

PRESENCE AND FATE OF THE ENDOCRINE DISRUPTER OCTYLPHENOL

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Abigail Elizabeth Wise

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Abigail Elizabeth Wise, Ph. D.

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Octylphenol (OP) and nonylphenol (NP) are structurally similar surfactant metabolites that have estrogenic properties. NP has been shown to undergo a type II *ipso* substitution degradation mechanism, however no gene(s) have been identified that encode this activity. *Sphingomonas* sp. PWE1 was isolated from sewage sludge enrichments based on its ability to grow on OP. PWE1 DNA was heterologously expressed in an *E. coli* fosmid library. Clone aew4H12 was identified and sequenced with transposon mutagenesis to reveal a single open reading frame, *opdA*, which had homology with FAD-dependent monooxygenases. When *opdA* was subcloned in *E. coli*, OP disappearance was accompanied by hydroquinone and 2,4,4-trimethyl-1-pentene formation. This demonstrates that the type II *ipso* substitution observed in PWE1 during OP degradation is encoded by *opdA*.

OP degradation in PWE1 was then compared with NP degraders *Sphingomonas* sp. TTNP3 and *Sphingobium xenophagum* Bayram. Both strains were found to contain homologs to *opdA*. When expressed in *E. coli*, the cloned genes were found to be necessary for OP disappearance and concomitant formation of both HQ and 2,4,4-trimethyl-1-pentene. Southern hybridizations showed differences between the wild-type strains when probed with *opdA*. Induction experiments with wild-type strains demonstrated that OP-degrading activity and *opdA* mRNA accumulation were inducible in Bayram, but not in PWE1 or TTNP3. Gene sequencing revealed 5 amino acid differences between the three strains. While the gene sequence was conserved,

there appeared to be differences in genetic context and activity in the wild-type strains, suggesting regulatory differences. These differences were most obvious with respect to degradation of OP-related substrates

NP degradation has been shown to be temperature-dependent in laboratory-scale studies so we wondered if sewage sludge collected during different seasons might have different levels of pollutants. NP and OP, as well as the antibacterial triclosan and fragrance compound HHCB, were monitored seasonally in anaerobically digested sludge samples from New York State. Samples from four sites were analyzed by accelerated solvent extraction and GC-MS. There were variations in the seasonal pollutant levels in sludge from the same site, but no correlations between sites, suggesting NP, OP, triclosan, and HHCB concentrations in sludge were not seasonally dependent.

BIOGRAPHICAL SKETCH

Abigail Wise Porter was born in Manheim, Pennsylvania. She grew up with an interest science, nature and the desire to understand why things happen. She attended Manheim Central Senior High School and graduated in 1998 with honors.

After graduation she attended Rutgers, the State University of New Jersey – New Brunswick, as a student at Cook College majoring in biotechnology. While there Abigail was a member of the honors fraternity Alpha Zeta, the Golden Key honors society, and the Cook College Leadership Program. She also selected as a participant in an undergraduate exchange program between Rutgers and the University of São Paulo and visited several campuses in Brazil during a week-long undergraduate research conference. Through her involvement in Cook College General Honors Program, she obtained a position as an undergraduate researcher in Dr. Lily Y. Young's laboratory in the fall of 1999. Initial work under the supervision of Dr. Craig Phelps produced a keen interest in the biodegradation of aromatic compounds. She became especially interested in biodegradation mechanisms of the endocrine disrupter nonylphenol, since growth on this compound did not readily occur under anaerobic conditions. The project then shifted to study anaerobic *p*-cresol biodegradation, which evolved into a George H. Cook Honors Thesis entitled "Anaerobic biodegradation of *p*-cresol". These findings were later presented at the 2002 American Society for Microbiology General Meeting in Salt Lake City, Utah.

Abigail graduated from Rutgers in May of 2002 and moved to Ithaca, NY, to attend Cornell University as a student in the Department of Microbiology. An exciting rotation in Dr. Anthony Hay's laboratory produced an isolated microorganism able to grow on the endocrine disrupter octylphenol, so she switched to the "dark side" and joined the Hay laboratory to study the aerobic biodegradation mechanism used by

that isolate. Abigail was an active participant in the Field of Microbiology Students organization, organizing prospective student recruitment weekends and serving a term as treasurer. While at Cornell, she received financial support for a United States Department of Agriculture Multi-Disciplinary Graduate Education Traineeship. The later part of her stay at Cornell was supported by an Environmental Protection Agency Science to Achieve Results fellowship, and included participation in two EPA-organized conferences.

Immediately after completion of her Ph.D. degree, Abigail will return to the Young laboratory at Rutgers University. Her new post-doctoral position will involve isolating novel anaerobic aromatic hydrocarbon degraders from hydrothermal vent sediments in the Guaymas Basin, Mexico, which she will help collect on a sampling cruise with collaborator Dr. Costa Vetriani.

To my family: thank you for believing in me

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CHAPTER 1
INTRODUCTION: PRESENCE AND FATE OF ESTROGENIC
ALKYLPHENOLS

1.1 WHAT ARE ALKYLPHENOLS?

Alkylphenol polyethoxylates are a widely used class of nonionic surfactants. These chemicals have diverse uses as wetting agents, plasticizers, dispersants, degreasers, institutional cleaners, and in the formulation of personal care products (50). The two main kinds of alkylphenol polyethoxylates are nonylphenol polyethoxylates and octylphenol polyethoxylates. Nonylphenol polyethoxylates are a mixture produced from 22 *para*-substituted congeners of nonylphenol (72) and are used in all of the applications mentioned above. They comprise 80% of the total alkylphenol polyethoxylates produced in the United States. Octylphenol polyethoxylates, which are mainly used in paints and resins, account for the remaining 20% (64). There are 200,000 tons of alkylphenol polyethoxylates produced in the United States annually (69). The large volume of use and production has resulted in a large influx of alkylphenol polyethoxylates into the environment via effluents from the wastewater treatment process.

Once they enter aquatic or terrestrial environments, microorganisms sequentially remove the terminal ethoxy units until alkylphenol mono- or di-ethoxylates are formed, but complete removal of the ether-linked residual side chain is unlikely in aerobic environments. In the anoxic environments like those found in anaerobic sludge digesters the polyethoxylate side chain can be completely removed to generate the stable intermediates octylphenol (OP) and nonylphenol (NP), collectively known as alkylphenols (28). Degradation of alkylphenols during anaerobic digestion does not readily occur, as the resulting NP and OP are very hydrophobic with pK_{ow} 4.3

and 4.1, respectively (1). Alkylphenols have been shown to accumulate in anaerobic sludge to concentrations 4 to 8 times higher than those in aerobic sludge (28). The octylphenol (OP) and nonylphenol (NP) that have been generated through wastewater treatment have been found to then enter the environment, either via disposal of biosolids or as trace contaminants in treated effluents from wastewater treatment.

1.2 ESTROGENIC ACTIVITY

NP and OP induce endocrine disrupting activity, specifically weakly mimicking estrogens. NP's role as an estrogen mimic was first reported by researchers who found that it was an additive in polystyrene laboratory products that induced cell proliferation in estrogen-sensitive human breast cancer cells (63). *Para*-substituted alkylphenols (AP) were shown to fully bind to recombinant human estrogen receptors in a dose-dependent fashion. NP consists of more than 22 *para*-isomers, all varying in the degree to which the alkyl side chain branches (72). The degree of estrogenicity correlates with these structural differences, as branched and unbranched isomers have different behavior patterns at the estrogen receptor. Receptor binding does not occur with alkanes or phenols with protected hydroxyl groups, indicating that receptor binding requires binding of both the phenol moiety and a portion of the alkyl chain (65). A study of estrogenicity in a commercial mix of NP isomers found that of the 7 isomers that induced estrogenic activity in a recombinant yeast screen, all had tertiary alpha carbons. The highest estrogenic response was caused by 4-(1,1,4-trimethyl-hexyl)-phenol, although its activity was 10,000 times weaker than the response caused by 17 β -estradiol. The isomer found to have the least estrogenic effect was NP with a linear side chain (39). OP, on the other hand, is a single isomer that has a fully substituted alpha carbon on the alkyl side chain. OP was found to be 10 times more estrogenic than NP mixtures, although it is

still 1,000 times weaker than 17 β -estradiol (73). OP is the most estrogenic alkylphenol, while there is a spectrum of estrogenicity in the commercial NP isomeric mix.

1.3 TOXICITY

Alkylphenols have different effects on different organisms, depending on the concentration, length of exposure, and stage of development. In studies with frogs (*Xenopus laevis*), NP injected at concentrations of 200 $\mu\text{g g}^{-1}$ body weight resulted in mortality of all frogs tested (46). Experimental exposure through NP-spiked seawater showed *Crassostrea gigas* (Pacific oyster) exposed for just 2 days during the larval stage at 1 or 100 $\mu\text{g L}^{-1}$ experienced changes in the sex ratio to female dominance and an increased frequency of adult hermaphrodites. *Crassostrea gigas* larvae produced from crosses where both parents were exposed to NP suffered 100% mortality (51). Similarly, salmon larvae exposed to aqueous levels of 100 $\mu\text{g L}^{-1}$ NP suffered 50% mortality within the first 60 days of exposure, while those exposed to 10 $\mu\text{g L}^{-1}$ still experienced mortality at a rate higher than controls (43). Exposure to as little as 10 $\mu\text{g L}^{-1}$ of NP was enough to cause significantly reduced hatching rates in rainbow trout (58).

Changes in sex ratios have been frequently associated with changes in vitellogenin. This protein is a biomarker of estrogenic exposure and is normally associated with egg production in females, but is not made by males. The threshold above which vitellogenin production in male rainbow trout was significant was 10 $\mu\text{g L}^{-1}$ for NP and about 3 $\mu\text{g L}^{-1}$ for OP (35). Numerous studies (7, 8, 35, 40) have reported that trout testicular growth is inhibited to varying degrees based on the dose of NP or OP and the developmental time of exposure. For example, testicular growth inhibition was seen in NP-treated trout during the gonad growth phase, but was absent

in OP-treated mature trout. Jobling *et al.* (34) found a very high incidence of intersexuality in wild freshwater fish (roach, *Rutilus rutilus*) downstream from sewage treatment plants in the United Kingdom, suggesting the cause was effluents following treatment. The percentage of intersexual individuals within the population was related to the amount of input from sewage treatment plants. This is consistent with laboratory studies of genetically all-male amago salmon, which showed that NP exposure changed the sex ratio to 38% females, 37% intersexual individuals, and 25% remaining males (49).

NP and OP effects have been studied repeatedly in fish due to the high instances of exposure in aqueous environments (19, 35, 49, 51). However, one study in mammals, specifically in pigs, found that animals treated with intrauterine OP during pregnancy had a longer pregnancy length. These F1 offspring, when treated with a low level of OP, had an accelerated onset of puberty. Mating between these offspring resulted in reduced litter size (5). This suggests that while there are obvious effects from acute alkylphenol exposure, there can also be trans-generational implications with possible effects on long-term population stability.

1.4 BIOACCUMULATION

Alkylphenols are hydrophobic compounds, and have the potential to bioaccumulate in tissue. OP has a bioconcentration factor of 1,190 in fat tissue in rainbow trout (19), whereas NP has a bioconcentration factor of 1,300 in sticklebacks (*Gasterosteus aculeatus*) (17). Male flounder (*Platichthys flesus*) exposed to OP had OP accumulated in liver and muscle tissues with a positive correlation to vitellogenin concentration, although more OP was found to accumulate in the liver than in muscle tissue (45). A survey of aquatic animals from Germany sampled between 1985 and 2001 showed NP concentrations in muscle tissue reach a maximum of 9.7 ng g⁻¹, but

started to decrease in samples taken after 1997 (71) as a result of a voluntary ban in alkylphenol polyethoxylate use in Europe. OP concentration remained relatively consistent. The maximum concentration in freshwater fish was 112 ng g⁻¹ for NP and 5.5 ng g⁻¹ for OP (71).

1.5 ENVIRONMENTAL PREVALENCE

NP is more prevalent than OP in the environment, mainly due to the higher use of nonylphenol polyethoxylates in commercial applications. The majority of the studies identifying NP in the environment relate to sewage treatment plants and riverine environments directly impacted by effluents from that process. NP was one of the 30 most frequently detected contaminants in a recent survey of 85 urban impacted rivers in the United States. NP was identified in 51% of the samples, while NP monoethoxylate, NP diethoxylate, OP monoethoxylate, and OP diethoxylate were detected less frequently, however, OP was not reported. The highest concentration of NP was found to be 40 mg L⁻¹, which was higher than the reported concentrations for NP mono- and diethoxylates. In the same survey, the highest concentrations reported for OP monoethoxylate and OP diethoxylate were found to be at much lower concentrations of 2 and 1 µg L⁻¹, respectively (41).

Lack of oxygen has been shown to slow the rate of NP and OP biodegradation (6, 18, 28, 36), resulting in the persistence of these compounds in anaerobic environments. The half-life of NP in anaerobic sediments from the Erren River ranged from 46 days to 69 days. Degradation rates in samples with NP-acclimated sediment showed complete disappearance of NP within 63 to 70 days, whereas samples without NP-acclimated sediment showed NP disappearance by 84 days. Degradation was much faster, with a half-life of 3.9 days, when enriching under sulfate-reducing conditions than under methanogenic or nitrate-reducing conditions (6).

NP has been found to be recalcitrant in the environment as demonstrated by its detection in surface sediments near a sewage treatment plant that had not been functional for twenty years prior to sampling (30). The concentration of NP in these sediments was reported to be 54 mg kg^{-1} . NP was detected in 45% of 75 sediments from diverse wastewater outfalls at concentrations of greater than $5 \text{ } \mu\text{g kg}^{-1}$ (31), illustrating that NP is a common environmental contaminant. Blackburn *et al.* found that in rivers where nonylphenol polyethoxylates were detected, NP was present in sediments at concentrations 1,000 times greater than the concentration in the water column (4).

NP has also been identified in food products and household environments. NP was repeatedly found in food products, regardless of fat content or packaging material, and ranged in concentration from 0.1 to $19 \text{ } \mu\text{g kg}^{-1}$. Based on these analyses, the calculated average daily intake for a German adult was $7.5 \text{ } \mu\text{g day}^{-1}$ (29). Another study demonstrated potential human exposure when NP was detected in indoor dust and air samples. NP was found at a maximum concentration of $8.9 \text{ } \mu\text{g g}^{-1}$ in household dust samples, whereas OP was detected at a maximum concentration of $0.09 \text{ } \mu\text{g g}^{-1}$. NP was detected in 100% of the household indoor air samples, with a maximum concentration of 420 ng m^{-3} (55). While present at low concentrations, the frequency at which NP was detected makes it a significant indoor air contaminant.

1.6 BIODEGRADATION

NP biodegradation by fungi

Both branched and linear isomers of NP have been tested for biodegradation by various fungal strains. The aerobic sludge isolate *Candida maltosa* was found to transform 4-*n*-nonylphenol through an attack on the alkyl side chain, with 4-acetylphenol identified as a metabolite (9). Two aquatic fungi, UHH 1-6-18-4 and

Clavariopsis aquatica, were found to attack the alkyl side chain of technical NP isomers. Specific metabolites identified from UHH 1-6-18-4 were 4-hydroxybenzoic acid and shortened alkyl chain alkylphenols, which suggested that UHH 1-6-18-4 utilized a pathway that hydroxylated the alkyl side chain. Both were able to degrade different branched isomers of NP, although *C. aquatica* was more selective toward certain isomers than UHH 1-6-18-4. However, when extracellular laccases isolated from both fungal strains were incubated with NP, the transformation products had a higher molecular mass than NP, demonstrating the capacity for two types of degradation in the fungal strains in question (37).

Linear alkylphenol biodegradation

Several isolates that degrade short-chain alkylphenols have been successfully characterized. For example, the *lap* operon was identified in *Pseudomonas* sp. strain KL28 as participating in the degradation of alkylphenols with linear side chains of up to five carbons in length. The operon consisted of genes encoding a multi-component phenol hydroxylase and a catechol 2,3-dioxygenase in a structure similar to that of the archetypical phenol degradation pathway in the *dmp* operon of *Pseudomonas putida* CF600 (33) (Figure 1.1). Similarly, *P. putida* MT4 was reported to contain an operon with architecture almost identical to that of the *lap* operon. The wild-type strain was restricted to degradation of the same alkylphenols described for strain KL28, but when a plasmid-borne copy of the phenol hydroxylase genes was expressed in a different *Pseudomonas* strain, the resulting recombinant strain could degrade alkylphenols with a straight side chain up to seven carbons in length (66). *Pseudomonas veronii* subsp. *inensi*, was limited to growth on *n*-alkylphenols with side chain lengths of three to six carbons, but was also able to degrade 4-*n*-nonylphenol co-metabolically with the addition of phenol. While the genes involved were not identified, there was evidence during growth of a yellow product associated with *meta*

ring fission (2). Although some mechanisms and associated genes have been identified for *n*-alkylphenol degradation, the environmentally relevant compounds NP and OP were not metabolized by the strains in question.

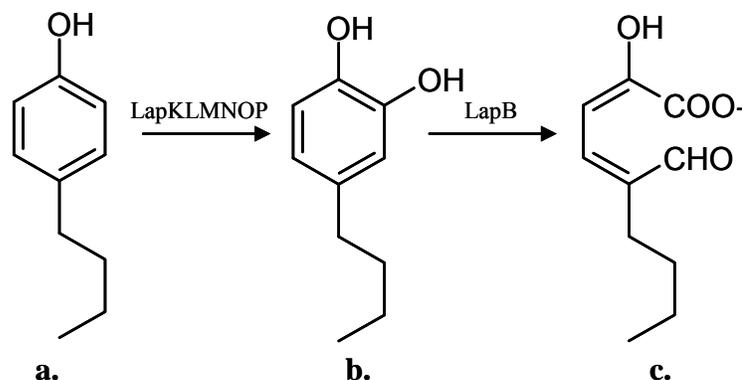


Figure 1.1. Degradation of short chain linear alkylphenols by the *lap* gene cluster. A catechol intermediate (b) is formed from hydroxylation by the multi-component hydroxylase LapKLMNOP. LapB then cleaves the ring to form the ring fission product (c) (33).

Branched chain alkylphenol biodegradation

Due to the large number and structural diversity of NP isomers, it is not surprising that different microorganisms preferentially metabolize certain isomers. *Sphingobium xenophagum* Bayram has been shown to readily degrade NP isomers containing a quaternary substituted alpha carbon, but was unable to transform less highly substituted isomers except through co-metabolism with branched isomers (26). When 4-(1,1,2,4-tetramethylpentyl)-phenol, 4-(1-ethyl-1,4-dimethylpentyl)-phenol, and 4-(1,1-dimethylheptyl)-phenol were tested, the rate of degradation and completeness of substrate disappearance positively correlated with the degree of side chain branching (26). *Sphingomonas* sp. TTNP3 showed a similar pattern for preferential degradation of three different branched isomers, 4-(3,5-dimethyl-3-

heptyl)-phenol (Figure 1.2d), 4-(3,6-dimethyl-3-heptyl)-phenol (Figure 1.2c), and 4-(2,6-dimethyl-2-heptyl)-phenol (Figure 1.2e) (11). Similarly, *Sphingobium amiense* showed preferential degradation for certain *para* isomers (16), as did the fungal isolate *C. aquatica* (37).

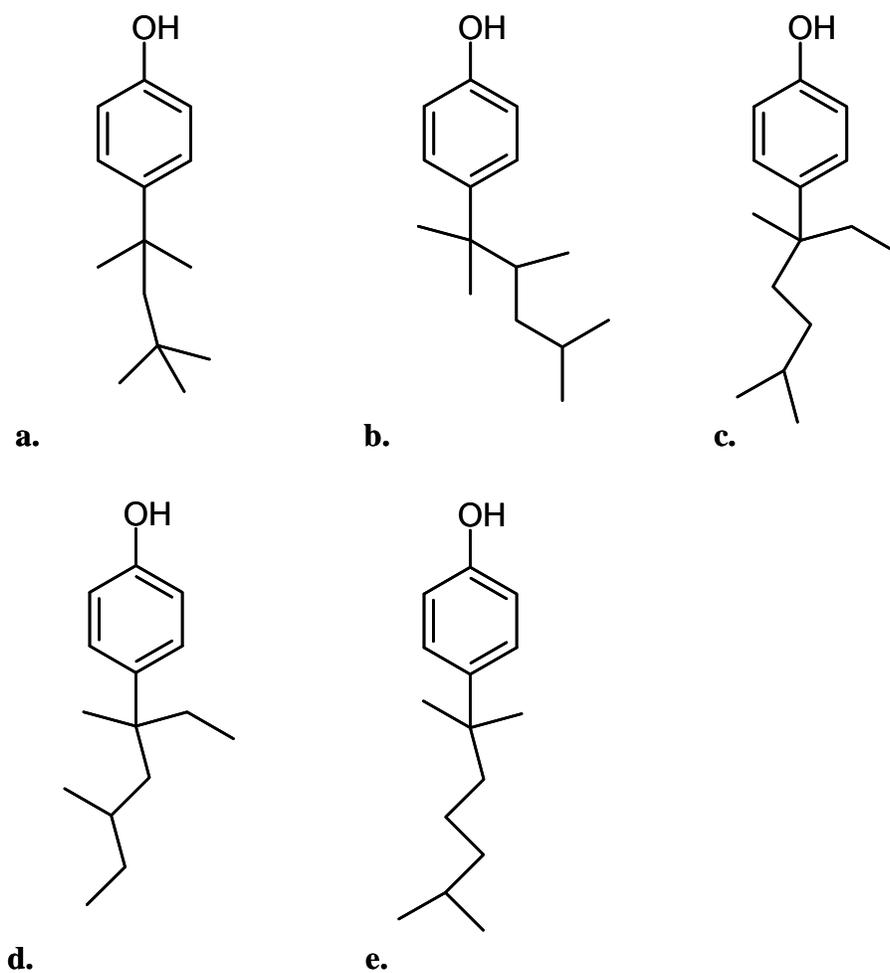


Figure 1.2. Structures of alkylphenols with quaternary alpha carbons. (a) OP; (b) 4-(2,3,5-trimethyl-2-hexyl)-phenol (c) 4-(3,6-dimethyl-3-heptyl)-phenol (d) 4-(3,5-dimethyl-3-heptyl)-phenol; (e) 4-(2,6-dimethyl-2-heptyl)-phenol.

Yuan *et al.* have suggested bacterial biodegradation of long-chain alkylphenols occurs via side chain modification (75). However, degradation examined in the greatest detail thus far involves ring hydroxylation and cleavage, without apparent side chain modification. The most common observations regarding NP and OP metabolism consist of the disappearance of UV-active phenol moieties and the appearance of alcohols corresponding in length and branching pattern to the alkyl side chain. *Sphingomonas cloacae* and *Sphingomonas* sp. strain TTNP3 are both organisms isolated from wastewater treatment plants with the ability to degrade NP (23). *Sphingomonas cloacae* was found to produce long chain alcohols that corresponded to the alkyl side chain formed in cultures with mixed isomers of NP, with no other detectable aromatic compounds (22). The side chain derived alcohols were detected in cultures of TTNP3 grown on both NP (14) and OP as the sole source of carbon (67). The metabolite 2,4,4-trimethyl-2-pentanol, the same alcohol found in TTNP3 cultures during growth on OP (67), was identified in enrichment cultures grown on octylphenoxyacetic acid (24). Similarly, *Sphingobium amiense* culture supernatants revealed long-chain alcohol formation and disappearance of the aromatic moiety. The authors hypothesized a degradation mechanism that proceeded first with ring fission (16). Another sewage sludge isolate, *Sphingobium xenophagum* Bayram, also produced alcohols from branched isomers of NP when grown (26). These same intermediates have been found with only a few exceptions from both isolates and consortia. One such exception is the unusual metabolite, 4-(3,5-dimethyl-3-heptyl)-2-nitrophenol, which was identified in complex mixture of sewage sludge and soil amended with 4-(3,5-dimethyl-3-heptyl)-phenol (68).

