Environmental fate of phenolic endocrine disruptors: field and laboratory studies

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Alkylphenolic compounds derived from microbial degradation of non-ionic surfactants became a major focus of environmental research in the early 1980s. More toxic than the parent compounds and weakly oestrogenic, certain metabolites of nonylphenol polyethoxylate (NPnEO) surfactants, especially nonylphenol (NP), raised sustained concern over the risk they pose to the environment and triggered legal measures as well as partly voluntary actions by the manufacturing industry. Continuous progress in the development of analytical techniques is crucial to understand how these alkylphenolic compounds behave in wastewater treatment, the aquatic environment and in laboratory experiments. Measured concentrations and mass flows of phenolic endocrine disruptors, particularly nonylphenolic compounds, bisphenol A and parabens in municipal wastewater effluents and in the Glatt River, Switzerland, show that rain events leading to discharges of untreated wastewater into rivers have a great impact on the riverine mass flows of contaminants. Biotransformation experiments in our laboratory with nonylphenoxyacetic acid and individual NP isomers enabled the elucidation of degradation pathways of these compounds. The finding that nonylphenoxyacetic acid is metabolized via NP further underscores the role of NP as the most relevant metabolite in the degradation of NPnEO. Several *Sphingomonadaceae* bacterial strains were found to degrade α-quaternary 4-NP isomers by an *ipso*-substitution mechanism, and to use only the aromatic part of the molecule. These reactions turned out to be isomer specific, meaning that rate and extent of transformation depend on constitution, and possibly also on the absolute configuration of the alkyl side chain of a specific isomer. The observation that NP isomers with distinct oestrogenic activities are differentially degraded has significant implications for risk assessment.

Keywords: nonylphenol; nonylphenoxyacetic acid; biotransformation;
oestrogenic activity; wastewater; rivers

1. Introduction and retrospect

The history of environmental chemical pollutants is dominated by several chemical compound classes of different characteristics such as petroleum hydrocarbons, polycyclic aromatic hydrocarbons, synthetic surfactants, polychlorinated

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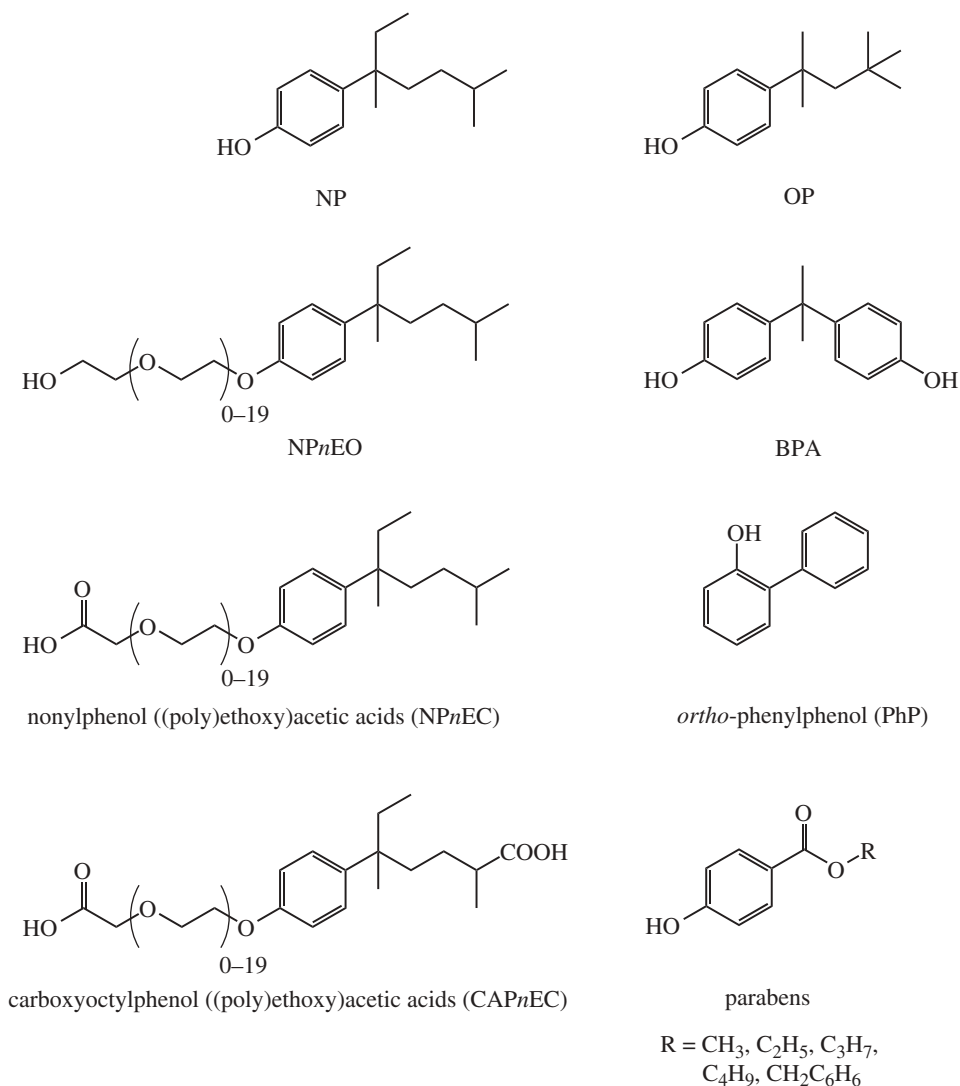One contribution of 12 to a Theme Issue 'Emerging chemical contaminants in water and wastewater'.

Figure 1. Structures and acronyms of nonylphenolic compounds and phenolic endocrine disruptors. Note that in the case of NP the *para*-substituted isomer, NP_{112}, is drawn as an example. In the case of OP, the only isomer contained in commercial surfactants is drawn.

insecticides, polychlorinated biphenyls, polychlorinated dibenzodioxins, arsenic, heavy metals, tin organics, etc. Among the technically produced surfactants, nonylphenol polyethoxylates (NPnEO) and their metabolites became a major focus of environmental research when it became evident in the early 1980s that alkylphenolic compounds derived from non-ionic surfactants of the alkylphenol polyethoxylate type are significant environmental contaminants. In figure 1, structural formulae and acronyms for the prevalent nonylphenolic substances are presented. Besides nonylphenol (NP) and octylphenol (OP), we discuss here some other weakly oestrogenic phenolic substances, among them bisphenol A (BPA) and parabens (figure 1).

Environmentally relevant nonylphenolic compounds, such as NP, NP mono-ethoxylate (NP1EO), NP diethoxylate (NP2EO), nonylphenoxyacetic acid (NP1EC) and nonylphenoxyethoxyacetic acid (NP2EC), do not appear in the environment because of direct use in chemical products, but they are formed in the course of the biological degradation of NPnEO. These chemicals are commercially important non-ionic surfactants still used in the USA and in many other countries as products for industrial, agricultural, household and institutional applications. However, NPnEO surfactants are banned for water relevant uses in the EU and in Switzerland. NP, NP1EO and NP2EO are known weakly endocrine disrupting chemicals (EDCs; Sumpter & Johnson 2008; Martin & Voulvoulis 2009). Reports on the environmental occurrence of polar organic water pollutants indicate that the NPnEO metabolite NP1EC is among the most abundant contaminants in European rivers (Reemtsma *et al.* 2006; Jonkers *et al.* 2009; Loos *et al.* 2009).

