Characterization of *para*-nitrophenol degrading bacterial communities in river water using functional markers and stable isotope probing

Agnieszka Kowalczyk[*1], *Özge Eyice*[1], *Hendrik Schäfer*[1], *Oliver R Price*[2], *Christopher J Finnegan*[2], *Roger A van Egmond*[2], *Liz J Shaw*[3], *Glyn Barrett*[3,4], *and Gary D Bending*[*1]

[1]School of Life Sciences, Gibbet Hill Campus, University of Warwick, Coventry, CV4 7AL, UK.

[2]Unilever, Safety and Environmental Assurance Centre, Sharnbrook, Bedfordshire, MK44 1LQ, UK.

[3]Department of Geography and Environmental Science, Russell Building, University of Reading, Whiteknights, RG6 6DW, UK.

[4]School of Biological Sciences, Knight Building, University of Reading, Whiteknights, Reading, Berkshire RG6 6AJ, UK.

* To whom correspondence may be addressed:

Dr Agnieszka Kowalczyk. Telephone: +44 (0) 7758572996. E-mail: AKowalcz@scj.com

Present address: SC Johnson, Frimley Green, Frimley, Camberley, Surrey, GU16 7AJ, United Kingdom

Summary

Microbial degradation is a major determinant of pollutant fate in the environment. *Para*-nitrophenol (PNP) is an EPA listed priority pollutant with wide environmental distribution but little is known about the microorganisms that degrade it in the environment. We studied the diversity of active PNP- degrading bacterial populations in river water using a novel functional marker approach coupled with [$^{13}C_6$]-PNP Stable Isotope Probing (SIP). Culturing together with culture independent Terminal Restriction Fragment Length Polymorphism analysis of 16S rRNA gene amplicons identified *Pseudomonas syringae* as the major driver of PNP degradation in river water microcosms. This was confirmed by SIP-pyrosequencing of amplified 16S rRNA. Similarly, functional gene analysisshowed that degradation followed the Gram-negative pathway involving *pnpA* from *Pseudomonas* spp. However analysis of maleylacetate reductase (*mar*), an enzyme common to late stages of both Gram negative and Gram positive bacterial PNP degradation pathways, identified a diverse assemblage of bacteria associated with PNP degradation, suggesting that *mar* had limited use as a specific marker of PNP biodegradation. Both *pnpA* and *mar* genes were detected in a PNP-degrading isolate, *P. syringae* AKHD2 which was isolated from river water. Our results suggest that PNP degrading cultures of *Pseudomonas* spp. were representative of environmental PNP degrading populations.

Biodegradation / Para-nitrophenol-degrading bacteria / Functional genes / Stable Isotope Probing / Para-nitrophenol

Introduction

Para-nitrophenol (PNP) is the most common and important mononitrophenol, in terms of quantities used by industry and extent of environmental contamination (1,2). PNP is used for synthesis of medicines, dyes, explosives and wood preservatives (1,3), and is also generated in the environment by the degradation of pesticides that contain the nitrophenol moiety (4). As a consequence PNP is released into a wide range of environments, and it has been detected in a range of compartments, including river water, street dust and air (5).

PNP has traditionally been used as a model compound by the chemical industry to understand factors controlling degradation of chemicals in the environment, and to develop and validate regulatory tests of chemical persistence (6). PNP represents a readily biodegradable chemical, so that within first tier OECD screening tests, natural microbial populations from a range of habitats are generally able to degrade it within a 28 day period without prior exposure to it. However there can be considerable variation in PNP biodegradation rates within and between studies, the microbial basis for which remains unclear (7).

Understanding of PNP biodegradation pathways is derived from studies with bacteria isolated from soil and sludge, and to date there is little understanding of how this translates into natural environmental substrates supporting complex communities, and particularly in regulatory tests which are based on the potential for degradation in water samples. Culturability in nearly all environments varies from 1 to 10% (8), because it is difficult to simulate complex environmental conditions essential for proliferation of specific groups of bacteria under any applied set of cultivation conditions. For example, more than 99.9% of the natural bacterioplankton community in seawater cannot be cultivated using marine agar (9).

PNP degrading isolates belong to several different genera including *Flavobacterium, Bacillus*, *Pseudomonas, Moraxella, Nocardia,* and *Arthrobacter* (10-13). The enzymes and

genes associated with PNP degradation pathways in bacterial isolates from soil and sludge have been well characterized (14-17). According to Chauhan *et al*. (18) the biodegradation of PNP may occur via two major catabolic pathways (Supporting Fig. 1). In Gram-negative bacteria such as *Pseudomonas* spp., initial degradation of PNP is catalysed by a 4-nitrophenol 4-monooxygenase which is encoded by the gene *pnpA* (17). The resulting 1,4-benzoquinone is further degraded via 4-hydroxymuconic semidaldehyde to maleylacetate. In Gram-positive bacteria such as *Arthrobacter* spp., PNP biodegradation proceeds via 4-nitrocatechol and involves 4-nitrophenol 2-monooxygenase which is encoded by the *npdA*2 gene (17). 4-nitrocatechol is then further degraded to 2-hydroxy-1,4-benzoquinone, 1,2,4-benzenetriol and maleylacetate. Both Gram negative and positive bacteria thus produce maleylacetate as a common intermediate metabolite in PNP degradation. This is degraded by maleylacetate reductase (MAR) to ketoadipate and further to acetyl-CoA, which is channelled into the citric acid cycle (18). MAR is involved in various bacterial catabolic pathways involving hydroquinone or hydroxyquinol as ring cleavage substrates (Supporting Fig. 2) (19), and a variety of genes encoding this enzyme have been identified in different bacterial strains e.g. the *pnpF* gene from *Pseudomonas* sp. WBC3 (17), the *clcE* gene from *Pseudomonas* sp. Strain B13 (20), the *macA* gene from *Rhodococcus opacus* 1CP (21) and the *tftE* gene from *Burkholderia cepacia* AC1100 (22). In the present paper we refer to these maleylacetate reductase genes collectively as *mar*.