These observations suggest ring cleavage as a mechanism for biodegradation, since the ultimate formation of a long-chain alcohol corresponding to the alkyl side chain indicates that additional side chain modification was not occurring and thus the

ring must be serving as the carbon source. Qualitative data in support of the ring cleavage hypothesis included almost complete NP degradation through uncharacterized aromatic intermediates in enrichment cultures (22). There have been many examples of NP biodegradation by consortia, including a culture that showed little evidence of phenolic intermediates as degradation products when monitored with HPLC analysis (21). Similarly, two different *Pseudomonas* isolates able to use NP as the sole carbon source showed monooxygenase activity that, coupled with a lack of short-chain alkylphenol formation, supports ring hydroxylation and subsequent cleavage as a mechanism for metabolism (62), although conclusive evidence of a specific pathway was not provided. However, Corvini *et al.* (12) characterized a dihydroxy intermediate produced from 4-(3',5'-dimethyl-3'-heptyl)-phenol metabolism in TTNP3 and showed that it was not nonylcatechol or nonylresorcinol, but rather a substituted hydroquinone (HQ), 2-(3',5'-dimethyl-3'-heptyl)-1,4-benzenediol (Figure 1.3g).

Additional data suggested that TTNP3 biodegradation of 4-(3',5'-dimethyl-3'-heptyl)-phenol proceeded via hydroxyl-induced shift to form 2-(3',5'-dimethyl-3'-heptyl)-1,4-benzenediol (14). TTNP3 cultures were screened to determine if 4-(3',5'-dimethyl-3'-heptyl)-phenol was metabolized to 2-(3',5'-dimethyl-3'-heptyl)-1,4-benzenediol, but Corvini *et al.* (12) found a smaller molecular weight metabolite instead. When HQ and *p*-benzoquinone standards were compared with the metabolite it was evident based on retention time and mass spectrum that the metabolite was HQ. TTNP3 was unable to grow on 4-chlorophenol and 4-nitrophenol, other compounds that have been shown to be metabolized via HQ intermediates (11). The same metabolite was detected when TTNP3 was grown with a different NP isomer, 4-(2,6-dimethyl-2-heptyl)-phenol (10). The combined formation of HQ and nonanol suggested *ipso* substitution as the biodegradation mechanism (Figure 1.3).

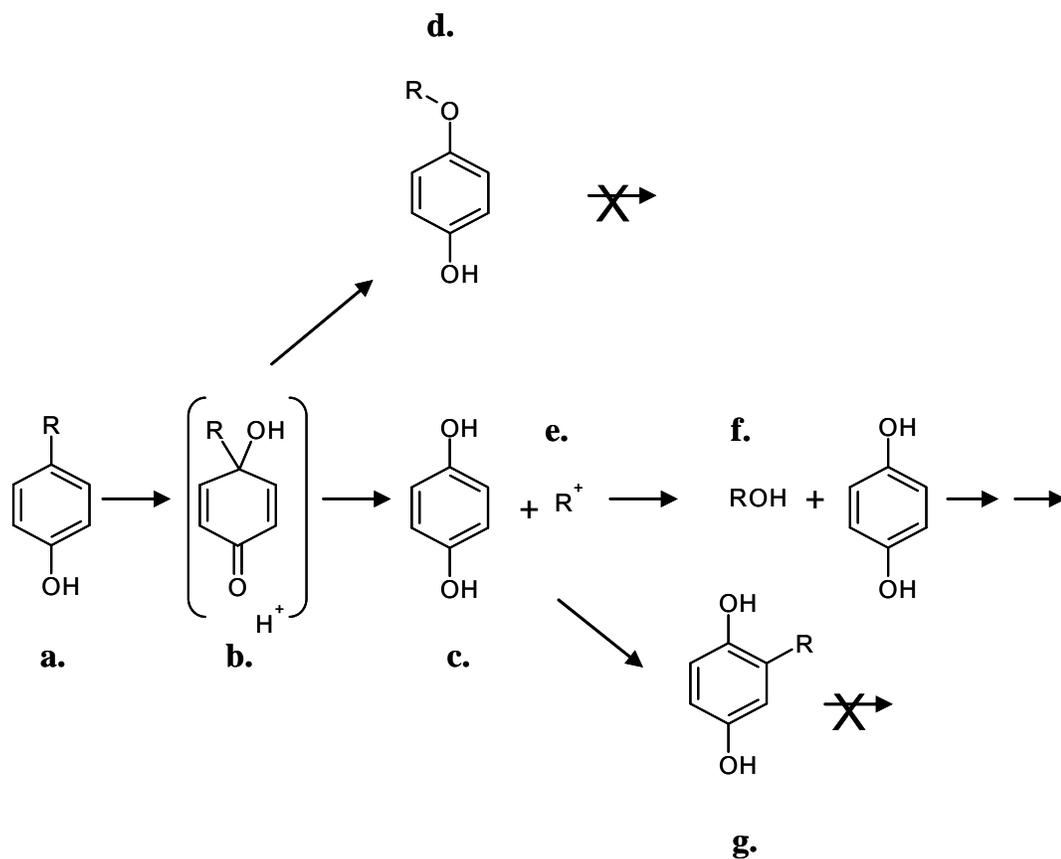


Figure 1.3. Evidence for type II *ipso* substitution in TTNP3. (a) alkylphenol isomer with a quaternary alpha carbon (b) putative *ipso* hydroxylation intermediate (c) HQ; (d) alkoxyphenol metabolite, which is thought to be a dead-end product in TTNP3; (e) putative carbocation intermediate; (f) the alcohol formed from a reaction of the carbocation with water; (g) 2-alkyl benzenediol (11).

Ipso substitution is a mechanism whereby by an intermediate is formed whose leaving group is not hydrogen and in which both groups temporarily share the same position during electrophilic substitution of an aromatic ring. With respect to NP metabolism, oxygen is added to a *para*-substituted phenol, with the replacement of the substituent by the oxygen atom. As demonstrated from isotope labeling experiments,

the oxygen in the reaction came from dioxygen (53). Either HQ or *p*-benzoquinone could form as a result, depending on whether or not the leaving group is an anion or cation. If the leaving group is an anion, then *p*-benzoquinone would form as an intermediate and is referred to as a type I *ipso* substitution. Conversely, a cationic leaving group and consequent HQ formation is a type II *ipso* substitution (53). In the case of cytochrome P450 liver microsomes, a hydroxyl group was required in the *para* position for elimination to occur (53). Substitution did not occur when 4-methylphenol was used as substrate, but instead the methyl and hydroxyl groups remained attached to the same carbon in the *para*-position of the ring structure (53). *Ips*o substitution has been shown to occur to a variety of substrates, including haloaromatics, nitroaromatics, *p*-alkoxyphenol, and *p*-phenoxyphenol (52).

HQ was identified as an intermediate when TTNP3 was grown on 4-(3',5'-dimethyl-3'-heptyl)-phenol and it has been suggested that degradation of this isomer proceeded via type II *ipso* substitution to form HQ, with the removal of the alkyl side chain as a carbocation (11). This was confirmed through ¹⁸O labeling experiments which showed that an oxygen atom from molecular oxygen was incorporated at the *para* position on the ring (42). TTNP3 was found to degrade a variety of branched NP isomers, as well as OP, but could not degrade short chain alkylphenols or a methoxylated NP isomer, indicating that the phenolic hydroxyl group needed to be free for biodegradation to occur (13). Additional data suggests that TTNP3 biodegradation of 4-(3',5'-dimethyl-3'-heptyl)-phenol, another branched isomer of NP, proceeds with a hydroxyl-induced shift to form 2-(3',5'-dimethyl-3'-heptyl)-1,4-benzenediol (Figure 1.3g) (14). This is similar to NP metabolites seen during co-metabolic studies with Bayram, suggesting a mechanism of ring hydroxylation that results in a hydroquinone intermediate prior to ring cleavage (11). TTNP3 intracellular extracts incubated with 4-(2,6-dimethyl-2-heptyl)-phenol, produced 2-

(3',5'-dimethyl-3'-heptyl)-1,4-benzenediol and 4-(2,6-dimethylheptan-2-yloxy)-phenol (Figure 1.3d). Occasionally 4-(3,5-dimethylheptan-3-yloxy)-phenol was also detected. Why 4-(2,6-dimethyl-2-heptyl)-phenol was not metabolized to HQ is unclear, although it is possible that the carbocation for 4-(3',5'-dimethyl-3'-heptyl)-phenol was not as reactive, and could point to differences in the mechanism for biodegradation between different isomers (10).

The *ipso* substitution mechanism initially postulated for Bayram differs slightly from that of *Sphingomonas* sp. strain TTNP3. Initial studies with Bayram suggested degradation via type I *ipso* substitution. While no metabolites were detectable from the addition of pure isomers, co-metabolic experiments with 4-(1-ethyl-1,4-dimethyl-pentyl)-phenol, 4-(1-methyl-octyl)-phenol (Figure 1.4a) yielded the following metabolites 2-(1-methyl-octyl)-benzene-1,4-diol (Figure 1.4b), 4-hydroxy-4-(1-methyl-octyl)-cyclohexa-2,5-dienone (Figure 1.4c), 4-hydroxy-4-(1-methyl-octyl)-cyclohex-2-enone (Figure 1.4d) as identified by MS and NMR. With 4-*n*-nonylphenol (Figure 1.4e) added, 4-hydroxy-4-nonyl-cyclohexa-2,5-dienone (Figure 1.4f) and 4-hydroxy-4-nonyl-cyclohex-2-enone (Figure 1.4g) were identified by MS as metabolites. Based on the formation of these metabolites, a type I *ipso* substitution with subsequent 1,2-C,O shift to detach the alkyl side chain was proposed as the mechanism (27). Later studies demonstrated that, like TTNP3, Bayram actually used type II instead of type I *ipso* substitution. Unlike TTNP3, transformation of both 4-alkylphenols and 4-alkoxyphenols by Bayram was dependent on the cells being first induced with NP (25).

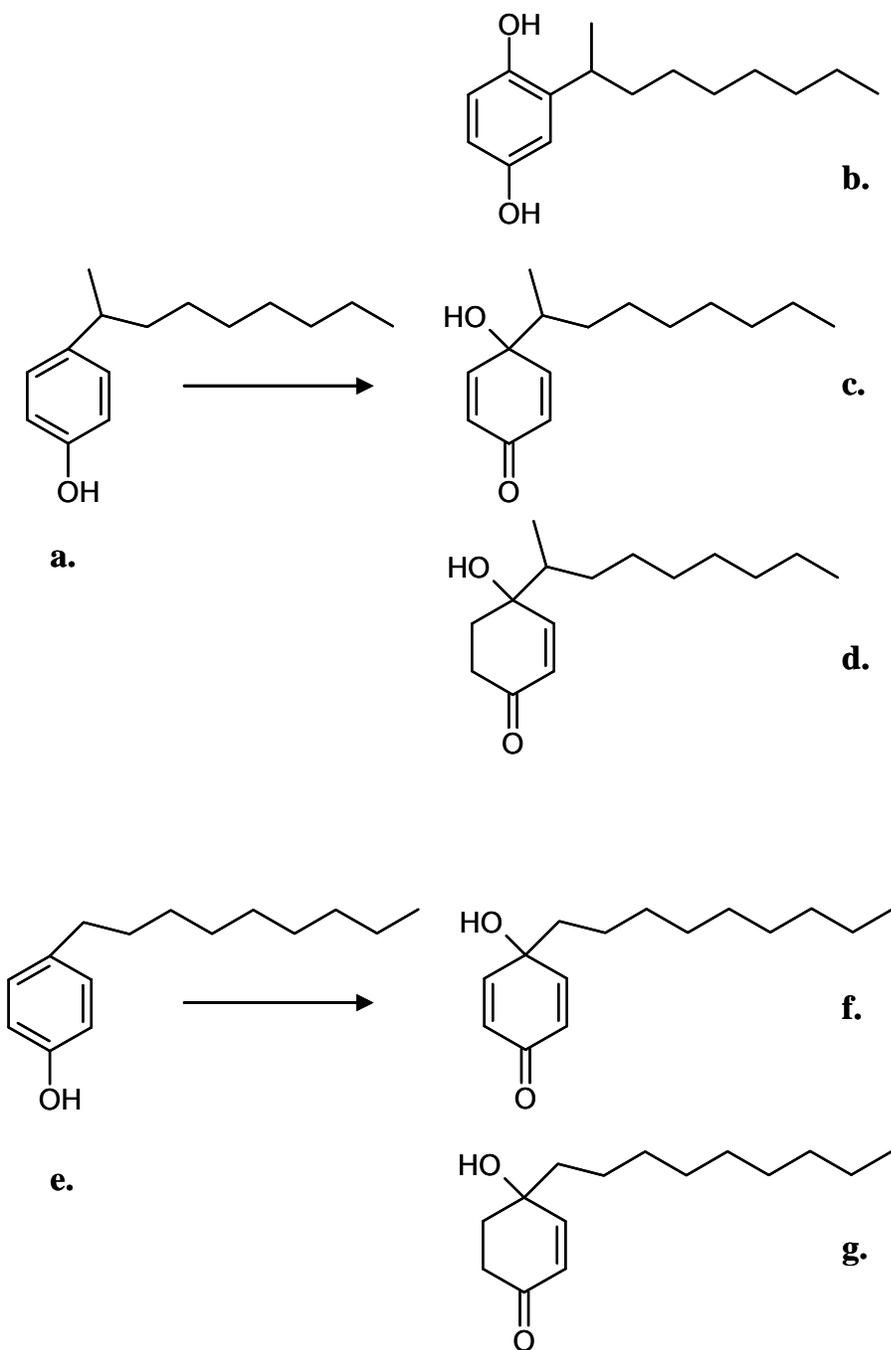


Figure 1.4. Metabolites identified in co-metabolic studies in Bayram. (a) 4-(1-methyl-octyl)-phenol; (b) 2-(1-methyl-octyl)-benzene-1,4-diol; (c) 4-hydroxy-4-(1-methyl-octyl)-cyclohexa-2,5-dienone; (d) 4-hydroxy-4-(1-methyl-octyl)-cyclohex-2-enone; (e) 4-n-nonylphenol; (f) 4-hydroxy-4-nonyl-cyclohexa-2,5-dienone; (g) 4-hydroxy-4-nonyl-cyclohex-2-enone.

As with TTNP3, Gabriel *et al.* (25) determined through isotopic labeling experiments in strain Bayram that an oxygen atom from ^{18}O -labeled molecular oxygen was incorporated in the ring, while the oxygen that was added to the carbocation (Figure 1.5a) intermediate came from ^{18}O -labeled water (Figure 1.5b). However, they also detected a small amount of the alcohol from 4-(1-ethyl-1,4-dimethyl-pentyl)-phenol having ^{18}O incorporated from molecular oxygen, suggesting that the carbocation reacted with the *ipso* hydroxyl group to a certain extent (25). However, the mechanism was slightly different during the degradation of 4-*t*-butoxyphenol, where ^{18}O labeled only the ring, without incorporation of ^{18}O from either H_2^{18}O or $^{18}\text{O}_2$ in the resulting alcohol. This suggests that the alcoholic hydroxyl group came from the ether linkage and would imply direct formation of HQ through the *ipso* substitution. Carbocations are also known to undergo E1 elimination reactions (Figure 1.5c), however none of the studies discussed here employed techniques, such as headspace analysis, that would have permitted detection of the volatile nonene degradation products expected to arise from an E1 elimination (25)

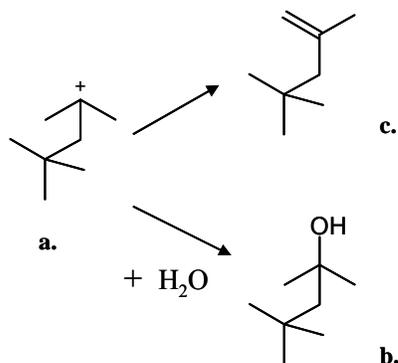


Figure 1.5. Side chain carbocation reactions. During type II *ipso* substitution, the side chain is removed as a carbocation (a). The carbocation has been shown to react with water via $\text{S}_{\text{n}}1$ reaction to form the corresponding alcohol (b) (25). Alternately, the carbocation could also undergo an E1 elimination reaction to form an alkene (c), although this has not been reported in the literature.

1.7 OTHER ENDOCRINE DISRUPTERS IN SEWAGE SLUDGE

The alkylphenols described above are all common contaminants with documented toxic effects and an affinity for sorbing to sewage sludge. There are numerous other personal care products that are repeatedly found in sludge, including triclosan and the polycyclic musk HHCB, which have frequently been detected (30, 38, 41, 44, 47, 60, 61, 74). These compounds also tend to associate with sludge particles and have been identified as having weak endocrine disrupting activity.

Triclosan fate and toxicity

Triclosan, 5-chloro-2-(2,4-dichloro-phenoxy)-phenol, is an antibacterial agent that acts by interrupting fatty acid synthesis in bacteria (48). It is a common additive in soaps, toothpaste, and other personal care products (15). Widespread use has led to approximately 300,000 kg year⁻¹ entering wastewater (30). Based on reported removal efficiencies, the amount of triclosan estimated to reach the environment after wastewater treatment via activated sludge is between 2,600 and 10,500 kg year⁻¹, with trickling filter systems estimated to be responsible for contributing 2,400 – 7,300 kg year⁻¹ into the environment (30). A high pK_{ow} of 4.8 indicates triclosan is hydrophobic and suggests the possibility for bioaccumulation (30).

There has been evidence for microbial degradation of triclosan, but the compound is still readily detected in the environment. It was one of 30 compounds most frequently detected in a survey of 85 urban rivers in the United States. It was detected in 58% of the samples and found at a maximum concentration of 2.3 µg L⁻¹ (41). Studies of Lake Greifensee in Switzerland found triclosan in sediment cores dating back almost forty years, and illustrated triclosan's recalcitrance in anoxic sediments (61).

Studies of triclosan fate in a Swiss wastewater treatment plant showed 79% removal through biodegradation, 15% removal through sorption to sludge, with the

remaining 6% being released into the environment (61). A survey of wastewater treatment plants in the United Kingdom found triclosan in all crude sewage samples and sewage effluent samples. Although there was great variability in removal efficiency, the authors found a general trend toward increased removal efficiency with increased retention time (38). Similar observations were made elsewhere, with removal efficiencies ranging from below detection in lagoon treatment systems (44) to 58% in trickling-filter systems (47). A study of Ohio wastewater treatment plants found the most variability in trickling-filter systems, with maximum removal of 86% (47). Removal by activated-sludge wastewater treatment plants was reported in several studies to be more consistent, with triclosan removal ranging from 90% (44, 47) to almost complete removal (30).

Triclosan has been shown to cause some toxic effects through decreased hatchability and time to hatching was delayed in fertilized *Oryzias latipes* eggs when exposed to triclosan (32). Triclosan has shown endocrine disrupting potential. Foran *et al.* (20) suggested that triclosan was potentially androgenic in studies with Japanese medaka (*Oryzias latipes*). There was a trend of lower testosterone levels in frogs (*Xenopus laevis*) injected with high doses of triclosan, as well as lower plasma vitellogenin than comparable controls (46). Triclosan is also a potent inhibitor of sulfotransferases, enzymes that are normally involved in phase II detoxification reactions. Thus organism exposed to triclosan may also be more vulnerable to the deleterious effects of other chemicals (70).

HHCB fate and toxicity

HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta- γ -2-benzopyran) is a synthetic polycyclic musk marketed under the trade name Galaxolide. Its pK_{ow} is 5.9 (60), which is much higher than that of either NP, OP, or triclosan. HHCB is one of four musks that represent 95% of the fragrance musks used in

Europe. The influent concentration in waste water treatment plants in one study ranged from 7,800 to 19,200 ng L⁻¹ and removal ranged from 39 % in a submerged aeration filter system to 93% in an activated sludge treatment system (38). The concentration of HHCB found in Canadian effluents were five to ten times less than reported values in studies from European wastewater treatment plants (44). Removal rates in Ohio wastewater treatment plants ranged from 83 – 92% (60). HHCB is a volatile compound and was found at detectable levels in the air over the city of Milwaukee, as well as over Lake Michigan, although at lower concentrations in the latter samples (54).

Both estrogenic and anti-estrogenic effects have been observed *in vitro* after HHCB administration, with responses varying based on the cell line and the type of estrogen receptor studied (57). In assays with mutant estrogen receptors, it was suggested that the binding site for HHCB was different than that of 17 β -estradiol (57). HHCB showed induction of estrogen receptor alpha, but did not have an effect on estrogen receptor beta. Overall estrogenicity was very weak, and was thought to be unlikely to elicit an estrogenic response at environmental concentrations (59). Conversely, HHCB was found to behave as an estrogen antagonist in transgenic zebrafish. The same zebrafish had HHCB concentrations in tissue samples that were 600 times higher than the nominal test dose (56). The bioconcentration factor was higher in *Chironomus riparius* (midge) exposed to HHCB and a cytochrome P450 inhibitor as compared to HHCB exposure alone. The bioconcentration factor and toxicity were both lower than predicted, suggesting the cellular metabolism of HHCB likely mediated by a cytochrome P450 (3).

1.8 CONCLUDING REMARKS

While there has been extensive research into the mechanism for NP biodegradation in *Sphingomonas* sp. TTNP3 and *Sphingobium xenophagum* Bayram, there are still unanswered questions. This work discusses identification of *opdA*, a gene identified first in *Sphingomonas* sp. PWE1 that encodes a putative flavin-dependent monooxygenase. As similarities arose between the degradation mechanism in PWE1 and those described for TTNP3 and Bayram, it seemed likely that the latter two strains could contain homologs to *opdA*. The second chapter of this work compares the sequence of *opdA* and the activity it encodes from all three strains of OP-degraders. The final chapter examines the prevalence of endocrine disrupting compounds, including NP, OP, triclosan, and HHCB, in anaerobic sewage sludge from four central New York State municipalities.

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CHAPTER TWO

IDENTIFICATION OF *opdA*, A GENE INVOLVED IN THE BIODEGRADATION OF THE ENDOCRINE DISRUPTER OCTYLPHENOL

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2.1 ABSTRACT

Octylphenol (OP) is an estrogenic detergent breakdown product. Structurally similar nonylphenols are transformed via type II *ipso* substitution resulting in the production of hydroquinone and removal of the branched side chain. Nothing is known, however, about the gene(s) encoding this activity. We report here on our efforts to clone the gene(s) encoding OP degradation activity from *Sphingomonas* sp. strain PWE1 which we isolated for its ability to grow on OP. A fosmid library of PWE1 DNA yielded a single clone, aew4H12, which accumulated a brown polymerization product in the presence of OP. Sequence analysis of loss of function transposon mutants of aew4H12 revealed a single open reading frame, *opdA* that conferred OP degradation activity. *E. coli* subclones expressing *opdA* caused OP disappearance, with the concomitant production of hydroquinone and 2,4,4-trimethyl-1-pentene, as well as small amounts of 2,4,4-trimethyl-2-pentanol. These metabolites are consistent with a type II *ipso* substitution reaction, the same mechanism described

for nonylphenol biodegradation in other sphingomonads. Based on *opdA*'s sequence homology to a unique group of putative flavin monooxygenases and the recovery of hydroxylated OP intermediates from *E. coli* expressing *opdA*, we conclude that this gene encodes the observed type II *ipso* substitution activity responsible for the initial step in OP biodegradation.

2.2 INTRODUCTION

Nonylphenol (NP) and Octylphenol (OP), collectively referred to as alkylphenols, are detergent breakdown products with highly branched side chains that act as endocrine disrupters, and are known to exhibit weak estrogenic activity (17). Tabira *et al.* (33) have shown that alkylphenols bind to recombinant human estrogen receptors in a dose-dependent fashion. Alkylphenols induce feminization in male amago salmon (26), cause changes in the sex ratio toward females, increase the frequency of intersexuality in Pacific oysters (27), and inhibit testicular growth in male rainbow trout during maturation (20).

Given their potential to cause harm to fish and other sensitive aquatic organisms, much interest has been focused on understanding the potential of microorganisms to degrade alkylphenols. Several organisms have been reported to degrade NP or OP when oxygen is available, including several fungal species (4, 22), psychrotrophic and psychrophilic *Pseudomonas* species (31), as well as the sewage sludge isolates *Sphingobium xenophagum* sp. Bayram (15), *Sphingomonas cloacae* (12), *Sphingomonas* sp. TTNP3 (35) and *Sphingobium amiense* strain YT^T (36).

The degradation mechanism for specific NP isomers has been examined biochemically in both *Sphingobium xenophagum* Bayram and *Sphingomonas* TTNP3. Both strains metabolize various NP isomers by type II *ipso* substitution. Growth on

NP appears to be limited to those isomers that contain fully substituted alpha carbons on the alkyl side chain (5, 14).