Table 1 lists milestones in the history of NPnEO surfactants as synthetic technical products and of nonylphenolic compounds as environmental contaminants. The increase in knowledge on the environmental occurrence and fate of nonylphenolic compounds was strongly promoted by the evolution in analytical methodology. Regarding an environmental risk assessment, it became apparent that the nonylphenolic metabolites of NPnEO surfactants, in particular NP, were more toxic than the parent substances (McLeese *et al.* 1980; Comber *et al.* 1993). In the early 1990s, several researchers discovered the significant oestrogenic effects of NP and other NPnEO metabolites (Soto *et al.* 1991; Jobling & Sumpter 1993), which greatly raised the concern and scientific interest in nonylphenolic compounds as environmental contaminants (for reviews, see Soares *et al.* (2008), Sumpter & Johnson (2008) and Martin & Voulvoulis (2009)).

Detailed risk assessments have been performed for some of the phenolic chemicals discussed in this article, particularly for NP and BPA. In the NP case, this has led to legal restrictions of the NPnEO use in many countries. In the European Water Framework Directive, NP is considered a Priority Substance and Environmental Quality Standards for surface waters have been set at 0.3 and 2.0 μg l^{-1} for annual average and maximum allowable concentrations, respectively.

The research on nonylphenolic substances at Eawag, the Swiss Federal Institute of Aquatic Science and Technology, started in the early 1980s with the identification and quantitative measurement of the NPnEO metabolites NP, NP1EO and NP2EO in treated municipal wastewaters (Giger *et al.* 1981; Stephanou & Giger 1982) using capillary gas chromatography (GC) with flame ionization (FID) and mass spectrometric (MS) detection. Subsequently, accumulation of the toxic NP in digested sewage sludge was observed (Giger *et al.* 1984). Municipal mechanical-biological wastewater treatment plants (WWTPs) were then investigated using a mass flow analysis or mass balance approach (Brunner *et al.* 1988; Ahel *et al.* 1994a). During his PhD studies, Ahel (1987) accomplished a series of substantial projects at Eawag and at the Rudjer Boscovic Institute in Zagreb that formed the basis for our current understanding of the environmental fate of these compounds (for references, see table 1). New analytical techniques based on liquid chromatography (LC) were developed, process-oriented field studies in WWTPs and in rivers carried out and laboratory experiments performed.

Table 1. Milestones in the history of nonylphenolic substances as environmental contaminants.

technological product development

1933	invention of polyethoxylate surfactants by C. Schoeller, BASF, Germany
1937	switch from vegetable oil based aliphatic raw material to coal derived phenolics because of supply problems

analytical methods

1960	collective determination of non-ionic surfactants	
1967	thin-layer chromatography of non-ionic surfactants	Patterson *et al.* (1967)
1980	GC/FID, GC/MS	Giger *et al.* (1981), Stephanou & Giger (1982)
1984	LC/UV absorption, LC/UV fluorescence	Ahel (1987), Ahel & Giger (1985*a*,*b*)
2001	LC/MS, LC/MSMS	Jonkers *et al.* (2001, 2009), Ferguson *et al.* (2001)
1995	high-resolution GC for NPs	Wheeler *et al.* (1997)
2009	comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry (GCGC/TOFMS)	Eganhouse *et al.* (submitted)

environmental occurrence

1981	NP, NP1EO and NP2EO in treated municipal wastewater	Giger *et al.* (1981), Stephanou & Giger (1982)
1984	NP in digested sewage sludge	Giger *et al.* (1984)
1985	NPnEC in wastewater	Ahel (1987), Ahel *et al.* (1987, 1994*a*), Field & Reed (2001)
1985	nonylphenolic compounds in rivers	Ahel (1987), Ahel *et al.* (1994*b*)
1985	nonylphenolic compounds in groundwater	Ahel (1987), Ahel *et al.* (1996)
2000	CAPnEC in wastewater	Di Corcia *et al.* (1998, 2000)

environmental fate and behaviour

1968	studies on non-ionic detergent degradation	Patterson *et al.* (1968, 1970)
1973	biodegradability of NPnEO	Rudling & Solyom (1974)
1988	mass flows in wastewater treatment	Brunner *et al.* (1988), Ahel *et al.* (1994*a*)
1987	physico-chemical behaviour	Ahel (1987)
2005	biotransformation of individual NP isomers	Gabriel *et al.* (2005*a*,*b*, 2007*a*,*b*, 2008), Kohler *et al.* (2008)

environmental effects and risk assessment

1980	lethality of Aminocarb components	McLeese *et al.* (1980)
1993	effects of NP on *Daphnia magna*	Comber *et al.* (1993)
1991	oestrogenic activity of NP	Soto *et al.* (1991)
1993	oestrogenic activity of NPnEO metabolites	Jobling & Sumpter (1993)
2003	EU risk assessment for NP	

legal measures

1986	partial ban of NPnEO surfactants in Switzerland
1987	voluntary agreement in Germany
2003	comprehensive ban of NPnEO surfactants in the EU
2007	environmental quality standard for NP within the EU Water Framework Directive

miscellaneous

2001	synthesis of individual NP isomers	Boehme *et al* (submitted)
2006	proposal of a numbering system for NP isomers	Guenther *et al.* (2006)

Table 1 includes an overview of the studies that have been performed since the early 1980s at Eawag, where the most recent research phase was based on two projects that were part of the Swiss National Research Programme on 'Endocrine Disruptors: Relevance to Humans, Animals and Ecosystems'. The bulk of this article summarizes the results of these projects. Analytical field studies were carried out using liquid chromatography coupled either to fluorescence or to mass spectrometric detection (Wettstein 2004; Voutsa *et al.* 2006; Jonkers *et al.* 2009). A relatively large project focused on a comprehensive river basin study in the Glatt valley in Switzerland encompassing WWTP effluents and rivers. A second focus was on biotransformation studies in the laboratory, which profited enormously from the availability of NP1EC (Wettstein 2004) and of individual NP isomers (Gabriel *et al.* 2005*a*,*b*, 2007*a*,*b*, 2008; Kohler *et al.* 2008). We present investigations on the biotransformation of nonylphenoxyacetic acid and individual NP isomers under laboratory conditions. Careful analysis of the results enabled us to significantly enhance the knowledge on the corresponding biotransformation pathways.

2. Field studies on occurrence and behaviour

(*a*) *Analytical methods*

During nearly four decades, the prevailing coverage in analytical environmental chemistry was on volatile and semivolatile non-polar/lipophilic contaminants, because GC as the predominantly employed analytical technique provided excellent separations for these types of pollutants. However, very polar and amphiphilic contaminants (e.g. anionic, non-ionic and cationic surfactants) could only be determined by GC after derivatization or transformation of the analytes into more volatile species. Amphiphilic substances (surfactants, surface-active agents) were for a long time determined almost exclusively by collective parameters without separation into individual components. The more volatile NP, NP1EO and NP2EO could still be reliably determined by GC (Giger *et al.* 1981; Stephanou & Giger 1982). The introduction of high-performance liquid chromatography (HPLC or LC) enabled the specific determination of several surfactant classes including the alkylphenolic compounds by ultraviolet absorption or fluorescence detection (Ahel 1987; Ahel & Giger 1985*a*,*b*; Ahel *et al.* 1987, 2000*a*; Marcomini & Giger 1987).

With the development of new ionization techniques such as electrospray ionization and atmospheric pressure chemical ionization, liquid chromatography directly coupled to mass spectrometry (LC/MS) has become since the mid-1990s a routinely applicable and robust method. LC/MS allows a far better coverage of polar/hydrophilic and amphiphilic contaminants (Giger 2009). Furthermore, the extremely high selectivity and sensitivity of multiple reaction monitoring techniques in tandem/multi-stage quadrupole and ion-trap mass spectrometry (MSMS) enable the analyses of trace constituents of complex mixtures. Typical examples of LC/MSMS application to alkylphenolic substances and other surfactants were published by Ferguson *et al.* (2001), Jonkers *et al.* (2001, 2009), Gonzalez *et al.* (2007) and Loos *et al.* (2007, 2008).

