was obtained by inoculating strain *Comamonas testosteroni* I2*gfp* (35). During 14 days, all 3-CA was completely degraded, but later on the degradation rate became slower and 3-CA started to accumulate in the reactor, probably due to the lower concentration of strain I2*gfp* in the reactor. The aim of this work was to obtain a prolonged enhanced degradation of 3-CA in a Semi-Continuous Activated Sludge-reactor by additionally inoculating a donor of a 3-CA catabolic plasmid together with strain *C. testosteroni* I2*gfp*. Plasmid pC1, recently described by us as the first reported plasmid to encode genes for deamination, dechlorination and ring-cleave of 3-CA (36), was used for this purpose.

MATERIAL AND METHODS

Bacterial strains

The used bacterial strains and plasmids are listed in Table 5.1.

Table 5.1. Strains and plasmids used in this study.

Strain or plasmid	Characteristic(s)[a]	Reference or source
Strain		
Comamonas testosteroni I2	Ani^{NC}, 3-CA^{NC}, Rif^{R}	(35, 36)
Comamonas testosteroni I2*gfp*	Ani^{NC}, 3-CA^{NC}, Rif^{R}, Km^{R}, GFP	(36)
Delftia acidovorans CA28	Ani^{NC}, 3-CA^{NC}, Rif^{R}	(189)
Ralstonia eutropha JMP228*gfp*	Rif^{R}, Km^{R}, GFP	(36)
Ralstonia eutropha JMP228*gfp* (pC1-3)	Rif^{R}, Km^{R}, GFP, 3-CA^{N}	(36)
Ralstonia eutropha JMP228n	Rif^{R}, Nal^{R}	(299)
Ralstonia eutropha JMP228n (pC1::gfp)	Rif^{R}, Nal^{R}, 3-CA^{N}	This study
Pseudomonas putida UWC3	Rif^{R}, Tet^{R}, ILV^{-}	(130)
Pseudomonas putida UWC3 (pC1::gfp)	Rif^{R}, Tet^{R}, ILV^{-}	This study
Escherichia coli S17-1 λpir (pUTgfp)	Amp^{R}, Km^{R}, expresses GFP	(36)
Plasmid		
pUTgfp	Amp^{R}, Km^{R}, expresses GFP	(297)
pC1-3 (from strain CA28)	3-CA^{N}	(36)
pC1::gfp (from strain CA28)	3-CA^{N}, GFP	This study

[a] GFP, Green Fluorescent Protein. Superscript "N" and "C" indicate that the strain can use that substrate as sole nitrogen source and sole carbon and energy source, respectively; Rif^{R}, Nx^{R}, Km^{R}, and Tet^{R}, resistance to rifampin, nalidixic acid, kanamycin, and tetracycline, respectively; ILV^{-}, auxotrophic for isoleucine, leucine, and valine.

The chromosomally *gfp*-marked strain *Comamonas testosteroni* I2*gfp* originates from strain *C. testosteroni* I2, which was isolated from a municipal wastewater treatment plant (35, 38). Strain I2*gfp* can mineralize 3-CA completely.

Ralstonia eutropha JMP228*gfp* (pC1-3) received the catabolic and self-transmissible plasmid pC1 by biparental mating with the 3-CA degrader *Delftia acidovorans* CA28 (36).

Since different types of JMP228*gfp* transconjugants were obtained, every type of pC1 plasmid (form the original host) was divided in three types of strains, i.e. pC1-1, pC1-2 and pC1-3. In this study we worked further with strain JMP228*gfp* (pC1-3). Due to the presence of the plasmid pC1, strain JMP228*gfp* (pC1-3) is able to use 3-CA as sole source of nitrogen but not carbon.

Marking of the plasmid pC1 with the *gfp* gene

In order to follow the fate of the catabolic plasmid pC1, the plasmid was marked with the mini-Tn*5* transposon containing the *gfp* gene and the *nptII* gene (coding for kanamycin resistance). A scheme of the selection procedure is shown in Figure 5.1.

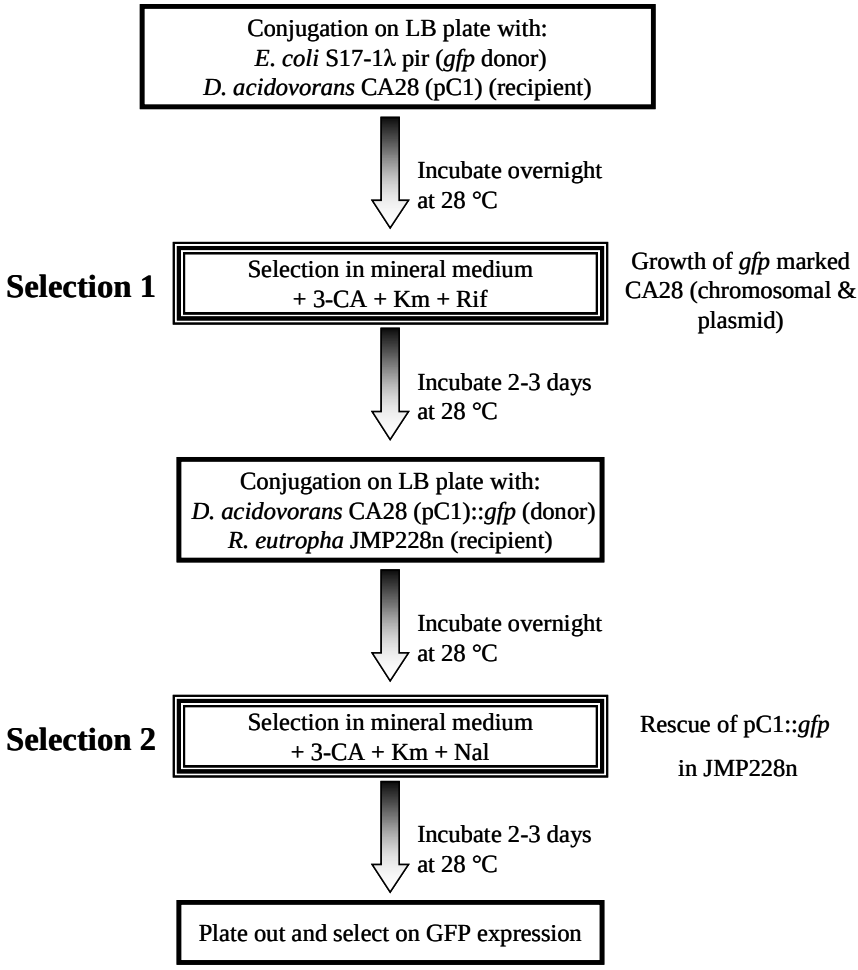

Figure 5.1. Selection procedure to mark the plasmid pC1 from strain CA28.

The construction principle of a GFP-tagged pC1 derivative was previously used for tagging the TOL plasmid, RP4 as well as other conjugative plasmids isolated from marine environments (53, 64, 105). In brief, the first step in the marking of the plasmid pC1 is the conjugation of strain CA28 with *E. coli* S17-1 λpir (pUTgfp). A mixture of both strains was incubated on LB agar plates and incubated overnight at 28 °C. The biomass was suspended in saline (0.85%) and 50 µl of this suspension was transferred to 5 ml of selective medium. Transconjugants were selected on liquid mineral medium with 3-CA as sole carbon or nitrogen source (MMN-CA or MMN-CAP respectively, see reference (36)), rifampin (100 mg/L) and kanamycin (50 mg/L) and incubated at a rotary shaker (140 rpm) at 28 °C. *E. coli* S17-1 λpir cannot grow in the presence of 3-CA and rifampin and non marked CA28 cells will be excluded by their kanamycin sensitivity. The selection on mineral medium with 3-CA allows us to eliminate the transconjugants with a 3-CA deficiency or vitamin or amino acid autotrophy. Thus the CA28 cells surviving the first selection received either the mini-Tn*5* cassette in the chromosome or the plasmid. When growth occurred in the medium, as could be monitored by an increased turbidity, the mixed *gfp*-marked CA28 population was transferred to new medium with the same composition. The second step in the selection of the marked plasmid is the transfer and selection of the marked plasmid. Therefore, the mixed *gfp*-marked population was conjugated with the naladixin resistant *Ralstonia eutropha* JMP228n on LB agar plates and incubated overnight at 28 °C. The biomass was suspended in saline and 50 µl of this suspension was transferred to 5 ml of selective mineral MMN-CAP medium, containing naladixin (200 mg/L) and kanamycin (50 mg/L). Strain CA28 was eliminated by the naladixin and strain JMP228n without a *gfp* containing plasmid was eliminated by the kanamycin and 3-CA. Thus, only *R. eutropha* JMP228n cells with a *gfp* marked pC1 plasmid (= pC1::gfp) would be able to grow in the selective medium. After growth in the selective liquid mineral medium, the culture was plated on agar medium of the same composition and the most green fluorescent bacteria (excitation at 396/476 nm, emission at 508 nm) under UV-light (TLD 18W/08, Philips, The Netherlands) were picked up. The purified colonies were tested in MMN-CAP medium and HPLC confirmed the 3-CA degradation capacity.

In a next conjugation experiment, the ability of transferring plasmid pC1::gfp from JMP228n to another species, i.e. *Pseudomonas putida* UWC3 was examined. A mixture of both strains was incubated on LB agar plates and incubated overnight at 28 °C. Selection of the transconjugants was done on LB plates supplemented with tetracycline (10 mg/L) and kanamycin (50 mg/L). The transconjugants obtained were again conjugated to JMP228n and selected on LB with naladixin and kanamycin.

Bacterial counts

Sludge flocs were dispersed by purging a 1 ml sample 20 times through a sterile 1 ml syringe with a sterile needle (1.2 × 40 mm). LB agar medium, supplemented with rifampin (100 mg/L) and kanamycin (50 mg/L) was used to count the I2*gfp* and JMP228*gfp* (pC1-3) cells. By using the GFP-fluorescence, it was possible to detect the inoculated strains against the background of other resistant micro-organisms. Moreover, both inoculated strains, I2*gfp* and JMP228*gfp* (pC1-3), could be distinguished based on a different colony morphology.

Semi continuous activated sludge (SCAS) reactors

The experiments were conducted with sludge freshly collected from a domestic wastewater treatment plant (Bourgoyen-Ossemeersen, Ghent, Belgium). The reactors (2 L plastic Erlenmeyer flasks), with an active volume of 1.2 L, were operated according to the SCAS (Semi-Continuous Activated Sludge) procedure at room temperature (ca. 21 °C), as described previously (33, 35). In a first experimental run, 4 different reactor set-ups were examined during 20 days: a first reactor inoculated with *C. testosteroni* I2*gfp*, the second reactor inoculated with *C. testosteroni* I2*gfp* and *R. eutropha* JMP228*gfp* (pC1-3), the third reactor inoculated with *R. eutropha* JMP228*gfp* (pC1-3) and finally a fourth reactor as non-inoculated control. In the second experimental run, duplicate reactors were inoculated either with *C. testosteroni* I2*gfp* alone(= reactors A), or with *C. testosteroni* I2*gfp* and *R. eutropha* JMP228*gfp* (pC1-3) (= reactors B). One reactor was used as non-inoculated control (= reactor C). No important differences could be observed between the two duplicate reactors. Hence, unless otherwise indicated, the data reported are averages of both duplicates. The reactors were operated without 3-CA for 16 days to allow the microbial community to adapt to the changed environment and growth conditions. The inocula were grown overnight in 5 ml LB medium, containing 100 mg 3-CA /L. Subsequently, these 5 ml cultures were used to inoculate 200 ml LB medium + 3-CA (100 mg/L). After shaking the cultures overnight, they were washed twice with saline and resuspended finally in saline. Reactors A and B were inoculated with *C. testosteroni* I2*gfp* to a final concentration of 9.0×10^7, 1.0×10^7 and 1.2×10^7 cells/ml respectively. Reactors B were additionally inoculated with *R. eutropha* JMP228*gfp* (pC1-3) to a final concentration of 4.4×10^7 cells/ml.

At different time intervals, the concentration of strains I2*gfp* and/or JMP228*gfp* (pC1-3) was determined. Every two days a sample was taken for HPLC-analysis and the suspended solids (SS) and the sludge volume index (SVI) were measured (115).

Plasmid transfer in batch experiments with activated sludge

Strain *Pseudomonas putida* UWC3 (pC1::*gfp*) was grown overnight in LB medium with kanamycin (50 mg/L) and tetracycline (5 mg/L) at 28°C. These cultures were centrifuged (7000 × g, 10 min.) and suspended in 5 ml of saline (0.85%). Activated sludge was freshly collected from a domestic wastewater treatment plant (Bourgoyen-Ossemeersen, Ghent, Belgium), 1 g/L milk powder was added and incubated overnight.

Four different conjugations of strain UWC3 (pC1::*gfp*) and activated sludge were performed in duplicate. A first set of conjugations was performed on LB agar plates and 30 µl of each bacterial mixture was plated on LB agar plates and incubated overnight at 28°C, either supplemented or not with 50 mg 3-CA/L. The second set of conjugations was performed in liquid medium, where 5 ml of activated sludge and 100 µl of UWC3 (pC1::*gfp*) were incubated with and without 50 mg 3-CA/L overnight at 28 °C (90 rpm).

Transconjugants were selected on MMN mineral medium (36), supplemented with 3-CA (50 mg/L), sodium pyruvate (1000 mg/L), and kanamycin (50 mg/L). Number of donors were determined on LB agar plates, supplemented with kanamycin (50 mg/L) and tetracycline (5 mg/L).

Analytical methods

Determination of the 3-CA concentration was analyzed by reversed phase HPLC as described previously (35).

RESULTS

Bioaugmentation of the SCAS-reactors in a first experiment with pC1

The sludge was adapted for 16 days to the operating SCAS system. At day 0, the different strains were inoculated and the supplementation of 3-CA started (Figure 5.2). In the reactors where I2*gfp* cells were present, the degradation of 3-CA started immediately. However after 10 days, the removal of 3-CA was not complete anymore in the reactor with only I2*gfp* cells. In the reactor where I2*gfp* and JMP228*gfp* (pC1-3) were inoculated together, almost all 3-CA was removed during the whole experiment. In the reactor with only JMP228*gfp* (pC1-3), an enhanced degradation was observed at day 6, but starting from day 8, 3-CA accumulated in the reactor. In the control reactor, no degradation of 3-CA by the indigenous bacteria was observed. Clearly co-inoculation of strains I2*gfp* and JMP228*gfp* (pC1-3) could extend the period of complete 3-CA removal, compared to the separate inoculations.

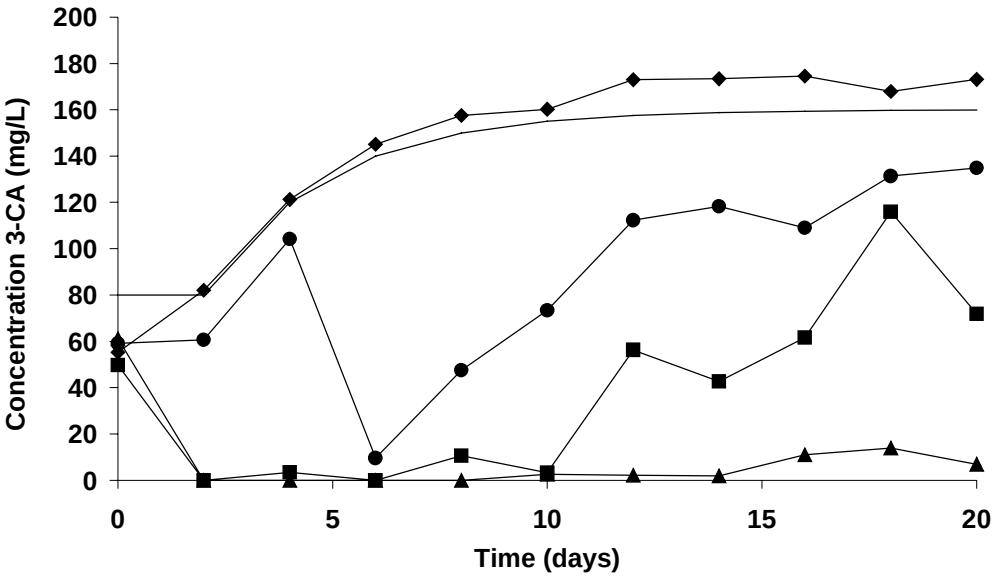

Figure 5.2. Concentration of 3-CA in the first test in the reactor with I2*gfp* (■), with I2*gfp* and JMP228*gfp* (pC1-3) (▲), with JMP228*gfp* (pC1-3) (●) and in the non-inoculated control reactor (◆), together with a simulation of the 3-CA concentration if no degradation would occur (-).

On different time intervals, the amount of *C. testosteroni* I2*gfp* and *Ralstonia eutropha*

JMP228*gfp* cells was determined by plating on selective LB medium with kanamycin and rifampin (Figure 5.3). In case of background growth by indigenous bacteria, the inoculated strains could be recognized by the GFP-protein auto-fluorescence under a long-wave UV-lamp and both strains could easily be distinguished from each other by the typical colony morphology. In the reactor with only I2*gfp*, cells number decreased about two log units towards the end of the experiment. The same was observed for strain I2*gfp* and JMP228*gfp* cells in the co-inoculated reactor. However, in the reactor where only strain JMP228*gfp* was inoculated, the number of cells declined rapidly form day 8 on and at day 20, no JMP228*gfp* cells were detectable anymore.

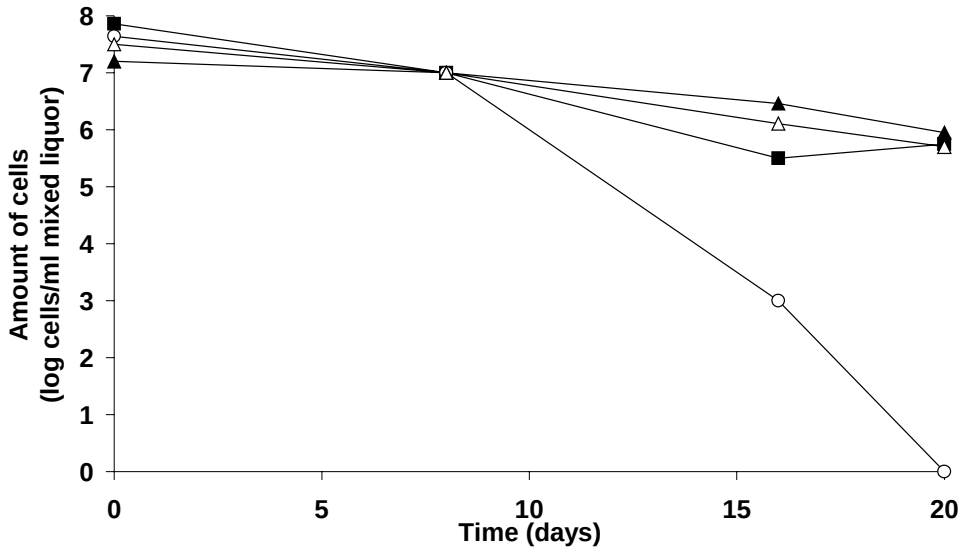

Figure 5.3. Cell counts of *C. testosteroni* I2*gfp* (closed symbols) and *Ralstonia eutropha* JMP228*gfp* (open symbols) in the first test in the reactor with I2*gfp* (■), with I2*gfp* and JMP228*gfp* (pC1-3) (▲, △) and with JMP228*gfp* (pC1-3) (○).

Bioaugmentation of the SCAS-reactors with pC1

To verify the successful 3-CA removal by co-inoculation for a longer time, as described above, a second bioaugmentation experiment was performed with 3 different reactors. In a first set of reactors A, a 3-CA metabolizing I2*gfp* was inoculated as such. In the second reactors B, strain I2*gfp* was inoculated together with strain JMP228*gfp* (pC1-3). The third reactor C was a non-inoculated control reactor. After the adaptation period of 16 days, the supplementation with 3-CA started and the strains were inoculated (day 0 on Fig. 2).

Starting from day 2 complete removal of 3-CA, in all inoculated reactors was obtained until day 30. At day 30, the 3-CA removal rate in reactors A became decreased and finally the concentration of 3-CA stabilized at 100 mg/L. In the reactors B, however, complete degradation was maintained during the rest of the experiment. A mass balance calculation of 3-CA in a theoretical control reactor (with 0 % degradation) showed that the concentration of 3-CA after 14 days stabilized at 160 mg 3-CA/L, due to the regular addition and wash-out of 3-CA. No significant removal of 3-CA occurred in the control reactor C during the first 25 days, while only a small amount of 3-CA was removed from day 25 until the end of the experiment.

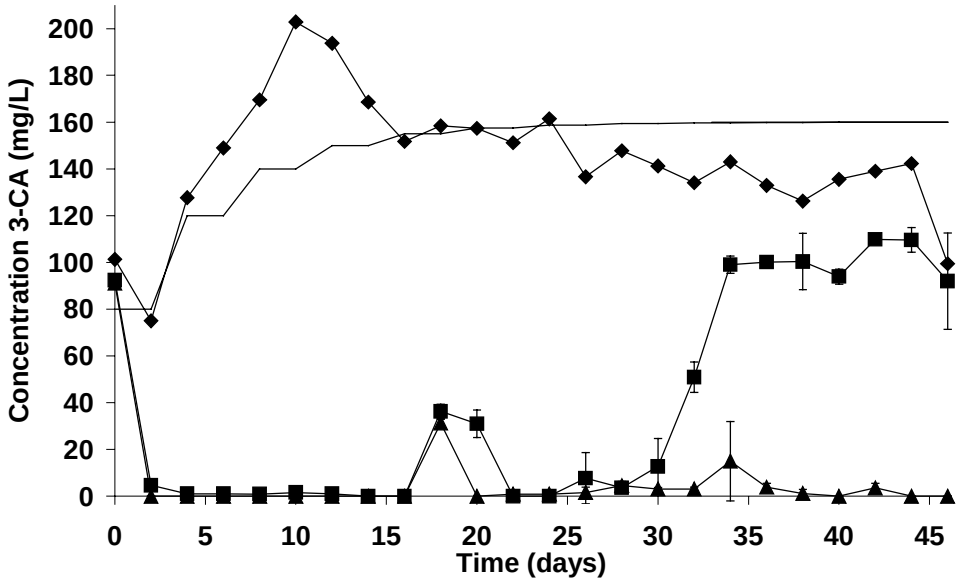

Figure 5.4. Concentration of 3-CA in the reactors A (I2*gfp*) (■), B (I2*gfp* and JMP228*gfp* (pC1-3)) (▲), and the control reactor C (♦), together with a simulation of the 3-CA concentration if no degradation would occur (theoretical control reactor) (-). Values represent the mean ± standard deviation (*n* = 2); in some cases, the standard deviations were too small to be visible.

The survival and behavior of *C. testosteroni* I2*gfp* and *R. eutropha* JMP228*gfp* (pC1-3) was monitored by plating the sludge on selective LB. During the first three weeks, the concentration of I2*gfp* cells in reactors A did not differ much from the initial value (10^7-10^8 CFU/ml), but from day 24 until the end of the experiment, the concentration of strain I2*gfp*

decreased from 10^6 to 10^5 CFU/ml. In reactor B the concentration of I2*gfp* first rose from 9.5 $\times$ 10^6 CFU/ml at day 0 until 5.4 $\times$ 10^8 CFU/ml at day 12. After 12 days, the concentration of I2*gfp* decreased until 5.4 $\times$ 10^5 CFU/ml. Strain JMP228*gfp* (pC1-3) was not detectable after 6 days because the concentration had already dropped below 1% of the concentration of I2*gfp*, which made it impossible to distinguish both colony morphologies (data not shown).

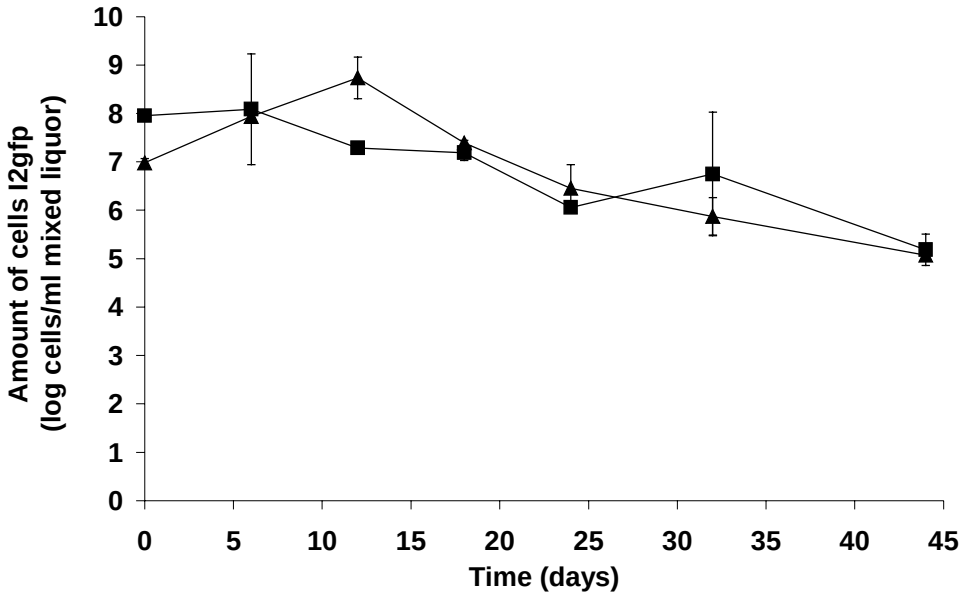

Figure 5.5. Cell counts of *C. testosteroni* I2*gfp* in the second test in the reactor with I2*gfp* (■), with I2*gfp* and JMP228*gfp* (pC1-3) (▲).

