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Abstract

Many river sediments have become polluted with various estrogenic compounds, which can cause toxicological effects on aquatic organisms, like the feminization of male fishes. On of these estrogenic compounds is nonylphenol (NP). Nonylphenol exists of a phenol group with a linear or a branched chain of nine carbon atoms, and mixtures of the branched isomers are mainly present as a pollutant in the environment. Sediments polluted with NP may act as a secondary source of NP for the river water due to desorption, where it can cause toxicological effects on aquatic organisms. The toxicological risk of NP in the sediment depends on the availability in the sediment, the mass transfer from the sediment to the river water and the biodegradation potential of NP. The aim of this thesis is to obtain insight into the availability, the mass transfer and the biodegradation potentials of NP in polluted river sediment.

An analysis method was developed to measure NP concentrations in samples with liquid and liquid and sediment (Chapter 2). Anaerobic and aerobic degradation experiments were performed with aged NP polluted sediment and the involved microorganisms were identified (Chapters 3 and 4, respectively). Furthermore, aerobic degradation of NP was combined with the availability and the estrogenic activity under optimized conditions for biodegradation (Chapter 5). In addition, a continuous flow-through experiment was performed under settled sediment and resuspended sediment conditions to mimic varying hydrodynamic conditions in a river system at lab scale (Chapter 6).

The developed method to analyse NP in samples with liquid and liquid and sediment was based on solid phase micro extraction with extraction from the headspace (Chapter 2). Sediment particles in the samples influenced the measured NP concentration. Dilution of the slurry samples below 1.8 g sediment$\cdot l^{-1}$ reduced this effect.

Under nitrate reducing conditions, linear NP could be degraded (Chapter 3). The involved microorganisms were related to alkane degrading species, which might indicate that degradation of linear NP under nitrate reducing conditions starts at the carbon chain. The branched isomers were persistent under nitrate reducing, sulphate reducing, and methanogenic conditions. Under aerobic conditions all isomers could be biodegraded (Chapter 4). During this aerobic biodegradation a nitro-NP isomer was formed and the involved microorganisms showed to be different from aerobic NP degrading microorganisms that are described in literature so far. This indicates that aerobic NP degradation can be performed by a wide range of microbial species.

The NP in the sediment was found to be almost completely available (~ 95%) and could desorb rapidly from the sediment under optimal mixed conditions (Chapter 5). Due to the aerobic biodegradation, the available NP fraction was completely biodegraded, which resulted in an equal reduction of the estrogenic activity. Besides NP, the sediment contained other estrogenic compounds, which showed an equal pattern in availability and biodegradation as NP. Therefore, NP might function as a model compound to predict the estrogenic activity of sediments.

Due to the persistence of branched NP under anaerobic conditions, NP will remain present in the anaerobic sediment for a long time (decades) because it will only be degraded at the interface between the anaerobic sediment and the aerobic river water and in the aerobic river water itself. Experiments in a flow-through experiment showed that NP desorbed continuously from the sediment bed into the bulk water, and this desorbing NP was degraded at the interface as long as sufficient oxygen was present (Chapter 6). Resuspension of the sediment increased the mass transfer of the NP from the sediment to the bulk water, and this resulted in higher NP concentrations in the bulk water compared to the settled sediment conditions. In this experiment, the amount of dissolved NP could not be completely biodegraded due to oxygen limitations. The results showed that resuspension of sediments loaded with NP and other estrogenic organic compounds will lead to enhanced environmental risks for effects on aquatic organisms. These findings need to be taken into account in managing the environmental quality of rivers and other surface waters, and besides the water quality, also the sediment quality should be monitored.

Nederlandse samenvatting

Veel riviersedimenten zijn in het verleden verontreinigd geraakt met estrogene verbindingen, die toxische effecten kunnen veroorzaken op aquatische organismen, zoals de vervrouwlijking van mannelijke vissen. Een van deze estrogene verbindingen is nonylfenol (NP). Nonylfenol is een organische verbinding die bestaat uit een fenolgroep met een lineaire of een vertakte keten van negen koolstofatomen. Voornamelijk mengsels van vertakte NP-isomeren komen voor als verontreiniging in het milieu. Sedimenten die verontreinigd zijn met NP kunnen functioneren als secundaire bron van verontreiniging van het rivierwater, waar het toxische effecten kan veroorzaken op aquatische organismen. Het risico van toxische effecten door NP, dat aanwezig is in het sediment, wordt bepaald door de beschikbaarheid van NP in het sediment, het massatransport vanuit het sediment naar het rivierwater en de mogelijkheid voor biologische afbraak van NP in het sediment of het rivierwater.

In dit promotieonderzoek is een analysemethode ontwikkeld om NP te meten in zowel vloeistofmonsters als monster waarin ook sediment aanwezig is (hoofdstuk 2). Er zijn biologische afbraakexperimenten uitgevoerd met sediment dat al enkele jaren verontreinigd was met NP. Deze experimenten zijn uitgevoerd onder anaërobe (hoofdstuk 3) en aërobe omstandigheden (hoofdstuk 4), waarbij tevens de micro-organismen zijn onderzocht die bij deze biologische afbraakprocessen betrokken zijn. Daarnaast is de beschikbaarheid van NP in het sediment bestudeerd en is de aërobe biologische afbraak van NP in het sediment onder optimale omstandigheden gecombineerd met de totale beschikbaarheid van het NP en de estrogene activiteit (hoofdstuk 5). Vervolgens zijn experimenten uitgevoerd in een continue doorstroomde reactor, waarbij de desorptie vanuit een sedimentbed en vanuit geresuspendeerd sediment in combinatie met de afbraak van NP is onderzocht (hoofdstuk 6).

Hoofdstuk twee beschrijft de ontwikkelde analysemethode waarbij NP uit de gasfase van het monster wordt geëxtraheerd met "solid phase microextraction". Aanwezige sedimentdeeltjes beïnvloeden de meting, maar door het verdunnen van het monster (<1.8 g sediment·l^{-1}) wordt dit effect gereduceerd.

De afbraakexperimenten onder anaërobe condities tonen aan dat alleen lineaire NP onder nitraatreducerende condities wordt afgebroken (hoofdstuk 3). De betrokken micro-organismen zijn lijken op alkaanafbrekende species, waaruit geconcludeerd wordt dat de afbraak van lineaire NP waarschijnlijk begint bij de koolstofketen. De vertakte NP isomeren daarentegen zijn persistent onder deze nitraatreducerende condities, evenals onder sulfaatreducerende en methanogene condities. In de aanwezigheid van zuurstof worden zowel de lineaire als de vertakte NP-isomeren afgebroken (hoofdstuk 4). Gedurende deze aërobe afbraak wordt nitro-nonylfenol gevormd als intermediair. Uit resultaten van de moleculaire analyse blijkt dat andere micro-organismen betrokken zijn dan de micro-organismen die tot dusver staan beschreven in de literatuur.

Nonylfenol in het sediment is bijna volledig beschikbaar (~ 95%) en kan snel desorberen optimaal gemengde omstandigheden van het sediment met de aanwezige vloeistoffase en de aanwezigheid van de adsorbent Tenax en (hoofdstuk 5). Deze beschikbare fractie kan volledig afgebroken worden door aërobe biologische afbraak. Dit resulteert in een vergelijkbare afname van de estrogene activiteit. Uit de analyses van de estrogene activiteit komt tevens naar voren dat naast NP ook andere estrogene stoffen in het sediment aanwezig zijn. Deze estrogene stoffen volgen een vergelijkbaar patroon als NP met betrekking tot de beschikbaarheid en de biologische afbreekbaarheid. Daarom zou NP mogelijk kunnen dienen als gidsstof voor de monitoring van estrogene activiteit in sedimenten.

Doordat de vertakte isomeren NP niet worden afgebroken onder anaërobe omstandigheden, blijft het gedurende langere tijd, in de orde van tientallen jaren, aanwezig in het anaërobe sediment. Uit het sedimentbed, onderzocht met behulp van een continue doorstroomde reactor, blijkt dat NP continue desorbeert van het sediment naar het bovenstaande water (hoofdstuk 6). Dit desorberende NP kan afgebroken worden in de grenslaag van het sediment met het bovenstaande water zolang er voldoende zuurstof aanwezig is in het water. Resuspensie van het sediment vergroot het massatransport van NP van het sediment naar het bulkwater. Dit resulteert in hogere concentraties in het water in vergelijking met de concentraties voor de resuspensie van het sediment. Tijdens het experiment, gedurende de resuspensie, daalde de opgeloste zuurstofconcentraties dusdanig dat aërobe biologische afbraak niet meer mogelijk was. Resuspensie van het verontreinigde sediment met NP en andere estrogene componenten geeft een verhoogd massatransport en daarmee een verhoogd risico op toxische effecten op aquatische organismen. Met name in het beheer van de ecologische kwaliteit van rivieren en andere oppervlaktewateren dient hier rekening mee gehouden te worden en zal naast de waterkwaliteit ook de kwaliteit van het sediment gemonitord moeten te worden.

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1 General Introduction

1. General Introduction

1.1 Background

This study deals with the quality of water and sediment systems in river basins. A river basin is the geographical area drained by a river and its tributaries. It is a complex system with interactions between air, water, soil and sediment. Within a specific river basin, all water that precipitates and does not evaporate, will be transported through the surrounding land to the central river and finally to the sea or ocean. As almost all people live in a river basin and depend on the water supply in that basin for drinking water and agricultural and natural systems, the quality of water is directly coupled to the quality of life. Unfortunately, 40% of the EU surface water bodies are identified as being at ecotoxicological risk according the EU Water Framework Directive (WFD) (SEC(2007)_363, 2007), and vast measures are needed to reduce this risk. The WFD states that member states must implement measures to achieve a "good ecological status" for their water systems by 2015. This should be done by preventing the deterioration of the status of the surface waters, by protecting, improving and restoring all water bodies. Also measures have to be implemented to reduce or phase out pollutions of priority substances as mentioned in the WFD (Directive_2000/60/EC, 2000).

Many river basins contain sediments that are often polluted with a large variety of chemicals. These chemicals have large impacts on the water quality and therefore impose a toxicological risk for the aquatic environment. It is expected that due to climate changes more frequent and more extreme changes in river systems will occur, like larger changes in temperature, flow conditions, and high and low water levels (Gibson et al., 2005). Due to these more frequent and more extreme changes, the interaction between sediments and surface water and related exchange processes of pollutants, may alter and possibly be intensified. Until now, it is not fully clear what the effects of these changes are on the aquatic system.

River sediments are mainly anaerobic. At the interface with the river water, only a thin layer of the sediment contains oxygen (Huttunen et al., 2006; Martin et al., 1998; Middeldorp et al., 2003). In case of clayey-peaty sediments, this layer is a few mm, and in sandy sediments this may reach a few cm. When chemical pollutants move from aerobic surface water to the anaerobic sediment or vice versa, the change in biogeochemical redox conditions affects their fate. Based on their availability and biodegradation behaviour, three types of chemical pollutants can be discriminated. The first group of compounds are the so called heavy metals like zinc, copper, lead, cadmium. They cannot be degraded, but can be immobilized in anaerobic sediments by reactions with sulphides (Vink, 2002; Vink et al., 2009). The result of such immobilization in sediment is that the water quality improves. However, redox conditions in the sediment can become more oxic due to the change of the river conditions, like the change from laminar to turbulent flow during a flooding. Then, the sediment particles are resuspended in the aerobic river water which can lead to remobilization of the heavy metals (Vink, 2002; Vink et al., 2009). The second group of chemicals are organic compounds which are degradable under anaerobic conditions, but more or less persistent under aerobic conditions, like polychlorobiphenyls and hexachlorobenzene (Brown et al., 1987; Mohn and Tiedje, 1992). The presence in or the adsorption of these type of compounds to anaerobic sediments may create optimal conditions for natural attenuation to decrease their environmental risk. The third group exists of organic compounds which are only biodegradable at aerobic conditions. This group contains non-halogenated hydrophobic organic pollutants, like polyaromatic hydrocarbons, total petroleum hydrocarbons, and natural hormones and synthetic chemicals used in industry, cosmetics and body-care products (Mes et al., 2005; Vermeulen et al., 2003). Anaerobic sediment functions as a sink for these compounds, because biodegradation in the sediment is limited under anaerobic conditions. At these conditions, the sediments will be loaded with non-halogenated hydrophobic organic pollutants and they will remain present for decades or longer. When the hydraulic condition of the river changes, these stored pollutants can be released, and thus these sediments will then function as a secondary source in future. Such a release creates a potential long lasting risk for pollution of the aquatic ecosystem. Enhanced release of these compounds may occur at flooding conditions when changes in the flow regime will lead to resuspension of the sediment particles with adsorbed chemicals in the water phase. In principle, these compounds can be biodegraded in the aerobic water phase, but information upon such events is lacking so far.

For accurate risk assessment and river basin management it is important to know the behaviour of the distinct group of pollutants present in sediment of river basins. Insight into the biodegradation properties is needed for the various groups of organic compounds at changing redox conditions as well as the desorption of these compounds from sediments at different river conditions.

An emerging compound of the third group, which grew in attention the recent years, is nonylphenol (NP). This compound is found in many river basins like the Ebro and the Elbe (Heemken et al., 2001; Navarro et al., 2009b). Insight into the biodegradation and availability behaviour of NP in sediment, and the possible effects of changing river conditions on the biodegradation and desorption behaviour is lacking. Such new knowledge is of scientific interest, and of importance for river basin managers. This knowledge will help them to make a realistic prediction of the toxicological risk of chemicals in rivers under changing environmental conditions and design appropriate measures. Therefore, NP was chosen as the compound to be researched in this study.

1.2 Nonylphenol

Nonylphenol is a man-made compound that is mainly used (65%) as intermediate in the production of nonylphenol ethoxylates (NPnE) (USEPA, 1990). These NPnEs are widely used as surfactant in cleaning products and industrial processes (Guenther et al., 2006). Nonylphenol itself is used as additive to manufacture antioxidants, emulsifiers and detergents. It exists of a phenol group with a linear or a branched chain of nine carbon atoms at the para-position of the aromatic ring (Figure 1.1).

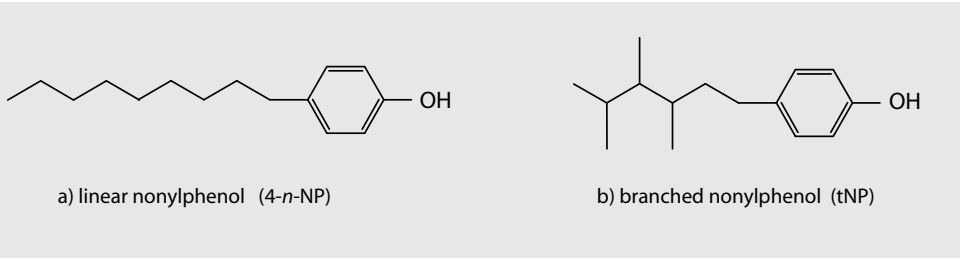


Figure 1.1: Molecular structure formula of a) linear nonylphenol and b) branched nonylphenol isomer.

The linear NP (4-*n*-NP) has a white crystal appearance whereas the technical mixture of branched NP (tNP) is a pale yellow liquid at room temperature. The technical mixture of nonylphenol is used for the production of NPnE, and is produced by the alkylation of phenol with branched nonenes. This production process results in a mixture of only branched NP isomers with the alkyl chain at the para-position (4th carbon atom) of the phenol ring. In addition, very low amounts of *ortho*-substituted isomers are formed with the alkyl chain at the 2nd carbon-atom of the phenol ring. The linear isomer of NP is not present in the technical mixture, since linear nonenes are not used for the synthesis of tNP. Technical mixtures of NP consist of over 100 isomers (Ieda et al., 2005). More than 85% of these isomers are α-quaternary carbon atom isomers (Figure 1.2).

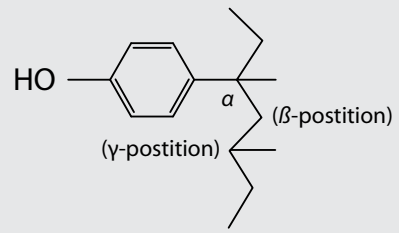


Figure 1.2: Molecular structure of 4-[1-ethyl-1,3-dimethylpentyl] phenol with a quaternary α-carbon on the branched nonyl chain. The ß-carbon atom and γ-carbon atom are mentioned as well.

In these isomers, the nonyl chain has a carbon atom, which is attached to the phenol group (the α-position of the chain) and three other carbon atoms (Wheeler et al., 1997). The physical-chemical properties of NP are presented in Table.1.1.

Table 1.1: Physical-chemical properties of nonylphenol.

Molecular formula	$C_{15}H_{24}O$
Molecular weight	220.34 g · mol^{-1}
Water solubility	4.9 mg · l^{-1}[a]
Vapour pressure (at 25 °C)	2.07 10^{-2} Pa[b]
Log octanol-water partition coefficient (log K_{ow})	4.48[c]
Log organic carbon partition coefficient (log K_{oc})	4.13-6.1[d]
[-10]log acid dissociation constant (pK_a)	10.28[e]

[a](Brix et al., 2001), [b](Muller et al., 1998) [c](Ahel and Giger, 1993), [d](Burgess et al., 2005; Heemken et al., 2001; Isobe et al., 2001; Navarro et al., 2009a) and [e](Muller et al., 1998).

Nonylphenol was first synthesized in 1940. Thereafter, the production increased per year up to 154,200 tones in the USA, 73,500 tones in Europe, 16,500 tones in Japan and 16,000 tones in China 2001, 2002 and 2004 respectively (Soares et al., 2008).

1.3 Regulatory aspects

After 40 years of its first production, the toxicity of NP on aquatic ecosystems was studied in 1981 (McLeese et al., 1981) and the earliest concerns about accumulation of NP in the aquatic environment emerged in 1983-84 (Giger et al., 1984). It turned out in the nineties that nonylphenol had endocrine disrupting properties and estrogenic effects were observed in fishes in the field caused by NP (Lee and Lee, 1996; Soto et al., 1991; White et al., 1994). Actions were taken to regulate the use of nonylphenolic compounds. Nonylphenol was identified as a priority hazardous compound in the Water Framework Directive (Directive 2000/60/EC, 2000), and in an EU directive (Directive 2003/53/EC), initiated in 2003, the marketing and use of NP and NPnE is regulated. This directive came into force in 2005, and states that products which contain 0.1% or more NP or NPnE are not allowed to be used or be placed on the market (Directive_2000/60/EC, 2000; Directive_2003/53/EC, 2003). Also the US Environmental Protection Agency (EPA) acknowledged the risk, and prepared a guideline for water quality (Brooke and Thursby, 2005). This guideline recommends that the NP concentration should not exceed a one-day average concentration of NP in freshwater of 28 µg·l^{-1} and a four-day average concentration of 6.6 µg·l^{-1} once every three years. Nowadays, NPnEs are being replaced by other surfactants like alcohol ethoxylates in most European countries, Canada and Japan, which are less toxic for the environment compared to nonylphenol ethoxylates (Soares et al., 2008). However, in many other countries like China and India, NP and NPnEs are still wide used.

1.4 Occurrence of nonylphenol in the environment

The main source of NP in the environment is the discharge of effluents and sludges from sewage treatment plants (Giger et al., 1984; Liber et al., 1999). Nonylphenol is an end product of the degradation of NPnE in anaerobic waste water treatment plants (Di Corcia et al., 1998; Giger et al., 1984) (Figure 1.3), and therefore the mixture of NP, which is found in the environment, consists mainly of branched isomers.

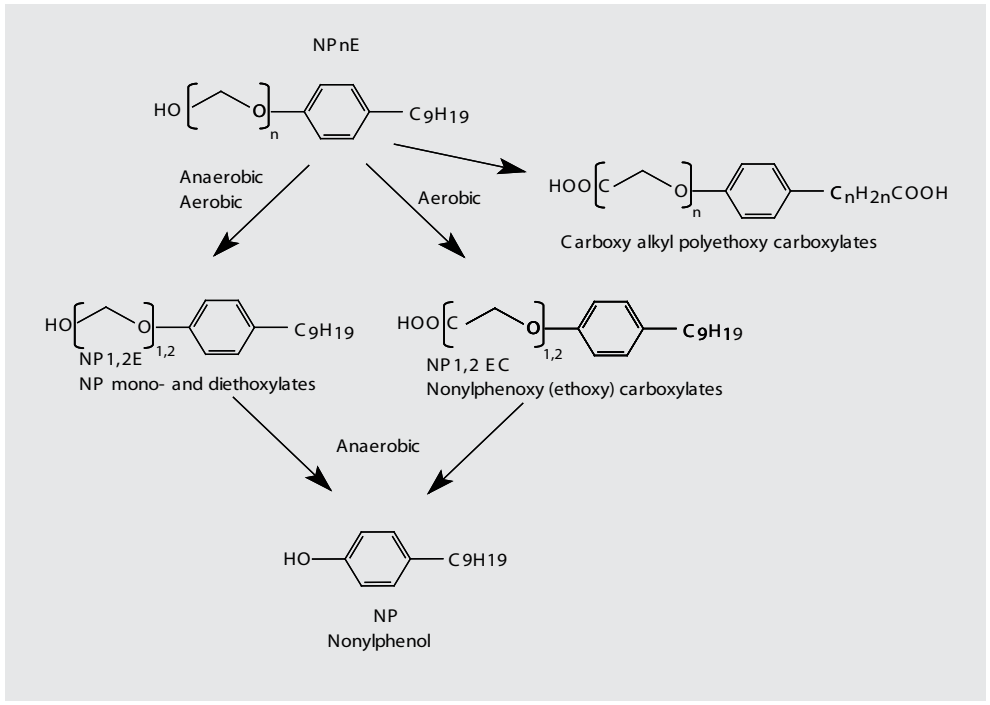


Figure 1.3: Degradation of nonylphenol ethoxylate (NPnE) in wastewater treatment plants (adapted from (Di Corcia et al., 1998; Giger et al., 1984))

Nonylphenol is detected in rivers, lakes and estuaries all over the world in the water phase and in sediments. Table 1.2 gives an overview of reported NP concentrations in European rivers and estuaries, and in rivers and lakes at other continents. For Spain, data from two reports are mentioned since the sediment used in this study originates from a tributary of the Ebro River in Spain. Due to the low water solubility (4.9 $mg \cdot l^{-1}$) and high hydrophobicity ($logK_{ow}$= 4.48), NP tends to sorb to the sludges, and to organic matter in sediments (Heemken et al., 2001; Isobe et al., 2001; Jonkers et al., 2003; Lacorte et al., 2006; Li et al., 2004b; Petrovic and Barcelo, 2000). Because of this sorption, nonylphenol accumulates in sediments in the aquatic ecosystem. In surface waters, NP concentrations were measured between values of 0.001 $\mu g \cdot l^{-1}$ (river water in Germany) and 32.8 $\mu g \cdot l^{-1}$ (lake water in China). In sediments, NP concentrations ranged between values of <0.0004 $mg \cdot kg^{-1}$ dry weight, as found in an estuary sediment in the Netherlands and 72 $mg \cdot kg^{-1}$ dry weight, as reported for a lake sediment in Canada (Table 1.2). The observed concentrations of NP in the environment are often related to anthropogenic activities such as discharge of waste waters from sewage treatment plants (STPs) and the presence of urban or industrial areas.

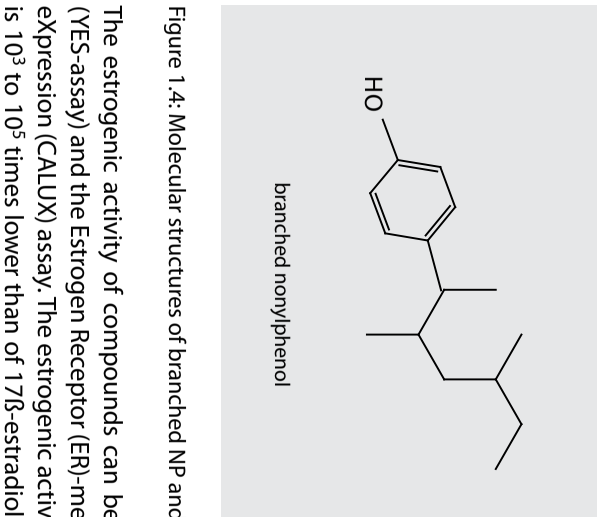
It is expected that the concentration of NP in river water of European river basins will decrease the coming years due to the ban on products which contain NP or NPnE, like industrial and household cleaning products. A decrease of the NP concentration in the river water was already observed at different locations in the Ebro River and its tributaries in Spain during 2004 to 2006 (Navarro et al., 2009b). However, measurements of the NP concentrations in the sediments at the same locations showed an increase of the sediment concentrations from the year 2005 to 2006 at most of the sampling locations in the Ebro. Due to sorption of NP to the sediment particles, the NP accumulates in the sediment and can remain present at high concentrations for a long time. Sediments which are polluted with NP can act as a secondary contamination source for the aquatic ecosystem. Part of the sediment bound fraction of NP can desorb and enter the river water, when the concentration in the river water is lower than the concentration in the sediment bed. This desorption of NP from the sediment to the river water is of concern for river basin management, since the water quality can be affected by NP in the sediment for a long time.

Table 1.2: Occurrence of nonylphenol in water and sediment of rivers, estuaries and lakes in different countries.

Country	Environment	Concentration water [µg· 1^{-1}]	Concentration sediment [mg· kg^{-1} dry weight]	Remarks	Reference
the Netherlands	Estuary	0.031 – 0.93	<0.0004 – 1.08	high concentrations in sediments nearby industrial areas	(Jonkers et al., 2003)
Spain	River	0.5 – 15	0.022 – 0.645	concentrations related to discharge waste water sewage treatment plants (STP)	(Petrovic et al., 2002)
Spain (Ebro and its tributaries)	River	2004: 0.16 – 24.3 2005: 0.16 – 0.83 2006: 0.04 – 0.84	2004: 0.07 – 6.0 2005: 0.02 – 4.9 2006: 0.15 – 3.66	-decreased water concentration and varying sediment concentration per same sampling location over the years	(Navarro et al., 2009b)
UK	River	<0.2 – 30	<0.01 – 1.5	concentrations related to STP discharge	(Blackburn et al., 1999)
Germany	River	0.001 – 0.17	0.39 – 1.38	concentration related to industries	(Heemken et al., 2001)
USA	River	0.1 – 0.5	0.075 – 0.34	concentrations related to STP discharge and urban areas	(Rice et al., 2003)
Canada	Lake and river	0.01 – 0.92	0.17 – 72	samples from industrial areas	(Bennie et al., 1997)
China	Lake	1.9 – 32.8	3.5 – 32.4	concentrations related to STP discharge	(Wu et al., 2007)
Korea	River	0.023 – 0.187	0.025 – 0.932	increase concentration nearby urban areas	(Li et al., 2004a)
Japan	River	0.051 – 1.08	0.5 – 13	seasonal fluctuation	(Isobe et al., 2001)

1.5 Endocrine properties of nonylphenol

Nonylphenol is an endocrine disruptor, which means that it can cause hormonal effects on organisms. The chemical structure of NP is comparable with that of the natural hormone 17ß-estradiol (Figure 1.4), and can compete with this natural hormone for the binding site of the receptor of 17ß-estradiol (Lee and Lee, 1996; White et al., 1994).



branched nonylphenol 17ß-estradiol

Figure 1.4: Molecular structures of branched NP and 17ß-estradiol.

The estrogenic activity of compounds can be determined by the Yeast Estrogen Assay (YES-assay) and the Estrogen Receptor (ER)-mediated Chemical Activated Luciferase gene eXpression (CALUX) assay. The estrogenic activity of the technical mixture of nonylphenol is 10^3 to 10^5 times lower than of 17ß-estradiol, depending on the used bioassay (Gabriel et al., 2008; Lee and Lee, 1996; Legler et al., 2002; White et al., 1994). This means that the concentration of NP should be 10^3 to10^5 higher compared to the concentration of 17ß-estradiol to cause the same estrogenic effect as 17ß-estradiol. Furthermore, the estrogenic activity differs between the different NP isomers (Gabriel et al., 2008; Kim et al., 2004; Preuss et al., 2006; Routledge and Sumpter, 1997). Most studies showed higher estrogenic activity for isomers with a more branched chain, especially with substitutions at the carbon atom at the ß- position of the chain (Figure 1.2), and at the carbon atom at the γ-position of the chain (Gabriel et al., 2008; Shioji et al., 2006). It was suggested that the chemical structures of these isomers are most comparable with the chemical structure of 17ß-estradiol, and therefore they might be able to interact with active cavity of the estrogen receptor protein. More linear structures are less similar to 17ß-estradiol and show therefore less estrogenic activity (Shioji et al., 2006).

The estrogenic effects of nonylphenol on various aquatic organisms are very diverse as recently reviewed by Soares et al. (Soares et al., 2008). Estrogenic effects like feminization, increased levels of vitellogin (female-specific protein) and low values of testosterone are observed in male fish. These effects were observed in laboratory studies, as well as in different studies downstream of sewage treatment plants (Soares et al., 2008).

Nonylphenol can also cause reduction of embryo survival of fish and daphnids, decreased reproduction of springtails, and reduced growth of the microorganisms *Azobacter* sp. Furthermore, NP has shown to accumulate in aquatic organisms as algae, fish, water birds (Soares et al., 2008). Finally, long time exposure to low concentrations of NP (0.33 - 2.36 $\mu g \cdot l^{-1}$) can lead to accumulation in fish (Snyder et al., 2001).

Other estrogenic compounds like estrone, 17ß-estradiol and organochloro pesticides can be also present in the aquatic system. The single concentration of these compounds can be below the so called no-observed-effect concentration, nevertheless the presence of two or more endocrinic compounds can be additive or synergistic, even at low concentrations (Rajapakse et al., 2002; Silva et al., 2002). Because of this mixture effect, NP may cause adverse effects to aquatic organisms in a river system even when it is present at concentrations below its no-observed-effect-concentration.

1.6 Biodegradation of nonylphenol

1.6.1 Aerobic biodegradation of nonylphenol

Biodegradation of NP by microorganisms leads to a decrease in the NP concentration, and if NP is degraded to non-toxic compounds, this can reduce the estrogenic risk for the environment. Biodegradation of NP (branched and linear) is frequently observed in soils, sediments and sludges as long as sufficient oxygen is present and supplied (Ekelund et al., 1993; Gabriel et al., 2005a; Hesselsoe et al., 2001; Langford et al., 2005; Tanghe et al., 1999). Several aerobic NP degrading microorganisms are isolated from different environments such as waste water treatment plants, soils and sediments (Table 1.3). These isolates mainly belong to the *Sphingomonas* genus (Fuiji et al., 2001, Vries et al., 2001; Gabriel et al., 2005a; Tanghe et al., 1999), although some of them are renamed and are now known as *Sphingobium* species (Fuiji et al., 2001, Vries et al., 2001; Gabriel et al., 2005a). Other strains haven been obtained that belong to the isolates *Stenotrophomonas* sp. and *Pseudomonas mandelii* species (Soares et al., 2003). Most strains are able to degrade NP as sole carbon and energy source, except *Sphingobium amiense*. This strain can degrade NP cometabolically in the presence of yeast as carbon and energy source.

Table1.3: Nonylphenol degrading microorganisms isolated from various environments.

Isolate	Environment	growth on NP	Remarks	Reference
Sphingomonas sp. TTNP3	Waste water treatment plant	+		(Tanghe et al., 1999)
Sphingobium xenophagum Bayram	Waste water treatment plant	+	formerly known as *Sphingomonas xenophaga* Bayram	(Gabriel et al., 2005a)
Sphingobium cloacae	Waste water treatment plant	+	formerly known as *Sphingomonas cloacae*	(Fuiji et al., 2001)
Stenotrophomonas sp.	Soil	+	cold-adapted biodegradation	(Soares et al., 2003)
Pseudomonas mandelii	Soil	+	cold-adapted biodegradation	(Soares et al., 2003)
Sphingobium amiense	Sediment	- (cometabolic conversion with yeast)	formerly known as *Sphingomonas* sp. YT	(Vries et al., 2001)

Five possible biodegradation pathways of NP are suggested in literature, namely via i) 2-nitro-nonylphenol (Figure 1.5), ii) phenol-oxidation (Figure 1.6), and iii - v) via *ipso*-hydroxylation (Figure 1.7). These five different pathways are briefly discussed below.
The first suggested pathway for the biodegradation of NP is via the formation of 2-nitro-nonylphenol (Figure 1.5) (Telscher et al., 2005; Zhang et al., 2009).

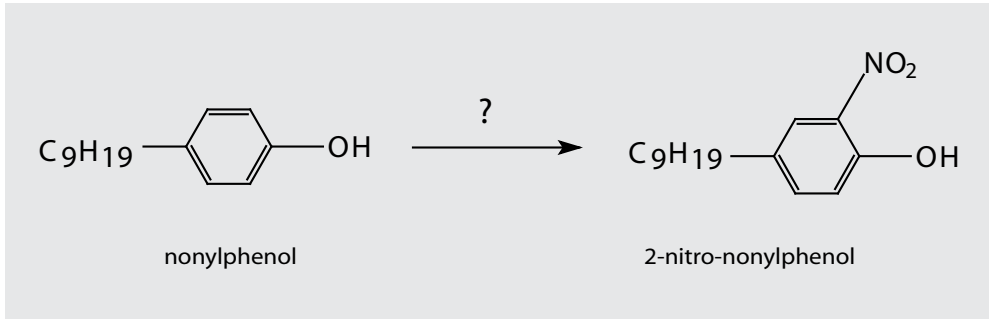


Figure 1.5: Aerobic degradation of nonylphenol with the formation of 2-nitro-nonylphenol.

Nitro-nonylphenol compounds were observed during the biodegradation of tNP in agricultural soil and in a mixture of agricultural soil and sludge from a public waste water treatment plant. The formed nitro-phenol in the experiments with only agricultural soil was further degraded. Details about involved microorganisms are not known and similar biodegradation rates were found for the various NP isomers (Telscher et al., 2005).

The second proposed pathway is the phenolic pathway, in which the phenolic ring is oxidized by monooxygenases as was shown for a *Pseudomonas* spp. (Soares et al., 2003) (Figure 1.6). Further details about this biodegradation pathway are not known.

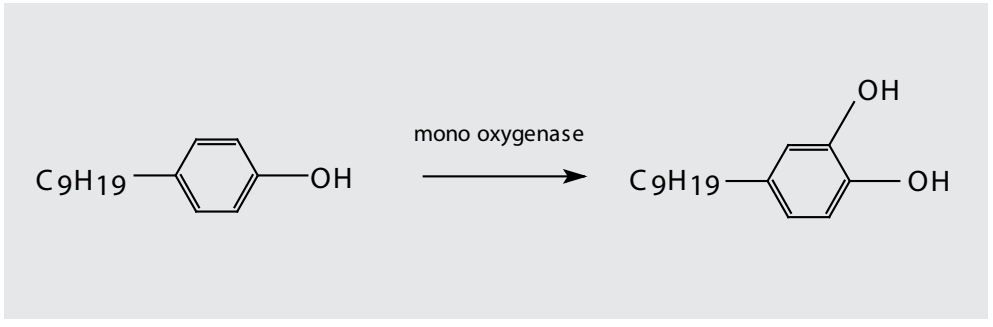


Figure 1.6: Proposed biodegradation pathway of NP via the phenolic degradation pathway by Pseudomonas spp.

The other three suggested pathways (iii – v), via *ipso*-hydroxylation, have been studied more intensively, and mainly in *Sphingobium xenophagum* Bayram and *Sphingomonas* sp. TTNP3 (Corvini et al., 2005; Corvini et al., 2006a; Corvini et al., 2007; Corvini et al., 2006b; Gabriel et al., 2005a; Gabriel et al., 2005b; Gabriel et al., 2008). The first step is the *ipso*-hydroxylation and this step is independent of the structure of the carbon chain (Figure 1.7) (Kohler et al., 2008). All NP isomers are initially hydroxylated at the position in the NP isomers where the alkyl chain is attached to the phenol group (*ipso*-position). The subsequent biodegradation steps depend on the structure of the carbon chain, and three further pathways are possible, namely via iii) the hydroquinone metabolite, iv) the NIH shift, v) dihydroxygenation. For pathway iii, isomers with an α-quaternary carbon atom are further metabolized via a hydroquinone metabolite, and for the specie *S. xenophagum* Bayram was demonstrated to gain energy from the further biodegradation of these isomers. Isomers without an α-quaternary carbon atom like linear NP can be further degraded via an NIH-shift (pathway iv) or via dihydroxygenation (pathway v).