(*b*) *Municipal wastewater, wastewater treatment and rivers*

Since the pioneering reports on nonylphenolic compounds in municipal wastewater and wastewater treatment effluents by the Eawag group (table 1), many authors have published original reports (e.g. Ferguson *et al.* 2001; Jonkers *et al.* 2001) and reviews on this topic (e.g. Soares *et al.* 2008; Staples *et al.* 2008; Martin & Voulvoulis 2009). Ahel *et al.* (2000*b*) revisited the earlier Eawag findings from the 1980s and documented the effects of risk reduction measures introduced in 1986 and of the improved performance of wastewater treatment. The situation is complicated, because we have to consider a complex technical product with mixtures of polyethoxymers and isomers as well as several metabolites with shorter polyethoxy substituents and possibly one or two carboxylate groups, NPnEC and CAPnEC, respectively (figure 1).

It is generally accepted that the carboxylated metabolites are at least partly formed during activated sludge treatment in the WWTPs, which may lead to rather high levels in secondary effluents and subsequently in receiving ambient waters (Ahel *et al.* 1987; Di Corcia *et al.* 2000). This particular situation is the reason why in a more recent investigation at Eawag, Wettstein (2004) studied NPnEOs and their respective metabolites with an emphasis on NP1EC. That study was performed in two parallel treatment trains at the wastewater treatment facility in Kloten/Opfikon, Switzerland: (i) the full-scale conventional wastewater treatment train using activated sludge treatment and a sand filter and (ii) a pilot-scale membrane bioreactor. Only small concentration differences were observed among effluent samples originating from either treatment train. NP, NP1EO and NP2EO elimination rates ranged from 82 per cent to 96 per cent. The NP1EC elimination rate reached 46 per cent in the membrane reactor and 58 per cent in the conventional plant. Metabolite concentrations decreased by up to 75 per cent during passage of the wastewater through the sand filter. Conventional wastewater treatment without sand filter achieved a lower elimination rate compared to that of the membrane plant. These data indicate that a relatively simple additional treatment like a sand filter provides an increased bioelimination of contaminants such as NP1EC.

The study by Jonkers *et al.* (2009) focused on the occurrence and behaviour of several phenolic EDCs including alkylphenolic compounds, BPA, phenylphenol (PhP) and parabens in municipal wastewaters and in the River Glatt near Zurich. A strong emphasis was put on discussing not only concentrations but also mass flows and comparing measured river data with the results of calculations based on a mass balance approach.

Table 2 shows the concentrations for the investigated EDCs. Both WWTP influents and effluents as well as river water were dominated by the alkylphenolic compounds, which were present at several micrograms per litre in WWTP influents, several 100 to 1000 ng l^{-1} in WWTP effluents and up to several 100 ng l^{-1} in the Glatt. OP, BPA and PhP concentrations in river water were a few nanograms per litre, whereas WWTP influent levels were several 100 ng l^{-1} for BPA and PhP and a few tens of nanograms per litre for OP. As a result of the phasing-out of alkylphenol polyethoxylates since the late 1980s and improvements in the performance of the municipal wastewater treatment, concentrations of alkylphenolic compounds were more than an order of magnitude lower than the values found in the River Glatt in 1984 (Ahel 1987; Ahel *et al.* 1994*b*).

Table 2. EDC concentrations ($ng\,l^{-1}$) in WWTP influents and effluents as well as in the Glatt River (adapted from Jonkers *et al.* 2009).

	WWTP influent			WWTP effluent			average removal in	Glatt River		
	median	min.	max.	median	min.	max.	WWTP (%)	median	min.	max.
NP1EO	1140	201	7030	34	15	139	95.1	18	<7.3	42
NP2EO	1890	596	4060	40	16	335	97.0	9.4	2.8	110
NP2–7EO	6820	1450	21 700	119	15	579	97.7	71	<0.9	624
NP8–10EO	257	14	1740	0.6	<0.9	16	99.3	0.8	<0.9	16
NP1EC	2650	465	9160	444	126	3280	77.0	135	69	335
NP2EC	1570	351	6050	394	183	4270	70.0	53	23	168
NP3,4EC	252	91	914	227	120	2680	−5.8[a]	36	24	101
NP	473	70	1240	123	<29	281	76.2	64	<29	195
OP	19	<1.2	3860	1.3	<1.2	8.8	89.7	2.4	<1.2	6.9
BPA	414	226	1640	24	1.3	707	74.5	9.4	2.0	46
PhP	254	98	640	15	<2.1	171	92.7	6.9	<2.1	16
methylparaben	724	65	9880	11	4.6	423	96	5.0	3.1	17
ethylparaben	129	2.2	719	0.2	<0.3	17	98.5	0.1	<0.3	1.6
propylparaben	430	43	1540	1.3	<0.5	28	99.5	0.6	<0.5	5.8
butylparaben	211	9.7	864	0.3	<0.2	12	99.5	0.3	<0.2	2.8
benzylparaben	0.2	<0.2	4.1	0.1	0.2	16	—[b]	0.0	<0.2	4.4

[a]For NP3,4EC, an average formation was observed.
[b]As many samples showed concentrations below limit of detection, a removal efficiency could not be determined.

The current concentrations of NP and BPA in the Glatt are similar to those found in 2004 (Voutsa *et al.* 2006). High concentrations above $1\,\mu g\,l^{-1}$ were found for parabens in WWTP influents, whereas their concentrations in river water were in the low nanogram per litre range. The relative concentrations of the five measured parabens reflect their consumption pattern, with methyl- and propylparaben being most abundant.

Based on WWTP effluent and influent concentrations, the efficiencies of EDC removal from the dissolved phase were calculated (table 2). For PhP and parabens, high removal percentages (averages of 93% and 98%, respectively) were observed at all WWTPs. Median removal percentages for NPnEO (greater than 97%) were high in all WWTPs, but NPnEO metabolites were not always removed with high efficiency, and at two WWTPs the authors even observed an increase in concentrations of NPnEC and NP, metabolites derived from NPnEO.

Water flows in the study area varied considerably during the sampling period. Two large rain events caused a strong increase in both wastewater and river flows during days 2, 3, 10 and 11. During these high-flow days, the wastewater flow exceeded the WWTP capacities and all WWTPs had to discharge some of the raw wastewater influx directly, without treatment into the Glatt. For most compounds, mass flows in WWTP effluents and in the river became greater with increasing water flows, indicating that pollutants were not diluted in proportion to the increase in flow. In an attempt to achieve mass balances for EDCs in the Glatt River, the total discharge of all known EDC sources (Greifensee outlet and

WWTP effluents) and the mass flow actually measured at the end of the river were compared. Throughout the low water flow period, mass flows predicted on the basis of the known inputs were similar to the actual mass flows at the end of the river for most analytes. This indicates that during low-flow days, all sources were accounted for, and no significant in-stream removal occurred in the Glatt River. In the periods with high water flows, however, mass flows of NPnEC in the river were much higher than could be explained by the initially defined sources. Clearly, on those days, not all NPnEC sources were accounted for. It is likely that at least part of the additional NPnEC mass during high-flow days originated from the untreated wastewater discharged into the Glatt on these days. With these additional sources included, the predicted mass flows for NPnEC, BPA and PhP were rather similar to the actual ones. Considering discharge of untreated wastewater for OP, NPnEO and methylparaben, however, led to an overprediction of the riverine mass flows, which may mean that these substances were partly degraded in overflow basins. It is even possible that in-stream removal is more efficient during days on which highly biologically active wastewater influent is discharged. Yet during dry periods, only BPA showed a significant in-stream removal, as indicated by comparison of actual and predicted riverine mass flows.