Transconjugant formation in activated sludge

The marked variant of plasmid pC1-3, i.e., pC1::gfp, was used in mating experiments with activated sludge to evaluate its intrinsic capacity to transfer from the donor strain to unknown recipient strains in the sludge. the previously used donor strain *R. eutropha* JMP228*gfp* is not a suitable donor strain, since it is also *gfp* marked and can thus not be easily differentiated from possible transconjugants. Therefore *Pseudomonas putida* strain UCW3 was chosen as a donor. This strain is auxotrophic for three amino acids and counterselection can be easily achieved by plating on a mineral medium without these amino acids.

Two different conjugation experiments were performed, i.e. a plate mating and a liquid culture mating, both in the presence and absence of 3-CA. The donor was UWC3 (pC1::gfp) and the sludge bacteria were the potential recipients. After overnight incubation,

the transconjugants were selected on the mineral medium with 3-CA as nitrogen source, sodium-pyruvate as carbon source, and kanamycin. The bacteria which were green fluorescent under a UV lamp were considered as transconjugants, since the donor, strain UWC3 (pC1::gfp) was unable to form colonies on the selective plates. Plating activated sludge that has not been in contact with UWC3 (pC1::gfp) showed that some kanamycin resistant indigenous bacteria could also grow on the selective plates. However, these colonies did not show the GFP fluorescence. In Table 5.2 the number of donors, transconjugants and transfer frequency are shown.

Table 5.2. Number of CFU /ml and error for transconjugants and donors (n=2), and the transfer frequency of plasmid pC1::gfp.

	3-CA	Transconjugants	Donors	Transfer frequency
Plate mating	+	$(5.3 \pm 0.5) \times 10^{2}$	$(1.0 \pm 0.2) \times 10^{8}$	5.3×10^{-6}
	-	$(4.1 \pm 0.5) \times 10^{2}$	$(4.0 \pm 0.4) \times 10^{7}$	1.0×10^{-5}
Liquid mating	+	$(1.7 \pm 0.3) \times 10^{2}$	$(2.8 \pm 0.4) \times 10^{7}$	6.1×10^{-6}
	-	$(3.6 \pm 1.8) \times 10^{1}$	$(9.5 \pm 2.1) \times 10^{6}$	3.8×10^{-6}

+ : 3-CA added to the mating; -: no 3-CA added to the mating

The frequencies of transfer are very similar for the agar based conjugation and the conjugation in the liquid medium. 3-CA did not have a positive effect on the transfer frequencies. The determination of the transfer frequency in the liquid medium without 3-CA was probably not very accurate, since the number of transconjugants and donors showed a large standard deviation. It seems that the number of donor cells decreased rapidly without the selective advantage of 3-CA. Moreover, the number of transconjugants and the number of donors were very low after 24 hours in the liquid mating without 3-CA added.

In conclusion the batch experiments showed that plasmid pC1::gfp could be transferred to the indigenous sludge bacteria. In liquid batch culture, the addition of 3-CA during the mating had a slightly positive effect on the number of transconjugants formed after 24 h.

DISCUSSION

The co-inoculation of *Comamonas testosteroni* I2*gfp* with *Ralstonia eutropha* JMP228*gfp*, harboring a 3-CA catabolic plasmid, significantly prolonged the 3-CA degradation capacity of the activated sludge community. Thus, the two strains together could improve the 3-CA removal compared to I2*gfp* alone. We hypothesize two possible reasons to explain the success of co-inoculation: (i) transfer of plasmid pC1-3 occurred from strain JMP228*gfp* (pC1-3) and allowed the indigenous sludge bacteria to degrade 3-CA, even though pC1-3 codes only for the first steps; (ii) an as yet unknown synergistic effect between the two strains caused a better degradation than when both strains were inoculated separately.

Successful bioaugmentation mainly depends on the behavior of the inoculated strain in the environment where it is introduced (35, 110, 328). The donor of the plasmid pC1-3, *Ralstonia eutropha* JMP228*gfp*, did not survive well in the activated sludge microbial community. This was in contrast with the good survival of strain I2*gfp* in the SCAS reactors. The origin and the type of inoculated strain plays an important role in the survival of the strain. *R. eutropha* JMP228*gfp*, originating from *R. eutropha* JMP134 (79), was isolated as a herbicide degrading soil bacterium and may therefore be less adapted to an activated sludge ecosystem. Tchelet et al (293) used *Pseudomonas* sp. P51, originally isolated from sediments, for a bioaugmentation experiment in a soil column and sewage sludge. The survival and activity of strain P51 in the soil column was successful, but the strain was not able to maintain itself in the sludge reactors and thus no degradation was observed. McClure et al. (199) showed that a sludge isolate AS2 was able to reach a stable level after inoculation and was able to increase the degradation rate of 3-CB, in contrast to other inocula tested. The authors suggested that adaptation to a particular ecosystem is an important condition for survival. Strain I2*gfp*, used in this study, was originally isolated from activated sludge and showed the capability of changing its cell surface (35). This cellular response probably improved its survival in activated sludge. Thus, a strains survival in a particular ecosystem mainly depends on its ability to adapt to the particular environmental conditions.

In this study, a new bioaugmentation strategy was tested to improve enhanced 3-CA removal in activated sludge by co-inoculating a 3-CA metabolizing *C. testosteroni* strain I2*gfp* and *R. eutropha* JMP228*gfp* (pC1-3), able to use 3-CA as sole nitrogen source, encoded on the plasmid. The initial sludge microbial community was not able to effectively degrade 3-CA, as was clearly demonstrated by the non-inoculated control reactors from both experimental runs and confirmed previous results (35). Apparently the natural level of the

indigenous 3-CA degraders was too low to substantiate the degradation of 3-CA. To distinguish the inoculated strain from similar indigenous sludge bacteria, both strains were chromosomally marked with the *gfp* gene. The plating method, combined with the *gfp* visualization with UV light, allowed a sensitive and reliable monitoring of the survival of the inoculated strains. Both inoculants could be distinguished based on its colony morphology. The visual discrimination was confirmed by testing randomly both colony types on mercury resistance (data not shown), since strain I2*gfp* is mercury resistant and strain JMP228*gfp* is not (35). The use of different fluorescent markers, such as GFP and DsRed (152, 296), or different antibiotic resistance genes would be more useful to discriminate between both inoculants in the future.

In both experimental runs, complete degradation of 3-CA was obtained in the co-inoculated reactors with I2*gfp* and JMP228*gfp* (pC1-3) for at least 20 en 45 days respectively in the first and second experimental run. No degradation at all occurred in the non-inoculated control reactor. In the reactors with I2*gfp* alone inoculated, complete removal of 3-CA was achieved for only a limited time. Compared with the theoretical control reactor, no degradation of 3-CA occurred in the non-inoculated control reactor C. However, during the sixth week of the control reactor in the second run, some degradation was observed in the reactor, probably due to the enrichment of indigenous bacteria with degradative capacities. It has been reported that in some cases indigenous bacteria become capable of removing xenobiotics after a long exposure time, either by metabolism or co-metabolism (200, 218, 309, 330). However, in our parallel SCAS reactors, the differences in degradation capacities between the inoculated and control reactors were striking and stable over a prolonged time period, suggesting that the bioaugmentation was effective and not ephemeral.

The improved degradation of 3-CA in the co-inoculated reactor is possible due to transfer of the catabolic plasmid pC1-3 to indigenous bacteria. The mating experiments with the marked pC1 plasmid with *gfp* showed that transfer can occur from a donor to the indigenous bacteria. Geisenberg et al. (105) showed that the broad host-range plasmids can be transferred in activated sludge at transfer frequencies ranging from 4.0×10^{-6} to 1.0×10^{-5}. These transfer frequencies are very similar with our findings. When catabolic genes are spread by plasmid seeding, the indigenous bacteria can take over the function of the inoculum and thus a more stable and complete removal of pollutants could be obtained. Recently Dejonghe et al. (77) demonstrated that accelerated degradation of xenobiotics in soil can be obtained by transconjugants, which acquired the catabolic plasmid from the inoculated donor. Springael et al also showed that inoculation of a 3-chlorobenzoate degrading *Pseudomonas*

putida BN210 carrying the self-transferable clc-element containing the clc-genes for 3CBA metabolism in a conventional activated sludge system (CAS) and a membrane separation bioreactor (MBR) lead to an increased resistance towards shock loading of 3CBA in terms of improved COD removal. The group of Springael showed that inoculation of a 3-chlorobenzoate (3CBA) degrading *Pseudomonas putida* BN210 carrying the self-transferable clc-element containing the genes for 3CBA metabolism in a conventional activated sludge system and a membrane separation bioreactor (MBR) resulted in an increased resistance towards shock loadings (107). The same group showed in a 3-chlorobenzoate degrading membrane biofilm reactor the involvement of *in situ* horizontal transfer of the clc-element from an inoculum to contaminant bacteria (278). However this improved pollutant degradation was not yet shown for catabolic plasmids in activated sludge. During the first weeks in the co-inoculated reactors, when I2*gfp* degraded all 3-CA, the plasmid pC1-3 could have been transferred to indigenous bacteria, which then took over the metabolic activity from day 30 on. Based on estimations of our previous results, the amount of 3-CA degraded by the I2*gfp* cells was estimated to be 33 mg/L•day starting from day 33 (33). This would mean that a potential degradation rate of the transconjugants of only ca. 4 mg 3-CA/L•day is sufficient to degrade the 37 mg/L 3-CA added to the reactor per day, and thus to obtain complete degradation. A second possibility is that plasmid pC1-3 is transferred to strain I2*gfp*. Mating experiments between *P. putida* UWC3 (pC1::gfp) and the wild type I2 cells resulted in transconjugant I2 (pC1::gfp) cells (data not shown). The transconjugants however showed very low 3-CA removal rates. The co-existence of a chromosomally encoded *meta*- (35) and a plasmid (pC1::gfp) encoded *ortho*-cleavage pathway (36) in strain I2 could be suppressing the 3-CA degradation. A similar suppression of the *meta* pathway by the introduction of an *ortho* cleavage pathway was earlier observed for methylanilines by Latorre et al. (175).

Marking the catabolic plasmid pC1 from CA28 was necessary for the monitoring potential plasmid transfer. However multiple attempts were necessary to obtain a stable insertion of the mini-Tn*5* transposon with the *nptII* (Km^R) and *gfp* genes in the plasmid without losing its degradation capacities. During the first step of the selection generally no problems occurred. CA28 derivates with the mini-Tn*5* transposon were obtained, capable of degrading 3-CA. In the second selection step, i.e. transferring the marked plasmid to the recipient strain JMP228n, in most cases no growth occurred on mineral medium with 3-CA as nitrogen source. At this stage, our method differed of that of Geisenber et al. (105) since the authors plated the transposon tagged cells after the first selection. Subsequently 5000 different transconjugants were tested to discriminate the strains with the transposon in the chromosome

or the plasmid. In our approach the relatively labor intensive second selection step was overcome by using the mixture of transposon tagged cells. In conclusion the GFP method is a complement to the traditional detection of transconjugants with selective plates containing antibiotics or heavy metals. The advantages of the GFP method are i) that it is a unique marker gene that is not found in activated sludge organisms; ii) that the total bacterial community can be assessed when epifluorescence or confocal scanning laser microscopy is used; and iii) that the detection of gene transfer directly, *in situ*, will make it possible to determine accurately the actual transfer rates of plasmids in natural environments.

In conclusion, the present study showed that co-inoculation of the catabolic strain I2*gfp* and plasmid pC1 prolonged drastically the total degradation of 3-CA in the SCAS reactors. This positive effect is possibly due to plasmid transfer from the donor strain towards the indigenous bacteria or to the I2gfp cells. Further research will focus on the monitoring of plasmid transfer of the newly marked plasmid pC1::gfp in the sludge and on the diversity of the new 3-CA degrading transconjugants.

ACKNOWLEDGMENTS

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CHAPTER 6

6. EFFECTS OF A 3-CHLOROANILINE SHOCK LOAD ON THE STRUCTURE AND FUNCTION OF AN ACTIVATED SLUDGE MICROBIAL COMMUNITY [††]

ABSTRACT

Bioaugmentation of bioreactors focuses on the removal of xenobiotics with little attention paid to the recovery of disrupted reactor functions such as nitrogen removal. Chloroanilines are widely used in industry as a precursor to a variety of products and are occasionally released into wastewater streams. This work evaluated the effects of a 3-CA shock load and a 3-CA degrading inoculant on activated sludge reactor functions such as nitrification, carbon removal, and sludge compaction. Changes in function were compared with the sludge community structure, in particular the nitrifying populations. DGGE and FISH characterized and enumerated the ammonium oxidising microbial community of SCAS reactors immediately after a 3-CA shock load. Two days after the 3-CA shock, the bacterial richness and numbers of ammonium oxidising bacteria decreased, ammonium accumulated and the nitrification activity did not recover over a 12-day period. In contrast, in the bioprotected reactor, nitrification was also completely inhibited at day 2, but started to recover from day 4 on. This recovery was linked to a differing community structure in the bioprotected reactor compared to the unprotected reactor. An additional organism, identified as an uncultured ß-Proteobacterium, was initially present in the bioprotected reactor as nitrification recovered but disappeared upon full recovery. The settleability of the activated sludge was negatively influenced by the 3-CA addition, with the Sludge Volume Index increasing by 230% and the filament index increasing from 1 to 3. Two days after a 3-CA shock, chemical oxygen demand (COD) removal efficiency decreased by 36% but fully recovered by day 4. In contrast, in the bioprotected reactor, no decrease of the COD removal efficiency was observed. This work found that 3-CA changed the nitrifying community structure and function. A transient community structure existed which appeared to initiate functional recovery but upon full recovery, the community resumed its initial structure.

[††] Redrafted after **Boon, N., E. M. Top, W. Verstraete, and S. D. Siciliano.** 2002. Effects of a 3-chloroaniline shock load on the structure and function of an activated sludge microbial community. Submitted.

INTRODUCTION

The biological treatment of industrial wastewaters by mixed microbial communities is often disrupted by organic (e.g. chlorinated organics, phenolic compounds, surfactants, herbicides) and inorganic (e.g. heavy metals, sulfides, ammonia) chemicals present in the waste water stream (28, 275). This disruption of biological processes results in inhibited nitrification, reduced carbon removal and modification of sludge compaction properties (28, 123). Little is known about the composition of mixed microbial communities in reactors when biological processes are disrupted by, or are recovering from xenobiotic shocks. Most investigators focus on xenobiotic degradation but in terms of the day to day functioning of the reactors, the restoration of biological activity such as ammonia removal, is of primary importance.

The aim of a wastewater treatment plant is to convert an incoming waste stream to N_2 and CO_2 as well as reduce the amount of suspended solids entering the environment. The first and most critical step in the conversion of incoming ammonia to nitrogen is performed by a defined sub-group of bacteria, namely the ammonia oxidizing bacteria. The removal of heterogeneous carbon from the wastewater stream is performed by diverse heterotrophic bacterial populations. The most important group seems to be the Proteobacteria, as they account for ca. 80% of all active bacteria found in the activated sludge (321). Sludge bulking leading to suspended solids entering the environment is a serious problem in 40-50 % of all activated sludge plants and is associated with high numbers of filamentous bacteria in the mixed liquor (319). The community composition of these diverse functional groups can be assessed by analysis of 16S rRNA (29, 183, 321) and genes, such as ammonia monooxygenase (122, 145, 169). Typically, phylogenetic analysis of reactors assesses the DNA encoding the 16S rRNA. Information on the active microbial community can be obtained by examining rRNA diversity (168) and prevalence by fluorescent *in situ* hybridisation (321).

The restoration of activity in wastewater treatment plants is a time-consuming and costly process. Thus, wastewater treatment plants contain buffering tanks or specialized microbial strains to protect the plant from chemical shocks (84) but there is no known process to protect reactors from chloroaniline shocks. Chloroanilines are released during the production of polyurethanes, rubber, azo-dyes, drugs, photographic chemicals, varnishes and pesticides (106, 157). The effect of chloroaniline on activated sludge microbial communities is not known but the non-chlorine, aniline, inhibits nitrification (95, 123). We hypothesized

that 3-CA shock loads in wastewater treatment plants will have a negative impact on wastewater treatment processes. The spontaneous removal of aromatic pollutants proceeds slowly and in the case of reactors, is not rapid enough to prevent reactor upset. In our previous work, a 3-CA degrading strain *Comamonas testosteroni* I2 was isolated from activated sludge, marked with the *gfp* gene and used in a Semi Continuous Activated Sludge (SCAS) system to biodegrade a continuous stream of 3-CA contaminated wastewater. For more than 14 days, strain I2*gfp* removed all 3-CA (35). Although strain I2*gfp* was never tested in a wastewater treatment system where a shock load occurred, we used I2*gfp* to protect SCAS reactors from 3-CA shocks.

Few reports investigated the recovery of disrupted reactor functions after bioaugmentation. Therefore, the aim of this work was to evaluate the effects of a 3-CA shock load and a 3-CA degrading inoculant on reactor functions such as nitrification, carbon removal, and sludge compaction, and to relate these possible changes in function with the structure of the activated sludge community, in particular the nitrifying populations.

MATERIAL AND METHODS

Bacterial strains

The chromosomally *gfp*-marked strain *Comamonas testosteroni* I2*gfp* originates from strain *C. testosteroni* I2 isolated from a municipal wastewater treatment plant (35, 36). Strain I2*gfp* was marked with the a mini-Tn*5* transposon, containing a wild type *gfp* and a kanamycin resistance gene (70), mineralises 3-CA, fluoresces green under UV-light and is rifampin (100 µg/ml) as well as kanamycin (50 µg/ml) resistant.

Semi-Continuous Activated Sludge (SCAS) reactors

The experiments were conducted with sludge freshly collected from a domestic wastewater treatment plant (Bourgoyen-Ossemeersen, Ghent, Belgium), according to a modified SCAS (Semi-Continuous Activated Sludge) procedure (35). In brief, every two days 200 ml of the mixed liquor was removed from the reactors (2 L plastic Erlenmeyer flasks with an active volume of 1.2 L), and 1.0 L was allowed to settle for 30 minutes of which 400 ml of the effluent was removed for analysis. 600 ml of synthetic influent (skimmed milkpowder; Gloria, Nestlé, Belgium; volumetric loading rate of 1 g COD/L·d; COD/N/P ratio equal to 100/6/1) was added to obtain again an active reactor volume of 1.2 L. The SCAS reactors operated with a hydraulic retention time (HRT) of 4 days, a sludge retention time (SRT) of 12 days and contained 4 g/L suspended solids (SS). To analyze sludge volume (SV) one litre of the mixed liquor settled for 30 minutes in an Imhoff cone (115).

All reactors were operated for 6 days before the experiment to allow the reactors to stabilize. At day 0, Reactors A (n=2) continued to receive only milk powder and were control reactors. Reactors B (n=2) and C (n=2) both received at day 0 a shock load of 300 mg 3-CA (Fluka AG Chemische Fabrik, Buchs, Switzerland; 99% pure), resulting in a final concentration of 250 mg /L in the reactor mixed liquor. Reactors C were also inoculated with *C. testosteroni* I2*gfp.*

The inocula were grown overnight at 28 °C in 5 ml Luria Broth (LB) medium (1 L contains 5 g NaCl, 10 g trypton and 5 g yeast extract) containing 100 mg 3-CA /L Subsequently, 5 ml cultures were used to inoculate 200 ml LB medium + 3-CA (100 mg/L). After shaking (140 rpm, New Brunswick Scientific) the cultures overnight at 28 °C, they were centrifuged (1 min. at 5000 × g) and washed twice with saline (0.85% NaCl) and resuspended finally in saline. Reactors C were inoculated with *C. testosteroni* I2*gfp* to a final concentration of $(5.4 \pm 0.37) \times 10^8$ cells/ml.

Sampling

Every two days a sample was taken for HPLC-analysis, for DNA- and RNA-extraction, for FISH analysis, for plate counts of strain I2*gfp*, as well as the determination of suspended solids (SS) and sludge volume index (SVI) (115). For DNA and RNA isolation, aliquots of the samples were immediately frozen at -20°C and -80°C respectively. For *in situ* hybridization, a subsample of activated sludge was fixed overnight with 4% paraformaldehyde (8).

Bacterial counts

Sludge flocs were dispersed by purging a 1 ml sample 20 times through a sterile 1 ml syringe with a sterile needle (1.2 × 40 mm). LB agar medium, supplemented with rifampin (Ducheva, Haarlem, The Netherlands) (100 mg/L) and kanamycin (Ducheva, Haarlem, The Netherlands) (50 mg/L) was used to count I2*gfp*. By using the GFP-fluorescence (excitation at 396/476 nm, emission at 508 nm) it was possible to detect the *gfp*-marked inoculated strains under long-wavelength UV light (TLD 18W/08, Philips, The Netherlands).

Analytical methods

The effluent was analysed for 3-CA content by reversed phase HPLC after centrifugation at 5000 × g for 10 min. The Summit HPLC system (Dionex, Wommelgem, Belgium) consisted of a Dionex Pump Series P580, a Dionex Autosampler Model ASI-100 (injection volume is 20 µl), a STH585 Column Oven (at 28°C), a Dionex UV/VIS Detector UVD 340S and a Chromeleon software system version 6.10. A Hypersil Green Env column (150 mm × 8 mm ID; 5 µm particle size; Alltech, Deerfield, IL, USA) was used. The mobile phase consisted of CH_3OH/ 0.1% H_3PO_4 (ratio 70/30), with a flow rate of 0.8 ml/min. The UV-detector was used at 210 nm.

The effluent was analysed for nitrite, nitrate and ammonium content by ion chromatography after centrifugation at 5000 × g for 10 min. and filtering through a 0.45 µm filter. The DX-600 system (Dionex, Wommelgem, Belgium) consisted of a Dionex Pump GP50, a Dionex Autosampler Model AS50 (injection volume is 100 µl), a Dionex ED50 Electrochemical Detector and a PeakNet 6 software system version 6.10. Ionpac AS9-HC (250 mm × 4 mm ID; 9 µm particle size; Dionex) column and Ionpac CS12-HC (250 mm × 4 mm ID; 8 µm particle size; Dionex) were used for anion and cation separation respectively. The mobile phase consisted of Na_2CO_3 (9 mM) and methanesulfonic acid (20 mM) for anion

and cation analysis respectively, with a flow rate of 1 ml/min. The residual COD and SVI of the effluent were determined following standard methods (115).

DNA and RNA extraction from activated sludge

Total DNA extraction from the sludge samples and PCR conditions were based on the protocols described previously (35). The total RNA extraction protocol was adapted from Griffiths et al. and Kowalchuk et al. (116, 167). Briefly, in a 2 ml Eppendorf tube 0.5 g RNase-free 0.1-mm diam. Zirconia/silica beads, 0.5 ml activated sludge, 0.5 ml CTAB buffer (Hexadecyltrimethylammonium bromide 5% wt/vol, 0.35 M NaCl, 120 mM potassium phosphate buffer, pH 8.0) and 0.5 ml of phenol – chloroform – isoamyl alcohol mixture (25:24:1) were homogenized 3 × 30 s at 5000 rpm in the Beadbeater with 10 s between shakings. Eppendorf tubes were spun (5 min, 3000 × *g)* and 300 µl of the supernatant transferred to RNase free Eppendorf tube. Another 500 µl of CTAB buffer was added to the sludge suspension and homogenized again for 3 × 30 s at 5000 rpm in Beadbeater with 10 s between shakings. 300 µl of the supernatant was added to the 300 µl taken from the first extraction for a total of 600 µl in a RNase free Eppendorf tube. The phenol was removed by mixing with an equal volume of chloroform-isoamyl alcohol (24:1), inverted and centrifuging for 10 sec. The aqueous phase was transferred to a new Eppendorf tube and nucleic acids precipitated with 2 volumes of 30% (wt/vol) polyethylene glycol 6000- 1.6 M NaCl for 2 h at room temperature. Subsequently the Eppendorf tube was centrifuged at 18000 × g in a refrigerated centrifuge at 4 °C for 10 min. The nucleic acid pellet is then washed in ice cold 70% (vol/vol) ethanol and dried under vacuum for 10 minutes prior to resuspension in 100 µl RNase free water. To obtain pure RNA a RQ1 RNase-Free Dnase treatment was performed according to the manufactures instructions (Promega, Madison, WI, USA). The average RNA yield was 3,4 ng/ml of activated sludge.

RT-PCR and PCR

The RNA was reverse transcribed to cDNA using the M-MLV Reverse Transcriptase (Promega). Briefly, for each sample, 1 µl of 10 mM dNTP's (Sigma-Aldrich Chemie, Steinheim, Germany), 1 µl of random primers (Sigma), 1 µl of extracted RNA and 7 µl of Dnase and Rnase free filtersterile water (Sigma) were mixed and incubated at 70 °C for 10 min, and placed on ice. To each sample, a mixture was added containing 4 µl 5 × first strand buffer (Promega), 2 µl 0.1 M DTT (Promega), 1 µl M-MLV Reverse Transcriptase enzyme

(Promega), 0.5 µl Rnase inhibitor (Sigma) and 2.5 µl of Dnase and Rnase free filter sterile water (Sigma). The sample, containing a final volume of 20 µl was incubated subsequently at 25 °C for 10 min., 37 °C for 3 h. and 94 °C for 10 min. The cDNA was stored at 4°C (short storage) or -20°C (long storage).