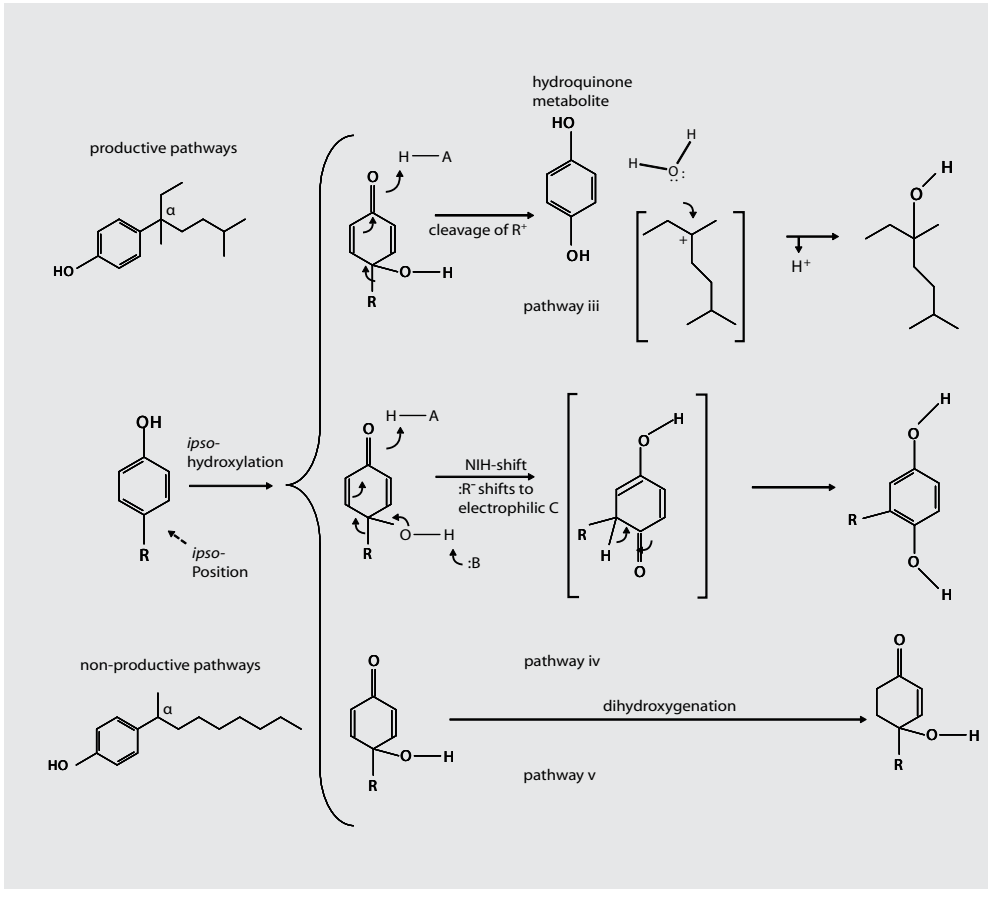


Figure 1.7: Proposed aerobic biodegradation pathways of nonylphenol in Sphingobium xenophagum Bayram via ipso-hydroxylation (adapted from (Kohler et al., 2008)).

With a NIH-shift, the alkyl chain migrates from the *ipso*-position to the adjacent carbon atom of the phenol ring, and with dihydroxygenation a double bond in the ring structure is removed in addition of hydrogen (Kohler et al., 2008). The bacteria can gain no or less energy out of these two reactions compared to further biodegradation of isomers with an α-quaternary carbon atom (Kohler et al., 2008). *Sphingomonas* sp. TTNP3 degrades NP also via *ipso*-hydroxylation, and α-quaternary C-atom isomers are also completely degraded via the formation of a hydroquinone (Corvini et al., 2006a). However, this strain is not able to degrade 4-*n*-NP.

The biodegradation of isomers via the NIH-shift and dehydroxygenation is slower compared to the biodegradation of α-quaternary carbon atom via the hydroquinone pathway (Gabriel et al., 2005a). Therefore, biodegradation by *ipso*-hydroxylation leads to a shift in the isomeric composition of the NP mixture. Due to the variation in estrogenic activity of different isomers, this shift also leads to a shift in estrogenic activity of the mixture of the isomers (Gabriel et al., 2008). Whether the estrogenic activity will increase depends on the isomeric composition of the mixture and the related biodegradability of the isomers via the *ipso*-hydroxylation. Because of the possible shift in estrogenic activity, research on single isomers instead of research on the total mixture can be necessary to investigate the fate of NP in the environment.

1.6.2. Anaerobic biodegradation of nonylphenol

Anaerobic biodegradation of tNP isomers has not been published so far, but anaerobic biodegradation with 4-*n*-NP has been reported. The anaerobic biodegradation of 4-*n*-NP is observed in sediment from the Erren River in China, in sludge from a waste water treatment plant and in soil in Taiwan (Chang et al., 2007a; Chang et al., 2005; Chang et al., 2004). 4-*n*-nonylphenol was degraded under methanogenic, sulphate reducing and nitrate reducing conditions, with the highest degradation rate under sulphate reducing conditions in sediment, sludge and soil. This was comparable with the degradation reported for 4-*n*-NP in a mixed culture in soil under aerobic conditions (Chang et al., 2007b). The most active anaerobic 4-*n*-NP degrading strains isolated from the sludges were closely related to *Bacillus cereus* and *Acinetobacter*, and the most active strain isolated from the soil to *Bacillus niacini* (Chang et al., 2007a; Chang et al., 2005). Details about the biodegradation pathways of 4-*n*-NP of these strains have not been mentioned in the literature.

1.6.3 Biodegradation in the aquatic environment

Pollution with NP in the aquatic environment is mainly originating from tNP, which can be degraded under aerobic conditions only. Various redox conditions are simultaneously present in river water and sediment systems. River water is mainly aerobic and sediment is to the largest extent anaerobic. In an interface layer of the sediment with a thickness of a few mm to a few cm contacting the river water, conditions change from aerobic to anaerobic with increasing depth (Bradley et al., 2008; Huttunen et al., 2006; Martin et al., 1998; Middeldorp et al., 2003). However, oxygen depletion in river water can take place due to eutrophication or biodegradation of organic matter (Paerl et al., 1998; Smith et al., 1999; Taft et al., 1980) resulting in anoxic water. Within the anaerobic part of the sediment, different redox conditions are present, such as denitrifying conditions or sulphate reducing conditions, depending on the presence of chemicals as nitrate and sulphate. Biodegradation of NP is most likely to occur in the aerobic part of the sediment, or in the water phase. To what extent and at what rate this NP biodegradation can occur at the water-sediment interface is to our knowledge not studied before.

1.6.4. Biodegradation of other estrogenic compounds

Besides NP, sediments can also contain other estrogenic compounds, as mentioned in paragraph 1.5. The presence of estrogenic pollutants in sediments is often related to discharges from STPs. The natural hormones 17ß-estradiol (E2), estrone (E1) and the synthetic hormone 17α-ethynylestradiol (EE2) are identified as main contributors to the estrogenic activity of waste water. The occurrence and fate of these estrogenic compounds was reviewed by Mes et al. (Mes et al., 2005). E2, E1 and EE2 are hydrophobic like NP and can therefore also accumulate in the sediment. The biodegradation properties of E2, E1 and EE2 are comparable to the biodegradation properties of NP. E2 is very rapidly converted into E1 under aerobic conditions, sometimes with a half life of a few minutes, and E1 is further degraded within hours or days. The biodegradation of EE2 is slower compared to the conversion of E2 and E1 and occurs through a co-metabolic process. E2 can be converted into E1 under anaerobic conditions but further biodegradation will not take place. EE2 is also persistent under anaerobic conditions. Because E1, E2 and EE2 adsorb to sediments, like NP, and can also only be degraded under aerobic conditions, sediments can also act as a secondary source for these estrogenic compounds.

1.7 Bioavailability

Bioavailability is an elegant concept to describe the fraction of a contaminant in soil or sediment that can affect specific species in the environment as well as the uptake for biodegradation of these contaminants. The part of the NP fraction that desorbs from the sediment into the surrounding water, becomes bioavailable for aquatic organisms. Bioavailability is the result of several processes including partitioning and desorption of a contaminant. Bioavailability depends on compound and sediment properties, organism characteristics and physicochemical properties of the surrounding aqueous environment.

Bioavailability has been used in the literature in different ways depending on the research area. Bioavailability can represent the accessibility of a chemical for assimilation as well as its possible toxicity. But it is also defined as the degree to which a compound is free to move into or onto an organism. In this second definition bioavailability is used in relation to specific organisms since availability differs between organisms and species (Alexander, 2000; Reichenberg and Mayer, 2006; Reid and Semple, 2000).

1.7.1 Potential bioavailability

The potential bioavailability of a chemical in a sediment is defined in this study as the fraction that can desorb from the sediment, and can be taken up or transformed by (micro)organisms via the water phase (Semple et al., 2003). This bioavailable fraction is considered as the total bioavailable or bioaccessible fraction, because it describes the total mass of contaminants that can become available (Reichenberg and Mayer, 2006). This is therefore also the amount of a chemical that can desorb from the sediment and can cause a toxicological effect. The potential available fraction can be determined with a chemical method like solid phase extractions (SPE). A solid sorbent is used to bind the compound, it adsorbs the compound out of the water phase, reducing the compounds concentration to extremely low values, and subsequently enhancing desorption from the sediment to the water phase. Tenax is such a sorbent which is frequently used for the determination of the potential available fraction (Cornelissen et al., 2001; Cuypers et al., 2001). During the extraction with Tenax, the mass transfer from the sediment to the bulk water is optimized. The sediment, water phase and solid sorbent are intensely mixed, which maximises the exchange surface of the polluted sediment particles and the water phase. The sorbent keeps the concentration in the water phase low to establish a high concentration gradient for optimal desorption.

The potential available fraction consists of a rapid available and a slow available fraction. The rapid available fraction can desorb easily to the water phase in a relevant time frame where it is available for biodegradation but it can also cause harmful effects to aquatic organisms. The rapid desorbing fraction is the most important fraction for risk assessment. The slow desorbing fraction will also desorb to the water phase, however rate constants of this slow desorbing fraction showed to be about a factor 1000 lower (Cornelissen et al., 1997) and is less relevant for the environment in terms of toxicological risk. The rapid and slow desorbing fractions can be described with a two-compartment model (Equation 1.1) (Cornelissen et al., 1997).

$$S_t = F_{rap} \cdot S_0 \cdot e^{-k_{rap} \cdot t} + F_{slow} \cdot S_0 \cdot e^{-k_{slow} \cdot t} \quad \text{(Equation 1.1)}$$

S_t = remaining extractable concentration of the contaminant in the sediment at a specific time of extraction [mg•kg^{-1}]
S_0 = initial total extractable concentration of the contaminant in the sediment [mg•kg^{-1}]
F_{rap} = rapid desorbing fraction [-]
F_{slow} = slow desorbing fraction [-]
k_{rap} = rate constant for the fast desorbing fraction [h^{-1}]
k_{slow} = rate constant of the slow desorbing fraction [h^{-1}]
t = desorption time [h].

In addition, also a very slow available fraction is reported, which is representing the sorption to soot and soot-like particles present in the sediment (Jonker et al., 2005). This very slow fraction can be added to Equation 1.1, and modelled with a three compartment model.

It is known that hydrophobic compounds that adsorb to organic matter, such as phenanthrene, 4-nitrophenol, DDT and atrazine, become less available with time by a process called aging or sequestration (Alexander, 2000; Chung and Alexander, 1998; Hatzinger and Alexander, 1995; Park et al., 2004). Although the processes that cause aging are not fully understood, it has been suggested that a sorption retarded diffusion of the organic pollutant into the organic matter and sorption to surfaces within nano- and micro pores are important mechanisms (Semple et al., 2003). Parameters as the hydrophobicity and the type of organic matter expressed as log K_{OW} and log K_{OC}, play a role in the adsorption and availability of compounds. Aging can decrease the associated environmental risk in field situations because the amount of contaminant that can desorb to the water phase reduces with time. So far, bioavailability of NP has been studied only in freshly spiked organic materials like humic substances (Vinken et al., 2004) and cellulose (Burgess et al., 2005), using a short contact time of respectively 30 h and 48 h. Short term experiments in range of a few days with freshly spiked sediment can give an overestimation of the bioavailable fraction because aging is a long term process. Bioavailability experiments with field aged NP polluted sediments give a more realistic prediction of the bioavailable fraction of this compound. Aging of NP was observed in marine samples where sediments at the surface and at various depths up to 140 cm were studied (Jin et al., 2008). In deeper sediment layers, the non-available fraction increased up to 99%. Potential availability of NP in river sediments is not investigated as far as we know. Insight into the potential available fraction and especially the fast desorbing fraction of NP is important since this is the fraction that causes the risk in the aquatic environment.

1.7.2 Mass flux approach

Many environmental studies and legislations on contaminants in river systems are based on concentrations of pollutants. One may question if such a static parameter is suitable to assess the quality of highly dynamic systems such as rivers. River systems represent ever changing conditions with continuously changing inflows of water by the operation of the hydrological cycle. For chemical engineering, more dynamic phenomena are already studied for decades in continuous reactor systems, focussing on driving forces for mass transfer. The flux based approach can be used to quantify the transport of NP at different hydrological conditions of a river system. In principal, the environmental load of NP on the river system can be forecasted when insight is obtained on the mass flux of NP from the sediment into the river water, as well as the microbial NP conversion rates. Insight will then be obtained to what extent a river system is at risk under various conditions and to what extent natural attenuation processes are able to mitigate NP mass transfer to the aquatic system and help to reduce the environmental risk.

Two main driving forces that control the mass transfer of compounds from the sediment to the water phase are the concentration gradient between the sediment particle and the surrounding water and the second is the contact surface of sediment particles and the

surrounding water (Birdwell et al., 2007; Cheng et al., 1995).

The total mass flux of a compound Φ [µg.d^{-1}] from the sediment to the water can be described for a reactor system by the following equation (Booij et al., 1992):

$\Phi_{sed} = k \cdot A_{sed} \cdot (C_{sur} - C_{bw})$ (Equation 1.2)

Φ = mass flux [µg·d^{-1}]
k = mass transfer coefficient [m·d^{-1}]
A_{sed} = contact surface of the sediment and the surrounding water [m^2];
C_{sur} = NP concentration at the solid surface [µg·m^{-3}]
C_{bw} = concentration in the bulk water [µg·m^{-3}]

When the concentration at the solid surface (C_{sur}) is larger than the concentration in the bulk water (C_{bw}), the sediment will act as a secondary source and the compound is transported from the sediment to the bulk water. The risk of NP in the sediment for effects on aquatic organisms in the bulk water depends largely on the mass transfer of NP from the sediment to the water phase. The mass transfer is different under various conditions in river systems that can vary strongly with changing seasons. Seasonal patterns of river hydrology are currently changing due to changes in land use and climate. In case of a gently flowing river with settled sediment, the contact area between the sediment and river water is limited which leads to a limited flux of the pollutant to the river water. Under more turbulent conditions, like a flooding, heavy rainfall or dredging of the river, the sediment can be resuspended. The desorption area is then enlarged and more pollutant can be transported to the water phase which enhances the toxicological risk for the aquatic system. Biodegradation of NP can reduce this risk. Most favourable, NP biodegradation occurs at the aerobic interface of the sediment and the river water, and prevents the pollutant to migrate to the river water. Thus far, these processes, and the possible effects of changing system conditions have not been studied as far as we know. Information about the mass transfer from the sediment to the water phase under different river conditions and the possible biodegradation of the released NP is necessary for a better understanding of chemicals in rivers and for river basin management. With this new information more realistic predictions of the environmental risk of NP present in the sediment under changing conditions can be made and possible remediation and risk reduction measures can be evaluated in a better way.

1.8 Scope of this thesis

Many river sediments have become polluted with NP by waste water discharges in the past. These sediments may act as a secondary source for NP to the river water due to desorption, and may cause a toxicdogical effect on aquatic organisms due to its estrogenic properties. Insight into the risks of NP in the sediment is necessary for accurate river basin management. The estrogenic risk for the environment depends on the availability of the NP, the mass transfer of NP from the sediment to the river water and the amount of biodegradation which can decrease the aqueous concentration.

The aim of this research is obtain quantitative and generic insight into the availability, the mass transfer and the biodegradation potentials of NP in polluted river sediment. Therefore, this thesis focuses on the biodegradation of NP combined with its availability under optimized conditions in laboratory experiments. In addition, an experimental set-up was used to mimic the dynamics and changing conditions in a river system at lab scale. All experiments were performed with the same aged NP polluted sediment, and was used for the biodegradation, availability, and mass transfer experiments to obtain information about the most important processes, which are relevant for the fate and toxicological effects of NP in river-sediment systems. The sediment originated from the Huerva River in Spain, a tributary of the Ebro River which was contaminated with tNP.

A fast and easy method was developed to analyze NP concentrations in a small sample volume, with sediment and water and with water only. A new method for the addition of NP in a low volume as an aid for biodegradation experiments with NP as a single carbon source was also developed. These two methods and the effect of the presence of sediment in the samples on the analyzed NP concentration are described in Chapter 2.

In Chapter 3, the study on the anaerobic biodegradation of 4-*n*-NP and tNP under various redox conditions is presented. Biodegradation of 4-*n*-NP under denitrifying conditions was investigated by further enriching the involved microorganisms. The microbial population was identified and the development of the microbial diversity during the enrichments was studied. By using molecular techniques such as polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), cloning and sequencing.

Chapter 4 describes the aerobic biodegradation of NP in the sediment. The formation of nitro-nonylphenol as intermediate in the aerobic biodegradation of branched and linear NP. The origin of the nitro group attached to the nonylphenol and the involved microorganisms were investigated in more detail by the use of molecular and other techniques.

The potential availability of the NP in the sediment is elaborated in Chapter 5. The available fractions were related to the amount of NP that could be biodegraded and to the estrogenic activity of the sediment analyzed by the ERα-luc bioassay. Bioconversion behaviour and reduction of estrogenic activity of NP and other estrogenic pollutants present in the sediments where compared.

The knowledge obtained in chapters 3, 4 and 5 was used for the mass transfer experiments as presented in Chapter 6. Mass transfer and biodegradation of NP was studied in a reactor set up that mimics the changing conditions that can occur in a river system, namely non mixed, and turbulent mixed conditions.

Finally, the conclusions of the experimental work and scientific consequences are summarized and discussed in Chapter 7 and placed in a broader and applied perspective for river basin management.

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2 Improved methods for biodegradation studies with nonylphenol and sediment: effects of suspended solids on SPME and GC-MS analysis and techniques for addition of nonylphenol as single carbon source.

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Abstract

Nonylphenol (NP) is an estrogenic compound and therefore harmful for the aquatic biota. Biodegradation of NP can significantly reduce toxic concentrations and the ecological risks of NP. To perform biodegradation experiments, a simple method to analyse NP in small volumes of water and sediment and an appropriate method to add the poorly water soluble NP to the biodegradation batches are needed.

A simple method was developed for the analysis is the extraction of NP from the headspace of samples with solid phase micro extraction (SPME) and analysis with gas chromatography-mass spectrometry (GC-MS). Also samples with liquid and sediment could be analyzed. Sediment present in the samples lead to an underestimation of the concentration, and this effect is larger for linear NP (4-*n*-NP) than for branched NP (tNP). Dilution of the slurry samples below 1.8 g sediment·l^{-1} was needed to avoid underestimation of NP concentrations.

An appropriate method was developed for the addition of NP (max. water solubility 5 mg NP·l^{-1}) to biodegradation batches as single carbon source without remaining solvent residues. Acetone was used to prepare stock suspensions of NP in demineralised water with NP concentrations hundred times the maximum water solubility of 5 mg NP·l^{-1}. Nonylphenol could be added from the suspensions to biodegradation experiments in a low volume (500 µl stock suspension to 50 ml medium) without the provision of other carbon sources than NP. Due to possible variation in the added concentration, analysis of the start concentration was required.

2.1 Introduction

Nonylphenol (NP) is a hydrophobic compound with a low vapour pressure (<10 Pa, 20°C), which enters the aquatic environment via the sludges of anaerobic waste water treatment plants and industrial effluents. In the aquatic environment NP is known to adsorb to sediment due to its chemical properties (Kravetz, 1983; Sturm, 1973). Because nonylphenol has endocrine disrupting effects on aquatic ecosystems (Nimrod and Benson, 1996), processes that reduce the ecotoxicological risk of NP, like biodegradation, are of great interest (Corvini et al., 2006).

Biodegradation is mainly studied in laboratory batch experiments. To be able to perform these experiments two requirements should be fulfilled; i) a reliable analysis of the NP concentration to investigate the progress of the biodegradation in the presence of sediments should be available and ii) the addition and re-addition of the poorly water soluble NP in a low volume to the biodegradation batches needs to be conducted without the addition of an extra carbon source. These two issues are presented here.

To address the first issue, an ideal NP analysis method is fast, and uses small volumes of sample and minimum of materials such as solvents. A method which meets these requirements is analysis with solid phase micro extraction (SPME) of NP from the headspace combined with gas chromatography-mass spectrometry (GC-MS). The benefit of extracting the NP from the headspace is that samples with solid particles can also be analyzed. However, the solid particles might influence the extraction, therefore the effects of sediment in the sample on the SPME extraction was investigated as well.

The second issue addresses the need to add and re-add NP to batch experiments when biodegradation and depletion of NP has occurred. This is required to cultivate enrichments and eventually select pure cultures. To study the conversion of NP as a single carbon source, no other carbon source should be present in the cultivation medium. Due to the high hydrophobicity (log K_{OW} = 4.48) and a low water solubility (22 µM; 5 mg·l^{-1}) of NP (Ahel and Giger, 1993; Brix et al., 2001), often solvents are used to spike NP to biodegradation experiments. Various organic solvents such as acetonitrile, acetone, methanol and chloroform are used for this addition (Chang et al., 2004; Langford et al., 2005; Lee Ferguson and Brownawell, 2003; Tanghe et al., 1998). To overcome the presence of the organic solvent as second carbon source, the organic solvent are generally flushed from the system at the beginning of the experiment (Corvini et al., 2007; Soares et al., 2003). However, flushing of the solvent after re-addition of NP during a biodegradation process could lead to disturbance of the degradation process and is therefore undesired. When no flushing occurs, the organic solvent remains in the system (Chang et al., 2004; Langford et al., 2005). In this case, the organic solvent may act as an additional carbon source for the microorganisms, which will influence the NP biodegradation process, for example by changing the lag phase, the degradation rate or by initiating other processes such as co-metabolic degradation of NP. This may result in misinterpretations and erroneous conclusions. As NP has a low water solubility, application of NP in water solutions for enrichments and selection studies is not realistic, because large volumes of NP solution need to be re-added to reach detectable concentrations as soon as NP is degraded. Addition of large volumes may influence the degradation conditions by severe dilutions, and is therefore unwanted. For these reasons, a method for NP addition by oversaturation was developed to prepare sterile suspensions of 4-*n*-NP and tNP in demineralised water with a concentration above the maximum water solubility of 22 µM.

2.2 Materials and methods

2.2.1 Chemicals and sediments

Linear NP (4-*n*-NP) (pestanal, purity >99%) and nonylphenol technical mixture (tNP) (purity > 94%) were purchased from Riedel de Haën (Seelze, Germany). Acetone (gas chromatography grade) and ethanol absolute (purity > 99.8%) were purchased from Merck (Darmstadt, Germany). Other used chemicals were of the highest analytical grade. Seven different sediments were collected from several rivers in Europe between August 2004 and June 2005 (Table 2.1).

Table 2.1: Collected sediment with locations and sampling date

Sediment	River	Location	Sampling date
S1	Elbe	Schönberg, Germany	October 2005
S2	Cinca (tributary of Ebro)	Monzon, Spain	June 2005
S3	Danube	Budapest, Hungary	August 2004
S4	Ebro Delta (rice field)	Sant Jaune d'Enveja, Spain	June 2005
S5	Ebro Delta (estuary)	Sant Jaune d'Enveja, Spain	June 2005
S6 (polluted)	Huerva (tributary Ebro)	Zaragoza, Spain	June 2005

Sediments S1 to S5 were not polluted with NP, whereas sediment S6, from the Huerva River nearby Zaragoza, was heavily polluted with tNP (20 mg·kg^{-1} dry weight). Sediment S6 is further used for the biodegradation and availability experiments preformed and described in this thesis.

2.2.2 Acetone analysis
Acetone was analyzed with a Varian 3800 gas chromatograph (GC) with a flame ionization detector (FID) equipped with a Varian CP Porabond Q fused silica column (length, 25 m; inner diameter, 0.32 mm, nominal film thickness, 5 µm; Middelburg, the Netherlands). Two ml sample was added to a capped 20 ml headspace vial and the vials were placed in a Combi Pal auto sampler. The samples were continuously stirred at 80°C for 20 min before a 0.5 ml sample was taken from the headspace and injected on the column with a split ratio of 5. The flow through the column was 1.4 ml·min^{-1}, the injector temperature was 200°C and the detector temperature was 300°C. The initial oven temperature was set at 35°C for 3 minutes, followed by an increase to 250°C at a rate of 10°C·min^{-1}, and maintained for 5.5 minutes.

2.2.3 Chemical analysis of NP with SPME
To analyse the 4-*n*-NP and tNP concentrations, samples were taken from the incubation bottles with a syringe. Depending on the (expected) concentration in the bottles, 200 µl or 500 µl samples were taken and added to a capped 20 ml headspace vial with 1.8 or 1.5 ml MilliQ water (Millipore BV, Amsterdam, the Netherlands) containing 7 mg·l^{-1} $HgCl_2$. The headspace vial was capped with a magnetic crimp cap with blue silicon and teflon coated septum (Grace Davison Discovery Science, Deerfield, Il, USA). The NP was extracted from the headspace of the samples with a SPME-fiber (polyacrylate 85 µm; Supelco, Bellefonte, PA, USA) by a Varian Combi Pal autosampler. Extraction with the SPME fiber took place from the headspace instead of in the liquid phase to avoid disturbing effects by direct contact of the fiver with the solid particles. The NP was extracted for 25 min at 100°C. During the extraction, the vial with the sample was constantly shaken. After adsorption to the SPME-fiber, the NP was thermally desorbed at 300°C for 5 min from the fiber in the injection port of a Varian 3800 GC. The GC was equipped with a Varian CP-Sil 8 CB low bleed/MS column (length, 50 m; inner diameter, 0.25 mm, nominal film thickness, 0.25 µm) and connected to a Varian Saturn 2000 ion-trap mass spectrometer of 200°C. The NP was injected splitless. The injector pressure was constantly 1.15 bar and helium was used as a carrier gas, with a flow of 1.0 ml·min^{-1}. The initial column oven temperature was set at 80°C for 1 min, after which the temperature was increased to 200°C at a rate of 30°C·min^{-1}, and then maintained for 2 min. Finally, the temperature increased at a rate of 10°C·min^{-1} to 260°C and then held for 10 min. The ionization was electron impact at 70 eV, and the detection was full scan.

2.2.4 Effect of sediment type on measured NP concentration by SPME
The effect of the presence of sediment in the samples in the headspace vials on the extraction of the NP by the SPME fiber was tested. Slurries of the sediments S1 to S5 were used in 120 ml crimp cap bottles with 50 ml demineralized water and 1 g (wet weight, ± 0.9 g dry weight) sediment. From these bottles, 1.9 – 2 ml slurry was taken with a syringe of 500 µM and transferred to 20 ml headspace vials. These slurry samples were used to prepare calibration lines of 4-*n*-NP and tNP with concentrations of 0 µM, 0.23 µM, 0.45µM and 2.3 µM NP. The 4-*n*-NP and tNP were added to the slurries from a concentrated solution of 45 µM 4-*n*-NP and tNP to a final sample volume of 2 ml. The concentrated solution of 45 µM for the preparation of the calibration samples was made in MilliQ water containing 7 mg·l^{-1} $HgCl_2$ by using a 9 mM 4-*n*-NP and tNP solution in ethanol. A calibration line with MilliQ water was also analyzed. The samples were extracted by SPME and analyzed by GC-MS.

2.2.5 Effect of amount of sediment on measured tNP concentrations by SPME
To measure the effect of the amount of sediment in the slurry samples on the analytical SPME method, slurry samples were prepared with a concentration of 0.45 µM or 2.3 µM 4-*n*-NP. For this experiment, the tNP polluted sediment S7 was used, because the tNP was already present in this sediment and this sediment was used in further experiments. The slurry was prepared in 120 ml crimp cap bottles with 50 ml demineralized water and 1 g wet weight sediment (= approximately 0.9 g dry weight). For the slurry samples, 0.2 – 2 ml slurry was taken with a syringe and transferred to a 20 ml headspace vials. MilliQ water was added to dilute the slurry samples to a final liquid volume (slurry and MilliQ) of 2 ml containing 100% slurry (undiluted), 50% slurry (two times diluted), 20% slurry (five times diluted) and 10% slurry (ten times diluted). The samples were prepared in triplicate, and the 0.45 µM or 2.3 µM linear NP (4-*n*-NP) was added to the headspace vials from the concentrated solution of 45 µM. The samples were extracted by SPME and analyzed by GC-MS.

2.2.6 Preparation of NP suspensions in water
Nonylphenol suspensions were prepared by weighing 19.5 mg 4-*n*-NP or 39 mg tNP in a sterile 120 ml crimp cap bottles. The pure 4-*n*-NP and tNP were sterilized by UV for 30 minutes before use. The chemical composition of the chemicals was not changed as checked by GC-MS. Acetone (200 µl) was added to the crimp cap bottles to better suspend the 4-*n*-NP and tNP in the liquid, and the bottle was capped with a sterile viton stopper. The acetone was flushed out of the bottle under a sterile flow of N_2-gas, via two needles through the stopper. One needle was used as influent for the N_2-gas and one needle as effluent. As soon the acetone was evaporated, 50 ml of sterile anaerobic demineralised water was added. This resulted in final suspensions of 1.8 mM 4-*n*-NP and 3.5 mM tNP, respectively. Before using the suspensions, the bottles with the suspensions were heated under a stream of tap water of 60°C and shaken to homogenize the suspension.
To test the homogeneity of the suspensions, four sets of triplicates samples in 20 ml headspace vials were extracted by SPME and analyzed by GC-MS. Because the concentration of the suspensions (1.8 – 3.5 mM) was above the upper detection limit of GC-MS method (4.5 µM), the suspensions were diluted before being analyzed on the GC-MS. Four individual samples with a hundred times dilution of the suspensions were prepared first. From each diluted sample, triplicate samples of 2 ml were prepared in 20 ml headspace vials with another fifty times dilution. The average measured concentrations of these four sets were used to

calculate the average concentration of the samples. The concentrations of acetone in the original undiluted NP suspensions were analyzed by GC-FID.

2.2.7 NP concentrations in sterile controls

Nonylphenol is hydrophobic and has the tendency to adsorb, e.g. to glassware and viton, in lab experiments. The adsorption of NP added from the stock suspension without solvent was investigated in 120 ml glass bottles containing only medium. The bottles were filled with 50 ml aerobic medium. The composition of the medium was described by Tros et al. (Tros et al., 1996). The bottles were capped with viton stoppers and autoclaved for 15 min. at 121°C. After sterilization, 4-*n*-NP or tNP was added to the batch bottles in a final concentration of 9 µM. Bottles with medium and 4-*n*-NP or tNP were prepared in triplicate and were stored in the dark at 30°C, and horizontally shaken at 110 rpm. The concentrations of 4-*n*-NP and tNP were analyzed in time. In addition to the behaviour of NP in liquid, the behaviour of NP in 120 ml bottles with 40 ml medium and 2 g wet sediment (0.78 g dry weight) was also tested to study the adsorption effect as sediment was present as well. The medium and sediment were autoclaved for one hour at 121°C to prevent degradation. Thereafter a concentration of 16 µM 4-*n*-NP and 7 µM tNP was added, and the bottles were stored at the dark at 30°C, and horizontally shaken at 110 rpm. Samples were taken in time, and depending on the concentration 100 or 200 µl sample was added to a 20 ml headspace vial containing 1.8 or 1.9 ml MilliQ water with 7 $mg \cdot l^{-1}$ $HgCl_2$. The samples were extracted by SPME and analyzed by GC-MS.

2.3 Results and discussion

2.3.1 Effect of sediment type on measured NP concentration by SPME

In the presence of sediment, the measured concentrations of 4-*n*-NP and tNP were lower than the measured concentrations in samples with only MilliQ water (Figure 2.1), as measured by headspace analysis with SPME. The difference in the measured 4-*n*-NP concentration between the samples with sediment and without sediment (only MilliQ water, Figure 2.1a) was larger than the difference between the measured tNP concentrations in the samples with and without sediment (Figure 2.1b). Measured NP concentrations in samples with sediment were underestimated with a factor of 2 to 7 for 4-*n*-NP and a factor of maximal 1.6 for tNP. The decrease in NP concentration when sediment is present is most probably caused by sorption of 4-*n*-NP and tNP to the sediment material.

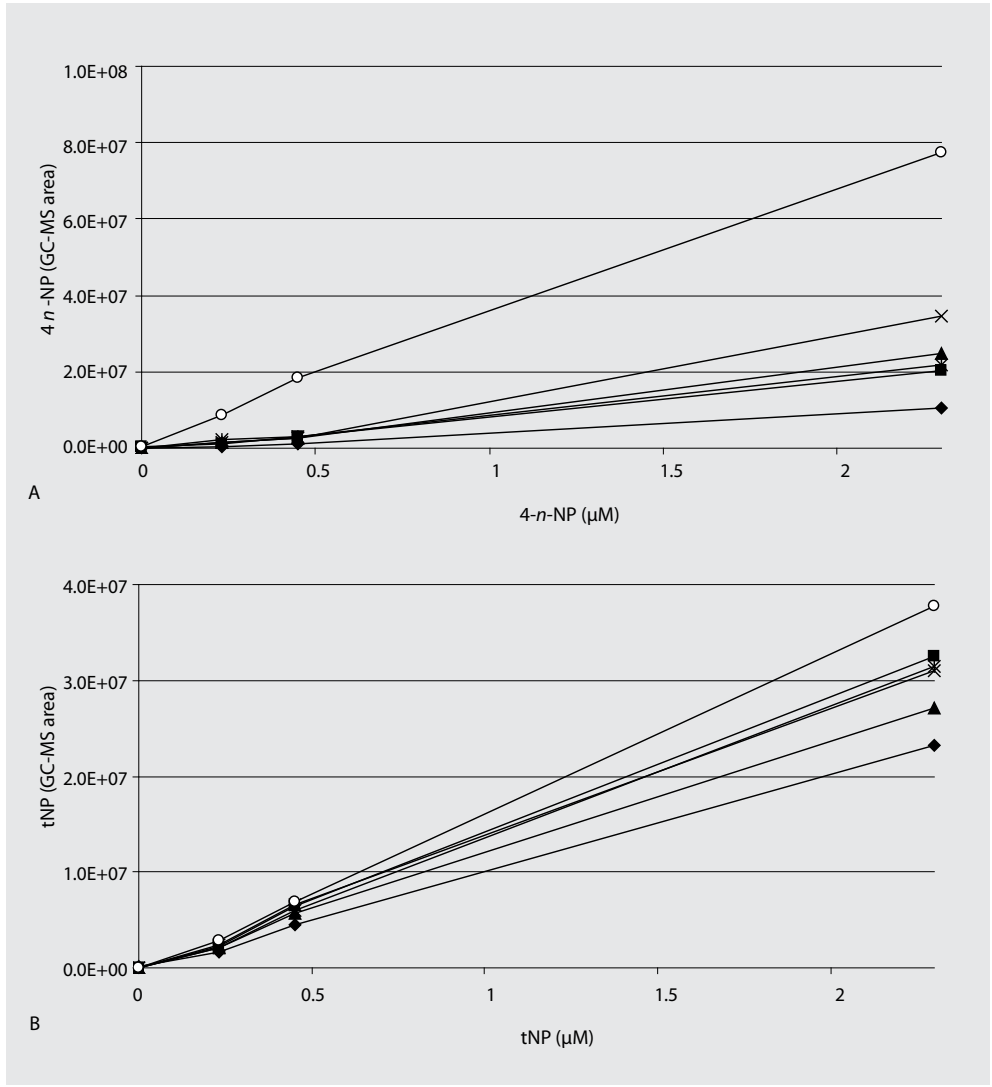

Figure 2.1: Influence of sediments S1-S5 on the measured concentrations of A) 4-n-N P and B) tNP. S1 = ■, *S2 =* ▲, *S3 =* ◆, *S4 =* X , *S5 =* ∗, *MilliQ water =* O

The effect of sediments on the measured 4-*n*-NP and tNP concentrations differed with type of sediment. However, there was no correlation between the amount of TOC in the sediments and the effect on the measured concentration as indicated by variation in the slopes of the calibration curves with sediment compared to those of MilliQ water (Table 2.2).

Table 2.2: Relative slope of calibration lines of linear NP (4-n-NP) and branched NP (tNP) of samples containing water and sediment compared to calibration line prepared with MilliQ water

Sample	TOC-value (%)	Relative slope (%) 4-*n*-NP	Relative slope (%) tNP
S1	3.01	27.0	86.1
S2	1.09	33.3	71.1
S3	2.26	14.4	61.1
S4	3.64	47.2	81.5
S5	3.92	28.8	83.6
MilliQ	--	100	100

2.3.2 Effect of amount of sediment on measured tNP concentrations by SPME

Dilution of the slurry samples showed that a decrease in the amount of sediment per sample corresponded to an increase in measured tNP concentrations (Figure 2.2). Higher tNP concentrations were measured when the samples were diluted with MilliQ water and less sediment is present. Dilution of the sediment samples reduces the effect on the measured concentrations and overcomes the underestimation in measured tNP concentrations.