As a conclusion, rain events and corresponding discharges of untreated wastewater appear to be of key importance when considering mass flows of contaminants in the aquatic environment. The study of Jonkers *et al.* (2009) also conclusively demonstrates that actual chemical monitoring of the water is necessary, as current hydrological fate models based on general consumption data of chemicals do not give any information with regard to the situation during the frequently occurring rain events.

3. Laboratory studies on biotransformation and effects

Primary biodegradation of NPnEO in WWTPs is quite efficient. However, overall biodegradation seems to be complicated by several bottlenecks that enhance the formation of metabolites, such as NP2EO, NP1EO, NP, NP1EC and NP2EC, and by unspecific oxidation reactions yielding metabolites with oxidized alkyl and ethoxy side chains (CAPnEC) (Di Corcia *et al.* 1998, 2000). These metabolites are not as easily transformed as the parent compounds and transiently accumulate so that they can be found in sewage treatment effluents (Brunner *et al.* 1988; Ahel *et al.* 1994*a*; Field & Reed 1996; Di Corcia *et al.* 2000), sewage sludges, rivers (Giger *et al.* 1984; Ahel *et al.* 1994*b*, 2000*a,b*; Field & Reed 1996; Jonkers *et al.* 2001), estuaries (Dachs *et al.* 1999; Potter *et al.* 1999; Ferguson *et al.* 2001; Jonkers *et al.* 2003), coastal waters (Kvestak *et al.* 1994) and sediments (Ferguson & Brownawell 2003; Ferguson *et al.* 2003).

Transformation of NPnEO to NP2EO and NP1EO metabolites is well established and bacteria able to grow on NPnEO as sole carbon source have been isolated (Maki *et al.* 1994; John & White 1998). Such bacteria degrade NPnEO by stepwise *exo*-scission of single glycol units with the accumulation of NP2EO and NP1EO as major end products. The proposed mechanism involves an oxygen-independent hydroxyl shift from the terminal to the penultimate carbon of the terminal ethoxylate unit of NPnEO and a

dissociation of the resulting hemiacetal to release acetaldehyde and the next-lower homologue. This $NP(n-1)EO$ metabolite then undergoes further cycles of the same reaction until the ethoxy chain is reduced to two or one ethoxy units (John & White 1998).

Until recently, transformation reactions of $NPnEO$ and its metabolites other than the ones mentioned above were not very well understood in terms of mechanisms and micro-organisms involved. The reaction schemes were mainly based on measuring concentrations of the various metabolites in environmental compartments and microcosm experiments. Such data usually do not allow deciding whether the detected metabolites arise as a consequence of a steady-state situation with relatively high turnover or are slowly accumulating dead end products formed by non-specific side reactions. For selected sewage treatment plants in Switzerland, it has been estimated that—on a molar basis—about 60 per cent of the $NPnEO$ were removed and about 40 per cent were released to ambient receiving waters (Ahel *et al.* 1994*a*) in the form of NP, $NPnEC$, NP1EO, NP2EO and untransformed $NPnEO$. About one-third of the removed $NPnEO$ was in the form of NP attached to digested sludge and about two-thirds might have been mineralized.

Here we discuss the most recent research findings that help to better understand the microbial metabolism of $NPnEO$ and NP and to shed better light on the role of NP in further transformation of NP1EC. As technical NP is a complex mixture of more than 100 isomers, which differ in the structure and the position of the alkyl moiety attached to the phenol ring (Ieda *et al.* 2005), the availability and the use of single NP isomers became of utmost importance for the elucidation of the degradation pathway. Throughout this paper, the various 4-NP isomers are numbered according to the systematic numbering systems proposed by Guenther *et al.* (2006).

(*a*) *Biotransformation of nonylphenol isomers*

First hints that micro-organisms are able to metabolize alkylphenolic compounds such as NP, OP and octylphenoxyacetic acid under aerobic conditions came from studies in which 2,4,4-trimethyl-2-pentanol was detected as a metabolite when OP (Tanghe *et al.* 2000) and octylphenoxyacetic acid (Fujita & Reinhard 1997) were incubated with *Sphingomonas* TTNP3 and groundwater enrichment cultures, respectively. Later, it could be shown that several *Sphingomonas* strains were able to use NP as sole carbon and energy source (Tanghe *et al.* 1999; Fujii *et al.* 2001; Gabriel *et al.* 2005*a*), and incubation experiments with these strains and individual NP isomers laid ground for the elucidation of the mechanism of the degradation pathway of NP.

Degradation experiments with defined mixtures of NP isomers indicated that *Sphingobium xenophagum* strain Bayram differentially transformed these isomers. The degradation rate showed a positive correlation with the branching degree of the alkyl substituent—the more highly branched, the faster the degradation (Gabriel *et al.* 2005*a*). These findings strongly contrasted with what had been the prevailing view on the degradation of technical NP mixtures (Tanghe *et al.* 1999; Topp & Starratt 2000; Corvini *et al.* 2004*b*) and emphasized the importance of considering isomeric effects.
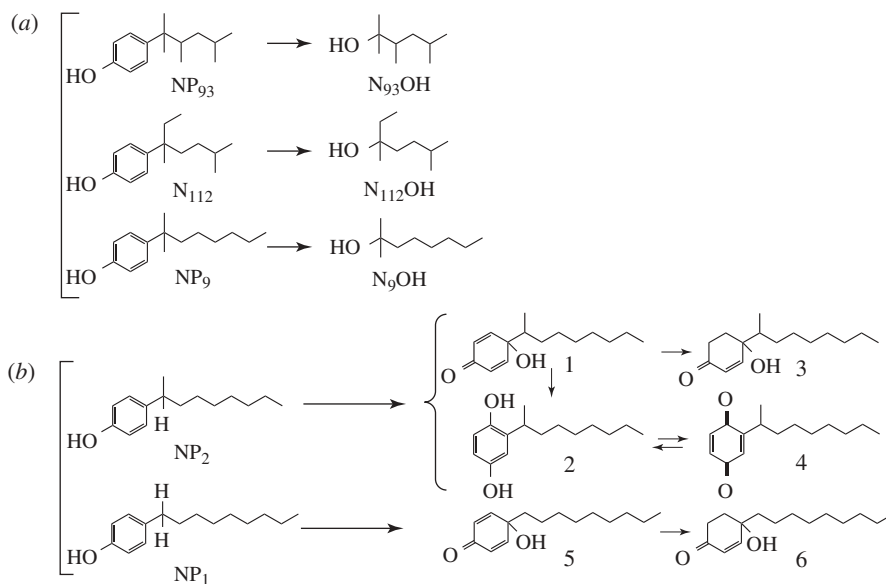
Figure 2. Co-metabolic transformation of NP_1 and NP_2 and formation of metabolites by strain Bayram in the presence of NP isomers that serve as growth substrates. (*a*) NP isomers that serve as substrates. (*b*) Formation of metabolites from NP isomers that do not serve as growth substrates and are only co-metabolically transformed. For details, see Gabriel *et al.* (2005*b*, 2007*a*) and Kohler (2008). Adapted from *CHIMIA*. Copyright © 2008 (Swiss Chemical Society).

Further incubation experiments with a mixture of five isomers led to the detection and identification of nonylalcohols that originated from the isomers with α-quaternary carbon atom at the benzylic position (figure 2). However, no traces of potential nonanol metabolites derived from NP isomers with hydrogen atoms at the benzylic position were detected in these experiments, although the corresponding NP isomers were partly degraded. This was an indication that NP isomers with hydrogen atoms at the benzylic position were metabolized differently from the more highly branched isomers. It was proposed (Gabriel *et al.* 2005*a*,*b*; Kohler *et al.* 2008) that although such isomers do engage in the main degradation pathway, they are only incompletely degraded, because they are unable to undergo the reaction in which the alkyl group is detached from the aromatic part. These data further indicated that the ring moiety had to be cleaved from the alkyl substituent to be used and that the alkyl moieties of the alkylphenol isomers that serve as growth substrates are transformed to alkyl alcohols with retention of the structures of the original alkyl side chains. Conventional pathways of alkylphenol metabolism could not explain the formation of such metabolites.