Functional gene assays show utility to identify and quantify catabolic genes involved in biodegradation of organic compounds *in situ*. For instance, Ní Chandhain *et al.* (23) determined microbial dioxygenase gene shifts during polycyclic aromatic hydrocarbon biodegradation while Zhang *et al*. (24) studied the changes of key catabolic genes and microbial community structure during the degradation of nonylphenol ethoxylates and nitrophenol in natural water microcosms. Functional gene based methods are advantageous over 16S rRNA-

based methods in that they can reveal biodegradation potential within an environment. However, for many chemicals, pathways for complete degradation may involve transformations by multiple taxa, so that functional gene markers inform only on taxa contributing to specific steps in the pathway (25). Definitive evidence of the actual microbial populations responsible for chemical biodegradation *in situ* can be obtained usingstable-isotope probing (SIP), which allows the identification of active microorganisms that utilize enriched compounds (26). To date, SIP has been applied with several ^{13}C-labelled organic compounds such as methane, naphthalene, polycyclic aromatic hydrocarbons, 2,4-dichlorophenoxyacetic acid, phenol, benzene and pentachlorophenol (27-32). These studies have revealed that microbial populations actively utilising chemicals in the environment are distinct from those found using culture-dependent methods.

The major aims of this work were to identify active PNP-degrading bacteria in river water and to gain insight into their metabolism. Due to the polyphyletic nature of PNP degrading bacteria, analysis of 16S rRNA genes is not sufficient for revealing the actual diversity of PNP-degrading bacteria. Therefore, we developed complementary approaches to investigate the diversity of PNP-degrading bacteria using cultivation-independent methods based on the functional genes involved in PNP biodegradation and stable isotope probing complemented with ribosomal RNA gene analysis. We developed and applied two different functional markers that are involved in early (*pnpA*, *npdA2*) and late (*mar*) stages of the PNP biodegradation pathway and which together could enable identification and comparison of taxa responsible for different parts of the pathway. DNA-SIP using [$^{13}C_6$]-PNP allowed the identification of the bacterial populations that were actively utilising PNP *in situ*. In addition, PNP-degrading bacteria were isolated from enrichment cultures to reveal whether the same microorganisms were responsible for PNP degradation in the natural environment. We used these approaches to characterise the PNP-degrading bacterial populations in river water in rural

Warwickshire (England, UK) to better understand the role and abundance of PNP degraders in the environment and their function in the ecosystem.

Experimental Procedures

Sampling of river water and PNP degradation experiment

Surface river water was obtained from the River Dene, in Warwickshire, UK on March 8, 2010 downstream of the Wellesbourne Wastewater Treatment Plant (WWTP) effluent discharge point (52˚11'58.54''N and 1˚36'44.94''W). The location is a rural catchment and there was no known previous exposure to industrial pollutants, including PNP. Analysis, using methods described below, indicated that no PNP was detectable in the freshly collected river water. The water had a temperature of approximately 10°C and was filtered (38 μm sieve) in order to remove particulate matter and large protozoa. River water for the abiotic controls was sterilized in three autoclaving cycles (121 ˚C, for 15 minutes, 1.1 atm). Treatments and controls were prepared in triplicate. Bottles (500 ml) were filled with 300 ml of river water and PNP, ≥99.5% purity (SigmaAldrich, UK), was added to give a concentration of 14 μM. A positive control was set up consisting of sterile river water with PNP inoculated with the PNP-degrading bacterial strains *Pseudomonas putida* SoT01 (see Supporting Information) or *Arthrobacter* sp. JS443 (obtained from Jim Spain and Shirley Nishino, School of Civil & Environmental Engineering, Georgia Institute of Technology, 311 Ferst Drive, Atlanta, GA 30332-0512), which had been grown on LB medium with PNP (0.4 mM) for 48 hours at 25˚C prior to use. Positive controls were used to investigate validity of the degradation assay, should there have been failure to degrade PNP, since PNP degradation can be highly variable within and between studies (7). Negative controls consisted of sterile river water with PNP. Vessels were incubated

at 20±2 ˚C, under constant motion using rotary shaker (MK V Shaker, L.A. Engineering Co., UK) at 50 rpm.

HPLC analysis

Daily, 1 ml of water was removed from each bottle and PNP was quantified by HPLC using a 20 µl injection volume. The analysis was carried out using a Hewlett Packard 1100 HPLC system with a LiChrosphere (5 µm) C-18 column (MerckMillipore, UK). The mobile phase was a mixture of water: methanol (HPLC grade, Fisher Scientific, UK) with a ratio of 40:60 (v/v) and a flow rate of 0.50 ml/minute. Analysis was performed with UV detection at 254 nm, with column pressure of 115 bar and column temperature of 25 ˚C. The concentration of PNP was quantified using a set of standards with known PNP concentration.