One µl of the extracted DNA or cDNA was amplified by PCR in a volume of 50 µl PCR mixture with a 9600 thermal cycler (Perkin-Elmer, Norwalk, CT, USA). The PCR mixture contained 0.5 µM of each primer, 200 µM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 10 µl of Thermophilic DNA Polymerase 10X Reaction Buffer ($MgCl_2$-free), 2.5 U of *Taq* DNA Polymerase (Promega, Madison, WI, USA), 400 ng/µl of bovine serum albumin (Boehringer), and Dnase and Rnase free filter sterile water (Sigma) to a final volume of 100 µl. The 16S rRNA or rDNA for all Eubacteria were amplified by PCR using the forward primer P338F (5'-ACT-CCT-ACG-GGA-GGC-AGC-AG-3'-forward) and the reverse primer P518r (5'-ATT-ACC-GCG-GCT-GCT-GG-3'-reverse) (208, 221). A GC-clamp of 40 bp (208, 221) was added to the forward primer. The PCR program consisted of 10 min 95°C; 30 cycles of 1 min. 94°C, 1 min. of 53°C, 2 min. of 72°C; and a final elongation for 10 min. at 72°C. The 16S rDNA for the ammonium oxidizing bacteria of the β-Proteobacteria were amplified by PCR using the forward primers CTO189fAB (5'-GGA-GRA-AAG-CAG-GGG-ATC-G-3'), CTO189fC (5'-GGA-GGA-AAG-TAG-GGG-ATC-G-3') and reverse primer CTO653r (5'-CTA-GCY-TTG-TAG-TTT-CAA-ACG-C-3') (167). The PCR program consisted of 10 min 95°C; 35 cycles of 1 min. 94°C, 1 min. of 57°C, 2 min. of 72°C; and a final elongation for 10 min. at 72°C. In order to increase the sensitivity and to facilitate the DGGE by analyzing fragments of the same length, a nested PCR technique was applied as described previously (34). During the second PCR round, the fragments amplified with the CTO primers were re-amplified by using the Bacterial primers P338F and P518r (see above). Since the ammonium oxidizing bacteria of the β-Proteobacteria belong to the domain of the Bacteria, the Bacterial primer set P338F and P518r used in the second PCR round reamplifies all fragments obtained after the first PCR round. In the first PCR round, a clearly visible band was present (first PCR product was at least 0.3 % of the total extracted DNA or cDNA), so that no aspecific amplification is expected (34). As a negative control, the DNA'se treated RNA was subjected to a PCR, and no amplification was observed, indicating that all DNA was removed from the samples. After each PCR amplification round, the size of the PCR product was verified on a 1 % agarose gel.

DGGE analysis

DGGE (Denaturing Gradient Gel Electrophoresis) based on the protocol of Muyzer et al. (208) was performed using the Bio-Rad D Gene System (Bio-Rad, Hercules, CA, USA). PCR fragments were loaded onto 8% (w/v) polyacrylamide gels in 1 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4). The polyacrylamide gels were made with denaturing gradient ranging from 50 to 65 % (where 100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run for 17 hours at 60°C and 38 V. Staining and analysing the gels were done as described previously (35). The processing of the DGGE gels was done with the Bionumerics software 2.0 (Applied Maths, Kortrijk, Belgium). The bandclasses of the DGGE patterns were exported to EstimateS (57), a program which allows statistical analysis of species richness from samples by calculating the Chao2 index (58) The formula used to calculate the index is Chao2 = S_{obs} + ($L^2/2M$), where S_{obs} is the observed number of species in a sample, L is the number of species that occur only in one sample ('unique species'), and M is the number of species that occur in exactly two samples. This is a incidence-based nonparametric estimator that uses presence-absence data and can be used with DGGE data to estimate total richness, making it a suitable index for PCR based analysis (142, 289).

***In situ* hybridization**

Sludge samples were fixed with fresh 4% paraformaldehyde solution, washed with phosphate-buffered saline (PBS), and stored in PBS-ethanol (1:1) at -20°C until further processing (8). *In situ* hybridisations were performed with fluorescent labelled, rRNA-targeted oligonucleotide probes according to the method of Manz et al. (194) and Biesterfeld et al (27). The following oligonucleotide probes were used: (i) NSO190, specific for the ammonia oxidizers in the ß subclass of *Proteobacteria* (204); and (iii) a mix of EUB338, EUB338-II and EUB338-III, specific for the domain *Bacteria* (65). Probes labelled with fluoresceine and the sulfoindocyanine dye Cy3 were obtained from Interactiva (Ulm, Germany).

Microscopy

Samples were analyzed by standard epifluorescence microscopy on a Zeiss Axioskop II microscope (Carl Zeiss, Jena, Germany). The microscope was equipped with a Peltier cooled single chip digital colour CCD camera (Hamamatsu Orca IIIm, Hamamatsu, Massy

Cedex, France) connected to a PC to obtain digital images. Image analysis was performed with MicroImage 4.0 (Media Cybernetics, Silver Spring, USA).

The abundance of filamentous micro-organisms was quantified by the Filament Index (FI) (85). The FI index is a measure of the number of filaments in activated sludge and it has a scale of 0 to 5 (from none to very many filaments). There is a difference of approximately a factor 10 between the consecutive classes and the FI is established by comparing the microscopic image with a series of reference photographs of the various FI classes.

DNA sequencing

16S rDNA gene fragments were cut out of the DGGE gel, reamplified and sent out for sequencing. DNA sequencing of the ca. 180 bp fragments was carried out by ITT Biotech-Bioservice (Bielefeld, Germany). Analysis of DNA sequences and homology searches were completed with standard DNA sequencing programs and the BLAST server of the National Center for Biotechnology Information (NCBI) using the BLAST algorithm (5).

Nucleotide sequence accession numbers

Nucleotide sequences for bands 1 to 5 have been deposited in GenBank database under accession from AF479281 to AF479285.

RESULTS

SCAS reactors performance

Two days after inoculation, the I2*gfp* cells had removed all detectable 3-CA (Figure 6.1A). The I2*gfp* cell numbers decreased approx. 3 log units after inoculation, and stabilized after 6 days at 10^5 colony forming units (CFU)/ml (Figure 6.1B).

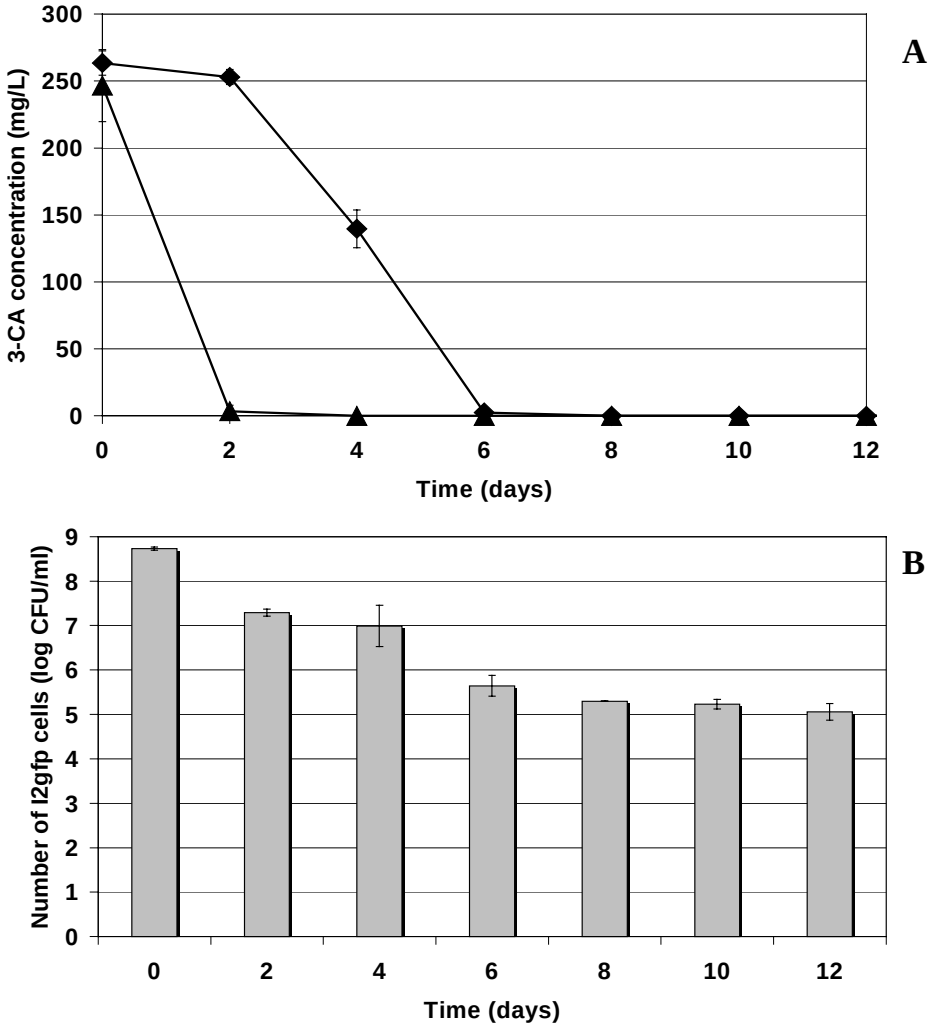

Figure 6.1. Reactors B were not inoculated but exposed to a 3-CA shock load (♦), bioprotected reactors C were inoculated and exposed to a 3-CA shock load (▲). Panel (A) concentration of 3-CA in reactors B and C in the effluent; (B) survival of *C. testosteroni* I2*gfp* cells in reactors C.

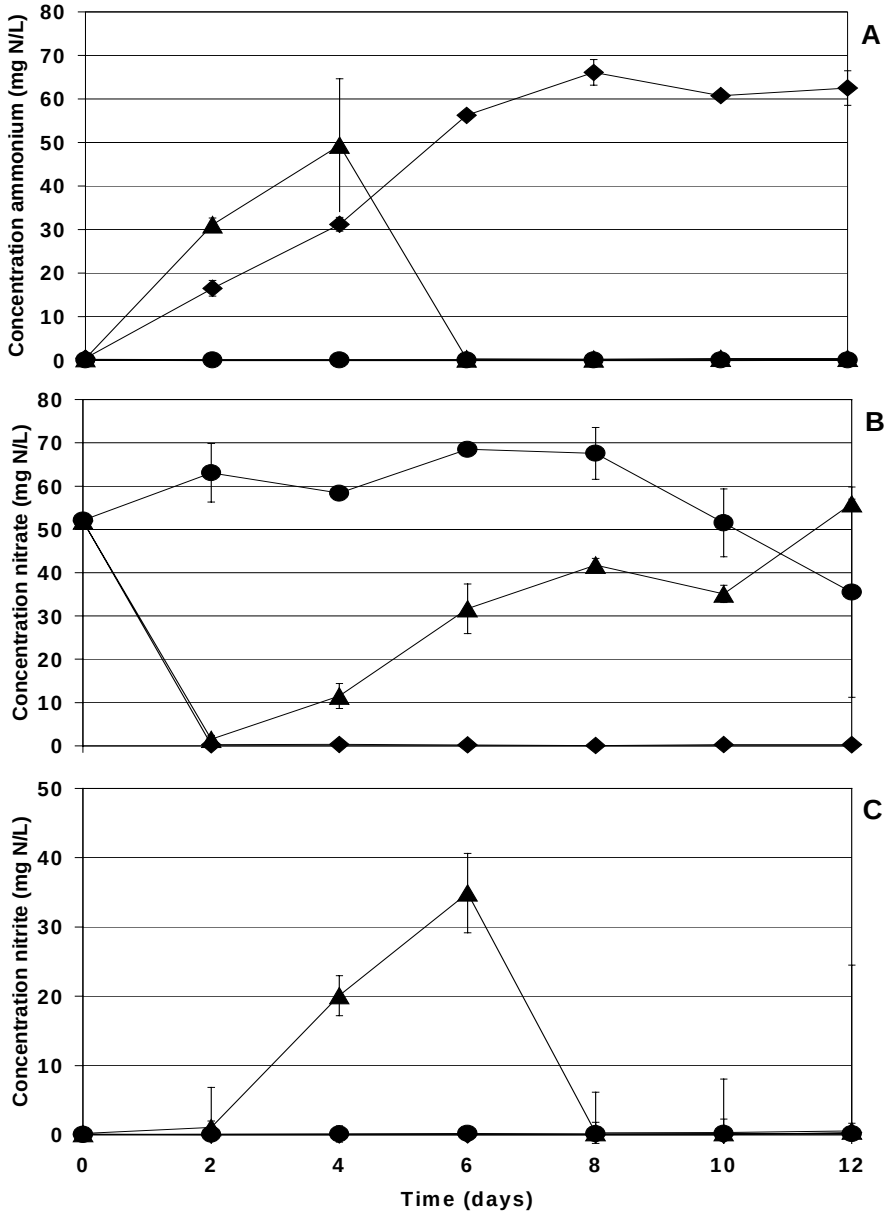

Figure 6.2. Reactors A were not inoculated and no 3-CA shock was applied (●), reactors B were not inoculated but exposed to a 3-CA shock load (♦), bioprotected reactors C were inoculated and exposed to a 3-CA shock load (▲). Panel (A) ammonium concentration in the effluent; (B) nitrate concentration in the effluent; (C) nitrite concentration in the effluent. Values represent the mean ± error bars of two reactors (n = 2); in some cases, the error bars were too small to illustrate.

In reactors without I2*gfp*, 3-CA persisted until day 6 and this removal was due solely to the wash out (hydraulic retention time of the system is 4 days) and not to (bio)degradation. The total dose of 3-CA to which the microorganisms were exposed, was calculated as the concentration of 3-CA, multiplied with its contact time (time·concentration). The bioprotected reactors were exposed three times less (6 g·h/L) compared to the non-protected reactor (19 g·h/L).

The 3-CA shock inhibited nitrification for an extended period of time despite its disappearance by day 2 in the protected and day 6 in the non-protected reactors (Figure 6.2). In non-protected reactors B, nitrite and nitrate concentration remained very low and ammonium accumulated for the duration of the experiment. From day 4 on, nitrification in the bioprotected reactors C recovered resulting in the accumulation of nitrate (Figure 6.2B) and nitrite (Figure 6.2C). The nitrite was present in reactor C until day 6 and at day 8, all the nitrite was removed. The restored nitrification activity resulted in the complete removal of ammonium from day 6 on (Figure 6.2A).

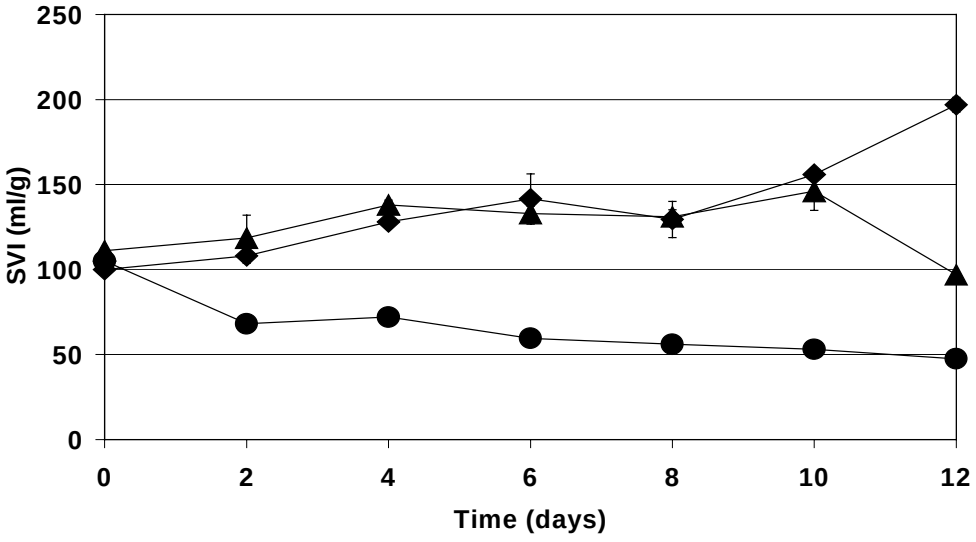

Figure 6.3. Settlement of the activated sludge, expressed as sludge volume index (SVI) of reactors A, not inoculated and no 3-CA shock applied (●), reactors B not inoculated but exposed to a 3-CA shock load (♦), bioprotected reactors C inoculated and exposed to a 3-CA shock load (▲). Values represent the mean ± error bars of two reactors (*n* = 2); in some cases, the error bars were too small to illustrate.

In both reactors that received the 3-CA pulse the sludge volume index (SVI) increased, indicating that the settlement of the activated sludge was poor (Figure 6.3). In contrast to nitrification, the SVI did not recover in the bioprotected reactor. However, at the last day of the experiment, the SVI of the bioprotected reactor C tended to go down, while the SVI of the non-bioprotected reactor was increasing further.

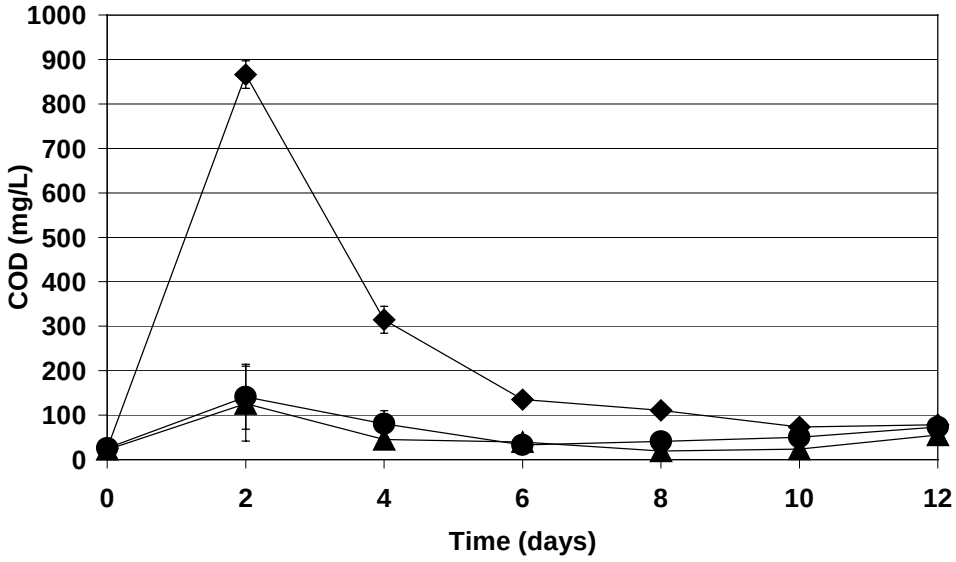

Figure 6.4. Chemical oxygen demand (COD) concentration in the effluent of reactors A, not inoculated and no 3-CA shock applied (●), reactors B not inoculated but exposed to a 3-CA shock load (♦), bioprotected reactors C inoculated and exposed to a 3-CA shock load (▲). Values represent the mean ± error bars of two reactors (*n* = 2); in some cases, the error bars were too small to illustrate.

The removal of carbon sources was evaluated by the Chemical Oxygen Demand (COD) concentration in the effluent (Figure 6.4). The non-bioprotected reactor B accumulated high amounts of COD, however from day 4 on the COD removal rate increased again. Still, some COD removal was not completely inhibited at day 2 in the non-bioprotected reactor B. The bioprotection of reactor C was very effective, since no differences in COD removal with the control reactors A were observed.

DGGE analysis

In order to relate the observed changes in function to changes in the community structure and diversity, DGGE analysis based on DNA as well as RNA was performed using bacterial primers and primers, specific for the AOB community. Since days 0, 2 and 8 were considered to be typical for the microbial community with day 0 representing the community just before disruption, day 2-after distortion (inhibition of certain functions) and day 8-recovery, these samples were analyzed in detail. On day 8, in the bacterial RNA-DGGE patterns of the non-disturbed reactors (A) and the bioprotected reactors (C), there is a prominent band (band 1, Figure 6.5), with a sequence having low similarity to members of the Cytophaga-Flavobacter group (146/168 bp with the uncultured bacterial clone AF369713.1; data not published; and 98/104 with the *Cytophaga* sp. AJ224415.1 (187), a fragment isolated from a nitrifying reactor system). Another band, band 2, only present in reactors receiving 3-CA (B and C), shows high similarity with *Nostocoida limicola* AF255736.1 (177/177; data not published; sequence isolated from a bulking filamentous bacterium in a reactor treating industrial waste), a Gram positive Actinomycete.

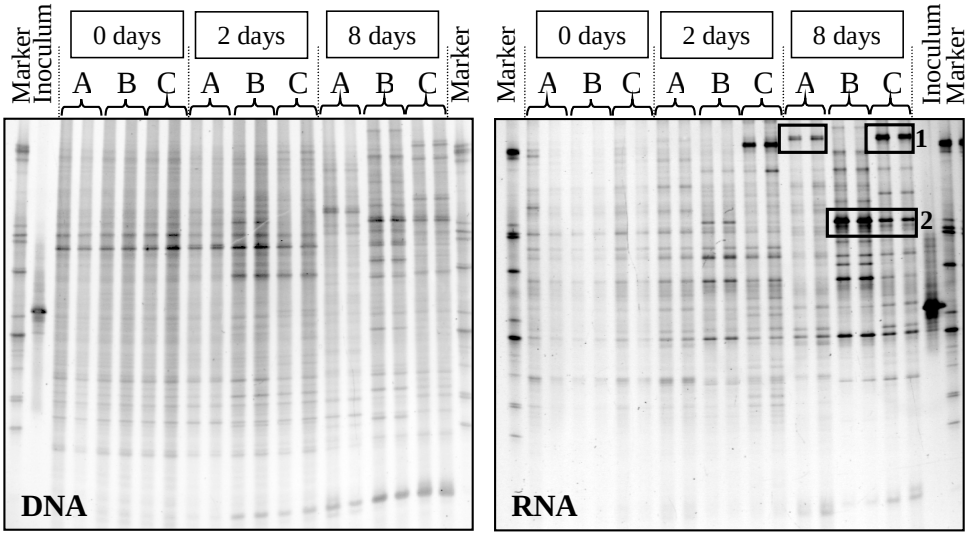

Figure 6.5. Analysis of the DGGE profiles of the different reactors at days 0, 2 and 8, using partial Bacterial 16S rRNA gene fragments (338-518), based on DNA and RNA. Bands 1 and 2 were excised and sequenced for further analysis. A, reactors without inoculant and without 3-CA shock; B, non-inoculated reactors with 3-CA shock load; and C, the bioprotected reactors with 3-CA shock load.

Since the 3-CA shock and the bioprotection had a major influence on the nitrification activity in reactors B and C, a more detailed analysis of the ammonium oxidizers was performed. At day 0 (good nitrification activity before the shock), DGGE patterns obtained with primers for ammonium oxidizing bacteria (AOB-DGGE) are very similar, even between the RNA and DNA samples (Figure 6.6). Two bands appeared to dominate the AOB community (Figure 6.6). The upper band 3 showed similarity with an uncultured *Nitrosomonas* AJ245752.1 (173/173; data not published; sequence obtained from a selective enrichment of ammonia-oxidizing bacteria from a sediment) and with an uncultured ß-Proteobacterium AJ299051.1 (177/177; (32); sequence of an ammonia oxidizer enriched from a freshwater sediment), while the lower band 4 matched with a *Nitrosococcus/Nitrosomonas* (174/177 bp identity with *Nitrosococcus mobilis* AF287297.1 (234), AJ298728.1 (3), M96403.1 (119) and *Nitrosomonas* sp. AF272415.1 (234)). Some other bands were also present, however they seemed to play only a minor role since no effect of the treatments were visible. Two days after the shock load, nitrification was inhibited in reactors B and C, but the AOB community structure differed between these two reactors. On day 2 in reactor B, band 3 on the RNA-DGGE had decreased from a relative intensity of 12,5 % (day 0) to 4,8 % (intensity of the band, divided by the sum of all band intensities of the respective lane). Band 4 decreased from a relative intensity of 22,4 (day 0) to 16,7 (day 2). Moreover, after 8 days, both the bacteria corresponding to band 3 and 4 remained at low ribosomal concentrations and were hardly visible on the DGGE patterns of reactors B. At day 2 in the bioprotected reactor C, both bacteria of bands 3 and 4 kept a high number of ribosomes (relative intensities of 11,8 and 14,5 respectively), however a third species (band 5), identified as an uncultured ß-Proteobacterium became more active (relative intensity of 18,2). This band was highly similar (171/172 bp identity) with a sequence submitted in Genbank by Speksnijder, A.G.C.L. and Laanbroek, H.J (AJ245760.1: Diversity of indigenous and competition with introduced ammonia-oxidising bacteria in freshwater sediments as revealed by selective enrichment and 16S rDNA analysis; unpublished data),. By day 8, nitrification was restored in this reactor, RNA-DGGE profiles were similar to their original pattern and the additional band 5 of the uncultured ß-Proteobacterium disappeared.

The DGGE patterns for all bacteria, based on RNA, contain less bands than the DNA based DGGE patterns (Figure 6.5). Based on the Chao2 richness estimation, the DNA patterns of the control reactor on day 0 had a Choa2 richness of 55,1 ± 3,7, while the RNA patterns of the same day only had a Chao2 index of 34,1 ± 3,8.