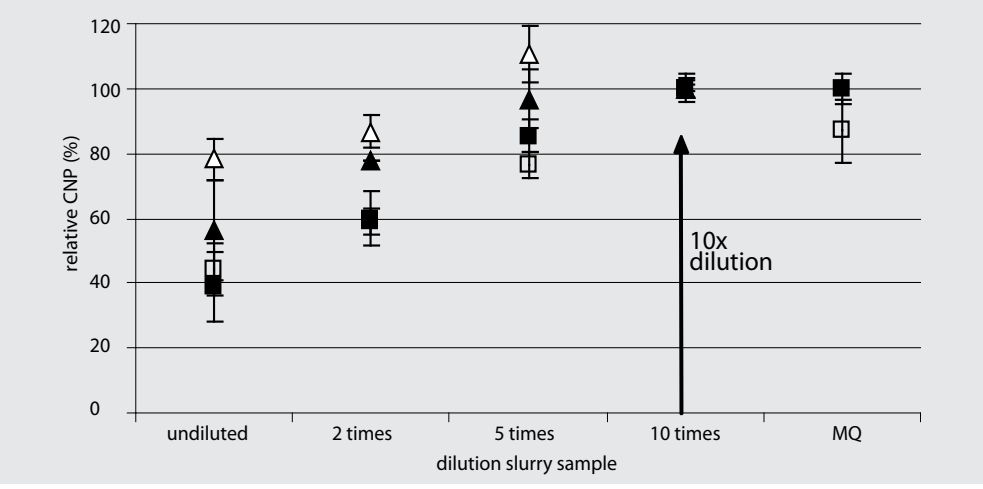

Figure 2.2: Relative measured 4-n-NP (□and ■) and tNP (△and▲) concentrations slurries with various dilutions. 0.45 µM 4-n-NP (closed symbols) or 2.3 µM 4-n-NP (open symbols) was added to samples with tNP polluted sediment S6. 4-n-NP was also measured in samples with only MilliQ water. Given results are averages of triplicates with standard deviations.

This may be caused by some competition between 4-*n*-NP and tNP in sorption on the SPME fiber and at this higher 4-*n*-NP concentration this would explain a lower measured tNP concentrations. This was not further investigated. The measured 4-*n*-NP concentrations increase with less sediment particles in the samples, and the measured concentrations in ten times diluted samples were comparable with the measured concentration in the samples with only MilliQ water. The optimal dilution rate for tNP varied with the added 4-*n*-NP concentration. Based on these results, we used a ten fold dilution to values less than 1.8 g sediment·l^{-1} water in the NP analyses as method for future biodegradation experiments. The use of a ten times dilution results in an acceptable variation of measured concentrations in samples with sediment. Diluting the sediment samples ten times in combination with the GC-MS analysis method with SPME leads to an easy and fast reproducible method to analyze NP concentrations in biodegradation studies in the presence or absence of sediment.

2.3.3 Stock suspensions of NP in water

Sterile stock suspensions of 4-*n*-NP or tNP were prepared in water with concentrations above the maximum water solubility. The measured NP concentration of the 4-*n*-NP suspension prepared with acetone was 0.7 times the calculated concentration of 1.8 mM (23 % standard deviation), and the analyzed NP concentration of the tNP suspension 0.3 times the calculated concentration of 3.5 mM (24 % standard deviation). The relatively high NP concentration in the sterile stock suspensions of 4-*n*-NP and tNP (1.8 and 3.5 mM, respectively) makes it also possible to add and re-add 4-*n*-NP or tNP in a small volume. Only 435 µl 4-*n*-NP suspension or 523 µl tNP suspension needs to be added to 50 ml medium to set a concentration in the biodegradation batches of 11 µM NP. The addition of these small suspension volumes leads to insignificant changes in the cultivation medium, and can therefore repeatedly be applied to one batch when needed.

The acetone concentrations in the 4-*n*-NP and tNP stock suspensions were 0.5 µM and 2 µM respectively, which results in approximately 2500 and 500 times lower concentrations of acetone compared to the 4-*n*-NP or tNP concentration in the biodegradation batches when 11 µM NP is added. Addition of NP from these sterile stock suspensions gives a variation of 23 and 24% of the added 4-*n*-NP and tNP concentrations in the batches. Despite this variation, an insignificant amount of solvent residues is added, and therefore the biodegradation process will not be influenced, i.e. by preventing the supply of a second carbon source. In this way, misinterpretations related to the use of NP as single carbon source by microorganisms will be prevented. The variation in concentration can be controlled by measuring the concentration at the start of the experiment.

2.3.4 NP concentration in sterile controls after addition from stock suspensions

Using the stock suspensions of 4-*n*-NP or tNP without almost no solvent in sterile controls shows that the concentration of 4-*n*-NP decreased immediately with 38% after addition of 4-*n*-NP to the sterile incubation bottles with only medium (Figure 2.3). Within 7 days only 15% of the added NP concentration was measured and after 64 days a concentration of only 6% is present in the liquid phase.

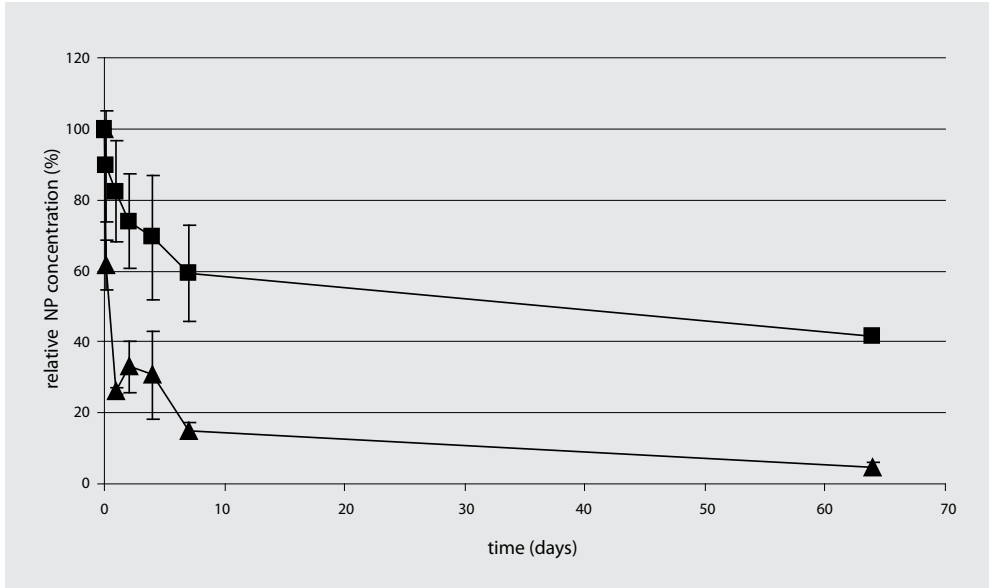

Figure 2.3: Relative linear nonylphenol (4-n-NP, ▲) and branched nonylphenol (tNP, ■) concentrations in abiotic batches with only medium after NP addition from a stock suspension prepared with acetone

The concentration of tNP in the medium decreased less compared to the concentration of 4-*n*-NP. Directly after addition, 90% of the original tNP concentration was measured. After 7 days, 59% was still present in the sample and after 64 days, a concentration of 42% of the original concentration was analyzed. 4-*n*-NP adsorbs more to the glass and stopper than tNP, since higher concentrations of tNP are measured in the water phase compared to 4-*n*-NP. The linear structure of 4-*n*-NP makes it apparently easier to adsorb compared to the branched NP, which has a branched carbon chain. Biodegradation of 4-*n*-NP and tNP usually takes place within 8 days. Thus, distinguishing the abiotic and biotic processes is possible. Nevertheless, the adsorption must be taken into account when performing biodegradation studies or sediment adsorption studies. Therefore, a control bottle was always be used during the biodegradation experiments to distinguish between the decrease of the concentration due to biodegradation, and to adsorption to the glass and the stopper.

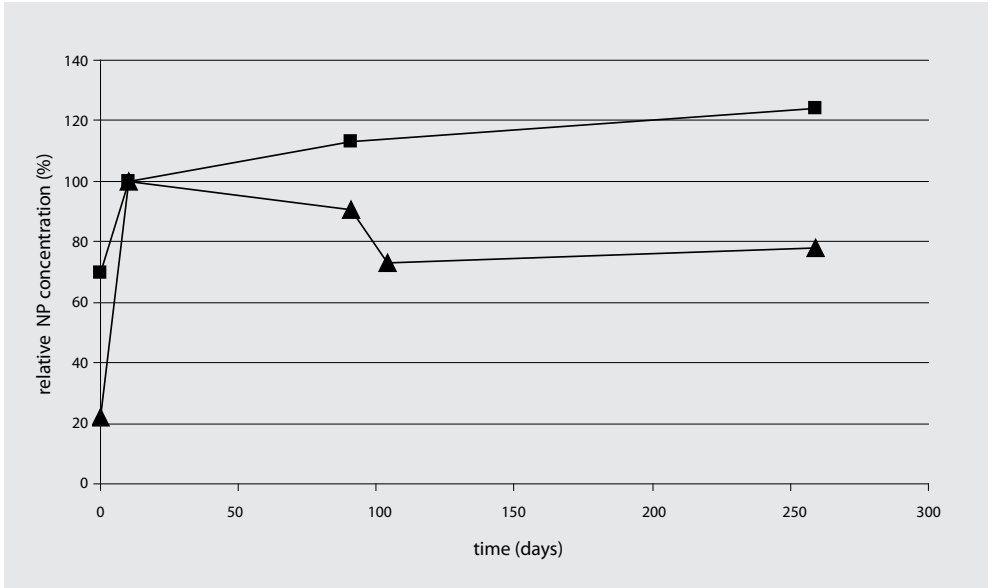

Figure 2.4: Relative linear nonylphenol (4-n-NP, ▲) and branched nonylphenol (tNP, ■) concentrations in time in abiotic batches with medium and sediment.

In the presence of sterile sediment, the measured 4-*n*-NP and tNP concentrations decreased much less compared to the batches without sediment (Figure 2.4). The measured concentration directly after addition was 70% and 20% for 4-*n*-NP and tNP respectively. These lower concentrations in t=0 samples are presumable caused by equilibration processes in the incubation bottle after addition of the stock suspensions. After 259 days concentrations of 124% of 4-*n*-NP and 78% of tNP of the original added concentrations were measured in 2 ml sample. The sediment has a large sorption area compared to the glass of the batch bottles. The strong decrease of NP concentrations is not observed in the batches with sediment, indicating that NP seems to prefer to adsorb more to the sediment than to the glass walls of the system. The analysis method with SPME extraction allows analyzing samples of water with dissolved NP as well as samples with water, sediment, dissolved and partly adsorbed NP.

2.4 Conclusion

Nonylphenol concentrations in taken samples from biodegradation batches containing sediment and NP can be analysed for NP by GC-MS with SMPE extractions, even when these samples contain sediment. The benefits of this method are that it is fast, does not need solvent extraction, and needs only small sample volumes of 100-500 µl. Solid particles in the samples affect the measured concentration. This can be overcome by diluting the sediment samples below 1.8 g sediment·l^{-1} water.

Stock suspension of 4-*n*-NP or tNP in water with NP concentrations above its maximum water solubility to add or re-add NP to biodegradation batches can be prepared with acetone. This will avoid the presence of an additional carbon source in the biodegradation batches due to transfer of solvent residuals. Degradation of NP as a single carbon source can thus be adequately investigated. Adsorption to glass and viton stopper of the 4-*n*-NP or tNP added from these stock suspensions has to be taken into account when drawing conclusions about the decrease of NP in batch experiments, especially when no sediment is present.

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3 Degradation of 4-*n*-nonylphenol under nitrate reducing conditions

Jasperien De Weert, Marc Viñas, Tim Grotenhuis, Huub Rijnaarts, Alette Langenhoff

Abstract

Nonylphenol (NP) is an endocrine disruptor present as a pollutant in river sediment. Biodegradation of NP can reduce its toxicological risk. As sediments are mainly anaerobic, degradation of linear (4-*n*-NP) and branched nonylphenol was studied under methanogenic, sulphate reducing and denitrifying conditions in NP polluted river sediment. Anaerobic bioconversion was observed only for linear NP under denitrifying conditions. The microbial population involved herein was further studied by enrichment and molecular characterization. Bacterial DNA sequences related to denitrifiers, denitrifying alkane-degraders and linear alkyl benzene sulphonate (LAS) degraders were detected. The molecular structures of alkanes and LAS are similar to the linear chain of 4-*n*-NP which might indicate that the biodegradation of linear NP under denitrifying conditions starts at the nonyl chain. Initiation of anaerobic NP degradation was further tested using phenol as a structure analogue. Phenol was chosen instead of an aliphatic analogue, because phenol is the common structure present in all NP isomers while the structure of the aliphatic chain differs per isomer. Phenol was degraded in all cases, but did not affect the linear NP degradation under denitrifying conditions and did not initiate the degradation of branched NP and linear NP under the other tested conditions.

3.1 Introduction

Nonylphenol (NP) is a hydrophobic compound which is widely used as intermediate in the production of nonylphenol ethoxylates (Giger et al., 1984; Liber et al., 1999). Nonylphenol ethoxylates are used in household and cleaning products as a surfactant, and are transformed in anaerobic waste water treatment plants with NP as an end product. Nonylphenol is also present in effluents of waste water treatment plants with aerated sludge (Johnson et al., 2005). Nonylphenol is a mixture of isomers with a hydrophilic phenol group and a linear (4-*n*-NP) or a branched (tNP) carbon chain with nine carbon atoms (Figure 3.1).

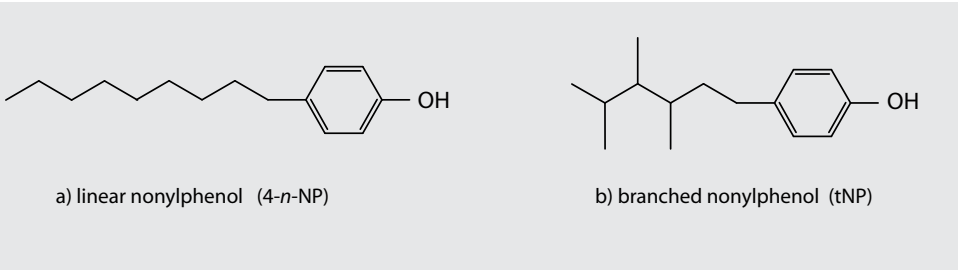

Figure 3.1: Molecular structure of (a) linear nonylphenol and (b) a branched nonylphenol isomer.

Nonylphenol enters the aquatic environment via the sludges of anaerobic waste water treatment plants and industrial effluents. In the aquatic environment, it adsorbs to sediment due to its chemical properties (Kravetz, 1983; Sturm, 1973). Nonylphenol has been shown to accumulate up to concentrations of 22 to 2230 µg NP·kg^{-1} dry weight in sediments of the Ebro River in Spain (Lacorte et al., 2006). Nonylphenol is also found in river sediments in Taiwan and Germany (Ding et al., 1999; Fries and Püttmann, 2003). Since NP is an endocrine disruptor, degradation of NP in the sediment to non toxic compounds is of great interest (Nimrod and Benson, 1996). Biodegradation under aerobic conditions is well described, as reviewed by Corvini et al. (Corvini et al., 2006). However, anaerobic degradation of NP is of greater importance towards potential natural attenuation since sediments are mainly anaerobic. Molecular oxygen is only present in the first mm at the interface of the sediment and the water phase (Middeldorp et al., 2003). So far, branched NP seems to be persistent under anaerobic conditions. Degradation without oxygen is not observed and the biodegradation rate is reduced with very low oxygen concentrations (Ekelund et al., 1993; Hesselsoe et al., 2001). Anaerobic biodegradation of 4-*n*-NP has only been reported in sediment from the Erren River in Taiwan (Chang et al., 2004). In that study, observed 4-*n*-NP half live values were 3.9, 16.1 and 20.4 days under sulphate reducing, methanogenic and nitrate reducing conditions, respectively. Anaerobic degradation of 4-*n*-NP in soil and sludge from a waste water treatment plant was also reported. The isolated 4-*n*-NP degrading strains in the soil and sludge were closely related to *Bacillus niacini*, *Bacillus cereus* and *Acinetobacter baumanni* (Chang et al., 2007; Chang et al., 2005).

To our knowledge, degradation of branched NP has not been described so far. Stimulation or initiation of the branched NP degradation process under anaerobic conditions with a structure analogue of NP is a possibility, as reported in other studies for the degradation of polychlorinated biphenyls initiated by terpenes (Focht, 1995; Tandlich et al., 2001). For NP, such initiation might occur via the aromatic ring of NP, since this is the common structure in all NP isomers. Thereby, the isomeric composition of the nonyl chain for the various isomers seemed to play an important role whether an isomer can be degraded. Anaerobic degradation of isomers with a linear nonyl chain is observed so far, whereas isomers with a branched chain are persistent under anaerobic conditions. Phenol can be a possible structure analogue for initiation of the degradation of 4-*n*-NP and the mixture of branched NP isomers via the aromatic ring. Degradation of phenol under various anaerobic conditions such as denitrifying conditions and sulphate reducing conditions has often been mentioned in literature (Bak and Widdel, 1986; Kuever et al., 2001; Li et al., 1996; Tschech and Fuchs, 1987). An aliphatic chain as structure analogue for the mixture of NP isomers is complicated because of the chemical structure of the nonyl chain in the branched NP isomers is rather variable.

This chapter describes the anaerobic degradation of 4-*n*-NP under denitrifying conditions, as well as the involved microbial populations during the enrichment process. Furthermore, the anaerobic degradation of branched NP was tested in the presence and absence of the structure analogue phenol under various anaerobic conditions.

3.2 Materials and methods

3.2.1 Chemicals and materials

Nonylphenol technical mixture (tNP) (branched, > 94% purity) and 4-*n*-NP (linear, pestanal, > 99% purity) were purchased from Riedel de Haën (Seelze, Germany). Phenol (purity > 99%) was ordered from Merck (Darmstadt, Germany). Other used chemicals were of the highest purity (> 99%).

Sterile stock suspensions of 4-*n*-NP (1.6 mM) and tNP technical mixture (3.3 mM) in demi water were prepared as described in Chapter 2. Briefly, 4-*n*-NP and tNP technical mixture were dissolved in acetone in a sterile bottle. The bottle was capped and the acetone was evaporated with N_2-gas. After evaporation, sterile demi water was added. The stock suspensions were heated (60°C) and shaken before use.

3.2.2 Biodegradation of NP

Polluted sediment with NP was collected in June 2005 from the Spanish Huerva River in Zaragoza (41° 37′ 23″N, 0° 54′ 28″W), which is a tributary of the Ebro River. Sediment was taken anaerobically with stainless steal cores, and transported on ice to the laboratory. Cores were opened in an anaerobic glove box with ± 1 % H_2-gas and ± 99 % N_2-gas to maintain anaerobic conditions, and the sediment was put in a glass jar. The glass jar was stored at 4°C in an anaerobic box which was flushed with N_2-gas. The sediment contained

a mixture of branched isomers of NP (20 mg·kg^{-1} dry weight). The chromatogram of the gas chromatography – mass spectrometry (GC-MS) of the NP mixture found in the sediment was comparable to the chromatogram of the tNP technical mixture ordered from Merck. The single isomers were not identified and 4-*n*-NP was not present in the sediment. The total organic carbon fraction of the sediment was 3.5% and contained mainly clay particles with a diameter size < 32 µM.

Biodegradation of 4-*n*-NP and the mixture of branched NP in the sediment were studied under methanogenic, sulphate reducing and denitrifying conditions with or without the addition of phenol. Briefly, 60 ml bottles were filled with 2 g dry weight sediment and 50 ml methanogenic medium (Holliger et al., 1993), sulphate reducing medium (Stams et al., 1993) or nitrate reducing medium (Evans et al., 1991) was added. Little modifications were performed: 5 mM Na_2SO_4 instead of 28 mM was used in the sulphate reducing medium, and 4 mM $NaNO_3$ instead of 20 mM KNO_3 was used in the nitrate reducing medium. Yeast extract was not used in the media. The bottles were filled with sediment and medium in the anaerobic glove box. All bottles were closed with a viton stopper and aluminium cap in the glove box, and the headspace was changed to N_2/CO_2 (80% : 20%). Two biotic and one abiotic bottle were prepared per treatment. The abiotic controls were performed by autoclaving one of the three bottles per treatment for 15 min at 121°C and adding 50 mg·l^{-1} $HgCl_2$. Both 4-*n*-NP and branched NP were added in a concentration of 9 µM (2 mg·l^{-1}) to the bottles. Since the used sediment was polluted with branched NP, the batches contained a final concentration of 2 mg·l^{-1} 4-*n*-NP and 2.8 mg·l^{-1} branched NP. Phenol was added in a concentration of 53 µM (5 mg·l^{-1}). The bottles were incubated in the dark at 30°C, and periodically sampled to measure the NP concentrations. The bottles with the medium and sediment were defined as the first generation enrichments.

3.2.3 Enrichments

Second to 5th generation enrichments were made of the 4-*n*-NP degrading batches with nitrate reducing medium. Branched NP was not added since degradation of branched NP was not observed. Phenol was not added for further enrichment. The enrichments originating from the first generation enrichment with 4-*n*-NP were encoded as "NP" and the enrichments originating from the first generation enrichment with 4-*n*-NP plus phenol were encoded as "NP+P". Besides transfers also dilutions series were performed to further enrich the 4-*n*-NP degrading population. Figure 3.2 shows an overview of the enrichments and dilution series.

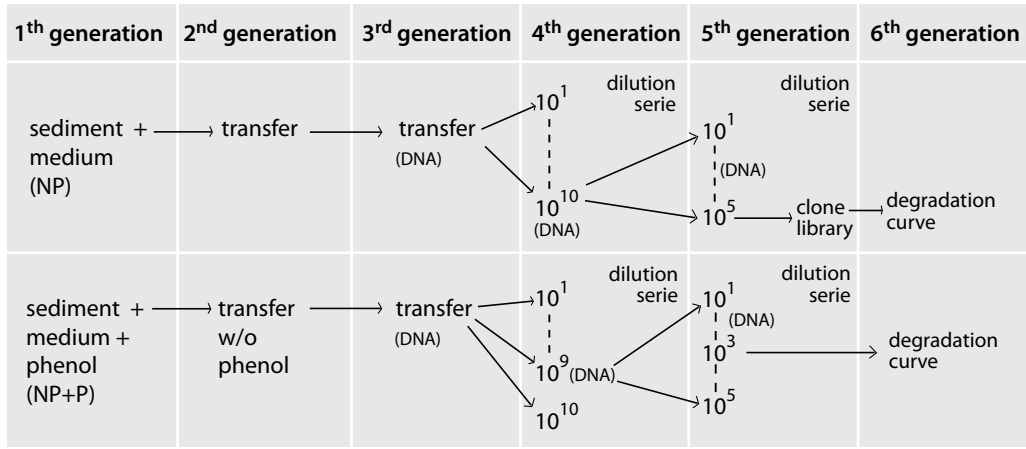

Figure 3.2: overview of enrichments, dilution series, DNA samples and clone library sample of batches originating without phenol (NP) and with phenol (NP+P).

Before the transfer to the second generation, 11 µM 4-*n*-NP was re-added to the first generation enrichment at t=104 days and this enrichment was transferred at t=259 days of incubation into fresh nitrate reducing medium with 20 mM of nitrate, 11 µM (2.5 mg·l^{-1}) 4-*n*-NP and 10% inoculum (v/v). The headspace was formed by pure N_2, and 4-*n*-NP was spiked from a 4-*n*-NP stock solution.

Dilution series were made to further enrich the 4-*n*-NP degrading population from the 3rd generation NP and NP+P enrichments with a maximum dilution of 10^{10} (encoded as 4th generation 10^{10}). To these dilution batches, 11 µM 4-*n*-NP was added. The 10^{10} dilution of the NP enrichments and the 10^9 dilution of NP+P enrichments were the most diluted 4-*n*-NP degrading enrichments, and were used for a second ten fold dilution series with a maximum dilution of 10^5 (5th generation). Degradation was observed in this 5th generation up to the 10^5 dilution of the NP enrichment and up to the 10^3 dilution of the NP+P enrichments.

Degradation studies were performed in duplicate in nitrate reducing medium with 10% inoculum (v/v) of the most diluted active enrichments and 2 µM 4-*n*-NP (6th generation). Two sterile controls containing medium, 10% inoculum (v/v) and 4-*n*-NP were also measured. The medium with inoculum was autoclaved for 15 min. at 121°C and thereafter 4-*n*-NP from the sterile stock suspension was added. Degradation rate constants and half live times of the NP degradation were calculated with first order kinetics. Student's t tests were used for comparison of the data. Probability of significance was set at $p < 0.05$. The batches were sampled daily for 8 days and the samples were analyzed immediately for the 4-*n*-NP concentration by GC-MS analyses.

3.2.4 Chemical analyses

Linear nonylphenol (4-*n*-NP) and branched NP concentrations were analyzed by GC-MS. A 200 µl slurry sample was taken from batches with sediment for analysis and added to a capped 20 ml headspace vial with 1.8 ml MilliQ water containing 7 mg·l^{-1} $HgCl_2$. From the enrichments without sediment, 200 or 500 µl sample was taken depending on the expected NP concentration. The samples were added to a capped 20 ml headspace vial with 1.8 or 1.5 ml MilliQ water containing 7 mg·l^{-1} $HgCl_2$ to a final volume of 2 ml. The headspace vial was capped with a magnetic crimp cap with blue silicon and teflon coated septum (Grace Davison Discovery Science, Deerfield, Il, USA) and analysed with GC-MS by solid phase micro extractions as described in Chapter 2.

Phenol concentration was analysed by high pressure liquid chromatography (HPLC) with a C18 5µm reverse column (Alltech, Breda, the Netherlands) and a UV-VAR detector (Varian Chrompack International BV, Middelburg, the Netherlands) at a wave length of 268 nm. A sample of 0.5 ml was taken from the batches with phenol and centrifuged for 1 min. (16.1 rpm). After centrifugation, the supernatant was transferred to a HPLC vial. The eluent consists of 40% acetonitrile and 60% 0.05M KH_2PO_4 (pH = 3) with a constant flow rate of 0.8 ml·min^{-1}. The injection volume was 25 µl.

3.2.5 DNA extraction and nested PCR

DNA was extracted from the 3rd generation, the 10^{10} dilution NP enrichment of the 4th generation, the 10^9 dilution NP+P enrichment of the 4th generation, the 10^1, 10^3 and 10^5 dilution NP enrichment of the 5th generation, and the 10^1 and 10^3 dilution NP+P enrichment of the 5th generation (Figure 3.2). To extract total DNA, 5 ml culture volume from the enrichments was filtrated through a 0.2 µm pore size cellulose acetate filter (Whatman GmbH, Dassel, Germany). DNA was extracted from the filters by bead-beating protocol by using a BIO101 Systems Fast DNA®Kit for Soil (Qbiogene, Inc, CA) according to the manufacturer's instructions. Bead-beat step was modified from 30 s to 45 s. DNA was stored at -20°C until further analysis.

A nested polymer chain reaction (PCR protocol), with two PCR steps, was performed to investigate the development and population dynamics of the microbial populations in the different enrichment and dilution steps by denaturing gradient gel electrophoresis (DGGE).

3.2.6 Clone library construction

A 16S rRNA gene clone library was constructed from the 5th generation 10^5 dilution NP enrichment genomic DNA. This enrichment was used because it was the most enriched batch which degraded 4-*n*-NP. The first PCR reaction was performed to amplify the complete 16S rRNA genes with two bacterial primer mixtures, fD1/fD2 and rP1/rP2, based on the method as described by Weisburg et al. (Weisburg et al., 1991). These PCR amplicons of the complete 16S rRNA genes were used to make a clone library. The 50 µl reaction mixture contained 0.4 U Taq-polymerase (Fermentas International inc, Burlington, Canada), 5 µl 10x Taq-buffer, 2 mM $MgCl_2$, 200 µM deoxynucleoside triphosphate, 0.6 µM bacterial primer fD1/fD2, 0.6 µM bacterial primer rP1/rP2 (Eurofins MWG Operon, Ebersberg, Germany)) and 1 µl aliquot of the 10 times diluted total DNA extracts. Amplifications were carried out with a ICycler (Bio-Rad laboratories, Hercules, CA) using the following temperature program: After 3 min of initial denaturation and enzyme activation at 94°C, a touchdown thermal profile protocol was performed by decreasing the annealing temperature 1°C per cycle from 65°C to 55°C, at which temperature 33 additional cycles were performed. Amplification was carried out with 30 sec of denaturation at 94°C, 30 sec of primer annealing, and 1 min of primer extension at 72°C, followed by 5 min of final extension at 72°C.

The PCR amplicons of the 16S rRNA genes were purified with the QIA Quick® PCR purification kit (Qiagen, Hilden, Germany), and cloned by using the TOPO TA cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The plasmids were purified with the Qiagen Qia prep® spin mini prep kit and sequenced by Eurofins MWG Operon (Ebersberg, Germany) with M13 uni (-21) sequencing primer. Sequences were inspected for ambiguous base assignments and were compared with sequences deposited in public accessible databases using the NCBI BLAST search tool at htpp://www.ncbi.nlm.nih.gov/blast. Alignment of the sequences was carried out by using Clustalw online software at http://www.ebi.ac.uk/tools/clustalw2.

3.2.6 Denaturing Gradient Gel Electrophoresis (DGGE)

The amplicons of the 16S rRNA genes (amplified by the primer mixtures fD1/fD 2 and rP1/rP2) were amplified in a second (nested) PCR step by using the primers F341-GC and R534 to perform DGGE as described by Muyzer et al. (Muyzer *et al*., 1993). For this second PCR reaction, 1 µl from 10-fold diluted PCR amplicons from 16S RNA gene were used as template DNA. The reaction mix had the same composition as for the amplification of the 16s rRNA gene and the PCR conditions were the same as described above.

A DGGE was performed as described by Muyzer et al.(Muyzer *et al*., 1993), with small modifications. Approximately 400 ng of nested PCR product (25 µl) were loaded on an 8% (w/v) polyacrylamide gel with a denaturing gradient of 30 to 70% denaturant (100% denaturant contained 7 mM urea and 40% (v/v) formamide). DGGE was performed in a 1x TAE buffer (40 mM tris, 20mM sodium acetate, 1 mM EDTA; pH 7.4) using a DcodeTM Universal Mutation Detection System (BioRad) at 100V at 60°C for 16h. The gels were stained for 45 min with SybrGold (Molecular Probes, Inc, Eugene, OR) in 20 ml of 1xTAE, and viewed under UV light. DGGE bands were processed using Quantity-one version 4.6.2 image analysis software (Bio-Rad Laboratories, Hercules, CA) and corrected manually when needed. After normalization of the gels, bands with a relative peak area intensity above 2% were included in further analyses, and were digitalized and analyzed.

Bacterial diversity, based on DGGE gel analysis, were assessed by using Shannon-Weaver diversity index ($H'=-\Sigma P_i \log P_i$) (Shannon and Weaver, 1949). P_i is the relative peak intensity of a DGGE band, calculated as $Pi= n_i/N$, where ni is the peak area of the band and N the

sum of all the peak areas in DGGE lane. Furthermore, a covariance principle component analysis (PCA) was performed on band type and peak height with the Excel application StatistiXL, version 1.4 to identify significant shifts and similarities in microbial populations during the enrichment of batches originating from NP with and without phenol.

Predominating present bands of DNA, which show differences in the pattern between the 5th generation 10^5 dilution of the NP enrichment and the 5th generation 10^3 dilution of the NP+P enrichment, were excised from the gel. A sterile razor blade was used to excise the bands. The bands were resuspended in 50µl sterilized MilliQ water and incubated overnight at 4°C. Desorbed DNA from the excised bands was re-amplified by PCR by using the F341 primer, without a GC-clamp and R534 primer. The 50 µl reaction mixture of the first PCR reaction on the 16S rRNA genes contained 2.5 U Takara Ex Taq DNA polymerase (Takara Bio, Otsu, Shiga, Japan), 5 µl 10x Takara Ex Taq Buffer, 100 µM deoxynucleoside triphosphate, 0.6 µM primer F341, 0.105 µM primer R534 (Eurofins MWG Operon, Ebersberg, Germany) and 1 µl aliquot of the DNA from the excised bands. The PCR amplicons were purified with the QIA Quick® PCR purification kit (Qiagen, Hilden, Germany) and stored at -20°C for further use. The reamplified PCR amplicons of part of the excised bands were sequenced directly by Eurofins MWG Operon (primer F341). Bands that contained DNA of more than one specie were cloned first by using the TOPO-TA cloning Kit. The plasmids were purified with the Qiagen Qia prep® spin mini prep kit and send for sequencing to Eurofins MWG Operon (primer M13 uni (-21)). Sequences were inspected for ambiguous base assignments with Bio Edit software, version 4.8.7 and were compared with sequences deposited in publicly accessible databases using the NCBI BLAST search tool at htpp://www.ncbi.nlm.nih.gov/blast. Alignment of the sequences was done by using Clustalw on line software at http://www.ebi.ac.uk/tools/clustalw2.

3.2.9 GenBank accession numbers

The 16S rRNA gene nucleotide sequences determined in this study have been deposited into the GenBank database under accession numbers FJ626744-FJ626782.

3.3. Results

3.3.1 Anaerobic degradation of 4-*n*-NP, branched NP and phenol

Degradation of 4-*n*-NP, branched NP and phenol was investigated under methanogenic, sulphate reducing and denitrifying conditions during 703 days. Degradation of 4-*n*-NP was detected in nitrate reducing medium with and without phenol addition (Table 3.1).

Table 3.1: Degradation of linear nonylphenol (4-n-NP), branched nonylphenol (tNP) and phenol in sediment under various anaerobic conditions during 703 days. Batches were prepared with 4-n-NP and branched NP, phenol, 4-n-NP and branched NP or only phenol. += degradation, - = no degradation, n.a. = not added, ()=time for complete degradation.

	4-n-NP	**tNP**	**Phenol**
4-*n*-NP + branched NP			
Methanogenic	-	-	na
Sulphate reducing	-	-	na
Nitrate reducing	+ (<91-104 d)	-	na
Phenol + 4-*n*-NP + branched NP			
Methanogenic	-	-	+ (19 d)
Sulphate reducing	-	-	+ (13 d)
Nitrate reducing	+ (10-91 d)	-	+ (< 6 d)
Phenol			
Methanogenic	na	na	+ (19 d)
Sulphate reducing	na	na	+ (10 d)
Nitrate reducing	na	na	+ (< 6 d)

In the absence of phenol, complete degradation was achieved in 91 to 104 days. The presence of the structure analogue phenol leads to complete degradation of the 4-*n*-NP degradation in the duplicate between 10 to 91 days.

Enrichments and dilutions series were made for 4-*n*-NP degrading bacteria in denitrifying medium which were started with NP, and with NP and phenol, respectively. The 6th generation enrichments NP (originating on NP) and NP+P (originating on NP and phenol) degraded 4-*n*-NP within 4 to 6 days (Figure 3.3). Decrease of the concentration in the first two days is partly due to adsorption of 4-*n*-NP to the incubation bottles, which is also observed in the sterile controls. The decrease in the sterile controls due to adsorption in the first two days was 0% and 20% in sterile control A and B, respectively, and over the whole incubation period 47% ± 19%. However, after a lag phase of two to three days the decrease of the 4-*n*-NP concentration in the active incubates was faster than the decrease of 4-*n*-NP in the sterile controls. The rate with which the concentration in the active incubates decreased was significantly higher compared to the rate in the sterile controls. The NP and NP+P enrichments degraded 4-*n*-NP with a half life time of 0.43 ± 0.2 day and 0.38 ± 0.06 day, respectively. No intermediate metabolites were detected by GC-MS during the degradation of 4-*n*-NP.

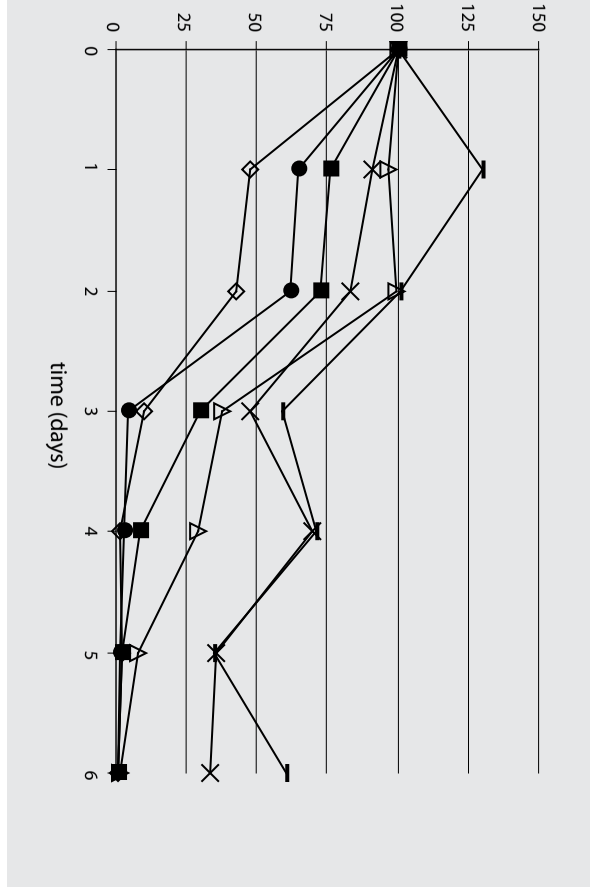

Figure 3.3: Degradation of 4-n-NP as C/C_0 in nitrate reducing medium in 6th generation enrichments from river sediment from batches originating without phenol (A:■ and B: ●), with phenol (A:◇ and B:Δ) and sterile controls (A: - and B: X).

Branched NP was not degraded under any of the tested conditions, whereas phenol was degraded under all tested conditions. The lag phase of phenol degradation in the batches with phenol and nonylphenol or with only phenol was comparable.

3.3.2 Microbial composition of a 4-*n*-NP-degrading enrichment

The clone library made from 10^5 diluted enrichment from the 5th generation on 4-*n*-NP contained 61 clones enclosing 13 different species (Table 3.2).