Isolation and identification of PNP-degrading bacteria

Aliquots of river water were taken from bottles after complete biodegradation of PNP. Serial dilutions were spread onto plates with solidified mineral medium (33) and PNP (0.4 mM) as a sole source of carbon. Colonies around which the yellow colour of PNP had disappeared after 48 hours were isolated and subcultured repeatedly to purity on Luria-Bertani agar (LB agar, Sigma-Aldrich, UK) with PNP (0.4 mM) for 48 hours at 25 ˚C. Pure cultures of isolates which degraded PNP were identified by colony PCR. Details about PCR conditions and sequencing can be found in Supporting Information.

Terminal Restriction Fragment Length Polymorphism (TRFLP)

DNA was extracted from freshly collected river water (T_0) and microcosm water samples taken after complete PNP degradation (T_{end}) using a PowerWater DNA Kit (MoBio Laboratories, Cambio, UK) according to the manufacturer's handbook. The quantity and

quality of DNA in extracts were analysed using a NanoDrop ND-1000 spectrophotometer, and by agarose gel electrophoresis, respectively. For TRFLP, bacterial 16S rRNA genes were amplified by PCR using primers 63F (34) and 1087R (35), and *MspI* and *HhaI* used for restriction digestion. Capillary electrophoresis and analysis of TRFLP data were performed as described by Hilton *et al*. (36).

Clone libraries of *pnpA* and *mar*

PCR products of triplicate samples, from river water at T_0 and T_{end}, were pooled, analysed by agarose gel electrophoresis, purified by gel extraction (Qiagen gel extraction kit) and cloned using the QIAGEN PCR cloning plus kit (Qiagen, Crawley, UK). Details about the clone library preparation and analysis are given in the Supporting Information.

Pyrosequencing of *pnpA* and *mar* genes

DNA aliquots (25 µl) extracted from triplicate samples of river water (T_0) and river water which had degraded PNP (T_{end}), were used for pyrosequencing of *pnpA, npdA2* and *mar* genes. Forward and reverse fusion primers were constructed from *pnpA, npdA2* and *mar* primers (SI Table 1), the (5'-3') Roche A linker (CCATCTCATCCCTGCGTGTCTCCGACTCAG), an 8-10 bp barcode for the forward primer, the (5'-3') biotin molecule and the Roche B linker (CCTATCCCCTGTGTGCCTTGGCAGTCTCAG). Amplifications were performed in 25 µl reactions with Qiagen HotStart Taq master mix (Qiagen Inc, Valencia, California), 1 µl of each 5 µM primer, and 1 µl of template. Reactions were performed on an ABI Veriti thermocycler (Applied Biosytems, Carlsbad, California) under the following thermal profile; 95 ˚C for 5 minutes, then 35 cycles of 94 ˚C for 30 seconds, 54 ˚C for 40 seconds, 72 ˚C for 1 minute, followed by one cycle of 72 ˚C for 10 minutes and a 4 ˚C hold. Strains *Pseudomonas putida*

SoT01 and *Arthrobacter* sp. JS443 were used as positive controls for *pnpA* and *mar*, and *npdA2* respectively.

There was no amplifaction of *npdA2*, but amplification products of *pnpA* and *mar* were used to create single stranded DNA following Roche 454 protocols (454 Life Sciences). Single stranded DNA was diluted and used in emPCR reactions. Sequencing followed established manufacture protocols (454 Life Sciences) using Roche 454 FLX instrument with Titanium reagents and Titanium procedures, and was performed at the Research and Testing Laboratory (Lubbock, TX, USA) (RTL, 2012). Sequences could not be obtained from the T_0 samples due to low amplification of functional genes. High-throughout sequence data have been submitted to the NCBI read archive with a project number of PRJNA271567.

The high throughput data were analysed using the Quantitative Insights into Microbial Ecology (QIIME) software, version 1.7.0, according to Caporaso *et al*. (37). The representative sequences from operational taxonomic units (OTUs) were analysed using an NCBI BLAST search (38) and those that were not *pnpA* or *mar* were excluded from downstream analysis. To assign taxonomy to OTUs, RDP Classifier 2.2. was used according to Wang *et al.* (39). Representative DNA sequences from OTUs were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) at 95% similarity according to Caporaso *et al*. (40). Representative sequences from OTUs were uploaded to the *pnpA* and *mar* ARB database and translated and aligned as described above. Representative sequences from OTUs were added to existing neighbour-joining phylogenetic trees with *pnpA* and *mar* reference sequences and sequences derived from cloning.

Quantitative PCR of *pnpA* and *mar* genes

Cloned *pnpA* and *mar* amplicons were used to generate dilutions and standards for calibration. Plasmid extraction and purification was carried out using the Qiagen HiSpeed

Plasmid Midi Purification Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. QPCR was carried out using the designed primers for *pnpA* and *mar* (Supporting Table 1) and the following cycling conditions; 95 ˚C for 10 minutes, followed by 40 cycles of 95 ˚C for 15 seconds, 60 ˚C for 1 minute and 95 ˚C for 15 seconds. Components of the reaction were as described by Hilton *et al*. (36). The products were sequenced to confirm the presence of *pnpA* and *mar* sequences. A standard curve of a fivefold dilution of each plasmid was made from 0.002-800 pg/µl. QPCR reactions for experimental samples, standards and blank were performed in triplicate. An average of the triplicate results was taken. The quantities of DNA obtained were converted to copy numbers of target DNA/µg total sample DNA. Details about the statistical analysis of the QPCR data are given in the Supporting Information.