There have been examples of *ipso* substitution as a mechanism for degradation of halogenated phenols by cytochrome P450s (2, 29), although there is no direct evidence that similar enzymes act upon alkyl substituted substrates. Kolvenbach *et al.* recently reported that *Sphingomonas* sp. TTNP3 appears to use a monooxygenase to transform NP, but no information regarding the gene coding for this activity was given (23). The present study describes the cloning of a putative flavin monooxygenase from *Sphingomonas* sp. strain PWE1 whose expression conferred on *E. coli* the ability to transform OP to hydroquinone .

2.3 MATERIALS AND METHODS

Chemicals

OP (4-(2',4',4'-trimethyl-pentyl)-phenol) and 1,2,4-benzenetriol were purchased through Sigma-Aldrich (St. Louis, MO). Ascorbate, hydroquinone (HQ), nonylphenol (NP), and 2,4,4-trimethyl-1-pentene were purchased from Acros (Morris Plains, NJ). All solvents were HPLC-grade and purchased through Fisher Scientific (Pittsburgh, PA).

Isolation

Activated sludge from the municipal Wastewater Treatment Plant in Ithaca, New York was spiked with 1000 mg L⁻¹ NP and incubated at room temperature while shaking at 150 rpm. After 7 days, 1ml of this enrichment was then transferred to 100 ml of minimal salts medium (MSM) (24) containing 1000 mg L⁻¹ NP and allowed to grow for an additional 7 days. This process was repeated 3 more times. On the fourth transfer OP (1000 mg L⁻¹) was used as a growth substrate rather than NP since this

single isomer is available commercially. This enrichment was subjected to 3 more transfers on OP and then plated onto MSM agar plates containing 1000 mg L⁻¹ OP. A single strain able to use OP as the sole carbon and energy source was isolated from these OP minimal medium plates and designated PWE1. The phylogenetic relatedness of PWE1 to other bacteria was determined by analyzing a portion of the 16S rRNA gene which had been PCR amplified using universal primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 1492R (5' TAC GGY TAC CTT GTT ACG ACT T 3'), then sequenced at the Cornell University BioResource Center.

Growth on OP

Growth was monitored in triplicate flasks of 100 ml MSM with 1000 µg ml⁻¹ OP at 24°C. Samples were taken in triplicate from each flask and the absorbance was measured at 600 nm using a MicroQuant spectrophotometer from BioTek Instruments (Winooksi, VT).

Fosmid library

PWE1 DNA was isolated via phenol-chloroform extraction and used to generate a fosmid library with the CopyControl Fosmid Library Production Kit (EPICENTRE Biotechnologies, Madison, WI) as per kit instructions. Fosmid clones were visually screened for the accumulation of putative ring-hydroxylated OP intermediates as indicated by the production of a brown polymerization product (BPP) when grown with OP in the presence of *p*-toluidine and FeCl₃ (30).

Transposon mutagenesis of fosmid clones

A BPP-producing fosmid clone labeled aew4H12 was mutated with the EZ::TN5 <R6K γ ori/Kan-2> transposon mutagenesis kit (Epicentre Biotechnology, Madison, WI) in order to obtain loss of function mutants. Briefly, the fosmid was extracted using a modified alkaline lysis method, and then subjected to transposon mutagenesis according to the manufacturers instructions. The reaction mixture was

then transformed into TransforMax EPI300 Electrocompetent *E. coli* (Epicentre Biotechnology, Madison, WI) and screened for loss of the BPP phenotype as described above. Fosmids from BPP⁻ mutants were extracted by alkaline lysis and were then sequenced with outward-facing transposon primers to determine the site of transposon insertion. Sequences were aligned using the DNASTar program suite (DNASTar, Madison, WI) to identify open reading frames.

Further information was gathered by using PCR to amplify fragments of the fosmid that lay between the site of Tn5 insertion and the fosmid multicloning site. This was done using transposon-specific primers (R6KAN-2 RP-1 Reverse Primer 5'CTA CCC TGT GGA ACA CTA CAT CT 3' and KAN-2 FP-1 5' ACC TAC AAC AAA GCT CTC ATC AAC C 3') and a fosmid-specific primer (pCC1/pEpiFOS Reverse Sequencing Primer 5' CTC GTA TGT TGT GTG GAA TTG TGA GC 3'). These additional amplicons were also sequenced.

In silico DNA analyses

Sequence analysis of the mutant fosmids that had lost the ability to confer BPP production was done with DNASTar and BLAST (1). A putative open reading frame which was common to all the mutants was identified and named *opdA*. This open reading frame was PCR-amplified using primers *opdA* forward (5' TTC ATC CTG AAA GAC ACT GCC GGA 3') and *opdA* reverse (5' ACG CGC TTC CAG ACC AAC CTA TTT 3') and subcloned into pGEM-T Easy (pGEM) (Promega, Madison, WI). The plasmid was designated pAW1 and transformed into *E. coli* JM109. Activity was assessed by monitoring formation of 2,4,4-trimethyl-1-pentene in the headspace of sealed cultures (see below).

Detecting hydroquinone formation

Overnight PWE1 cultures were diluted 1:1 with fresh medium and brought to a starting OP concentration of 480 μ M. The fresh culture was incubated at room

temperature while shaking. After 1 hour, the culture was filtered through glass wool to remove residual OP and then centrifuged to pellet the cells. The resulting supernatant was adjusted to pH 9 with 1.5% K₂CO₃. Acetic anhydride at 0.5% was then added to derivatize aromatic hydroxyls and the supernatant was incubated while shaking at room temperature for 1 hour. The derivatized supernatant was then extracted with 30 ml of ethyl acetate. The extract was dried using anhydrous Na₂SO₄ then evaporated under N₂ at 40°C. The residue was re-dissolved in 1 ml of ethyl acetate for analysis via GC-MS using an HP 6890 GC equipped with an HP-5MS column (5% phenyl methyl siloxane; 30 m by 0.25 mm; 0.25- μ m film thickness) using helium as the carrier gas with a flow rate of 1 ml/min. The temperature program included a hold at 40°C for 1 minute, followed by an increase of 5°C/minute to 150°C and a hold for 5 minutes. This was followed by an increase of 40°C/minute to 300°C and a hold for 5 minutes. The detector was an HP 5973 MSD with the quadrapole and source set at 150°C and 230°C, respectively.

The accumulation of HQ in the supernatant of *E. coli* clones expressing *opdA* was also confirmed via HPLC using a mobile phase of 20% methanol and 80% of 40 mM acetic acid. The solvent was pumped at a rate of 1 ml min⁻¹ using a Waters Model 590 pump through a Varian Microsorb-MV C₁₈ column (250 mm by 4.6 mm). Samples were injected by a Shimadzu SIL-10AD AP autoinjector and detected with a Shimadzu SPD-10A VP UV-Vis detector by monitoring absorbance at 290 nm. Quantitation was accomplished by comparison with a standard curve of authentic HQ.

Detecting side chain metabolites

For pGEM subclones in *E. coli*, 500 μ l of an overnight culture were added to 4.5 ml aliquots of 1/10th LB in 25 ml Balch tubes. The medium was supplemented with 150 μ g ml⁻¹ ampicillin and the cultures were incubated with shaking at 37°C for 2 hours, at which time *opdA* expression was induced by the addition of 0.2 mM IPTG.

Uninduced controls did not receive IPTG, but rather, had 50 mM glucose added upon inoculation in order to repress expression from the *lac* promoter of pGEM. After 2 more hours of incubation, OP in a methanol solution was spiked into cultures and the tubes were immediately sealed with rubber stoppers and crimped. Given the apparent toxicity of HQ to *E. coli*, only 1/4th the amount of OP (120 μ M) added to PWE1 was added these *E. coli* cultures. Headspace samples of 0.25 ml were periodically removed and analyzed by GC-MS as follows: 40°C hold 1 minute, then increased by 5°C /minute to 100°C and held for 3 minutes. The temperature was then increased by 10°C /minute to 165°C and finally increased at 60°C /minute to 240°C. All other GC-MS conditions were as described above. The appearance of 2,4,4-trimethyl-1-pentene was quantified by comparison with dilutions of an authentic standard made in similar Balch tubes.

Quantifying OP disappearance

After 70 hours the above Balch tube cultures were sacrificed for further chemical analysis. The tubes were unsealed and 500 μ l of culture was removed and diluted with an equal volume of methanol. The methanol-amended culture was centrifuged to remove cellular debris and the resulting supernatant was filtered through a 4 mm 0.2 μ m regenerated cellulose syringe filter (Corning, Corning, NY). The filtrate was analyzed for both HQ and OP via HPLC. OP was resolved with a mobile phase of 80% methanol and 20% 80 mM acetic acid and detected at 220 nm, whereas HQ was resolved using the method described above.

Nucleotide sequence

The full sequence of the putative OP monooxygenase (*opdA*) and a partial sequence of the *Sphingomonas* sp. strain PWE1 16S rRNA gene have been deposited in GenBank under accession numbers EU002557 and EU004850, respectively.

2.4 RESULTS

PWE1 Growth on OP

Sphingomonas sp. PWE1 was isolated based on its ability to grow with OP as a sole carbon and energy source. Nucleotide sequence analysis of the 16S rRNA gene in PWE1 showed 99% homology with *Sphingomonas cloacae*, a known NP-degrading microorganism (12). In minimal medium, stationary phase was reached by 72 hours and the optical density at 600 nm did not exceed 0.2, as determined in a 96-well plate spectrophotometer (Figure 2.1). TTNP3 showed a similar growth pattern in minimal medium with OP over a similar time span, reaching an optical density at 550 nm of only 0.23 (34). PWE1 grew to a much higher optical density in a complex rich medium (data not shown), just as TTNP3 growth increased with the addition of sodium acetate to the OP-degrading cultures (34).

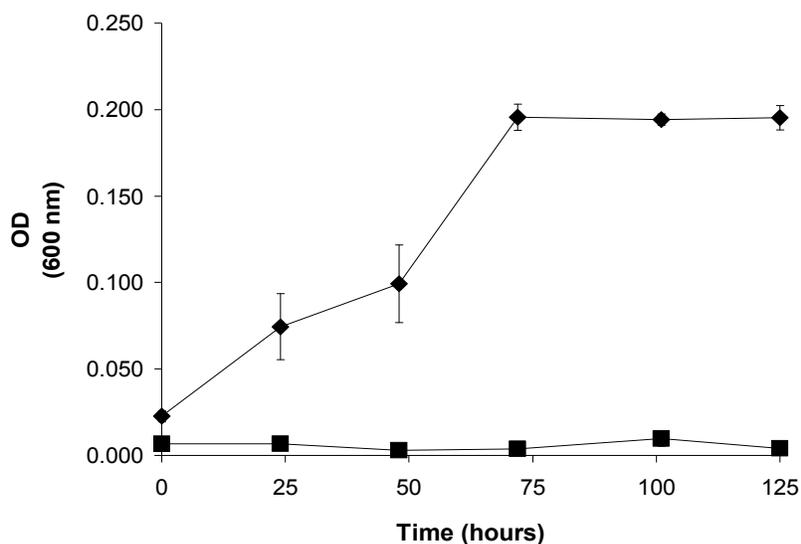


Figure 2.1. Growth of *Sphingomonas* PWE1 on OP. Triplicate values of a representative culture flask of PWE1 grown in MSM with OP (◆) compared to PWE1 grown in MSM without OP (■).

Identification of opdA

The reported production of ring hydroxylated intermediates by other *Sphingomonas* strains lead us to hypothesize that a screen dependent upon the polymerization of these intermediates would yield a diagnostic brown polymerization product (BPP) in the presence of OP. BPP production could then be used to identify PWE1 fosmid clones harboring the gene(s) which encoded this activity. More than 900 fosmid library clones were screened on 1/10th LB with OP. *p*-Toluidine and FeCl₃ were also added to enhance polymerization (30). One clone accumulated BPP when incubated with OP. It was designated aew4H12. No BPP was observed in the supernatant of *E. coli* harboring aew4H12 in the absence of OP, nor in the supernatant of any other fosmids in the presence of OP. Sequence analysis of BPP⁻ transposon mutants of aew4H12 was used to target a putative open reading frame that appeared to encode OP degradation and was labeled *opdA*. When *opdA* was subcloned from aew4H12 into pGEM-T Easy to give pAW1 and expressed in *E. coli* JM109, it conferred BPP production but the vector control did not.

In silico analyses of *opdA* performed using PSI-BLAST (1) identified a conserved monooxygenase domain and an FAD-binding domain that shared homology with those found in UbiH from *E. coli* (Figure 2.2). BLASTx analysis of *opdA* showed it to have weak predicted amino acid sequence similarity (32% identity, 48% similarity over 535 amino acids) to a putative polyketide hydroxylase from *Stigmatella aurantiaca* DW4/3-1 (RefSeq accession number ZP_01459560.1). Other putative genes that showed some predicted amino acid similarity with *opdA* included those encoding FAD-binding monooxygenases such as the PheA/TfdB family FAD monooxygenase from *Myxococcus xanthus* DK1622 (RefSeq accession number YP_635433), 2-polyprenyl-6-methoxyphenol hydroxylase from *Burkholderia*

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Consensus      1  MNAPLPLSSMXXMRPXXXXXXQVDVXIVGXGPVGLXXANLLGQYGXXVLVXEREEXTH
BAA13052.1    -----MAIQHPDIQPAVNHSVQVALAGAGPVGLMMANYLGQMGIDVLVVEKLDKLI
YP_980856.1   MNAPLPLSSLPLAARPSGASSGGHFPVVIIGAGPTGLTLANLGTQGIPVLVIERSHDTV
YP_635433.1   -----MGCDSQQTDTQVDVIISGCGPVGALTGNLLGLMGVRTLLERDVAPH
ZP_01459560.1 -----MSAETVDVIIVGCGPVGGITANYLGQDGVRTLIIDRELHAH
CAD19095.1    -----MDVAIVGGGPVGLMAANLLSYGVGVLVLERESSPH
ABC00744.1    -----MTRPLETPPDIEVPVLIVGGSMVGLSTLFLSHYGIQAMAVERHERTA
NP_417383.1   -----MSVIIVGGGMAGATLALAISRLSHGALPVHLIEATA
PWE1          -----MENEMTTEPGDRASQFDVAIVGCGPVGALLANLLKQYGHKVAVLEREPDIF

Consensus     61  XEPRAXSXDEXLRTLOSXGLDXXXXXXZSLAMXYXXGT      GKXFASISPXXXD
BAA13052.1    DYPRAIGIDDEALRTMQSVGLVDDVLPHTTPPWHAMRFLTPK-----GRCFADIQPMTDE
YP_980856.1   GEPRAVSIDDESLRTLTAGLIGEVLPPHVVOGYGVHYYSWR-----NKEFARIEPSVMD
YP_635433.1   GEPRAFSCDDEGLRIYOSTGLLDLLQSDMRESRFAEYVGGT-----GKRFAEIHTGETD
ZP_01459560.1 TQPRAFSCDDETQRNFQAAGLANELAVNLYHCPKMDYIDQQ-----RNVLASAIFKGLD
CAD19095.1    TYPRAVALYDDALRTFOAVGLVEKQASEMMMDLEMQIVSGT-----GQLLIGVNPAVAS
ABC00744.1    IHPRAGHFHLRTLELLRSVGLEEVVARTSAEAFFPNGGINAVQSLAGGETASFISNLNAG
NP_417383.1   PESHAHPGFDGRAIALAAGTCQQLARIGVWQSLADCATAIT-----TVHVSDRGHA
PWE1          YAPRGMGFDDSTRIMQSAGILDLRLKAEGHIYQADLELIDRSGKRLGCFDRRSVGEDLLS

Consensus     276  VEIXRXXVYTFHARLAXRWRSGRVFLAGDAAHLMPPFXGQGLNSGVRDAVNLAWKLALXX
BAA13052.1    VELIHRVYTHNARLAQFRIDRVLLAGDAAHIMVWQQGYNSGMRDAFNLAWKLALVI
YP_980856.1   LEITRKVVYAFHARVAERWKSGRVFLAGDAAHLTPPFAGQGMNSGVRDAVNLAWKLAAVV
YP_635433.1   VRIERASVYTFECLNAARWRHGNVFLLGDAAHTMPPFMGQGLVSGMRDASNLAWKLKRVL
ZP_01459560.1 VTIVAAVFTFSKVAQWRSGRVFLLGDAAHLMPFLGQCMCSGVRDAVNLTWKLLAV
CAD19095.1    YEIVRHRVYIHYRLAERYRDRIFLLGDAAHLVPPFGAQLSSGIRDAVNLAWKVAMAV
ABC00744.1    VEIQDVAHWEATAALADRYGGRIFLAGDAAHVPPYGGFGNTGVQDAHNLAKLLVL
NP_417383.1   THAGKRSAYPLALTHAARSITHITVLVGNAQTLHPIAGQCFNLGMRDVMSIAETLQAQ
PWE1          FRVISVSYTFHAGMPSKWRVGRLMVAGDAAHLTPWSGQGLNSGVRDAVNLSFKLNLAL

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Figure 2.2. Alignment of OpdA with single-component monooxygenases. The predicted amino acid sequence of OpdA is compared with BLASTx matches *Escherichia coli* W3110 MhpA (BAA13052.1) (GenBank or RefSeq accession numbers are in parentheses), *Polaromonas naphthalenivorans* CJ2 putative FAD-binding monooxygenase (YP_980856.1), *Myxococcus xanthus* DK1622 PheA/TfdB family putative FAD-binding monooxygenase, (YP_635433.1), *Stigmatella aurantiaca* DW4/3-1 putative polyketide hydroxylase (ZP_01459560.1), *Stigmatella aurantiaca* putative cytochrome P450 dependent monooxygenase (CAD19095.1), and *Burkholderia* sp. DNT 4-methyl-5-nitrocatechol monooxygenase (ABC00744.1). *Escherichia coli* K12 UbiH (NP_417383.1) is also part of the alignment, based upon identification of a conserved region in OpdA that was shared with UbiH using PSI-blast. Highlighted residues are those shared in common with OpdA. Underlined regions designate the ADP-binding and flavin-binding motifs, with the bold-face residues in the consensus line being the specific conserved residues. Residues 26-65 in the consensus correspond with an ADP-binding motif (10, 37). Residues 295 - 335 in the consensus correspond with a flavin-binding motif (8, 10).

cenocepacia PC184 (GenBank accession number EAY67308), and 4-methyl-5-nitrocatechol monooxygenase from *Burkholderia* sp. DNT (GenBank accession number ABC00744). The closest related gene encoding a protein of known function was *mhpA*, 3-hydroxyphenylpropionate 2-monooxygenase, from *E. coli* W3110 (GenBank accession number BAA13052). A multi-sequence alignment of OpdA from PWE1, MhpA, UbiH, and close BLASTx matches showed regions of conserved residues (Figure 2.2). Specifically, two motifs recognized for ADP-binding and flavin-binding that had been identified in MhpA (10) were also found to be in OpdA.

Table 2.1. OP conversion to HQ and 2,4,4-trimethyl-1-pentene in an *E. coli* subclone harboring *opdA*. *OP was not detectable in the 70 hour culture samples that were used for HQ quantification.

	Starting mass (μ moles)	Expected mass (μ moles)	Observed mass (μ moles)	Percent (%) Expected
OP	0.61	0	n.d. *	
HQ	0	0.61	0.15	25
2,4,4-trimethyl-1-pentene	0	0.61	0.35	57

Testing opdA activity

Based on similarities with other sphingomonads that can grow on alkylphenols (11), we expected that JM109 pAW1 would metabolize OP to HQ. The complete disappearance of OP (0.61 μ moles) added to 5 ml cultures of JM109 pAW1 (final concentration of 120 μ M OP) after 70 hours of incubation, was accompanied by the production of HQ, which reached a maximal detectable amount of 0.15 μ moles (final concentration of 30 μ M HQ) (Table 2.1). HQ was not produced in cultures that lacked OP, or by either JM109 pAW1 without IPTG, or the vector control. OP

disappearance in media inoculated with JM109 pAW1 without polymerizing agents was highly variable. Further analysis of cell viability suggested that this was likely due to the toxicity of the accumulating HQ, as 90 μ M HQ was sufficient to reduce *E. coli* colony forming units by a factor of 1000 (data not shown). Addition of FeCl₃ and toluidine, which facilitated HQ polymerization, alleviated this toxicity somewhat (data not shown). As further evidence of toxicity, when pAW1 was maintained in *E. coli* DH5 α (which lacks the *lac* repressor), the BPP phenotype was hypervariable and rapidly lost during subculturing. Interestingly, sequence analysis of *opdA* amplified from BPP⁻ DH5 α pAW1 revealed the presence of one silent and three coding mutations. The latter resulted in the following substitutions: N163S, Q205E, and A241C.

The long-chain alcohol expected to form as a result of OP metabolism (13, 34) was not detectable under the extraction conditions used to identify metabolites in wild-type PWE1 or in JM109 pAW1. GC-MS analysis of the headspace of JM109 pAW1 cultures was therefore used to capture any volatile metabolites, and surprisingly revealed two distinct peaks. One peak corresponded in retention time (1.8 minutes) and mass spectrum to a commercial standard of 2,4,4-trimethyl-1-pentene, with a base peak *m/z* (relative abundance) of 57 (100), and yielded additional fragments at *m/z* 55 (28.7), 69 (8.64), 97 (17.6), and 112 (18.6). The other peak had a retention time of 3.8 minutes and its mass spectrum revealed a base peak *m/z* 59 (100), as well as additional fragments at *m/z* 55 (34.9), 57 (93.3), 97 (21.0) and 115 (7.49). The mass spectrum of this second peak was identical to the mass spectrum Fujita and Reinhard (13) described for 2,4,4-trimethyl-2-pentanol. The peak area of the pentene was approximately 20 times greater than that of the pentanol although we could not quantify the pentanol since no commercial standards were available. Over the course of 24 hours, there was increased formation of both side chain products in the

headspace of *E. coli* pAW1 cultures, although only 2,4,4-trimethyl-1-pentene is shown in Figure 2.3. There was very little change in the headspace concentration past 24 hours. The final amount of 2,4,4-trimethyl-1-pentene at 70 hours was 0.35 μ moles (Table 2.1).

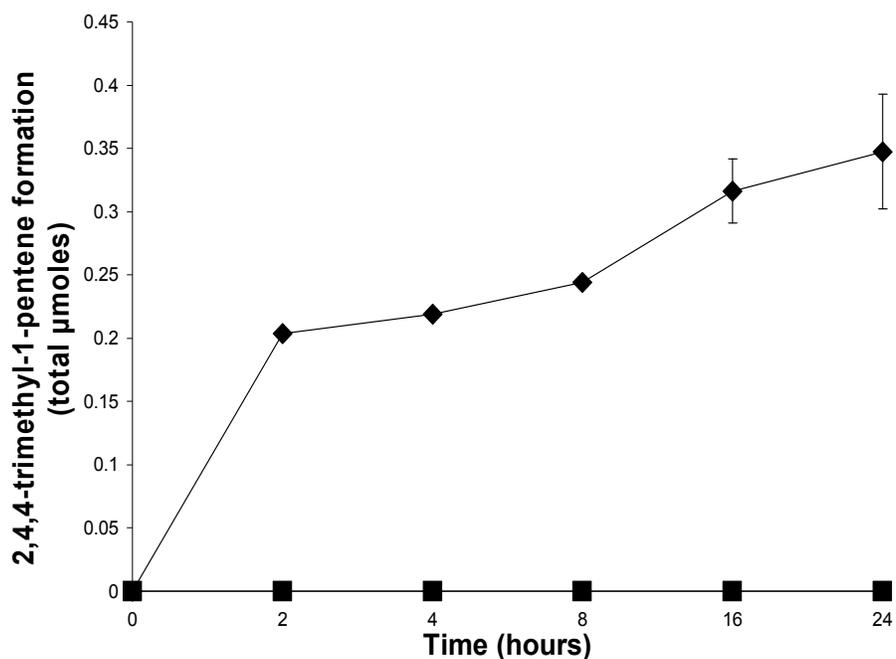


Figure 2.3. 2,4,4-trimethyl-1-pentene formation in the headspace of *E. coli* expressing *opdA*. Sealed culture tubes with 0.61 μ moles OP in the aqueous phase were monitored by headspace sampling for the evolution of 2,4,4-trimethyl-1-pentene in the gas phase over time. *E. coli* pAW1 (■) and the vector control (◆).