Table 3.2: Phylogenetic affiliations and frequencies of bacterial 16S rRNA gene clones in the clone library of the 5th generation 10^5 dilution enrichment which degrades 4-n-NP under denitrifying conditions.

No. clones	Closest related organisms in Gen Bank (accession no.)	% similarity	Accession no.	Phylogenetic group
20	Uncultured bacterium clone OTU_8 (EU083486)	96-100	FJ626754-FJ626765	*Phyllobacteriaceae; Parvibaculum* (α)
16	Uncultured bacterium clone OTU_10 (EU083488)	98-99	FJ626744-FJ626752	
4	*Pseudomonas stutzeri* (U65012)	95-99	FJ626774-FJ626777	*Pseudomonadaceae* (*y*)
5	*Pseudomonas* sp. 42(2008) (EU883660)	99	FJ626780-FJ626781	
1	Uncultured bacterium clone OTU_9 (EU083487)	94	FJ626753	
3	*Ochrobactrum anthropi* ATCC 49188 (CP000759)	99	FJ626766-FJ626767	*Brucellaceae* (α)
1	*Ochrobactrum tritici* strain LMG 2320(t1) (AJ865000)	99	FJ626768	
3	*Thauera* sp. R-25071 strain R-25071 (AM084033)	99	FJ626772-FJ626773	*Rhodocyclaceae* (ß)
2	*Stenotrophomonas* sp. YC-1 (DQ537219)	99	FJ626769-FJ626770	*Xanthomonadaceae* (*y*)
2	Uncultured *Beijerinckia* sp. clone 31 (EF584507)	97	FJ626778	*Beijerinckiaceae* (α)
1	*Chelatococcus daeguensis* strain K106 (9AY921846)	98	FJ626779	
2	Uncultured alpha proteobacterium clone AKYG1898 (AY921846)	97	FJ626771	*Proteobacteria* (α)
1	*Rhizobium* sp. TKW1 (AF345860)	94	FJ626782	*Rhizobiaceae* (α)
total = 61				

From the 61 clones, 36 clones were closely related to uncultured α-proteobacteria, strains OTU_8 (96-100% similarity) and OTU_10 (98-99% similarity). Strains OTU_8 and OTU_10 are identified in a hexadecane degrading denitrifying consortium (Genbank accession numbers EU083486 and EU083488) (Callaghan et al., 2009). The closest hit with a cultured bacterium was with *Parvibaculum lavamentivorans* DS-1 (94-98% similarity). From the 61 clones, 9 clones were related to γ-proteobacteria *Pseudomonas stutzeri* (95-99% similarity) and *Pseudomonas* sp. 42 (99% similarity). These denitrifying strains are also possibly involved in the biodegradation of 4-*n*-NP under denitrifying conditions. Also other denitrifying microorganisms, like the α-proteobacterium *Ochrobactrum anthropi* and the ß-proteobacteria of the *Thauera* genus (Heylen et al., 2006) were identified in our enrichments.

3.3.3 DGGE profiles of total bacterial community

DGGE profiles were made of the 3rd generation, the 10^{10} dilution 4th generation, and the 10^{1}, 10^{3} and 10^{5} dilution 5th generation of the NP enrichment and the 3rd generation, the 10^{9} dilution 4th generation, and the 10^{1} and 10^{3} dilution 5th generation of the NP+P enrichment (Figure 3.4).

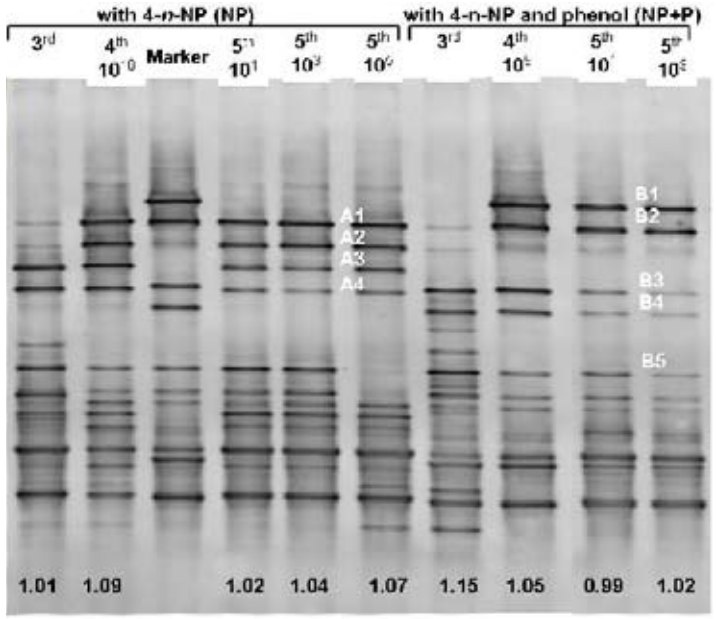

Figure 3.4: Denaturing gradient gel electrophoresis of PCR-amplified 16s rRNA gene fragments of 4-n-NP degrading enrichments of various generations and dilutions originating from samples with 4-n-NP, and with 4-n-NP and phenol. The marker is equal to 4th generation 10^{9} sample originating with phenol. A1-A4 and B1-B5 are excised bands, and the number below the lanes is the Shannon-Weaver diversity index.

Band quantity and Shannon-Weaver diversity index (H') of the NP enrichments (originating with 4-*n*-NP), as analyzed by DGGE (figure 3.4), varied between 12 and 14 bands and 1.01 to 1.09 respectively. This small variety in diversity index indicates that transferring and diluting the cultures did not affect the microbial diversity in these enrichments. This indicates that the population remained stable. The amounts of bands in the DGGE of the NP+P enrichments (originating with 4-*n*-NP and phenol) varied between 12 and 16, and the H' index varied between 0.99 and 1.15. The largest shift in diversity was observed between the 3rd generation and 4th generation enrichment, as this enrichment step lowered the diversity from 1.15 to 1.05. Further enrichment did not affect the population that much. The H' index in the 5th generation enrichments was 0.99 to 1.02.

Changes in microbial composition were monitored by principal-component analysis (PCA). The PCA scatter plot (Figure 3.5) shows that the NP enrichments and the NP+P enrichments form two different clusters. Within a cluster can be seen that transfer to a new generation had a larger effect on the population than making dilutions series. Dilution of inocula did not change the population significantly in both types of enrichments. However, the population change between the enrichments and dilutions from the batch originating with 4-*n*-NP and phenol was larger compared to the samples originating with 4-*n*-NP, mainly on the first component (PC1) which explains 48.3% of the variation in the data.

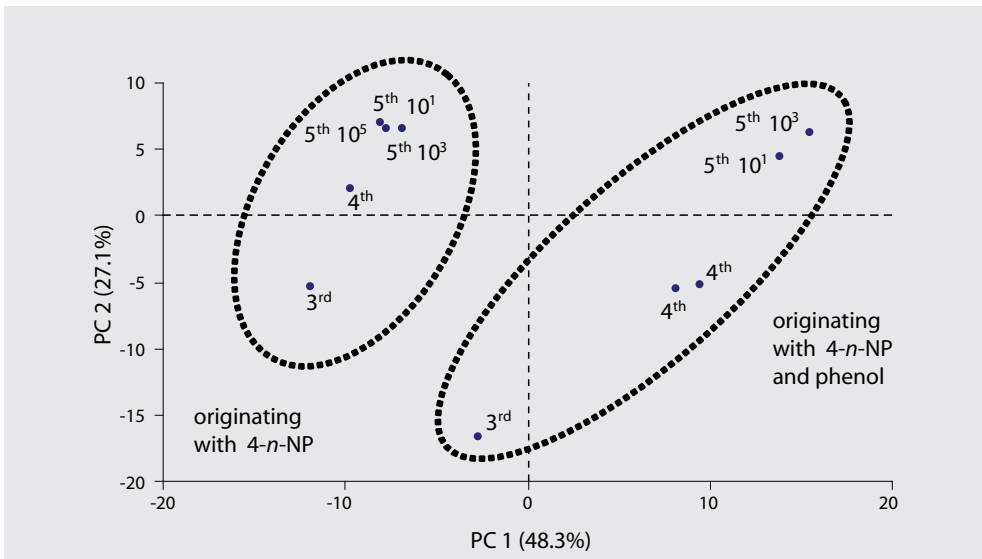

Figure 3.5: Principal component analysis scatter plot of denaturing gradient gel electrophoresis profiles (Figure 3.3) of a 4-n-NP degrading enrichments of various generations and dilutions originating from samples with 4-n-NP, and with 4-n-NP and phenol.

In the DGGE pattern from enrichments with NP, and with NP and phenol, different bands were detected. The main differences were the unique presence of bands A2 and A3 in the enrichments NP, and the unique bands B1 and B4 in enrichments NP+P (Figure 3.4). Band A1 and B2 were dominantly present in both enrichments. Band B5 was dominantly present in both enrichments but disappeared in the 10^5 dilution of the 5th generation NP enrichment. Band A1 and A2, which are dominantly present in the NP enrichments, and which became more relatively intense from the 10^{10} dilution of the 4th generation. Sequencing of the PCR amplicons of bands A1 to A4 and B1 to B5 showed that the sequence of band A1 belonged to species closely related to *Pseudomonas stutzeri* isolate st104 (Table 3.3).

Table 3.3: Identity of excised bands from denaturing gradient gel electrophoresis (Figure 3.4) with phylogenetic group, accession number Gen Bank and % of similarity to closest related organism.

	DGGE band	Phylogenetic group	Closest related organism in GenBank (accession no.)	% similarity
originating with 4-*n*-NP	A1	*Pseudomonadaceae* (γ)	*Pseudomonas stutzeri*, isolate st104 (AM905852)	99
	A2	*Pseudomonadaceae* (γ)	*Pseudomonas stutzeri*, isolate st104 (AM905852)	98
		Phyllobacteriaceae; Parvibaculum (α)	Uncultured bacterium clone OTU_10 (EU083488)	98
	A3	*Phyllobacteriaceae; Parvibaculum* (α)	Uncultured bacterium clone OTU_8 (EU083486)	99
	A4	*Phyllobacteriaceae; Parvibaculum* (α)	Uncultured bacterium clone OTU_8 (EU083486)	99
originating with 4-*n*-NP and phenol	B1	*Pseudomonadaceae* (γ)	*Pseudomonas aeruginosa* strain H13 (AY074894)	99
		Phyllobacteriaceae; Parvibaculum (α)	Uncultured bacterium clone OTU_10 (EU083488)	98-99
		Alcaligenaceae (ß)	*Alcaligenes* sp. 22-27 (AY999035)	100
	B2	*Rhizobiaceae* (α)	*Ochrobactrum* sp. HPC481 (AY074894)	98
		Pseudomonadaceae (γ)	*Pseudomonas aeruginosa* strain H13 (AY074894)	98
		Phyllobacteriaceae; Parvibaculum (α)	Uncultured bacterium clone OTU_10 (EU083488)	98-100
	B3	*Phyllobacteriaceae; Parvibaculum* (α)	Uncultured bacterium clone OTU_8 (EU083486)	99
	B4	*Phyllobacteriaceae; Parvibaculum* (α)	Uncultured bacterium clone OTU_8 (EU083486)	99
	B5	*Phyllobacteriaceae; Parvibaculum* (α)	Uncultured bacterium clone OTU_8 (EU083486)	99

Band A2 contained DNA related to the sequence of two different species; *Pseudomonas stutzeri* isolate st104 (98% similarity) and uncultured bacterium OTU_10 (98% similarity). Alignment of the sequences of the clones of band A1 (235-239 bp) and A2 (193-197 bp) which are closely related to *Pseudomonas stutzeri* isolate st104 showed 99% similarity (240 bp). Band A3 and A4 were dominantly present in all NP enrichments. These bands belong to species closely related to uncultured strain OTU_8 (both 99% similarity). Alignment of the sequences of band A3 (115 bp) and A4 (116 bp), which were both closely related to uncultured bacterium OTU_8, showed 100% similarity to each other, although these bands appear at different positions in the DGGE gel. The different band migration could be explained based on PCR bias and by DGGE bias caused by denaturing of duplex DNA into single-chained DNA, generating different DGGE bands.

Band B1 was not present in the DGGE pattern of the 3rd generation NP+P enrichment, and appeared in the 4th enrichment 10^{10} dilution as a very dominant band. Band B2, had a weak signal in the gel pattern of the 3rd generation NP+P enrichment and became more intense in the enrichment of the 4th generation 10^9 dilution. These two bands (B1 and B2) contained DNA of three different species. Both bands contained DNA of species closely related to *Pseudomonas aeruginosa* strain H13, and uncultured bacterium clone OTU_10. Band B1 contained also DNA from species related to *Alicaligenes* sp.22-27 and band B2 of species closely related to *Ochrobactrum* sp. HPC481. Alignment showed 99% similarity (194 bp) between the sequences related to *Pseudomonas aeruginosa* strain H13 of band B1 and B2. Due to further enrichment and dilution of the 4th generation NP+P enrichment, the previous dominant bands B3, B4 and B5 became less dominant. Bands B3, B4 and B5 were identical to each other and related to uncultured bacterium OTU_8 (99% similarity). The different band migration could be explained by PCR bias and DGGE bias as described before.

3.4 Discussion and conclusions

Linear NP (4-*n*-NP) was degraded under nitrate reducing conditions. The degradation rate increased during the enrichment of the 4-*n*-NP degrading population. Enhancement of 4-n-NP degradation due to adaptation to 4-*n*-NP was also reported in sediment from the Erren River (Chang et al., 2004). Degradation in our enrichments is approximately 40 times faster than degradation under denitrifying conditions in batches with sediment from the Erren River. Degradation of 4-*n*-NP was not observed in batches under sulphate reducing or methanogenic conditions.

Degradation of branched NP was not observed after 703 days of incubation of the sediment under the different tested conditions, even not under nitrate reducing conditions. Linear NP and the branched NP isomers vary in molecular structure of the nonyl chain. As degradation of 4-*n*-NP was shown under nitrate reducing conditions in our sediment, but not of the branched NP, it is likely that the degradation of 4-*n*-NP starts with the degradation of the linear alkyl chain and not with the phenol ring. This is confirmed by the microbial population in our enrichments, as the clone library is dominated by clones which are most closely related to uncultured hexadecane degrading bacteria

under denitrifying conditions. Hexadecane is a linear alkane like the nonyl chain of 4-*n*-NP. It is worth mentioning that the closest similar sequence of a cultured strain to these dominant present sequences was *Parvibaculum lavamentivorans* DS-1. This bacterium converts linear alkylbenzene sulfonate (LAS) to sulfophenylcarboxylate as end product. LAS is a compound which has a similar chemical structure as 4-n-NP with a SO3-- group instead of an OH-group attached to the aromatic ring. *Parvibaculum lavamentivorans* DS-1 degrades LAS under aerobic conditions (Schleheck and Cook, 2005; Schleheck et al., 2000). Degradation under (facultative) anaerobic conditions by *Parvibaculum lavamentivorans* DS-1 is not reported. However, our identified sequences were more related to the sequences of uncultured denitrifying strains than to the sequences of *Parvibaculum lavamentivorans* DS-1.

The microbial population of the enrichments changed most significantly between the 3rd and 4th generation enrichment as seen in the DGGE profile. Further transfers and dilutions led only to minimal differences in population diversity. The population remained stable and approximately 13 bands were present in the DGGE. This variety of DGGE bands in the 5th generation enrichments shows that different microorganisms are involved in the degradation of 4-*n*-NP under denitrifying conditions.

DNA sequences from DGGE excised bands from enrichments which started either with 4-*n*-NP, or with 4-*n*-NP and phenol were closely related to the uncultured hexadecane degrading bacterium OTU_8. Although most of the bands related to this uncultured bacterium are present at different positions in the gel, they show 100% similarity to each other. The different band migration can be explained by PCR bias and by DGGE bias caused by denaturing of duplex DNA into single-chained DNA, generating different DGGE bands. Alignment of the sequences of all the bands related to uncultured bacterium OTU_8 (Bands A3, A4, B3, B4 and B5) did not show any differences. Bands A1 and B2 appear at the same position in the gel, and the sequences related to a *Pseudomonas* strain of these two bands show a similarity of 98% (191 bp). To elucidate to what extent additional microbial species are involved in 4-*n*-NP biodegradation, further enrichments should be made to isolate the microorganisms responsible for the degradation of 4-*n*-NP under denitrifying conditions.

It is worth mentioning that sequences related to *Pseudomonas* species are detected both in the clone library and in the dominant DGGE bands of the enrichments of the batches originating with 4-*n*-NP, and with 4-*n*-NP and phenol. Sequences related to *Pseudomonas stutzeri* are identified in the clone library and in the dominating DGGE bands of the enrichment originating with 4-*n*-NP. A wide variety of diverse denitrifying strains belong to *Pseudomonas stutzeri* species which can degrade many different xenobiotic compounds and have been identified in natural sources. The species *Pseudomonas stutzeri* contain strains which are able to degrade alkanes and phenol (Lalucat et al., 2006). Furthermore, in the enrichment originating with 4-*n*-NP and phenol, DNA of strains related to *Pseudomonas aeruginosa* species are present in the dominant bands. *Pseudomonas aeruginosa* species are commonly present in soils and sediment (Gamble et al., 1977), and are able to degrade phenol and alkanes under denitrifying conditions (Chayabutra and Ju, 2000; Thu *et al*., 1999). Although species related to *Pseudomonas aeruginosa* are only identified in the dominating DGGE bands of the NP+P enrichment, it is unclear if this is caused by the presence of phenol in the first generation enrichment or by other unknown factors.

As seen in the study, branched NP could not be degraded under anaerobic conditions, although the sediment was polluted with branched NP and the present bacteria could already have been adapted to it. Our results agree with other studies, which did not observe anaerobic degradation of branched NP either (Ekelund *et al*., 1993; Hesselsoe *et al*., 2001). Phenol could be degraded under all three redox conditions, but could not function as a structure analogue to initiate the degradation of the branched NP, despite the partly similar chemical structure.

In conclusion, 4-*n*-NP can be degraded under denitrifying conditions. Enrichments were obtained that degraded 2 µM 4-*n*-NP within 6 days. Most of the sequences obtained in our 4-*n*-NP degrading nitrate reducing enrichments are closely related to sequences of uncultured alkane denitrifying microorganisms. The linear chain of 4-*n*-NP is important, and the degradation of 4-*n*-NP most probably would start with degradation of the linear chain. Branched NP is recalcitrant to biodegradation under all tested anaerobic conditions. For future studies into the effect of structure analogues to enhance or initiate the degradation of all branched NP isomers one should take into account the complicating fact that a large variety of branched alkanes is needed. This study shows that branched NP will not degrade under anaerobic conditions, which implies that the branched NP will remain in sediment systems, which are generally anaerobic (Huttunen et al., 2006; Martin et al., 1998; Middeldorp et al., 2003). These amounts of branched NP will function as a long lasting secondary source for surface water pollution, which may be remobilised under changed hydrologic conditions posing risks for the environment due to is estrogenic properties.

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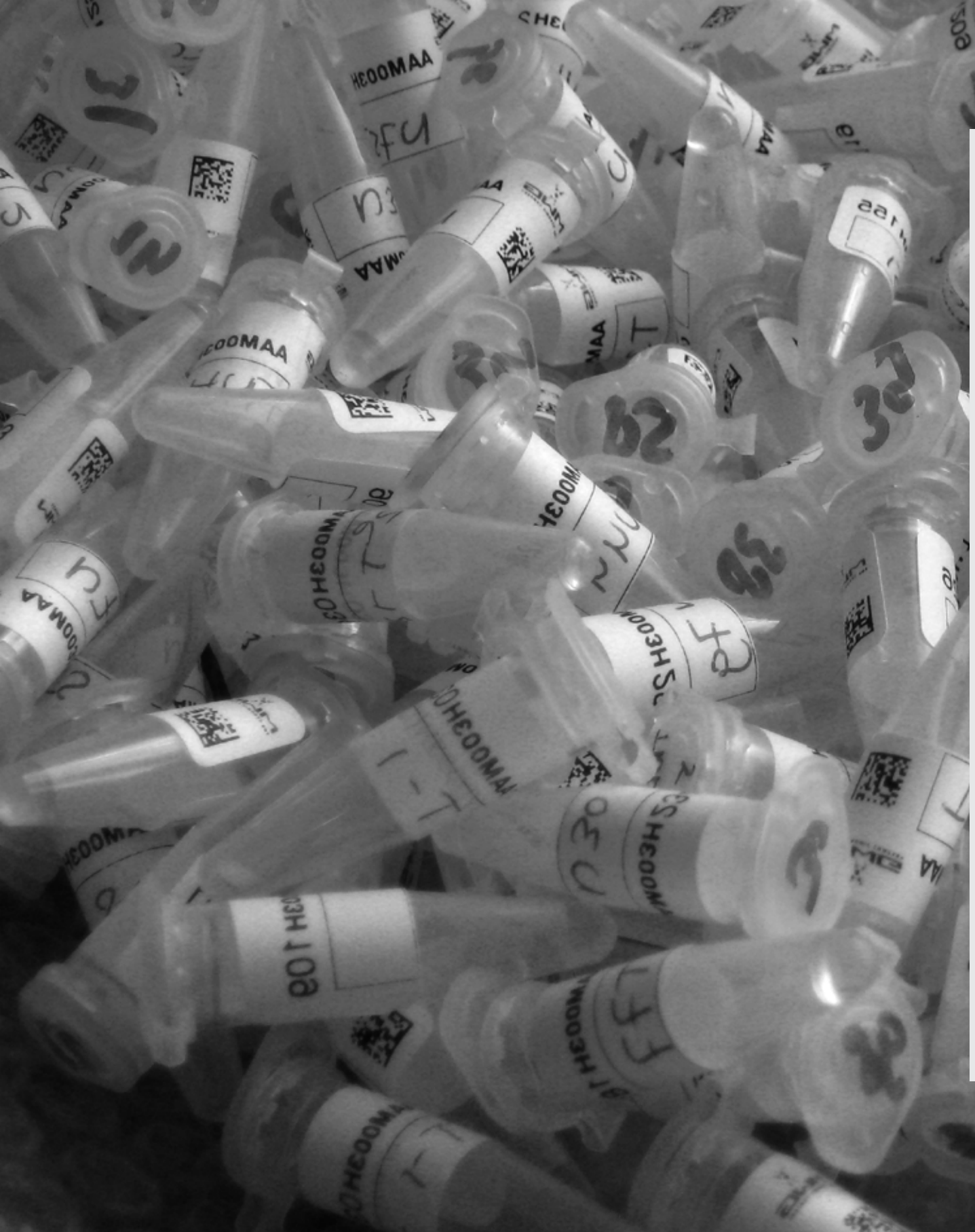

4 Aerobic nonylphenol degradation and nitro-nonylphenol formation by microbial cultures from sediments

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Abstract

Nonylphenol (NP) is an estrogenic pollutant which is widely present in the aquatic environment. Biodegradation of NP can reduce the toxicological risk. In this study, aerobic biodegradation of NP in river sediment was investigated. The sediment used for the microcosm experiments was aged polluted with NP. The biodegradation of NP in the sediment occurred within 8 days with a lag phase of 2 days at 30°C. During the biodegradation nitro-nonylphenol metabolites were formed, which were further degraded to unknown compounds. The attached nitro-group was originated from the ammonium present in the medium.

Five subsequent transfers were performed from original sediment and yielded a final stable enrichment. In this NP degrading enrichment culture, the micro-organisms involved in the biotransformation of NP to nitro-nonylphenol were related to ammonium oxidizing bacteria. Besides the degradation of NP via nitro-nonylphenol, bacteria related to phenol degrading species which degrade phenol via ring cleavage, are abundantly present.

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4.1 Introduction

Nonylphenol (NP) is an estrogenic surfactant which is wide spread in the aquatic environment. Nonylphenol exists of phenol group with a linear or a branched chain of nine carbon atoms. A technical mixture of branched nonylphenol (tNP) isomers is used for the production of nonylphenol polyethoxylates (NPnE) (Thiele et al., 2004; Wheeler et al., 1997). This technical mixture does not contain linear NP (4-*n*-NP), and the linear form is therefore generally not present in natural samples. At anaerobic conditions in waste water treatment plants, NPnE degrades partially to NP, which is a persistent end product in sewage sludges and the effluent of waste water treatment plants (Giger et al., 1984; Stephanou and Giger, 1982). When these NP contaminated sludges and effluents are discharged, NP ends up in the environment as a pollutant.

Due to its chemical properties, NP adsorbs to sediments and accumulates in anaerobic sediments of various rivers such as the Llobregat River (Petrovic et al., 2002) and the Ebro River in Spain (Lacorte et al., 2006), and the Elbe River in the Czech Republic and Germany (Heemken et al., 2001). Nonylphenol can desorb from the sediment and re-enter the water phase where it may bioaccumulate in fishes and be harmful for other aquatic organisms (LeBlanc and Rider, 2000; Servos, 1999; Snyder et al., 2001; Tyler et al., 1998; Yang et al., 2005). Biodegradation of NP can reduce the toxicological risk of NP in the environment.

Under aerobic conditions, biodegradation of NP (branched and linear) has frequently been shown (Corvini et al., 2006b; Ekelund et al., 1993; Hesselsoe et al., 2001; Yuan et al., 2004). However, anaerobic biodegradation of branched NP is not observed so far. Only anaerobic biodegradation of linear NP has been described to occur in sediment from the Erren River in China under sulphate reducing, nitrate reducing and methanogenic conditions (Chang et al., 2004). Aerobic NP degrading bacterial strains are isolated from various environments. For example, *Sphingomonas* sp. TTNP3 and *Sphingobium xenophagum* Bayram, formerly known as *Sphingomonas xenophaga* Bayram, are isolated from waste water treatment plants (Gabriel et al., 2005a; Tanghe et al., 1999). *Stenotrophomonas* sp. and *Pseudomonas mandelii* are isolated from polluted soils (Soares et al., 2003), and *Sphingobium amiense*, formerly known as *Sphingomonas* sp. YT (Ushiba et al., 2003; Vries et al., 2001) from sediment. Most isolates are able to use NP as a sole carbon and energy source (Gabriel et al., 2005a; Soares et al., 2003; Tanghe et al., 1999), whereas some strains can only degrade NP co-metabolically in the presence of yeast extract as carbon and energy source like *Sphingobium amiense* (Vries et al., 2001).

Three possible biodegradation pathways of NP are mentioned in literature. One suggested pathway is the degradation of NP via a nonylphenol metabolite with a nitro-group at the *ortho*-position of the aromatic ring (Figure 4.1). This metabolite was observed in the degradation experiments with soil and sewage sludge (Telscher et al., 2005), and in agricultural soils (Zhang et al., 2009).

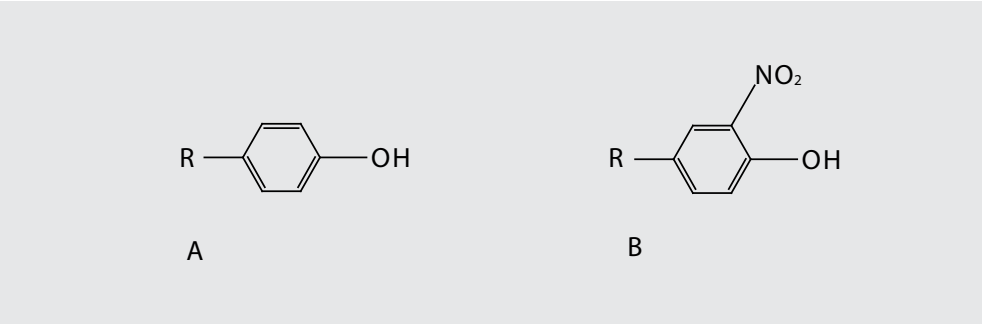

Figure 4.1: Molecular structure of A) nonylphenol and B) ortho-nitro-nonylphenol

A second postulated pathway starts with the cleavage of the phenolic ring via the phenolic degradation pathway. This pathway is suggested for the biodegradation of NP by *Pseudomonas* spp, which was isolated from contaminated soil from a former industrial site (Soares et al., 2003). A third pathway of NP degradation starts with the hydroxylation of the C-4 atom of the aromatic ring to which the nonyl chain is attached, also called *ipso*-hydroxylation. This pathway is intensively studied in *Sphingobium xenophagum* Bayram and *Sphingomonas* sp. TTNP3. These strains were isolated from activated sludge from a municipal waste water treatment plant and a lab-scale activated sludge reactor fed with NP, respectively (Corvini et al., 2006a; Corvini et al., 2007; Gabriel et al., 2005a; Gabriel et al., 2005b; Kohler et al., 2008; Tanghe et al., 1999).

This chapter describes the aerobic biodegradation of the technical mixture of branched NP (tNP) in NP polluted river sediment. During the biodegradation process, the formation of nitro-nonylphenol was observed. The origin of the nitro-group was studied by using medium containing labelled ammonium nitrate ($^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$), and the involved microorganisms were identified by culture-independent approaches.

4.2 Materials and methods

4.2.1 Chemicals and stock solutions

Nonylphenol technical mixture (tNP) (purity > 94%) and linear NP (4-*n*-NP) (pestanal, purity >99%) were purchased from Riedel de Haën (Seelze, Germany). Labelled $^{15}NH_4NO_3$ (98% labelled) was ordered from Sigma-Aldrich (Zwijndrecht, the Netherlands) and $NH_4{}^{15}NO_3$(98% labelled) from Isotech (Miamisburg, Ohio, USA). Solvents and chemicals were of the highest purity available and were used without further purification.

For the addition of tNP to the degradation experiments (22 µM of final concentration) without the addition of unwanted solvents, a high concentrated suspension of tNP (3.5 mM) in water was prepared as described in Chapter 2. For the sterile addition of pure tNP, the chemical was UV-sterilized for 30 minutes. No photo oxidized products or metabolites were formed during the UV-sterilization as confirmed by GC-MS analyses. Before using the

suspension, the bottle with the suspension was heated at 60°C and shaken to homogenize the suspension.

4.2.2 Biodegradation experiments of tNP in slurries with polluted sediments

Nonylphenol polluted sediment was used for the biodegradation experiments, and was collected in June 2005 from the Huerva River in Zaragoza (Spain) (41° 37' 23"N, 0° 54' 28"W), a tributary of the Ebro River. During the sampling the temperature of the river water was 25.1°C. Sediment was taken with stainless steel cores to 50 cm depth, and transported on ice to the laboratory. In the laboratory, the sediment was sieved aerobically at mesh < 2 mm and stored at 4°C for a year until use. The sediment was polluted with 14 mg·kg^{-1} dry weight sediment of branched NP isomers. The individual branched isomers were not identified. However, the GC-MS peak pattern of NP in the sediment sample was comparable with the peak pattern of the technical mixture of branched isomers. Linear NP was not present in the polluted sediment.

The aerobic degradation of the branched NP present in the polluted sediment was studied in 250 ml serum bottles with 2 g wet weight tNP polluted sediment and 50 ml of aerobic medium. Based on the concentration tNP in the sediment, the batches contained 2.5 µM tNP. The headspace of the bottles (200 ml) was filled with air. The aerobic medium was prepared as described in Tros *et al*., but without $Na_2SeO_3.5H_2O$ (Tros et al., 1996). The bottles were closed with a viton stopper with crimp cap and incubated horizontally shaken (110 rpm) in the dark at 30°C. These aerobic degradation experiments were performed in triplicate and these batches were defined as the 1st generation enrichments (Figure 4.2). Duplicate sterile controls were included, which were autoclaved for 1 hour at 121°C and 50 mg·l^{-1} $HgCl_2$ was added to prevent biological activity during the experiment. To follow the NP degradation, the batches were sampled ten times in duplicate in time. For routine analyses of NP, a slurry sample (200 µl) was taken from the bottles and added to a capped 20 ml headspace vial with 1.8 ml MilliQ water with 7 mg·l^{-1} $HgCl_2$ as described in Chapter 2.

4.2.3 Enrichments

Enrichments of the NP degrading cultures were made, and incubated at 30°C (Figure 4.2).

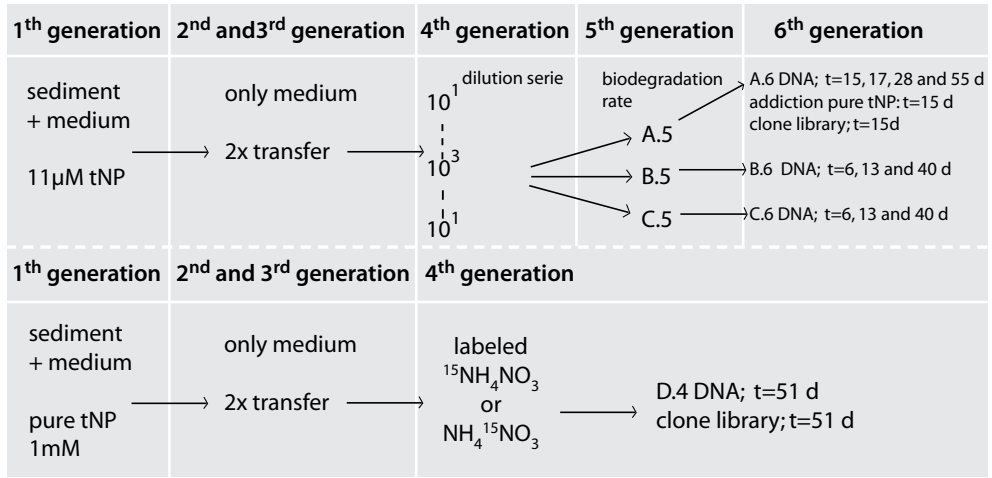

Figure 4.2: overview of enrichments, dilution series, DNA samples and clone libraries of samples with 11 µM and pure branched nonylphenol

Each transfer was considered as a new generation enrichment. All enrichments were performed at 11 uM tNP, except the 5th and 6th generation batches. These were performed at 6 uM and 15uM, respectively. The 2nd generation batches were prepared in fresh sterile mineral medium with ten fold diluted inoculum of the sediment slurry batches, which are described above. After two transfers, a ten fold dilution serie was performed with fresh aerobic medium and 10% (v/v) of inoculum to further enrich the NP degrading population. The dilution serie was carried out to a maximum dilution of 10^{10}. The 10^3 dilution of this 4th generation enrichment was used in further biodegradation experiments, since this was the most enriched active incubation. Transfers of this batch were made in triplicate (5th generation batches coded as A.5, B.5 and C.5) in fresh aerobic medium with 10% (v/v) of inoculum to determine the degradation rate of the tNP. Degradation rate constants and half life times were calculated from 5th generation batches by using first order kinetics. After complete degradation of tNP, the triplicates were transferred again to fresh aerobic medium to investigate the formation of intermediates in the enrichments (6th generation batches coded as A.6, B.6 and C.6). After 15 days of incubation of batch A.6, and complete degradation of tNP, 52 µmol tNP (= 11.5 mg tNP; oversaturated) was added to this batch to study the effect of a high concentration of tNP on the microbial population. In enrichment B.6, the added NP was degraded by day 13, and 4.5 µM tNP was re-added. For molecular analyses, samples from batch A.6 were taken before the addition of pure tNP (t=15 days), and also after 17, 28 and 55 days. Samples for molecular analyses from batches B.6 and C.6 were taken after 6, 13 and 40 days of incubation.

4.2.4 Metabolite formation

To study the formation of the metabolites, degradation experiments were performed with tNP concentrations above the maximum water solubility. These experiments were encoded as experiment D. Batches with 2 g dry weight of the original tNP polluted sediment and 50 ml of aerobic medium batches were prepared. After degradation of the NP present in the sediment, 52 µmol UV-sterile pure tNP (= 11.5 mg tNP) was added under sterile conditions, to obtain a suspension of 1 mM tNP. A sterile control was included and the batches were incubated horizontally shaken (110 rpm) in the dark at 30°C. The batches were sampled four times in duplicate in time to follow the formation of intermediates of the tNP degradation. Samples were analyzed by GC-MS at the start of the experiment to ensure no metabolites were present in the batches. Per analyzed sample, 100 µl slurry was taken from the bottles and added to a capped 20 ml headspace vial with 1.9 ml MilliQ water with 7 $mg \cdot l^{-1}$ $HgCl_2$ as described in Chapter 2.

4.2.5 Degradation experiments with ^{15}N-labelled NH_4NO_3

Inoculum from the 3th generation enrichments (Figure 4.2) were used for the degradation batches in medium with labelled $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$ to study the origin of the incorporated NO_2-group of the formed intermediate. Batches were prepared with 50 ml of aerobic media as previously described with 1 mM tNP (above saturation), and 10% (v/v) of inoculum. To the aerobic medium, 12.5 mM of either $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$ was added instead of non-labelled NH_4NO_3. Three batches were prepared with each labelled nitrogen source medium. The batches were incubated horizontally shaken (110 rpm) in the dark at 30°C. Samples were collected for GC-MS analysis at the beginning of the experiment, and after 12, 21, 30 and 49 days of the start of the experiment to follow the formation of intermediates. Samples were collected for molecular analyses after 51 days of incubation from one of this 4th generation enrichments grown with $^{15}NH_4NO_3$ (batch coded as D.4).

4.2.6 Chemical analyses

To analyse the concentration of tNP and the formation of metabolites in the batches, the collected liquid samples in the 20 ml headspace vials were measured by GC-MS with solid phase micro extraction (SPME) injections. The NP and the metabolites were extracted with a 85 µm polyacrylate SPME fiber from the headspace of the samples and analyzed by GC-MS as described in Chapter 2.