Growth experiments with strain Bayram and several pure α-quaternary 4-NP isomers that could serve as growth substrates (4-NP_{93}, 4-NP_{112} and 4-NP_9) did not reveal any metabolites except the C_9 alcohols derived from the nonyl side chains (figure 2*a*). However, experiments in which non-growth isomers, such as 4-NP_2 and 4-NP_1, were co-metabolically transformed in the presence of the growth substrate 4-NP_{112} resulted in the detection of metabolites that could be isolated in milligram quantities (figure 2*b*). Their chemical structures were elucidated by NMR spectroscopy and mass spectrometry (Gabriel *et al.* 2005*b*).
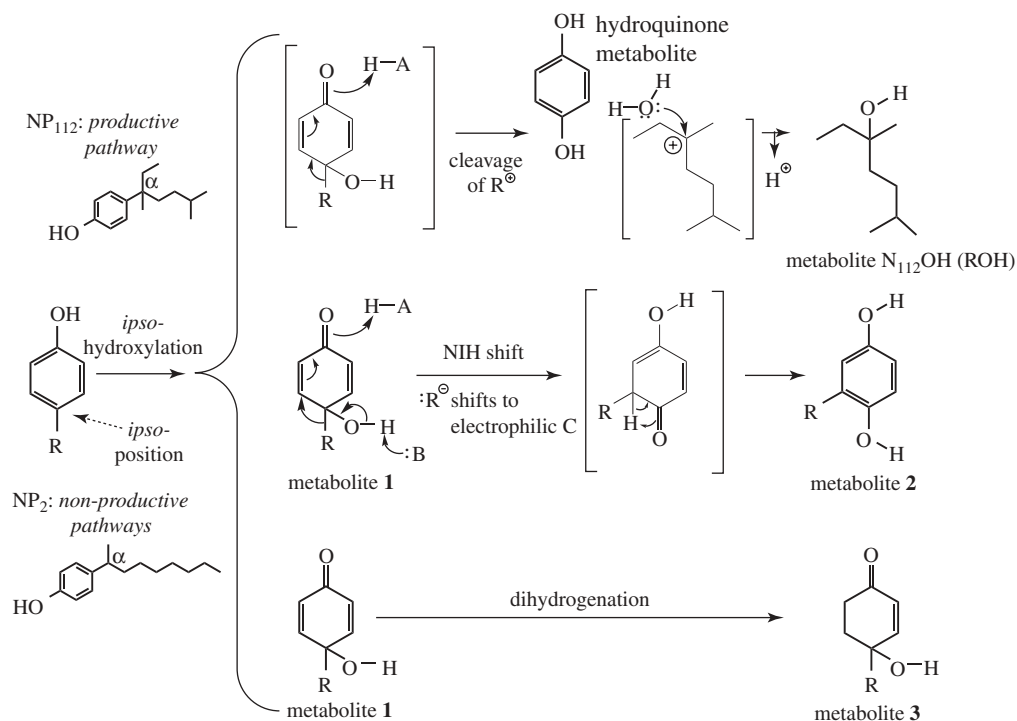
Figure 3. Pathways proposed for the degradation of NP isomers in *S. xenophagum* Bayram. NPs are transformed by an initial *ipso*-hydroxylation to 4-alkyl-4-hydroxycyclohexadienone intermediates (e.g. metabolite **1**). As an example for a productive pathway (large arrow) NP isomer NP_{112} and as an example for a non-productive pathway isomer NP_2 are shown. α-Quaternary intermediates dissociate by releasing the alkyl moiety as a cation, which is stabilized by α-alkyl branching. The formed hydroquinone is then further metabolized. Degradation experiments with ^{18}O-labelled oxygen and water clearly showed that the detached cation preferentially reacts with the nucleophilic oxygen atom of a water molecule forming the corresponding nonanol (e.g. $N_{112}OH$), which most likely cannot be further metabolized. 4-Alkyl-4-hydroxycyclohexadienone intermediates with hydrogen at the α-position are not able to dissociate at all or they undergo an NIH shift yielding metabolites that cannot be further degraded. A–H and B denote a proton donor and acceptor, respectively. For details, see Kohler (2008). Adapted from *CHIMIA*. Copyright © 2008 (Swiss Chemical Society).

Figure 2 shows these metabolites, and figure 3 shows an NP degradation scheme that is based on the results of these growth experiments. The structures of metabolites **1** and **5** (figures **2** and **3**), both with an additional hydroxy substituent at the 4-position of the ring, were strong indicators that the initial reaction was a hydroxylation at the anchor carbon atom of the alkyl substituent—an *ipso*-attack—yielding 4-alkyl-4-hydroxycyclohexadienones. By analogy, the authors concluded that the growth substrate isomers also underwent an initial *ipso*-hydroxylation. However, intermediates derived from growth substrate isomers did not accumulate, as they spontaneously broke down to the corresponding alkyl cation and hydroquinone (figure 3; Gabriel *et al.* 2005*b*, 2007*a*).

Experiments with ^{18}O-labelled dioxygen and ^{18}O-labelled water clearly indicated that the *ipso*-hydroxy group was derived from molecular dioxygen and the resulting nonanol metabolite contained an oxygen atom from

water (Gabriel *et al.* 2007*a*). These results clearly proved that the initial *ipso*-hydroxylation reaction in the degradation pathway was catalysed by a monooxygenase and they led to the conclusion that the alkyl cation that was released from the substituted cyclohexadienone intermediate preferentially reacted with water (figure 3, productive pathway) to form the corresponding alcohol. In *Sphingomonas* sp. TTNP3, nonanols are formed from NP by a similar mechanism (Corvini *et al.* 2006; Kolvenbach *et al.* 2007). The breakdown of the 4-alkyl-4-hydroxycyclohexadienone intermediate represents an S_N1 reaction, whereby the carbocation reacts with the solvent and the dissociation energy is provided by rearomatization of the leaving carbon ring. In contrast to the substituted cyclohexadienones derived from α-quaternary NPs, metabolites **1** and **5** did not undergo detachment of the alkyl moiety because the corresponding α-carbocations were not sufficiently stabilized by alkyl substituents in α-position, but they might serve as substrates for side reactions (figures **2** and **3**).

The formation of metabolite **2** (figures **2** and **3**), a 2-alkylhydroquinone, involves a (1,2–C,C)-shift of the alkyl substituent, a reaction well known as NIH shift (Gabriel *et al.* 2005*b*), and most likely represents a non-productive side reaction that only becomes relevant when other reactions are unfavourable as is the case for 4-NP isomers with hydrogen atoms at the benzylic α-carbon. Small amounts of NIH-shift products have also been detected in cell extracts of other NP degrading strains (Corvini *et al.* 2004*a*).

Laboratory experiments revealed that *S. xenophagum* strain Bayram significantly changed the isomer distribution pattern of technical NP when growing with this substrate as the sole carbon and energy source (Gabriel *et al.* 2008). Figure 4 depicts the outcome of such an 'ageing' experiment, in which about 86 per cent of 4-NP was consumed during 9 days of incubation. A strong correlation between transformation of individual isomers and their α-substitution pattern was observed. As a rule, isomers with less bulkiness at the α-carbon and those with an optimally sized main alkyl chain (four to six carbon atoms) were degraded most efficiently. The authors concluded that differential microbial metabolism of technical NP mixtures, as observed in the laboratory experiments, will ultimately lead to changes in the relative composition of partially degraded material in the environment. They argued that environmental NP isomer patterns as observed in Tokyo Bay (Horii *et al.* 2004) and in composted biosolids (Das & Xia 2008) may be well explained by the *in situ* activity of bacterial strains that metabolize NPs by *ipso*-substitution, as strain Bayram does. In the future, detailed analysis and comparison of NP isomer patterns in different environmental compartments will certainly be a valuable means of helping to reveal the dominating transformation processes in such compartments.