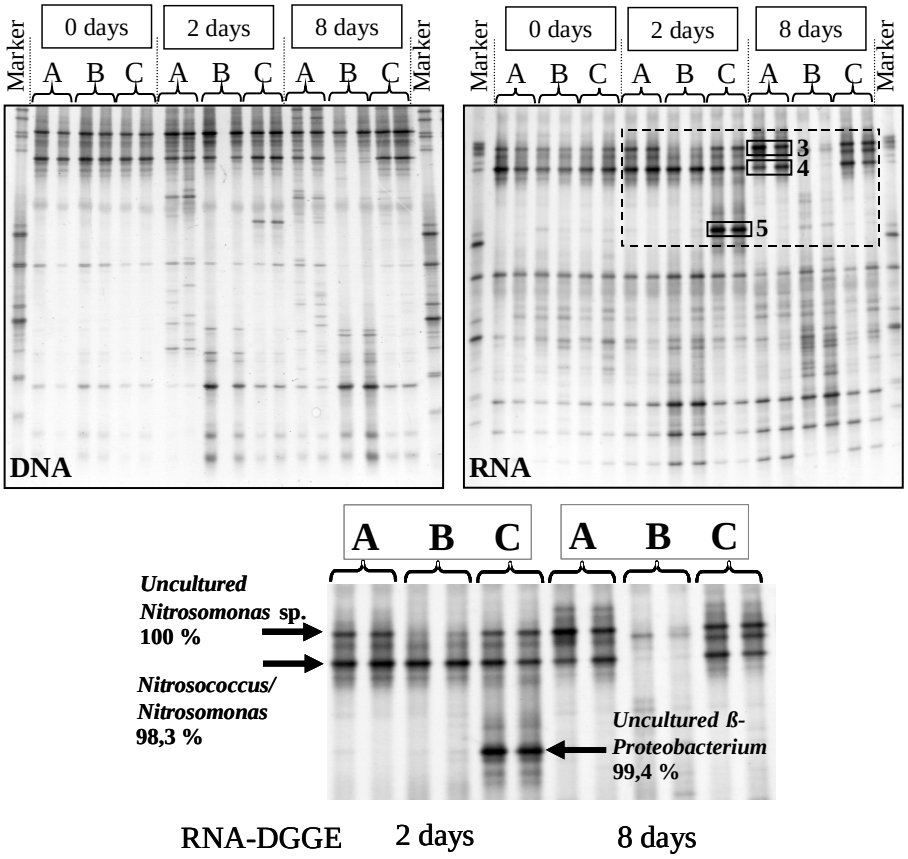

Figure 6.6. Analysis of the DGGE profiles at days 0, 2 and 8 of the different reactors, using partial 16S rRNA gene fragments, obtained from extracted DNA and RNA. The gene fragments were obtained by using an AOB specific PCR with CTO primers, followed by a second PCR with bacterial primers (338-518). Bands 3, 4 and 5 were excised and sequenced for further analysis. A, reactors without inoculant and without 3-CA shock; B, non-inoculated reactors with 3-CA shock load; and C, the bioprotected reactors with 3-CA shock load. Bands 3, 4 and 5 were excised and sequenced for further analysis. The area, surrounded by the dotted rectangle, is shown in detail at the bottom. The accession numbers of the most similar sequences in Genbank are mentioned in the text.

The same was observed for days 2 ($Chao2_{DNA}$= 37,2 ± 2,3; $Chao2_{RNA}$= 20,0 ± 0,2) and 8 ($Chao2_{DNA}$= 40,8 ± 3,2; $Chao2_{RNA}$= 23,3 ± 0,8). The differences between DNA and RNA indices suggest that ca. 55 % of the bacteria present in the activated sludge were not active. It

is also possible that the RNA DGGE analysis is confounded by a single strain that produces large amounts of rRNA and thus many active species fall below the 1 % detection limit (208). In contrast, on day 0 the RNA samples of the AOB-DGGE's show a higher Chao2 richness than the DNA samples (Chao2 index of 14,0 ± 0,5 versus 7,0 ± 0,0) (Figure 6.6). The $Chao2_{RNA}$ index of the AOB community (on day 8) increased in the inhibited reactor (B), from 14,0 ± 0,5 to 24,6 ± 2,9 compared to 16,2 ± 0,7 and 15,0 ± 0,0 for reactors A & C respectively. These differences in richness may be controlled by the relative decrease of the dominant bands, so that the minor rRNA sequences became detectable by the PCR amplification and DGGE analysis.

Microscopy and Fluorescent in situ Hybridisation (FISH)

FISH was used to examine the prevalence of the AOB in the activated sludge (Figure 6.8). At day 0, clusters of AOB could be observed in the activated sludge flocs and an area based calculation estimated that ca. 2 % of the area were AOB (Figure 6.7). By day 8, AOB clusters were only detected in the reactors where nitrification was observed, i.e., reactors A (1,7 %) and C (1,8 %), whereas in reactor B, almost no AOB flocs were observed. This indicates that the number of AOB dropped drastically (to less than 0,4 %), which confirms the interpretation formulated above based on the DGGE patterns, that the community had not recovered.

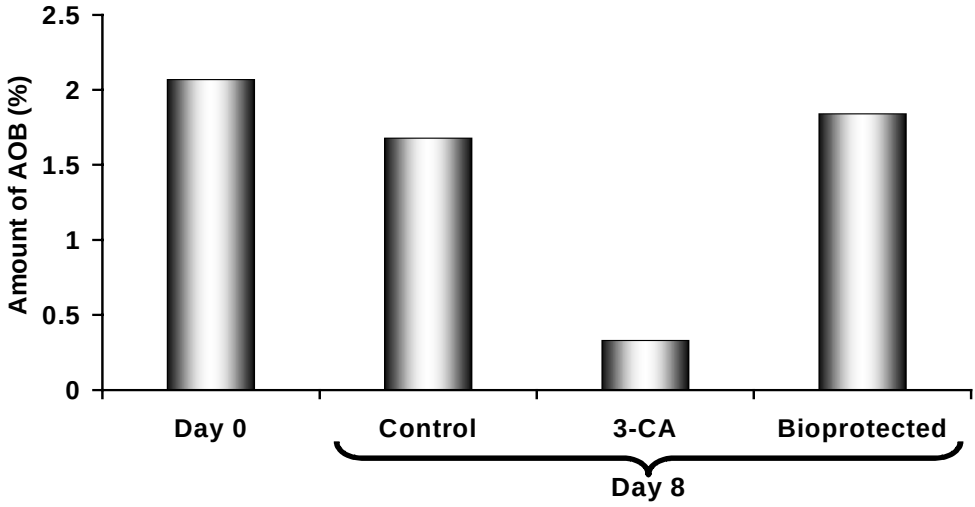

Figure 6.7. Relative amount of AOB prevalence (as % AOB area towards the total EUB area), determined by FISH analysis.

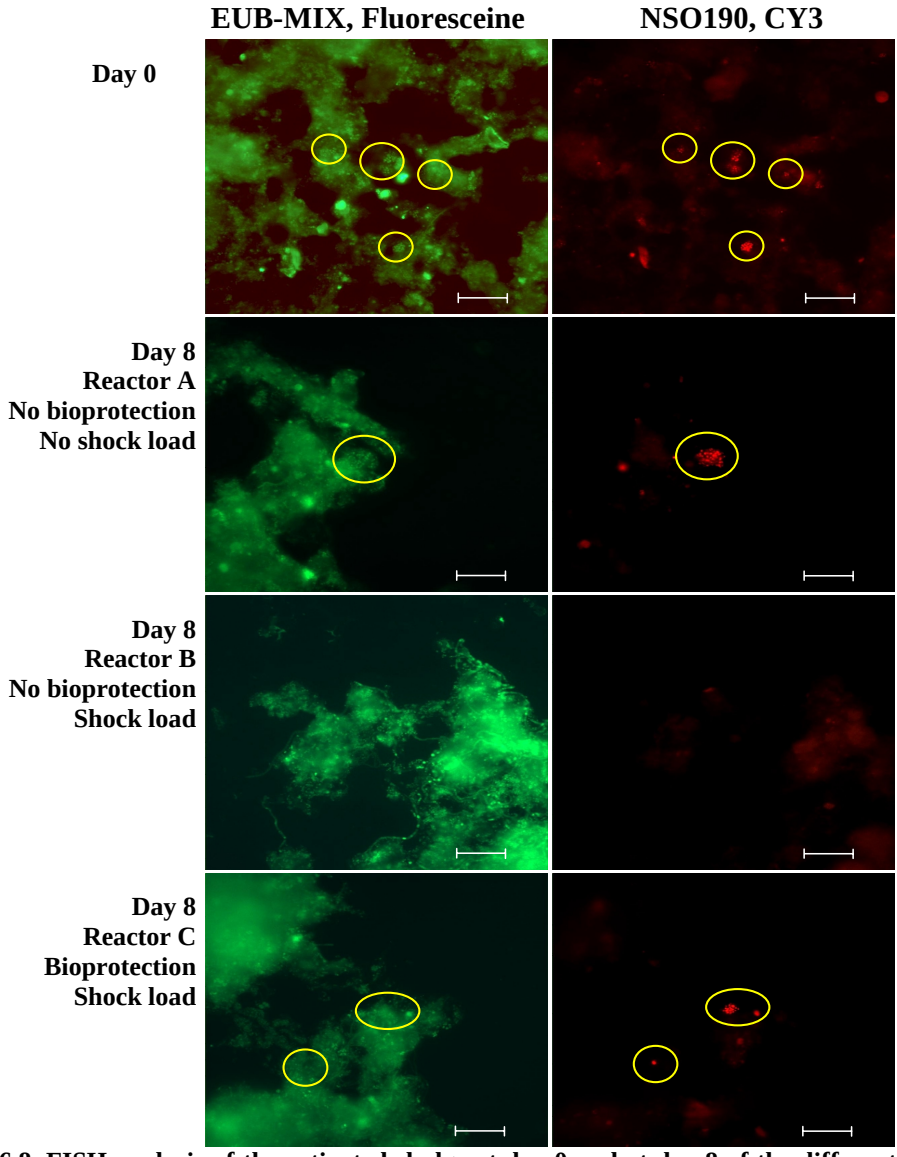

Figure 6.8. FISH analysis of the activated sludge at day 0 and at day 8 of the different reactors: the Eubacteria are labeled with fluoresceine (EUB-MIX) and the AOB with CY3 (NSO190); bar = 25 µm.

Light microscopy explained the higher SVI values of reactors B and C (Figure 6.10). The number of filaments in these reactors increased, as seen by an increase in Filament Index (FI) from 1 (day 0) to 3 (day 8), compared to a FI of 1 in the control reactor (Figure 6.9).

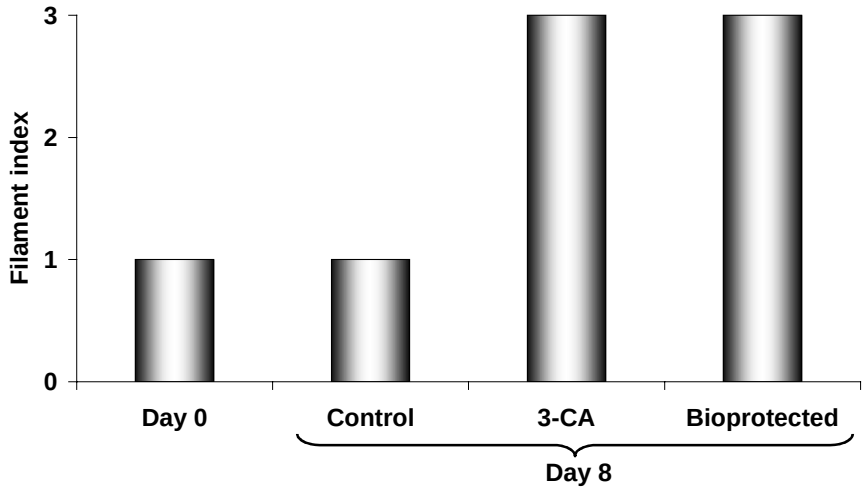

Figure 6.9. Filament index of the different reactors at days 0 and 8.

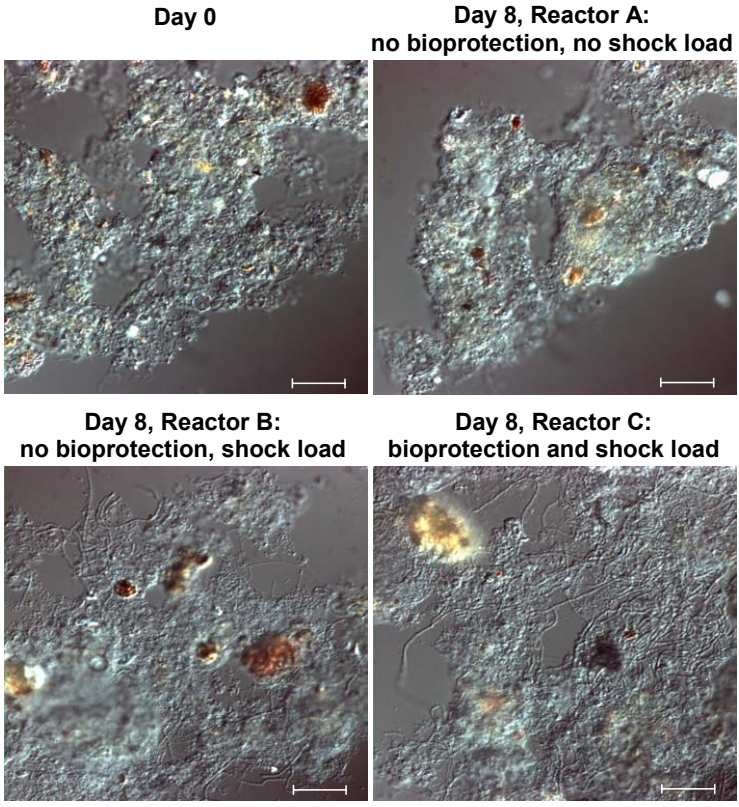

Figure 6.10. Light microscopic images of the activated sludge at day 0 and at day 8 of the different reactors. Notice increased number of filaments in reactors B and C at day 8; bar = 25 µm.

DISCUSSION

We investigated the effects of a 3-chloroaniline (3-CA) shock load on the basic functions of a wastewater treatment reactor and focused on the functional recovery. Moreover, bioaugmentation was used to investigate if rapid 3-CA removal could decrease the recovery period. The most drastic effect of the 3-CA shock load was observed on the nitrification activity. Initially, nitrification was totally inhibited in the two reactors B and C where a 3-CA shock load was applied. The non-substituted form of 3-CA, aniline is known to inhibit nitrification (95, 106) and like most inhibitors, aniline inhibits nitrification activity by acting as a suicide substrate for the ammonia monooxygenase (198). It seems likely that 3-CA will work in a similar fashion (D.J. Arp, personal communication). As a consequence, the re-establishment of the nitrification activity in the activated sludge after the loss of the inhibitor requires *de novo* synthesis of the enzymes. After two days in the bioprotected reactors C, 3-CA was degraded completely, allowing the slow recovery of the nitrification, visible from day 4 on (Figure 6.2), however during four days nitrite accumulated. The temporal accumulation of nitrite is normal during the start-up period of nitrification (178, 316).

In the RNA-DGGE profile of the AOB community, the additional band 5 appeared at day 2 in the bioprotected reactor C and was dominant. Based on DNA-DGGE patterns of that day, the species corresponding to this extra band never became the most dominant but could be observed as a clear band. The exact reason why the corresponding species became active is not clear, however it is possible that the rRNA content of the two initial dominating AOB (bands 3 and 4) decreased, so that the new band became visible (band 5). Probably just after the 3-CA removal, this species benefited from the inhibition of initial dominating AOB (bands 3 and 4) and became more active, since a suitable niche became available, i.e. ammonium oxidation. However the activity or the number of cells of the extra species was too low to see the effect on nitrification. From day 4 on, the nitrification activity was restored by the original AOB (bands 3 and 4) and the species corresponding to band 5 appeared to be out-competed. In the non-bioprotected reactor B this additional species was never visible. In the reactors B the 3-CA was present for 4 days and thus the whole nitrifying community was inhibited during this time. Although all the 3-CA was washed out by day 6, no nitrification recovery could be observed. A contact time of 4 days with 3-CA seemed to be critical for the AOB and on the RNA-DGGE, a total collapse of the AOB community at day 8 was visible. Also the FISH analysis showed the effect of the 3-CA exposure, since hardly any AOB cluster could be detected in reactors B at day 8. The autotrophic nitrifying community appeared to be

so much affected by the four-days ongoing inhibition, that the amount of AOB cells was reduced, resulting in a loss of nitrification for the rest of the experiment.

After the 3-CA shock, a higher COD concentration in the effluent of the non-bioprotected reactors B was observed. The heterogeneous nature of wastewaters allows the development of diverse heterotrophic bacterial populations. From day 4 on, the COD removal was already restored; the higher COD concentration in these reactors at day 4 was in part due to the residual 3-CA concentration (ca. 150 mg/L of the extra 200 mg/L), which was gradually washed out. The effect of inoculation of *Comamonas testosteroni* I2*gfp* in the shock-loaded reactors was striking: COD removal capacity was not lowered, resulting in a full bioprotection of the activated sludge. There are different factors that can explain the increased COD in the effluent of the non-bioprotected reactor: (i) by the long 3-CA shock, the indigenous bacteria, responsible for the COD removal, could be temporarily inhibited; (ii) the protozoa, important predators of bacteria, could be inhibited, resulting in an increased number of dispersed bacteria in the effluent; (iii) the toxicity of the 3-CA resulted in the lysis of a part of the biomass; (iv) in the bioprotected reactor, strain I2*gfp* protected the other bacteria by degrading 3-CA quickly and therefore there was no visible inhibition of COD removal observed. A combination of all these factors is a possible explanation for the increased COD at day 2 in reactor B.

The higher SVI in reactors B and C was directly or indirectly a result of the 3-CA shock load. Microscopy revealed a higher number of filaments in both reactors and sequencing of a dominant band in the RNA-DGGE profiles in reactors B and C revealed the presence of an Actinomycete, i.e. *Nostocoida limicola*. The latter species has been related to the decline of the sludge settling properties in a high number of studies (179, 325) and an increased number of filaments correspond with higher SVI values (179, 192, 214).

In this study, we used DGGE analysis based on extracted DNA and RNA to evaluate changes in the sludge microbial communities. It is clear from the bacterial DGGE patterns that the number of species present was higher than the number of active ribotypes: at day 0, the bacterial richness in the RNA-DGGE gels was remarkably lower than in the DNA-DGGE. This suggests a lower bacterial richness among the active numerically dominant populations than among the total (dead, dormant, active) dominant populations. Also some very active ribotypes, visible in the RNA-DGGE, could hardly be detected in the DNA-DGGE patterns. This shows that DNA based analysis does not always reflect the real metabolic state of the microbial communities: DNA obtained form environmental samples could originate from dormant or dead cells (148), or even from free DNA. This is also reflected in the analysis of

the AOB: the RNA-DGGE reflects the nitrification activity more accurately. Based on the DNA-DGGE, only some differences in the minor bands were visible at days 0 and 2, while the RNA-DGGE clearly showed differences in the dominant bands, as discussed above. Without the knowledge of the RNA-DGGE at day 8, it would seem that only the bacterium of band 4 is responsible for the nitrification, since this band almost disappeared on the DNA-DGGE pattern when no nitrification was present. Similar observations were made for RNA-TGGE (Temperature Gradient Gel Electrophoresis) fingerprints of grassland soil bacterial communities, which were less dense than the DNA-TGGE (91). Therefore it is appropriate to be prudent when linking DNA-DGGE patterns to bacterial activity.

Previous work demonstrated that a decrease of bacterial diversity goes along with a decrease in metabolic function (84). In that study the addition of substituted phenols to an activated sludge reactor resulted in a decrease in oxygen uptake rate. Two to four days later the toxic effects were visible on DNA-TGGE: the bacterial community collapsed and the diversity (calculated by the Shannon diversity index based on the TGGE profiles) dropped drastically from 1,0 to 0,2-0,4. We did not see such a drastic effect on the bacterial community of our activated sludge, possibly because the lower toxicity of 3-CA. However, the disastrous effect of 3-CA on the nitrifying community was visible and a loss of nitrification activity resulted in a higher bacterial richness (the Chao2 index almost doubled from day 0 to day 8). In case of the AOB community of our activated sludge reactors, the following scenario could be envisioned. A stable nitrifying system contained only two or three numerically dominant ammonium oxidizing species, and thus showed a low Chao2 diversity index based on DGGE patterns. When this AOB community was disturbed, previously less fit nitrifiers (and thus invisible in the DGGE patterns) appeared to become able to compete with the dominating species and thus the apparent richness of the community increased. After the disturbance, the AOB community and function was restored, again resulting in a decreased richness with only a few dominant species.

In conclusion this study has demonstrated that a 3-CA shock load decreased the basic metabolic functions of activated sludge. Inoculation with a bacterial strain that is capable of 3-CA mineralization, protected the reactors and allowed recovery of nitrification. Some of these functional changes were explained by visible changes in the structure of the overall bacterial community and specifically of the AOB community. This work indicates that bioprotection of wastewater systems against shock loads allows a fast recovery of the metabolic functions.

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CHAPTER 7

7. EVALUATION OF NESTED PCR-DGGE (DENATURING GRADIENT GEL ELECTROPHORESIS) WITH GROUP-SPECIFIC 16S rRNA PRIMERS FOR THE ANALYSIS OF BACTERIAL COMMUNITIES FROM DIFFERENT WASTEWATER TREATMENT PLANTS [‡‡]

ABSTRACT

The diversity of different bacterial groups of activated sludge samples that received wastewater from four different types of industry was investigated by a nested PCR-DGGE (denaturing gradient gel electrophoresis) approach. Specific 16S rRNA primers were chosen for large bacterial groups (*Bacteria* and α-*Proteobacteria* in particular), which dominate activated sludge communities, as well as for actinomycetes, ammonium oxidizers and methanotrophs (Types I and II). In addition primers for the new *Acidobacterium* group were used to observe their community structure in activated sludge. After this first PCR amplification, a second PCR with Bacterial primers yielded 16S rRNA gene fragments that were subsequently separated by DGGE, thus generating "group specific DGGE patterns". The community structure and diversity of the bacterial groups from the different samples was further analyzed using different techniques, such as statistical analysis and Shannon diversity index evaluation of the band patterns. By combining the seven DGGE gels, cluster analysis, Multidimensional scaling (MDS) and Principal Component Analysis (PCA) clearly clustered two of the four activated sludge types separately. It was shown that the combination of molecular and statistical methods can be very useful to differentiate activated sludge microbial communities.

[‡‡] Redrafted after **Boon, N., W. De Windt, W. Verstraete and E.M. Top** 2002. Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. FEMS Microbiol. Ecol.: 39:101-112.

INTRODUCTION

Activated sludge of aerobic wastewater treatment plants consists of a complex mixture of microorganisms that are either generalists or specialists. For years, researchers have examined the microbial populations of these activated sludge communities in order to understand their specific biological processes (6). The presence of certain microbial groups in wastewater treatment systems can cause problems such as poor solid separation, bulking and foaming (29) and needs a more thorough evaluation. When biodiversity is studied by conventional techniques, such as cultivation of bacteria on solid media, these results are quite biased, because a majority of microorganisms are not culturable using standard techniques (10). For activated sludge the percentage of culturable bacteria in comparison with total cell counts is estimated to range between 1 and 15% (321, 322).

During the last decade, methods based on direct PCR amplification and analysis of ribosomal RNA genes were developed and allowed a more comprehensive analysis of microbial communities in comparison with cultivation based techniques. The amplified fragments of 16S or 18S rRNA genes and especially the analysis of these genes by denaturing or temperature gradient gel electrophoresis (DGGE/TGGE) have been frequently used to examine the microbial diversity of environmental samples and to monitor changes in microbial communities (62, 84, 209, 310). In a DGGE gel the number and precise position of the bands in a gel track give an estimate of the number of numerically dominant ribotypes in the sample. This approach allows a comparison of different microbial communities, but not without specific problems. The banding patterns of highly diverse microbial communities, present in soils, activated sludges and sediments, are usually very complex when Bacterial primers are used. Moreover, only the major constituents of the analyzed community are represented on these DGGE patterns and thus relatively less abundant but potentially very important species may not be detected by this molecular method (126).

A recent evolution in the direct amplification and analysis of ribosomal RNA genes is the use of specific primers, which allows to amplify and analyze the 16S rRNA genes of defined groups within a complex microbial community. The analysis of these group-specific PCR-fragments on a DGGE gel provides a valuable tool for monitoring the structure and dynamics of the microbial subpopulations over time or under the influence of environmental changes. This approach has already been used in a few studies, which investigated specific microbial groups such as methanotrophic members of the α- and γ-Proteobacteria (122), actinomycetes (126), α- and β-Proteobacteria (111), ammonia-oxidizing bacteria (167), Archaea (221) and

fungi (273).

In this study, we aimed to simplify the use of DGGE for different group-specific PCR generated 16S rRNA gene fragments, and apply this method to analyze the structure of specific bacterial groups in activated sludge systems of different wastewater treatment plants. This standardization was made possible by performing a nested PCR approach. In a first PCR round, specific fragments were amplified by using group specific primers for methanotrophic members of the α- and γ-Proteobacteria, actinomycetes, α-Proteobacteria, ammonia-oxidizing bacteria and *Acidobacterium*, and in parallel a first set of Bacterial primers was used to amplify all members of the domain Bacteria. The second PCR round was performed with a second set of Bacterial primers, which amplified an internal fragment, and served to reduce and equalize the length of the specific fragments and to add a GC-clamp, necessary for DGGE analysis. Since all above mentioned groups belong to the domain of the Bacteria, the Bacterial primers used in the second PCR round should reamplify all fragments obtained after the first PCR round. The use of this group-specific approach can extend the possibilities of DGGE in microbial community analysis because it can better reveal subtle changes within or differences between microbial communities. To detect and quantify these small differences, the power of statistical tools, such as clustering analysis, Multidimensional scaling (MDS), Principal Component Analysis (PCA), diversity index evaluation and regression analysis were evaluated. We chose to limit the analysis to those groups for which specific 16S rRNA primers have been designed and published previously, and which are known to be present and even important in activated sludge systems.

MATERIALS AND METHODS

Activated sludge samples

Activated sludge samples were taken at 15 wastewater treatment plants (WWTP) from different origins (Flanders, Belgium), i.e. domestic wastewater (samples A), carbohydrate rich wastewater from paper and starch related industries (samples B), protein and fat rich wastewater from food and meat related industries (samples C) and wastewater from textile industry (samples D). A list of the samples together with some operational parameters (SVI = Sludge Volume Index; SRT = Sludge Retention Time; Bx = Sludge Loading Rate) is given in Table 7.1.