Metabolites from wild-type PWE1

GC-MS analysis of acetylated extracts from PWE1 cultures revealed the presence of a metabolite with the same mass spectrum and retention time (20.2

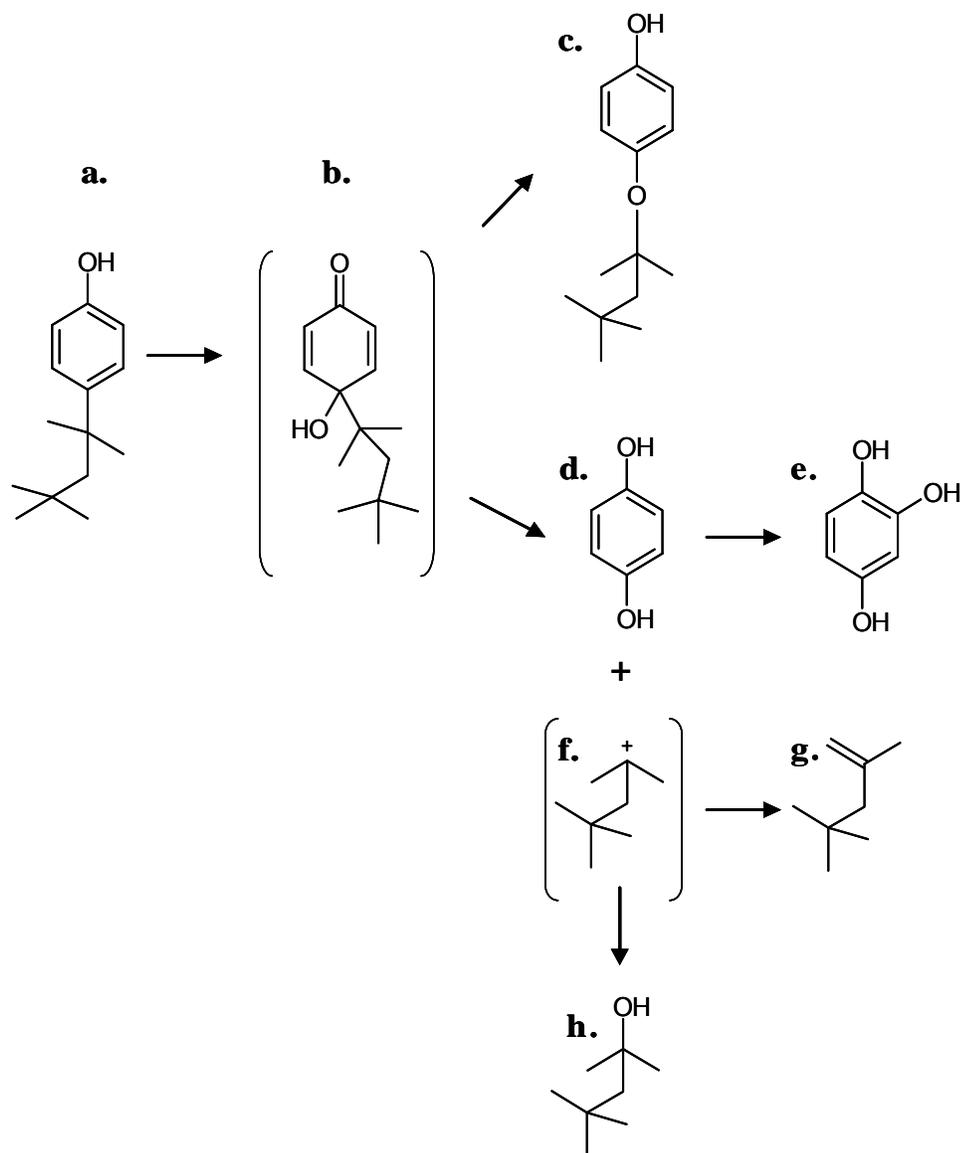


Figure 2.4. Proposed pathway for OP mono-oxygenation encoded by *opdA* in *Sphingomonas* sp. PWE1. (a) OP; (b) putative *ipso* hydroxylation intermediate; (c) tentatively identified octyloxyphenol; (d) HQ; (e) 1,2,4-trihydroxybenzene; (f) putative alkyl side chain carbocation; (g) 2,4,4-trimethyl-1-pentene; (h) 2,4,4-trimethyl-2-pentanol.

minutes) as a similarly derivatized sample of authentic HQ. A putative trihydroxylated intermediate was also detected and found to have the same mass spectrum and retention time (28.7 minutes) as authentic 1,2,4-benzenetriol when

derivatized by acetylation. Another putative metabolite was also detected in ethyl acetate extracts from both the acetylated (30.1 minutes) and underivatized supernatants (29.5 minutes) of PWE1 cultures. Without acetylation the parent ion had an m/z of 222 (23.1), with a base peak of m/z 151 (100), and an additional fragment at an m/z of 123 (37.8). After aqueous acetylation the metabolite yielded a weak molecular ion with an m/z of 264 (10.0) and showed a loss of m/z 42, yielding a fragment with an m/z of 222 (28.0), a base peak with an m/z of 151 (100), and another fragment with an m/z of 123 (26.5). The underivatized spectrum was consistent with either hydroxyoctylphenol (1,2-dihydroxy-4-octylbenzene) or octyloxyphenol (Figure 2.4, compound c), although the addition of only one acetyl group during derivatization strongly suggests a single free hydroxyl group on the ring and is therefore more consistent with octyloxyphenol (Figure 2.4, compound c).

2.5 DISCUSSION

We report here on the successful cloning and expression of a gene (*opdA*) whose product is capable of transforming OP to HQ. Although several thorough recent reports have described in detail the mechanism whereby specific NP isomers are degraded to HQ by sphingomonads closely related to PWE1 (5, 14, 16, 23), no genes have previously been identified that code for such ring hydroxylating and dealkylating activity. It appears that PWE1 utilizes the same enzyme to accomplish both these activities, as expression of *OpdA* was both necessary and sufficient for the transformation of OP to HQ and for the production of the side chain products in *E. coli*. However, we cannot yet rule out the possibility that an endogenous *E. coli* protein catalyzes the dealkylating activity.

While other aromatic monooxygenases, such as phenol monooxygenase in *Pseudomonas putida* CF600, contain multi-component monooxygenases with different

genes encoding the FAD-binding domain and the catalytic monooxygenase subunit, *opdA* likely encodes a single-component enzyme containing both domains. This was suggested from PSI-BLAST analyses indicating homology with the respective conserved regions of an *E. coli* ubiquinone synthesis enzyme, 2-octaprenyl-6-methoxyphenol hydroxylase (UbiH). Interestingly, as with OP, the ubiquinone alkyl side chain is also highly branched, however unlike OpdA, UbiH acts on an unsubstituted carbon *para* to an existing hydroxyl group (38). By contrast, the apparent site of hydroxylation for OpdA is the *para* carbon already occupied by the octyl side chain. OpdA also shared homology with MhpA, which catalyzes the hydroxylation of 3-(3-hydroxyphenyl)-propionate to 3-(2,3-dihydroxyphenyl)-propionate. However, MhpA-associated hydroxylation results in a catecholic intermediate (10), not HQ. Importantly, neither UbiH nor MhpA activities result in removal or rearrangement of the alkyl side chains.

NP and OP degradation were initially thought to occur through ring hydroxylation adjacent to the phenolic hydroxyl group (7, 35) as was shown for the degradation of 3- and 4-*n*-alkylphenols, yielding catecholic intermediates with subsequent *meta*-cleavage (19). More recently Corvini *et al.* (6) showed that degradation of alkylphenols with branched side chains occurs via oxidation at the quaternary alpha carbon in NP isomers p353NP and p262NP in *Sphingomonas* sp. TTNP3. Based on the formation of HQ and the detection of side-chain alcohol products (5), it has been determined that strain TTNP3 transforms NP via type II *ipso* substitution (5). *Ips*o substitution in general is characterized by an intermediate whose leaving group is not hydrogen and in which both groups temporarily share the same position during electrophilic substitution of an aromatic ring. For alkylphenols type I and II *ipso* substitutions differ in the charge of the leaving group and the nature of the resulting ring products: OP degradation via type I *ipso* substitution would result in an

anionic leaving group and the formation of *p*-benzoquinone whereas type II *ipso* substitution of OP would result in the formation of HQ (29). In the case of NP degradation by Bayram and TTNP3, a putative bisubstituted intermediate is thought to decompose and ultimately result in the formation of hydroquinone and a 9-carbon carbocation that then undergoes a S_n1 reaction with water to produce the observed alcohol (15, 25). Kolvenbach *et al.* (22) have shown using ¹⁸O oxygen, that the new hydroxyl group of HQ is derived from molecular oxygen. The HQ is then further metabolized and serves as the true growth substrate.

The branching pattern of the alkyl side chain of NP isomers that serve as growth substrates for TTNP3 and Bayram are different than that of OP, however, those isomers and OP share in common a fully substituted alpha carbon on the alkyl side chain. This feature seems to be a prerequisite for side chain removal (5, 14). Based on the similarity of the intermediates detected in the supernatant of PWE1 and of *E. coli* expressing *opdA* to those produced by Bayram and TTNP3, we propose that PWE1 uses a similar type II *ipso* substitution mechanism to degrade OP. However, in PWE1 we found that a large portion of the putative carbocation was converted to 2,4,4-trimethyl-1-pentene (Figure 2.4). This is likely the result of an E1 elimination reaction and actually strengthens the case for a carbocation intermediate since carbocations are known to undergo both S_n1 and E1 reactions (9). Although we did not confirm that similar alkenes were produced during NP degradation, any that were produced would likely have escaped detection previously as none of the earlier work describing NP degradation employed headspace analysis (6, 14).

Despite OP disappearing to levels below detection in induced cultures of *E. coli* pAW1, we could only account for 57% of the side chain as the pentene and 25% as HQ (Table 2.1). This may have been due to the formation of the octyloxyphenol, a tentatively identified product we previously detected via GC-MS in samples from

wild-type cultures incubated with OP. However, octyloxyphenol was not detectable in *E. coli* pAW1 cultures. This may have been due to the significantly lower concentration of OP used in those assays compared with the wild-type PWE1 experiments. Others have shown that similar NP metabolites accumulate in culture supernatants and do not undergo further metabolism (6, 7, 16). It is also possible that some of the HQ produced in *E. coli* was further transformed to alleviate toxicity or may have polymerized and was therefore not detected using our methods.

HQ is a metabolic intermediate in a variety of aromatic catabolic pathways, including that of *p*-nitrophenol (32), 4-chlorophenol (28), pentachlorophenol (3), γ -hexachlorocyclohexane (25), and 4-ethylphenol (21). Unlike TTNP3, PWE1 was unable to grow on HQ even when ascorbate was added to prevent HQ polymerization. However, resting cells of PWE1 incubated with HQ produced a yellow color that disappeared upon acidification and had a maximum absorbance at 320 nm, which is characteristic of HQ *meta* ring fission product formation (18). HQ has been reported to be directly cleaved via *meta*-cleavage in some instances (25), but in other instances HQ was found to be further transformed to 1,2,4-trihydroxybenzene which then served as a substrate for ring fission (21, 28). In contrast to the results reported for TTNP3, we were able to detect 1,2,4-trihydroxybenzene in ethyl acetate extracts of PWE1 supernatant, but only when the supernatant was first derivatized with acetic anhydride. 1,2,4-trihydroxybenzene did not serve as a growth substrate when supplied exogenously, and was not readily cleaved via *meta* cleavage by PWE1 resting cells so it is unclear if this is a true metabolic intermediate or a dead-end product. Approximately 3.5 kilobases of DNA on either side of *opdA* were sequenced, but did not appear to encode any genes for putative ring cleavage enzymes, as has often been found for other ring hydroxylating enzymes.

Although the additional steps whereby HQ is degraded by PWE1 require

further investigation, we have presented evidence that the degradation of OP to HQ is mediated by a putative flavin monooxygenase encoded by *opdA* from *Sphingomonas* sp. strain PWE1. This is the first example of a gene associated with the ring oxidation and side chain removal of branched chain alkylphenols.

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CHAPTER 3
A COMPARATIVE ANALYSIS OF THE PUTATIVE OCTYLPHENOL
MONOOXYGENASE *opdA* IN THREE *SPHINGOMONAS* STRAINS

3.1 ABSTRACT

The same type II *ipso* substitution mechanism identified for nonylphenol biodegradation by *Sphingomonas* sp. TTNP3 and *Sphingobium xenophagum* Bayram was found in *Sphingomonas* sp. PWE1 cultures grown on octylphenol (OP), where it was associated with a putative flavin monooxygenase encoded by *opdA*. The goal of this work was to determine if similar *opdA* homologs existed in TTNP3 and Bayram, as well as to compare OP-degrading activity in all three strains. Wild-type strains analyzed with *opdA*-specific probes hybridized to DNA of different molecular sizes, in both pulsed-field gel electrophoresis and Southern hybridization. Unlike Bayram or TTNP3, PWE1 had two apparent sites of hybridization in both assays, suggesting that *opdA* is found in a different genetic context in each of the three strains.

PCR fragments encoding *opdA* were cloned from each *Sphingomonas* strain and expressed in *E. coli* where they conferred the ability to degrade OP with concomitant formation of hydroquinone and 2,4,4-trimethyl-1-pentene. Each subclone degraded similar amounts of OP, but the amount of metabolites detectable varied. Incubating these clones with related alkoxyphenolic substrates also led to hydroquinone formation, although the actual amount of hydroquinone detected varied among the three subclones. Cell-free extracts made from clones over-expressing *opdA* showed hydroquinone formation only when the extracts were amended with both FAD and NADPH.

OP induction experiments showed increased *opdA* mRNA accumulation in Bayram, as well as significantly higher 2,4,4-trimethyl-1-pentene production. PWE1

and TTNP3 *opdA* genes did not appear to be inducible as they showed no changes under the same conditions. When *opdA* was sequenced from these strains we found only 5 predicted amino acid differences, 4 of which occurred in TTNP3. Near identical nucleotide sequence similarity within *opdA*, as well as the upstream region, suggests that the differences in activity may be related to differences in transcriptional regulation.

3.2 INTRODUCTION

The endocrine disrupter nonylphenol (NP) consists of at least 22 *para*-isomers (17) that have varying degrees of branching in their alkyl side chain. Degradation activity was previously shown for linear NP, although it was a co-metabolic process that did not allow growth (1, 7). Degradation of specific branched isomers of nonylphenol (NP) has been examined in *Sphingomonas* sp. TTNP3 and *Sphingobium xenophagum* Bayram, and the initial steps of degradation were found to proceed by a type II *ipso* substitution mechanism (4, 6). This contrasts with the more common degradation mechanism described for short-chain linear alkylphenols (9, 16) which proceeds through catecholic intermediates. Strains TTNP3 and Bayram require a fully substituted alpha carbon on the alkyl side chain in order for growth to occur (5, 7). Such quaternary substituted alkylphenols represent a new group of substrates shown to undergo *ipso* substitution (4), though *ipso* substitution had previously been reported as the pathway for chlorophenol and nitrophenol catabolism by cytochrome P450 (13, 14).

Octylphenol (OP) is related to NP, but consists of a single branched isomer with a dimethyl substituted alpha carbon on the side chain. We previously demonstrated that *Sphingomonas* sp. PWE1 utilizes type II *ipso* substitution for OP biodegradation (15). The putative FAD-dependent monooxygenase we cloned from

PWE1, encoded by *opdA*, was directly responsible for the conversion of OP to hydroquinone (HQ) and 2,4,4-trimethyl-1-pentene in *Sphingomonas* sp. PWE1 (15). This was the first report of a gene encoding type II *ipso* substitution of alkylphenols. The similarities in the transformation mechanisms led us to hypothesize that Bayram and TTNP3 were likely to contain homologs of *opdA* that might be involved in alkylphenol biodegradation. The goal of this work was to determine if *opdA* homologs exist in TTNP3 and Bayram, if they encoded similar activity, and if the substrate range was similar.

3.3 MATERIALS AND METHODS

Chemicals

OP, FAD, HQ and NADPH were obtained from Sigma-Aldrich (St. Louis, MO). 4-*t*-Butylphenol, 4-*t*-butoxyphenol, and 4-*n*-octyloxyphenol were obtained from Fisher Scientific (Pittsburgh, PA). IPTG was acquired from Gold Biotechnology (St. Louis, MO). All solvents used were HPLC-grade and were purchased through Fisher Scientific. DTT, lysozyme, and proteinase K were obtained from Acros (Morris Plains, NJ).

Growth on OP

All cultures were maintained in MSM medium (12) with 1,000 mg L⁻¹ OP. For higher density cell growth the strains were grown in rich medium, Luria Bertani (LB) for Bayram and 218 (5 g L⁻¹ peptone, 5 g L⁻¹ tryptone, 2 g L⁻¹ yeast extract, 1 g L⁻¹ MgSO₄ x 7H₂O) (3) for PWE1 and TTNP3, which was supplemented with 100 mg L⁻¹ OP.

Amplification and sequencing of opdA

Primers for *opdA* forward (5' TTC ATC CTG AAA GAC ACT GCC GGA 3') and reverse (5' ACG CGC TTC CAG ACC AAC CTA TTT 3') (14) were used in

PCRs with DNA from Bayram and TTNP3 to determine if they amplified fragments that were similar in size to those amplified from PWE1. Amplification products were sequenced with the PCR primers, as well as internal forward (5' TTT GAT GTG GCT ATT GTC GGC TGC GG 3') and reverse (5' GCG CTC TTC ATC ATA TGT GTC CAG G 3') primers at the Cornell University BioResource Center. Sequences were aligned with the MegAlign program (DNA Star, Madison, WI). Putative promoter regions were identified with the BPROM program (Softberry, Inc., Mount Kisco, NY).

Pulsed-field gel electrophoresis

Cultures were grown in either 218 or LB, as well as in MSM with OP as the sole carbon source. Cultures grown on rich media were concentrated 10-fold via centrifugation whereas those grown on MSM OP were concentrated 20-fold, both were resuspended in phosphate buffer (30 mM pH 7.4). The concentrated cells suspensions were fixed in low-melt agarose and the agarose-fixed cells were then lysed as per Basta *et al.* (2). Briefly, solidified agarose-cell plugs were RNase-treated and lysed with lysozyme at 37°C for 1 hour. The plugs were further incubated with proteinase K at 50°C overnight, before digestion with 7 units of S1 nuclease, after which the plugs were loaded into a 1% agarose gel. The DNA was separated using in a CHEF DRI-II system (Bio-Rad, Hercules, CA) with a pulse interval of 15-50 seconds and 6 V cm⁻¹ for 24 hours at 14°C.

The gel was then transferred to a Nytran SPC membrane (Whatman, Florham Park, NJ) using downward neutral transfer. The membrane was probed using a 650 base internal fragment of *opdA* that was amplified with forward (5' TCA ATC ACG AGG TCA CTG GCA TCA) and reverse (5' GCG CTC TTC ATC ATA TGT GTC CAG G) primers specific for PWE1. The probe was labeled with α -³³P dATP (Perkin Elmer, Waltham, MA) using DECAprime II kit (Ambion, Austin, TX). The membrane was prehybridized at 42°C for 2 hours with UltraHyb (Ambion), then

hybridized with the probe for 20 hours at 42° C. The membrane was washed twice with 2X SSPE, 0.1% SDS for 5 minutes, then twice with 0.1X SSPE, 0.1% SDS for 15 minutes. The membrane was exposed overnight to a phosphor screen and the signal was quantified with a Molecular Dynamics PhosphoImaging system and IMAGEQUANT software.

Southern hybridizations

The cell plugs used above for pulsed-field gel electrophoresis were also used as the DNA source for Southern gels. The plugs were removed from storage buffer and equilibrated with two 30-minute washes of TE buffer. The buffer was removed and plugs were incubated for 30 minutes in 100 µl of restriction enzyme buffer to ensure diffusion through the agarose. The plugs were then incubated in a fresh aliquot of 100 µl of restriction enzyme buffer and 30 U of either *ApaI* (Promega, Madison, WI) or *PvuII* (Fisher Scientific, Pittsburgh, PA) at 37°C overnight. Following digestion, the buffer was removed and the plugs were incubated at room temperature in TE buffer. The plugs were then loaded into a 1% agarose gel, and the gel was run at 2 volts cm⁻¹ overnight. The DNA was transferred to a Zeta-Probe membrane (Bio-Rad) using a TurboBlotter (Whatman) via downward neutral transfer as per the manufacturer instructions. The membrane was then probed with a 650 base fragment of *opdA* as described above in the pulsed-field gel electrophoresis section.

Assessing the inducibility of OP degradation

All three wild-type strains were grown for 48 hours in rich medium with or without 100 mg L⁻¹ OP. The cultures grown without OP had previously been transferred 3 consecutive times without OP to insure no residual OP was carried over. The cells were pelleted via centrifugation, washed twice in 30 mM phosphate buffer, and resuspended in the same buffer. OP was added at a concentration of 25 mg L⁻¹ to 5 ml cell suspensions in Balch tubes. Duplicates were set-up using cultures that had

been grown with OP. Headspace samples of 0.25 ml were taken at 0, 3, and 6 hours to look for 2,4,4-trimethyl-1-pentene formation by GC-MS as described below. Cultures were sacrificed after 6 hours by the addition of an equal volume of methanol. Aliquots of 1-ml were removed and prepared for OP quantification by HPLC analysis as described below.

To determine if the inducibility of OP degradation correlated with changes in *opdA* mRNA levels, we grew cells in MSM with 1000 mg L⁻¹ OP or rich medium without OP. Rich media was employed because no single carbon source other than OP supported growth of all three strains. The OP-grown cultures were filtered through glass wool to remove any insoluble OP particles then concentrated via centrifugation. The cell pellets were then stored in RNAlater (Ambion) according to manufacturer's instructions. After removing RNAlater, cells were lysed using a combination of 10 mg ml⁻¹ lysozyme and 1 mg ml⁻¹ proteinase K. RNA was purified from the lysate with the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA).

RNA was quantified using RiboGreen (Molecular Probes, Eugene, OR) and then diluted in DEPC-treated water to a 0.002 µg µl⁻¹ total RNA concentration. The dilutions were applied in duplicate to a Zeta-Probe membrane (Bio-Rad) using a Bio-Rad Slot Blot apparatus. The membrane was cut in half, preserving one set of dilutions for probing with an *opdA*-specific probe and the other for probing with a 16S rRNA gene-specific probe. Probes were generated as described above, and an additional 16S rRNA probe was made with PWE1 DNA as template using universal primers 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3'). Hybridizations were carried out and quantified under the conditions described above.

Heterologous expression of opdA

We had previously cloned *opdA* from strain PWE1 to create JM109 pAW1 (15). In order to determine if the putative *opdA* amplicons obtained from TTNP3 and Bayram encoded similar OP degradation activity, we cloned them into pGEM-T Easy Vector (pGEM) (Promega). The resulting plasmids were designated pAW2T and pAW3B, respectively, and transformed into *E. coli* JM109. Overnight cultures were diluted 1:10 by adding 500 μ l of culture to 4.5 ml 1/10th LB with 150 mg ml⁻¹ ampicillin and were incubated at 37°C for 2 hours in Balch tubes. Controls received 50 mM glucose upon inoculation to suppress the *lac* promoter and did not contain IPTG. All other samples had 0.2 mM IPTG added 2 hours post inoculation. After 2 hours of induction at 37°C 25 mg L⁻¹ OP was added and the cultures were immediately sealed with butyl rubber stoppers and crimped to trap volatile metabolites. OP degradation activity was assessed by measuring 2,4,4-trimethyl-1-pentene formation as described previously (15). Samples of 0.25 ml of headspace were analyzed by GC-MS using an HP 6890 GC equipped with an HP-5MS column (5% phenyl methyl siloxane; 30 m by 0.25 mm; 0.25- μ m film thickness) using helium as the carrier gas with a flow rate of 1 ml min⁻¹. Initially, the temperature was held at 40°C for 1 minute, then increased by 5°C per minute to 100°C and held for 3 minutes. The temperature was then increased by 10°C per minute to 165°C and finally increased at 60°C per minute to 240°C. The detector was an HP 5973 MSD with the quadrupole and source set at 150°C and 230°C, respectively.

After 24 hours the cultures were sacrificed by the addition of an equal volume of methanol for further chemical analysis. The methanol-amended culture was centrifuged to remove cellular debris and the resulting supernatant was filtered through a 4 mm 0.2 μ m regenerated cellulose syringe filter (Corning, Corning, NY). The filtrate was analyzed for both HQ and OP via HPLC. OP was resolved with a

mobile phase of 80% methanol and 20% 80 mM acetic acid. The solvent was pumped at a rate of 1 ml min⁻¹ using a Waters Model 590 pump through a Varian Microsorb-MV C₁₈ column (250 mm by 4.6 mm). Samples were injected by a Shimadzu SIL-10AD AP autoinjector and detected with a Shimadzu SPD-10A VP UV-Vis detector by monitoring absorbance at 220 nm. HQ was similarly quantified, although the mobile phase consisted of 20% methanol and 80% of 40 mM acetic acid. Absorbance was measured at 290 nm.