(b) Biotransformation of nonylphenoxyacetic acid

Although NP1EC and NP2EC are the dominating NP*n*EO metabolites in secondary effluents of sewage treatment plants (Ahel *et al.* 1987, 1994*a*), not much is known about further metabolism of these compounds. However, two recent studies (Wettstein 2004; Montgomery-Brown *et al.* 2008) gave indications that NP1EC is metabolized via NP. In one study, the authors performed aerobic and microaerobic microcosm experiments, in which they incubated NP1EC with organic carbon-poor soils from Arizona as well as with organic carbon-rich
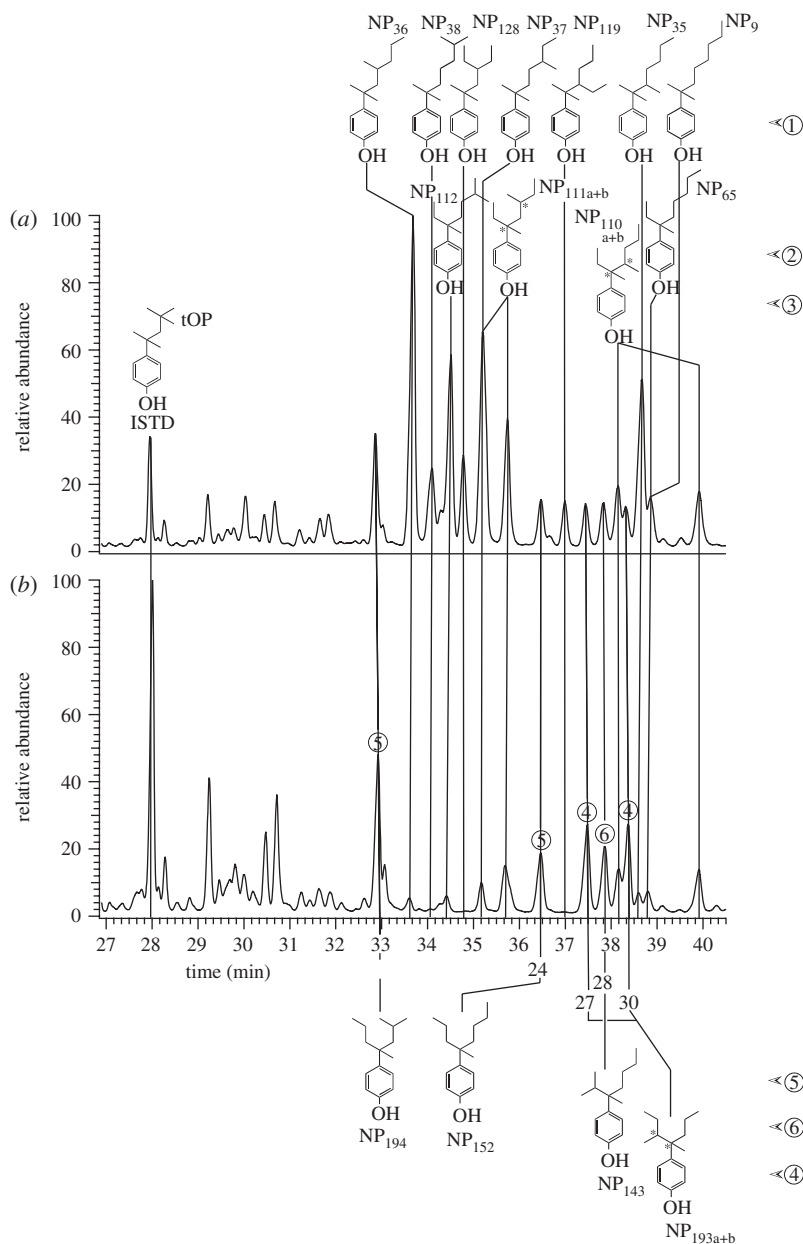
Figure 4. Ageing of technical NP as a result of degradation by *S. xenophagum* Bayram in minimal medium. GC/MS chromatograms (total ion current) of the culture extracts correspond to (*a*) the start of the experiment and (*b*) day 9 of the incubation. Note that differential degradation leads to a significant change in the isomer distribution of the NP mixture. The isomer pattern in the non-inoculated control was identical to that of non-degraded technical NP (*a*). Peaks of recalcitrant isomers (groups 4, 6, and 5) are highlighted (encircled numbers). The great majority of peaks eluting before peak 1 most likely correspond to *ortho*-isomers. Signals were numbered according to their retention time. The vertical position of a mass spectrometric group indicates the recalcitrance of its members to biodegradation by strain Bayram (group numbers—encircled numbers at the far right—are listed according to increased recalcitrance). Adapted from *Environmental Science and Technology*. Copyright © 2008 (American Chemical Society).
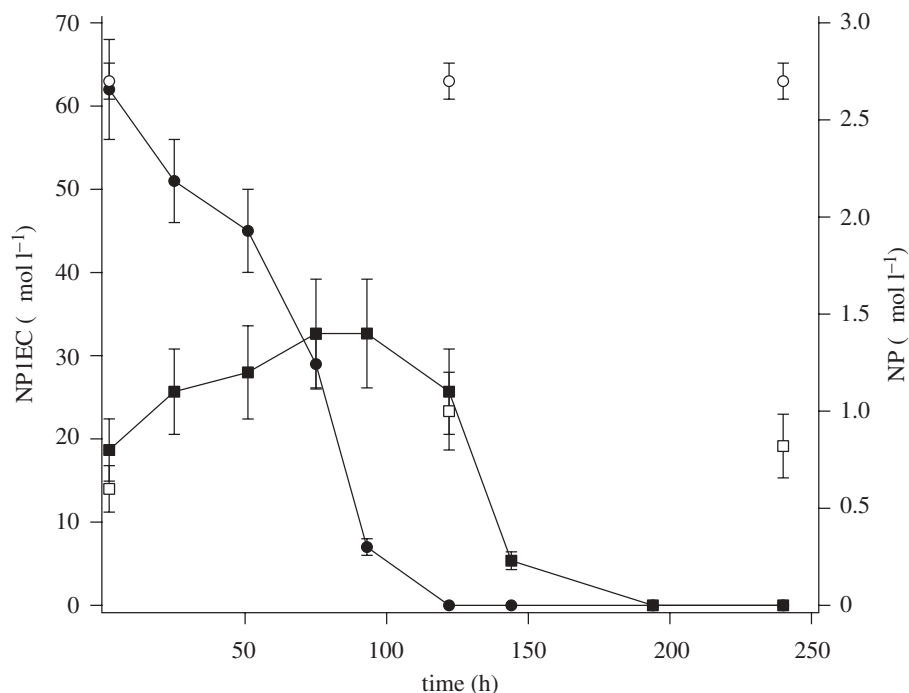
Figure 5. Aerobic degradation of NP1EC incubated with activated sludge from a sewage treatment plant. Besides the progress curves for NP1EC (filled circles) and NP (filled squares), values for the sterile controls for NP1EC (open circles) and NP (open squares) are shown. For details, see Wettstein (2004).

sediments from California (Montgomery-Brown *et al.* 2008). The results suggested that the oxygen tension determined the dominant biodegradation pathway. Under fully aerobic conditions (Arizona soils), ether cleavage with formation and subsequent degradation of NP was favoured, while under microaerobic conditions (California sediments) alkyl chain oxidation with formation of CAPnEC was favoured. The authors clearly showed that in the microcosms with Arizona soils (high oxygen tension) *para*-NP1EC was transformed to *para*-NP, which was then quickly turned over to nonyl alcohols by *ipso*-hydroxylation (Montgomery-Brown *et al.* 2008).