Phylogenetic analysis of *pnpA and mar* gene sequences

Phylogenetic analysis of *pnpA* and *mar* functional markers was based on the conserved amino acid sequences obtained from clone libraries and 454 sequencing, which were aligned in the ARB database and exported to MEGA 5.1 (41). The evolutionary history was inferred using the neighbour-joining method (42). Details about the phylogenetic analysis can be found in the Supporting Information.

Stable Isotope Probing

Stable-isotope probing (SIP) of PNP-degrading bacteria was carried out according to Neufeld *et al*. (28) using 99% pure [$^{13}C_6$]-PNP (Sigma Aldrich, UK). An initial study was done in order to check [$^{12}C_6$]- or [$^{13}C_6$]-PNP uptake by the river water bacterial community using microcosms prepared in triplicate. River water samples (300 ml) obtained on January 10, 2013 were incubated with four µmoles of [$^{13}C_6$]-PNP (24 µmoles of carbon) as described above. [$^{12}C_6$]-PNP incubations were also set up as controls in order to confirm the stable isotope

enrichments in the [$^{13}C_6$]-PNP incubations. Additionally, positive and negative controls were run using sterilized river water. The positive controls were inoculated with *Pseudomonas putida* SoT01, and amended with [$^{12}C_6$]- or [$^{13}C_6$]-PNP to verify $^{13}C_6$-PNP utilization by bacteria while negative controls had only [$^{12}C_6$]- or [$^{13}C_6$]-PNP. The amount of PNP in incubation bottles was monitored by HPLC as described above.

Upon completion of the initial PNP-uptake study, a time-course DNA-SIP experiment was carried out. Three sets of replicated microcosms (9 in total) were prepared using 400 ml of river water collected on February 23, 2013 and amended with approximately 1.66 μmoles of [$^{12}C_6$]- or [$^{13}C_6$]-PNP (10 μmoles of carbon) initially. HPLC measurements were taken daily and additional PNP was provided to the bottles when it was depleted. Incubations were sacrificed when 1.66, 4 and 6.66 μmoles of PNP (10, 24 and 40 μmoles of carbon, respectively) had been degraded in the microcosms.

DNA extraction and gradient fractionation

River water samples from the incubation bottles were filtered through 0.20 μm sterile membrane filters (Merck Millipore, UK) and the filter papers were used to extract DNA using the Fast DNA soil extraction kit (MP Bioscience, UK) according to the manufacturer's instructions. The concentration of the total DNA from each sample was estimated using a spectrophotometer (NanoDrop ND-1000) and found to be in the range of 90-135 ng/μl. Approximately 2.7 μg of DNA was added to the caesium chloride/gradient buffer and centrifuged (28). DNA was then recovered via gradient fractionation. 12 or 13 fractions were obtained from the samples and a digital refractometer (Reichert 110 AR200, Reichert Analytical Instruments) was used to measure the densities of each fraction. DNA was purified from fractions and suspended in 50 μl of nuclease-free water as described by Neufeld *et al.* (28). In order to investigate whether differences in labelling patterns in ^{12}C and ^{13}C amended

microcosms occurred as a result of stable isotope incorporation into PNP degraders in the [$^{13}C_6$]-PNP fed microcosms, 16S rRNA gene PCR and denaturing gradient gel electrophoresis (DGGE) were applied as described in the Supporting Information.

Pyrosequencing of 16S rRNA genes in SIP fractions and data analysis

Partial bacterial 16S rRNA genes were amplified and sequenced using primers 27F and 519R through Roche 454 GS Junior workflow (Roche 454 Life Sciences, Branford, CT, USA) according to the manufacturer's instructions at Micropathology Ltd. (Coventry, UK).

Pyrosequencing reads were quality filtered and denoised using Acacia, version 1.52.b0 (43). QIIME was used for downstream analyses (37). OTUs were determined against the Greengenes database at ≥97% identity (44, 45) and alignment of the sequences to the Greengenes Core reference set was achieved using PyNAST (40). Chimeras were detected using BLAST (38) and removed before further analysis. The RDP Classifier 2.2 (39) was used for taxonomy assignment of the OTUs against the Greengenes reference database (46). Sequences have been submitted to the NCBI read archive (PRJNA271567).

Results

Biodegradation of PNP and isolation of PNP-degraders

Complete biodegradation of PNP occurred within eight days for river water incubated with PNP (Fig. 1). The PNP biodegradation curves were similar between replicates with the following times to PNP biodegradation obtained: DT_{50} = 5.19 (± 0.54), and DT_{90} = 5.90 (± 0.08) days.

Strains capable of PNP biodegradation (Supporting Table 2) were isolated from river water after biodegradation of PNP (isolates AKHD1, AKHD2, AKHD3). Analysis of 16S rRNA gene sequences using BLASTN against the Genbank database (38) revealed that all three

strains were related (≥98% sequence identity) to bacterial strains belonging to the genus *Pseudomonas.* PCR amplification of functional genes from DNA extracts obtained from bacterial isolates indicated the presence of *pnpA* and *mar* in all isolates, but not *npdA2* (Table S2).