Table 7.1. Overview of the analyzed activated sludges from the wastewater treatment plants and their operational parameters.

Sample	Source	SVI *(ml/g)*	Bx *(g COD/g.d)*	SRT *(d)*
Domestic wastewater influent				
A1	Municipal waste	107	0.1	10
A2	Hospital waste	120	0.2	17
A3	Municipal waste	160	0.3	4
A4	Municipal waste	220	0.4	5
Carbohydrate rich influent				
B1	Starch production	190	0.1	38
B2	Paper production	100	0.3	8
B3	Potato processing	100	0.1	15
B4	Paper production	30	0.3	6
Protein and fat rich influent				
C1	Food production	109	0.2	5
C2	Food production	160	0.05	50
C3	Slaughterhouse	110	0.2	20
C4	Meat production	70	0.1	21
Textile influent				
D1	Textile	48	0.2	31
D2	Textile	114	0.2	52
D3	Textile	140	0.1	31

COD = Chemical Oxygen Demand; SVI = Sludge Volume Index; Bx = Sludge Loading Rate; SRT = Sludge Retention Time

The respective companies reported the operational parameters. All samples were collected from the aerated mixed liquor and 50 ml of sample was frozen at -20 °C until use. Upon thawing, the total community DNA was extracted and purified as described previously (35).

DNA quantification

DNA was measured by staining it with SYBR Green I nucleic acid gel stain (1:10,000 dilution; FMC BioProducts, Rockland, ME, USA) and the emission intensity was measured with a microplate spectrofluorometer (Perkin-Elmer,Shelton, USA).

PCR-DGGE analysis

All the used primers and PCR conditions are listed in Table 7.2, and for more information about these primers and PCR conditions, we refer to the original papers. In order to increase the sensitivity and to facilitate the DGGE by analyzing fragments of the same length, a nested PCR technique was applied. In the first round different group specific primers and one set of Bacterial primers were used, each with their own corresponding PCR protocol. During the second PCR round, the obtained fragments were reamplified by using the Bacterial primers P338F and P518r in one and the same PCR protocol (Table 7.2). Since all mentioned groups in Table 7.2 belong to the domain of the Bacteria, the Bacterial primers P338F and P518r used in the second PCR round should reamplify all fragments obtained after the first PCR round. After PCR, samples were stored at 4°C (few hours) or at -20°C (days).

The final concentrations of the different components in the mastermix were: 0.2 µM of each primer, 200 µM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 1X Taq DNA Polymerase 10X Reaction Buffer ($MgCl_2$-free), 1.25u/50µl of *Taq* DNA Polymerase (Promega, Madison, WI, USA), 400 ng/µl of bovine serum albumin (Hoffmann-La Roche, Basel, Switzerland), and DNase and RNase free filter sterilized water (Sigma-Aldrich Chemie, Steinheim, Germany). During the first PCR round, 1 µl of extracted DNA was added to 24 µl of PCR mastermix and in the second PCR round, 1 µl of amplified product from the first round was added to 49 µl of PCR mixture. After each PCR amplification round, the size of the PCR product was verified on a 1 % agarose gel.

DGGE (Denaturing Gradient Gel Electrophoresis), based on the protocol of Muyzer et al. (208), was performed using the Bio-Rad D Gene System (Bio-Rad, Hercules, CA, USA). The PCR products of the second round were loaded onto 8% (w/v) polyacrylamide gels in 1xTAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4). The polyacrylamide gels were

made with denaturing gradient ranging from 50 to 65 % (actinomycetes, methanotrophs, *Acidobacterium* and α-Proteobacteria) or 45 to 60 % (*Bacteria* and ammonium oxidizers) (where 100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run for 16 hours at 60°C and 37 V (Bacteria, ammonium oxidisers and α-Proteobacteria) or 40 V (actinomycetes, methanotrophs, *Acidobacterium*). After the electrophoresis, the gels were soaked for 5 min in fixation buffer (10% ethanol, 0.5 % acetic acid) (optional), and subsequently 10 min in SYBR Green I nucleic acid gel stain (1:10,000 dilution; FMC BioProducts, Rockland, ME, USA). The stained gel was immediately photographed on a UV transillumination table with a Video Camera Module (Vilbert Lourmat, Marne-la vallé, France).

Table 7.2. PCR primers used in this study.

Target	Primers	PCR conditions							References
		Nr of cycles	Denaturation		Annealing		Elongation		
			°C	min	°C	min	°C	min	
First PCR round									
Bacteria	P63F, R1378r	30	95	1	53	1	72	2	(221)
Actinomycetes	F243, R1378r	35	95	1	63	1	72	2	(126)
Ammonium oxidisers	CTO189fABC, CTO653r	35	94	1	57	1	72	2	(167)
Acidobacterium	31f, R1378r	30	95	1	53	1	72	2	(22, 126)
Type I Methanotrophs	MB10γ, R1378r	35	94	1	60	1	72	2	(122, 126)
Type II Methanotrophs	MB9α, R1378r	35	94	1	60	1	72	2	(122, 126)
α-Proteobacteria	F203a, R1378r	35	94	1	56	1	72	2	(111, 126)
Second PCR round									
Bacteria	P338F*, P518r	30	95	1	53	1	72	2	(221)

* A 5' GC-clamp was added for DGGE analysis (208); Before each PCR run, the temperature was held at 95°C for 10 min and after each run the temperature was kept at 72°C for 12 min for final template elongation.

Table 7.3. Sequences of the primers used in this study

Primer	Target	Sequence (5'-3')	Reference
P63F	All *Bacteria*	CAGGCCTAACACATGCAAGTC	(221)
P338F	All *Bacteria*	ACTCCTACGGGAGGCAGCAG	(221)
P518r	Universal	ATTACCGCGGCTGCTGG	(221)
R1378r	All *Bacteria*	CGGTGTGTACAAGGCCCGGGAACG	(126)
F243	Actinomycetes	GGATGAGCCCGCGGCCTA	(126)
CTO189AB	Ammonium oxidizers	GGAGRAAAGCAGGGGATCG	(167)
CTO189C	Ammonium oxidizers	GGAGGAAAGTAGGGGATCG	(167)
CTO653r	Ammonium oxidizers	CTAGCYTTGTAGTTTCAAACG	(167)
31f	Acidobacterium	GATCCTGGCTCAGAATC	(22)
MB10γ	Type I Methanotrophs	AAGCGGGGGATCTTCGGACC	(122)
MB9α	Type II Methanotrophs	GTTCGGAATAACTCAGGG	(122)
F203a	α-Proteobacteria	CCGCATACGCCCTACGGGGGAAA GATTTAT	(111)
GC-clamp	-	CGCCGGGGGCGCGCCCCGGGCGGG GCGGGGGCACGGGGGG	(208)

Cloning and sequencing analysis

The specific 16S rRNA gene fragments of the first PCR round were cloned by using the TOPO TA cloning kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. DNA sequencing was carried out by IIT Biotech - Bioservice (Bielefeld, Germany). Analysis of DNA sequences and homology searches were completed with the BLAST server of the National Centre for Biotechnology Information (NCBI) using the BLAST algorithm (5) for the comparison of a nucleotide query sequence against a nucleotide sequence database (blastn).

Analysis of DGGE patterns

The processing of the DGGE gels was done with the Bionumerics software 2.0 (Applied Maths, Kortrijk, Belgium). The calculation of the similarities is based on the Pearson (product-moment) correlation coefficient (227) and results in a distance matrix. The Pearson correlation is an objective coefficient that does not suffer from typical peak/shoulder mismatches as often found when band-matching coefficients are used. Clustering analysis,

Multidimensional scaling (MDS) and Principal Component Analysis (PCA), were performed with Bionumerics 2.0. The clustering algorithm of Ward (326) was used to calculate the dendrograms of each DGGE gel and a combination of all gels. By using MDS and PCA analysis, the different data of the complex DGGE patterns of one sample could be reduced to one point in a three dimensional space. MDS does not analyze the original data set, but the distance matrices of each DGGE using a similarity coefficient (Pearson correlation). A PCA analysis is different from MDS, because the data are directly analyzed. For PCA analysis, all bands are divided into classes of common bands and for each pattern, a particular band class can have two states: present or absent (binary matrix).

The structural diversity of the microbial community was examined by the Shannon index of general diversity H (265). H was calculated on the basis of the bands on the gel tracks, using the densiometric curves. The intensity of the bands was reflected as peak heights in the densiometric curve. The equation for the Shannon index is:

$$H = -\Sigma\ (n_i/N) \log (n_i/N)$$

where n_i is the height of the peak and N the sum of all peak heights of the densiometric curve. Regression analysis was performed with SPSS for Windows release 7.5.2. to investigate correlation between the Shannon index and operational parameters.

RESULTS

The use of DGGE with 16S rRNA gene fragments, regenerated with group-specific primers, was evaluated by comparing the specific DGGE patterns of activated sludge samples from wastewater treatment plants that receive different types of influents. To be able to correlate certain industrial activities with specific microbial populations, a rough classification was made into four influent types (Table 7.1).

Validation of the nested PCR procedure

In the first PCR round, group specific primers were used (Table 7.2). These specific forward and reverse primers were located before and after the 16S rRNA *E. coli* positions 338 and 518 respectively. Therefore it was possible to use the Bacterial primer set P338F and P518r in a second PCR round. The result of this nested PCR approach was that a fragment of the same 16S rRNA gene region and the same length was obtained for all specific bacterial groups.

In our study, the size of the amplified fragments of first and second PCR rounds was evaluated on an agarose gel. All the PCR products were of the expected length. Although these primers were tested rigorously before (Table 7.2), we evaluated the specificity of the PCR amplification by cloning and sequencing a few fragments after the first PCR run for each specific primer set (Table 7.4).

Table 7.4. BLAST results of some 16S rRNA gene fragments, obtained by cloning PCR fragments from the group-specific PCR reactions.

Primer target (Table 7.2)	**Clone nr.**	**Organism in Genbank with highest sequence similarity**	**Phylum**	**Accession number**	**% similarity (nr. identical bp/ blasted bp)**
Bacteria	Eub-1	Not cultivated	ß-Proteobacteria	AF314448.1	99 (519/522)
	Eub-2	Unidentified	ß-Proteobacteria	X73223.1	95 (524/549)
Actinomyceten	Act-1	Unidentified	Actinobacteria	AB021332	98 (599/608)
	Act-2	*Nocardiodes* sp.	Actinobacteria	X76179.1	96 (538/555)

Table 7.4. Continued.

Acidobacterium	Aci-1	Uncultivated sludge bacterium	-	AF234731.1	97 (453/463)
	Aci-2	Unknown organism	-	X84575.1	96 (397/413)
Ammonium oxidisers	Amo-1	Unidentified	ß-Proteobacteria	AJ003760.1	98 (459/466)
	Amo-2	Uncultivated *Nitrosomonas*	ß-Proteobacteria	AJ245752.1	98 (435/440)
	Amo-3	*Nitrosomonas oligotropha*	ß-Proteobacteria	AF272422.1	96 (452/466)
Methanotrophs I	MTI-1	*Methylobacter psychrophilus*	γ-Proteobacteria	AF152597.1	98 (561/569)
Methanotrophs II	MTII-1	*Methylocystis* sp.	α-Proteobacteria	AB015608.1	97 (389/409)
	MTII-2	*Methylosinus trichosporium*	α-Proteobacteria	Y18947.1	93 (509/543)
α-Proteobacteria	A-Pr-1	α-Proteobacterium	α-Proteobacteria	AF236000.1	98 (406/414)
	A-Pr-2	Uncultivated *Eubacterium*	α-Proteobacteria	AF050531.1	95 (537/564)
	A-Pr-3	*Xanthobacter flavus*	α-Proteobacteria	X94206.1	95 (327/342)

After the first PCR round, the concentration of the PCR products sometimes differed, depending on the initial amount of specific template DNA in the sample. However, after the second PCR round, no differences in PCR-product concentration were visible, based on band intensities.

Determination of the minimum concentration of first PCR product required to obtain a specific second PCR product

In addition to sequencing amplified fragments, as described above, another way to insure that the DGGE patterns consisted of only group-specific bands, is the following. We examined what the minimum ratio of amplified product over template DNA should be after the first PCR round in order to avoid amplification of non-group-specific template DNA, still present as background in the second PCR round. This second template indeed consists not only of the specific first PCR fragment, but also still contains some extracted genomic DNA (25 times diluted). Thus if no or very little specific fragment would be amplified in the first PCR round, the genomic DNA could serve as template in the second PCR round, resulting in

an aspecific second PCR fragment and thus an incorrect DGGE-pattern. Hence, the lowest concentration of first PCR fragment required to avoid aspecific bands in the final DGGE pattern has to be determined. The evaluation was based on the amplification of ammonium oxidizing bacteria with the CTO-primers. As template DNA, DNA extracted from wastewater treatment plant A1, which was characterized by good nitrification, was chosen.

The first DNA template concentration was 0.12 µg/µl, and since 1 µl was added to 25 µl of PCR mastermix, the final genomic DNA concentration in the mastermix was 4.8 ng DNA/µl. Using the CTO primers, a group specific PCR product of 15.75 ng DNA/µl was obtained. In order to examine the influence of the group specific PCR product, a tenfold dilution series of the first specific PCR product was made in water, containing 4.8 ng total genomic DNA/µl. A sample containing 4.8 ng DNA/µl was regarded as infinitely diluted. The concentration of total genomic DNA was thus constant while the concentration of specific PCR fragment varied. From this dilution series, 1 µl was added to 49 µl mastermix for the second PCR round, containing the general bacterial primers 338GC and 518r. A DGGE of these final PCR fragments was run for 17 hours at 38V on a gel with a denaturing gradient of 50-65%. The DGGE patterns showed that non-specific Bacterial fragments only appeared when the PCR product of the first round was diluted more than 10^3 times in the template background (Figure 7.1).

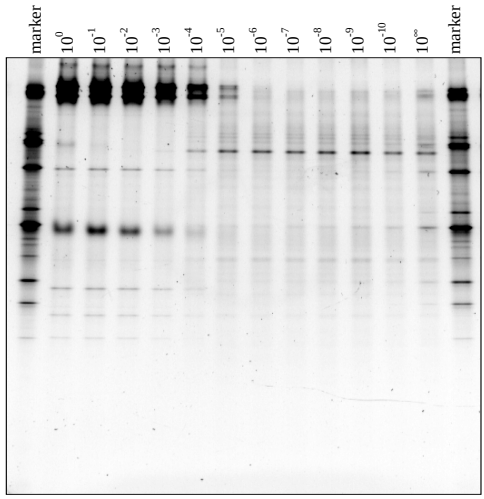

Figure 7.1. DGGE of the PCR fragments of the second PCR run with the general bacterial primers. At the top of the gel the dilution is mentioned of the first group specific PCR product CTO primers) with diluted DNA from sludge sample A1.

This corresponded with a PCR product concentration of less than ca. 16 pg of amplified product per µl (or 0.3% of the total template DNA). These results strongly indicate that as long as the PCR product (a few µl) of the first round is visible on the gel, there should be no problem with amplification of aspecific ribosomal RNA genes in the second PCR round.

Validation of the nested PCR approach

A potential effect of the second PCR round on the number and relative intensities of the different bands in the final DGGE profile was examined by comparing this profile with that obtained after only the first PCR with the specific primers. In a first PCR reaction, a *Acidobacterium* specific PCR fragment was obtained in a single PCR run with primer 31f with GC-clamp (31fGC) in combination with primer P518r (lanes 1 of Figure 7.2). The nested PCR fragment was generated by a first PCR with primer 31f (specific for the *Acidobacterium* group), without GC-clamp, and primer P518r, followed by a second PCR round with the Bacterial primers P63FGC (a GC-clamp was added to primer P63F), and 518r (lanes 1 of Figure 7.2).

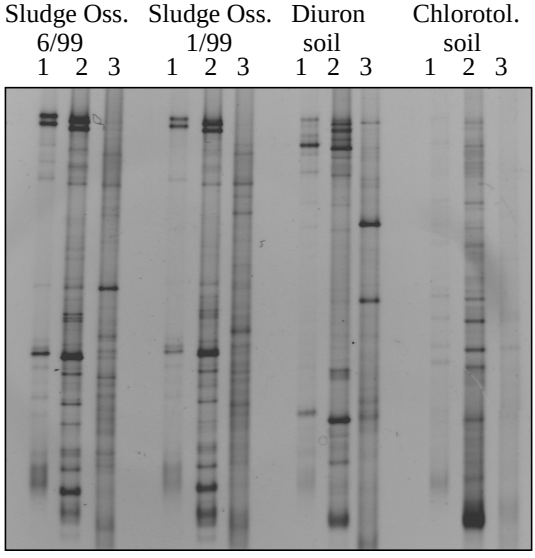

Figure 7.2. Analysis of the DGGE profiles of the different samples: (1) fragments of PCR with total DNA and *Acidobacterium*-primers; (2) fragments of a nested PCR on (1) with bacterial primers; (3) fragments of PCR with total DNA and bacterial-primers. The different DNA samples were from a wastewater treatment plant at two different dates (Sludge Oss.) and from two soils, treated with diuron and chlorotoluron (87).

These primer sets were chosen such that both fragments would only differ 40 bp in length and thus could both be easily analyzed and compared on the same DGGE gel. In lanes 3 of Figure 7.2 PCR fragments obtained with primers P63FGC-518r are showed. For 4 samples tested, both patterns were very similar, except that the positions of the P63FGC-518r PCR fragments were all shifted downwards in comparison to the 31fGC-518r fragments, due to the slightly larger length of the latter fragment (Figure 7.2). These data suggest that the second PCR round did not drastically change the number nor the intensities of the DGGE-bands, compared to a 1-step PCR protocol with specific primers.

DGGE analysis of PCR-amplified 16S rRNA gene fragments

Figure 7.3 shows the seven DGGE gels for all the activated sludge samples and for all the different bacterial groups. The DGGE patterns obtained with the Bacterial primers did not show many intensive bands (Figure 7.3a). A few dominant bacteria were present in some samples, but in all samples the high number of weak bands resulted in a smear. This is probably due to the high number of different Bacterial species present in the sludge. Also the DGGE patterns of the α-Proteobacteria were very complex (Figure 7.3g). The DGGE pattern obtained with the *Acidobacterium* primers contained a relatively high number of bands as well (Figure 7.3c), which indicates that this group is also highly diverse. Every activated sludge sample seemed to have a unique *Acidobacterium* community and common bands were exceptional. The actinomycete, methanotrophs and ammonium oxidizing communities in all sludge samples seemed to consist of only a limited number of dominant species, without a smear of weak bands (Figure 7.3b, d, e and f). Striking were the patterns of the ammonium oxidizing populations for the sludge samples of groups A and B. The four DGGE patterns within each of these two types of sludge showed great similarity among each other, although they came from four different wastewater treatment plants. This was not the case for patterns from the sludge groups C and D.

Analysis of DGGE banding patterns

Highly diverse ecosystems, such as activated sludge, sediments and soils, have DGGE banding patterns that are very complex to interpret. Therefore, computer-aided analyses are necessary to examine these patterns. To be able to perform such analyses on each gel, three standard patterns (markers) were included to allow a normalization of the gels. The normalized gels were then used to perform the calculations for the different statistical analyses and for the calculation of the Shannon diversity index.

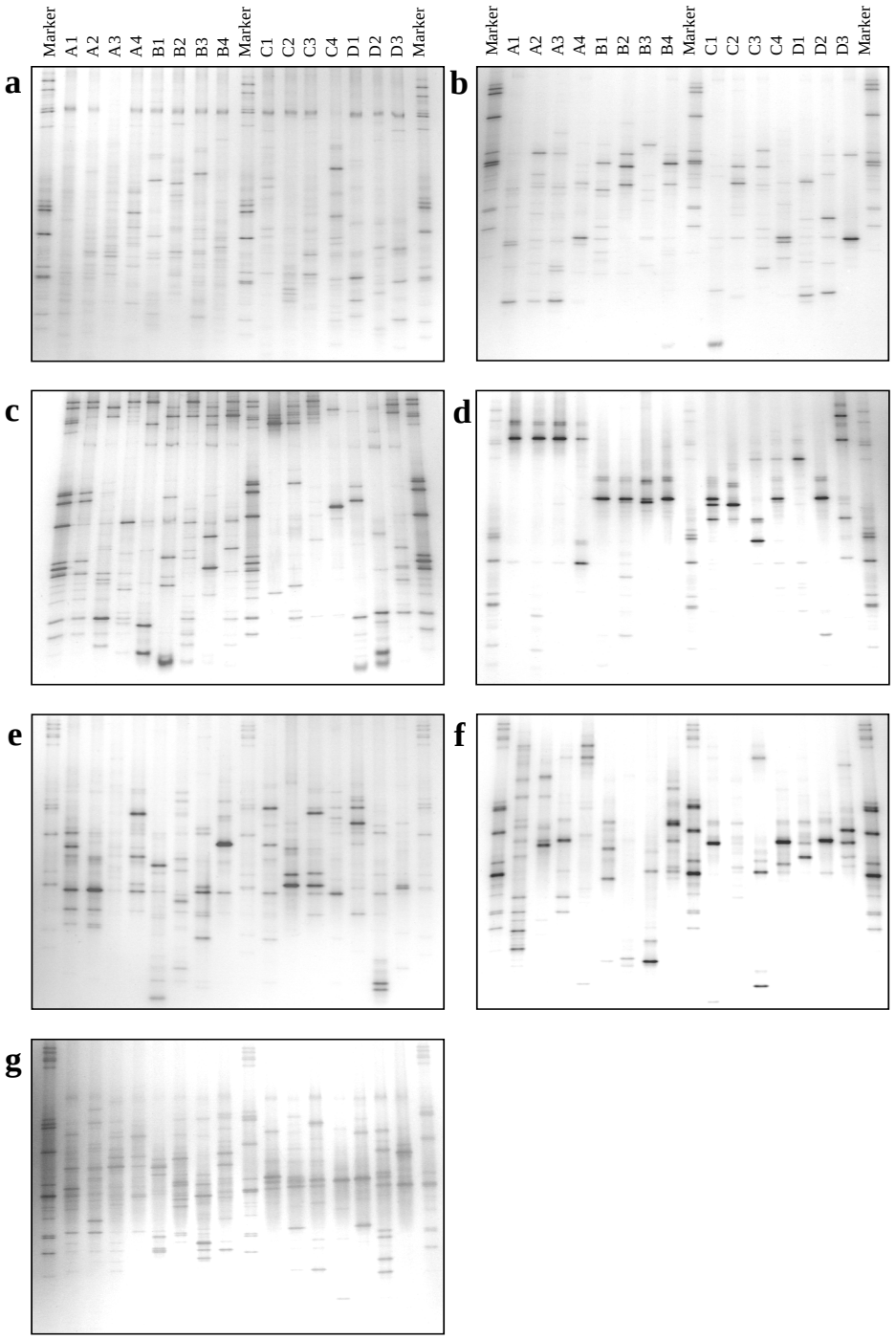

Figure 7.3 DGGE analysis of the 15 activated sludge samples, listed in Table 7.1: Bacteria (a); actinomycetes (b); *Acidobacterium* (c); Ammonium oxidizers (d); Methanotrophs - type II (e) and type I (f); α-Proteobacteria (g).

The different DGGE banding patterns were examined in three ways, i.e. clustering, analysis by dimensioning techniques (MDS and PCA) and Shannon diversity index analysis. First, the information of the different tracks was analyzed for one type of group-specific fragments by calculating a distance matrix of all the possible gel tracks within the DGGE patterns by using the Pearson correlation. Based on the values of the resulting matrix, a cluster analysis was performed and the 15 different samples were visualized in dendrograms (Figure 7.4). In the dendrograms, sometimes several sludge samples (3 or 4) of the same type of wastewater were located in one cluster, such as for samples A (Figure 7.4a, b, d, e, g), samples B (Figure 7.4a, b, d, g), samples C (Figure 7.4a, b, d, g) and samples D (Figure 7.4a, b, d, f, g). At first sight, no real conclusions or correlations could be made when Bacterial patterns were compared, while on the group specific level, the clustering analysis in some cases led to interpretable results. Finally, a dendrogram was created based on the combination of all data from the seven different gels (Figure 7.4h). Remarkably, this resulted in a dendrogram with two clusters, which clearly separated the patterns of the different activated sludge groups A and B. This was not the case for the activated sludge groups C and D, where more variation was present within each group of samples.

Multidimensional Scaling (MDS) and Principal Component Analysis (PCA) are two alternative grouping techniques that can both be classified as dimensioning techniques. These techniques produce two or three-dimensional plots in which the entries are spread according to their relatedness. Unlike a dendrogram, a MDS or PCA plot does not provide clusters and thus the interpretation is more subjective. To perform the MDS and PCA analysis, all DGGE patterns available for the different activated sludge samples were included. MDS just replaces the clustering step and it is an alternative to the dendrogram methods, which often oversimplifies the data available in a similarity matrix and tends to produce overestimated hierarchies. The MDS analysis shows that the different activated sludge groups in the three-dimensional plot are not grouped together (Figure 7.5a). The samples of the fat and protein rich influents (C) and of the textile industry (D) are more distributed over the whole plot, while the other sample types are more or less grouped. This corresponds to the results of the overall cluster analysis of all DGGE patterns. A PCA analysis distinguishes itself from a MDS, because a PCA analyses the data directly by a binary band-matching table.