To assess OP degradation activity in cell free lysates, subclones were prepared as above, but the cultures were pelleted at 7,000 rpm after 2 hours of induction. After removing the supernatant, the pellet was stored at -80°C overnight. The pellets were resuspended in 1 ml of 30 mM phosphate buffer and sonicated 10 times in 15 second intervals. Crude extracts were treated with 1 mM DTT before centrifugation at 16,000 x g at 4°C for 30 minutes to remove residual cell debris. The supernatant was then filtered through a 0.2 um filter. Extracts were amended as described by Kolvenbach *et al.* (10) with 1 mM FAD, 20 mM NADPH, and 50 mg L⁻¹ OP in a total reaction volume of 400 µl. The extracts were incubated at room temperature for 4 hours, at which time an equal volume of methanol was added to stop the reactions. The extracts were analyzed for HQ production by HPLC as described above.

HQ formation in subclones treated with alternate carbon sources

Three other possible substrates for *ipso* substitution were tested with the subclones pAW1, pAW2T, and pAW3B in JM109. The cultures were set up as described above, but *t*-butylphenol, *t*-butoxyphenol, or *n*-octyloxyphenol were added at 25 mg L⁻¹ instead of OP. The cultures were sacrificed after 24 hours and HQ concentrations were measured by HPLC as described above.

3.4 RESULTS and DISCUSSION

Southern hybridization

As expected, PWE1, TTNP3, and Bayram DNA digested with *Pvu*II showed hybridization to a fragment of approximately 1.6 kb in size (Figure 3.1b). The fact that similar fragments were produced from *Pvu*II sites flanking *opdA* even though they had only been identified in PWE1 suggested that the immediate genetic context surrounding *opdA* is similar for all three strains. To assess the broader genetic context, *Apa*I was chosen because *in-silico* analyses revealed that it did not cut within the immediate vicinity of *opdA*. *Apa*I cut within Bayram DNA more frequently than in PWE1 or TTNP3. As a result, the probe hybridized to a smaller fragment of Bayram DNA than TTNP3 or PWE1. Both TTNP3 and PWE1 had hybridization at the wells, but PWE1 also had hybridization to a very large fragment of DNA that had migrated out of the well. The size of the fragments that hybridized following the *Apa*I digest were not quantified, as the bands did not migrate as far as the high molecular weight markers of the DNA ladder.

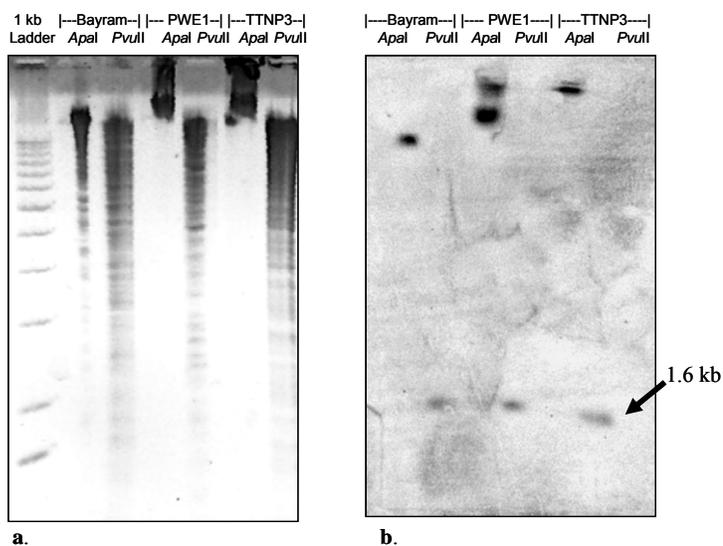


Figure 3.1. Southern hybridization of wild-type strains. (a) Wild-type OP-degraders were digested with *Apa*I and *Pvu*II. (b) Corresponding membrane hybridized with an *opdA*-specific probe to show the location of *opdA*.

Pulsed-field gel electrophoresis with wild-type OP degraders

There were two bands visible on the pulsed-field gel lanes for PWE1 and TTNP3 that could be plasmids. The bands appeared to be about 430 and 360 kb in size (Figure 3.2a). While the DNA profile obtained via pulsed-field gel electrophoresis was similar for TTNP3 and PWE1, the regions that hybridized with the *opdA* probe appeared to be different. PWE1 appeared to have two faint regions of hybridization, one corresponding to a plasmid band of 360 kb and the other corresponding to what was likely intact genomic DNA. It is possible that *opdA* is duplicated in the PWE1 genome, which may explain the two points detected in the Southern blot of *ApaI* digested DNA.

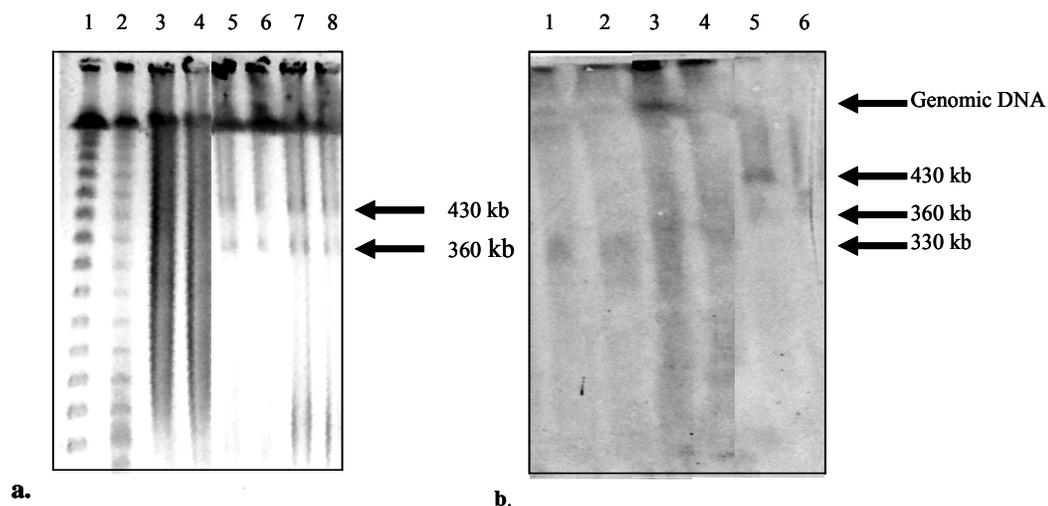


Figure 3.2. Pulsed-field gel electrophoresis with wild-type OP-degraders. (a) Pulsed-field gel electrophoresis. Lane 1 is a high molecular weight ladder and lane 2 is a lower molecular weight ladder. Lane 3 and 4 are Bayram digested with S1 nuclease and not digested, respectively. Lane 5 is PWE1 digested with S1 nuclease and lane 6 is undigested PWE1 DNA. Lane 7 is TTNP3 DNA that has been digested with S1 nuclease and lane 8 is TTNP3 DNA that has not been digested. (b) Hybridization of the corresponding membrane with a probe specific for *opdA*. Lane 1 and 2 contain Bayram DNA, 3 and 4 are PWE1 DNA, and 5 and 6 have TTNP3 DNA.

There was weak hybridization in TTNP3 that corresponded to a plasmid band of approximately 430 kb. Consistent with the presence of a plasmid in TTNP3, the signal was strongest in lanes where the DNA had been digested with S1 nuclease.

It was difficult to determine if there were mega plasmids in Bayram, as endogenous nuclease activity appeared to result in smearing so that the PFGE separation did not reveal distinct bands. There appeared to be some weak hybridization in Bayram at approximately 330 kb (Figure 3.2b), although no distinct band was visible at that weight in the original pulsed-field gel.

OP disappearance in pGEM subclones

Consistent with probing experiments, a copy of *opdA* was readily amplified via PCR from both Bayram and TTNP3. When the PCR product was cloned and expressed in *E. coli* there was noticeable 2,4,4-trimethyl-1-pentene formation in within 2 hours, although the concentration was higher in JM109 pAW2T and JM109 pAW3B than in JM109 pAW1 (Figure 3.3). The trend continued for the duration of the experiment (24 hours), with final amounts of 2,4,4-trimethyl-1-pentene found to be 0.11 μ moles in JM109 pAW1 clones, 0.29 μ moles in JM109 pAW2T clones, and 0.20 μ moles in JM109 pAW3B clones. There was more variability among the duplicate samples of JM109 pAW3B as compared to the other two subclones. After 16 hours there were detectable amounts of 2,4,4-trimethyl-1-pentene formation in subclones JM109 pAW2T and JM109 pAW3B (0.021 and 0.017 μ moles, respectively, after 24 hours) that had been treated with 50 mM glucose to repress expression, although this would be expected due to the leaky tendency of the *lacZ*-based pGEM cloning vector. 2,4,4-Trimethyl-1-pentene was not detected JM109 pAW1 subclones treated with glucose, nor was it detected in any of the induced cultures that were not supplemented with OP.

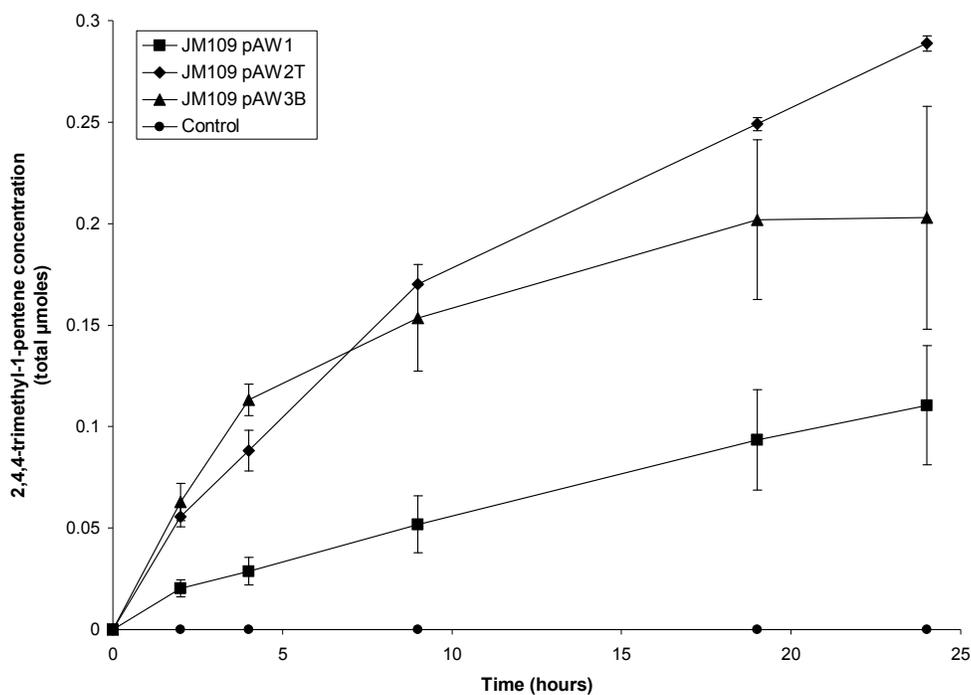


Figure 3.3. Formation of 2,4,4-trimethyl-1-pentene in *opdA* subclones. Headspace samples were taken from subclones JM109 pAW1, JM109 pAW2T, JM109 pAW3B, and the vector control that had been induced with IPTG and supplied with 25 mg L⁻¹ OP.

Table 3.1 demonstrates that there was no statistical difference ($P > 0.05$) in the amount of OP remaining in JM109 pAW1 culture after 24 hours as compared to JM109 pAW2T. The average calculated OP disappearance based on the amount of residual OP was basically the same for all three subclones, with 82% disappearance for both JM109 pAW2T and JM109 pAW3B and 76% for the JM109 pAW1. However, there was much more variation regarding the amount of HQ and 2,4,4-trimethyl-1-pentene that were formed. The JM109 pAW3B subclone produced 79% of the anticipated HQ and 33% of the expected 2,4,4-trimethyl-1-pentene. The JM109 pAW2T subclone had 67% and 48% of HQ and 2,4,4-trimethyl-1-pentene, respectively, whereas the JM109 pAW1 subclone produced 48% of the expected HQ and only 18% of the expected 2,4,4-trimethyl-1-pentene.

Table 3.1. Mass-balance of OP disappearance in subclones JM109 pAW1, JM109 pAW2T, and JM19 pAW3B. Cultures were sacrificed after 24 hours and OP, HQ, and 2,4,4-trimethyl-1-pentene values were compared to the calculated expected μ moles. Standard deviations for the average actual final concentrations are designated by parentheses.

	Starting mass (μ moles)	Expected final mass (μ moles)	Actual final mass (μ moles)	Percent (%) Expected
JM109 pAW1				
OP	0.61	0	0.15 (0.0075)	76
HQ	0	0.61	0.29 (0.044)	48
2,4,4-trimethyl-1-pentene	0	0.61	0.11 (0.029)	18
JM109 pAW2T				
OP	0.61	0	0.11 (0.0074)	82
HQ	0	0.61	0.40 (0.10)	67
2,4,4-trimethyl-1-pentene	0	0.61	0.29 (0.0038)	47
JM109 pAW3B				
OP	0.61	0	0.11 (0.025)	82
HQ	0	0.61	0.48 (0.12)	79
2,4,4-trimethyl-1-pentene	0	0.61	0.20 (0.055)	33

Previously we had reported recovering 25% of the expected HQ and 57% of the expected 2,4,4-trimethyl-1-pentene from JM109 pAW1 after 70 hours of incubation (15). That decreased HQ recovery relative to what we report here was likely due in part to extended incubation time (70 h) which facilitated self-polymerization of accumulating HQ as was evident by the accumulation of a brown polymerization product in the supernatant (14). Despite higher HQ yields, the amount of pentene recovered over 24 hours (Figure 3.3) was lower in each subclone than was

previously reported for JM109 pAW1 (15). The reason for this remains unclear. Variation from culture to culture was observed for all three subclones in previous experiments (data not shown), even though the seed cultures for all experiments were inoculated from the same frozen stocks.

HQ formation in subclones treated with structurally related substrates

HQ was produced from three structurally related substrates, 4-*t*-butoxyphenol, 4-*n*-octyloxyphenol, and 4-*t*-butylphenol, by JM109 pAW1 and JM109 pAW3B, whereas JM109 pAW2T subclones produced HQ from 4-*t*-butoxyphenol and 4-*n*-octyloxyphenol but not from 4-*t*-butylphenol (Table 3.2). In all cases, the amount of HQ that formed was much less than the amount formed when OP was added as substrate. Wild-type Bayram had previously demonstrated *ipso* substitution activity with 4-*t*-butoxyphenol and these results corroborate those observations (6). Oxygen uptake was reported to have been stimulated in Bayram by 4-*t*-butylphenol (6), which supports the observed formation of HQ in JM109 pAW3B. Our result showing that HQ served as substrate for the Bayram *opdA* subclone contrast with those of Gabriel *et al.* who found that 4-*n*-octyloxyphenol did not stimulate oxygen uptake in wild-type Bayram cultures (6). Given the low levels of HQ we observed and the high levels of endogenous oxygen uptake by resting cells, this apparent discrepancy is likely due to the different detection limits of these methods.

The formation of branched octyloxyphenol has been hypothesized as a dead-end product in JM109 pAW1 cultures (15) with a similar product, 4-(2,6-dimethylheptan-2-yloxy)-phenol accumulating in NP treated cultures of TTNP3 (3). The formation of HQ from 4-*n*-octyloxyphenol was therefore somewhat surprising since branched side chain removal from the ring requires a fully substituted alpha carbon (5, 7). However, it is possible that constraints on the branching of the side chain are different for *ipso* substitution from the oxygen of an aryl ether than it is

directly from the ring. The data in table 3.2 also demonstrate that the ether substrates are transformed less efficiently than OP, thus the apparent dead-end nature of similar metabolites may be an issue of kinetics.

Table 3.2. Transformation of structurally related substrates to HQ. Subclones JM109 pAW1, JM109 pAW2T, and JM109 pAW3B were tested with either (a) 4-*t*-butylphenol, (b) 4-*t*-butoxyphenol, or (c) 4-*n*-octyloxyphenol at starting concentrations of 170, 150, and 110 μM , respectively. The actual HQ formed after 24 hours was compared to the expected HQ concentration. Standard deviations for the average actual HQ concentration are designated by parentheses.

a. 4-<i>t</i>-Butylphenol	Starting concentration (μM)	Expected HQ (μM)	Actual HQ (μM)	% of Expected
JM109 pAW1	170	170	37 (7.3)	22
JM109 pAW2T	170	170	0 (0)	0
JM109 pAW3B	170	170	50 (3.4)	30
vector control	170	170	0	0

b. 4-<i>t</i>-Butoxyphenol	Starting concentration (μM)	Expected HQ (μM)	Actual HQ (μM)	% of Expected
JM109 pAW1	150	150	12 (3.6)	8
JM109 pAW2T	150	150	8.2 (0)	5.5
JM109 pAW3B	150	150	31 (10)	20
vector control	150	150	0	0

c. 4-<i>n</i>-Octyloxyphenol	Starting concentration (μM)	Expected HQ (μM)	Actual HQ (μM)	% of Expected
JM109 pAW1	110	110	23 (0)	20
JM109 pAW2T	110	110	3.7 (1.9)	3.2
JM109 pAW3B	110	110	14 (0)	13
vector control	110	110	0	0

Activity in cell-free extracts

HQ was detectable in cell-free extracts of the *E. coli* subclones expressing *opdA* from PWE1, Bayram, and TTNP3 (Table 3.3), but only when both NADPH and FAD were added to the reaction mixture (data not shown). The amount of HQ produced by the extract from TTNP3 subclones was 2-3 fold less than what was detected for Bayram and PWE1 subclones. The reason for this remains unclear since they all degraded similar amounts *in vivo*. One possibility is that OpdA from TTNP3 is kinetically slower than the enzyme from the other two strains. Such differences might not have been picked up in the *in vivo* assay which lasted 24h, but would be more obvious over the shorter time frame (4h) used in the *in vitro* assay. Differences in kinetics are also supported by data in table 3.2 which show much lower production of HQ from *t*-butoxyphenol and *n*-octyloxyphenol by pAW2T extracts.

Table 3.3. HQ formation in cell-free extracts of *E. coli* expressing *opdA* from different *Sphingomonas* strains. Extracts of subclones JM109 pAW1, JM109 pAW2T, and JM109 pAW3B were treated OP. The resulting HQ was measured at the end of the experiment and compared to the expected concentration. Each subclone was measured individually, not as replicates.

	Expected HQ (μ M)	Actual HQ (μ M)	% of Expected
JM109 pAW1	240	150	63
JM109 pAW2T	240	41	17
JM109 pAW3B	240	110	46

Assessing induction in wild-type cultures

Bayram produced 30% more 2,4,4-trimethyl-1-pentene in resting cells from OP exposed cultures as compared to resting cells from cultures without prior OP exposure (Figure 3.4). This difference was statistically significant ($P < 0.05$) and is

supported somewhat by earlier reports which showed a difference between NP degradation in induced and uninduced cultures of Bayram resting cell (6). Unlike Gabriel *et al.*, however, we did see a high level of constitutive activity in uninduced cultures. It is possible that the difference we observed between induced and uninduced Bayram cultures was not as great as that observed by Gabriel *et al.* because the 100 mg L⁻¹ of OP used as an inducer was consumed early in the 48 h incubation, or because OP is not as effective an inducer as the NP used previously (6).

Although resting cell cultures of PWE1 grown in an absence of OP actually produced 15% more pentene than cultures exposed to OP prior to the experiment and TTNP3 cultures grown without OP showed 7% less pentene formation, neither of these differences were statistically significant.

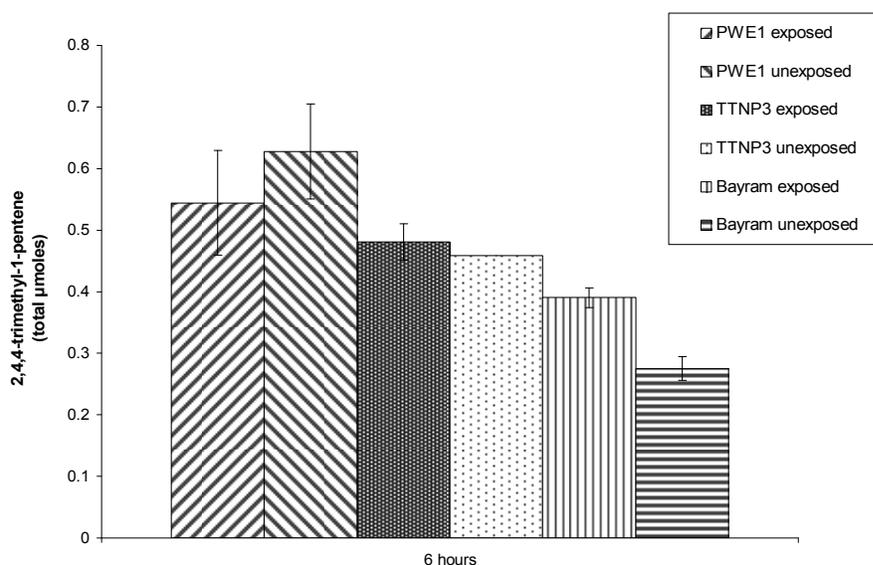


Figure 3.4. 2,4,4-trimethyl-1-pentene formation in wild-type resting cells. Resting cell cultures of the wild-type strains grown in OP or transferred 3 times without the addition of OP were tested for 2,4,4-trimethyl-1-pentene formation in the culture headspace. Only Bayram showed a statistically significant difference in 2,4,4-trimethyl-1-pentene formation when comparing resting cells with and without previous OP exposure ($P < 0.05$).

The effect of OP exposure on comparative *opdA* mRNA expression was determined by quantifying the ratio of 16S rRNA-normalized hybridization signal from cultures with and without OP exposure. Consistent with our observations regarding 2,4,4-trimethyl-1-pentene formation by whole cells, Bayram accumulated higher relative amounts of *opdA* mRNA when grown in the presence of OP than either PWE1 or TTNP3 (Figure 3.5). This suggests that *opdA* homologs in PWE1 and TTNP3 do not appear to be inducible, while *opdA* expression in Bayram is inducible.

	0.2 µg RNA Relative intensity	Normalized Intensity (Induced/Uninduced)
PWE1 Exposed to OP	16S 	1.94
	<i>opdA</i> 	
PWE1 Not exposed to OP	16S 	
	<i>opdA</i> 	
TTNP3 Exposed to OP	16S 	1.69
	<i>opdA</i> 	
TTNP3 Not exposed to OP	16S 	
	<i>opdA</i> 	
Bayram Exposed to OP	16S 	2.95
	<i>opdA</i> 	
Bayram Not exposed to OP	16S 	
	<i>opdA</i> 	

Figure 3.5. *OpdA* mRNA induction in wild-type cells. RNA slot-blot with RNA extracted from wild-type cells either growth with or without OP were hybridized with a probe designed for *opdA*. The hybridization intensity was normalized to the intensity of signal from a 16S rRNA probe hybridized to total RNA from the samples. The ratio of the OP-exposed to unexposed samples was compared for each strain.

Our observations regarding *opdA* mRNA abundance and OP degradation induction are consistent with Gabriel's work which has shown that NP degradation is inducible in Bayram (6). They are also consistent with Corvini's observation that NP degradation is not inducible in TTNP3 (4). PWE1 is similar to TTNP3 in this regard. The results from the activity induction experiment (Figure 3.4) correlate well with our observations regarding *opdA* mRNA abundance in both TTNP3 and PWE1 (Figure 3.5). Although the differences in OP induced levels in *opdA* mRNA are small, they suggest the possibility of regulatory differences between Bayram and the uninducible strains TTNP3 and PWE1.

In an effort to understand the mechanism behind differences in the level of inducibility between the three strains, we sequenced the DNA more than 1000 base pairs upstream of *opdA*. Surprisingly, this upstream region was 100% identical for all three strains. This demonstrates that regulatory differences were not due to sequence divergence in the promoter, although it is possible that far upstream regulatory sites which we have not sequenced may be involved. *In silico* analyses predicted the presence of a putative promoter region 200 bases upstream of the *opdA* start site for all strains. Although no regulatory protein was identified, it is possible that the differences observed in the mRNA abundance and OP degradation activity of wild-type TTNP3, Bayram, and PWE1 could be due to differences in the regulatory protein.