TRFLP analysis of bacterial community structure

Analysis of Similarities (ANOSIM) revealed significant differences between TRFLP profiles of river water before the incubation (T_0) and after complete degradation of PNP (T_{end}) (Fig. 2). The 245 nt TRF was present in the river water before incubation (mean relative abundance 8.51% ± 2.87%) and increased significantly ($P<0.01$) after complete degradation of PNP (mean relative abundance 51.33% ± 8.22%).

TRFLP analysis was performed for the 16S rRNA genes of the PNP-degrading river isolates *Pseudomonas syringae* (AKHD2 and AKHD3)*, Pseudomonas fluorescens* (AKHD1) (Supporting Table 2). Only the 16S rRNA gene from *Pseudomonas syringae* AKHD2 resulted in a TRF of 245 nt in the analysis.

Functional marker analysis

With the newly designed primers (Supporting Table 1), the presence of the PNP degradative genes *pnpA*, *npdA2* and *mar* were investigated by PCR using DNA extracted from environmental samples and isolates. The *npdA2* gene fragment (589 bp) was not detected in the *Pseudomonas* isolates AKHD1, AKHD2, AKHD3 or *Pseudomonas* SoT02 DNA or from river water DNA extracts. PCR products were obtained for *pnpA* (approx 267 bp) and *mar* (approx 384 bp) from DNA extracted from river water at T_0 and after complete PNP biodegradation, as well as in the PNP-degrading isolate *Pseudomonas syringae* isolates, but not *Pseudomonas* SoT2 (Supporting Table 2).

Sequencing of *pnpA* and *mar* genes

The *pnpA* functional marker analysis revealed that two main clades (Fig. 3) of *pnpA* gene sequences were present in river samples before (T_0) and after (T_{end}) incubation with PNP. The main clades were characterized as *Pseudomonas syringae*-clade 1 with *pnpA* sequences from *P. syringae* AKHD2, and clade 2 with *pnpA* sequences from previously undetected *Pseudomonas*-like bacteria. Chao 1 analysis was carried out for the *pnpA* clone library to determine if biodegradation of PNP had a significant impact on diversity. Chao 1 estimate of population size indicated that 41 and 55% of the predicted total diversity of *pnpA* sequences had been represented within the *pnpA* clone library for T_0 and T_{end} samples, respectively. The Mann-Whitney U-test showed that overall there was no significant difference in *pnpA* community structure from river water at T_0 and following PNP degradation (P=0.066).

Analysis of *mar* sequences from T_0 river water and river water after complete degradation of PNP (T_{end}) resulted in the identification of 18 different lineages of *mar*, eleven of which were represented by single sequences (Fig. 4, Table 1) within the *mar* clone libraries. Sequences of *mar* genes grouped not only within *γ-Proteobacteria* such as *Pseudomonas syringae*, but also within *α-Proteobacteria* (e.g. *Rhizobium leguminosarum* bv. *viciae* 3841), *β-Proteobacteria* (e.g. *Burkholderia cenocepacia* J2315, *Burkholderia multivorans*) and *Actinobacteria* (e.g. *Rhodococcus opacus* RHA1). Additionally, marked differences in the number of clones detected between T_0 and T_{end} suggested proliferation during PNP degradation of taxa related to *Rhizobium leguminosarum bv. viciae* 3841, *Agrobacterium vitis* S4, *Bordetella petrii* and *Pseudomonas syringae* AKHD2 (Table 1). Chao 1 estimate of population size indicated that 23 and 11% of the predicted total diversity of *mar* sequences had been represented within the *mar* clone library for T_0 and T_{end} samples, respectively. The Mann-

Whitney U-test revealed a significant difference ($P<0.0001$) in *mar* community structure from river water at T_0 and following PNP degradation.

Analysis of *pnpA* and *mar* gene pyrosequences

pnpA and *mar* gene sequences were obtained from river samples after complete PNP degradation (T_{end}) using 454 high-throughput sequencing. In total, 65 459 sequences were obtained and 40 463 *pnpA* gene sequences were used for downstream analysis of *pnpA* genes after quality filtering and non-target gene sequences (e.g. alcohol dehydrogenase, haloacid dehalogenase, and some hypothetical proteins) were discarded. The average read count was 14 513 (± 5244) sequences per sample. Sequences were clustered at 86% similarity into three OTUs, however there was a high variation in the fractional abundances of these OTUs between replicates (Fig. 5). The first replicate included only OTU1 whilst OTU2 constituted 16.6 and 9.8% of the sequences from the second and third replicates, respectively. Also, OTU3 was only represented in the second replicate (2%). BLAST analysis of representative *pnpA* sequences from the three OTUs showed that they were related to the *para*-nitrophenol 4-monooxygenase gene from *Pseudomonas* sp. WBC-3 (Supporting Table 3). Translated amino acid sequences derived from representative OTU sequences were are in the phylogenetic tree (Fig. 3). OTU1 clustered within clade 1 which was previously described as a *P. syringae* clade, while OTUs 2 and 3 grouped within clade 2 (previously undetected *Pseudomonas*-like).