Accurate mass weight analysis was used to identify the formed metabolites. With this method only one single metabolite isomer can be identified. Since tNP consists of a mixture of branched NP isomers, tNP was not useful for this analysis. Therefore, we decided to use the linear NP isomer, and new batches were incubated in duplicate with the single linear NP isomer (4-*n*-NP) in concentrations above the maximum water solubility. Inoculum (10% v/v) was used from the 1st generation tNP degrading slurry batch. The batches were transferred twice with 10% v/v inoculum and one of these 3th generation batches was sampled and used for the analyses of the metabolite. The metabolite was extracted from 15 ml medium with 10 ml dichloromethane and hexane (1:1) by horizontally shaking (110 rpm) for 2 hours at 30°C. The dichloromethane/ hexane was completely evaporated, the extracted metabolite was resuspended in 40 µl dichloromethane and analyzed via direct insert probe mass analysis on a Finnigan MAT95 mass spectrometer. An amount of 2 µl sample was load into the mass spectrometer. The analysis started at room temperature and the temperature of the sample was increased in steps of 10°C until enough sample evaporated for analysis. Data were acquired in the electron impact mode (70 eV) at a resolution of 5400, scanning range m/ z 24–600 at 2 s per decade. Molecular ion peak m/z 265 was used for accurate mass weight analyses.

4.2.7 DNA extraction and PCR

To extract the DNA from the enrichments, 5 ml of the 6th generation batches A.6, B.6 and C.6 were collected at different time periods, and were filtrated through a 0.2 µm pore size cellulose acetate filter (Whatman GmbH, Dassel, Germany). From the 4th generation batch D.4, enrichment material that was attached to the viton rubber stopper was used and put on a filter as well. Thereafter, the DNA was extracted from the filters by using a BIO101 Systems Fast DNA®Kit for Soil (Qbiogene, Inc, CA) according to the manufacturer's instructions. Bead-beat step was modified from 30 s to 45 s. The extracted DNA was stored at -20°C until further analysis.

Nested-PCR approach encompassing two PCR steps was performed with the extracted DNA to investigate the development and population dynamics of the microbial populations in the different enrichment samples in time. The first PCR on the DNA samples was performed to amplify the complete 16S rRNA genes with the bacterial primer mixture fD1/fD2 and rP1/rP2 as based on the method described by Weisburg *et al*. (Weisburg et al., 1991). Ten fold diluted PCR amplicons of the complete 16S rRNA genes were used to carry out a clone library. Amplifications were performed with 1.25 U Takara Ex Taq DNA polymerase (Takara Bio, Otsu, Shiga, Japan).

For the denaturing gradient gel electrophoresis (DGGE), direct amplification of the DNA with the F341-GC and R534 primers was not possible. Therefore a nested PCR approach was performed. These nested amplifications were performed with 0.4 U *Taq*-polymerase (Fermentas International inc, Burlington, Canada). To do that ten fold diluted PCR amplicons from first PCR of the whole 16S rRNA genes were amplified in a nested step (second PCR reaction). The primers F341-GC, including a GC-clamp and R534 were used in the nested PCR based on the method described by Yu and Morrison (Yu and Morrison, 2004).

4.2.8 Denaturing gradient gel electrophoresis (DGGE)

A DGGE was performed based on the method described by Yu and Morrison (Yu and Morrison, 2004). Approximately 400 ng of nested PCR product (25 µl) was loaded on an 8% (wt/vol) polyacrylamide gel with a denaturing gradient of 30 to 70% denaturant using a DcodeTM Universal Mutation Detection System (BioRad) at 100V at 60°C for 16 h. The gels were stained for 45 min with SybrGold (Molecular Probes, Inc, Eugene, OR) in 20 ml of

TAE, and viewed under UV light. DGGE bands were processed using Quantity-one version 4.6.2 image analysis software (Bio-Rad Laboratories, Hercules, CA) and corrected manually when needed. After normalization of the gels, bands with the relative peak area intensity above 1% were included in further analyses, and were digitalized and analyzed.

Bacterial diversity, based on DGGE gel analysis, were assessed by using Shannon-Weaver diversity index (H'=-Σ P_i log P_i) (Shannon and Weaver, 1949). Pi is the relative peak intensity of a DGGE band, calculated as P_i= n_i / N, where ni is the peak area of the band and N the sum of all the peak areas in a DGGE lane. Furthermore, a covariance principle component analysis (PCA) was performed on band type and peak height with the Excel application StatistiXL, version 1.8, to consider possible shifts and similarities in microbial populations during the incubation of batches A.6, B.6, C.6 and D.4.

4.2.9 Cloning and sequencing

Clone libraries of 16S rRNA genes were constructed from the DNA of batch A.6 at 15 days, cultured with 15µM tNP, and of batch D.4 cultured with 1 mM tNP (saturated). The PCR amplicons of the 16S rRNA genes were purified with the QIA Quick® PCR purification kit (Qiagen, Hilden, Germany). Almost complete 16S rRNA gene fragments were cloned by using the TOPO-TA cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The plasmids were purified with the Qiagen Qia prep® spin mini prep kit and sent for sequencing to Eurofins MWG Operon (Ebersberg, Germany) with M13 uni (-21) sequencing primer. Sequences were inspected for ambiguous base assignments and were compared with sequences deposited in public accessible databases using the NCBI BLAST search tool at htpp://www.ncbi.nlm.nih.gov/blast. Alignment of the sequences was done by Clustralw on line software at http://www.ebi.ac.uk/tools/clustralw2.

4.2.10 GenBank accession numbers

The 16S rRNA gene nucleotide sequences determined in this study have been deposited into the GenBank database under accession numbers FJ591135-FJ591150, FJ609321-FJ609385 and FJ626783-FJ626794. If sequences were identical, the longest sequence was submitted.

4.3 Results

4.3.1 Nonylphenol degradation

Experiments were performed to degrade tNP that was present as pollutant in the sediment. The biodegradation of tNP in the batches with medium and sediment (1st generation) started after a lag phase of two days and within 8 days 95% (3.3 ± 0.4 µM) of the NP present in the sediment was biodegraded (data not shown). The half life time of tNP in the sediment was 1.5 ± 0.4 d. In the batches of the 10^{-3} diluted 5th generation enrichments, a decrease in tNP concentration in enrichments A.5, B.5 and C.5 was observed from t=0, which was also observed in the sterile control (Figure 4.3).

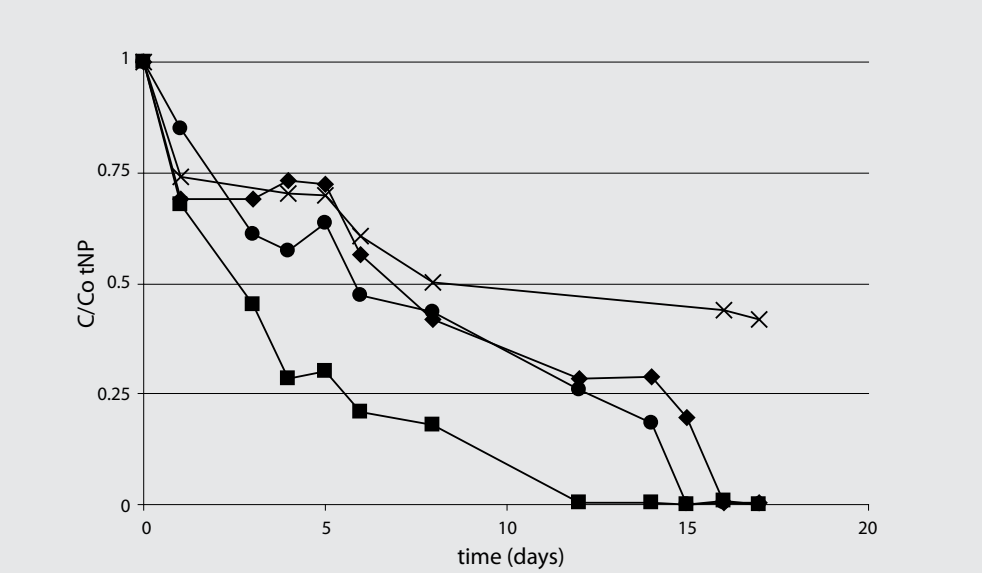

Figure 4.3: Degradation of branched NP (tNP) as C/C0 in 5th generation enrichments from Huerva River sediment, Spain. In triplicate, ■ = A.5, ● = B.5, ◆ = C.5 and X = sterile control

This decrease was due to other processes than degradation e.g. adsorption to the glassware and the viton stopper. A faster decrease in tNP concentration in the active batches and compared to the decrease in the sterile control was observed as soon as the biodegradation of tNP started. In the 5th generation batch A.5, the biodegradation started after 1 day, and within less than 12 days 3 µM tNP was degraded. The biodegradation in the 5th generation batches B.5 and C.5 started after 5 days of incubation, and 4 µM tNP was biodegraded within 10 and 11 days, respectively. The half life time of tNP in these enrichments varied from 4 to 5 d, although the data are limited for calculating the half life times. Enrichment enlarged the lag phase of the biodegradation in the 5th generation compared to the lag phase of the first generation enrichment with medium and sediment and also the half life times of tNP increased.

4.3.2 Metabolite formation

Addition of pure tNP at a concentration of 1 mM (above saturation) to biodegradation batches resulted in the formation of metabolites as seen in the GC-MS chromatogram (Figure 4.4). The metabolites were not present at the start of the experiment and were not observed in the sterile control indicating that these metabolites were formed during the degradation of NP. The peak pattern of tNP in the GC-MS chromatogram was seen between retention times 7.5 to 8.5 minutes (Figure 4.4).

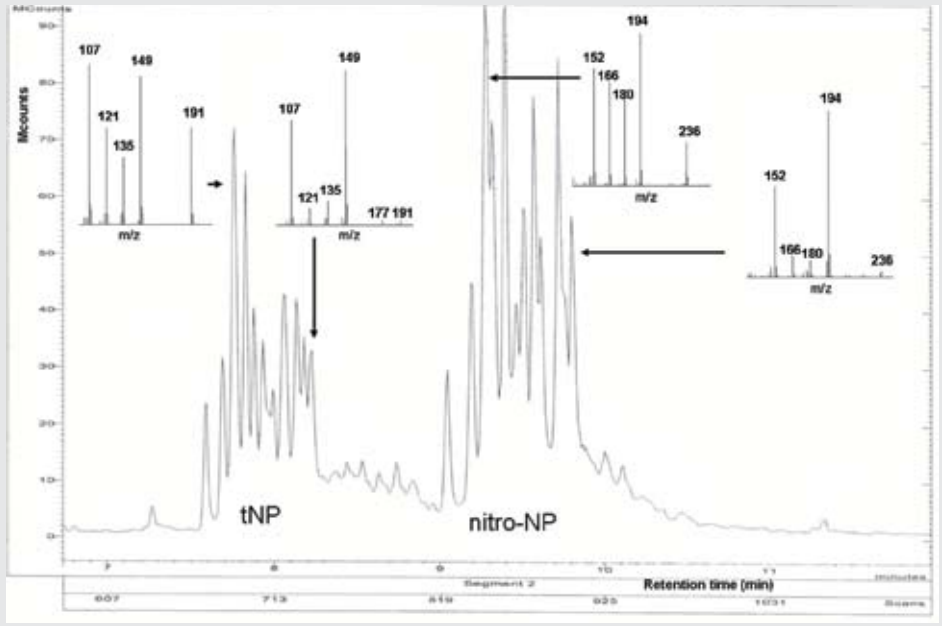

Figure 4.4: GC-MS chromatogram of tNP and nitro-nonylphenol metabolite, including mass spectra.

The pattern consists of several peaks, since tNP is a mixture of branched NP isomers. The peak pattern of the metabolites was seen between 9 to 10 minutes, and showed the same peak pattern as the tNP. The fragmentation pattern of tNP contains ions of m/z 107, 121, 135, 149 and 191 whereas the fragmentation patterns of the metabolites showed ions of m/z 152, 166, 180, 194 and 236. This shift in mass weight of 45 m/z indicated that an extra group was attached to the tNP. The same mass shift was observed in the batches growing on 4-*n*-NP. The fragmentation pattern of 4-*n*-NP contained ions of m/z 107 and 220 whereas the fragmentation pattern of the metabolite showed ions of m/z 152 and 265.

The similarity of the chromatogram pattern of the tNP and the metabolite, the mass shift of 45 m/z, and the typical ions of m/z 152, 166, 180, 194 and 236 with even mass weights indicated the substitution of a hydrogen atom by a nitro-group at the ring (Posthumus, 2008). This was confirmed by the results of the accurate mass weight analysis in extract of the sample of the 4-*n*-NP degradation. The observed mass spectrum contained m/z 43 (11% relative intensity), 57(9), 106(8), 135(11), 152(100) and 265(56), which was in agreement with a 2- or 3-nitro-4-nonylphenol. Nuclear Magnetic Resonance (NMR) analysis could not be performed to identify the position of the nitro-group in the molecular structure, as the concentration of the metabolite in the extract was too low.

Incubation of enrichments grown with tNP above the maximum solubility and labelled $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$ medium was performed to investigate the origin of the nitro-group. The nitro-group attached to the NP in the batches with labelled $^{15}NH_4NO_3$ gave a shift in mass weight of 46 m/z while the formed metabolite in the incubates with labelled $NH_4{}^{15}NO_3$ showed the earlier observed shift in mass weight of 45 m/z. This demonstrates that the attached nitro-group in the metabolites originated from the ammonium in the medium. Enrichments with nonylphenol and nitrite under abiotic conditions did not show the formation of nitro-nonylphenol (data not shown).

The nitro-nonylphenol metabolites accumulated in the enrichments with tNP concentrations above the maximum water solubility. Nitro-nonylphenol was also detected in the 6th generation enrichments A.6, B.6 and C.6 cultured with only 11µM tNP. Figure 4.5 shows this formation in enrichment A.6, before the addition of 1 mM tNP (above saturation) at t=15 and of batch B.6.

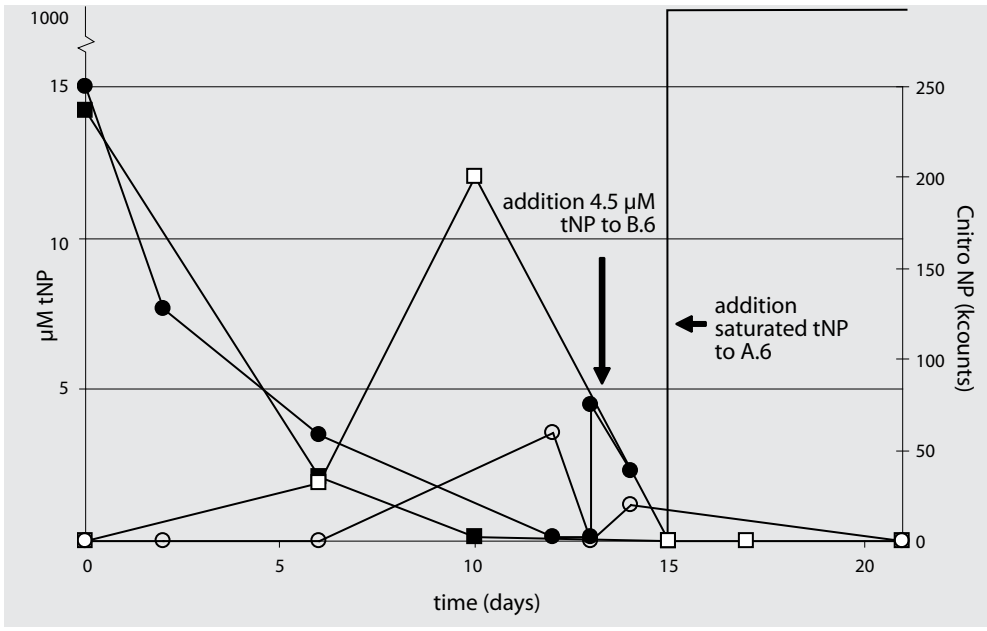

Figure 4.5: Concentration profiles during branched NP (tNP) degradation in a batch system of the 6th generation enrichment. tNP in µM (tNP; left Y-axis, filled symbols) and nitro-nonylphenol in kcounts (right Y-axis, open symbols). ■ and □ = A.6, ● and ○ = B.6

Nitro-nonylphenol was produced during the first 10 days in enrichment A.6, followed by a decrease in concentration indicating that the metabolite was further degraded. After the addition of 1 mM tNP to this enrichment, no nitro-nonylphenol metabolites were measured

in this enrichment. In enrichment B.6, nitro-nonylphenol was produced between the 6th and 12th day of incubation. At t=13, the concentrations of tNP and the nitro-nonylphenol decreased below the detection limit (18 nM). After re-addition of 4.5 µM tNP at t=13 an increase of nitro-nonylphenol was observed again at t=14 followed by a decrease, again indicating the biodegradability of the formed nitro-nonylphenol.

4.3.3 Microbial diversity of the enrichments

The effect of the presence of 11µM tNP or tNP in a concentration above the maximum solubility on the microbial population was analyzed by DGGE. The band patterns in the DGGE of the four DNA samples at different times during the incubation from the 6th generation enrichment A.6 contained 15 to 18 DGGE bands (Figure 4.6). The Shannon-Weaver diversity index obtained from enrichment DGGE profiles varied between 1.09 and 1.10, indicating that the diversity did not change during the incubation.

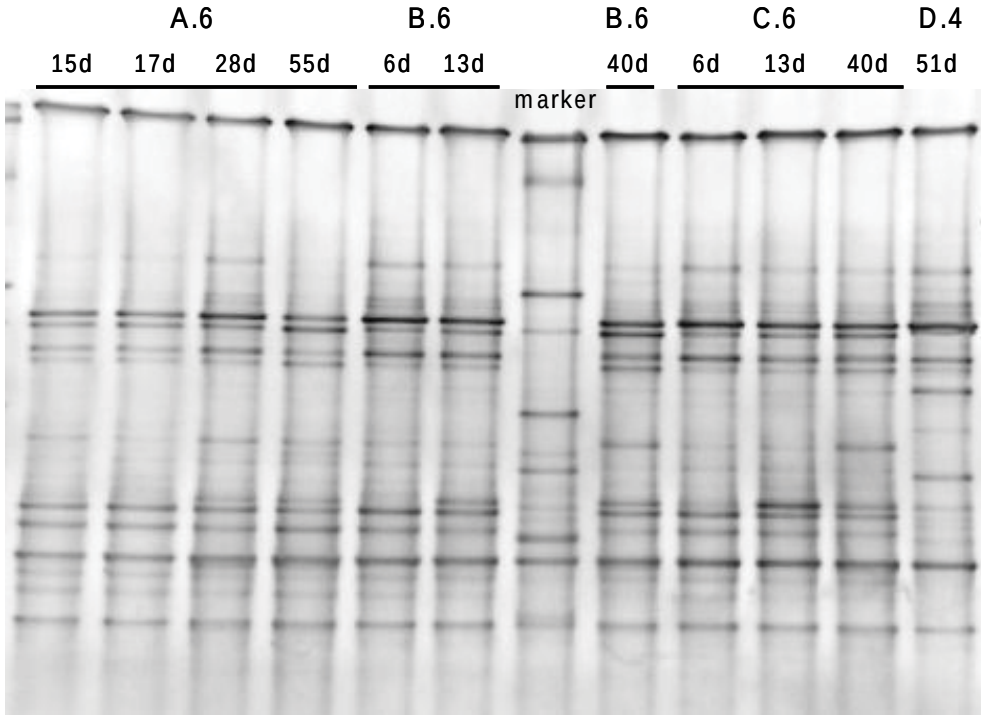

Figure 4.6: Denaturing gradient gel electrophoresis of 6th generation enrichments A.6 cultured with 11 µM tNP till t=15 d and there after with 1 mM tNP, B.6 and C.6 incubated with 11µM tNP and 4th generation enrichment D.4 incubated with 1 mM tNP at different time intervals.

Also no change in diversity was observed after the addition of a tNP concentration above the maximum solubility at day 15, and the population remained stable. The DGGE patterns of the samples at different times of the 6th generation enrichments B.6 and C.6, cultured with only 11µM NP, contained both 16 to 18 bands and looked similar to the patterns of the samples of enrichment A.6. The Shannon-Weaver diversity index for the enrichments B.6 and C.6 ranged from 1.08 to 1.12 respectively. This indicated that the microbial diversity of enric hments B.6 and C.6 did not change either during the incubation of 40 days. This was confirmed by principal component analysis (PCA) (Figure 4.7). In fact, the DGGE profiles of the enrichments A.6, B.6 and C.6 had almost the same PC1 and PC2 value in the scatter plot. During the incubation of enrichments A.6, B.6 and C.6 no changes were observed in the PC1 and PC2, indicating an only small change in the microbial diversity.

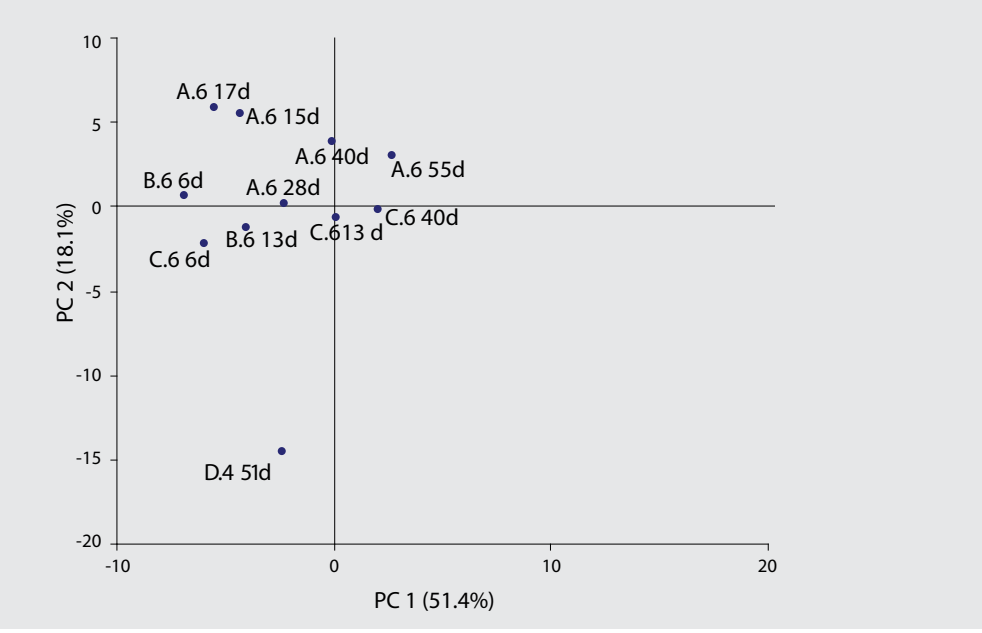

Figure 4.7: Principle component analysis scatter plot of DGGE profiles (Figure 6) of 6th generation enrichments A.6 cultured with 11 µM tNP till t=15 d and there after with 1 mM tNP, B.6 and C.6 incubated with 11µM tNP and 4th generation enrichment D.4 incubated with 1 mM tNP at different time intervals.

The DGGE pattern of the sample of enrichment D.4, which is a 4th generation transfer cultured with 1 mM tNP (above saturation), contained 14 bands. The Shannon-Weaver index for microbial diversity of sample of D.4 was 1.05. This indicates that the microbial diversity in sample D.4 was less than in the samples of enrichments A.6 to C.6. The pattern showed similarities with the patterns of the samples of enrichments A.6 to C.6, but there were also some different dominating bands present. The PCA results of the DGGE profiles (Figure 4.7) show that the data point of sample D.4 did not cluster with the results of enrichments A.6, B.6 and C.6. This indicates that incubation with a tNP concentration

above maximum solubility gives a different microbial population compared to incubation with 11 µM tNP concentration.

4.3.4 Microbial population composition of the enrichments

As the microbial composition of A.6 did not change during the incubation time and was similar to enrichments B.6 and C.6, a clone library was made of enrichment A.6. The DNA extract from t=15 days was used when nitro-nonylphenol was formed. A second clone library was made from enrichment D.4 at t=55 days which showed a different microbial composition on the DGGE pattern. Enrichment D.4 was cultured with 1 mM NP whereas A.6, B.6 and C.6 were cultured with 15 uM tNP. Respectively, 70 and 49 clones were obtained from enrichments A.6 and D.4 (Table 4.1). The most dominant clones of A.6 were related to *Nitrosomonas* genus (36 out of 70 clones), which are ammonium oxidizing bacteria (Stein et al., 2007). Of these 36 clones, 18 were closely related to *Nitrosomonas euthropha* (GenBank accession number AY123795) with a maximum 16S rRNA gene identity of 91-100%. Another 16 clones were closely related to *Nitrosomonas euthropha* C91 with a maximum identity of 99%. Alignment of the sequences of *Nitrosomonas euthropha* (GenBank accession number AY123795) and *Nitrosomonas euthropha* C91 showed a 99% similarity between these two strains. In the clone library of enrichment D.4, clones were also identified that are related to *Nitrosomonas euthropha* (2 out of 49) and *Nitrosomonas euthropha* C91 (4 out of 49), however they were less abundant present than in the clone library of enrichment A.6. Three clones were related to *Nitrosomonas* sp. GC22 with a similarity of 96-99%.

Clones related the sequence of *Beta proteobacterium* C14 JRPA-2007 (8 out of 70 clones) were also dominant present in the clone library of enrichment A6. *Beta Proteobacterium* C14 JRPA -2007 sequence belongs to the *Alcaligenacea* family and is closely related to Alcaligenes sp. Ic4, a 3-chlorobenzoate degrading micro organism (Krooneman et al., 1996). Clones (4 out of 49 clones) related to *Beta proteobacterium* C14 JRPA-2007 were also present in de clone library of enrichment D.4.

The most dominating clones in the clone library from enrichment D.4 belonged to the family *Comamonadacea* (28 out of 49 clones). Thirteen clones were closely related to Acidovorax sp. PD-10 (92-99% similarity) and 11 clones to *Alicycliphillus* sp. R-24604 (96-99% similarity). *Acidovorax* sp. PD-10 is closely related to the species *Acidovorax avenae* isolate C1 (AF508114), which is a phenol-degrading, nitrate-reducing bacterium (Baek et al., 2003).

Table 4.1: Phylogenetic affiliations and frequencies of bacterial 16S rRNA gene clones in clone library of 6th generation enrichment cultured with 11µM branched nonylphenol (A.6) and the 4th generation enrichment cultured with a NP concentration above maximum water solubility) (D.4).

Phylogenetic group	Closest related organism GenBank (accession no.)	Accession no.	% similarity	no. of clones	% similarity	no. of clones
Alcaligenaceae (ß)	Beta proteobacterium C14 JRPA-2007 (EF599312)	FJ609349-FJ609350, FJ609379-FJ609381, FJ626785, FJ626786	97-99	8	95-99	4
Bradyrhizobiaceae; Bradyrhizobium (α)	*Bradyrhizobium japonicum* strain HMJ-03 (EU481826)	FJ609338-FJ609339	98-99	3	--	--
	Bradyrhizobium sp. MOB-4 (EF681762)	FJ609336-FJ609337	99	3	--	--
Bradyrhizobiaceae; Afipia (α)	*Afipia felis* (AF338177)	FJ609333-FJ609335	96-99	4	--	--
Bradyrhizobiaceae; Oligotropha (α)	Uncultured bacterium clone RO229 (AB099660)	FJ609351	95	1	--	--
Comamonadaceae (ß)	*Acidovorax* sp. PD-10 (AB195159)	FJ609345-FJ609348, FJ609356-FJ609363, FJ626783	96-99	4	92-99	10
Comamonadaceae; Alicycliphillus (ß)	*Alicycliphilus* sp. R-24604 (AM084015)	FJ609353-FJ609354 FJ626790-FJ626794, FJ626787	97-99	2	95-99	11
	Uncultured bacterium clone 181up (AY212633)	FJ609384-FJ609385, FJ626783, FJ626784	--	--	96-97	2
	Uncultured bacterium clone EC2-2 (EU741793)	FJ609369-FJ609370	--	--	94-99	2
Micrococcaceae (Acetinobacter)	Uncultured bacterium clone M40C11 (EU331390)	FJ609352	91	1	--	--
Nitrosomonadaceae (ß)	*Nitrosomonas eutropha* (AY123795)	FJ591139-FJ591150, FJ609364-FJ607368	91-100	18	93-99	2
	Nitrosomonas eutropha C91 (CP000450)	FJ609322-FJ609331 FJ609371-FJ609373	99	16	98-99	4
	Nitrosomonas sp. GH22 (AB000701)	FJ609321	94	1	96-99	3
	Nitrosomonas sp. clone 74 (AJ224410)	FJ609332	90	1	--	--

Continue Table 4.1

Phylogenetic group	Closest related organism GenBank (accession no.)	Accession no.	% similarity	no. of clones	% similarity	no. of clones
Rhodocyclaceae (ß)	Uncultured *Azoarcus* sp. (AY098637)	FJ609355	--	--	97	1
Rhodocyclaceae; Thauera (ß)	Uncultured bacterium clone 189up (AY212641)	FJ609374	--	--	95	2
	Thauera sp. R-26885 (AM084104)	FJ609375	--	--	97	1
	Uncultured bacterium clone: nsmpV06 (AB210048)	FJ609376	--	--	96	1
Rhodospirillaceae (α)	Uncultured alpha proteobacterium clone MKC26 (EF173357)	FJ609341-FJ609343	91-98	3	--	--
	Uncultured *Rhodospirillaceae* bacterium clone Amb_16S_1106 (EF018478)	FJ609344	93	1	--	--
Xanthomonadaceae (γ)	Gamma proteobacterium RIB 1-20 (EF626688)	FJ609377	--	--	99	2
	Uncultured bacterium clone 010B-A05 (AY661995)	FJ609382	--	--	92	1
	Luteimonas sp. Q-1 (AB246805)	FJ609383	--	--	96	1
	Luteimonas sp. CHNTR31 (DQ337595)	FJ626789	--	--	98	1
	Uncultured beta proteobacterium clone 100M2_E5 (DQ514071)	FJ609378	--	--	92	1
Xanthomonadaceae; Cibimonas (γ)	Uncultured bacterium clone IYF16 (DQ984577)	FJ591135	96	2	--	--
Xanthomonadaceae; Rhodanobacter (γ)	*Rhodanobacter lindaniclasticus*, strain: Gsoil 3028 (AB245366)	FJ591137	100	1	--	--
	Uncultured bacterium clone 2B25 (EU528240)	FJ591136	92	1	--	--
Total Clones				**70**		**49**

Alignment of the sequences of *Alicycliphillus* sp. R-24604 (AM084015) and *Acidovorax avenae* isolate C1 (AB195159) gave 99% similarity of these two strains. This indicates that *Alicycliphillus* sp. R-24604 is very closely related to *Acidovorax avenae* isolate C1 as well. Clones related to *Acidovorax* sp. PD-10 and *Alicycliphillus* sp. R-24604 were also identified in the clone library of enrichment A.6, and were less dominantly present than in the clone library of enrichment D.4.

4.4 Discussion and conclusions

The biodegradation experiments with the tNP polluted sediment showed that microorganisms in the used sediment are able to transform tNP under aerobic conditions. Comparing our results with an aerobic biodegradation study with sediment from the Erren River in China, the biodegradation of NP in our sediment was much faster (Yuan et al., 2004). Within 8 days approximately 4.5 µM NP was biodegraded whereas biodegradation of 9 nM in Erren River sediment took 84 days. Adaptation of the Erren sediment increased the biodegradation to almost complete biodegradation within 28 days. A possible reason for the faster biodegradation in our sediment is the adaptation of the bacteria to NP. Our sediment was already polluted with NP, and the biodegradation started after a short lag phase of 2 days. The sediment of the Erren River was originally not polluted with NP, resulting in a low biodegradation rate of NP. After adaptation of the microorganisms in the batches, a faster biodegradation was observed, but not as fast as in our sediment. The presence of NP degrading microorganisms and the short lag phase in our sediment is favourable for biodegradation of tNP and thus reducing the toxicological risk of tNP in the sediment. This will only occur when the conditions for biodegradation are appropriate, like the presence of oxygen.

Enrichment of the microbial population of our sediment enlarged the lag phase compared to the first generation enrichment, and increased the half life time of tNP. Normally, a transfer results in a decrease in the lag phase, an increase in the degradation rate, and thus a decrease in the half life time. The microbial population in the enrichments originated from sediment, but the 5th generation enrichment did hardly contain any sediment particles due to the transfers. Possibly, the tNP degrading microorganisms used the sediment particles as a carrier material and were mainly present on the solid phase. By transferring the population into fresh medium, the sediments particles were diluted, resulting in less carrier material and less bacteria in the next generation.

During the degradation of tNP in our enrichments, a nitro-nonylphenol metabolite was formed by the substitution of an H-group at the ring by a nitro-group. We could not distinguish whether the nitro-group was attached to the *ortho*- or *meta*-position of the phenol group as not sufficient metabolite was formed for NMR-analyses. In other biodegradation studies of tNP, the formation of nitro-nonylphenol was also observed (Telscher et al., 2005; Zhang et al., 2009). In these studies the nitro-group was attached to the *ortho*-position of the aromatic ring. Besides the formation of nitro-nonylphenol during the NP degradation, we observed that the nitro-nonylphenol is degraded further.

The biodegradation of a tNP isomer in agricultural soils via the formation and further biodegradation of the nitro-nonylphenol metabolite was also observed (Zhang et al., 2009). The formed nitro-nonylphenol was not completely degraded, 13% of the initial applied NP remained in the sediment as a nitro-metabolite. In our experiments the nitro-nonylphenol was only completely degraded when 11 µM tNP was applied. Higher concentrations (1 mM) led to accumulation of the nitro-metabolites.

The biodegradation of tNP in the presence of 12.5 mM ^{15}N-labelled NH_4NO_3 showed that the attached NO_2-group in the formed nitro-nonylphenol originates from ammonium. A chemical reaction between nonylphenol and added nitrite (12.5 mM), resulting in the formation of nitro-phenol, was not observed (data not shown). This indicates that the formation of nitro-nonylphenol is a biological process. This process is relevant in the biodegradation of tNP, because the formation of nitro-nonylphenol occurred in our sediment and is also observed in two different agricultural soils (Telscher et al., 2005; Zhang et al., 2009).

Sequences closely related to *Nitrosomonas eutropha* and *Nitrosomonas eutropha* C91 strains were detected in the clone library of both enrichments. *Nitrosomonas eutropha* and *Nitrosomonas eutropha* C91 are ammonium oxidizing bacteria. Probably, these organisms were responsible for oxidation of the ammonium to NO_2-, followed by the attachment of the NO_2- group to the aromatic ring of NP. The sequences of N*itrosomonas eutropha* C91 is closely related to the sequence of *Nitrosomonas europaea* (Koops et al., 1991). *Nitrosomonas europaea* is a strain that oxidizes non substituted alkanes (Hyman et al., 1988), and which is able to co-oxidize aromatic compounds in the presence of ammonium (Keener and Arp, 1994). In our enrichments, NP with branched alkyl chains was degraded, and a nitro-group was attached to the aromatic ring, followed by further degradation of the nitro-nonylphenol. Evidence for ring fission of aromatic compounds by *N. europaea* has not been described, indicating that these organisms are not involved in the degradation of the aromatic ring in our experiments. However, it is suggested that this strain may initiate the degradation of aromatic compounds to provide products which can be used or transformed by other bacteria (Keener and Arp, 1994). If *Nitrosomonas* species form the nitro-nonylphenol, and are not able to cleave the aromatic ring structure, other species must be involved in further biodegradation of the *nitro-nonylphenol*. Both clone libraries contain sequences of species related to *Beta proteobacterium* C14 JRPA-2007 (95-99% similarity). *Beta proteobacterium* C14 JRPA-2007 belongs to the *Alcaligenes genus*, and this *Alcaligenes* genus contains species able to degrade *ortho*-nitrophenol (Xiao et al., 2007). Other strains are not identified in both enrichments, which can be related to nitro-phenol degradation. Therefore, it is possible that the strains related to these *Beta proteobacterium* C14 JRPA-2007 are responsible for further biodegradation of the formed nitro-nonylphenol.

Besides species related to ammonium oxidizing and nitrophenol degrading microorganisms, both clone libraries of the enrichments with 11 µM tNP and 1mM (saturated) tNP contain sequences related to *Acidovorax* sp. PD-10 and *Alicycliphillus* sp. R24604. These two species are abundantly present, particularly in the enrichment with 1mM tNP. Both species are closely related to *Acidovorax avenae* isolate C1, a nitrate reducing, phenol-degrading bacterium (Baek et al., 2003). Biodegradation of phenol by *Acidovorax avenae* isolate C1 is suggested to occur via cleavage of the ring structure (Baek et al., 2003). Although clones in both enrichments are related to these denitrifying strains, that reduce NO_3^- to NO_2^-, this process is not likely to occur, because of the aerobic conditions in our enrichments. Furthermore, the results showed that the NO_2-group in the formed nitro-nonylphenol originate from ammonium instead of nitrate. Biodegradation of NP via cleavage of the ring structure like suggested for phenol by *Acidovorax avenae* isolate C1 was proposed for NP degradation by *Pseudomonas* species (Soares et al., 2003). The presence of species related to the phenol-degrading *Acidovorax avenae* isolate C1 after several enrichments, and the presence of NP as only carbon source suggests that the biodegradation of NP occurs via the phenolic-degradation pathway. Unfortunately, we have no direct evidence for this pathway and this should be further investigated.