In contrast to the 5' region of *opdA*, we have indirect evidence that there are differences in the downstream sequence of Bayram. A 469 base pair region downstream of *opdA* was PCR-amplified in TTNP3 with primers designed from PWE1, and found to be 100% identical in TTNP3 and PWE1. These same primers did not produce an amplicon from Bayram template DNA. This suggests nucleotide sequence differences, at least in the target site of the reverse primer located downstream from *opdA*, since we know from sequence analysis that the forward

primer, targeted within *opdA* itself, was a perfect match.

It is possible that these indirectly observed differences downstream of *opdA* could possibly lead to differences in message stability (11). Alternatively differences in genomic context may account for the observed differences in inducibility. Southern hybridization did show differences in the location of hybridization for all three strains. Both Bayram and TTNP3 had single distinct regions of hybridization, but PWE1 appeared to have two points of hybridization. It is not clear if this implies gene duplication, the presence another gene with a region homologous to the *opdA* probe, or is simply the result of an incomplete enzyme digest.

OpdA sequence alignment

The nucleotide sequence for *opdA* was almost identical for all three strains. There were 7 total nucleotide differences between these strains in the *opdA* coding region, but only 5 had an effect on the amino acid composition. The predicted amino acid sequence of OpdA differed by 1 amino acid when comparing PWE1 and Bayram, but the predicted sequence from TTNP3 differed from PWE1 by 4 amino acids (Figure 3.6).

The resulting residue differences between the three strains were not located within the predicted FAD-binding motif previously identified in the PWE1 OpdA sequence (15). Previously, we had identified three point mutations, N163S, Q205E, and A241C, in a copy of OpdA that no longer conferred OP degradation activity in *E. coli* (15). Interestingly, two of the four predicted amino acid differences between TTNP3 and PWE1 corresponded to the residues identified in loss of function mutants. The sequence in the loss of function mutants was identical to that of TTNP3 in residue Q205 and A241, suggesting that those residues are not absolutely necessary for OP degradation activity. However, residue N163 was conserved in all three strains, suggesting it may play a role in activity. There was a single difference in predicted

amino acid sequence between Bayram and PWE1, which does not appear to effect activity, as differences between Bayram and PWE1 with regard to transformation of structurally related substrates were not statistically significant ($P < 0.05$).

Bayram	1	MENEMTTEPGDRASQFDVAIVGCGPVGALLANLLKQYGHKVAVLEREPDIFYAPRGM G FD	60
PWE1	1	MENEMTTEPGDRASQFDVAIVGCGPVGALLANLLKQYGHKVAVLEREPDIFYAPRGM G FD	60
TTNP3	1	MENEMTTEPGDRASQFDVAIVGCGPVGALLANLLKQYGHKVAVLEREPDIFYAPRGM A FD	60
Bayram	61	DESTRIMQSAGILDRLKAEGHIYQAD L ELIDRS GKRLGGFDRRSVGEDLLSGLHGHRHLT	120
PWE1	61	DESTRIMQSAGILDRLKAEGHIYQAD L ELIDRS GKRLGGFDRRSVGEDLLSGLHGHRHLT	120
TTNP3	61	DESTRIMQSAGILDRLKAEGHIYQAD S ELIDRS GKRLGGFDRRSVGEDLLSGLHGHRHLT	120
Bayram	121	LFHQPSLEATLREEFATGENAATAYFNHEVTGITDQGDRVELNCKDRATDEEHSLIAKYV	180
PWE1	121	LFHQPSLEATLREEFATGENAATAYFNHEVTGITDQGDRVELNCKDRATDEEHSLIAKYV	180
TTNP3	121	LFHQPSLEATLREEFATGENAATAYFNHEVTGITDQGDRVELNCKDRATDEEHSLIAKYV	180
Bayram	181	VGCDGARSTVRKTMNVPRIDLKYT Q KYLVDVAIVDDPVYFRTMIPQGGYIILLDGKEAGVL	240
PWE1	181	VGCDGARSTVRKTMNVPRIDLKYT Q KYLVDVAIVDDPVYFRTMIPQGGYIILLDGKEAGVL	240
TTNP3	181	VGCDGARSTVRKTMNVPRIDLKYT T EKYLVDVAIVDDPVYFRTMIPQGGYIILLDGKEAGVL	240
Bayram	241	A KGLHGHVRFDFLQHSETIGQELKTDEDYQKAARDLIRSRGFDPENFRVIRSVSYTFHAG	300
PWE1	241	A KGLHGHVRFDFLQHSETIGQELKTDEDYQKAARDLIRSRGFDPENFRVIRSVSYTFHAG	300
TTNP3	241	C KGLHGHVRFDFLQHSETIGQELKTDEDYQKAARDLIRSRGFDPENFRVIRSVSYTFHAG	300
Bayram	301	MPSKWRVGRMLVAGDAAHLTPPWSGQGLNMGVRDAANLSFKLNLAALRGKSSDRILDYDE	360
PWE1	301	MPSKWRVGRMLVAGDAAHLTPPWSGQGLNMGVRDAANLSFKLNLAALRGKSSDRILDYDE	360
TTNP3	301	MPSKWRVGRMLVAGDAAHLTPPWSGQGLNMGVRDAANLSFKLNLAALRGKSSDRILDYDE	360
Bayram	361	ERRPQSLETIQAAVDMGIRMQNTSPLQIGLRNLAYALSRSKSKFVNRLLFKNWIRKPSYKS	420
PWE1	361	ERRPQSLETIQAAVDMGIRMQNTSPLQIGLRNLAYALSRSKSKFVNRLLFKNWIRKPSYKS	420
TTNP3	361	ERRPQSLETIQAAVDMGIRMQNTSPLQIGLRNLAYALSRSKSKFVNRLLFKNWIRKPSYKS	420
Bayram	421	GLLGLQHRLSGGPMFQPWVETAEGKRVRMDDLI GLNFALISTDSPTGPEVRQFVSELGGV	480
PWE1	421	GLLGLQHRLSGGPMFQPWVETAEGKRVRMDDLI GLNFALISTDSPTGPEVRQFVSELGGV	480
TTNP3	421	GLLGLQHRLSGGPMFQPWVETAEGKRVRMDDLI GLNFALISTDSPTGPEVRQFVSELGGV	480
Bayram	481	VLKLCDFDFPSETVCKWYDEHRINAVLLRPDRVIYDAGR DGQALCR S LLAELRK	535
PWE1	481	VLKLCDFDFPSETVCKWYDEHRINAVLLRPDRVIYDAGR DGQALCR S LLAELRK	535
TTNP3	481	VLKLCDFDFPSETVCKWYDEHRINAVLLRPDRVIYDAGR DGQALCR S LLAELRK	535

Figure 3.6. Putative amino acid differences between OpdA in wild-type OP-degraders. The 5 positions where the amino acid sequence differed are identified in bold and underlined.

The formation of HQ and 2,4,4-trimethyl-1-pentene in *E. coli* subclones from all three strains further supports the assertion that carbocation formation and type II *ipso* substitution is mediated by OpdA (14). Previously described activity in $^{18}\text{O}_2$ atmospheres with wild-type TTNP3 and Bayram have shown the incorporation of a

single atom of ^{18}O into HQ formed from NP biodegradation (6, 10), suggesting monooxygenase activity. There was no incorporation of ^{18}O into the corresponding alcohol that evolved as a result of NP biodegradation by either strain (6, 10), suggesting that it formed via a carbocation intermediate that later reacted with H_2O in the surrounding medium to become nonanol. These findings correlate well with the identification of OpdA as a putative FAD-dependent monooxygenase (15).

Despite small sequence difference, subclones of *opdA* from all three strains produced HQ when *t*-butoxyphenol and *n*-octyloxyphenol were added as substrates. However, only subclones JM109 pAW1 and JM109 pAW3B produced HQ when 4-*t*-butylphenol was added, and the HQ formed was much less for all three substrates in JM109 pAW2T than expected. It is unclear if the difference in amino acid residues between TTNP3 and the other two strains played a role in this observed difference in substrate range. While there was a high level of sequence similarity in *opdA* and surrounding DNA between Bayram, TTNP3, and PWE1, there were distinct differences in both substrate range and inducibility that should be examined in greater detail to increase understanding of alkylphenol biodegradation.

Recent work in strain TTNP3 suggests a broad substrate range for the enzyme responsible for type II *ipso* substitution. A mutant of TTNP3 that was unable to transform NP, but still able to grow on HQ could no longer degrade the estrogenic xenobiotic bisphenol A to HQ (10). This suggests that the NP degradation and related *ipso* substitution activity observed in bisphenol A are encoded by a single loci in TTNP3 (10). As we have shown that OpdA from TTNP3 heterologously expressed in *E. coli* is directly responsible for the conversion of OP to HQ, we surmise that the loss of activity described for the TTNP3 mutant must be due to the loss of *opdA* or a mutation therein.

3.5 CONCLUSIONS

In this work we have compared the OP-degrading activity of two different *Sphingomonas* strains to PWE1, which was described in Chapter 2. We found that both TTNP3 and Bayram harbor open reading frames nearly identical to *opdA*, which we previously identified as a putative FAD-dependent monooxygenase in PWE1 (15). When expressed in *E. coli* all three *opdA* variants conferred the ability to degrade OP and structurally related analogs to varying degrees.

The presence of a single highly conserved open reading frame responsible for the observed activity is consistent with what is known mechanistically about alkylphenol degradation. Strain TTNP3 had been shown previously to use a type II *ipso* substitution mechanism to convert 4-(3,5-dimethyl-3-heptyl)-phenol to HQ and a putative carbocation (4). Although Bayram was initially reported to degrade alkylphenols via type I *ipso* substitution (8), this was later shown to also proceed via type II substitution (6). Most recently, we have shown that *Sphingomonas* strain PWE1 also employs type II *ipso* substitution during OP biodegradation, producing HQ and 2,4,4-trimethyl-1-pentene (15).

The predicted amino acid sequences differed in five total amino acids when comparing the three strains. Four of differences occurred in TTNP3, two of which corresponded to point mutations identified in loss of function *opdA* subclones from PWE1 (15). It is possible that the amino acid differences found in OpdA are involved in substrate binding, which would help to explain the observations that related substrates were not transformed as readily by the TTNP3 subclones as they were by Bayram and PWE1 subclones. Overall, HQ formation with substrate analogs was less than with OP, which would suggest that OP was a preferred substrate.

The upstream and downstream DNA did not contain any open reading frames that would suggest genes associated with formation of lower pathway products, so it

seems unlikely that *opdA* is part of an operon. The 1000 bases upstream of *opdA* had identical sequences in PWE1, TTNP3, and Bayram. The same was found to be true for the downstream 469 bases in PWE1 and TTNP3, although we were unable to determine the nucleotide sequence for this region in Bayram. It does appear that the larger genetic context is different for each of the strains, as was observed from Southern hybridization and pulsed-field gel electrophoresis. The latter results suggest the presence of at least one large plasmid in all three strains, although the locations where hybridization occurred with *opdA*-specific probes were different for all three strains.

Our finding of closely related *opdA* homologs in PWE1, Bayram, and TTNP3, coupled with similar metabolic evidence from both the wild type strains and from the heterologous expression of the *opdA* variants, strongly support that assertion that *opdA* encodes a flavin monoxygenase that catalyzes alkylphenol degradation via type II *ipso* substitution.

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CHAPTER 4
SEASONAL TRENDS OF ALKYLPHENOLS, TRICLOSAN, AND A
FRAGRANCE COMPOUND IN ANAEROBIC SEWAGE SLUDGE

4.1 ABSTRACT

Octylphenol (OP), nonylphenol (NP), triclosan, and 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta- γ -2-benzo-pyran (HHCB) are common components of personal care products and as such are common wastewater contaminants. All have been shown to cause toxic effects and have weak endocrine disrupting properties. Previous work has shown NP biodegradation to be temperature dependent, so the goal of this work was to determine if there are seasonal trends in the concentration for NP, OP, triclosan, and HHCB in sewage sludge. Sludge samples from Syracuse, Ithaca, Cortland, and Cayuga Heights municipal wastewater treatment facilities in central New York State were collected periodically from 2001 to 2003. These were analyzed through accelerated solvent extraction and GC-MS. While there was seasonal concentration variability at some of the sites, these differences did not translate to a common trend across the four sampling sites. NP and OP concentrations were especially high compared to reports in the literature, with a maximum NP concentration found to be 2.2×10^3 mg kg⁻¹ in sludge from Ithaca. The maximum OP concentration was 1.7×10^2 mg kg⁻¹ in sludge from Cayuga Heights. Combined NP and OP concentrations translated to estimated equivalent amounts of estrogen ranging from approximately 0.12 mg kg⁻¹ in Cortland samples to 0.33 mg kg⁻¹ in Cayuga Heights samples. As one method of sewage sludge disposal is land application, these high concentrations of potentially toxic and estrogenic compounds could have deleterious effects on the surrounding biota.

4.2 INTRODUCTION

Alkylphenol Fate and Toxicity

Widespread public use of personal care products results in their continuous release into the wastewater treatment systems, and subsequently into the environment. Nonylphenol polyethoxylates and octylphenol polyethoxylates are nonionic surfactants that have numerous and varied uses. Nonylphenol ethoxylates account for 80% of total alkylphenol polyethoxylates, with the remaining 20% being octylphenol polyethoxylates (33). When they are released into wastewater treatment plants, microorganisms found in anaerobic digesters facilitate degradation to the stable intermediates nonylphenol (NP) and octylphenol (OP), which tend to accumulate in anoxic conditions (10). NP and OP have a tendency to sorb to solids (7), which results in their frequent detection in sewage sludge, although they are also routinely found in effluents from wastewater treatment plants. NP and OP are both weakly estrogenic, but OP is 10 times more estrogenic than NP (41). NP exposure has been shown to result in a visually discernable feminization of males (23, 24), but has also been shown to be toxic to frogs (20), reduce gamete viability (24) and bioaccumulate in muscle tissue (40, 41).

Triclosan Fate and Toxicity

Triclosan, 5-chloro-2-(2,4-dichloro-phenoxy)-phenol, is an antibacterial agent that acts by interrupting fatty acid synthesis in bacteria (22) and is commonly added to soaps, toothpaste, and other personal care products (5). Widespread use has led to approximately 300,000 kg annually entering wastewater (11). Based on reported removal efficiencies, the amount of triclosan estimated to reach the environment after wastewater treatment via activated sludge is between 2,600 and 10,500 kg year⁻¹ (11). Triclosan was one of 30 compounds most frequently detected in a survey of 85 urban rivers in the United States (17). Studies of sediment cores dating back almost forty

years illustrated triclosan's widespread use and recalcitrance in anoxic sediments (31). Triclosan has been shown to cause diverse effects, from decreased hatchability and delayed hatching in fertilized *Oryzias latipes* eggs (14) to androgenic effects in Japanese medaka (*Oryzias latipes*) (9) and in frogs (*Xenopus laevis*) (20). It has also been shown to inhibit sulfotransferases and glucuronosyltransferases involved in phase II metabolism and therefore may affect the level of other contaminants in the body (39).

HHCB Fate and Toxicity

HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta- γ -2-benzopyran) is a polycyclic musk known by the trade name Galaxolide. HHCB is one of 4 musks that represent 95% of the fragrance musks used in Europe. The influent concentration in wastewater treatment has been reported to range from 7,800 to 19,200 ng L⁻¹, with significant variability in removal efficiency (39% to 93%), depending on the wastewater treatment process (15). Both estrogenic and anti-estrogenic effects have been observed *in vitro* after HHCB administration, although variations in response have been noted with different cell lines and depend on the type of estrogen receptor used (29). There is also evidence for bioaccumulation in zebrafish as tissue samples were 600 times higher than the nominal test dose (28). While this would be expected given the pK_{ow} of 5.9 (30), studies with *Chironomus riparius* (midge) exposed to HHCB had lower than predicted bioconcentration factor and toxicity (1). These findings suggest species variability in metabolism of HHCB, which is likely mediated by differences in cytochrome P450-type enzymes.

Sewage Sludge Pollutant Loads

Since the ban on ocean dumping in 1991 the practice of sewage sludge disposal via application to agricultural land has become common and is now employed for the disposal of more than 60% of the 6.2 million metric tons (dry weight) of

sludge produced annually in the United States (38). The compounds described above are all common contaminants with documented toxic effects and an affinity for sludge. Of these compounds, only NP has been examined for temperature related degradation and was found to have slowed degradation rates at lower temperatures during laboratory experiments (36, 37). NP in sludge that is land-applied might be expected to have seasonal trends in degradation and have greater soil recalcitrance in areas with colder climates. This hypothesis is supported by Yuan *et al.* (43) who observed that there was more complete biodegradation of riverine contaminants at higher water temperatures: more specifically, as the water temperature increased, the concentration of NP decreased. The effects of seasonal temperature fluctuations on pollutant load have not yet been studied in sludge directly from wastewater treatment plants, so the goal of this study was to examine the temporal concentrations of NP, OP, triclosan, and HHCB in anaerobically digested sludge collected seasonally from four wastewater treatment plants in central New York State from 2001 to 2003.

4.3 MATERIALS AND METHODS

Chemicals

NP and OP were obtained from Sigma-Aldrich (St. Louis, MO). Sodium sulfate, phenyldodecane, and di-tert-butylethylphenol (DTBEP) were purchased from Acros (Morris Plains, NJ). HHCB was a kind gift from International Flavors and Fragrances (New York, NY). Triclosan was from Ciba Specialty Chemicals (Tarrytown, NY). All solvents were HPLC-grade and purchased from Fisher Scientific (Pittsburgh, PA).

Sludge Preparation and Extraction

Dewatered sludge samples were collected quarterly from anaerobic digesters in Ithaca, Cortland, Cayuga Heights and Syracuse wastewater treatment plants in central New York State from 2001 until 2003. Sludge samples were air-dried and stored at 4°C until ready for processing. Air-dried sludge was ground to a powder-like consistency using a mortar and pestle to prepare for extraction. All sludge samples were processed in triplicate. Extractions were performed with 2 grams of sludge mixed with an equal amount of anhydrous sodium sulfate. The mixture was then placed in a solvent-washed Accelerated Solvent Extraction (ASE) cell. The surrogate, DTBEP, was dissolved in ethyl acetate and added at a concentration of 8 mg kg⁻¹ sludge. The space in the cell not filled by the sludge mixture was filled with solvent-cleaned glass beads. Each sample was then extracted via accelerated solvent extraction with a Dionex ASE-200 (Dionex, Sunnyvale, CA) as described by La Guardia *et al.* (18) using ethyl acetate as the extraction solvent. Briefly, the extraction process consisted of 2 cycles of 5 minutes each with a pressure of 1000 psi and 100°C followed by 5 minutes static, and purge of 120 seconds.

Resulting extracts were completely evaporated under a stream of high purity N₂ gas at 40°C. Each sample was resuspended in 5-ml methanol by sonication for 30 minutes. Extracts were cleaned via solid-phase extraction using Supelco C-18 6-ml cartridges (Supelco, Bellefonte, PA) with a vacuum manifold set-up to remove particulate matter resulting from the extraction process. The extract was eluted twice with 5-ml methanol applications. The effluent and eluant were pooled and evaporated briefly to a volume of approximately 10-ml. This was then transferred to a 25-ml volumetric flask, 2000 µg ml⁻¹ phenyldodecane in ethyl acetate was added as the internal standard and the flask was then brought up to volume with methanol.

Sludge Extract Analysis

A 1-ml aliquot of the final extract was analyzed by GC-MS using an HP 6890 GC equipped with an HP-5MS column (5% phenyl methyl siloxane; 30 m by 0.25 mm; 0.25- μ m film thickness) using helium as the carrier gas with a flow rate of 1 ml min⁻¹. The temperature program included a hold at 40°C for 1 minute, then an increase of 10°C/minute to 250°C and a 10 minute hold. This was followed by an increase of 30°C/minute to 300°C and a 5 minute hold. The detector was an HP 5973 MSD with the quadrupole and source set at 150°C and 230°C, respectively.

Selected Ion Monitoring was employed for detection of these four compounds (Table 4.1). As NP is actually a mixture of congeners, the NP concentration was determined as the sum of the peaks for the ions 135 and 149. The concentration was calculated based upon standard curves of pure chemicals in ethyl acetate and normalized to the extraction efficiency of the surrogate, DTBEP.

Table 4.1. Monitoring ions and retention time used to detect sludge pollutants. The criteria for positive identification of these compounds were the presence of both the quantification and confirmation ions at the correct retention time.

Compound	Quantification Ion (<i>m/z</i>)	Confirmation Ion (<i>m/z</i>)	Retention Time (minutes)
DTBEP	219	234	14.4
NP	135	149	16.0-16.9
OP	135	206	14.8
HHCB	243	258, 213	17.6
Triclosan	288	290, 292, 218	20.0
Phenyldodecane	246	92	17.8

Statistical methods

Concentrations were determined to be significantly different when the *P* value of the average concentrations was less than 0.05 in a one-tailed *t* test.

4.4 RESULTS

Alkylphenols

NP was present in the highest concentrations of all compounds examined by at least one order of magnitude (Figure 4.1, 4.2). OP was present in concentrations approximately 10-fold lower (Figure 4.3, 4.4), which was not surprising given the production volume differences between the parent compounds nonylphenol polyethoxylate and octylphenol polyethoxylate (35). OP was not detectable in some of the sludge samples from Syracuse, Cortland, and Ithaca, although the dates on which this occurred was different for all three locations. This was unique to OP, as NP, triclosan, and HHCB were detectable in sludge from each location at all sampling times.

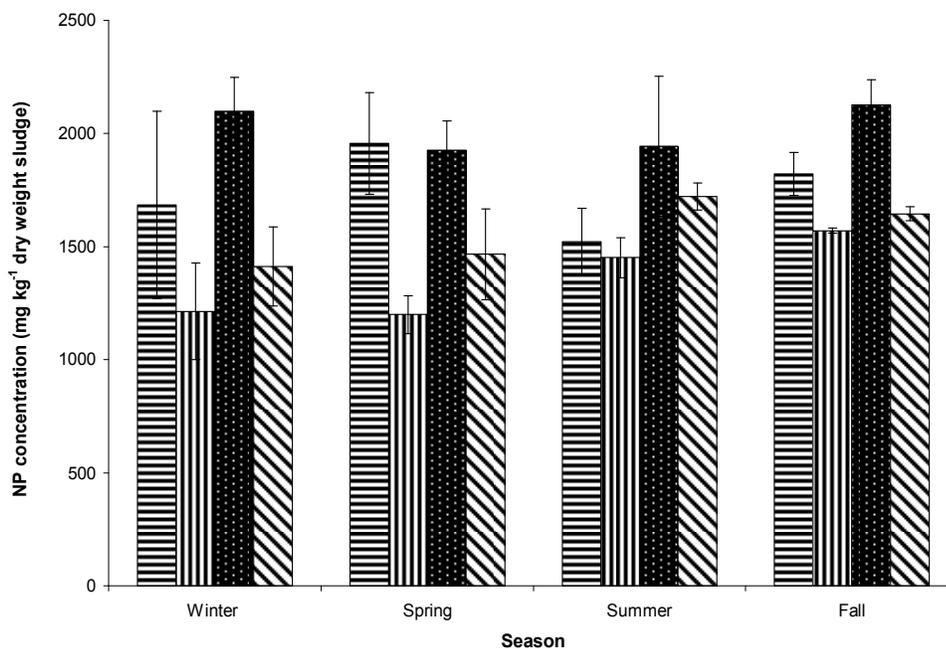


Figure 4.1. Seasonal average concentrations of NP. Seasonal averages of Cayuga Heights (▨), Cortland (▧), Ithaca (■), and Syracuse (▩) anaerobic sludge samples from 2001 to 2003.