In the second study, degradation experiments with activated sludge from a municipal WWTP in Switzerland were performed under aerobic conditions (Wettstein 2004). Figure 5 shows that in such incubations NP transiently increased during degradation of NP1EC. At the end of the experiment, both NP1EC and NP were completely metabolized. The fact that the transient accumulation of NP was not very pronounced indicates that under such conditions the rate limiting process most likely was the transformation of NP1EC to NP. This also might explain why the formation of NP from NPnEC under aerobic conditions has not been observed previously in studies on the environmental fate of NPnEC (Fujita & Reinhard 1997; Potter *et al.* 1999; Wild & Reinhard 1999).
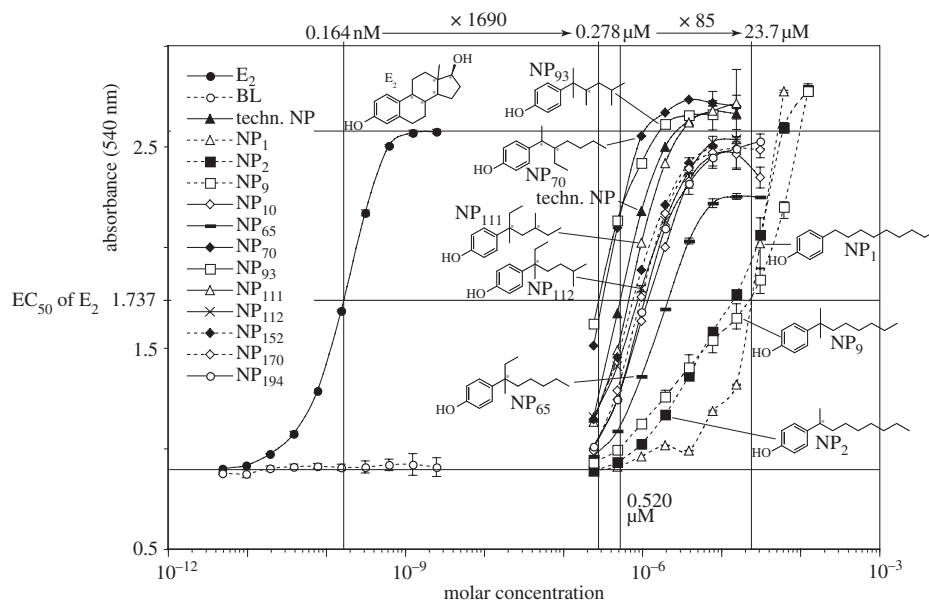
Figure 6. Endocrine activity (YES assay) of a range of NP isomers. The graph depicts the logarithmic concentration of 17β-oestradiol (E$_2$) serially diluted from 2.5 nM to 4.9 pM and of NP isomers diluted from 0.13 to 0.24 μM, plotted against the absorbance of the medium after a 3-day incubation at 32°C. The blank (BL) shows the response of the assay in the absence of test compound. Values represent mean±standard deviation from duplicate wells. Structures of selected test compounds are shown. The vertical lines depict the concentrations used to determine the potencies of NP$_{93}$, NP$_1$ (the most and least potent isomers of our test series, respectively) and 17β-oestradiol, relative to that of technical NP. For details, see Gabriel *et al.* (2008). Adapted from *Environmental Science and Technology*. Copyright © 2008 (American Chemical Society).

(c) Oestrogenic activity of nonylphenol isomers

Three recent studies clearly demonstrate that NP isomers differ in oestrogenic activity (Preuss *et al.* 2006; Shioji *et al.* 2006; Gabriel *et al.* 2008). Figure 6 shows the response of the yeast oestrogen screen (YES) to various concentrations of a range of NP isomers (Gabriel *et al.* 2008). The NP isomers generally produced full dose response curves over a range of two orders of magnitude, with the most potent isomer (NP$_{93}$) being only about 1700 times less potent than 17β-oestradiol, and nearly twice as potent as the technical mixture of isomers. Despite the difference in cell reporter systems used and except for one NP isomer, the oestrogenicity rankings of the three studies were in excellent agreement. The results of the three studies also well support the hypothesis of Shioji *et al.* (2006) that an optimal length of the main alkyl chain (four to six carbon atoms) together with bulkiness around the β- and γ-position is necessary for high oestrogenicity, whereas the presence of α-substituents is less important.

Differential degradation of NP isomers together with the fact that NP isomers have different oestrogenic activities has significant implications for future risk assessments. Analysis of the degradation experiment with technical NP indicated that isomers with sterically hindering α-substituents tend

to be recalcitrant. This holds well for, for example, NP_{193a} and NP_{193b}, which have bulky α-CH_3, α-$CH(CH_3)C_2H_5$ substitutions and are the most recalcitrant among the main isomers in technical NP (Gabriel *et al.* 2008). However, they are also characterized by β-substitution and by a main alkyl chain of four carbon atoms, which implies high oestrogenic activity. It is clear that if recalcitrant components of technical NP prove to be highly oestrogenic, then there is a substantial risk that microbial degradation by *ipso*-substitution will increase the specific oestrogenicity of the remaining material. Further research on varying isomer composition and oestrogenicity of NP mixtures during microbial degradation will be needed to establish whether certain highly oestrogenic isomers are recalcitrant and whether microbial transformation indeed increases the specific oestrogenicity of technical NP in the environment.

(*d*) *New views on microbial transformations of nonylphenolic compounds*

Figure 7 shows a schematic overview of biotransformation reactions that have been described to date (see also Montgomery-Brown & Reinhard 2003; Kohler *et al.* 2008, and references therein). As pointed out in this paper, it is now well established that certain bacteria are able to degrade NP by *ipso*-substitution (figure 7, reaction 1). These bacteria are able to grow at the expense of the aromatic part of the molecule and leave behind the alkyl side chain as the corresponding alkyl alcohol, whose ultimate fate still remains unknown. We also presented new information on the microbial transformation of NP1EC, as there are now strong indications that NP1EC is metabolized via NP (figure 7, reaction 2). However, we now also realize that certain transformation reactions in the reaction network shown in figure 7 (especially reactions 1 and 3) are isomer specific. This means that the rate or the extent of the transformation or both are dependent on the isomeric structure of the alkyl side chain of a specific NP isomer. To be transformed by *ipso*-substitution NP isomers require α-quaternary carbon atoms as structural components; however, the bulkiness of these α-substitutions is another critical factor that determines transformability by *ipso*-substitution. The degradation ranking established for differential degradation of technical NP by strain Bayram clearly indicated that degradation was more effective when the α-position was less bulky. The length of the main alkyl chain also seems to play an important steric role, as isomers with little bulkiness at the α-position but lengthy alkyl chains were rather slowly transformed (Gabriel *et al.* 2008). From these findings, we can safely conclude that differential microbial metabolism of technical NP mixtures by *ipso*-substitution will ultimately lead to changes in the relative composition of the remaining material. We therefore suggest that NP residues in different environmental matrices will be characterized by distinct isomeric fingerprints that depend on the grade of degradation and on the dominating degradation process.