Sequences related to *Sphingomonas* and *Sphingobium* species, which degrade NP via *ipso*-hydoxylation were not identified in our enrichments. We did not observe a shift in the isomeric composition of the mixture of tNP isomers, which occurs when the tNP is degraded by the Sphingomonas and Sphingobium species via *ipso*-hydroxylation due to different degradation rates of the various isomers (Gabriel et al., 2005a; Kohler et al., 2008). The absence of strains involved in the NP degradation via *ipso*-hydroxylation and absence of the shift in isomeric composition gives a strong indication that biodegradation of NP via *ipso*-hydroxylation in our enrichments could not be dominant.

The microorganisms identified in this study are not related to known NP-degrading bacteria such as *Sphingomonas, Sphingobium, Stenotrophomonas* or *Pseudomonas* species (Gabriel et al., 2005a; Soares et al., 2003; Tanghe et al., 1999; Ushiba et al., 2003). This indicates that aerobic NP degradation can be performed by a wide range of microbial species and via different degradation pathways. The involvement of a wide range of microorganisms related to NP biodegradation enlarges the possible risk reduction of NP in the environment by biodegradation under aerobic conditions. The toxicological effect is also reduced when degradation of NP occurs via the formation of nitro-nonylphenol, even when this metabolite is not completely degraded. The nitro-nonylphenol metabolites are less toxic (Kammann et al., 2009) and have an estrogenic activity of 85% of that of the parent compounds (Zhang et al., 2009).

In conclusion, this study shows that tNP in our polluted sediment is degraded in the presence of oxygen. Degradation in the enrichment cultures occurred via the formation of nitro-nonylphenol. The identified microorganisms in the enrichments are different from other tNP degrading microorganisms described so far. Up to date, this is the first study that describes both a nitro-nonylphenol metabolite formation and the putative microbial diversity related on tNP degradation.

As nonylphenol can be degraded aerobically by a wide range of microorganisms, this enlarges the possibility for risk reduction of tNP in the environment by biodegradation

under aerobic conditions. Although more insight has been obtained by this study, further investigations into the aerobic biodegradation of NP in this sediment are needed to elucidate the degradation pathways, to obtain more information about the specific involved microorganisms and their role in the biodegradation process, and to identify important environmental parameters that control these processes. This information can help us to better predict the fate and effects of NP in aquatic systems, and to design bioremediation measures for specific field situations.

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5 Bioavailability and biodegradation of nonylphenol in sediment determined with chemical and bioanalysis

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Abstract

The surfactant nonylphenol (NP) is an endocrine disrupting compound that is wide spread throughout the environment. Although environmental risk assessments are based on total NP concentrations, only the bioavailable fraction posses an environmental risk. The present study describes the bioavailability and biodegradability of NP over time in contaminated river sediment of a tributary of the Ebro River in Spain. The bioavailable fraction was collected with Tenax TA® beads, and biodegradation was determined in aerobic batch experiments. The presence of NP was analyzed chemically using gas chromatography-mass spectrometry and indirectly as estrogenic potency using an in vitro reporter gene assay (ER_α-*luc* assay). Of the total extractable NP in the sediment, 95% ± 1.5% (mean ± standard error) desorbed quickly into the water phase. By aerobic biodegradation, the total extractable NP concentration and the estrogenic activity were reduced with 97% ± 0.5% and 94% ± 2%, respectively. The easily biodegradable fraction equals the potential bioavailable fraction. Only 43 to 86% of the estrogenic activity in the total extractable fraction, as detected in the ER_α-*luc* assay, could be explained by the present NP concentration. This indicates other estrogenic compounds were present and that their bioavailability and aerobic degradation were similar to that of NP. Therefore, we propose to use NP as an indicator compound to monitor estrogenicity of this Ebro River sediment. To what extent this conclusion holds for other river sediments depends on the composition of the contaminants and/or nature of these sediments and requires further testing.

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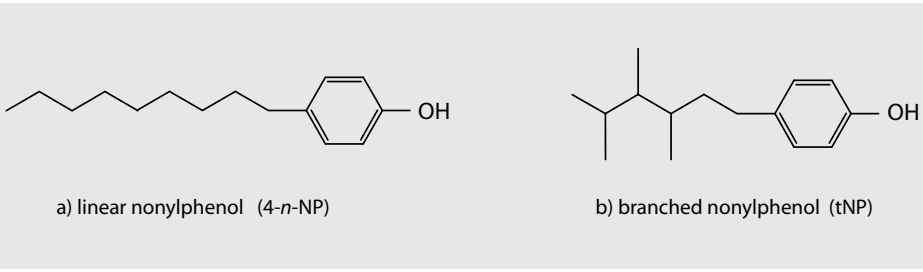
5.1 Introduction

Many (xeno)estrogenic compounds enter the aquatic environment through discharges of domestic and industrial waste water treatment facilities, sewage sludge disposals and runoff from agricultural land fertilized with manure (Mes et al., 2005; Topp and Starratt, 2000; Tyler et al., 1998). These compounds can accumulate in sediment organic matter largely because of their high hydrophobicity (Reid and Semple, 2000).

The present study focuses on the estrogenic compound nonylphenol (NP). Nonylphenol is a man made product that is used mostly as intermediate in the production of nonylphenol ethoxylates, which are used in cosmetics and in household and cleaning products as a surfactant. Nonylphenol itself is also used in the production of resins, chemical stabilizers and antioxidants. During anaerobic degradation of nonylphenol ethoxylates in waste water treatment plants, NP is formed as a persistent intermediate in sewage sludge (Giger et al., 1984; Stephanou and Giger, 1982). Nonylphenol is a mixture of isomers with a hydrophilic phenol group and a linear or branched carbon chain with nine carbon atoms (Figure 5.1).



a) linear nonylphenol (4-*n*-NP) b) branched nonylphenol (tNP)

Figure 5.1: Molecular structure of (a) linear nonylphenol and (b) branched nonylphenol isomer.

Mainly branched NP isomers are found as pollutants in the environment, because these are used commercially and are formed as a product of anaerobic degradation processes of nonylphenol ethoxylates (Kravetz, 1983; Sturm, 1973). Nonylphenol is an endocrine disruptor (Nimrod and Benson, 1996) that can cause endocrine effects in fish, leading to modification of development and reproduction (Ashfield et al., 1998; Tremblay and Kraak, 1998; Tyler et al., 1998). Endocrine effects can be induced by NP in frogs (Yang et al., 2005) and daphnids (LeBlanc et al., 1999) as well.

Nonylphenol has shown to accumulate up concentrations of 22 to 2,230 µg/kg dry weight in sediments of the Ebro River and its tributaries as well as in other rivers in Spain (Lacorte et al., 2006; Petrovic et al., 2002). Such sediments can function as a secondary contamination source for the aquatic ecosystem because part of the sediment-bound fraction of NP can desorb and dissolve into the water phase again, becoming more bioavailable for gill-breathing organisms. Bioavailability has been used in the literature in different ways (Alexander, 2000; Reichenberg and Mayer, 2006; Reid and Semple, 2000).

We define the bioavailable fraction of a chemical in a sediment as that fraction that can be taken up or transformed by (micro)organisms via the water phase (Semple et al., 2003). In sediments polluted with compounds such as hexachlorocyclohexane and polycyclic aromatic hydrocarbons (PAH), the biodegradable fraction equals the bioavailable fraction given optimal biodegradation conditions (Cuypers et al., 2002; Smit et al., 2005) for non persistent hydrophobic organic compounds. Bioavailability of a compound can be related to its hydrophobicity expressed as the log octanol-water partition coefficient (K_{OW}) (Reid and Semple, 2000). The amount and type of organic matter play an important role as well. The partitioning of a compound to organic carbon is expressed by the log organic carbon partition coefficient (K_{OC}) (Burgess et al., 2005; Kleineidam et al., 1999). Nonylphenol has a log K_{OW} value of 4.48 (Ahel and Giger, 1993) and the log K_{OC} value ranges from 4.7 to 6.1 (Burgess et al., 2005; Heemken et al., 2001; Isobe et al., 2001).

From older sediments with historical contamination of hydrophobic compounds, the bioavailability of the compounds decreases with time, which in turn decreases the associated environmental risk. This aging of the sediment, as reviewed by et al. (Semple et al., 2003), has been shown for compounds including phenanthrene, 4-nitrophenol, DDT and atrazine (Alexander, 2000; Chung and Alexander, 1998; Hatzinger and Alexander, 1995; Park et al., 2004). So far, bioavailability of NP has been studied in freshly spiked organic materials like humic substances (Vinken et al., 2004) and cellulose (Burgess et al., 2005), using a short contact time (30 and 48 h respectively). To our knowledge, no bioavailability studies have been performed with sediment and aged NP pollution. The results of short-term experiments with freshly spiked sediment are expected to overestimate the bioavailable fraction and, therefore, overestimate the associated environmental risk in field conditions.

The bioavailable fraction of NP can be reduced in an aquatic ecosystem by microbial biodegradation, reducing the toxicological risk. Nonylphenol is easily degradable under aerobic conditions but is persistent under anaerobic conditions, as reviewed by Corvini et al. (Corvini et al., 2006). Because sediments are often anoxic whereas surface waters are aerobic, insight regarding biodegradation of NP in field samples of sediments is needed to improve the environmental risk assessment for aquatic ecosystems. To our knowledge, however, no data are available regarding biodegradability and bioavailability of NP in aged sediments.

Nonylphenol pollution in the sediment consists of several NP isomers with varying estrogenic potencies (Preuss et al., 2006). Not all single NP isomers and other estrogenic compounds, which are likely are present in the sediment as well, are detectable with current analytical techniques. Although the individual contribution of single compounds can be low, a mixture of these compounds can result in significant estrogenicity (Rajapakse et al., 2002). The total estrogenic activity of these mixtures can be quantified by using an in vitro bioassay.

The present study aims at determination of the bioavailability of NP from aged NP contaminated sediment and its biodegradation. The total and bioavailable fraction of NP

is determined via chemical analysis of NP-isomers and an in vitro reporter gene assay (ER_α-*luc* assay) is applied for estrogen receptor activation to quantify the total estrogenic activity in the samples.

5.2 Materials and methods

5.2.1 Chemicals and materials

Petroleum ether (40-60ºC, ≥90% purity) and acetone of high-performance liquid chromatography quality were from Merck (Darmstadt, Germany); Tenax TA® porous polymer based on 2,6-diphenyl-p-phenylene oxide (20-35 mesh) was from Buchem BV (Apeldoorn, The Netherlands). The NP technical mixture for calibration standards (purity, > 94%) was from Riedel de Haën (Seelze, Germany). Dimethyl sulfoxide (DMSO) was from Across (Landsmeer, The Netherlands). Other used chemicals were of the highest purity (> 99%).

All used glassware and microwave tubes were pre cleaned by washing three times with acetone and two times with petroleum ether.

5.2.2 Sediment

The sediment was collected during June 2005 from the Spanish Huerva River in Zaragoza (41º37'23"N, 0º54'28"W), which is a tributary of the Ebro River (Spain). Several kilograms were taken with a stainless-steel core and then stored on ice for transport. In the laboratory, the sediment was sieved (mesh size < 2 mm) and divided over two sealed glass pots (sample 1 and sample 2). The samples were stored at 4ºC. Sample 2 was used four months later than sample 1. Before the use of the samples, the dry weight, the organic matter content, and the total extractable NP concentration were measured (Table 5.1). The samples differ in dry weight (sample 1, 39% ; sample 2, 46%).

*Table 5.1: Characteristics of the sediment samples**

Parameters	Sample 1	Sample 2
Dry weight (%)	39 ± 0.1	46 ± 0.3
Organic matter (%)	8.4 ± 0.4	8.0 ± 0.3
Nonylphenol concentration (mg.kg^{-1} dry weight)	13.1 ± 0.8	13.6 ± 1.8

** Values are presented as the mean ± standard error*

5.2.3 Bioavailability experiments

The bioavailable fraction of NP and other estrogens from the sediment was extracted with Tenax, based on the method described by Cornelissen et al. (Cornelissen et al., 2001). The suggested extraction time of 6 hours in this method was changed into a long extraction time of 432 hours (18 days) to obtain insight regarding the total bioavailable fraction of NP and other estrogenic compounds. The equivalent of 2 g of dry sediment was added to a precleaned separatory funnel with 40 ml aerobic medium as described in Tros et al. (Tros et al., 1996), but $Na_2SeO_3.5H_2O$ was excluded. To prevent biodegradation during the Tenax extraction, 0.5 ml $HgCl_2$ (10 g·l^{-1}) was added to the funnels. Finally, 1.5 g Tenax was added and the funnels were shaken horizontally at 110 strokes per minute at 30ºC. Tenax was refreshed after 1, 2, 3, 5, 24, 72 and 288 h by transferring the sediment and medium to a funnel with fresh Tenax. After 18 days, the Tenax extraction was finished. The Tenax extractions were performed in triplicate and compared to a control without sediment.

5.2.4 Aerobic biodegradation experiments

Aerobic degradation of NP in the sediment was performed in 250-ml serum bottles with 5 g of wet sediment and 35 ml of the aerobic medium. The bottles were closed using a viton stopper with crimp-cap seal and incubated in the same way as the funnels for Tenax extraction. The content of the bottles with sediment from sample 1 were each transferred after 16 days to a 1-l bottle with a screw cap and a viton inlayer to have enough oxygen to keep the batches aerobic during further incubation. The aerobic degradation experiments were performed in triplicate. To follow the NP degradation, the bottles were sampled in duplicate over time. Per sample, 100 µl of slurry was taken from the bottles and added to a capped 20-ml headspace vial with 1.9 ml Milli-Q® (Millipore BV, Amsterdam, the Netherlands) with 7 mg·l^{-1} of $HgCl_2$. The samples were measured by gas chromatography – mass pectrometry (GC-MS) with solid phase micro extraction injections.

5.2.5 Exhaustive NP extraction from sediment and Tenax

The total extractable fraction of NP and other estrogens from the original untreated sediments and the residual fractions in the sediments after aerobic degradation and/or Tenax extraction were extracted by microwave-assisted solvent extraction (Ethos 1, Milestone, Sorisole, Italy). Of the original sediment, an equivalent of 2 g of dry sediment was placed in a 100-ml Teflon® microwave tube. For the aerobic degradation and/or Tenax extraction, the sediments were first separated from the medium by centrifugation (10 min, 4,000 rpm); thereafter, the pellet was added to a microwave tube. Acetone (15 ml) and petroleum ether (15 ml) were added to all sediments. The sediments were extracted in the microwave at 80ºC for 25 min. Thereafter, the acetone and petroleum ether were separated by shaking the extract with 100 ml water. The petroleum ether was collected and stored in the dark in 20-ml crimp cap vials with a polytetrafluoroethylene/butyl rubber septum. Before filling, the vials and septa were pre cleaned in ethanol. To extract the NP from the Tenax, the Tenax was transferred to the microwave tubes and treated as described above.

The extracts were split into two equal portions, based on weight; one portion was used for GC-MS and the other for estrogenic potency analysis. The samples for GC-MS were concentrated under a gentle flow of nitrogen gas at room temperature if necessary and

stored in the freezer. The samples for the estrogenic potency analysis were concentrated under a gentle flow of nitrogen gas and transferred to a conical vial precleaned with ethanol. The petroleum ether was evaporated completely from the conical vials, and the extracts were re-dissolved in DMSO. The samples in DMSO were stored at room temperature.

5.2.6 Experimental set up

Sediment sample 1 was used to perform aerobic degradation of NP for 183 days (Figure 5.2). The extractable NP concentration of the untreated sediment was determined before the aerobic degradation started and after the aerobic incubation of 183 days. From sediment sample 2, the total extractable NP concentration was determined from the untreated sediment sample as well.

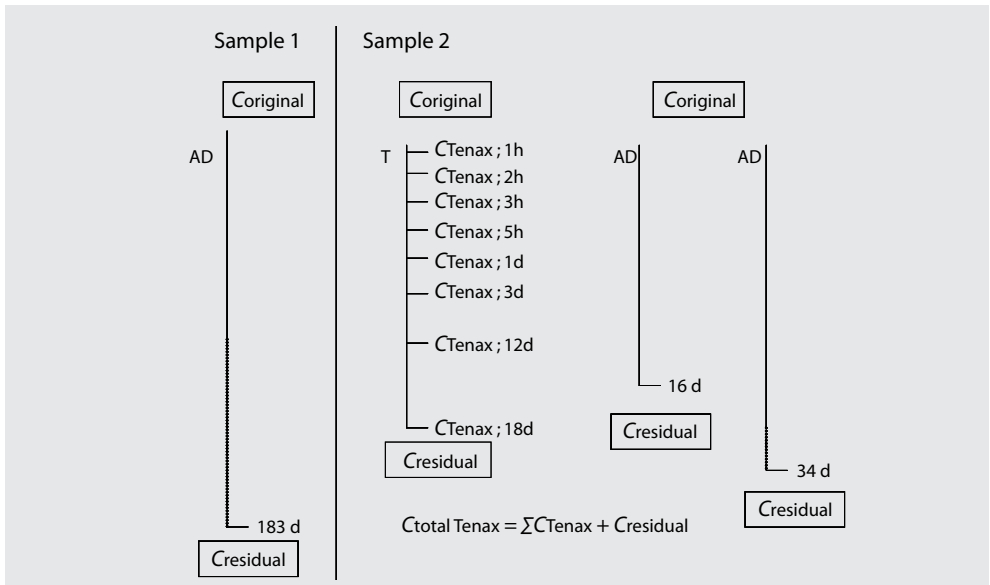


Figure 5.2.Experimental setup to study the bioavailability and aerobic biodegradation of nonylphenol (NP) in two sediment samples. AD = aerobic degradation of NP; $C_{original}$ = microwave assisted extraction of total extractable NP of the untreated original sediment sample; $C_{residual}$ = microwave assisted extraction of the residual NP from the sediment; C_{tenax} = Tenax extraction (T) with eight extraction steps.

Sample 2 was used for the Tenax extraction experiment of 18 days and aerobic biodegradation of 16 and 34 days. After the Tenax extraction or the aerobic incubation, the residual NP concentration also was extracted from the sediment. The total concentration of the Tenax extraction experiment was calculated as the sum of all NP concentrations of the single Tenax extraction steps and the extractable NP concentration of the untreated sediment after the Tenax extraction. The total concentration in the Tenax should be equal to original concentration of sediment sample 2.

5.2.7 GC-MS analysis

An 1-µl amount of petroleum ether extract was injected splitless at 250°C with an Interscience (Breda, the Netherlands) AC 2000 auto sampler on an Interscience Trace GC 2000 gas chromatograph, equipped with an CP-Sil 8 CB low bleed/MS column (length, 50 m; inner diameter, 0.25 mm; film thickness, 0.25 µm; Varian, Middelburg, the Netherlands) and connected to an Interscience Polaris Q 2000 ion-trap mass spectrometer of 300°C. Helium was used as carrier gas with a constant flow of 1 ml·min^{-1}. The initial column oven temperature was held at 40 °C for 4 min. Then the temperature was increased to 300°C with 10°C·min^{-1} and then held at 300 °C for 10 min. The ionization was electron impact at 70 eV, and the detection was full scan.

5.2.8 GC-MS analysis with solid phase micro extraction injection

The samples of the aerobic degradation experiment were analysed by a Varian (Middelburg, the Netherlands) 3800 gas chromatograph equipped with a CP-Sil 8 CB low bleed/MS column (length, 50 m; inner diameter, 0.25 mm, nominal film thickness, 0.25 µm and connected to a Varian Saturn 2000 ion-trap mass spectrometer of 200°C. Samples were extracted with a solid phase micro extraction fiber (polyacrylate 85 µm; Supelco, Bellefonte, PA, USA) from the headspace in the vial for 25 min under stirring conditions at 100°C with a Varian Combi Pal autosampler. The fiber was thermally desorbed (5 min, 300°C) within the GC injection port and injected splitless. The injector pressure was constantly 1.15 bar and helium was used as a carrier gas, with a flow of 1.0 ml·min^{-1}. The initial column oven temperature was set at 80°C for 1 min, after which the temperature was increased to 200°C at a rate of 30°C·min^{-1}, and then maintained for 2 min. Finally, the temperature increased at a rate of 10°C·min^{-1} to 260°C and then held for 10 min. The ionization was electron impact at 70 eV, and the detection was full scan.

5.2.9 Estrogenic activity analysis

The estrogenic activity of the extracts was determined with a reporter gene assay (ER_α-*luc* assay) based on U2OS-ER_α cells, with luciferase as reporter (Quadackers et al., 2001). The method to culture and expose the cells and to measure the luciferase activity was described earlier by Ter Veld et al. (Ter Veld et al., 2006). In short, the cells were seeded in a 96-well plate at a density of approximately 10 x 10^{-4} cells per well in 100 µl of Dulbecco modified Eagle medium-F12 without phenolred (Gibco BRL, Breda, the Netherlands) supplemented with 5% dextran-coated charcoal - fetal calf serum (Perbio Science NV, Etten-Leur, the Netherlands). After 24 h, 50% of the assay medium was replaced by fresh assay medium. Forty-eight hours after seeding, the cells were exposed to the extracts in triplicate, not exceeding a 0.4% DMSO concentration. At every 96-well plate, assay medium controls, DMSO controls and 17ß-estradiol calibrations points (60, 6, and 0.6 pM, respectively) were included. After 24 h of incubation, the medium was removed and the cells were

washed with 100 µl/well 0.5x phosphate-buffered saline, and lysed in 30 µl hypotonic low-salt buffer, pH 7.8 (10mM Tris, 2mM DTT and 2mM 1,2-diaminocyclohexanetetraacetic-acid in nanopure water). Plates were put on ice for 10 min and subsequently frozen at -80°C for at least 30 min. Plates were thawed to room temperature and shaken for 10 min before measurement. Luciferase activity was measured at room temperature in a Luminoskan Ascent luminometer (Thermo Fisher Scientific, Inc. Waltham, MA, USA). After measurement of the background light emission, 100 µl of Flash mix (20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM $EDTA{\cdot}2H_2O$, 2 mM dithiothreitol, 0.47 mM D-luciferin, 5 mM adenosine triphosphate) was added. The light emission was measured for 2 s and extinguished with 50 µl/well of 0.2 M NaOH. The measured relative light units were corrected for the corresponding background light emission

A complete standard curve for 17ß-estradiol was performed to determine the median effective concentration and the detection limit. The standard curve also was used to determine the reporter gene response of the sediment extracts by interpolation. The estrogenic activity of the samples was expressed in estrogenic equivalents. The detection limit was calculated as the luciferase activity of the solvent control plus twofold the standard deviation of the solvent control. The reported estradiol equivalence factor of 2.3 x 10^{-5} for NP was used to calculate the contribution of the NP to the estrogenic response (Legler et al., 2002).

5.2.10 Two-compartment model

The results of the Tenax extractions were used to model the rapidly and slowly desorbing fractions of NP and the estrogenic activity, with the often-used, two-compartment model of Cornelissen et al. (Cornelissen et al., 1997):

$$S_t = F_{rap} \bullet S_0 \bullet e^{-k_{rap} \cdot t} + F_{slow} \bullet S_0 \bullet e^{-k_{slow} \cdot t} \quad \text{(Equation 5.1)}$$

S_t = remaining extractable concentration of the contaminant in the sediment at a specific time of extraction [mg•kg^{-1}]
S_0 = initial total extractable concentration of the contaminant in the sediment [mg•kg^{-1}]
F_{rap} = rapid desorbing fraction [-]
F_{slow} = slow desorbing fraction [-]
k_{rap} = rate constant for the fast desorbing fraction [h^{-1}]
k_{slow} = rate constant of the slow desorbing fraction [h^{-1}]
t = desorption time [h].

Excel® 2003 (Microsoft Corporation, Redmond, Washington, USA) was used to fit the model (Equation 5.1) to the data of every single experiment. The parameters were estimated by minimizing the sum of the difference between the data and the model. The rates for the rapidly and slowly desorbing fractions give insight regarding how fast a compound desorbs from the sediment.

5.2.11 Statistics

Student's *t* tests were used to for comparison of the data. Probability of significance was set at $p < 0.05$. All data are reported as the mean ± standard error.

5.3 Results and discussion

5.3.1 Bioavailability of NP and estrogenic activity

The amount of NP and estrogenic activity, extracted with Tenax from polluted sediment, rapidly decreased over time (figures 5.3a and b). Within the first 5 hours, 75% ± 2% of the NP was already desorbed from the sediment, which was paralleled by a fast decrease in estrogenic activity of 66% ± 2%. After 24 hours, the desorption of NP and estrogenic activity slowed and after 3 days, almost no desorption took place (figures 5.3c and d). A residual NP concentration of 5% ± 1.5% could still be extracted from the sediment after 18 days of Tenax extraction compared to the total extractable concentration (Figure 5.4); the same was found for the residual extractable estrogenic activity (5% ± 1%). Both the detected NP and the extractable estrogenic activity in the sediment were approximately 95% bioavailable.

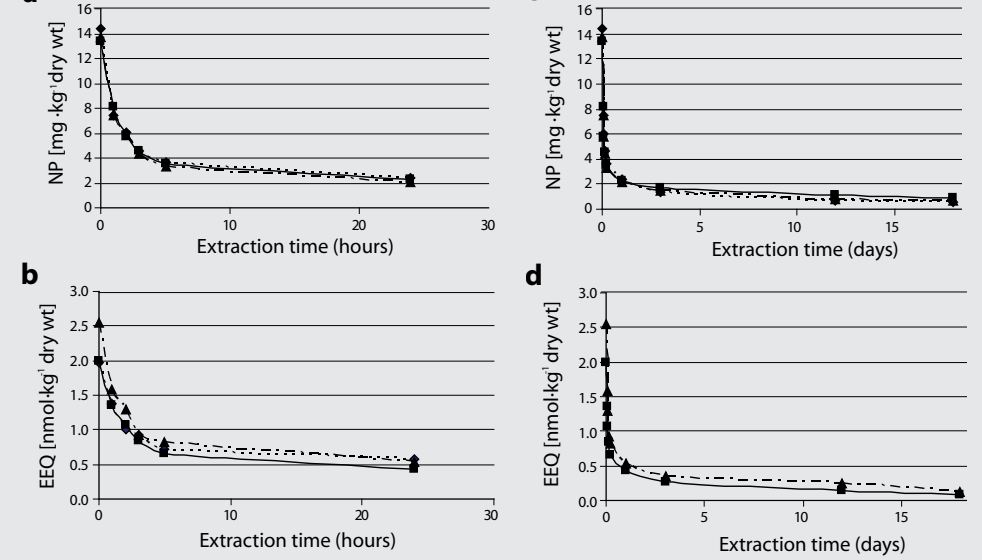


Figure 5.3: Concentration of nonylphenol (NP; a and c) and estrogenic activity (EEQ; b and d) as extracted by Tenax TA® (Buchem BV, Apeldoorn, The Netherlands) during the first 24 hours (a and b) or 18 days (c and d). Each line represents the result of an individual batch. The three batches are triplicates of one another.

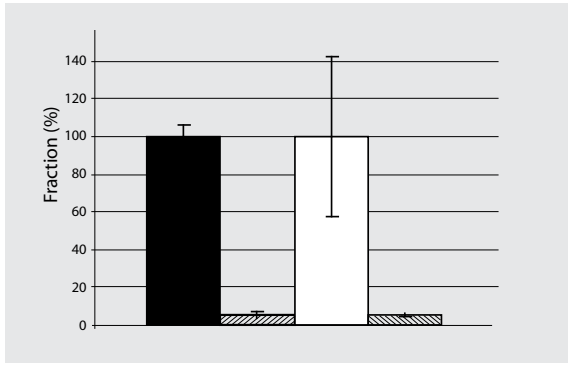


Figure 5.4: Residual microwave-assisted extractable nonylphenol (NP) or estrogenic activity in estradiol-equivalents (EEQs) after 18 days of Tenax TA® (Buchem BV, Apeldoorn, The Netherlands) extraction of NP (Cresidual) and as fraction of the total microwave assisted extractable amount in the original sediments (Coriginal). Error bars represent standard deviations of the triplicates.
■ = NP $C_{original}$; ▨ = NP, $C_{residual}$; □ = EEQ, $C_{original}$; ▧ = EEQ ,$C_{residual}$.

The results demonstrate that Tenax extractions can be used to assess the bioavailability of NP. Furthermore, the extracts of the Tenax experiment can be used to determine the bioavailable estrogenic activity with the ER_{α}-*luc* assay. So far, a receptor gene assay for estrogenic activity, like the ER_{α}-*luc* assay, has only been used to measure the total extractable estrogenic activity in sediments (Legler et al., 2002), and no combination was ever made with the bioavailability by using the extracts from Tenax extraction. Determining the estrogenic potency of the bioavailable fraction gives, in our opinion, a more realistic estimation of the ecotoxicological risk compared with measuring the total extractable estrogenic activity.

Our results also show that almost all extractable NP (~95%) is readily bioavailable from aged sediment, which based on the log K_{OW} of NP is an unexpected high fraction. For comparison, another study found that the bioavailability of the three-ring polycyclic aromatic hydrocarbons phenanthrene and anthracene (log K_{OW} = 4.57 and 4.54, respectively) (Mackay and Callcott, 1998) in aged sediments was only 65 to 80% after 11 days of Tenax extraction (Cuypers et al., 2002). Besides the physicochemical properties of a compound, the amount and type of organic matter in the sediment are important for bioavailability. The log K_{OC} of NP (4.7-6.1) is in the same range as the three ring three-ring polycyclic aromatic hydrocarbons phenanthrene and anthracene (Jonker and Smedes, 2000; Kleineidam et al., 1999), but higher than the log K_{OC} of atrazine (2.0-2.7) (Park et al., 2004). Phenanthrene and atrazine in soil with the same amount of organic carbon as our sediment became 40% less available during an aging period of 200 days (Chung and Alexander, 1998). Our sediment was naturally aged for a much longer period but approximately 95% of the extractable NP remained bioavailable. We have no explanation for the fact that NP in this specific sediment was more bioavailable than other compound/sediment combinations with similar log K_{OW} and/or K_{OC} values. We speculate that sediment-related parameters (e.g., particle size distributions) or compound related properties of NP (e.g., the detergent nature, an apolar tail, or the charged/hydrophilic head) cause this difference in behaviour.

5.3.2 Biodegradability versus bioavailability

After an aerobic degradation period of 183 days for sample 1, a residual concentration of NP of 3% ± 0.6% was still present compared to the total extractable concentration in the original sediment (Figure 5.5a).

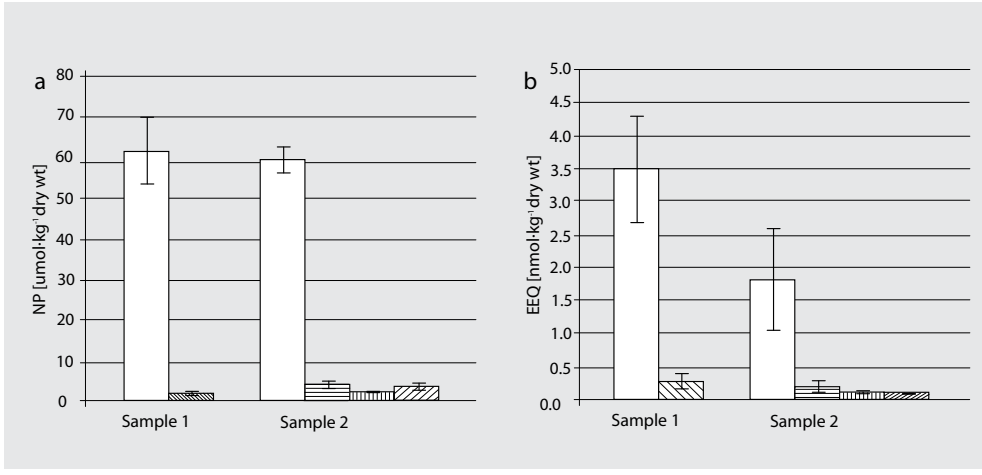


Figure 5.5: Total microwave extractable nonylphenol (NP; a) and estrogenic activity in estradiol-equivalents (EEQs; b) in untreated sediment samples 1 and 2 and residual microwave extractable amounts in the samples after aerobic degradation during 183 days of incubation (sample 1) and during 16 days and 34 days (sample 2). For sample 2, the residual concentration after Tenax TA® (Buchem BV, Apeldoorn, the Netherlands) extraction of 18 days is given. Error bars represent the standard deviations of the triplicates. □ = Untreated; ▧ = 183 days; ▤ = 16 days; ▥ = 34 days; ▨ = 18 days.

The residual extractable NP concentration in sample 2 was 8% ± 1.3% and 3% ± 0.5 % after aerobic degradation of 16 days and 34 days, respectively. After 16 days of incubation, the bioavailable NP was not completely degraded. After 34 days, however, all the bioavailable NP was degraded and the residual extractable fraction was equal to the residual extractable fraction after a Tenax extraction of 18 days. The residual concentration of NP after 183 days was not different from that after 34 days of incubation. The residual extractable fractions of the estrogenic activity showed the same results (Figure 5.5b). The residual estrogenic activities determined in sample 1 after aerobic degradation of 183 days and in sample 2 after aerobic degradation of 16 days and 34 days and after Tenax extraction of 18 days were

not significantly different. After aerobic degradation for more than 16 days, the estrogenic activity extractable from the sediment was reduced by 94% ±2%. The bioavailable fraction is the fraction that can be biodegraded because the residual fraction after Tenax extraction is equal to the residual fraction after aerobic degradation. This is stated with the conclusion that Tenax extractions can be used to determine the bioavailable fraction of NP and the estrogenic activity in the used sediment. Although 95% of the present NP and the estrogenic activity can become bioavailable in the environment, the ecotoxicological risk will be reduced with 95% because of aerobic degradation.

5.3.3 Two compartment model fit

The F_{rap} of NP and the relative decrease in estrogenic activity are 0.78 ± 0.05 and 0.74 ± 0.04, respectively (Table 5.2), which are not significantly different by Student's *t* test ($p<0.05$).

Table 5.2: Calculated rapidly desorbing fractions (Frap) and rate constants of rapidly and slowly desorbing fractions (krap and kslow, respectively) of nonylphenol (NP) and the estrogenic activity (EEQ).*

	NP	EEQ
F_{rap}	0.78 ± 0.05	0.74 ± 0.04
K_{rap} [h^{-1}]	0.75 ± 0.18	0.53 ± 0.05
K_{slow} [h^{-1}]	0.008 ± 0.07	0.0044 ± 0.0001

** Values are presented as the mean ± standard error*

The calculated rates K_{rap} and K_{slow} of the rapidly and slowly desorbing fractions of NP are 0.75 ± 0.18 h^{-1} and 0.008 ± 0.007 h^{-1}, respectively. The calculated rate for the rapidly desorbing fraction of the estrogenic activity is 0.53 ± 0.05 h^{-1} and that for the slowly desorbing fraction was 0.004 ± 0.001 h^{-1}. The rates of the NP desorption and the decrease in estrogenic activity are insignificantly different for the rapidly and slowly desorbing fractions. Both NP and the estrogenic activity become available with the same rates (predicted $K_{slow} \approx 0.01\ K_{rap}$). This is also found for other hydrophobic compounds and seems to be related to the type of organic matter and particle size matrix parameters of the sediment (Cuypers et al., 2002; Smit et al., 2005).

The two-compartment model of the Tenax extractions leads to a good fitting of the rapidly desorbing fractions. In contrast, the model underestimates the residual NP concentration and residual estrogenic activity. At t=183 days (4,392 hours), the average calculated NP fraction remaining in the sediment (S_t/S_o) is 1.6×10^{-7}, and that for estrogenic activity is 3.56×10^{-9}. These calculated values are much smaller than the observed residual fractions of 0.03 for both NP and the estrogenic activity (Figure 5.5). Apparently, either a very slow fraction or a residual bound fraction, which not desorbs from the sediment, is present. The two box model does not account for these two phenomena; however, the rapidly desorbing fractions for NP and the estrogenic activity are well predicted by the model. These rapidly desorbing fractions contain the highest amount of NP and other estrogens, which are most relevant for ecological risk assessment.

5.3.4 Monitoring NP to predict the estrogenic activity

Calculation of the contribution of NP to the estrogenic activity in the original sediment samples with an estradiol equivalence factor for NP of 2.3×10^{-5} (Legler et al., 2002) yields 42% ± 14% and 86% ± 41% for samples 1 and 2, respectively. The high error margin in sample 2 resulted from one of the triplicates that gave a low response in the ER_α-*luc* assay, whereas the NP concentration was as high as in the other two. We found no experimental cause to exclude the lower response. Because the estrogenic activity is not fully caused by the present NP, this implies that in addition to NP, other estrogenic compounds also are present in the sediment which contribute to the estrogenic activity. In addition, these estrogenic compounds are aerobically degraded in both samples to the same level as NP. Because the biodegraded fraction is equal to the bioavailable fraction, these other estrogenic compounds are as bioavailable as NP. Which estrogenic compounds other than NP are present in the sediment could not be determined. The most common estrogenic compounds - estradiol, ethynylestradiol and estrone - were not identified by chemical analyses. Therefore, other unknown estrogenic compounds must be present in the sediment.

The NP measured in the sediment used consists of a mixture of various branched NP isomers, which are all summed as total NP. In the ER_α-*luc* assay, only the estrogenic isomers respond, but the assay does not differentiate between the estrogenic isomers. The GC-MS chromatograms of NP gave a certain specific peak pattern for the sum of isomers for this sediment that did not change in the extracts during the experiments. This indicates that the different isomers were extracted and degraded equally.