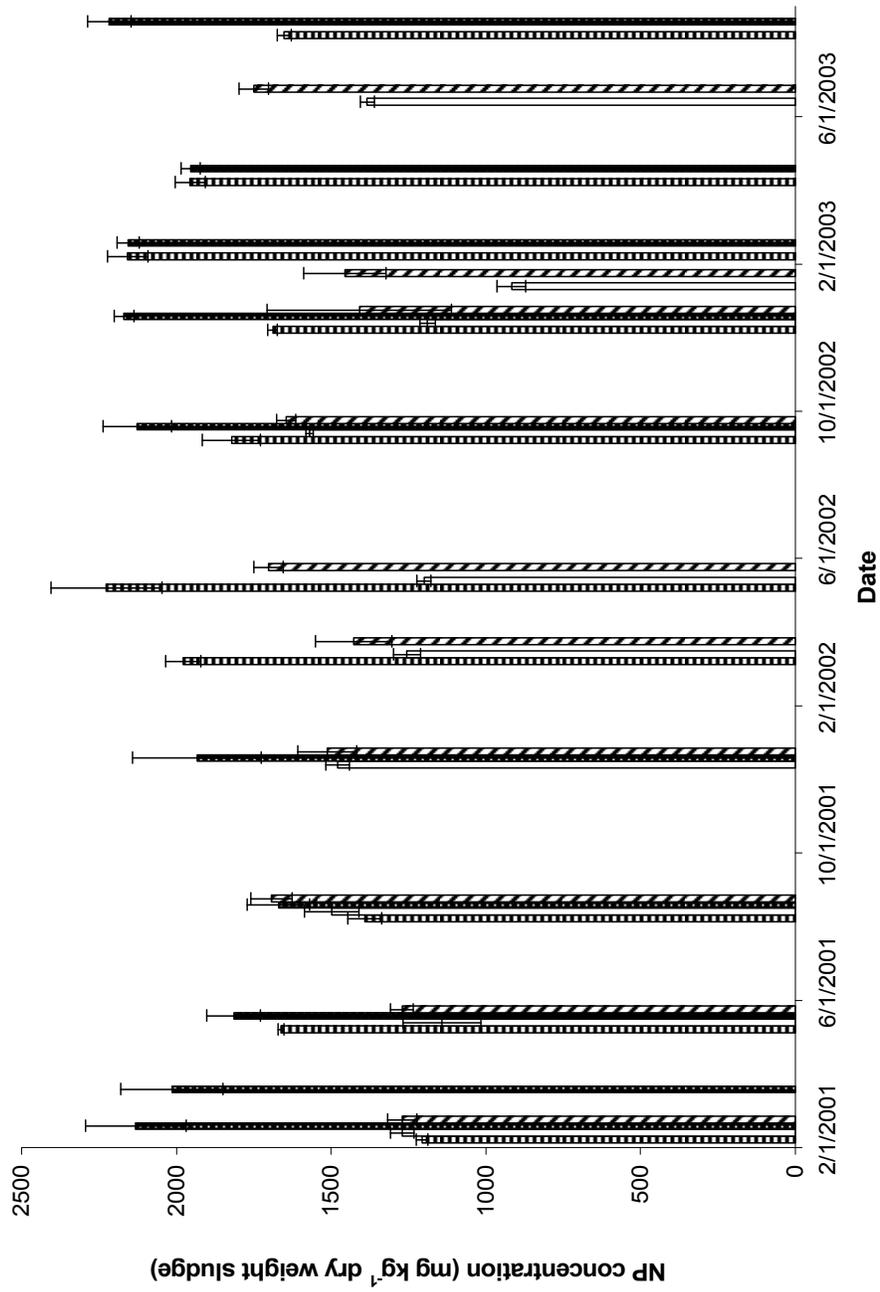


Figure 4.2. Individual concentrations of NP from 2001 to 2003. Cayuga Heights (▨), Cortland (□), Ithaca (▤), and Syracuse (▧) sludge samples taken in triplicate for each individual time point over the course of the sampling period.

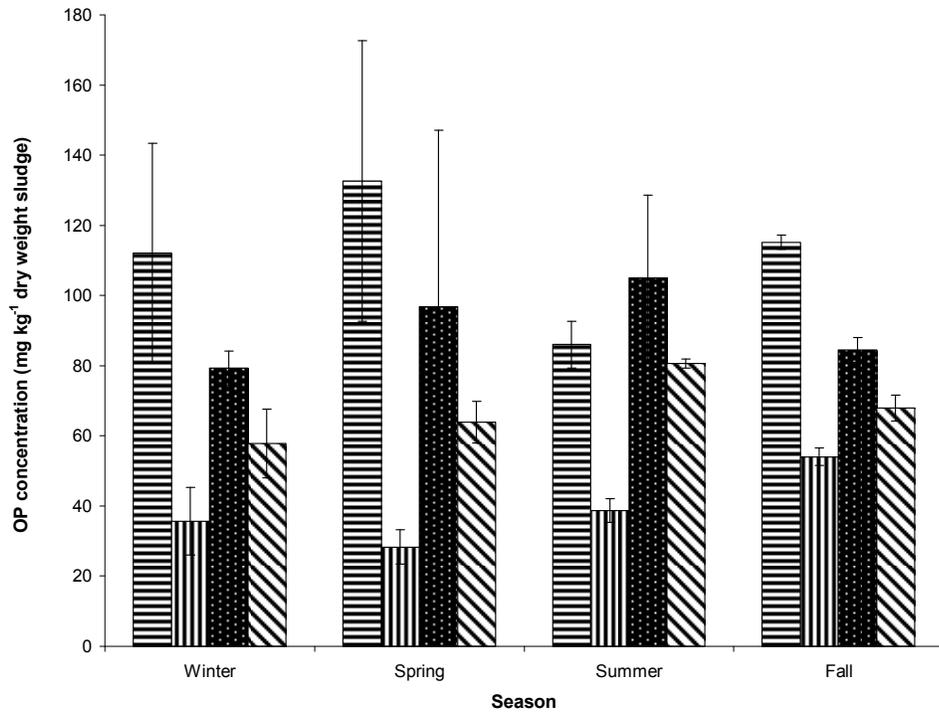


Figure 4.3. Seasonal average concentrations of OP. Seasonal averages of Cayuga Heights (▨), Cortland (▩), Ithaca (■), and Syracuse (▧) anaerobic sludge samples from 2001 to 2003.

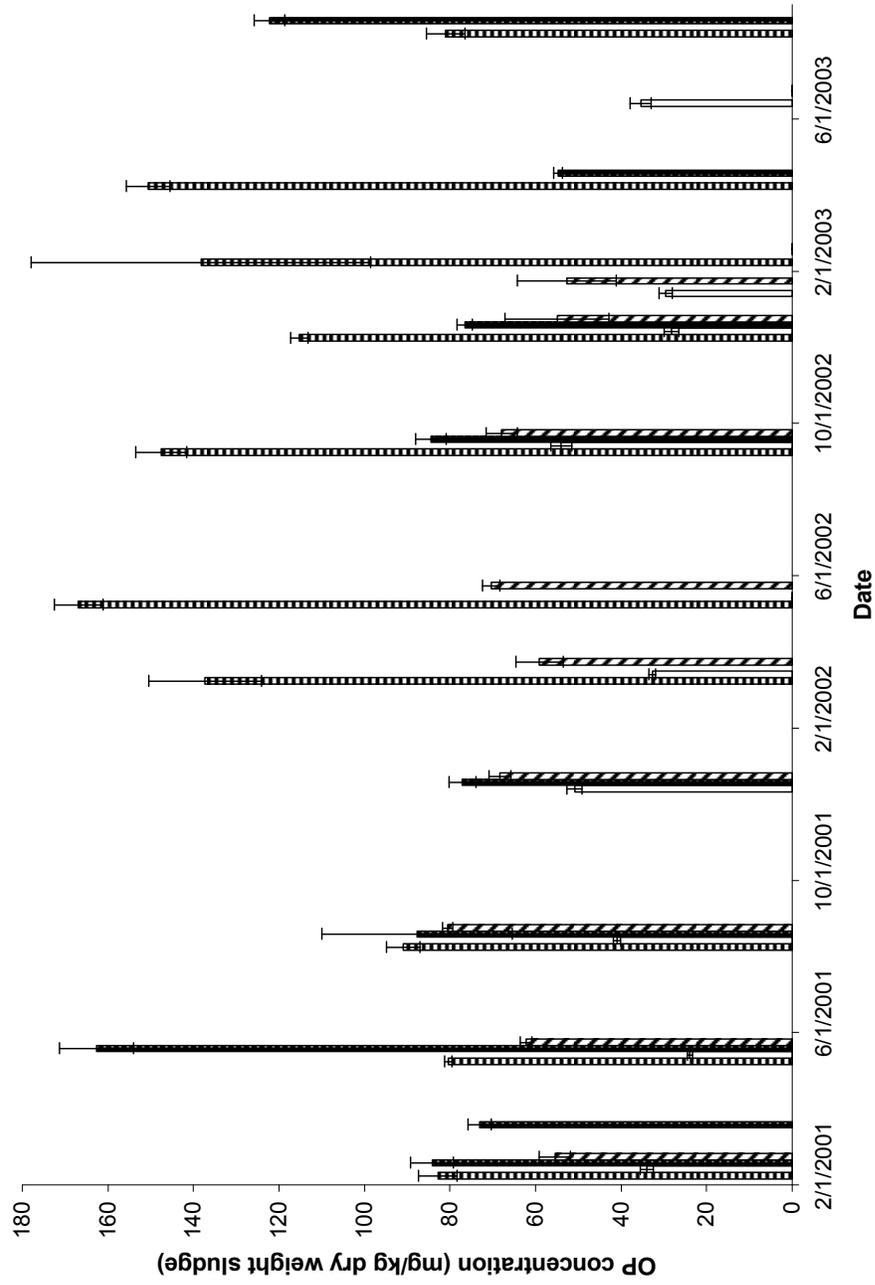


Figure 4.4. Individual concentrations of OP from 2001 to 2003. Cayuga Heights (▨), Cortland (▤), Ithaca (■), and Syracuse (▧) sludge samples taken in triplicate for each individual time point over the course of the sampling period.

Ithaca sludge samples showed slight but not statistically significant decreases in average NP concentrations during the spring and summer. However, there were statistically significant differences between the NP concentration in Ithaca sludge and that from both Cortland and Syracuse sludge samples for all seasons (Figure 4.1). NP in sludge averaged $2.2 \times 10^3 \text{ mg kg}^{-1}$ in spring samples from Cayuga Heights, which was not statistically different from $1.9 \times 10^3 \text{ mg kg}^{-1}$ NP found in spring samples from Ithaca. However, the average concentrations of alkylphenols in Ithaca samples taken during the other seasons were statistically higher than those found in Cayuga Heights.

The biggest differences in NP concentrations were between those from Cortland and Syracuse that had been collected in the spring and fall. Cortland samples showed a seasonal trend of increasing concentrations from winter to fall, although the winter and spring seasonal averages were not statistically different. Syracuse followed a similar trend, but the summer NP average concentration was slightly higher than the fall average. In general, Cayuga Heights and Ithaca had relatively higher concentrations of NP in the summer and fall, whereas Syracuse and Cortland samples had increasing concentrations starting with the winter and spring. As with the seasonal concentrations, there were no strong trends in NP concentrations at individual time points (Figure 4.2).

There was a large difference in OP seasonal averages with the lowest average concentration being found in Cortland and the highest being found in Cayuga Heights (Figure 4.3). The greatest difference, approximately 4-fold, for these two installations was in the spring. There was greater variability in the replicates, as well as within the seasonal averages, in both Cayuga Heights and Ithaca samples, but there was no significant difference in Ithaca seasonal concentrations.

Triclosan

Seasonal triclosan averages from Ithaca were not statistically different, but

there was a lot of variability within each season, especially spring and summer. Cayuga Heights samples had their highest concentration (140 mg kg^{-1}) in the spring which was statistically higher than the other seasons (Figure 4.5). Syracuse samples showed little variation in the winter, spring, and summer, but the fall average concentrations was statistically lower than the other seasons. Cortland samples also showed little variation until the fall, though unlike the Syracuse samples there was a statistically significant concentration increase in the fall.

Individual Cortland samples had triclosan concentrations that were not significantly different from those taken in Syracuse over the same time period (Figure 4.6). Ithaca showed a general trend of increasing triclosan concentrations that peaked in the summer of 2001 at 160 mg kg^{-1} , followed by a steady decrease through the end of the sampling period. Cayuga Heights showed a decreasing trend from a maximum of 155 mg kg^{-1} in 2001 to 117 mg kg^{-1} detected in the final sample of 2003.

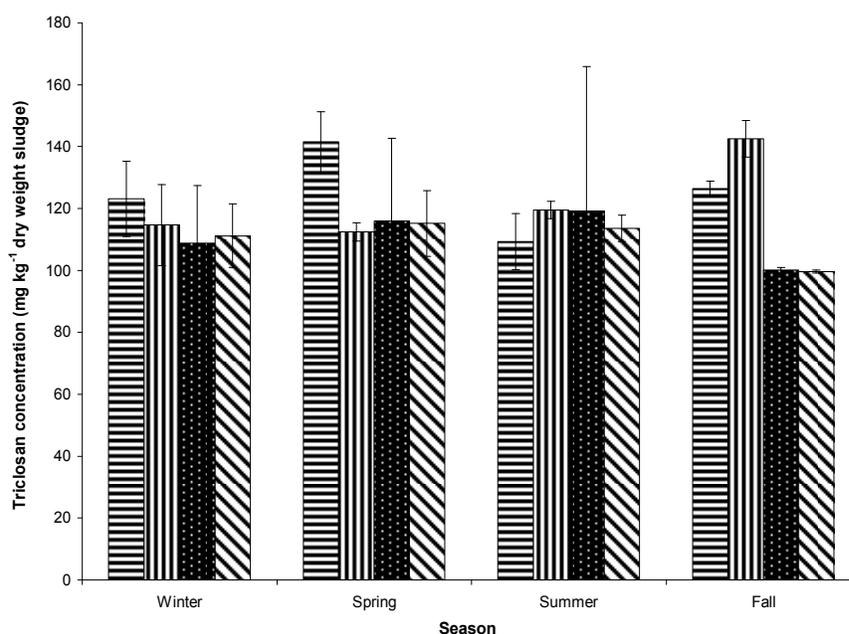


Figure 4.5. Seasonal average concentrations of triclosan. Seasonal averages of Cayuga Heights (▤), Cortland (▨), Ithaca (■), and Syracuse (▩) anaerobic sludge samples from 2001 to 2003.

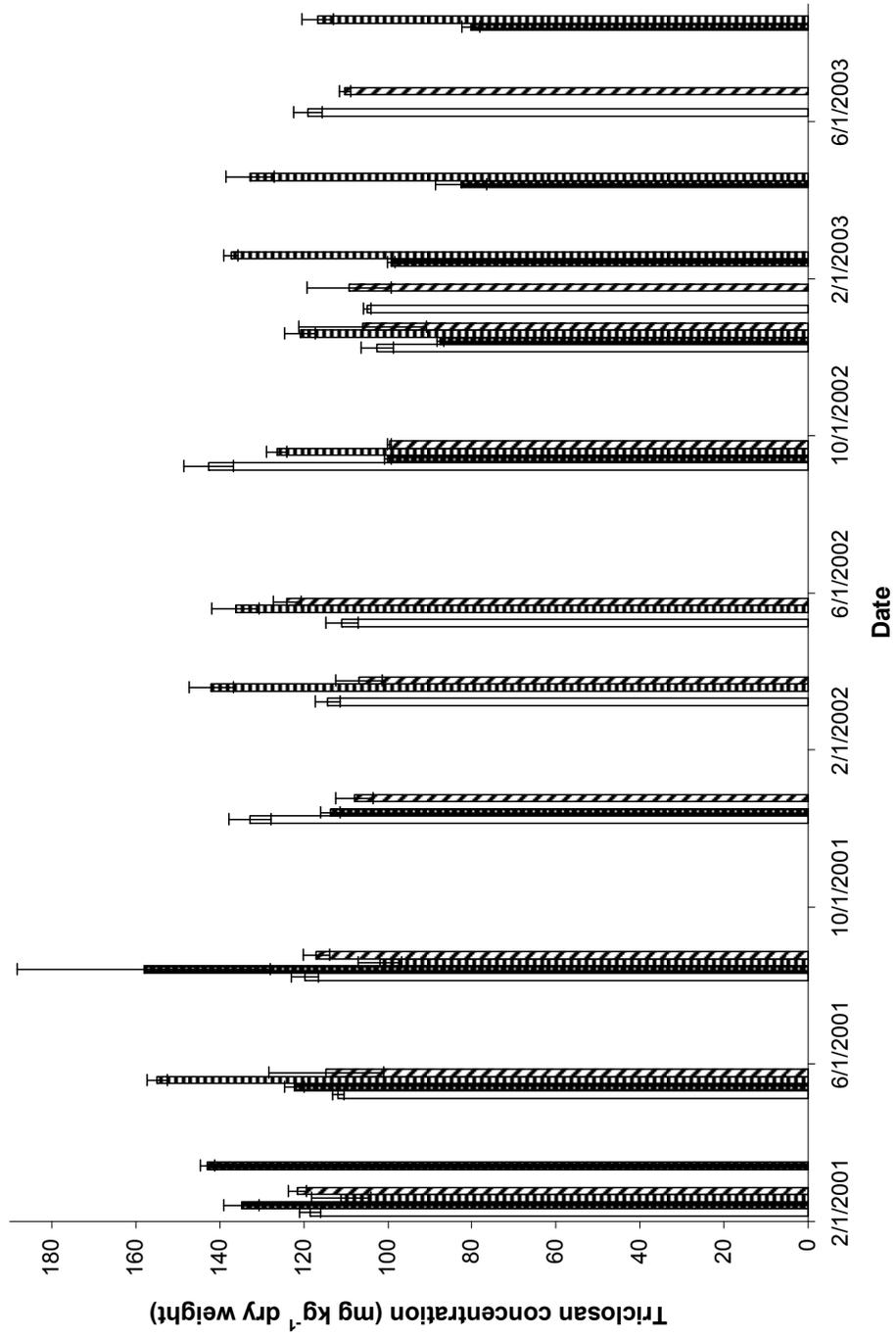


Figure 4.6. Individual concentrations of triclosan from 2001 to 2003. Cayuga Heights (▨), Cortland (□), Ithaca (■), and Syracuse (▩) sludge samples taken in triplicate for each individual time point over the course of the sampling period.

HHCB

There was no variation in HHCB concentration in Ithaca or Syracuse averages regardless of season, nor was there a statistically significant difference ($P < 0.05$) between the highest and lowest concentrations from either site (Figure 4.7, 4.8). The Cayuga Heights samples showed lower HHCB concentrations in the summer, but in general did not show a seasonal trend. Cortland, however, did have a general increasing trend from the spring to the fall. These samples showed a maximum HHCB concentration of 31 mg kg^{-1} that was reached in the fall of 2002.

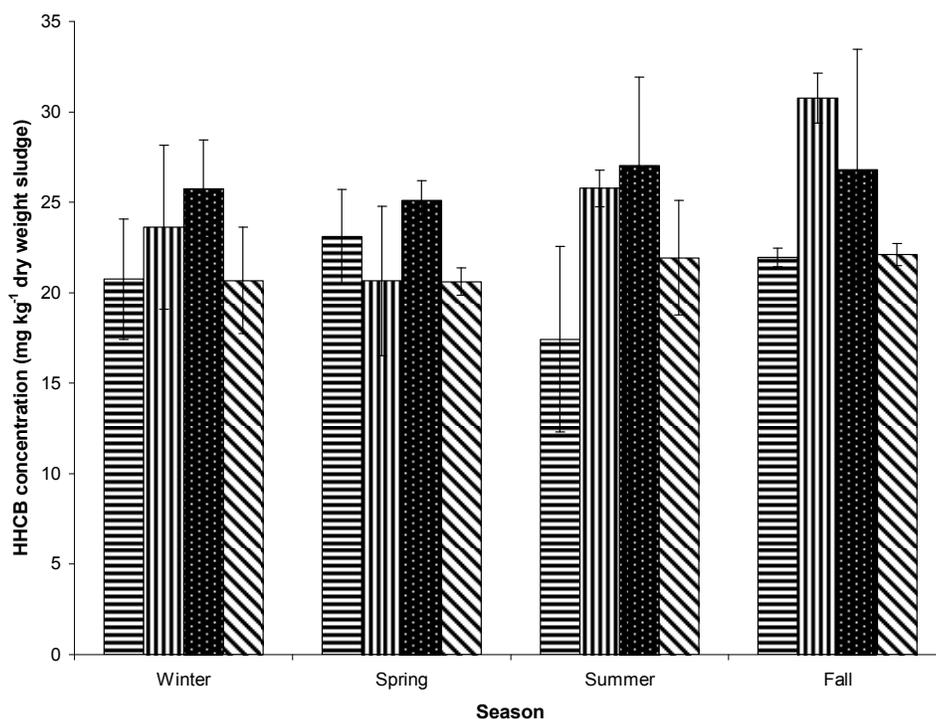


Figure 4.7. Seasonal average concentrations of HHCB. Seasonal averages of Cayuga Heights (▨), Cortland (▧), Ithaca (■), and Syracuse (▩) anaerobic sludge samples from 2001 to 2003.

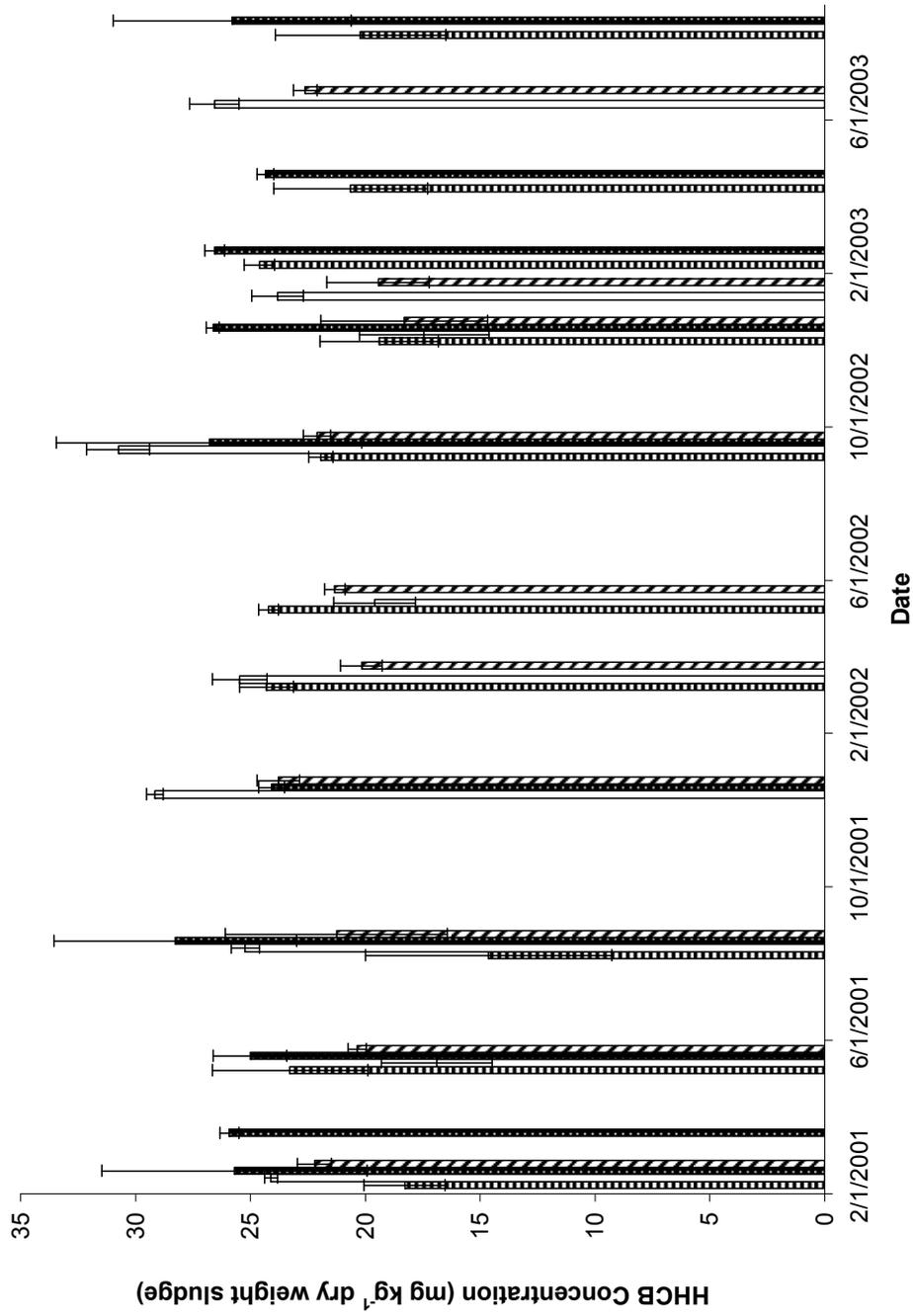


Figure 4.8. Individual concentrations of HHCB from 2001 to 2003. Cayuga Heights (▨), Cortland (□), Ithaca (■), and Syracuse (▩) sludge samples taken in triplicate for each individual time point over the course of the sampling period.