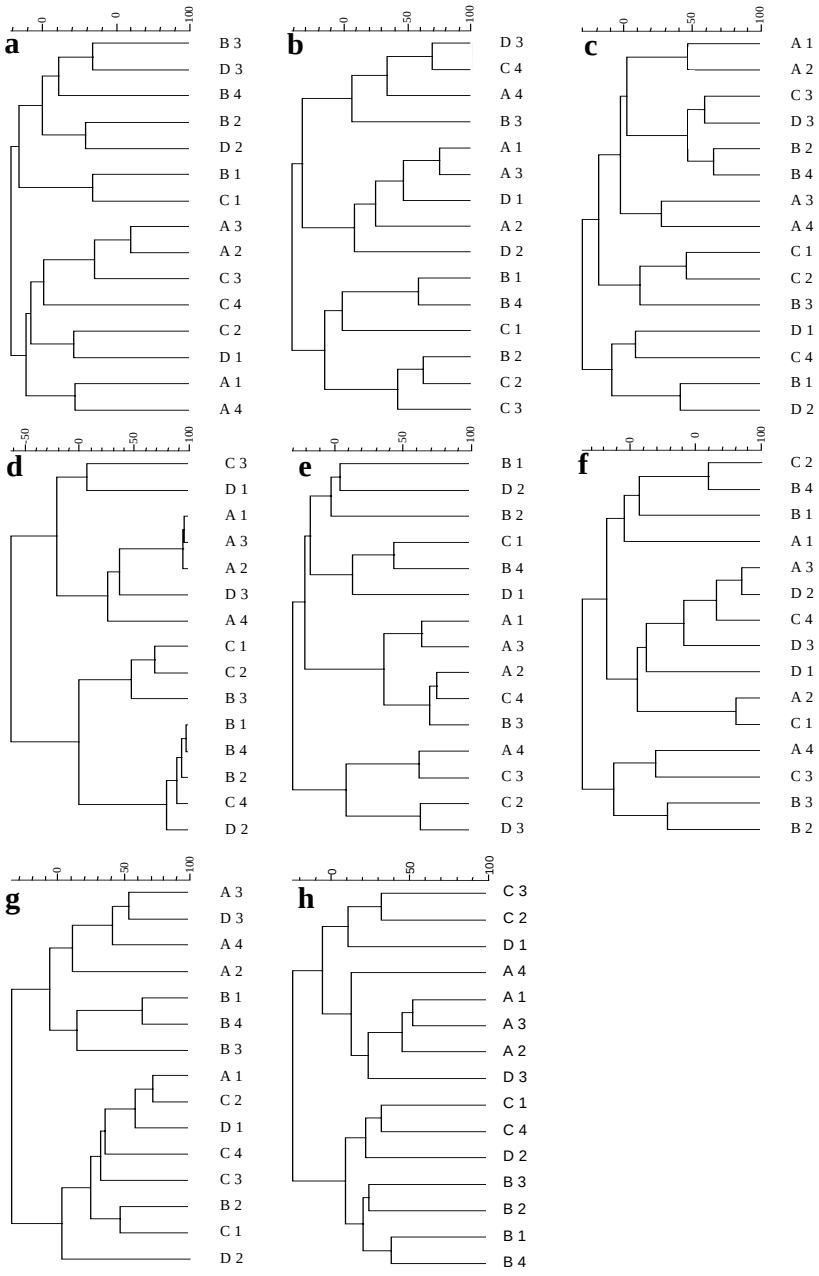

Figure 7.4. Clustering analysis of the DGGE patterns of the 15 samples, listed in Table 1: Bacteria (a); actinomycetes (b); *Acidobacterium* (c) ammonium oxidizers (d); methanotrophs - type II (e) type I (f); α-Proteobacteria (g); all different DGGE-tracks combined (h).

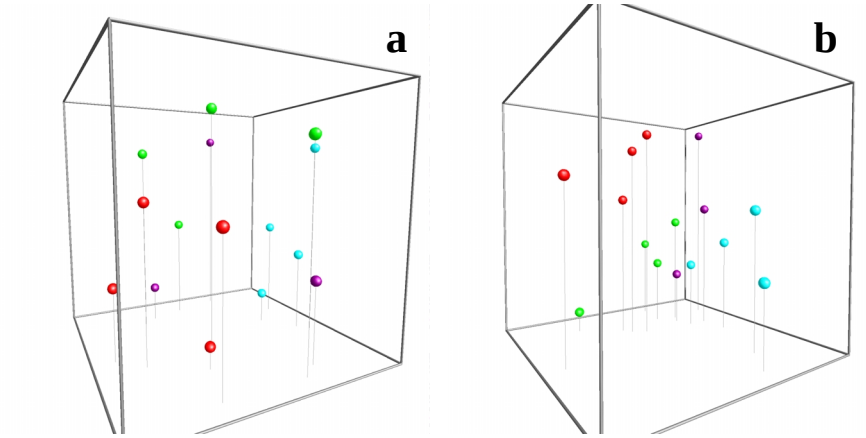

Figure 7.5. Representation of two dimension techniques, calculated for each track of each DGGE gel with group specific 16S rRNA gene fragments: Multi-Dimensional Scaling (a) and Principal Component Analysis (b): samples A (●); samples B (●); samples C (●); samples D (●).

Therefore, the same sets of data will result in a different PCA-and MDS-plot (Figure 7.5b). The three principal components (PC) explained a very low and almost equal percentage of the total variation (PC 1 = 12 %; PC 2 = 10 %; PC 3 = 9 %). The PCA did not separate the different groups completely, but all the sludge samples of one wastewater type were localized together in the same area.

The third method that was used to compare the bacterial communities of the different samples, was the calculation of the Shannon diversity index *H*, based on the DGGE banding patterns of the specific groups. Diversity indices are useful as a first approach to estimate the diversity of microbial communities, i.e. the higher *H*, the greater the diversity of the microbial community. A diversity index consists of two components: (i) the total number of species present or species richness and (ii) the distribution of the number of individuals among those different species, called species evenness, or species equability (160). The averages and the standard deviations of the Shannon index *H* values for each activated sludge group and for all samples are listed in Table 7.5.

Table 7.5. Mean and standard deviation of the Shannon Diversity Indices for the sludges of the different wastewater treatment plant-groups.

	Samples A	Samples B	Samples C	Samples D	Average
Bacteria	1.08 ± 0.08	1.07 ± 0.06	1.06 ± 0.06	0.98 ± 0.09	1.05 ± 0.08
Actinomycetes	0.81 ± 0.14	0.70 ± 0.11	0.66 ± 0.12	0.59 ± 0.27	0.70 ± 0.16
Acidobacterium	1.04 ± 0.10	1.05 ± 0.07	0.91 ± 0.25	0.97 ± 0.09	1.00 ± 0.15
Ammonium oxidisers	0.45 ± 0.09 ^{D}	0.45 ± 0.16 ^{D}	0.58 ± 0.14	0.76 ± 0.09 ^{AB}	0.54 ± 0.17
Type I Methanotrophs	0.91 ± 0.21	0.71 ± 0.14	0.78 ± 0.21	0.62 ± 0.19	0.86 ± 0.20
Type II Methanotrophs	0.95 ±0.06 ^{C}	0.88 ± 0.13	0.80 ± 0.05 ^{A}	0.83 ± 0.10	0.76 ± 0.10
α-Proteobacteria	1.08 ± 0.13	1.07 ± 0.13	0.97 ± 0.14	0.85 ± 0.20	1.00 ± 0.16

A, B, C, D: samples which are significantly different (t-test), with P<0.05

The Anderson-Darling test for normality showed that the Shannon Diversity Indices *H* for the different groups were normal distributed. A two-tailed t-test was performed to investigate if the *H* values of different activated sludge groups were significantly different. The diversity index of ammonium oxidizers was significantly higher in textile wastewater activated sludge types (samples D) than in the activated sludge types A and B. The Shannon index for the methanotrophs Type II population was significantly different between sludge groups A and C. The Bacteria and the α-Proteobacteria showed the highest average index *H* of the sludge samples. Remarkably, the standard deviation of the Bacterial indices was very low, in comparison with those of the other bacterial groups. The ammonium oxidizer community had the lowest average Shannon index *H*. This is a result of the very limited number of bands, visible in the gel (Figure 7.3d).

The Shannon diversity indices of the different groups and the operational parameters were subjected to a correlation analysis. The diversity indices for the ammonium oxidizers correlated positively with the sludge retention time (SRT) ($P=0.05$; $R^2=0.31$), while the index of the Bacteria ($P=0.05$; $R^2=0.30$) had a negative correlation. The Shannon index of the actinomycetes was positively correlated to the sludge volume index (SVI) ($P=0.10$; $R^2=0.22$).

DISCUSSION

Nested PCR

In this study a nested PCR approach was chosen to facilitate the analysis of the 16S rRNA gene fragments of different bacterial subgroups by DGGE. A comparable approach has been used earlier by Heuer et al (126) to monitor actinomycete community changes in potato rhizosphere and to investigate actinomycete diversity in different soils. They used the actinomycete specific primer F243 (same as in this study), followed by the Bacterial primers F984GC and R1378. An advantage of the nested PCR approach in our study is that the final group-specific DGGE patterns can be directly compared (under the same DGGE conditions) to all the other group-specific and Bacterial patterns of the same sample, because the same 16S rRNA gene fragment was amplified in the second PCR. As a result, only small optimization of the denaturing gradient and electrophoretic conditions is needed to obtain a good separation for all the fragments of the different bacterial groups. A second advantage of using nested PCR with specific primers, is the increased sensitivity, which allows to visualize also those species that are present in lower numbers. Phillips et al. (230) detected ammonium oxidizers by using a nested PCR approach with the specific primers, while the abundance of these ammonium oxidisers was maximum 0.01 % of the total bacterial soil community.

The number and intensity of bands in a DGGE gel do not necessarily give an accurate picture of the number and abundance of the corresponding species within the microbial community. One organism may produce more than one DGGE band because of multiple, heterogeneous rRNA operons (55, 217, 236). On the other hand, partial 16S rRNA gene sequences do not always allow discrimination between species, such that one DGGE band may represent several species with identical partial 16S rRNA gene sequences (305). Some bacteria have more copies of the rRNA genes than others, and some lyse more easily than others. In addition, in a mixture of target rRNA genes present at very different concentrations, the less abundant sequences are not amplified sufficiently to be visualized as bands on a DGGE gel. Therefore, the banding pattern reflects only the most abundant ribosomal RNA types in the microbial community (208).

A problem with the application of group-specific primers for the analysis of natural microbial communities is that the prediction of their specificity relies on the available cultured isolates and on known sequences in the database, which may not adequately reflect the entire pool of 16S rRNA gene sequences in nature. The study of Purkhold et al. (234) clearly demonstrated that specific primers for ammonium oxidizers are, in many cases, not

sufficiently specific. Also primer F243 might not be the ideal primer for all studies of actinomycetes because it does not match the 16S rRNA of all actinomycetes and it matches 16S rRNA of a few nonactinomycetes (126). Nevertheless, the primer is useful to enrich actinomycete 16S rRNA gene fragments in order to improve the detection of this group from environmental samples (126). Also in our study, there is no absolute certainty that all the amplified bands in the DGGE gels represent true members of the respective group. However, sequence analysis of three randomly chosen DGGE bands for each primer set confirmed that the sequences corresponded to bacteria belonging to the respective amplified group. In the future, thanks to the ever expanding sequence information, improved primer design will allow a more accurate development of group-specific PCR-DGGE approach.

DGGE analysis of different bacterial groups

To obtain an optimal separation of the PCR fragments on DGGE, slight modifications of the running voltage and the gradient had to be performed. After this optimization it would be possible to analyse the seven different groups of one sludge sample on one gel, using a voltage of 40 V and a gradient of 45-65% (data not shown). A slight disadvantage of this approach is a less optimal separation of certain bands in some groups.

For many sludge samples, several bands of some of the analyzed specific groups were not visible in the corresponding Bacterial pattern. Heuer et al. (126) made a similar observation when they examined actinomycetes in soil samples with the same approach. This indicates that species, other than the specific groups, analyzed in this study, were more abundant in these activated sludges, and therefore masked the presence of some of the members of these subgroups. The appearance of new bands when group-specific primers were used, confirms that diversity reflected by Bacterial DGGE patterns only relates to the numerically dominant species and not at all to the total number of different species in the environmental sample. Muyzer et al. (208) showed that the presence of a few dominant species leads to a simple pattern, and that species of less than 1% of the analyzed community were not represented in the microbial community pattern. This will also be true for PCR-DGGE for certain very diverse bacterial groups: only species that are dominant within this specific group will be visible. For subgroups that still contain a large number of different 16S rRNA types, further subdivison using more specific primers could be useful if more detailed analysis is desired.

In this study, only one activated sludge sample of each wastewater treatment plant was investigated. To monitor the reproducibility in time, two samples of the wastewater treatment

plant A1 were taken with an interval of 6 months, and their DGGE profiles of the Bacterial and *Acidobacterium* populations did not show much variation (Figure 7.2). Other investigators also did not find evidence for variation in the wastewater treatment plants they studied by PCR-DGGE and concluded that a single sample of an activated sludge plant was sufficient for a plant to plant comparison (62).

Fluorescent *in situ* hybridization (FISH) analysis has shown before that on average ca. 70-90% of the observable micro-organisms in activated sludge are binding with the Bacteria probe EUB (321). In the same study the authors observed that the α-Proteobacteria accounted for 60 to 75% of the Bacteria. Whereas members of the β subclass were common in both high- and low-load aeration basins, members of the α subclass were more common in low-load basins (321). Earlier work has also demonstrated that the α-Proteobacteria are numerically dominant in activated sludge (174, 206) and that some species have been shown to form filaments in activated sludge systems (212, 214). Our DGGE patterns of the *Bacteria* and α-Proteobacteria are very complex, because these groups seem to have not only a high number of cells in the activated sludge microbial community, but also a high number of different species (ribotypes). This was also reflected in the Shannon diversity index. The Bacteria and the α-Proteobacteria have the highest average index *H* of the investigated samples. The high indices are the result of the high number of bands and the absence of a few very dominating bacteria. Within the profiles of the Bacteria, the variance of *H* was very low. Therefore DGGE analysis with a focus on specific rather than all bacterial groups would be better to compare different ecosystems.

The kingdom *Acidobacterium* is a recently discovered bacterial lineage and at this time contains only a few cultured representatives (22, 269). The *Acidobacterium* group seems to be present in many ecosystems, particularly in soils, while only one study so far has reported its presence in wastewater (190). The exact role and the ecological significance of these bacteria is still unknown, however recent developments indicate that members of the kingdom *Acidobacterium* are involved in methanol metabolism (235). Barns et al. (22) have suggested that members of the *Acidobacterium* kingdom could be as genetically and metabolically diverse, as environmentally widespread and, perhaps, as ecologically important as the well-known Proteobacteria and Gram-positive bacterial kingdoms. To study the diversity of this kingdom, the latter authors designed a specific forward primer to clone *Acidobacterium* 16S rRNA gene sequences (22), but until now, DGGE analysis of this new bacterial kingdom has not yet been published. Remarkably, none of the DGGE patterns in the

different samples were comparable. This was also observed by the absence of real groups in the clustering analysis. The Shannon diversity index of the *Acidobacterium* group was as high as the Bacteria and α-Proteobacteria, which could indicate that also in activated sludge a very diverse *Acidobacterium* community is present.

The analysis of actinomycetes was included in this study, because these bacteria can cause severe bulking and foaming in activated sludge plants (67, 192, 264). Their hydrophobic cell surface is supposed to support adherence and stabilisation of interfaces and thus promote sludge flotation, leading to a higher Sludge Volume Index (SVI) (179). In our activated sludge samples only a few species were dominating the actinomycete community, based on the DGGE patterns, and the type of industrial influent seems to determine its species composition. A weak positive correlation was found between the Shannon index of diversity of the actinomycetes, and the Sludge Volume Index (SVI). Although the amount of samples investigated in this study was too low to draw firm conclusions from this observation, the correlation could be related to the role of these bacteria in sludge bulking.

Methanotrophs have already been isolated from different activated sludges (4, 56, 108) and they were also clearly present in the samples examined in this study. They are a phylogenetically heterogeneous group, belonging to the α- or γ-Proteobacteria. Seven genera of type I (γ-Proteobacteria) and type II (α-Proteobacteria) methanotrophs have been proposed (44). These bacteria can utilize C-1 molecules, such as methane, methanol and formaldehyde. The DGGE patterns of the samples clustered differently when they were analyzed with the two different sets of group specific primers, i.e., for the Type I and II methanotrophs. This phenomenon has been observed before in groundwater, where both types of methanotrophs reacted differently on changing conditions, such as biostimulation (145). Similarly, in a soil treated with herbicides, the diversity of type I methanotrophs decreased, while the diversity of type II increased (261).

The 16S rRNA gene fragments related to the β-Proteobacteria subgroup of the ammonium oxidizers were examined because of their importance in the nitrogen removal process. The most prominent members of this β-subgroup in activated sludge are usually related to the genera *Nitrosomonas* or *Nitrosospira* (21). Within this group of bacteria, clustering analysis of their DGGE patterns showed a clear similarity among the samples of domestic wastewater (group A) and also among those of the carbohydrate rich wastewater (group B). The ammonium oxidizers in the activated sludge samples of the textile industry (group D) were significantly more diverse than groups A and B, as observed by Shannon

diversity index evaluation. In textile wastewaters, azo-dyes and their metabolites, i.e. chloroanilines, can be present and these compounds have a negative influence on the nitrification (337). This could lead to a lower density of common ammonium oxidizers, which might result in a more diverse ammonium oxidizing community. More work would have to be done however to confirm this hypothesis.

More and more often, the interpretation of complex DGGE patterns is carried out with techniques such as MDS (145, 254) and PCA-plots (207, 237). We also tried to analyze our data with both techniques, but no clear defined groups could be observed. Activated sludge populations are very complex systems and the reduction into four groups based on influent type is too simplistic. The operational parameters and nutrient removal efficiencies were different for every plant and may have an important impact on the composition of the microbial communities. An indication for the latter interaction was given by the correlation analysis between the Shannon diversity indices of the different groups and the operational parameters. The regression model was significant, however the low R^2 values make it impossible to draw real conclusions. To know the effect of the parameters on the different bacterial subpopulations, experiments with one type of sludge and controlled changes of one parameter should be performed.

This study clearly shows that it is possible to obtain a view on activated sludge bacterial communities that was previously inaccessible. The integration of the fingerprinting data with statistical tools can be used to show the biological relationships between different activated sludges. A greater understanding of activated sludge microbiology is then expected to lead to improvements in analysis and control of activated sludge treatment processes. In the future, when specific primers will be designed for an increasing number of groups, a more complete picture of bacterial communities will be obtained. DGGE with group specific 16S rRNA primers is not only useful to compare different microbial communities, but also to monitor microbial communities in function of time. Combination of qualitative (such as DGGE with group specific 16S rRNA primers) and quantitative techniques (such as FISH or real time PCR (7)) would be a next step in acquiring a good descriptive tool for microbial community analysis of activated sludges.

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CHAPTER 8

8. GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

INTRODUCTION

The main focus of this work was to investigate the diversity of bacteria that are involved in the degradation of herbicides and to apply these specialized bacteria for the bioaugmentation of activated sludge systems (314). As a model compound 3-chloroaniline was used, a residue of the herbicide chloropropham (156) and a common precursor for a variety of chemicals (106, 157). During the last 4 years, the major accomplishments of this work were:

1. Isolation and identification of new chloroaniline degrading bacteria from activated sludge and soil (Chapter 2).
2. Characterization of plasmids, encoding the transformation of aniline and/or chloroaniline (Chapter 2).
3. Development of a DGGE method to investigate the diversity of an aniline catabolic gene (Chapter 2).
4. Successful bioaugmentation of 3-CA receiving activated sludge for a limited time (Chapter 3).
5. Improvement of the bioaugmentation by a slow release approach (Chapter 4) and by co-inoculation with a donor of a 3-CA degradative plasmid (Chapter 5).
6. Bioprotection of functions of an activated sludge wastewater system receiving a 3-CA shock load by the inoculation of a 3-CA metabolizing strain (Chapter 6).
7. Evaluation of a rapid and easy nested PCR-DGGE approach to examine the diversity bacterial groups (Chapter 7).

The importance of our findings will be briefly discussed in the following paragraphs and suggestions for future work will be made.

DIVERSITY OF CHLOROANILINE DEGRADING BACTERIA

In this study, 3-chloroaniline degrading bacteria were isolated and described. All

isolates were identified as *Delftia* or *Comamonas* and belong to the *Comamonadaceae* family. Remarkably, all isolates, able to degrade chloroanilines, described in literature belong to the Proteobacteria (Table 8.1).

Table 8.1. Overview of the isolated chloroaniline degrading species.

Genus and species	Strains	Taxonomic group[a]	Reference
Achromobacter sp.	PA4, PB31	β-Proteobacteria	(76)[b]
Acidovorax sp.	TA2, TA35	β-Proteobacteria	(76)[b]
Aquaspirillum sp.	2CA	β-Proteobacteria	(285)
Aquaspirillum itersonii	1CA, 10CA	β-Proteobacteria	(285)
Brevundimonas diminuta	INMI KS-7	α-Proteobacteria	(284)
Comamonas testosteroni	I2	β-Proteobacteria	This work
Comamonas testosteroni	TB1, TB18, TB30	β-Proteobacteria	(76)[b]
Comamonas testosteroni	WDL7	β-Proteobacteria	(74)[b]
Delftia acidovorans	BN3.1	β-Proteobacteria	(46)
Delftia acidovorans	CA26, CA28, CA37, CA45	β-Proteobacteria	(189)
Delftia acidovorans	LME1, B8c	β-Proteobacteria	This work
Delftia acidovorans	WDL34	β-Proteobacteria	(74)[b]
Delftia sp.	MA14, MA22, MA34	β-Proteobacteria	(76)[b]
Moraxella sp.	G	γ-Proteobacteria	(339)
Pseudomonas sp.	JL2, JL3, JL4, JL5	γ-Proteobacteria	(175)
Pseudomonas sp.	TA8, TA12	γ-Proteobacteria	(76)[b]
Pseudomonas sp.	FRB4.5	γ-Proteobacteria	(46)
Paracoccus denitrificans	3CA	α-Proteobacteria	(285)

[a] Taxonomic classification as determined by the taxonomy browser of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov)

[b] Unpublished data from recently isolated chloroaniline degrading bacteria

It is rather extraordinary that all chloroaniline degrading isolates belong to this one, although large, phylogenetic group. The latter could have two meanings, namely that during evolution only Proteobacteria evolved into effective chloroaniline degraders or that the isolation procedure selects for these organisms. The selective isolation of Proteobacteria is not only valid for chloroaniline degrading bacteria. In the Biodegradative Strain Database (**www.cme.msu.edu**), almost 75 % of all deposited strains are Proteobacteria (Figure 8.1). A high number of degradative strains are however member of the high G+C Gram-positives, but no chloroaniline degrading members of this group have yet been isolated.

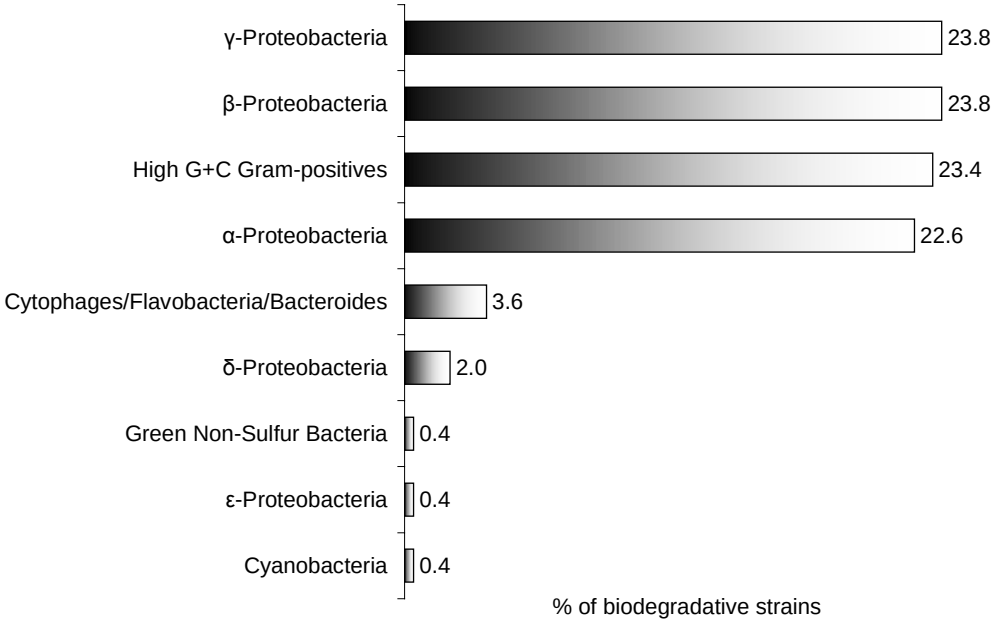

Figure 8.1. Relative distribution of the different phylogenetic bacterial groups among the published biodegradative strains (n=249), submitted at the Biodegradative Strain Database website (www.cme.msu.edu).

A strategy to overcome the cultivation bias is to investigate *in situ* the microbial component which is responsible for the degradation of certain compounds. However this is a difficult approach. Bacterial communities can be studied easily with the current molecular techniques. For example, the microbial community of a polychlorinated biphenyl-polluted soil was studied by preparing clone libraries of 16S rRNA and ribosomal DNA (216). The library sequencing results clearly showed that 62 % of all clones belonged to the Proteobacteria. The *Holophaga-Acidobacterium* division counted for 18 % of all clones. The kingdom

Acidobacterium is a recently discovered bacterial lineage and at this time contains only a few cultured representatives (22, 269). Also 3% of the sequences were similar to the proposed candidate division OP10. This division was first found back in Obsidian Pool (OP) of Yellowstone and no cultured representatives have been described yet (141).