Our approach, determining the Tenax-extracted NP or estrogenic activity to calculate the bioavailable estrogenic fraction prevents overestimation of ecotoxicological risk and is useful for designing appropriate and effective remediation strategies. Because the behaviour of the bioavailability and biodegradation of the estrogenic compounds and the single NP isomers in this sediment are equal to the behaviour of the total concentration of all NP isomers, the NP concentration can be used as an indicator compound to monitor the estrogenic activity of this river system. For this specific case, only the total microwave-extractable NP concentration has to be measured to monitor the estrogenic activity in sediment, because almost all NP is bioavailable and the model predicts the rapid desorbing fraction well.

This approach, based on our results for sediment of the Ebro estuary, possibly can be applied to other sediments with NP and other estrogenic compounds as well. To do so, the relationship between NP and estrogenic activity need to be studied further.

5.4 References

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6 Nonylphenol mass transfer from field aged sediments and subsequent biodegradation in reactors mimicking different river conditions

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Abstract

Sediments can function as secondary source for water pollution of aerobically biodegradable non-halogenated organic compounds, which are persistent in anaerobic sediments. The mass transfer of compounds from sediment to bulk water depends on hydraulic conditions. In this study desorption, mass transfer and biodegradation are investigated under settled and resuspended sediment conditions for branched nonylphenol (NP), which was used as model compound for aerobically biodegradable and anaerobic persistent compounds. Continuous flow through reactor experiments were performed in duplicate with aged NP polluted sediment under sterile and non-sterile conditions, to investigate the mass transfer and the combined mass transfer and biodegradation, respectively. In the presence of a sediment bed, the mass transfer of NP from the sediment bed to the bulk water decreased to an almost constant value. The desorbed NP in the non-sterile reactors was biodegraded. Upon resuspension, the NP concentration in the bulk water increased instantaneously in all reactors. This immediate increased mass transfer of NP from the sediment was larger than the amount that can be biodegradation under optimal conditions. Longer periods of stirring lead to a decreased mass transfer, to amounts that can be biodegraded under optimal environmental conditions. Nonylphenol desorbs continuously at low concentrations from the sediment bed into the bulk water, which can almost be completely biodegraded. Resuspension of NP polluted sediment initial led to an increase of the environmental risk, and can be followed by a subsequent reduction of the environmental risk due to biodegradation under optimal environmental conditions.

This chapter is accepted for publication in a slightly modified version in Journal of Soils and Sediments

6.1 Introduction

River basins contain many xenobiotic compounds, which can be toxic for the aquatic environment. Depending on their chemical properties, these chemicals are either dissolved or adsorbed to sediment particles. In the case of strong adsorption, sediment particles scavenge pollutants out of the water phase. Settling of these sediment particles results in a pollutant sink in the beds of rivers, lakes and estuaries. These sinks may change into sources for water contamination when the compound desorbs from the sediment under changes in river conditions, such as the turbulence of the river water. These changes can be caused by seasonal effects, extreme rainfall combined with peak river discharges (Gibson et al., 2005), or nautic and dredging activities. Changing hydraulic regimes of rivers, like the change from calm low flow conditions to high flow turbulent conditions, can result in resuspension of sediment particles into the river water. Then, adsorbed compounds can be released into the water phase and become more available for uptake by aquatic organisms posing renewed ecotoxicological risks to aquatic life. However, in this situation, pollutants become also more available for biodegradation that can decrease the amount of desorbed compounds in the water phase. When the rate of biodegradation in the aerobic river water is higher than the rate of desorption, the compounds are generally biodegraded in the sediment – river water interface and will not reach the bulk water. This results in a limited risk for the aquatic environment. For adequate prediction of the fate of pollutants and the risks of polluted sediments in river basins, these effects of changing conditions on desorption and biodegradation processes need to be better understood and taken into account.

Sediment beds are mainly anoxic with a thin aerobic layer of only a few mm at the interface with the aerobic surface water (Huttunen et al., 2006; Martin et al., 1998; Middeldorp et al., 2003). When chemicals move from the anaerobic sediment to the aerobic river water or vice versa, the changed biogeochemical conditions affect their fate. Most heavy metals are immobilised in anaerobic sediments (Vink, 2002; Vink et al., 2009). This immobilization improves the chemical and ecological quality of water systems. However, metals become mobilised again when sediments are resuspended in aerobic surface water. Biodegradation rates of highly halogenated organic compounds, like polychlorinated biphenyls and hexachlorobenzenes, are higher in anaerobic organic rich sediments compared to degradation under aerobic conditions (Brown et al., 1987; Mohn and Tiedje, 1992). Therefore, storage and natural attenuation (naturally occurring biodegradation) of these halogenated compounds in anaerobic sediments may offer a way to reduce their concentration and environmental risk. Many non-halogenated hydrophobic organic pollutants, like heavy poly aromatic hydrocarbons, petroleum hydrocarbons and natural hormones are less or non-biodegradable under anoxic conditions (Mes et al., 2005; Vermeulen et al., 2003). Storage of these non-halogenated organic compounds in anaerobic sediments slows down or even stops their biodegradation, thus creating a long lasting potential source of pollution for the aquatic ecosystem in the future.

This study focuses on the fate of branched nonylphenol (NP) during changing river conditions. Branched nonylphenol was used as model compound for aerobically biodegradable non–halogenated organic pollutants that are persistent under anaerobic conditions, because i) it is a pollutant found in many river systems (Bennie et al., 1997; Heemken et al., 2001; Navarro et al., 2009b; Wu et al., 2007), ii) it has significant toxicological effects on aquatic biota because of its estrogenic activity (Soares et al., 2008), iii) it adsorbs to and desorbs from sediments (Chapter 5), and iv) is biodegradable under aerobic conditions, but persistent under anaerobic conditions (Corvini et al., 2006, chapters 3 and 4)

Pollutant mass transfer from the anaerobic sediment through the interface between the sediment and the water to the aerobic bulk water is mainly driven by the concentration gradient between the sediment particle and the surrounding water and is affected by the sediment-water contact area (Birdwell et al., 2007; Cheng et al., 1995). The total mass mass transfer Φ_{sed} [µg·d^{-1}] from the sediment to the bulk water can be described by the following equation:

$$\Phi_{sed} = k \cdot A_{sed} \cdot (C_{sur} - C_{bw}) \qquad \text{(Equation 6.1)}$$

Φ_{sed} = mass flux [µg·d^{-1}]
k = overall mass transfer coefficient [m·d^{-1}]
A_{sed} = contact surface of the sediment and the surrounding water [m^2]
C_{sur} = concentration at the solid surface [µg·m^{-3}]
C_{bw} = concentration in the bulk water [µg·m^{-3}]

From this equation can be seen that there is a flux from sediment to the bulk water when the concentration at the solid surface (C_{sur}) is larger than the concentration in the bulk water (C_{bw}). Thus, the sediment will act as a source for water pollution. The contact area enlarges during resuspension, and this enlarges the mass transfer, followed by an increased rate of NP desorption. Biodegradation of the desorbed NP can reduce the ecotoxicological risk of this compound (Chapter 5). However, if the biodegradation rate is lower than the desorption rate, the pollutant will remain at elevated concentrations in the water phase after resuspension, where it may cause toxicological effects on aquatic organisms.

Understanding of the interaction between sorption, mass transfer, and biodegradation under changing conditions is needed for a quantitative prediction of the toxicological risk in rivers. Therefore, the above mentioned processes were studied for our model compound NP under two different conditions, namely i) settling sediment conditions and ii) resuspended sediment conditions, as schematically presented in Figure 6.1.

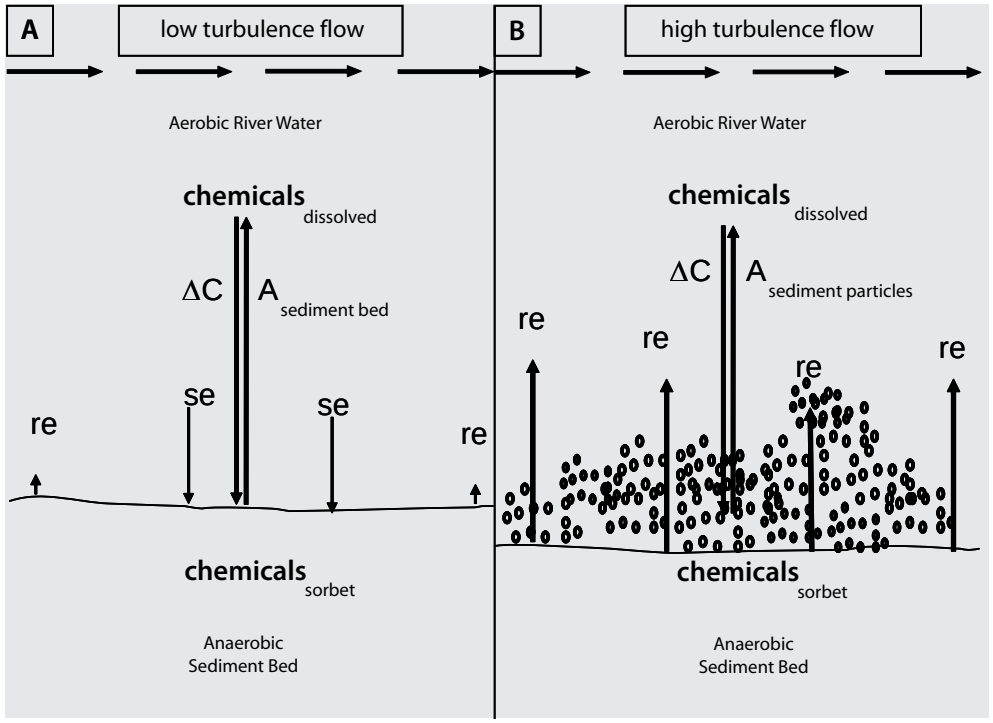

Figure 6.1: Conceptual model for exchange processes of hydrophobic chemicals dissolved in the river water and sorbed to sediments under A) settling sediment conditions with low resuspension and high settling of the sediment and B) resuspended sediment conditions. se = settling; re = resuspension.

A river has a low water flow velocity during settling sediment conditions without resuspension. The sediment is predominantly present as a sediment bed, and the area of the sediment bed (A_{sed}) controls the mass transfer from the sediment to the bulk water. Biodegradation of the desorbing NP can only occur in the aerobic bulk water and at the interface with the sediment bed. During resuspended sediment conditions (e.g. during a flood event), a vast amount of sediment particles is resuspended into the bulk water. Under this condition the desorption area A_{sed} becomes enlarged compared to the desorption area of the sediment bed. As a result, the mass transfer of NP into the bulk water will be enhanced, resulting in higher NP concentrations in the bulk water. This increases the toxicological risk of NP. On the other hand, biodegradation may be stimulated upon resuspension as the redox conditions will shift to aerobic. This stimulation of biodegradation can reduce the risk of ecological effects and uptake in the food chain.

In this study, quantitative desorption and biodegradation of NP were determined using aged NP polluted sediment. Continuous reactor experiments were conducted to mimic natural and changing mass transfer conditions in a river system.

6.2 Materials and methods

6.2.1 Chemicals and materials

Tenax®-TA (20-35 mesh, pre cleaned) was purchased from Buchem B.V. (Apeldoorn, the Netherlands). Petroleum ether 40-60°C (≥ 90% purity) and acetone (HPLC grade) were obtained from Merck (Darmstadt, Germany). A nonylphenol technical mixture (> 94% purity) for calibration standards was purchased from Riedel de Haën (Seelze, Germany). Other used chemicals were of the highest purity available and were used without further purification. All used glassware and microwave tubes were pre cleaned by washing three times with acetone and two times with petroleum ether.

6.2.2 Sediment

Sediment with approximately 20 mg·kg^{-1} dry weight NP was collected in June 2005 from the Huerva River, a tributary of the Ebro River in Spain. The sampling location was located on the boundary of the city Zaragoza (Spain, 41°37'23"N, 0°54'28"W). Sediment samples were taken with stainless steel cores to 50 cm depth and collected in a ten litre vessel. The vessel was completely filled with sediment to avoid a gaseous head space, and was transported on ice to the laboratory. The sediment was sieved at mesh < 2 mm in the laboratory and stored at 4°C until use. The NP in the sediment consisted of a mixture of branched isomers as analysed by GC-MS, and the single isomers were not identified. Previous experiments showed that 95% of the NP in this sediment was potentially available and could be biodegraded under aerobic conditions (Chapter 5). The TOC-content of the sediment was 3.5% as determined by Al-West (Deventer, the Netherlands). The dry matter content was determined by drying the sediment overnight at 105°C. The organic matter content was 8.4%, determined gravimetrically by combustion of dried samples for 6h at 550°C. The sediment contained two main particle size fractions, namely <32 µm (52%) and 32-125 µm (34%). These fractions were determined by sieving with Retsch sieves with mesh sizes of 32, 125, 500 µm, and by using a Beckman Coulter LS 230 laser diffraction particle size analyzer with Polarization Intensity Differential of Scattered Light (PIDS). The Fraunhofer theory of light scattering was used to calculate the particle size distribution. Prior to particle size analysis, sediment samples were dispersed in demineralized water and thoroughly mixed. The injected slurry volume was controlled to obtain a total obscuration level of 10±3% and a PIDS obscuration of 50±10% (Buurman et al., 1997).

6.2.3 Reactor set up

Desorption from the polluted sediment and aerobic biodegradation of NP were studied in duplicate in a reactor set up that mimics changing conditions in a river system (Figure 6.2), based on the set up as described by Smit et al. (Smit et al., 2008). The set up was open on top. Non-mixing and mixing conditions were studied in duplicate under both sterile

and non-sterile conditions. Before the start of the experiment, the reactors were rinsed with acetone and petroleum ether and autoclaved at 121°C for 15 minutes. Thereafter, the reactors were filled with 100 g sediment (wet weight) and 650 ml mineral medium. The mineral medium was prepared as described in Tros et al., but without $Na_2SeO_3.5H_2O$ (Tros et al., 1996). To establish a sediment bed, the sediment was shortly mixed with the medium before the start of the experiment. The influent was pumped into the reactor (approximately 1 $l \cdot d^{-1}$) just above the surface of the sediment and the effluent was pumped from the liquid surface with a glass tube with the same pumping rate as the influent (Figure 6.2). Only liquid was removed from the reactor and the sediment remained permanently in the reactor. The hydraulic retention time (HRT) of the medium in the reactors was approximately 15 h. The effluent was pumped from the medium surface via the glass tube through a glass column with approx. 0.6 g Tenax. The glass column contained a glass filter beneath, and a 21 µm stainless steel filter with a thickness < 0.5 mm on top of the Tenax, to keep the Tenax in the glass column. The effluent passed the Tenax, where all NP in the effluent sorbed to the Tenax. After passing through the Tenax, the effluent was pumped into a waste vessel. The Tenax in the columns was refreshed at selected times during the experiments. Before the refreshment of the Tenax, 15 ml effluent samples were taken to control the efficiency of the Tenax extraction of the NP from the water phase. During the change of the Tenax, the pump was switched of for 1–2 min for practical reasons. With the Tenax, the absolute desorbed amount of NP was determined. The concentration in the water phase was calculated with the absolute amount of NP and the volume of medium that had passed through the Tenax.

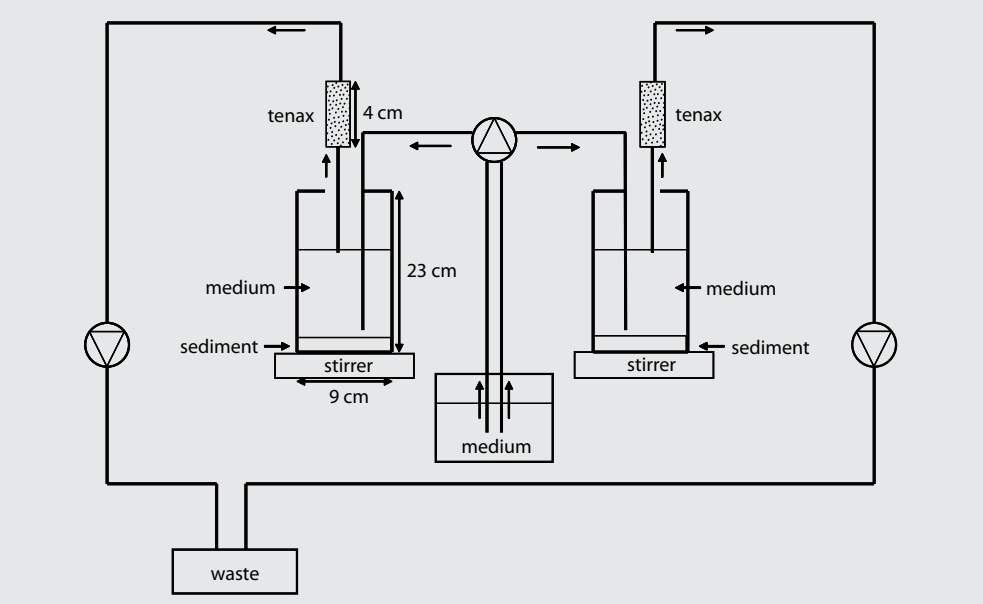

Figure 6.2: Schematic set up of the duplicate reactors.

Sediment samples (2 g dry weight) were taken to determine the NP concentration in the sediment before the start and at the end of the experiments. All experiments were performed at 20°C and during the experiments the dissolved oxygen in the water phase, as well as in the sediment was measured with an OX-500 micro electrode (Unisense, Aarhus, Denmark). The experiment lasted for 59 days. The settling sediment condition was maintained in the first 35 days of the experiment, simulating a gently flowing river (part I). The sediment was not mixed with the medium, and a sediment bed was formed with a bed thickness of approximately 2.5 cm. Thereafter, the experiment continued with the resuspended sediment condition for 14 days (part IIa and IIb), simulating a turbulent flowing river. The sediment and medium were mixed for 5 min every 8 hrs. During the mixing, the pumps were switched off to avoid removal of sediment from the reactor. The sediment was allowed to settle for 25 min. before the pumps were switched on again. After the mixing period of 14 days, the reactors were operated for another 9 days at the original settling sediment conditions (part III).

Table 6.1 shows the experimental conditions. A sterile experiment was performed in duplicate (reactor 1 and reactor 2) to study the desorption behaviour of NP from the sediment.

Table 6.1: Experimental conditions of the reactors during the sterile (reactor 1 and 2) and non sterile experiments (reactor 3 and 4)

Experimental conditions	**Sterile**		**Non sterile**	
	Reactor 1	**Reactor 2**	**Reactor 3**	**Reactor 4**
Sediment (g dry weight)	41.5	41.5	37.3	37.3
NP S_0 ($mg \cdot kg^{-1}$ dry weight)	22.6 ± 1.5	22.6 ± 1.5	19.1 ± 0.58	19.1 ± 0.58
Medium volume (ml)	649	653	651	680
Flow ($ml \cdot min^{-1}$)	0.70	0.63	0.68	0.64

To inhibit microbial activity, $HgCl_2$ (50 $mg \cdot l^{-1}$) was added to the medium. The combination of desorption and biodegradation of NP from the sediment was studied in the non-sterile experiment (reactor 3 and reactor 4). One of the duplicates of the non-sterile reactors (reactor 4) was started one day later due to technical reasons. However, the mixing period started at the same day, and the period with the sediment bed was therefore 35 days in reactor 3 and 34 days in reactor 4.

6.2.4 Biodegradation of NP in batch experiments

The biodegradation of the NP in the sediment was studied in batch experiments with the mineral medium under optimal conditions to determine the biodegradation rate. Degradation was also studied with aerobic river water. For this purpose, river water samples were taken in dark glass bottles at the same location and the same time as the sediment samples were collected (§ 2.2). These water samples were transported to the laboratory on ice and stored at 4°C until use. The degradation tests of NP were performed in triplicate including one sterile control. Volumes of 50 ml mineral medium or 50 ml river water and 5 g wet sediment (2 g dry weight) were added to 250 ml bottles, which were closed with a viton stopper. The bottles were incubated at 20°C, and horizontally shaken in the dark. The NP concentration was measured at various time intervals by taking 200 µl of slurry sample from the bottles. The samples were analyzed for NP concentrations by GC-MS with solid phase micro extraction (SPME) (Chapter 2)

6.2.5 Mass transfer calculations

The following mass balance was used to calculate the average mass transfer (Φ) in a certain time interval in the sterile experiment without biodegradation:

$$V \cdot dC/dt = \Phi - \varphi \cdot C \qquad \text{(Equation 6.2)}$$

V = volume medium in reactor [m^{-3}]
C = concentration in bulk water [$\mu g \cdot m^{-3}$]
t = time [d]
Φ = mass transfer [$\mu g \cdot d^{-1}$]
φ = flow [$m^3 \cdot d^{-1}$]

The following mass balance includes biodegradation and is used for the non-sterile experiment:

$$V \cdot dC/dt = \Phi - \varphi \cdot C - V \cdot k \cdot C \qquad \text{(Equation 6.3)}$$

V = volume medium in reactor [m^3]
C = concentration in bulk water [$\mu g \cdot m^{-3}$]
t = time [d]
Φ = mass transfer [$\mu g \cdot d^{-1}$]
φ = flow [$m^3 \cdot d^{-1}$]
k = first order degradation rate constant [d^{-1}]

Equation 6.3 assumes that the degradation of NP, expressed with the product V·k·C, takes place in the whole reactor volume, and is valid for fully resuspended sediment conditions. During the settled sediment conditions, degradation can only take place at the interface of the sediment and bulk water. Since the volume of the active layer is unknown, this mass balance is not used for the calculation of the average mass transfer in the non-sterile reactor experiment under the settling sediment conditions.

6.2.6 Pore water concentrations

In the inner part of the sediment bed, NP was present in the pore water and adsorbed to the sediment particles. As equilibrium in the sediment bed is assumed, the pore water concentration can be calculated with:

$$K_d = C_{sed} / C_{pw} \qquad \text{(Equation 6.4)}$$

K_d = partitioning coefficient between the concentration in the sediment [$l \cdot kg^{-1}$]
C_{sed} = concentration in the sediment [$mg \cdot kg^{-1}$]
C_{pw} = concentration in the pore water [$mg \cdot l^{-1}$]

6.2.7 Nonylphenol extraction, chemical analysis and statistics

Both Tenax samples and sediment samples were collected in 20 ml vials with 10 ml acetone. The vials were closed with a polytetrafluoroethylene (PFTE)/butyl rubber septum and stored at -20°C before extraction. The NP was extracted from the Tenax or sediment samples by micro wave extractions with acetone and petroleum ether. Before extraction, another 5 ml acetone and 15 ml petroleum ether were added to the Tenax and sediment samples. The petroleum ether extracts were analysed by GC-MS as described in Chapter 5. The detection limit of NP in the petroleum ether extracts was 100 $\mu g \cdot l^{-1}$.

For the analyses of NP concentrations in the effluent, 2 ml effluent was added to a 20 ml headspace vial. The headspace vial was capped with a magnetic crimp cap with blue silicon and teflon coated septum (Grace Davison Discovery Science, Deerfield, Il, USA) and analysed with GC-MS by solid phase micro extractions as described in Chapter 5.

Student's t tests were used for comparison of the nonylphenol concentration, and the probability of significance was set at $p < 0.05$.

6.3 Results and discussion

6.3.1 Reactor performance

The reactor experiments to measure the desorption of NP were performed for 59 days and the release of NP to the bulk water for the sterile and non-sterile experiments is shown in figures 3a en b. The conditions for the settling sediment and resuspended sediment are represented in four parts; parts I, IIa, IIb and III.

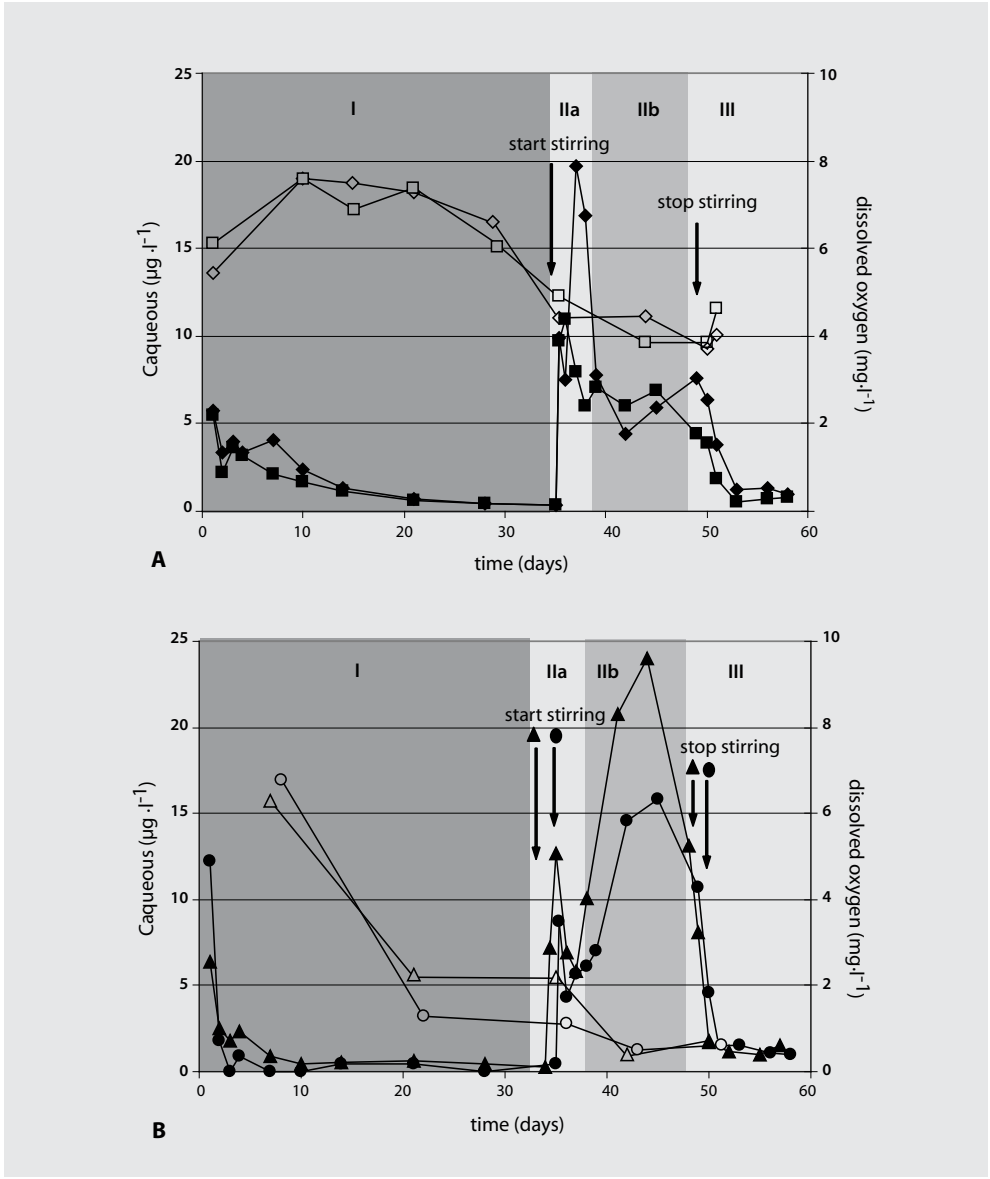

Figure 6.3: Nonylphenol concentration in aqueous phase (left Y-axis, filled symbols) and dissolved oxygen concentrations (right Y-axis, open symbols) during the experiment under A) sterile conditions ◆ = reactor 1 and ■ = reactor 2 and B) non sterile conditions ● = reactor 3 and ▲ = reactor 4. Stirring in reactor 4 started 1 day before reactor 3.

In part I (35 days), settling sediment conditions were applied. The resuspended sediment conditions were divided in parts IIa and IIb, based on the results. Part IIa contains the results of the first 2 to 4 days of the resuspended sediment period and part IIb the results of the remaining resuspended sediment period. In part III the stirring was stopped and settling sediment conditions were applied again (figures 6.3a and 6.3b).

6.3.1.1 Sterile experiment

Initially in part I of the sterile experiment, the NP concentration in the bulk water was high, due to addition of the sediment to the reactors, followed by stirring of the sediment and the medium (Figure 6.3a). The stirring stopped shortly after the additions of the sediment, the NP concentration decreased in both sterile reactors. The concentration in the bulk water in reactor 1 fluctuated between t =2 and t=7 days at an average concentration of 3.6 $\mu g \cdot l^{-1}$. Thereafter, between t=7 to t= 35 days, the NP concentration in the bulk water in this reactor decreased to 0.3 $\mu g \cdot l^{-1}$. In reactor 2, the average concentration between t=2 and t=4 days was 3.0 $\mu g \cdot l^{-1}$, and a similar decrease to 0.3 $\mu g \cdot l^{-1}$ was observed at t=35 days. As soon as the mixing of the sediment started at t=35 days (part IIa), the NP concentration in the bulk water in both sterile reactors increased to a concentration of 9.9 $\mu g \cdot l^{-1}$ in reactor 1 and 9.7 $\mu g \cdot l^{-1}$ in reactor 2 in the first 8 hours of part IIa. After reaching the maximum concentrations of 19.7 $\mu g \cdot l^{-1}$ and 11.0 $\mu g \cdot l^{-1}$ in reactor 1 and 2, respectively, the concentration decreased. The NP concentration in the bulk water remained at an average level of 6.4 ± 1.6 $\mu g \cdot l^{-1}$ in reactor 1 and 6.1 ± 1.2 $\mu g \cdot l^{-1}$ in reactor 2 in part IIb. When the stirring was stopped at t=49 days (part III), the NP concentration sharply decreased within 4 days to 0.9 $\mu g \cdot l^{-1}$ and 0.8 $\mu g \cdot l^{-1}$ in reactor 1 and 2, respectively. This NP concentration was slightly higher compared to the NP concentration at the end of part I. During the sterile experiment, the oxygen concentration remained stable at an average concentration of 6.9 ± 0.9 $mg \cdot l^{-1}$ and 6.8 ± 0.7 $mg \cdot l^{-1}$ during the first 29 days in reactors 1 and 2, respectively. During part IIa and IIb, the dissolved oxygen concentration decreased from 4.4 to 3.7 $mg \cdot l^{-1}$ with a rate of 0.05 $mg \cdot l^{-1} \cdot d$-1 in reactor 1 and from 4.9 to 3.8 $mg \cdot l^{-1}$ with a rate of 0.07 $mg \cdot l^{-1} \cdot d^{-1}$ in reactor 2. This decrease might be explained from chemical oxidation processes like pyrite oxidation as biodegradation is inhibited by $HgCl_2$ (Vermeulen et al., 2007).

6.3.1.2 Non-sterile experiment

At the start of the non-sterile experiment (reactors 3 and 4), a high initial NP concentration was observed in the bulk water, due to the stirring of the sediment at the start of the experiment (Figure 6.3b; part I). The NP concentration in reactor 3 decreased from t=1 day within 1 day to concentrations below the detection limit (100 $\mu g \cdot l^{-1}$ in the petroleum ether extracts). At t=4 days, a NP concentration of 0.9 $\mu g \cdot l^{-1}$ was measured, where after the NP concentration fluctuated between 0.5 $\mu g \cdot l^{-1}$ and a concentration below the detection limit. In reactor 4, the concentration decreased also between t=1 and t=2 days, and remained at an average concentration of 2.2 ± 0.4 $\mu g \cdot l^{-1}$ till t=4 days (Figure

3b; part I). This was followed by a concentration decrease to levels fluctuating between 0.3 and 0.7 $\mu g \cdot l^{-1}$ with the lowest concentration at t=34 days, just before stirring. As soon as the mixing of the sediment started (part IIa), the concentration in both reactors increased immediately to a NP concentration of 8.9 $\mu g \cdot l^{-1}$ and 12.7 $\mu g \cdot l^{-1}$ in reactors 3 and 4, respectively. Subsequently, the concentration decreased in both reactors, but after two days of stirring (part IIb) the concentration of NP in the bulk water increased again after 10 days of stirring. Concentrations up to 15.8 $\mu g \cdot l^{-1}$ and 24.0 $\mu g \cdot l^{-1}$ were measured in reactors 3 and 4, respectively. The concentrations in both reactors started to decrease just before the mixing was stopped and this decrease continued in part III, when the settling sediment condition was established again. The average concentration in the bulk water in part III was 1.2 $\mu g \cdot l^{-1}$ and 1.1 $\mu g \cdot l^{-1}$ in reactors 3 and 4, respectively. The dissolved oxygen concentration during the non-sterile experiment decreased from 6.8 to 1.3 $mg \cdot l^{-1}$ in reactor 3 and from 6.3 to 2.2 $mg \cdot l^{-1}$ in reactor 4 in part I. The dissolved oxygen concentration decreased further within part IIa and IIb to 0.5 $mg \cdot l^{-1}$ with a rate of 0.1 $\mu g \cdot l^{-1} \cdot d^{-1}$ and 0.3 $mg \cdot^{-1}$ with a rate of 0.3 $mg \cdot l^{-1} \cdot d^{-1}$ in reactors 3 and 4, respectively. The decrease of dissolved oxygen in the non-sterile reactors is most probably caused by chemically oxidation processes of sediment minerals and biological processes like organic matter transformation.

6.3.2 Mass transfer and biodegradation of NP in reactors

6.3.2.1 Settling sediment conditions (part I)

For part I, the measured NP concentrations in the bulk water in reactor 1 and 2 showed an exponential decrease in time. In Table 6.2, the constants a and b of the exponential trend line ($C_{bw} = a + e^{b \cdot t}$) and the correlation factor R^2 are given. The data in the first 7 days of the experiment diverged most from the trend line. This data scatter in the beginning of the experiment was also observed in previous performed try out experiments and this is due to the starting up of the reactors. The mass transfer (Φ) is calculated using the fitted exponential reduction of the concentration in the bulk water.

Table 6.2: Constants of exponential trend line $C_{bw} = a + e^{b \cdot t}$, with C_{bw} as bulk water concentration in $\mu g \cdot l^{-1}$ and t as time in days, and the correlation coefficients R^2 of the trend lines

Parameters	Reactor 1	Reactor 2
a	5.1	3.8
b	-0.085	-0.076
R^2	0.96	0.94

The Φ of NP from the sediment to the bulk water is 5.5 and 4.7 $\mu g \cdot d^{-1}$ at the beginning of the experiment in reactor 1 and 2, respectively, and decreases to 0.3 $\mu g \cdot d^{-1}$ in both reactors at the end of part I. The measured NP concentrations in the bulk water and the mass transfer level off to nearly constant values, which is to be expected according to Equation 6.2. This indicates that small amounts of NP will continuously desorb from the sediment into the bulk water. A stable mass transfer was not reached yet in our experiments. With a stable mass transfer of NP from sediments to the bulk water, there will be a constant emission of NP from the sediment into the bulk water as long as the pollutant is present in the sediment, even at non-turbulent conditions. Although the mass transfer from a sediment bed can be relatively low, and the resulting NP concentrations in the bulk water remain low, exposure to low NP concentrations for a long period can still lead to adverse effects in aquatic organisms (Snyder et al., 2001).

The non-sterile reactors 3 and 4 were operated similar as the sterile reactors reactor 1 and 2, but without the addition of $HgCl_2$. An initial rapid decrease in NP concentration in the aqueous phase is observed. This decrease is not due to biodegradation, because the batch experiments with mineral medium showed that the involved microorganisms need a lag phase of 2 days before they biodegraded NP (data not shown). Therefore, the decrease is a result of dilution of the medium after the start up of the experiment, comparable with the sterile reactors 1 and 2. The measured bulk water concentrations from t=2 till t=21 days are lower in reactor 3 and 4 than the concentrations measured in the sterile reactors in part I. This lower concentration indicates that the biological activity decreases the NP aqueous concentrations compared to the sterile experiment. This biodegradation of the desorbing NP can take place in the bulk water, but is most likely to occur at the aerobic interface between the anaerobic sediment and the medium, because the microorganisms are present in the sediment. The inner part of the sediment was anaerobic. This was confirmed by measurements with the micro-oxygen electrode, showing that within less than 1 mm from the top of the sediment oxygen became depleted (results not shown). As the NP concentration decreased due to biodegradation, the environmental risk of NP present in the sediment will thus be reduced, because the desorbing NP is degraded in this thin interface before it reaches the bulk water.

At the end of part I, the concentrations in the water phase under sterile and non-sterile conditions were comparable. We had expected lower concentrations in the non-sterile reactors due to biodegradation, but apparently hardly any biodegradation took place at the end of part I. The reason for this reduced biodegradation is most likely due to the low dissolved oxygen concentrations of less than 2.5 $mg \cdot l^{-1}$ in reactor 3 and 4 were apparently too low for NP degradation. As reported in literature, the presence of sufficient oxygen is crucial for NP biodegradation (Ekelund et al., 1993, Chapter 4).

6.3.2.2 Resuspended sediment conditions (part IIa)

Directly after mixing of the sediment bed (part IIa), the NP concentration increased in all four reactors. The mass transfer Φ had a value of 0.3 $\mu g \cdot d^{-1}$ in reactors 1 and 2 before

resuspension of the sediment and increased to 29 $\mu g \cdot d^{-1}$ in reactor 1 and 27 $\mu g \cdot d^{-1}$ in reactor 2 in the first 8 hours, directly after the first time of resuspending the sediment bed. Two mechanisms explain this sharp increase in mass transfer from the sediment to the bulk water. First, at t=35 days when the sediment bed is mixed, the NP in the pores of the sediment is released to the bulk water. The maximum pore water concentration at equilibrium leads to maximum concentrations varying from 1.0 $\mu g \cdot l^{-1}$ to 3.8 $\mu g \cdot l^{-1}$, based on the lowest and highest reported K_d values obtained for Ebro River sediments (Navarro et al., 2009a). The second mechanism is the increase of exchange surface area (A_{sed}) in Equation 6.1. The surface area of the sediment bed ($7.1 \cdot 10^{-3}$ m^2) is increased about 195 times for 70 µm diameter size particles and more than 680 times for 20 µm diameter size particles. According to Equation 6.1, the mass transfer would increase with a similar factor. In our experiment, the mass transfer increased by a factor of 100. This lower increase is caused by the rapid increase of the concentration in the bulk water (C_{bw}), and therefore a reduction of the driving force of the concentration difference in Equation 6.1.