After quality filtering and discarding non-target gene sequences, 20 230 *mar* gene sequences were obtained with read count between 6400 and 13 350 per sample. Sequences clustered within 12 OTUs which showed high variability in terms of fractional abundance between the replicates (Fig. 5). Representative sequences of the *mar* OTUs were related to *mar* genes from *Pseudomonas* sp. GM74, *Pseudomonas* sp. WBC-3, *Pseudomonas fluorescens* F113, *Cupriavidus taiwanensis* LMG 19424, *Herbaspirillum frisingense*, *Rhizobium*

leguminosarum bv. viciae 3841, *Burkholderia cepacia*, *Burkholderia* sp. SJ98, *Novosphingobium resirovorum*, *Hoeflea phototrophica* DFL-43, *Azospirillum lipoferum* 4B and *Sphingobium chlorophenolicum* L-1 (Supporting Table 3). *Sphingobium chlorophenolicum* L-1 was the most dominant in the first replicate (46.7%), however *Novosphingobium resirovorum* and *Pseudomonas* sp. WBC-3 were more abundant in the second and third replicates (57.4% and 45.3%), respectively. Moreover, *Burkholderia* sp. SJ98 was highly abundant in the third replicate (24.1%) compared to others (less than 1%). The phylogenetic tree, which includes translated amino acid sequences derived from representative *mar* sequences and sequences from clone libraries, consisted of seven major clades (Fig. 4).

qPCR for *pnpA* and *mar*

Gene copy numbers of *pnpA* and *mar* were determined for the environmental samples before (T_0) and after (T_{end}) complete degradation of PNP. The *pnpA* copy number was 27.9 ± 9.3 at T_0 and 946.0± 381.0 per µg DNA at T_{end}. The copy number of *mar* was 32.6 ± 10.7 at T_0 and 120 ± 25.6 per µg DNA at T_{end}. ANOVA showed that the copy number of *pnpA* had increased significantly ($P<0.001$) after degradation of PNP (T_{end}). Although an increase in the *mar* gene copy number was also recorded, it was not significant ($p>0.05$).

Analysis of bacterial diversity in SIP gradient fractions

During the initial study to check [$^{12}C_6$]- or [$^{13}C_6$]-PNP uptake by river water bacterial communities, four µmoles of PNP were consumed in the microcosms in five days regardless of the carbon isotope used (Supporting Fig. 3). Subsequently, during the time-course SIP experiment, one set of replicated incubations was sacrificed after approximately 1.66, 4 and 6.66 µmoles of PNP (10, 25 and 40 µmoles of carbon, respectively) had been consumed (Supporting Fig. 4). Initial analysis of SIP gradient fractions was done using DGGE on samples

390 of the second time-point (6.66 µmoles of PNP consumption) (see Figure S5). Banding patterns of the incubation with ^{13}C-PNP showed a distinct and dominant band in the 'heavy' fractions (F5 to F9), which was absent in the 'light' DNA fractions (F10-12). This pattern is consistent with a population becoming labeled with ^{13}C as a result of assimilation of ^{13}C-PNP, which shifts the DNA of that population into the 'heavy' fraction. A band at the same position in the
395 DGGE profiles was seen in all fractions (heavy and light) of the ^{12}C-PNP amended microcosm, which is consistent with growth of a ^{12}C PNP degrading population, and distribution of its DNA along the entire SIP gradient rather than a consequence of GC-content, as described by Neufeld *et al.* (28).

Quality and chimera filtering of the pyrosequencing reads yielded 57 444 sequences
400 which were clustered into 3094 OTUs at 97% identity. The average read count was 2127 ± 849 sequences per sample. 'Heavy' (F7) and 'light' (F12) DNA fractions from the SIP experiment revealed distinct taxonomic classification of bacterial populations. *Gammaproteobacteria* dominated the bacterial community in all the ^{13}C-labelled 'heavy' fractions (F7). At the family level, the 'heavy' fractions from the three time points were dominated by *Pseudomonadaceae,*
405 which accounted for between 51.4 and 99.8% of the sequences. Similarly sequencing of the DGGE band which became enriched in ^{13}C and ^{12}C PNP heavy fractions (SFig5) supported the identification of the dominant PNP degrader as being related to *Pseudomonas* (data not shown).

At the genus level, *Pseudomonas* was the most prevalent genus across all the ^{13}C-labelled 'heavy' fractions (Fig. 6). It constituted 51.4% and 62.2% of sequences in the two first
410 time-point replicates but it represented 99.1% of sequences in the third replicate. This showed the predominance of *Pseudomonas* species even when a low amount of PNP was provided as the carbon source (1.66 µmoles). In the second time-point replicates, *Pseudomonas* was highly abundant making up 95.8 ± 6.9 % of the sequences. In the third time-point incubations (6.66 µmoles of PNP consumption), *Pseudomonas* accounted for 89.5 ± 8.5% of the sequences. The

read abundances of *Pseudomonas* in the 'light' fractions (F12) of the [$^{13}C_6$]-PNP incubations were significantly different from the 'heavy' fractions ($p<0.001$).