Furthermore, isomer selectivity is also evident for reaction 3, the formation of CAP*n*EC from NP*n*EC (Montgomery-Brown *et al.* 2008). The authors observed accumulation of only certain CA_6PEC isomers in their microcosm experiments, which is a strong indication that CAP*n*EC formation or removal processes are isomer selective. As ω-oxidation with a subsequent β-oxidation step has been suggested to be the dominant process for the formation of CA_6PEC, it
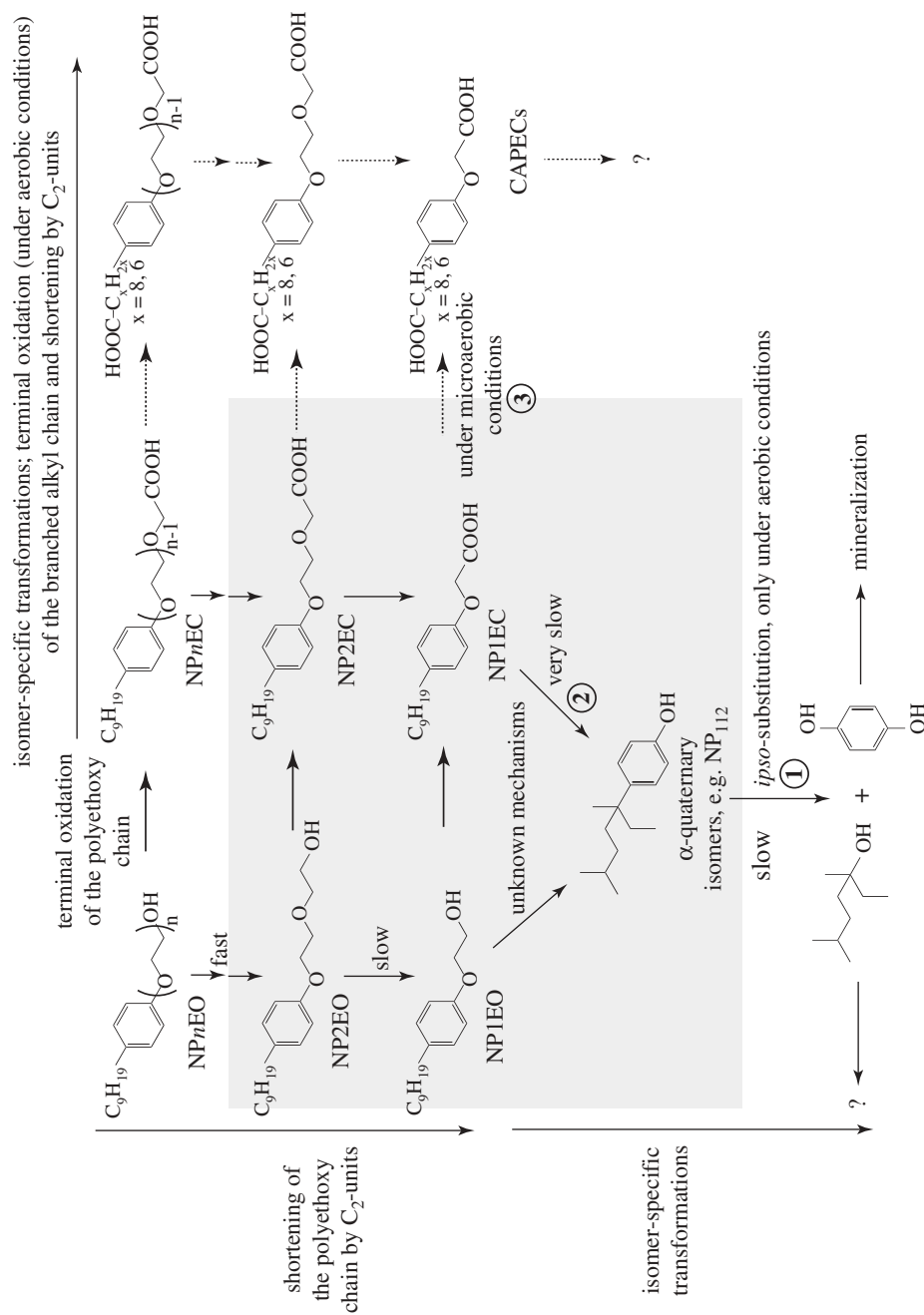
Figure 7. New views on the transformation reactions of nonylphenolic compounds. The compounds highlighted by the shading are all weakly oestrogenic. For encircled numbers assigned to specific reactions, refer to the main text.

is reasonable to assume with regard to technical NP that only isomers with a main alkyl chain length of more than three carbon atoms and without terminal or sub-terminal alkyl chain branching will be able to undergo chain shortening by β-oxidation; e.g. NP_9 and NP_{35} would be expected to be transformed into CA_6PEC under suitable conditions, whereas NP_{38}, NP_{37} and NP_{111} would not.

Figure 7 also conveys the notion that microbial transformation of $NPnEO$ comprises a network of transformation reactions, each being selective to a certain degree and dependent on certain conditions. For a specific nonylphenolic compound, the dominant reaction pathway and therefore the ultimate fate of the compound will depend on the compound's isomeric structure and other chemical properties as well as on the prevailing reaction conditions. Especially, oxygen availability is a significant environmental reaction parameter. Under strict anaerobic conditions, NP turned out to be the end product of $NPnEO$ transformation (Ahel *et al.* 1994*a*,*b*; Montgomery-Brown & Reinhard 2003; Wettstein 2004). Under aerobic conditions further transformation of certain NP isomers by *ipso*-substitution (Gabriel *et al.* 2007*a*,*b*, 2008; Kohler *et al.* 2008) and the formation of $CAPnEC$ from $NPnEC$ is possible. Moreover, oxygen tension also seems to be an important factor in determining the reaction pathway of $NPnEC$. Under low oxygen tension, formation of $CAPnEC$ is significant, whereas under high oxygen tension further metabolism via NP and subsequent *ipso*-substitution is favoured (Montgomery-Brown *et al.* 2008).

4. Conclusions and outlook

Our several decades long involvement in work on the environmental fate and effects of phenolic and in particular alkylphenolic chemicals has shown us many times the pivotal importance of multi-disciplinary cooperation of analytical and environmental chemists, microbiologists, environmental engineers and ecotoxicologists. Only through such close cooperation could the presented insights be achieved. Publications of projects deficient of such an interdisciplinary approach clearly lack optimum performance.

Steadily advancing analytical chemical methodology will continue to increase the knowledge obtainable from full-scale treatment plants, ambient waters and laboratory investigations. Important developments are on-line enrichment and large-volume injections into ultra-efficient LC systems coupled to multi-stage and high-resolution mass spectrometry. It can be expected that future routine methods will operate without time and labour consuming off-line enrichment of trace constituents in aqueous samples. But also multi-dimensional chromatographic systems such as comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry (GCGC/TOFMS) have started contributing to the efficient analyses of complex isomeric mixtures such as technical NP (Eganhouse *et al.* submitted).

In order to get reliably interpretable results from full-scale studies in wastewater treatment and particularly in ambient water systems, there is a clear need for sophisticated and extensive sampling concepts. Otherwise, the highly complex input situations and the impact of hydrological dynamics are not sufficiently considered. We very much doubt whether small numbers of grab samples from larger rivers or very different river types distributed all over

Europe and with no control on the hydrological situations can provide reliable databases. Our data from the Glatt River in Switzerland clearly show that such a relatively small river catchment system is already highly dynamic under different weather conditions.

All the new findings of the laboratory studies described in this paper help to better understand the microbial metabolism and the environmental fate of nonylphenolic compounds. But they also have led us to realize that a network of various reactions leads to a plethora of possible metabolites depending on isomeric structures and environmental conditions. Because of bottlenecks in reaction pathways, such metabolites might be present at significant steady-state concentrations in certain environments even when the prevailing reaction pathways ultimately lead to mineralization.

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