The results obtained by Nogales et al. (216) strongly suggest the involvement of the "unculturable" bacteria in pollutant metabolism, however the exact role and the ecological significance of these bacteria is still unknown. By a culture independent method it was shown that members of the kingdom *Acidobacterium* are involved in methanol metabolism (235). Barns et al. (22) suggested that members of the *Acidobacterium* kingdom could be as genetically and metabolically diverse, as environmentally widespread and, perhaps, as ecologically important as the well-known *Proteobacteria* and Gram-positive bacterial kingdoms. Nevertheless, the other - less characterized - bacterial divisions, such as *Verrucomicrobia* (121), GNS (Green non-sulfur) bacteria (335), and OP11 (141) are also considered to play significant roles in the environment (for a review on candidate divisions, see reference (139)). From these groups, some bacteria have already been described to play a role in the metabolism of xenobiotic substrates. For example *Dehalococcoides ethenogenes*, a member of the GNS bacteria, is able reduce chlorinated hydrocarbons (220) and sequences from a trichlorobenzene-transforming consortium appear to be related to OP10 (318).

The present data suggest that a lot of the intrinsic catabolic potential is still uncovered. The main bottleneck remains the cultivation of the micro-organisms. Thus, two possible strategies can be used to obtain the unknown functional genes. The first strategy is the cultivation of the "unculturable" organisms. The term "unculturable" is possibly not appropriate since these organisms can grow in their environment, , which probably means that scientists did not yet find the right growth conditions. The present evolution in automatization and robotizing allows to test a lot of different nutrient combinations. Molecular techniques can be used to monitor the enrichment of the desired micro-organism. The data can be used in a genetic algorithm to design the best suited growth medium (109). A second possible approach is to clone whole genomes from the environment and to use expression vectors to obtain gene expression (248). The resulting proteins can furthermore be tested for their catabolic properties and be used subsequently in cultivable genetically modified organisms.

MOBILE GENETIC ELEMENTS AND CATABOLIC GENES

Bacterial plasmids are extrachromosomal genetic units that replicate in step with the bacterial chromosome (294). They constitute from 1% to greater then 10% of the genome of many species and can be lost or acquired from other bacteria by conjugation, transformation or transduction.

The role of plasmids in bacteria is a bit controversial. Most plasmids carry genes that have potentially useful traits on their bacterial hosts. On the other hand, there is a theory that views plasmids as genetic parasites (for an overview, see reference (246)). It has been shown that carrying plasmids can result in reduced growth rates, and that plasmid-free variants thus have a growth advantage and therefore can become dominant when the plasmid encoded traits are not selected for. When high cell densities are present, plasmids show an infectious-like spread (181). Nevertheless, plasmids are important players in bacterial adaptation. Their traits are useful in certain specific environments, such as antibiotic resistance, host invasion or xenobiotic degradation, and many others (181).

The relative new halogenated organic compounds, such as pesticides, herbicides and solvents, are good examples to demonstrate the adaptative capacities of bacteria, mediated by mobile genetic elements. The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) has been used for only ca. 40 years and the first 2,4-D degradative plasmids were already isolated in 1981 (80). The *tfd* degradation genes for 2,4-D were present on this plasmid. Top et al. (1995) isolated seven plasmids, encoding 2,4-D degradation. The *tfd* genes (*tfdABCDEF*) were present on the different plasmids as mosaics, suggesting that the plasmids are acting as a shuttle vector.

Transposable elements play a role in the *in vivo* construction of plasmids. Catabolic pathways can be assembled by combining the genes for a particular peripheral pathway and for a central (chloro)catechol cleavage pathway (243). For example the first step of the peripheral pathway for 2,4-D degradation is encoded by *tfdA* and this gene is located away from the *tfdCDEFtdfB* cluster, encoding for the *modified ortho*-cleavage pathway for chlorocatechols (301). The simplest form of transposable elements are insertion sequences (IS), which are found on many plasmids (193). On the (chloro)aniline degradative plasmids too of this work, IS*1071*-like sequences were present. IS*1071* was first described as a the class II insertion sequence that brackets a chlorobenzoate-catabolic transposon (Tn*5271*) (210). When the transposon was introduced in microcosm with chlorinated aromatics, the populations of Tn*5271*-carrying bacteria were significantly higher in chloroaromatic-dosed

microcosms (103). These chemicals exerted a selective force on the Tn*5271*-carrying genotypes in natural systems. Moreover, the IS*1071* element has already been detected on other catabolic plasmids (47, 102, 150).

In conclusion, it is clear that plasmids play a major role in exchanging genetic material between bacteria, which may result in a better adaptation to changing environmental conditions. Patchwork assembly of existing pathways in novel combinations can occur by shuffling gene clusters from chromosomes to plasmids and *vice versa*.

INCREASED INTEREST IN BIOAUGMENTATION APPLICATIONS

Bioaugmentation as a tool for pollution prevention and cleanup is a relative new area in environmental microbiology and only during the last 10 years different research groups started to be interested in this area. As a result, the number of publications discussing bioaugmentation is increasing rapidly (Figure 8.2).

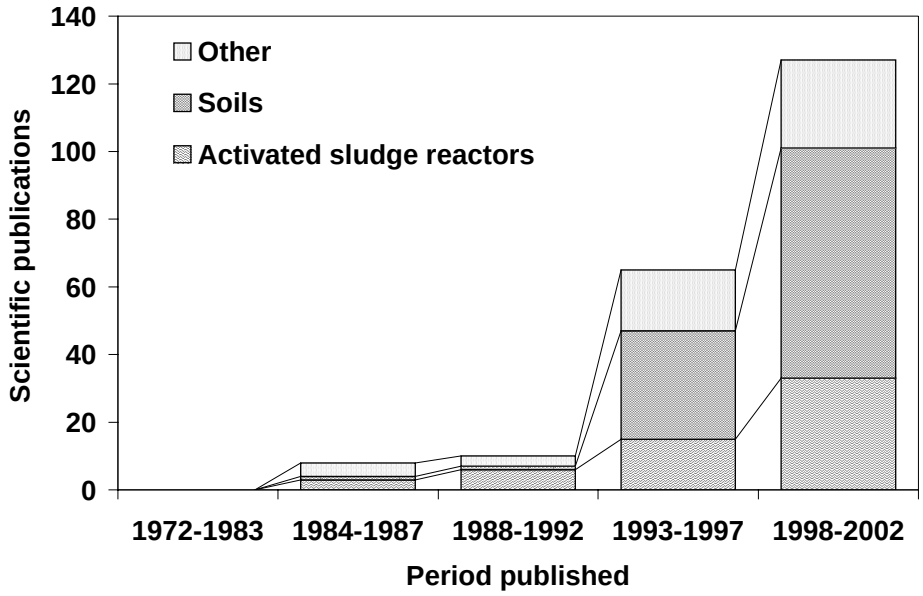

Figure 8.2. Number of scientific publications abstracted in the ISI database (source: www.websci.rug.ac.be).

Although the number of bioaugmentation reports of activated sludge reactors was higher in the eighties, during the nineties bioaugmentation of soils became more popular.

Bioaugmentation of bioreactors is not yet widely applied and only a few successful cases have been reported (311).

The use of bioaugmentation strategies for the remediation of polluted environments will increase the following years. Improper physical treatments of pollutants, such as incineration, can give rise to even more toxic products (144). Nowadays, the tendency exists to imply "green" technology in our society. Moreover, biotreatment is often a more economically feasible alternative than incineration (23).

NOVEL STRATEGIES TO PROLONG BIOAUGMENTATION IN ACTIVATED SLUDGE

The current technologies used for the bioaugmentation of activated sludge are not predictable and controllable enough compared to the physical or chemical destruction of pollutants. One of the causes of this lack of predictability is our incomplete understanding of survival and activity of the inocula. In our work, the 3-CA degrading *Comamonas testosteroni* strain I2 showed the best characteristics to be a successful strain for the inoculation of activated sludge. Other strains, such as *Delftia acidovorans* CA28 (data not shown) and *Ralstonia eutropha* JMP228*gfp* (pC1-3), poorly survived in the activated sludge, and minimal to no enhanced 3-CA degradation occurred in the activated sludge reactors. In contrast, our strain I2 remained at relatively high cell numbers in the reactors after inoculation, forming small clusters in the activated sludge flocs. Thus, strain I2 could return with the settled sludge and this probably aided its persistence in the reactor. The I2 cells were also protected by the integration in the sludge flocs from the predatory protozoa, which feed mainly on suspended bacteria (41, 200). However the metabolic activity declined during the experiments and resulted in an incomplete removal of 3-CA. This is probably the consequence of the harsh conditions that the bacteria had to cope with in de activated sludge system. Stress can lead to increased flocculation and aggregation behavior (40), but also to decreased catabolic activity towards specific compounds (327).

A possible solution for the decreased metabolic inoculum activity is the repeated inoculation of freshly grown metabolically active cells. Previous studies showed however that repeated inoculations of suspended cells would increase the population of predatory protozoa, resulting in a rapid decline of the inoculum and relevant indigenous bacteria, such as nitrifiers (41, 42). The repeated reinoculation was considered to be no option, since reactor failure could not be excluded and constant reinoculation was not a sustainable and feasible technology.

The continuous addition of small amounts of active 3-CA degrading bacteria was obtained by designing a new bioaugmentation principle, namely the slow release concept. The specialized inoculum was immobilized in an agar mixture that provided nutrients. The bacteria-agar mixture was subsequent injected into thin walled silicone tubes, which stayed open. In this protective niche, the inoculum could grow by using the nutrients from the agar and by metabolizing the 3-CA that diffused from the mixed liquor through the silicone walls. At the same time the bacteria were also released continuously at a constant rate and thus improved the bioaugmentation for a considerably longer time. Our approach combines the advantages of the "suspended" and "immobilized" bioaugmentation approaches used in previous studies (188, 199, 200). The immobilization of our cells made the retention in the activated sludge easier. The cells inside the silicone tubes were also able to degrade 3-CA. By the release of "not yet stressed" suspended cells from the open ends of the silicone tubes, high metabolic activities could be obtained. The second advantage of suspended cells compared to immobilized cultures is that the biodegradation rates at low concentrations are better (149). Modifications of the agar mixture and immobilization of consortia can extend the possibilities of the slow release principle.

Co-inoculation of strain I2 and a donor of the catabolic plasmid pC1-3 was another approach that prolonged the complete 3-CA removal greatly. The improved degradation capacity is probably due to the formation of indigenous transconjugants, although no transconjugants could be isolated. The number of intrinsic transconjugants was too low for complete 3-CA removal alone. But co-inoculation compensated for the 3-CA decreased degradation capacity of the I2 cells. To show that transfer of the plasmid pC1-3 to indigenous bacteria is possible, the plasmid was tagged with the *gfp* gene, coding for the green fluorescent protein. Plate matings and batch experiments showed that transfer occurred. Unfortunately there was insufficient time left to repeat the SCAS inoculation experiments with this marked plasmid, which would show the relation between the formation of transconjugants and the enhanced 3-CA removal.

In conclusion strain I2 can remove 3-CA completely for a limited period since its activity declines upon introduction in activated sludge. The amounts of chloroaniline added in our experiments are comparable with maximum concentrations measured in industrial wastewaters (185) and thus we can assume our bioaugmenting reactors were operated under the worst case scenario. In addition, two alternative methods, i.e. a slow release and a plasmid aided approach, were found to be effective for a complete 3-CA removal at high concentrations for longer periods.

MOLECULAR MICROBIAL ECOLOGY METHODS CAN HELP TO UNDERSTAND MICROBIAL COMMUNITIES

The problem of "unculturable" bacteria for the isolation of new catabolic strains was already discussed previously. The same problem exists when microbial diversity is investigated by methods based on cultivation and plating. In a review of Amann (1995), he describes the "great plate count anomaly" (10): huge discrepancies can exist between viable cell counts and direct microscopic counts (Table 8.2).

Table 8.2. Culturability determined as a percentage of culturable bacteria in comparison with total cell counts (after Amann, 1995 (10)).

Habitat	Culturability (%)[a]
Seawater	0.001–0.1
Freshwater	0.25
Mesotrophic lake	0.1–1
Unpolluted estuarine waters	0.1–3
Activated sludge	1–15
Sediments	0.25
Soil	0.3

[a] Culturable bacteria are measured as CFU.

Ribosomes are universal structures present in all prokaryotic and eukaryotic cells. This makes them a suitable tool to study bacteria which are not cultivable with the current technology. Pioneering work comprehended the direct extraction of 5S rRNA molecules, electrophoretic separation and sequencing (279). Since the 5S rRNA molecules are ca. 120 nucleotides long, the retrieved information was relatively small. Consequently, the larger rRNA molecules were used for studies in microbial ecology. An average bacterial 16S rRNA molecule has a length of 1,500 nucleotides, and 23S rRNA molecules are around 3,000 nucleotides long (10). The development of PCR allowed the rapid and selective amplification of 16S rRNA genes from mixed DNA. 16S and 23S rRNA genes have become the most used tool to analyze bacterial communities and identify its members.

A major disadvantage of using rRNA sequences is the fact that it is quite difficult to use rRNA similarity values for the identification at the level of a species or genus. Normally, bacterial taxonomists define a species on the basis of a DNA-DNA hybridization and strains

with DNA-DNA similarities of more than 70% are considered as the same species (255, 329). They also agree that rRNA sequence information alone should not be used define strains into species. Comparative studies involving both 16S rRNA sequencing and DNA-DNA hybridization indicated that a 50% DNA-DNA hybridization typically corresponds to about 99% 16S rRNA similarity (9). Therefore, interpretation of rRNA sequences in respect to taxonomic classification should considered carefully.

Denaturing gradient gel electrophoresis (DGGE) was the main molecular technique that was used in this study to analyze amplified PCR fragments. DGGE is a method that has long been used to identify single base mutations in DNA fragments (96). Muyzer et al. (1993) used DGGE for the first time in microbial ecology almost 10 years ago (208). Nowadays, DGGE has become a standard tool to investigate the composition and diversity of bacterial communities based on their 16S rRNA or the rRNA encoding genes. The patterns obtained by DGGE analysis are a fingerprint of the dominant members of the microbial community and richness, evenness and diversity indices can be calculated with these data. One has to bear in mind that the development of specific 16S rRNA primers for groups within the Bacteria allowed the development of group-specific DGGE's (111, 122, 126, 167). By only examining the community structure of bacterial subgroups, higher resolution of the DGGE patterns is obtained and specific responses of certain populations on environmental changes can be examined more in detail. The main disadvantage for the group specific approach was the optimization of the DGGE conditions for each primer set by changing percentage of acryl amide, denaturation gradient, running time and voltage.

In Chapter 7, the use of the group specific DGGE's was standardized by a nested PCR approach (Figure 8.3). The first PCR is used to amplify PCR fragments for the particular bacterial group with specific primers. This step is crucial for the specificity of the final DGGE patterns, for which a high [(first PCR fragment)/(total DNA)] ratio is required. A second PCR step uses general bacterial primers, of which the forward primer contains GC-clamp. During this second PCR step, the specific fragments of the first PCR round are reduced to a size suitable for DGGE analysis. Small PCR fragments have a better resolution on DGGE gels than large PCR fragments (37). The second PCR also adds the GC clamp to the PCR fragments.

PCR based techniques always suffer from some limitations. Primers for specific groups are designed based on the cultured members, and when these primers are used for the amplification of mixed bacterial communities these primers may be too specific or not specific enough (234). Continuous evaluation and redesign of primers is therefore necessary.

Another possible disadvantage of applying PCR is the introduction of bias due to preferential amplification (289). Quantification based on band intensities can give a skewed representation of the real bacterial distribution in the microbial community. Additional problems may be incomplete lysis of all cells (99) and different numbers of 16S rDNA copies in chromosome of different bacteria (55, 217, 236). In spite of these disadvantages, PCR-DNA based techniques have contributed a lot to the present knowledge of microbial communities.

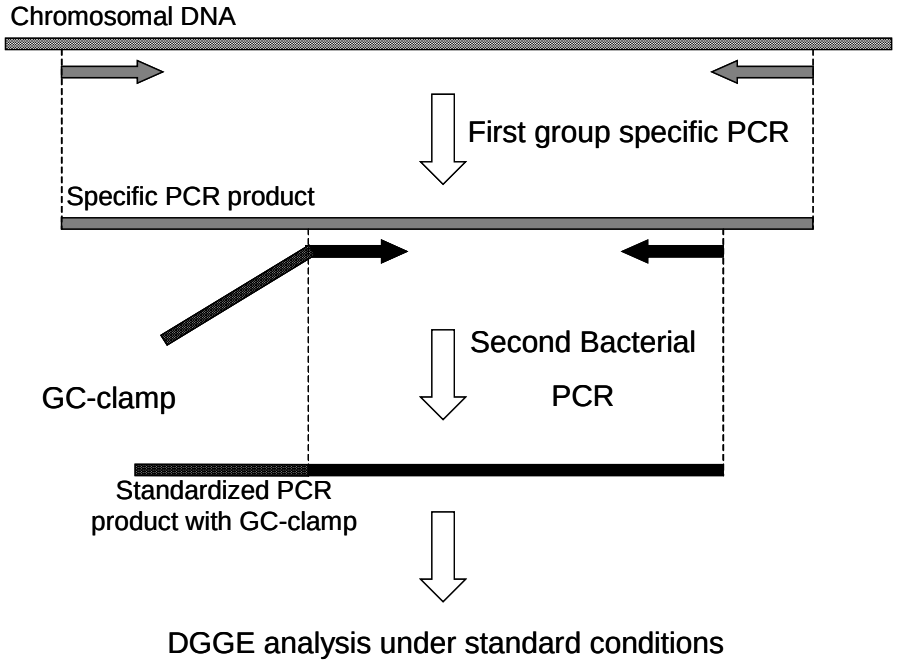

Figure 8.3. Scheme of the nested PCR-DGGE approach to standardize the use of DGGE for specific bacterial groups.

Fingerprinting techniques describing microbial communities have one major disadvantage: the data are qualitative. Some authors suggest that the intensity of the 16S rDNA bands in a DGGE gel may correspond at least semiquantitavely with the abundance of the corresponding species (126, 127). However, when considering the limitations mentioned above, this assumption may as well be wrong as correct. The fingerprinting techniques should not be used anymore as stand alone tools and the implementation of quantitative techniques will become indispensable. At the moment, Fluorescent *in situ* hybridization (FISH) is the most common technique for the quantification of bacterial groups in activated sludge systems and biofilms (67, 257). The DNA chips or micro-arrays are also intended to have some

quantification properties (271), however its use for environmental samples is still very limited. Real time PCR (124) and the bead-based method for multiplexed identification and quantification of DNA sequences using Flow Cytometry (276, 277) are novel and very promising techniques for the quantification of microbial populations in environmental samples. Future experiments should be designed in such a way that microbial communities are examined both quantitatively and qualitatively by combinations of molecular techniques.

PERSPECTIVES: NEW BIOAUGMENTATION CHALLENGES IN THE 21ST CENTURY

The study of biodegradative strains will change drastically during the 21st century. Costerton et al. (60) pointed out clearly that the current studies of planctonic monospecies cultures cannot be easily extrapolated to environmental conditions. This is probably one of the major causes of so many bioaugmentation failures. Microbiologists like to isolate a microbial strain from a very complex multi-species environment, cultivate it and learn it tricks, such as production of pharmaceuticals and fine chemicals or the degradation of environmentally undesired compounds. They can even provide the bacterial strains the genetic information for the desired metabolic process. However problems occur when these strains are introduced again into the natural environment: Cells, grown as a monoculture in nutrient rich media and at optimal temperatures, encounter nutrient limiting conditions, predation by protozoa, parasitism by phages and antagonism by the indigenous bacteria. These harsh conditions should be a quite stressful for the inocula (Figure 8.4).

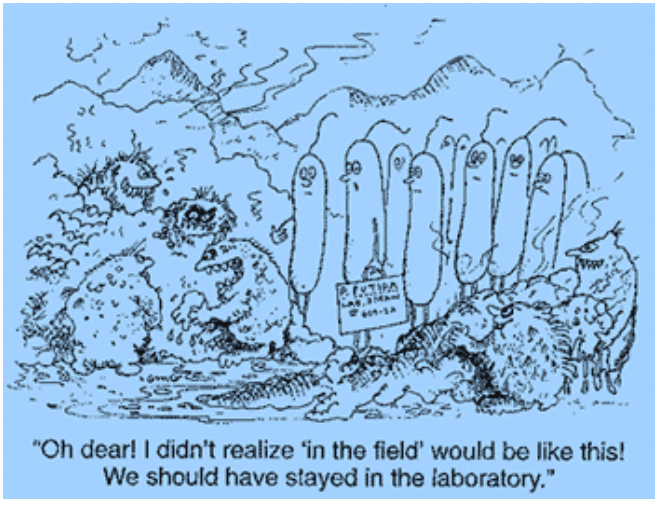

Figure 8.4. Figure, expressing the feeling of inoculated laboratorory-adapted bacteria into the environment (source unknown).

The main challenge will be the development of a frame to study the inoculants introduced in microbial communities. Molin described the SCIO (**s**tructure, **c**oordination, **i**nteraction and **o**rganism) model as a way to describe microbial communities (205). We believe that the same four parameters can be used as guideline for understanding and engineering inoculum introduction and bioaugmentation.

Structure refers to the position of the component elements in a 3-dimensional arrangement. The structural parameter is very important for activated sludge flocs. The structure of sludge flocs is very porous and the bacteria inside undergo the same nutrient flow compared to suspended bacteria. This means that flocs cannot be seen as dense solid particle of microorganisms (180, 274). Therefore the structure of activated sludge flocs resembles a biofilm structure, where canals are also present between mushroom-shaped secondary structures (166). Gradients of substrates exist (336) and the cells are entrapped in EPS layers (97). Fluorescent *in situ* hybridization techniques (320) or labeling inoculants with fluorescent proteins (83) and confocal scanning laser microscopy can reveal the structural organization within activated sludge flocs and the behavior of the inoculants. Inoculants can be selected on the basis of their cell surface properties and the hydrophobicity of the activated sludge flocs (342). Sludge flocs, which contain the majority of the bacteria present in activated sludge, are an ideal site for conjugations. It is not investigated in depth whether exopolymeric material is not an obstacle for conjugation (311). It must be noted that DNA has been regularly found in the EPS of biofilms and activated sludge from wastewater systems (215).

Coordination results in a microbial community where different structures and functions can coexist. To accomplish this, communication between individual cells and between cell clusters is required. This communication is established by chemical signals, so called quorum sensing signals, and these can be subdivided into two main categories: (1) amino acids and short peptides, commonly utilized by Gram-positive bacteria and (2) fatty acid derivatives, frequently utilized by Gram-negative bacteria (for a recent review, see reference (333)). While it has been shown that during biofilm formation quorum sensing is involved (68), to our knowledge no reports showed this was also the case for activated sludge flocs. It would however be very unlikely no if communication between cells in activated sludge flocs occurred. Unraveling the communication puzzle in activated sludge can lead to the modification and steering of introduced inocula, resulting in better fitness, survival and bioaugmentation activity. A lot of processes are controlled by quorum sensing mechanisms, such as plasmid transfer, enzyme production in pathogens and antibiotic production (333). Possibly also biodegradation is controlled by intercellular communication. As far, no

indications were found for this, but during the pathway of catechol degradation, also a lactone structure is formed (see Chapter 1, Figure 4) which resembles quorum sensing molecules. However, preliminary experiments with these lactonic structures did not show increased biodegradation rates (J.R. van der Meer, personal communication).

The ***interaction*** parameter refers to the direct observable activity of organisms reacting to signals and responses from the environment (biotic or abiotic). The reaction of micro-organisms should be studied *in situ* in the environment. Current work frequently uses monoculture planctonic cell cultures to examine bacterial responses. It was already mentioned that most bacterial cells do not live as planctonic cells in the environment but as cell aggregates. The physiological state of planctonic cells can be totally different with cells living as aggregates. For example *Pseudomonas aeruginosa* , grown as planktonic cells, have more than 800 proteins which have a six fold or greater change in expression level (over 50% of the proteome) when compared with maturation-2 stage biofilm cells (253). This was a monoculture experiment, so gene expression in mixed communities can be influenced even more. Modern molecular tools allow us to monitor *in situ* gene expression. A currently used method is Fluorescent *in situ* hybridization (FISH) of the rRNA, since the ribosome content reflects the physiological state of the cells (233). To monitor expression of metabolic genes, inoculants can be provided with a reporter gene, such as GFP, linked to a specific promoter (50). *In situ* PCR (135) or reverse transcriptase-PCR of extracted total RNA (262) are two other approaches for assessing the activity of cells within a mixed microbial community. All these observation techniques can be used to investigate which modifications can have a positive influence on the metabolic activity of inocula.

Finally the ***organism*** parameter refers to the types, characteristics and relative numbers of bacteria in an ecosystems. *In situ* identification is currently accomplished with FISH, using specific of general probes (320). As discussed previously, also the other molecular techniques, such as microbial population fingerprinting, can give an idea of the population composition. Isolation methods for degradative strains needs also some consideration. Future bioaugmentation experiments can be accomplished by well defined microbial consortia, of which the genetic information is fully characterized. The same techniques as has been used for the cloning of the metagenome of a soil, are suitable for the characterization of such consortia (248).

Bacteria in the environment cannot be considered anymore as lone rangers. Modern microbial ecologists like to think about bacterial populations as multicellular organisms (266).

Collectively and coordinately, bacteria act far more efficiently than they could as autonomous agents. The challenge will be the study these bacteria as multicellular communities and to examine how each individual cell receives, interprets, and responds to information from its neighbor cells. The following citation of J.A. Shapiro can serve both as a conclusion and further perspective for microbial ecology: “thinking about bacterial populations as multicellular organisms may help put microbiology at the top of the scientific agenda in the 21st Century” (266).