Discussion

Although PNP is the most common and important mononitrophenol and extensively used in industry, little is known about the abundance, diversity and activity of PNP-degraders in the environment. This is partly due to the fact that the previous work has used cultivation-based methods, and these have limitations in their capacity to provide a representative picture of microbial diversity and function in the environment. Overall, the results obtained using 16S rDNA-based TRFLP and DNA-SIP with ^{13}C-PNP, coupled with those from qPCR quantification of the *pnpA* gene), suggested that *Pseudomonas* spp. were the key bacterial population that degraded PNP in river water, and that PNP degradation showed typical kinetics and timescales for freshwater inoculum (49).

Pseudomonas spp. capable of degrading PNP have previously been isolated from soil and sludge (50, 12, 51). However, a range of other PNP strains have been isolated from soil and sludge, including *Sphingomonas* spp. (52), *Serratia* sp. (53), *Stenotrophomonas* sp. (54) and there is a lack of knowledge of how complex microbial communities conduct PNP biodegradation in the natural environment.

It is known that degradation of aromatic chemicals often requires diverse microbial communities with sufficient genetic potential to conduct the varied enzymatic reactions involved, including ring activation and finally ring cleavage to non-cyclic compounds that are converted to central metabolites (55, 56). This study presented a novel two functional gene approach that targeted the first and the last stage of the PNP biodegradation pathway to investigate the bacterial taxa involved at different stages of catabolism. It is noticeable that the *pnpA* functional marker revealed lower diversity of potential degraders than the more generic

mar functional marker (Fig. 3, Fig. 4 and Table 1). That more diverse bacterial populations consisting of *α, β* and *γ- Proteobacteria* were detected with the *mar* functional marker could support the possibility that different groups of bacteria are responsible for different stages of PNP biodegradation. Although the SIP analysis showed that a diverse assemblage of OTUs appeared in 'heavy' fractions, suggesting potential association with biodegradation of PNP, the identity of these taxa did not match those *mar* genes which became enriched during biodegradation. This suggests that the changes in *mar* abundance during degradation reflected the background shifts in the bacterial community which occurred during sample incubation, rather than reporting PNP degradation.

Sequencing of *pnpA* genes implied that the major pathway mediating the degradation of PNP was the Gram-negative pathway. Since the *npdA2* gene was not detected in the samples, it can be suggested that the PNP degradation pathway previously shown in Gram-positive bacteria did not play a role in PNP biodegradation in these experiments. One could argue that the abundance of the Gram-positive PNP-degrading bacteria could have simply be lower than the Gram-negative PNP-degraders, and therefore less readily detected using the coarse-level TRFLP and functional gene assays employed. However, SIP combined with pyrosequencing is an ideal tool to detect active bacterial populations even in low numbers. SIP-pyrosequencing results support the findings that *Pseudomonas* spp. were the most abundant PNP degrading bacteria in river water as revealed by the other methods used in this study.

According to the DGGE and pyrosequencing analyses of the SIP fractions, *Pseudomonas* spp. were the dominant PNP degraders in the river water microcosms. *Pseudomonas* spp. sequences were heavily enriched in the 'heavy' part of the [$^{13}C_6$]-PNP fractions at three time-points. In the third time-point, additional sequences apart from *Pseudomonas* spp. appeared. However results from SIP experiments need to be interpreted carefully as cross-feeding might lead to the identification of bacteria which do not degrade the

particular compound as the carbon source but use the metabolic intermediates (28). The pattern of *Pseudomonas* abundance across the time-point samples suggests their capability to degrade PNP rapidly and the slight decrease in their fractional abundance in the third time-point might indicate the likely start of cross-feeding. Another possibility might be that nutrient depletion led *Pseudomonas* cells to enter into the stationary phase or senescence, which reduced their abundance gradually.

An interesting result of this study was the agreement between the identity of PNP-degrading bacteria recovered using culture-dependent and –independent methods. Three cultures isolated from the river water after PNP degradation were identified as *Pseudomonas* strains (*P. fluorescens* and *P. syringae*). Although only bacteria that are adaptable to laboratory conditions can be cultured, the genus level identities of the PNP-degrading bacteria obtained by both approaches overlapped. This result contrasts with several studies which have identified clear differences between bacterial diversities that degrade chemicals using culture-dependent and –independent methods. For instance, Manefield *et al.* (57) demonstrated that *Thauera* dominated an industrial bioreactor degrading phenol while an earlier study using culture-dependent methods revealed the presence of only pseudomonads in the same bioreactor. Similarly, Jeon *et al.* (58) applied field-based SIP using labelled naphthalene and found that a *Polaromonas vacuolata*-like strain was responsible for *in situ* naphthalene metabolism although previous culture-based studies had indicated *Pseudomonas*-like strains as naphthalene-degrading bacteria. Furthermore, Shi and Bending (59) found that specific profiling of Sphingomonad communities revealed much greater complexity of taxa contributing to degradation of the herbicide isoproturon in soil, than had been revealed by culturing or profiling of general bacterial communities.

In the case of PNP, there is heterogeneity in the identity of PNP degraders and pathways of PNP degradation between environmental compartments, with strains isolated from different

soils and sludges found to degrade PNP via benzoquinone or nitrocatechol pathways, with both pathways present in some strains (60). The factors which control the distribution of PNP degradation pathways within and between environmental compartments remain to be resolved.