The mass transfer of NP from the sediment to the bulk water under sterile conditions directly after the first time of resuspending the sediment bed (t= 35 days), is about three times larger than the amount of NP that can be biodegraded under optimal conditions. This is calculated with the product V·k·C (Equation 6.3), using the first order degradation rate constant of NP of 1.5 d^{-1} as obtained from the results of the batch experiments with sediment and medium (data not shown). This high mass transfer indicates that resuspension of a sediment bed leads to an immediate increase of the mass transfer of NP to the bulk water due to the release of NP from the pore water and the sediment particles. The released amount of NP can not be fully degraded, and reaches the bulk water, which increases the toxicological risk for the aquatic environment.

6.3.2.3 Resuspended sediment conditions (part IIb)

The mass transfer in the sterile reactors during part IIb decreased, and levelled off to values of 5.7 ± 1.9 $\mu g \cdot d^{-1}$ and 5.3 ± 1.9 $\mu g \cdot d^{-1}$ in reactor 1 and reactor 2, respectively. This relatively stable mass transfer indicates that a steady state situation has been reached. However, the system is not at equilibrium yet, because the equilibrium concentration in the water phase should be between 11 and 44 $\mu g \cdot l^{-1}$, calculated with the range of K_d values (Navarro et al., 2009a), which is higher than the average bulk water concentration of 6.4 and 6.1 $\mu g \cdot l^{-1}$ reactor 1 and 2, respectively. The mass transfer in part IIb is significantly higher compared to the mass transfer at the end of part I. This is to be expected since the contact area of the sediment particles with the bulk water is much larger during stirring compared to the contact area in the presence of a sediment bed.

In the non-sterile reactors a decreased mass transfer of NP to the bulk water was expected, as part of the NP should be biodegraded. However, in part IIb of the non-sterile reactor a second increase in mass transfer is observed, instead of a constant mass transfer as seen in the sterile reactors. This second increase in mass transfer seems to be related to microbial activity, because the sterile and non-sterile reactors are operated equally and this phenomenon is not observed under sterile conditions. A possible explanation is that microorganisms are able to degrade organic matter in sediment under aerobic conditions (Cole et al., 1988) and this degradation of organic matter can lead to smaller organic matter particles, disintegration of organo-silica cemented aggregates, changes in organic matter structure or transformation of particulate organic matter to dissolved organic matter. These processes can lead to more desorption of NP from the sediment. Whether, and which of these processes really contributed to the observations in our reactors is unclear, especially as the organic matter in the non-sterile reactors did not significantly differ between the start and the end of the experiment (data not shown). However, oxygen is needed for the transformation of organic matter, and the dissolved oxygen concentration in the non-sterile reactors decreased faster than in the sterile reactors due to the combination of chemical oxidation processes and biological processes.

From the results of the NP concentrations in the non-sterile reactors during the resuspension in part IIb, we can not derive to what extent the extra released NP is degraded. However, the NP seemed not to be biodegraded at the end of part I due to low oxygen concentrations. The oxygen concentration during part IIa and IIb decreased further, which indicates that no or almost no NP could be biodegraded during part IIb. Therefore, the product V·k·C for biodegradation in Equation 6.3 can be assumed to be negligible. The maximum mass transfer in the non-sterile reactors during part IIa was 16 $\mu g \cdot d^{-1}$ and 21 $\mu g \cdot d^{-1}$ after 7 days of stirring in reactors 3 and 4, respectively, which is comparable to the amount of NP that could have been degraded as calculated with the product V·k·C (equation 6.3) under optimal conditions. This indicates that although more NP desorbs from the sediment under non-sterile conditions than under sterile conditions during longer periods of resuspension of the sediment, this extra released NP can be degraded under optimal environmental conditions for biodegradation.

In field situations, the conditions are often not optimal for biodegradation. This is shown by the results of the batch experiments performed with sediment and river water. Nonylphenol can be degraded in the presence of the river water of the same location as the sediment originates from. Within eight days, 89% of the NP was biodegraded (data not shown). However, in the batch experiment under optimal conditions with medium, the NP was almost completely degraded (97%) in less than four days. The biodegradation in the presence of river water was slower than the biodegradation of NP in the presence of medium. This indicates that biodegradation rates of NP under field conditions will be lower, and the desorbing NP may not be fully degraded. Then, this desorbing NP reaches the bulk water and leads to increased ecotoxicological risks.

6.3.2.4 Resumed settling sediment conditions (part III)

After the stirring was stopped (part III), the concentrations decreased in all four reactors as expected, because Ased decreased, and the sediment bed was established again. The comparable concentrations in the non-sterile and sterile reactors in part III indicate that

no biodegradation took place after stirring, probably caused by the low dissolved oxygen concentrations in the non-sterile reactors, which had been dropped further during the resuspension period to 0.7 mg·l^{-1}.

6.3.3 Mass balance of concentration in sediment

Before and after the experiments, the concentration of NP in the sediment (Csed) was measured. At the end of the sterile reactor experiment, respectively 72% and 64% of the original NP was present in the sediment in reactors 1 and 2 (Figure 6.4).

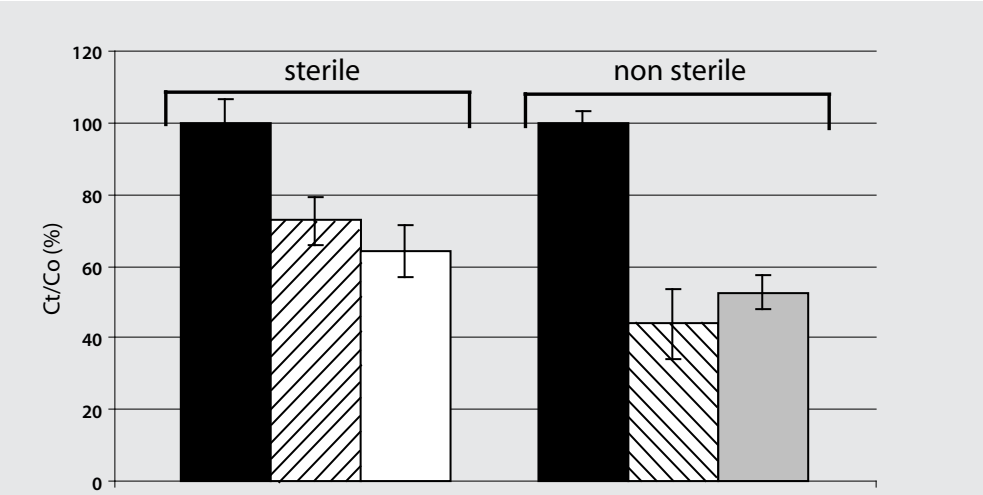

Figure 6.4: Relative NP concentration in the sediment at the start (C0) and the end (Ce) of the reactor experiments.

■ = C_0; ▨ = C_e *reactor 1;* □ = C_e *reactor 2;* ▧ = C_e *reactor 3;* ▩ = C_e *reactor 4*

From the desorbed NP, 62 ± 1%, is released during the resuspension of the sediment (part IIa and IIb). Under non-sterile conditions, respectively 56% and 47% of the original present NP remained in the sediment in reactors 3 and 4. This significant difference of NP concentration in the sediments at the end of the sterile and non-sterile experiments is caused by the biodegradation of the released NP during part I and the extra mass transfer of NP during part IIb under non-sterile conditions. From the results of the remaining NP in the sediment can be concluded that after two weeks of resuspension, 50 to 70% NP is still present and the sediment which still can function as source of contamination. The NP in the sediment is potentially available for 95% including a rapidly desorbing fraction of 75% that desorbs from the sediment within 5 hours, as determined by direct mixed sediment-Tenax extractions (Chapter 5). Compared to this, the desorption of the available fraction in our reactor set up is much slower.

Based on the reactor experiments we calculated that it will take 5 years to desorb the rapid available fraction (75 % of the NP) from the sediment under non-sterile conditions, as long as the sediment is present in a settled bed. To desorb all available NP (95%), it will take much longer under these conditions because of the lower driving force caused by lower concentration gradients at lowers NP concentrations. Resuspension leads to a faster release of the rapidly desorbing fraction. Nevertheless, it will still take at least 30 days before the rapidly desorbing fraction is released, as calculated with the maximum mass transfer of NP from the sediment to the water phase during resuspension under non-sterile conditions.

6.3.4 Perspectives of other estrogenic compounds

In this study we used NP as a model compound for aerobically biodegradable estrogenic compounds. Previously we have shown that NP has the same availability and biodegradation behaviour as the total mixture of other predominantly unknown estrogenic compounds in this sediment. This was assessed by measuring estrogenic activities before and after aerobic treatment of the sediment (Chapter 5). From the results from the reactor experiments with NP and our previous study, it can be expected that other estrogenic compounds present in this sediment will also desorb and be degraded like NP. In addition, a similar increased mass transfer to the bulk water during resuspension as found for NP can be expected for these other estrogenic compounds. As these estrogenic compounds generally also need oxygen for their degradation, oxygen depletion by processes such as eutrophication and degradation of organic matter (Paerl et al., 1998; Smith et al., 1999; Taft et al., 1980) lead to an increased estrogenic compound concentrations in bulk river water, because biodegradation cannot counter balance the desorption and mass transfer. More estrogenic compounds will enter the bulk water during resuspension than when the sediment is present in a sediment bed. The released estrogenic compounds can potentially cause estrogenic effects, especially upon sediment resuspension combined with low oxygen or (temporary) anoxic conditions. Therefore, anoxic conditions should be prevented to reduce the risk for effects of these estrogenic compounds for the aquatic systems.

6.4 Conclusions

From our reactor experiments can be concluded that the experimental set up can be used to study combined processes of NP desorption and NP biodegradation. Under settling sediment conditions, a continuous amount of NP is transferred from the sediment bed to the bulk water. Biodegradation reduces the NP concentrations in the bulk water by counter balancing desorption and mass transfer from the sediment bed to the bulk water. When changing to resuspended sediment conditions, the mass transfer of NP to the bulk water initially increases sharp under sterile and non-sterile conditions due to the release of NP from pore water and enlargement of the sediment desorption area. This initial mass transfer

is larger than the amount of NP that can be biodegraded with the optimal biodegradation rate. Therefore, resuspension leads to an initial increased environmental risk. After two days of resuspending the sediment, more NP is released under non-sterile conditions than under sterile conditions due to biological processes most likely affecting the adsorption properties of the sediment. The mass transfer becomes comparable to the amount of NP that can be biodegraded with an optimal biodegradation rate during a longer period of resuspension under non-sterile conditions. Finally we expect that the environmental risk can be reduced during longer periods of resuspension by biodegradation if the environmental conditions are optimal for biodegradation. Therefore, the environmental parameters should be optimized, and especially sufficiently high dissolved oxygen concentrations are important.

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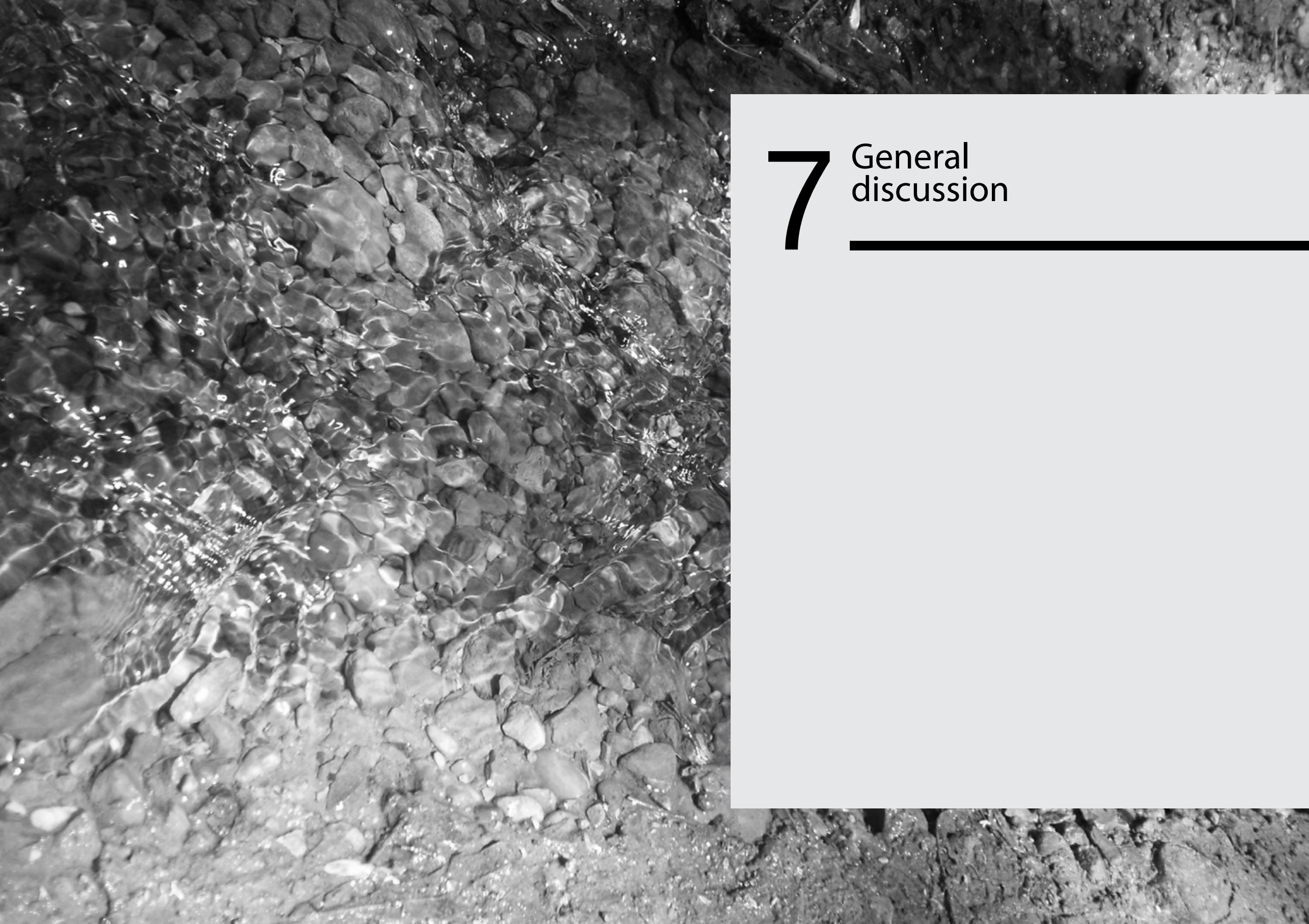

7 General discussion

7.1 Introduction

River basins contain many xenobiotic compounds. One of these compounds is nonylphenol (NP), which is abundantly present as pollutant, (Heemken et al., 2001; Isobe et al., 2001; Jonkers et al., 2003; Lacorte et al., 2006; Li et al., 2004; Petrovic and Barcelo, 2000). Nonylphenol exists of a phenol group with a linear or a branched chain of nine carbon atoms at the para-position of the aromatic ring (Figure 7.1). The NP found in the environment mainly consists of a mixture of branched isomers (Di Corcia et al., 1998; Giger et al., 1984).

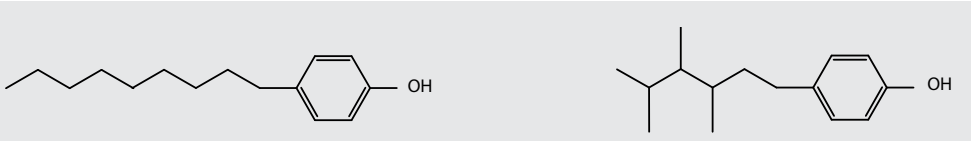

Figure 7.1: Molecular structure formula of a) linear nonylphenol and b) branched nonylphenol isomer.

Nonylphenol adsorbs to sediments due to its chemical properties like hydrophobicity and low water solubility. The adsorbed NP can desorb again from the sediment, and therefore, these NP polluted sediments may act as a secondary source in polluting river water. The desorbing NP may cause toxicological effects to aquatic organisms due to its estrogenic properties. The environmental risk of NP adsorbed to sediments depends on the combination of availability, the mass transfer from the sediment to the river water, and vice versa and the biodegradation of NP. Insight into the biodegradation and availability of NP in the sediment is necessary for accurate risk assessments of NP supporting river basin management. The aim of this research was to obtain qualitative and quantitative insight into the biodegradation potential and the effect of the biodegradation on the environmental toxicity, the availability and the mass transfer of NP and comparable compounds in polluted river sediments. Therefore, biodegradation and availability experiments were performed and transport of NP from sediment to water was mimicked in lab scale experiments with NP polluted sediment from the Huerva River, which is a tributary of the Ebro River, in Spain.

7.2. Biodegradation of nonylphenol

The most effective way for risk reduction is biodegradation of NP to non harmful compounds. Therefore, anaerobic and aerobic biodegradation of the technical mixture of branched NP isomers, known as tNP, present in the polluted sediment was studied (chapters 3 and 4). Besides tNP, the biodegradation of linear NP (4-*n*-NP) was investigated as well (Chapter 3), although 4-*n*-NP is hardly found in the aquatic environment and not present in the used sediment. This isomer was used to investigate possible biodegradation pathways.

In the literature, the biodegradation of 4-*n*-NP and tNP under aerobic conditions has been frequently described (Ekelund et al., 1993; Gabriel et al., 2005; Hesselsoe et al., 2001; Langford et al., 2005; Tanghe et al., 1999). However, under anaerobic conditions the biodegradation of 4-*n*-NP has only been described by the group of Chang. Biodegradation has been reported under nitrate reducing, sulphate reducing and methanogenic conditions (Chang et al., 2007; Chang et al., 2005; Chang et al., 2004). So far, no anaerobic biodegradation of tNP has been described. In this thesis we report that 4-*n*-NP can be degraded under nitrate reducing conditions in the sediment we used (Chapter 3). However, this NP isomer was found to be persistent under sulphate and methanogenic reducing conditions in our experiments. Furthermore, tNP was not biodegraded under all tested anaerobic conditions. Phenol was used as a structure analogue for the different NP isomers, but could not initiate the biodegradation of the branched isomers. The microbial populations involved in the biodegradation of 4-*n*-NP in our experiments showed that the most abundant present microorganisms were related to bacteria that biodegrade linear alkane structures. Such structures are similar to the structure of the nonyl chain of 4-*n*-NP. From this investigation was concluded that the biodegradation of 4-*n*-NP under nitrate reducing conditions starts at the nonyl chain of the 4-*n*-NP, and not at the phenol group. Because the degradation under nitrate reducing conditions started at the chain and not at the phenol group, phenol as structure analogue to initiate biodegradation is not useful. Initiation of the biodegradation with structure analogues similar to the branched nonyl-chain structures of NP is complicated, because there are more than 100 different NP isomers. The commercial technical mixtures of NP contain a couple of major isomers (Figure 7.2) (Russ et al., 2005).

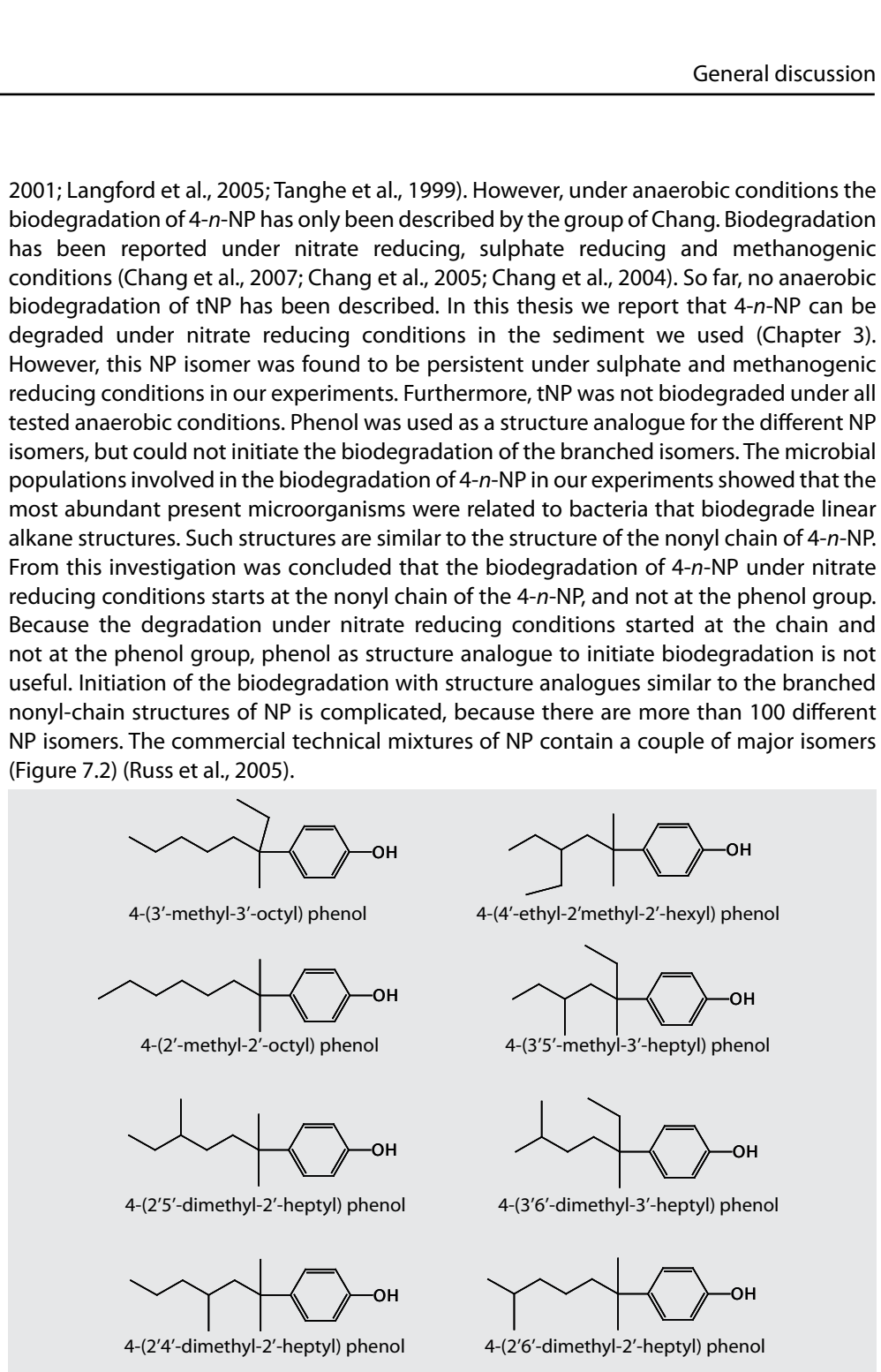

Figure 7.2: Structures of branched NP isomers which are major present in the technical mixture (adapted from Russ et al., 2005).

Structure analogues of these major present isomers are probably useful to initiate biodegradation, but they should be biodegradable under anaerobic conditions. However, no literature is available about the possibilities of anaerobic biodegradation of these branched alkane structures. Due to the persistence of these branched alkane structures under anaerobic conditions and that the anaerobic biodegradation of NP under anaerobic conditions starts at the chain, makes the biodegradation of tNP under anaerobic conditions not likely to occur.

Fast biodegradation of 4-*n*-NP and tNP under aerobic conditions was observed in our sediment. The tNP in the sediment was biodegraded within 6 to 8 days after a lag phase of 2 days (Chapter 4). The fast biodegradation indicated that the microorganisms were already adapted to tNP biodegradation under aerobic conditions even though the sediment was anaerobic. The biodegradation was not only observed in batches with sediment and medium but also in batches with sediment and river water from the same location (Chapter 6). This indicates that the tNP can be degraded under aerobic field conditions in the sediment at the sampling location in the Huerva River. A wide range of microorganisms is able to biodegrade 4-*n*-NP and tNP aerobically as the identified NP biodegrading microorganisms in the aerobic enrichments were different from those described in literature. This may lead to good opportunities for field bioremediation strategies.

Although a wide range of microorganisms is involved in aerobic biodegradation of tNP, this does not automatically indicate that tNP polluted sediments will be remediated by natural occurring biodegradation (Natural Attenuation). The reason is that sediments are mainly anaerobic (Huttunen et al., 2006; Martin et al., 1998; Middeldorp et al., 2003) and tNP is persistent under anaerobic conditions. If biodegradation occurs, this can only take place at the interface of sediments and the aerobic river water. Therefore, tNP will remain present as a pollutant in the sediment until all the tNP has been desorbed from the sediment to the aerobic river water. To biodegrade the tNP that enters the bulk water, oxygen is required. Therefore, depletion of the dissolved oxygen should be prevented. To prevent anoxic situations, it is very important for river basin management to overcome eutrophication by nutrients and organic matter run off to the river water, and to ensure refreshment of river water with oxygen rich and nutrient poor water (Bradley et al., 2008).

7.3 Availability and nonylphenol as model compound

The availability of NP is important for the potential risk of NP in the sediment and the possible bioremediation. Availability experiments, as described in Chapter 5, showed that almost all the NP (95%) in the used sediment was potentially available. The large availability of NP in the used sediment and the high NP concentrations compared to sediments in other parts of the Ebro or other rivers (Blackburn et al., 1999; Heemken et al., 2001; Isobe et al., 2001; Jonkers et al., 2003; Navarro et al., 2009b; Rice et al., 2003), results also in a high potential environmental risk. However, the potential available fraction in the sediment used in this research could be completely biodegraded and led to an equal reduction of the estrogenic activity of the sediment, and thus a reduction of the environmental risk of the sediment. The results of the estrogenic activity of the sediment showed that other estrogenic compounds are present in the sediment, since the estrogenic activity was more than only the contribution of NP to the estrogenic activity could account for. In general, E1, E2 and EE2 are the most common estrogens, although, they could not be identified in the sediment used in this research. The chemicals with estrogenic properties in the sediment were analyzed with the ER_{α}-*luc* assay as a sum parameter. For the response of the ER_{α}-*luc* assay, we found the same behaviour in availability and biodegradation of the mixture of estrogens as for NP. The available fraction and reduction in total estrogenic activity after aerobic treatment of the sediment was comparable to the available fraction of NP and the reduction of the NP concentration after aerobic biodegradation. Therefore, it can be concluded that NP in this specific sediment can be used as model compound for this sediment in monitoring the estrogenic activity of the total mixture of estrogenic compounds and the related toxicological risk for the aquatic environment.

The use of NP as a model compound to predict the estrogenic activity may also be useful for other locations, because estrogens such as E1, E2 and EE2 are present in many sediments as they mainly enter the environment via discharges from waste water treatment plants (Mes et al., 2005). The occurrence of NP is also often related to discharges of waste water treatment plants (Petrovic et al., 2002; Rice et al., 2003; Wu et al., 2007). The benefit of using NP compared to the other common estrogens is that NP is present in many river sediments in detectable concentrations, which makes monitoring easy. Other estrogenic compounds are often present in low concentrations, even below detection limits (Mes et al., 2005), but their contribution to the estrogenic activity of the sediment can be significant. For example, the estrogenic activity of E1, E2 and EE2 is approximately 10^5 times higher than that of NP (Legler et al., 2002a; Legler et al., 2002b). Compared to NP, these estrogens have the same estrogenic activity as NP at 10^5 times lower concentrations and can therefore cause more severe estrogenic effects because of their higher estrogenic activity. The often low concentrations of these compounds at or below detection limits for chemical analysis make these estrogens unsuitable as model compound for more detailed studies as we report here, and unsuitable as a guide compound for water and sediment monitoring. From the results of this study it is proposed to further develop water and sediment monitoring methods and programs with NP as guide compound, which can lead to a cost-effective approach to monitor river basins. Whether the correlation between availability and biodegradability of NP and other estrogens is valid for other sediments, as we demonstrated for the polluted sediment of the Huerva River, should be further investigated. A possible disadvantage of NP as model compound is that products with 0.1% or more NP or NPE have been banned for further use since 2005. With this restriction, the environment will be less or not further be polluted with NP. However, the amount of NP polluted sediments world wide is very large. The NP is already present in these sediments for years and, as we showed in this study, will remain there for a long time, presumably decades. Therefore, NP will remain a threat for the environment, and will

be a useful model guide compound for monitoring the group of aerobically degradable estrogenic organics.

7.4 Mass transfer of nonylphenol

The mass transfer of a compound that desorbs from the sediment to the bulk water, is mainly driven by the concentration gradient between the compound in sediment particles and the surrounding water, and the particle surface area (Birdwell et al., 2007; Cheng et al., 1995). To what extent anaerobic persistent and aerobic biodegradable compounds can really reach the bulk water and be harmful for the aquatic life in the river water, depends on the ratio between the mass transfer and the biodegradation rate at the sediment-water interface. When the biodegradation rate is larger than the mass transfer rate of a compound, the amount that desorbs from the sediment particles can be completely degraded. When the biodegradation rate is smaller, the compound enters the river water. The mass transfer of NP from the used sediment to the bulk water was minimal at settling sediment conditions compared to resuspended sediment conditions (Chapter 6). In the presence of a sediment bed over a longer period of time (5 weeks), a constant flux of NP enters the bulk water. Although the concentration in the bulk water, due to this desorption is low, long time exposure to low concentrations of NP in the river water can lead to accumulation in aquatic organisms (Snyder et al., 2001). This mass transfer will continue for a long time (several years to decades) before all NP has been desorbed from the polluted sediment. The NP that desorbs from the sediment bed can be biodegraded as long as the bulk water contains enough oxygen. This biodegradation reduces the environmental risk. Therefore, it can be stated that at settling sediment conditions, the biodegradation rate and the desorption rate in the sediment used in this research are balanced, and it can be assumed that at these conditions natural attenuation maintains minimal environmental risks as long as the environmental conditions are appropriate for biodegradation of NP. That means that at least high enough oxygen concentrations for aerobic NP biodegradation need to be present in the surface water.

Resuspension of the sediment leads to an immediate increase of the mass flux to the bulk water. This increase is due to the enlargement of the desorption area and the release of NP in the pore water that is mixed with the bulk water during resuspension. At this moment, the release of NP is much faster than the optimal biodegradation rates as measured in Chapter 6, and the natural attenuation can not counter balance the mass transfer of NP, which leads to an increase of the environmental risk. During longer periods of resuspension, the mass transfer of NP from the sediment particles becomes gradually comparable to the biodegradation rate under optimal conditions. However, biodegradation rates in the field are often lower compared to biodegradation rates obtained in lab experiments. Therefore, it can be expected that the NP that desorbs during resuspension will not be completely biodegraded, and will reach the bulk water where it can cause ecotoxicological effects. Insight into the mass transfer rate and the biodegradation rate under field conditions is therefore important for river basin managers to make a realistic risk prediction. This accounts not only for NP but also for other pollutants in the sediment, which are persistent under anaerobic conditions and can be biodegraded under aerobic conditions. These compounds will also desorb to the water phase, and resuspension will increase their mass transfer.

7.5 Perspectives for river basin management

Polluted sediments play an important role in the water quality, as seen in Chapter 6. The Water Framework Directive (WFD) does not consider sediment quality so far (Heise, 2008), and therefore only the water quality is monitored. Because polluted sediments influence the water quality, especially under changing hydraulic conditions, measures to reach the goals of the WFD may only be effective when transport of pollutants to and from sediments are fully taken into account. Furthermore, rivers and sediments will change due to changes in climate and land use. Therefore, it is not sufficient to take only the current status of water and sediment systems into account. Combined river morphology and environmental studies are required to develop an overall system approach to get insight into contaminant mass transport and biodegradation and to assess long term river water quality according to the WFD.

It is recommended to include monitoring of the sediment quality in the WFD, and take measures to improve the sediment quality. Resuspension of polluted sediment results in an increase of the contaminant mass transfer to the surrounding water, and this effect should be taken into account in natural or controlled floodings. A measure that can be taken is capping of the polluted sediment. With capping, the polluted sediment is covered with clean sediment, gravel, rock and/or synthetic materials. In this way, the polluted sediment is not in direct contact with the bulk water, will not get resuspended, and is not harmful for the environmental during floodings as long as the cap resists the water shear. If capping of the sediment is not possible, because of a large impact of the capping on hydraulic forces or the water depth, it is recommended to keep the conditions in the river water as optimal as possible for aerobic biodegradation.

For compounds which are persistent to biodegradation, mass transfer to bulk water will determine their risk. For sediments present in a bed, this means that small amounts of these persistent compounds continuously enter the bulk water for many years. For resuspended sediments, this transport will be larger. Although the concentrations may be low and will not cause acute toxic effects on the short term, these compounds end up in the ecosystem and may accumulate in the food chain. In addition, the presence of two or more toxic compounds can be additive or synergistic, even if the single concentration of these compounds is below the no-observed-effect concentration (Rajapakse et al., 2002; Silva et al., 2002). Therefore, monitoring sediment quality and taking measures to minimalize the pollutant mass transfer from sediments to water and to optimize the conditions for biodegradation are needed in sound environmental management of rivers.

Measures with the largest impact to improve the environmental quality are regulating primary and secondary polluting sources, and preventing further pollution of the

environment. Applying, respecting and enforcing these regulations is essential in good and adequate environmental protection. There are many persistent compounds in sediments posing ecotoxicological risks for the aquatic environment and humans for decades into the future. From the research presented here and by others, we know that reduction of ecotoxicological risks is possible. We understand the problem of contaminated sediments to a great extent, and we have gained insights into possible solutions. Let us bring that knowledge to practice and improve the quality of our rivers and sediments.

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List of abbreviations

4-*n*-NP	= linear nonylphenol
BLAST	= Basic Local Alignment Search Tool
DGGE	= denaturing gradient gel electrophoresis
E1	= 17ß-estradiol
E2	= estrone
EE2	= 17α-ethynylestradiol
ER-CALUX	= Estrogen Receptor -mediated Chemical Activated Luciferase gene eXpression
ER_α-*luc* assay	= in vitro reporter gene assay
FID	= flame ionization detector
GC	= gas chromatograph
GC-MS	= gas chromatography-mass spectrometry
HPLC	= high pressure liquid chromatography
K_{OW}	= octanol-water partition coefficient
K_{OC}	= organic carbon partition coefficient
LAS	= linear alkyl benzene sulphonate
NCBI	= National Center for Biotechnology
NMR-analysis	= Nuclear Magnetic Resonance analysis
NP	= nonylphenol
NPnE	= nonylphenol ethoxylate
PAH	= polycyclic aromatic hydrocarbon
SPE	= solid phase extraction
SPME	= solid phase microextraction
STP	= sewage treatment plant
tNP	= (technical mixture of) branched nonylphenol
US-EPA	= US Environmental Protection Agency
YES-assay	= Yeast Estrogen Assay
WDF	= Water Framework Directive

Curriculum Vitae

Jasperien Petra Antoinette, born on 16th of January 1976 in Loon op Zand, the Netherlands obtained in 1994 her "Atheneum" diploma at the Pauluslyceum in Tilburg. Thereafter, she studied Environmental Sciences at the Agricultural University Wageningen (now Wageningen University and Research centre) with the specialization Environmental Technology. She focused on soil remediation and microbiology, and carried out two MSc-projects at the laboratory of microbiology and at the sub department of environmental technology. At the laboratory of microbiology, she studied the effects of various electron acceptors on anaerobic tetrachloroethene degradation. The topic at the sub department of environmental technology was focused on the bioavailability of polycyclic aromatic hydrocarbons. She performed an internship in Japan at the National Institute of Bioscience and Human Technology, where she studied the aerobic biodegradation of 2,4-dichlorophenoxyacetic acid.

After her graduation in 2000, she started working in a consultancy firm HMB group in Maasbree, as project leader on soil research and soil remediation. During this work she supervised projects on soil pollution and advised stakeholders, provinces and local communities about soil remediation and use of polluted sites.
In June 2004, she started with her PhD project at TNO (now Deltares) and WUR, and was involved in the European 6th Framework project Aquaterra. The results of this PhD research are presented in this thesis.

At present, she is working at Deltares as researcher on soil remediation and sediment pollution.

List of Publications

Luijten, M., Weert, J. de, Smidt, H., Boschker, H., Vos, W. de, Schraa, G. and Stams, A. (2003)
Description of *Sulfurospirillum halorespirans* sp. nov., an anaerobic, tetrachloroethene-respiring bacterium, and transfer of *Dehalospirillum multivorans* to the genus *Sulfurospirillum* as *Sulfurospirillum multivorans*.
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Jasperien de Weert
September 2009

“Soms moet je een gevecht vaker dan een keer leveren om te winnen”
Margaret Thatcher (1925) in The Observer, 14 - 01 - 1979




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° Summer school: Biomonitoring, bioavailability and microbial transformation of pollutants in sediments and approaches to stimulate their biodegradation, University of Genova, 12 – 14 September 2005, Genova, Italy
° Summer school Chemicals in soil: Interactions, availability and residue formation, UFZ, 24 – 27 September 2009, Leipzig, Germany

Research and Management Skills:

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° AquaTerra Final Conference, 25 March 2009, Tübingen, Germany

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