SUMMARY

Chloroanilines are important precursors in chemical industry and a literature search showed that these types of molecules are found regularly in industrial wastewaters. Since chloroanilines are not removed by the wastewater treatment, these molecules are discharged in the environment. There is obviously a need for more research to enhance chloroaniline degradation in the wastewater treatment systems in order to prevent the release of these compounds in the environment. 3-chloroaniline (3-CA) is also a very good model compound for recalcitrant chloroaromatic compounds. As many of these compounds, it is often degraded via the common central molecule, chlorocatechol. Literature review indicates that the number of cases of successful bioaugmentation is limited. In addition, the influence of pollutants and bioaugmentation on the microbial communities of activated sludge has not yet been studied thoroughly.

A first part of this research was focused on the diversity of five different chloroaniline degrading bacteria. Two strains have been described and identified previously, i.e., *Delftia acidovorans* CA28 and BN3.1. Strains I2 and B8c were isolated in this study from a wastewater treatment plant and were identified as *Comamonas testosteroni* and *Delftia acidovorans*, respectively. Strain LME1, identified as *Delftia acidovorans*, was isolated from a linuron-treated soil. Both *Delftia* and *Comamonas* belong to the family of the *Comamonadaceae*. All five strains possess a large plasmid of ca. 100 kb, but the plasmids from only 4 strains could be transferred to a recipient strain by selecting on aniline or 3-CA as sole source of carbon and/or nitrogen. Plasmid transfer experiments and Southern hybridization revealed that the plasmid of strain I2 encodes total aniline but not 3-CA degradation, while the plasmids of strains LME1 and B8c were only responsible for the oxidative deamination of aniline. Several transconjugant clones that had received the plasmid from strain CA28 showed different degradative capacities: all transconjugants could use aniline and 3-CA as nitrogen source, while only some of the transconjugants could dechlorinate 3-CA. For all four plasmids, the IS*1071* insertion sequence of Tn*5271* was found to be located on a 1.4 kb restriction fragment, which also hybridized with a probe for the *tdnQ* gene, encoding the deamination of aniline. This suggests the involvement of this IS element in the dissemination of aniline degradative genes in the environment. Specific primers for the *tdnQ* gene from *Pseudomonas putida* UCC22, the diversity of the PCR amplified fragments in the five strains was examined by denaturing gradient gel electrophoresis (DGGE) and sequence analysis. Sequencing data showed that the *tdnQ* sequences of I2, LME1, B8c and

CA28 were very closely related, while the *tdnQ* fragment of BN3.1 and *P. putida* UCC22 were less similar. Northern hybridization revealed that the *tdnQ* gene is only transcribed in the presence of aniline and not when only 3-CA is present.

The *Comamonas testosteroni* I2 strain was subsequently tested for its ability to clean wastewater containing 3-CA upon inoculation in activated-sludge. To monitor its survival, the strain was chromosomally marked with the *gfp* gene and designated I2*gfp*. After inoculation into a lab-scale semicontinuous activated-sludge (SCAS) system, the inoculated strain maintained itself in the sludge at least 45 days and was present in the sludge flocs. After an initial adaptation period of 6 days, complete degradation of 3-CA was obtained during two weeks, while no degradation at all occurred in the non-inoculated control reactors. Upon further operation of the SCAS system, only 50 % 3-CA removal was observed. DGGE of 16S rRNA genes revealed a dynamic change in the microbial community structure of the activated-sludge. The DGGE patterns of the noninoculated and the inoculated reactors evolved after 7 days to different clusters, which suggests an effect of strain inoculation on the microbial community structure.

To obtain prolonged 3-CA degradation a slow release strategy was tested. *C. testosteroni* strain I2, was mixed with molten agar and encapsulated in 4 mm diameter open ended silicon tubes of 3 cm long. The tubes containing the immobilized bacteria represented about 1 % of the volume of the mixed liquor. The bioaugmentation activity of a reactor containing the immobilized cells was compared with a reactor with suspended I2*gfp* cells. From day 25-30 after inoculation, the reactor with only suspended cells failed to completely degrade 3-CA, probably due to both a decrease in cell concentration and metabolic activity. In the reactors with immobilized cells however, 3-CA continued to be removed. A mass balance indicated that ca. 10 % of the degradation activity was due to the immobilized cells. Slow release of the growing embedded cells from the agar into the activated sludge medium, resulting in a higher number of active 3-CA degrading I2 cells, was responsible for most of the degradation.

A second possible alternative to overcome the problem of decreased catabolic activity of an inoculum is the delivery of catabolic genes to the indigenous bacteria. We examined the introduction of the catabolic plasmid pC1-3, involved in deamination and dechlorination of 3-chloroaniline (3-CA), into activated sludge that received a daily 3-CA dose. The introduction of the plasmid donor did not result in sustained bioaugmentation. In contrast, co-introduction of the donor of pC1-3 and the 3-CA metabolizing strain *C. testosteroni* I2*gfp* resulted in a significant prolongation of complete removal of 3-CA from the reactors. Inoculation of strain

I2*gfp* alone could not support this prolonged 3-CA degradation after a few weeks. Since plasmid pC1-3 lacked suitable selection markers the possible formation of transconjugants could not been shown in the coinoculated reactor. Before a next inoculation experiment was set up, plasmid pC1-3 was marked with a mini-Tn*5* transposon, containing genes encoding for the green fluorescent protein (GFP) and kanamycin resistance. The *gfp* marked plasmid, pC1::gfp, was subsequently used in batch experiments to show that plasmid transfer towards the indigenous bacteria occurred. Transfer frequencies between 4×10^{-6} and 1×10^{-5} were detected. We postulate two possible reasons to explain the success of co-inoculation: (i) transfer of plasmid pC1-3 occurred and allowed the indigenous sludge bacteria to degrade 3-CA or (ii) an as yet unknown synergistic effect between the two co-inoculated strains caused a better degradation than when both strains were inoculated separately. In conclusion bioaugmentation of bioreactors can be improved by the co-inoculation of the metabolic strain I2gfp and a catabolic plasmid bearing donor strain.

As in the three previous chapters, most bioaugmentation studies focus on the removal of xenobiotics with little attention paid to the recovery of disrupted reactor functions such as nitrogen removal. Therefore the effects of a 3-CA shock load were evaluated on activated sludge reactor functions such as nitrification, carbon removal, and sludge compaction. One set of disturbed reactors was bioprotected by the inoculation with *C. testosteroni* I2*gfp*. Changes in function were compared with the sludge community structure, in particular the nitrifying populations. DGGE and Fluorescent *in situ* Hybridisation (FISH) characterized and enumerated the ammonium oxidising microbial community of SCAS reactors immediately after a 3-CA shock load. Two days after the 3-CA shock, the bacterial richness and numbers of ammonium oxidising bacteria decreased, ammonium accumulated and the nitrification activity did not recover over a 12-day period. In contrast, in the bioprotected reactor, nitrification was also completely inhibited at day 2, but started to recover from day 4 on. This recovery was linked to a differing community structure in the bioprotected reactor compared to the unprotected reactor. The settleability of the activated sludge was negatively influenced by the 3-CA addition, with the Sludge Volume Index increasing by 230% and the filament index increasing from 1 to 3. Two days after a 3-CA shock, chemical oxygen demand (COD) removal efficiency decreased by 36% but fully recovered by day 4. In contrast, in the bioprotected reactor, no decrease of the COD removal efficiency was observed.

Different bacterial groups each play a particular role in microbial communities. The present techniques do not allow the screening of high numbers of samples for different bacterial groups. Therefore an easy and fast nested PCR-DGGE based technique was

developed to examine the diversity of different bacterial groups of activated sludge samples that received wastewater from four different types of industry. Specific 16S rRNA primers were chosen for large bacterial groups (Bacteria and α-Proteobacteria in particular), which dominate activated sludge communities, as well as for actinomycetes, ammonium oxidisers and methanotrophs (Types I and II). In addition primers for the new *Acidobacterium* kingdom were used to observe their community structure in activated sludge. After this first PCR amplification, a second PCR with Bacterial primers yielded 16S rRNA gene fragments that were subsequently separated by DGGE, thus generating "group specific DGGE patterns". The community structure and diversity of the bacterial groups from the different samples was further analyzed using different techniques, such as statistical analysis and Shannon diversity index evaluation of the band patterns. By combining the seven DGGE gels, cluster analysis, Multidimensional scaling (MDS) and Principal Component Analysis (PCA) clearly clustered two of the four activated sludge types separately.

In conclusion, in this work several new 3-CA degrading bacteria were isolated and characterized. One of these strains was successfully used to bioaugment activated sludge by different strategies. Finally, a nested PCR-DGGE method was evaluated to examine the diversity of specific bacterial groups in activated sludge.

SAMENVATTING

Chlooranilines zijn belangrijke intermediairen in de chemische industrie en een literatuurstudie heeft duidelijk aangetoond dat deze moleculen geregeld teruggevonden worden in bepaalde industriële afvalwaters. Via de klassieke waterzuiveringssystemen worden chlooranilines niet verwijderd en als gevolg daarvan worden deze moleculen vrijgesteld in het milieu. 3-chlooraniline (3-CA) is een zeer goede modelcomponent voor recalcitrante chloroaromatische componenten. Zoals velen van deze moleculen wordt 3-CA dikwijls gemineraliseerd via het centraal intermediair chlorocatechol. Het is overduidelijk dat er een grote nood bestaat aan meer onderzoek om chlooraniline degradatie in waterzuiveringssystemen te bewerkstelligen zodat de vrijstelling van deze componenten in het milieu wordt verhinderd. Daarenboven is de invloed van polluenten en bioaugmentatie op microbiële gemeenschappen van actief slib nog onvoldoend onderzocht.

Een eerste deel van het onderzoek heeft zich toegespitst op het bestuderen van de diversiteit van chlooraniline degraderende bacteriën. Hiervoor werden vijf verschillende stammen onderzocht. Twee van deze stammen waren reeds vroeger beschreven en geïdentificeerd, namelijk *Delftia acidovorans* CA28 en BN3.1. De stammen I2 en B8c werden in deze studie geïsoleerd uit een waterzuiveringsinstallatie en ze werden geïdentificeerd als *Comamonas testosteroni* en *Delftia acidovorans* respectievelijk. De stam LME1, geïdentificeerd als *Delftia acidovorans* werd geïsoleerd uit een met linuron behandelde bodem. Zowel *Delftia* als *Comamonas* behoren tot de familie van de *Comamonadaceae*. De vijf stammen bevatten een groot plasmide van ongeveer 100 kb. Enkel de plasmides van vier stammen konden worden overgedragen naar een acceptorstam door de transconjuganten te selecteren op een medium met aniline of 3-CA als enige koolstof en/of stikstofbron. Deze transfer experimenten en Southern-hybridisaties toonden aan dat het plasmide van de stam I2 voor totale aniline degradatie, maar niet voor 3-CA degradatie codeerde, terwijl de plasmiden van de stammen LME1 en B8c enkel verantwoordelijk waren voor de oxidatieve deaminatie van aniline. Na transfer van het plasmide van stam CA28 werden er verschillende transconjuganttypes met verschillende metabolische capaciteiten gevonden. Alle transconjuganten konden aniline en 3-CA gebruiken als enige stikstofbron, maar sommige types konden 3-CA niet meer dechlorineren. Alle vier plasmiden bevatten een sequentie die gelijkaardig was met de IS*1071* insertiesequentie van Tn*5271*. Hetzelfde restrictiefragment hybridiseerde ook met een probe voor het *tdnQ*-gen, dat instaat voor de deaminatie van aniline. Deze bevindingen suggereren dat dit type IS-element een rol gespeeld

heeft in de verspreiding van aniline degraderende genen in het milieu. Specifieke primers voor het *tdnQ*-gen, werden ontwikkeld en via denaturing gradient gel electropheresis (DGGE) werden er drie verschillende clusters van het *tdnQ*-gen opgemerkt. Sequentie analyses toonden aan dat de *tdnQ*-genen van I2, LME1, B8c en CA28 zeer vergelijkbaar waren, terwijl de *tdnQ*-fragmenten van BN3.1 en UCC22 minder overeenkomst vertoonden met de andere sequenties. Northern-hybridisatie toonde aan dat het *tdnQ*-gen enkel afgeschreven werd in de aanwezigheid van aniline en niet wanneer 3-CA aanwezig was.

Er werd nagegaan of de stam *C. testosteroni* I2 na introductie in actief slib bruikbaar was om afvalwater te zuiveren dat gecontamineerd was met 3-CA. De stam werd gemerkt met het *gfp*-gen in het chromosoom om de overleving te kunnen opvolgen. Na de inoculatie van de stam in een labo-schaal semi continu actief slib (SCAS)-systeem, overleefde de stam gedurende 45 dagen in het slib als clusters in de slibvlokken. Na een initiële adaptatieperiode van 6 dagen werd er complete verwijdering van 3-CA bekomen gedurende twee weken, terwijl er geen degradatie optrad in niet-geïnoculeerde controlereactors. Na deze twee weken werd er nog maar 50 % 3-CA verwijdering waargenomen. DGGE van de 16S rRNA genen toonden een dynamische evolutie van de microbiële gemeenschap in het actief slib aan. De DGGE patronen van de niet-geïnoculeerde en geïnoculeerde reactoren differentieerden zich na 7 dagen in twee clusters. Dit toont aan dat de inoculatie van de stam een effect had op de structuur van de microbiële gemeenschap.

Een nieuwe ‘slow release’ strategie werd vervolgens getest om volledige 3-CA degradatie gedurende langere periodes te bekomen. *C. testosteroni* I2 werd gemengd met gesmolten agar en omringd door siliconen buisjes van drie cm lang en vier mm diameter. De tubes met de geïmmobiliseerde bacteriën maakten ongeveer 1 % uit van het volume van het reactorvolume. De bioaugmentatie activiteit van de reactor met de geïmmobiliseerde cellen werd vergeleken met de reactor met de gesuspendeerde cellen. Wegens een daling in celconcentratie en metabolische activiteit werd er na 25 à 30 dagen in de reactor met enkel gesuspendeerde cellen, onvolledige 3-CA verwijdering vastgesteld. Dit was in tegenstelling tot de reactors met de geïmmobiliseerde cellen, waar 3-CA continu werd verwijderd. Een massabalans toonde aan dat ongeveer 10 % van de degradatiecapaciteit te danken was aan de geïmmobiliseerde cellen. De trage vrijstelling in het reactormedium van de cellen die aan het groeien waren in de agar, waren verantwoordelijk voor het merendeel van de degradatie.

Een tweede mogelijk alternatief om de dalende katabolische activiteit te voorkomen, is de verspreiding van een katabolisch plasmide naar de endogene autochtone bacteriën. De introductie van de donor *Ralstonia eutropha* JMP228*gfp* met het katabolisch plasmide pC1-3

dat 3-CA kan deamineren en dechlorineren in actief slib dat een dagelijkse dosis 3-CA ontving, werd geëvalueerd . De introductie van het plasmide leidde niet tot een duurzame bioaugmentatie. De co-introductie van de donor voor het plasmide pC1-3 en de stam I2 resulteerde echter wel in een duidelijk langere periode van complete 3-CA verwijdering. De inoculatie van de stam I2 alleen kon enkel gedurende een paar weken 3-CA volledig degraderen. Transconjuganten konden in dit experiment niet worden aangetoond omdat het plasmide pC1-3 geen selectieve merker bevatte. Er viel geen onderscheid te maken tussen transconjuganten en de co-geïnoculeerde I2-stammen. Daarom werd het plasmide pC1-3 gemerkt met het mini-Tn*5* transposon waarop de genen lagen voor het groen fluorescerend proteïne (GFP) en voor kanamycine resistentie. In batch experimenten met een donor van het *gfp* gemerkte plasmide pC1::gfp en actief slib, werd vervolgens aangetoond dat plasmide transfer naar de autochtone bacteriën kon plaatsvinden aan detecteerbare frequenties.

De bioaugmentatie van bioreactors spitst zich vooral toe op de verwijdering van xenobiotica, maar slechts weinig aandacht wordt besteed aan het herstel van ontregelde reactorfuncties, zoals stikstofverwijdering. Daarom werden de effecten van een éénmalig toegediende dosis 3-CA op actief slib reactor functies zoals nitrificatie, koolstofverwijdering en slibcompactheid, geëvalueerd. Eén set van verstoorde reactoren werd geïnoculeerd met *Comamonas testosteroni* I2. De veranderingen in functie werden vergeleken met de structuur van de slibgemeenschap, en in het bijzonder die van de nitrificerende populaties. DGGE en Fluorescent *in situ* Hybridazation (FISH) werden gebruikt om de ammonium oxiderende populatie kwalitatief en kwantitatief te bestuderen na een piek van 3-CA. Twee dagen na de 3-CA piek daalden de bacteriële rijkdom en het aantal ammonium oxiderende bacteriën met als gevolg dat ammonium accumuleerde. De nitrificatie herstelde zich niet gedurende de duur van het experiment (12 dagen). In de geïnoculeerde reactor werd de nitrificatie weliswaar geïnhibeerd op dag twee, maar vanaf dag vier herstelde de nitrificatie zich. Dit herstel was gecorreleerd met een verschillende gemeenschapsstructuur in de geïnoculeerde reactor vergeleken met de niet-geïnoculeerde reactor. De bezinkbaarheid van het actief slib was negatief beïnvloed door de 3-CA dosis, wat resulteerde in een toename van de Sludge Volume Index van 230 % en de filament index steeg van 1 naar 3. Twee dagen na de 3-CA piek daalde de verwijdering van de chemische zuurstofvraag met 36 % maar deze herstelde zich vanaf dag vier. De geïnoculeerde reactor toonde geen daling in chemische zuurstofvraag verwijdering.

Verschillende bacteriële groepen vervullen een specifieke rol in microbiële gemeenschappen. De huidige technieken laten niet toe om een groot aantal stalen tegelijkertijd te screenen voor een aantal bacteriële groepen. Daarom werd er een eenvoudige

en snelle methode ontwikkeld, gebaseerd op een ‘nested’ PCR-DGGE techniek, om de diversiteit van verschillende bacteriële groepen te bestuderen van actief slib stalen van een viertal industriële types. Specifieke 16S rRNA primers werden gekozen voor grote bacteriële groepen (Bacteria en α-Proteobacteria) die actief slib gemeenschappen domineren en minder prominente groepen, zoals actinomyceten, ammonium oxideerders en methanotrofen (type I en II). Daarnaast werden primers gebruikt om het nieuwe *Acidobacterium* kingdom te bestuderen in actief slib. Na deze eerste PCR amplificatie werd er een tweede PCR met Bacteriële primers uitgevoerd. De bekomen 16S rRNA gen fragmenten werden vervolgens gescheiden via DGGE en op die manier werden er groep-specifieke DGGE patronen bekomen. De structuur en de diversiteit van de gemeenschap werd verder geanalyseerd via verschillende technieken, zoals statistische analyse en de Shannon diversiteits index. Via de combinatie van de zeven DGGE gels konden twee van de vier actief slib types van verschillende industrieën onderscheiden worden via cluster analyse, Multidimensional scaling (MDS) en Principale Componenten Analyse (PCA).

In conclusie, verschillende nieuwe 3-CA degraderende bacteriën werden geïsoleerd en gekarakteriseerd. Eén van deze stammen werd vervolgens met succes gebruikt voor de bioaugmentatie van actief slib via verschillende strategieën. Tenslotte werd een nested PCR-DGGE methode geëvalueerd die het toelaat om de diversiteit van bacteriële groepen in actief slib te bestuderen.

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PERSONALIA

Age: 26
Date of birth: 3 July 1975
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Nationality: Belgian

PROFESSIONAL ACTIVITIES

1998-2001	Doctoral research carried out at the Laboratory of Microbial Ecology and Technology (Ghent University) with Prof. Dr. ir. Eva M. Top and Prof. Dr. ir. Willy Verstraete as scientific promoters (project grant G.O.A. 12050797 of the "Ministerie van de Vlaamse Gemeenschap, Bestuur Wetenschappelijk Onderzoek" (Belgium))
2001-2002	Assistant position (AAP) at the Laboratory of Microbial Ecology and Technology (Ghent University)

WORK EXPERIENCES

1998-2002	Collaborator of research projects commissioned by Inexo, Fonck, Laborelec, Avecom and Procter & Gamble

1998-2002	Tutor of 4 students bio-engineer during their Master's thesis and 3 students laboratory technicians A1 during their training
1998-1999	Responsible for practical exercises of the course "Biotechnological Processes of Environmental Sanitation"
1999-2002	Responsible for practical exercises of the course “Microbial Ecological Processes”

EDUCATION

Koninklijk Atheneum Tienen (1989-1993)

Mathematics-Sciences

Ghent University (1993-1998)

Faculty of Agricultural and Applied Biological Sciences:

Bio-engineer Cell and Gene Biotechnology (Faculty of Agricultural and Applied Biological Sciences, Ghent University); Distinction

Master's thesis: "Isolation and characterisation of 3-chloroaniline degrading bacteria and their catabolic genes" with Prof. Dr. ir. Eva M. Top and Prof. Dr. ir. Willy Verstraete as scientific promoters; Great distinction

Ghent University (1998-2002)

Faculty of Agricultural and Applied Biological Sciences

PhD study programme

TRAINEESHIPS

- "International Fluorescent *In Situ* Hybridization (FISH) Course" at the Department of Microbiology TU München, Germany (M. Wagner), September 1999.
- "*In situ* monitoring of plasmid transfer" at the Technical University of Denmark, Lyngby, Denmark (E. Zechner and S. Molin), August 2000.

PUBLICATIONS WITH PEER REVIEW

- Boon, N., L. De Gelder, H. Lievens, S.D. Siciliano, E.M. Top and W. Verstraete. 2002. Bioaugmenting bioreactors for the continuous removal of 3-chloroaniline by a slow release approach. Env. Sci. Technol. Submitted.
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- Vincke, E., N. Boon and W. Verstraete. Analysis of the microbial communities on corroded concrete sewer pipes – a case study. Appl. Microbiol. Biot. 57:776-785.
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PUBLICATIONS WITHOUT PEER REVIEW (PROCEEDINGS)

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- Boon, N., H. Lievens, W. Verstraete and E. M. Top. 2001. Aniline and 3-chloroaniline degrading bacteria: genetics and possible applications. *In:* Proceedings 7th PhD symposium. Mededelingen Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Ghent. 66(4):87-91.
- Boon, N., H. Lievens, W. Verstraete and E. M. Top. 2000. Plasmid diversity in 3-chloroaniline degrading *Comamonadaceae* and their application in the bioaugmentation of activated sludge. *In:* Proceedings 6th PhD symposium. Mededelingen Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Ghent. 65(4):163-168.
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PARTICIPATION IN SYMPOSIA, CONGRESSES,…

- 12th Forum for Applied Biotechnology (FAB), Provincial Court, Brugge, Belgium. 24-25 September 1998. (poster presentation)
- Najaarsvergadering van de Belgische Vereniging voor Microbiologie "Apoptosis and Microorganisms", Leuven, Belgium. 6th November 1998. (poster presentation)
- 1st symposium of the EU-concerted action on Mobile elements' contribution to bacterial adaptability and diversity (MECBAD), Mont Ste. Odile, France. 23-27 April 1999. (poster presentation)
- 13th Forum for Applied Biotechnology(FAB), Het Pand, Ghent, Belgium. 22-23 September 1999. (poster presentation)
- 5th PhD symposium of the Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Ghent University , Ghent, Belgium. 13th Oktober 1999. (poster presentation)
- Najaarsvergadering van de Belgische Vereniging voor Microbiologie "Microbial Identification: an Integrative Approach", Leuven, Belgium. 17th December 1999. (poster presentation)
- 2nd symposium of the EU-concerted action on "Mobile genetic elements' contribution to bacterial adaptability and diversity" (MECBAD), Prague, Czech Republic. 15-19 September 2000. (oral and poster presentation)
- 6th PhD symposium of the Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen , Ghent University , Ghent, Belgium. 11th Oktober 2000. (poster presentation)
- Symposium on the Green Fluorescent Protein, Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, KUL, Leuven, Belgium. 18th May 2001.
- 9th International Symposium on Microbial Ecology (ISME-9) on "Interactions in the microbial world", Amsterdam, The Netherlands. 26-31 August 2001. (oral presentation)
- 3th symposium of the EU-concerted action on "Mobile genetic elements' contribution to bacterial adaptability and diversity" (MECBAD), Berlin, Germany. 15-19 September 2001. (oral and poster presentation)
- 7th PhD symposium of the Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen , Ghent University , Ghent, Belgium. 10 Oktober 2001. (poster presentation)

MISCELLANEOUS

- First place of the 4th "Vlaamse Biologie Olympiade" and "Prijs VOB", organised by the "Vereniging voor het Onderwijs in de Biologie, de Milieuleer en de Gezondheidseducatie". 15th May 1993, Beerse, Belgium
- Honourable Mention of the 4th International Biology Olympiad. 4-11 July 1993, Utrecht, the Netherlands
- "Prijs voor Biologie Jaques Kets 1993 with the thesis “Landpissebedden: Verspreiding en Ecologisch Onderzoek”; “Distribution and Ecology of Woodlice”, organized by "De Koninklijke Maatschappij voor Dierkunde van Antwerpen". 15th September 1993, Antwerpen, Belgium.

DE AFSLUITER

Het moet juli 1997 geweest zijn, toen ik de eerste stappen in LabMET zette. Na een thesisjaar kon ik daar blijven om te doctoreren en het werk van die vier jaar is nu eindelijk gebundeld. De moleculaire groep van LabMET - toen nog bestaande uit een tweetal personen - had net een eigen labo (labo 245) gekregen, ver weg van iedereen op eenzame hoogte (het tweede verdiep). Er is ondertussen daar al veel veranderd en er zal, zelfs heel binnenkort, nog veel veranderen...

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Nico

13 juni 2002