Table 4.2. Concentration ranges of each compound of interest. The maximum and minimum averages of triplicate samples and corresponding standard deviation (SD) were reported for (a) NP and OP, (b) triclosan, and (c) HHCB for all sampling locations. The values were reported in mg kg⁻¹ dry weight.

a.	OP		OP		NP		NP	
	min.	SD	max.	SD	min.	SD	max.	SD
Cortland	0	0	54.0	2.46	917	46.3	1570	11.9
Ithaca	0	0	163	8.63	1670	102	2220	69.9
Cayuga Heights	80.4	0.88	167	5.70	1390	55.5	2220	179
Syracuse	0	0	80.6	1.23	1270	48.4	1750	47.5

b.	Triclosan		Triclosan	
	min.	SD	max.	SD
Cortland	103	3.90	143	5.91
Ithaca	80.3	2.18	143	1.72
Cayuga Heights	102	5.27	155	2.43
Syracuse	99.6	0.443	124	3.38

c.	HHCB		HHCB	
	min.	SD	max.	SD
Cortland	16.9	2.42	30.8	1.37
Ithaca	24.1	0.554	28.3	5.28
Cayuga Heights	14.6	5.36	24.6	0.664
Syracuse	18.3	3.63	23.8	0.923

Estrogen Equivalents

Published reports detailing the estrogenicity of alkylphenols indicate that OP is a factor of 10 more potent than NP (41). Therefore, in order to compare the estrogenic impact of all alkylphenols, we converted OP to an equivalent concentration of NP that would give the same estrogenic response, added this number to the total NP concentration, and then multiplied this number by 0.0001 to determine the total amount of alkylphenol estrogen equivalents (APEEs). This allowed us to estimate that the total APEEs ranged from 0.124 mg kg⁻¹ in Cortland spring samples to 0.328 mg kg⁻¹ in Cayuga Heights spring samples.

The estimated APEEs were significantly higher in the Cayuga Heights and Ithaca sludge than in those from Cortland or Syracuse, although there was no significant difference between Cayuga Heights and Ithaca APEEs. Cortland concentrations were approximately half that of the Cayuga Heights samples in the winter and spring sampling seasons, and were significantly lower than the estimated APEEs for Syracuse sludge. The Ithaca APEEs did not vary from season to season (no statistical difference, $P < 0.05$). The highest concentration in Syracuse samples was in the summer season, but this value was still lower than those estimated for Ithaca or Cayuga Heights sludge. Cayuga Heights showed the highest concentration in the spring and the lowest concentration in the summer, while the fall and winter values were similar (Table 4.3).

Table 4.3. Seasonal estimated alkylphenol estrogen equivalents. The total alkylphenol concentrations were converted to equivalent concentrations of 17β -estradiol with the 0.0001 estrogen to NP conversion factor. Values were measured in mg kg^{-1} dry weight.

Estrogen equivalents	Winter	Spring	Summer	Fall
Cayuga Heights	0.28	0.33	0.24	0.30
Cortland	0.16	0.15	0.18	0.21
Ithaca	0.29	0.29	0.30	0.30
Syracuse	0.20	0.21	0.25	0.23

4.5 DISCUSSION

Alkylphenols

NP had previously been measured in anaerobic sludge from the same wastewater treatment plants examined in this study, although samples were taken a year earlier. The values determined from that single time point were 1840 mg kg^{-1} in

Syracuse, 1790 mg kg⁻¹ in Ithaca, 1480 mg kg⁻¹ in Cortland, and 1240 mg kg⁻¹ in Cayuga Heights (26). While the reported values for Ithaca and Cayuga Heights were low compared to our findings, the concentrations previously reported using Soxhlet extraction methods were within the range of concentrations reported here. In contrast, the NP average winter concentration reported here for Syracuse was 24% lower than the previously reported concentration, while the NP concentration reported for Cortland was within the range of concentrations we detected.

The NP concentrations reported here are much higher than those reported elsewhere. La Guardia *et al.* (18) found a range of NP concentrations to range from 5.4 to 887 mg kg⁻¹ dry weight in sludge samples collected from a number of locations in the eastern United States, which was similar to the maximum reported NP concentrations in anaerobically treated sludge from Switzerland (10). Those NP maximum concentrations are similar to the lowest concentrations we observed. On average, the maximum values we report here are almost 2.5-fold higher than the maximum values reported by others (10, 18). A broader survey of biosolids from wastewater treatment plants across the United States found total NP concentrations ranging from 2.2 to 1,500 mg kg⁻¹, with the highest concentrations being found in sludge from agricultural, not municipal, waste sources (16). It is therefore somewhat surprising that our municipal samples had NP concentrations exceeding, though closest to, those from agricultural sources.

Given that two different investigators (this work and Pryor *et al.* (28)) employing different extraction techniques have now found very similar NP concentrations in sludge samples from the same sources, we are inclined to believe that these numbers are real and not merely a methodological artifact. Although it is possible that the clean up methods used by others contributed to their low numbers: we found that the combined eluent and first wash from the Supelco solid-phase

extractions columns that have been discarded by other researchers (21) had nearly half of the APs in our samples which may account for some of the differences we see.

If, however, methodological differences cannot account for the high concentrations reported here, it begs the question as to why the concentration of NP is so much higher in these sludge samples than in sludge from other municipalities. This holds true even when locations with similar climatic conditions are compared, suggesting temperature is unlikely to play a role. Temperature can be further eliminated by looking internally at our data which shows no seasonal trend in sludge NP levels. Alternative explanations might involve regional differences in wastewater treatment practices and/or public consumption of products containing alkylphenols.

Within our study, the most variation across all four wastewater treatment plants was found in the OP samples. Only sludge samples from Cayuga Heights had detectable OP in all samples, whereas the other three sites had at least one time point where OP was not detected. The Cortland OP concentrations were much lower than the concentrations for the other three locations. There does not appear to be a consistent trend in concentration variation that could be attributed to seasonal fluctuation. The fluctuations vary by facility, as was seen for the NP concentrations. Interestingly, there were two samples, one from Ithaca in the winter and one from Syracuse in the spring, where OP was not detectable. Cortland also had a spring sample where OP was not detectable, but that sample was taken in different year. Previous reports of OP concentrations in biosolids from the United States ranged from 1.5 to 12.6 mg kg⁻¹ (18) in one study, and from 0.90 to 5.4 mg kg⁻¹ in another (16). These concentrations are much lower than the values we report here, which ranged from 0 mg kg⁻¹ to maximum concentrations of 54 mg kg⁻¹ in Cortland samples and 167 mg kg⁻¹ in Cayuga Heights samples.

Since both NP and OP are biodegradation products, their concentrations had to

be influenced by biodegradation rates of the parent polyethoxylate compounds. Previous studies showing temperature-based changes in NP concentration examined only NP degradation in NP spiked samples, and did not take into account the formation of NP from NPE as possibly being temperature dependent also (36). It seems likely that the degradation of NPE to NP would also be influenced in the same way by temperature, such that the net difference in concentration in real world samples with both NPE and NP might not change.

The most widely reported mechanism of AP toxicity results from their estrogenic potential. The highest estimated APEEs in this study was found in Cayuga Heights sludge (0.33 mg kg^{-1} dry weight) and was 13 to 55 times amount of 17β -estradiol reported in dry solid waste from dairy farms (27). This is of particular concern if the sludge is to be applied to agricultural land. Although APs can be degraded in soils (13, 19, 37), their fate will be influenced by a number of factors including sludge particle size, off-site transport, tillage methods, and soil temperature.

The degradation rate of AP is slow in anaerobic conditions, so the size of the soil-sludge particles on the field will determine the oxygen diffusion and will dictate the potential for biodegradation (13). Any surfaces containing alkylphenols that are exposed to oxygen would likely show disappearance, however large particles would be expected to retain these compounds (13). Other reports have shown periods of adaptation (3) are required for degradation under anaerobic conditions, as well as effects of temperature on biodegradation (3, 36). Since the application of these sludges would be in New York State, which has very pronounced seasons and corresponding variable soil temperatures, there is a chance that the alkylphenols could resist biodegradation during the cold winter months and accumulate over time. At the concentrations detected in Ithaca and Cayuga Heights sludge samples, accumulation of AP over time could be significant. There have been examples of NP detected in

leachate derived from biosolids (18), so there is also a potential risk of introducing NP into the aquatic environment through leaching or erosional transport of land-applied biosolids.

Triclosan

Triclosan was detected in all samples, with maximum concentrations ranging from 124 mg kg⁻¹ in Syracuse samples to 155 mg kg⁻¹ in Cayuga Heights samples. The values we reported here from New York State sludge samples are almost two orders of magnitude higher than concentrations reported for sludge samples from a German wastewater treatment plant that had an average triclosan concentration of 1.2 mg kg⁻¹ over a six month monitoring period, with little variation in concentration (2). The range in triclosan concentrations in samples from the mid-Atlantic region of the United States was found to be 20– 50 mg kg⁻¹ (12), while another study in the United States reported concentrations ranging from 1.2 – 33 mg kg⁻¹ (16). The triclosan concentrations reported here for central New York State sludge samples are well above that range, and are also considerably higher than the 17 mg kg⁻¹ reported in sludge from Australia (42) or 12 mg kg⁻¹ reported in Canadian sludge (4).

A previous study found that triclosan in anaerobically digested sludge was 10-fold higher than it was in aerobically digested sludge, indicating little removal under anaerobic conditions (21). A number of studies have reported on triclosan loss by monitoring the difference between influent and effluent concentrations and found variability based on the type of sewage treatment process (15, 21). There is also variability in the amount of triclosan reported to be removed during waste water treatment via sorption. An estimated 30% of the triclosan was lost through sorption to sludge (2). Federle *et al.* (8), however, found sorption to be negligible (less than 4.5%) compared to biodegradation (94%) in the removal of triclosan. This contrasts with a mass balance study at a wastewater treatment plant in the United States which

found that almost 50% of the influent triclosan could be account for in the sludge following anaerobic digestion (21).

HHCB

The maximum HHCB concentrations we found in sludge ranged from 23.8 mg kg⁻¹ in Syracuse samples to 30.8 mg kg⁻¹ in Cortland samples. In general HHCB concentrations were mostly static during the monitoring period with no significant differences between seasons or between wastewater treatment plants. HHCB was found to be at lower concentrations than alkylphenols or triclosan, ranging from a factor of 2 to 100 times less. However, our estimates for HHCB may be conservative, as our samples were air-dried for weeks before storage. This likely allowed some of the HHCB to escape. Different preparation methods have previously revealed differences in HHCB concentrations from samples prepared wet as compared to those that were dried (41). While there was a large range in HHCB concentrations in that study, the authors reported a statistically significantly 2-fold difference in the amount of HHCB detected in wet sludge samples compared with the amount in air dried samples (32). Unfortunately, our samples were collected and processed prior to the publication of that study.

HHCB was found in digested sludge in the United Kingdom at concentrations ranging from 1.9 to 81 mg kg⁻¹ dry weight. There was a weak association between catchment size and the total concentration, most likely due to larger input of personal care products in more urban areas (34). Syracuse, which has a larger contributing population, had a lower maximum HHCB concentration than Ithaca or Cortland, which have smaller populations. The concentration in biosolids from Los Angeles were 18 mg kg⁻¹ wet weight and the concentration for Las Vegas was 10 mg kg⁻¹ wet weight (25). The concentrations for HHCB reported here are as high or higher than the concentration in Los Angeles, however those measurements were per wet weight

of the biosolids and ours was in dry weight. Given that the average solids contents of wet sludge is approximately 20%, it is likely that the actual HHCB concentration present in those sludge samples, on a mg kg^{-1} dry weight basis, was 4-5 times higher than what was reported. When such a correction is made, the Los Angeles and Las Vegas numbers would be expected to be 2-3 times higher than the HHCB concentrations reported here.

Yet another report revealed that municipal waste sludge in China was found to have HHCB concentrations between 5.4 and 21 mg kg^{-1} , although much higher concentrations were found in association with waste from a cosmetic plant, which had sludge HHCB concentrations of 700 mg kg^{-1} (44). A survey of biosolids from wastewater treatment plants in the U. S. had similar trends in HHCB concentrations, with lower concentrations attributed to municipal waste and the highest concentrations coming from cosmetic-related waste (16). HHCB measured in digested sludge samples taken 2 years apart from treatment plants in Delaware showed a decrease in concentration by almost half (6).

To our knowledge, this is the first study to examine the concentrations of OP, NP, HHCB, and triclosan for an extended period of time in sludge from anaerobic digesters. Although we did detect some statistically significant variations, no clear trends emerged. This information is valuable in that it demonstrates that flux of these compounds does not change dramatically over time. The changes that did occur were not statistically linked to the season when all plants we compared.

4.6 CONCLUSIONS

While there was no seasonal trend in concentration, there was variation for each chemical at each site. In addition, all the compounds we measured were found at concentrations equal to or higher than those previously reported in sludge from other

locations. Differences in extraction methods, as well as characteristics of the wastewater treatment plants and the influent waste could all be variables to explain this. The highest concentrations of NP and OP were found in sludge samples from Cayuga Heights and Ithaca. Cayuga Heights employs a trickling filter, which may have lower overall removal efficiency than the activated sludge technologies employed at the other plants (21, 35).

OP was the only compound that was not detectable in all the samples and was present at widely variable concentrations. The concentration of HHCB was relatively constant over the sampling period, especially in Ithaca and Syracuse samples that showed no statistical difference between the samples with highest and lowest concentrations. Whether or not the apparent concentration stability was impacted by volatilization or not has yet to be determined. However, the concentrations reported here are within limits reported by other studies of sewage sludge contaminants in the United States. The triclosan concentrations were also within the ranges reported by others.

The data presented here shows NP and OP concentrations that are well above the reported values for these compounds in other locations. However, these values are well within the range reported by Pryor *et al.* (26), who used sludge from the same sites a year before this study. That analysis was also done with a different method than the one used in this work which strengthens our confidence in the reliability of the data reported here. Our rigorous methods following accelerated solvent extraction showed alkylphenols present in all fractions eluted during solid phase extraction, whereas others have analyzed only the final fraction (18), which may account for some of the differences.

Temperature has been reported to have an effect on NP and OP biodegradation, with seasonal trends especially apparent in sediment samples. Work with NP

amended activated sludge in bench-scale reactors has shown a decreased degradation with decreased temperatures (36). However, the same would also be expected of AP formation from APE, and thus would be expected to result in no net change in steady state AP levels. That could be one explanation as to why our work showed no seasonal influence on AP concentration. If seasonal temperature fluctuations affected biodegradation as a whole, one would also have expected them have had some impact on sludge levels of both triclosan and HHCB, however, we found no evidence for such an effect. In conclusion, while there was variability over time for all four sampling locations, these differences did not correlate with seasonal changes.

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CHAPTER 5

CONCLUSIONS

5.1 CHALLENGES ASSOCIATED WITH THE STUDY OF OP BIODEGRADATION

There are many methodological challenges associated with studying OP biodegradation. OP has a very low solubility in water and as a result created severe limitations with respect to experimental design and implementation. Crystalline OP would therefore not dissolve when directly added to bacterial culture, but rather readily formed large particles that had a tendency to sorb to the glassware and created problems when trying to consistently add the same amount of substrate to multiple replicates. OP readily dissolved in methanol, the preferred method for delivering substrate, but immediately precipitated upon contact with aqueous culture medium. The use of a carrier solvent allowed a more uniform concentration to be dispensed, but did not resolve the difficulty of evenly dispersing OP throughout the aqueous medium.

Undissolved OP had a tendency to sorb to the surfaces of glass or plasticware used to contain the cultures. Thus, in order to accurately calculate the amount of OP remaining in cultures, the entire culture was sacrificed with an equal volume of methanol to increase the recovery. This made it difficult to do a time-course of OP disappearance, as replicate cultures would need to be sacrificed at various time points which seemed to significantly increase variability. Our discovery of the production of 2,4,4-trimethyl-1-pentene improved things somewhat since it could be sampled in the headspace nondestructively instead of monitoring OP disappearance by sacrificing whole cultures.

It was also difficult to identify growth when OP was used in solid media. When magnesium sulfate was added to the MSM medium prior to autoclaving, the OP

had a tendency to crystallize more evenly than if magnesium sulfate was added after autoclaving. OP was added immediately after autoclaving in order for the crystallized compound to melt and form droplets in the medium. The plates were poured as soon as possible, as cooler liquids resulted in OP loss through adhesion to the glassware. Even though the liquid medium was vigorously shaken before pouring, there were likely differences in the distribution of OP in the plates. At high enough concentrations the crystallized OP could physically be removed from the surface of the plate during inoculation, either by direct adhesion to the lid of the plate or by sticking to the inoculation loop. Although some OP still remained in the medium, there was additional concentration variability from plate to plate. Unfortunately, adding OP at levels below the aqueous solubility limit would not have provided sufficient substrate for growth in minimal medium.

Subclones expressed in *E. coli* DH5 α showed variable activity after repeated transfers in the presence of OP. One PWE1 subclone lost the ability to transform OP which we think was due to mutations that were selected for because of the toxicity of the HQ it produced (6). In order to prevent mutations, pAW1 was moved from *E. coli* DH5 α to *E. coli* JM109 so that *opdA* was then expressed in a strain that contained the lac repressor, thus providing a way to control gene expression. While some variability existed after repeated transfers, activity no longer disappeared when expression was controlled. To minimize variability, aliquots of a single culture that had not been previously induced were stored at -80°C and individual aliquots were used to start cultures for each subclone experiment. There was still some variability in activity between batch cultures of the same subclone, but not to the degree that had been previously observed.

5.2 EVIDENCE FOR THE FATE OF OP IN THE ENVIRONMENT

In Chapter 2 we identified a putative FAD-dependent monooxygenase encoded by *opdA* in *Sphingomonas* sp. PWE1. OpdA, when heterologously expressed in *E. coli*, conveyed the ability to degrade OP with concomitant formation of hydroquinone (HQ) and 2,4,4-trimethyl-1-pentene. The formation of both intermediates supports a type II *ipso* substitution mechanism.

In Chapter 3 OP-degrading activity in PWE1 was compared with that from *Sphingomonas* sp. TTNP3 and *Sphingobium xenophagum* Bayram, two strains previously identified as degrading NP via type II *ipso* substitution. We found that Bayram harbors an open reading frame differing by only one predicted amino acid from that of OpdA, while TTNP3 contains an OpdA homolog that differs by four amino acids. Two of these differences in TTNP3 corresponded to point mutations identified in loss of function *opdA* subclones from PWE1, as described in Chapter 2. When expressed in *E. coli* all three *opdA* variants conferred the ability to degrade OP, although activity with structurally related analogs occurred in varying degrees. It is possible that the amino acid differences found in OpdA are involved in substrate binding, which would help to explain the observations that related alkoxyphenols were not transformed as readily by the TTNP3 subclones as they were by Bayram and PWE1 subclones. However, HQ formation from incubations with substrate analogs was less than with OP, suggesting that OP is a preferred substrate.

The sequences flanking *opdA* did not revealed further information regarding the lower pathway. The 1000 bases upstream of *opdA* were identical in PWE1, TTNP3, and Bayram, as was true for the downstream 469 bases in PWE1 and TTNP3. We were unable to determine the nucleotide sequence for this region in Bayram, which could suggest differences in the downstream sequence. However, this information, combined with Southern hybridization and pulsed-field gel

electrophoresis results, supports the assertion that PWE1, TTNP3, and Bayram differ with respect to the broader genetic context in which *opdA* appears. Genetic evidence of closely related *opdA* homologs in PWE1, Bayram, and TTNP3, coupled with metabolic data from the wild type strains and from the heterologous expression of the *opdA* subclones, strongly support the assertion that *opdA* encodes a flavin monoxygenase that catalyzes alkylphenol degradation via type II *ipso* substitution.

5.3 PRESENCE OF ALKYLPHENOLS, TRICLOSAN, AND HHCb IN SEWAGE SLUDGE

Anaerobically digested sewage sludge from four municipalities in central New York State was analyzed for the presence of OP, NP, triclosan, and HHCb. Samples were taken seasonally over a period of three years to assess whether seasonal temperature changes influenced concentrations of the target compounds. NP had previously been measured in anaerobic sludge from the same wastewater treatment plants examined in this study, although samples were taken a year earlier (7). Although different extraction methods were used, the values reported here were within the range of those concentrations (7). The highest NP concentration was found to be 2.2×10^3 mg kg⁻¹ in sludge from Ithaca. On average, the maximum values we report here for all four sites are almost 2.5-fold higher than the maximum values reported by others (1, 4).

OP showed the most variation of the compounds examined. Only sludge samples from Cayuga Heights had detectable OP in all samples, whereas the other three sites had at least one occasion where OP was not detectable. As with NP, the OP concentration varied and did not positively correlate with seasonal trends in temperature. The concentrations we report here, which ranged from 0 mg kg⁻¹ to maximum concentrations of 54 mg kg⁻¹ in Cortland samples and 167 mg kg⁻¹ in

Cayuga Heights samples, are much higher than those reported by others (3, 4).

Both NP and OP have been shown to mimic the effects of estrogen. The highest combined estimated estrogenic equivalency of NP and OP in this study was found in sludge from Cayuga Heights (0.33 mg kg^{-1} dry weight) and was 13 to 55 times amount of 17β -estradiol reported in dry solid waste from dairy farms (8) and is of particular concern if the sludge is to be used for agricultural land application.

Both triclosan and HHCB were detected in all samples. Triclosan maximum concentrations ranged from 124 mg kg^{-1} in Syracuse samples to 155 mg kg^{-1} in Cayuga Heights samples, values that are almost 10-fold higher than concentrations reported by others (11). The maximum HHCB concentrations ranged from 23.8 mg kg^{-1} in Syracuse samples to 30.8 mg kg^{-1} in Cortland samples. HHCB concentrations were mostly static during the monitoring period, with no significant differences between seasons. HHCB concentrations were considerably lower than alkylphenols or triclosan, although our estimates may be conservative as our samples were air-dried for weeks before storage and possibly lost HHCB through volatilization.

All the compounds we measured were found at concentrations equal to or higher than those previously reported in sludge from other locations. Variation in extraction methods, as well as characteristics of the wastewater treatment plants and the influent waste could all be variables to explain this. If methodological differences cannot account for the high concentrations reported here, the question remains as to why the concentration of NP is so much higher in these sludge samples than in sludge from other municipalities. Temperature did not appear to be a factor, as our data shows no seasonal trend in NP concentrations, at least not any trend that was consistent in all four sites. Alternative explanations might involve regional differences in wastewater treatment practices or public consumption of products containing alkylphenols.

5.4 CLOSING REMARKS

The objectives of this work have been to study biodegradation of the endocrine disrupter OP, as well as quantify the amount of OP, NP, HHCB, and triclosan in anaerobically digested sewage sludge from New York State. Chapter 2 is the first report of a gene associated with alkylphenol biodegradation. Specifically, *opdA* was found in *Sphingomonas* sp. PWE1 and encodes a putative FAD-dependent monooxygenase. When heterologously expressed in *E. coli*, this gene conveyed OP degrading activity with the concomitant formation of hydroquinone and the two side-chain intermediates 2,4,4-trimethyl-1-pentene and 2,4,4-trimethyl-2-pentanol in agreement with a type II *ipso* substitution mechanism. Formation of an alkene product had not been previously detected. The same intermediates were also produced by subclones generated from *opdA* homologs in the known NP degrading organisms *Sphingomonas* sp. TTNP3, and *Sphingobium xenophagum* Bayram, which were also identified as using a type II *ipso* substitution mechanism.

Chapter 4 is the first study to monitor the concentrations of OP, NP, HHCB, and triclosan over an extended length of time. This work has also demonstrated that the concentration variability of OP, NP, HHCB, and triclosan do not correlate with seasonal temperature changes. Interestingly, the concentrations of OP and NP found in sludge from New York State were significantly higher than those reported by others (1, 3, 4), but the NP concentrations were similar to those reported by Pryor *et al.* (7) using sludge from the same sites but with a different extraction method. To our knowledge, this is the first study to examine the concentrations of OP, NP, HHCB, and triclosan for a three year period of time in sludge from anaerobic digesters. Although we did detect some statistically significant variations, no clear trends emerged. This information is valuable in that it demonstrates that flux of these compounds was not statistically linked to seasonal changes. In conclusion, the

comprehensive results of this work demonstrates the seasonal presence of alkylphenols during the wastewater treatment process and also shows the first evidence of a gene associated with the fate of OP in the environment.

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