In conclusion, gaining insight into the diversity of active microbial populations in the environment as well as understanding their functional composition may improve our understanding of biodegradation processes occurring in the natural and engineered environments. Here, we showed that *Pseudomonas* spp. were active PNP-degraders in river water using a combination of different approaches. The findings of this study are useful in providing us with a better understanding of the bacterial populations that may drive different steps of PNP biodegradation in rivers and potentially other environmental compartments and suggest that *Pseudomonas* species are relevant and useful model organisms for optimizing bioremediation of PNP and understanding its degradation in the environment.

Acknowledgements

We thank Unilever and the Natural Environment Research Council (Grant NE/J014168/1) for financial support. We also thank Dr Robin Jackson (School of Biological Sciences, Knight Building, University of Reading, Whiteknights, Reading, Berkshire RG6 6AJ, UK) for his contribution to isolation of control strain *Pseudomonas* SoT02.

References

1. **Wan N, Gu JD, Yan Y.** 2007. Degradation of p-nitrophenol by Achromobacter xylosoxidans Ns isolated from wetland sediment. Int. Biodet. Biodeg. **59:** 90-96.

2. **Yi S, Zhuang WQ, Wu B, Tay, STL, Tay, WJH.** 2006. Biodegradation of p-nitrophenol by aerobic granules in a sequencing batch reactor. Environ. Sci. Technol. **40:** 2396-2401.

3. **Agency for Toxic Substances and Disease Registry (ASTDR).** 1992. Toxicological profile for nitrophenols: 2-nitrophenol and 4-nitrophenol. US Department of Health and Human Services, Public Health Service, Atlanta, USA.

4. **US EPA (United States Environmental Protection Agency**). URL http://www.epa.gov. (Accessed 10 October 2012).

5. **Japan Environment Agency.** 1995. Chemicals in the environment. Monitoring of the general environment in Japan 1994. Tokyo.

6. **Comber M, Holt M.** 2010. Developing a set of reference chemicals for use in biodegradability tests for assessing the persistency of chemicals. Report No: MCC/007.

7. **Godhead AK, Head IM, Snape JR, Davenport RJ.** (2014) Standard inocula preparations reduce the bacterial diversity and reliability of regulatory biodegradation tests. Environ. Sci. Pollut. Res. **21:** 9511-9521.

8. **Amman RI, Ludwig W, Schleifer KH.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143-169.

9. **Eilers H, Pernthaler J, Glöckner FO, Amann R.** 2000. Culturability and in situ abundance of pelagic bacteria from the North Sea. Appl. Environ. Microbiol. **66:** 3044-3051.

10. **Spain JC, Gibson DT.** 1991. Pathway for biodegradation of *p*-nitrophenol in *Moraxella* sp. Appl. Environ. Microbiol. **57:** 812-819.

11. **Malgoza RM, Buitrón G.** 2001. Degradation of *p*-nitrophenol in a batch biofilter under sequential anaerobic/aerobic environments. Wat. Sci. Tech. **44:** 151-157.

12. **Kulkarni M, Chaudhari A.** 2006. Biodegradation of p-nitrophenol by *Pseudomonas putida*. Biores. Technol. **97:** 982-988.

13. **Qiu X, Wu P, Zhang H, Li M, Yan Z.** 2009. Isolation and characterization of *Arthrobacter* sp. HY2 capable of degrading a high concentration of p-nitrophenol. Biores. Technol. **100:** 5243-5248.

14. **Kitagawa W, Kimura N, Kamagata Y.** 2004. A novel p-nitrophenol degradation gene cluster from a Gram-positive bacterium, *Rhodococcus opacus* SAO101. J. Bacteriol. **186:** 4894-4902.

15. **Perry LL, Zylstra, GJ.** 2007. Cloning of a gene cluster involved in the catabolism of p-nitrophenol by Arthrobacter sp. strain JS443 and characterization of the p-nitrophenol monooxygenase. J. Bacteriol. **189:** 7563-7572.

16. **Takeo M, Murakami M, Niihara S, Yamamoto K, Nishimura M, Kato D, Negoro S.** 2008. Mechanism of 4-nitrophenol oxidation in *Rhodococcus* sp. strain PN1: characterization of the two-component 4-nitrophenol hydroxylase and regulation of its expression. J. Bacteriol. **190:** 7367-7374.

17. **Zhang JJ, Liu H, Zhang XE, Zhou NY.** 2009. Identification and characterization of catabolic para-nitrophenol 4-monooxygenase and para-benzoquinone reductase from *Pseudomonas* sp strain WBC-3. J. Bacteriol. **191:** 2703-2710.

18. **Chauhan A, Pandey G, Sharma NK, Paul D, Pandey J, Jain RK.** 2010. p-nitrophenol degradation via 4-nitrocatechol in *Burkholderia* sp. SJ98 and cloning of some of the lower pathway genes. Environ. Sci. Technol. **44:** 3435–3441.

19. **Seibert V, Stadler-Fritzsche K, Schlömann M.** 1993. Purification and characterization of maleylacetate reductase from *Alcaligenes eutrophus* JMP134 (pJP4). J. Bacteriol. **175:** 6745-6754.

20. **Kaschabek SR, Reineke W.** 1992. Maleylacetate reductase of *Pseudomonas* sp. strain B13: dechlorination of chloromaleylacetates, metabolites in the degradation of chloroaromatic compounds. Arch. Microbiol. **158:** 412-417.

21. **Seibert V, Kourbatova EM, Golovleva LA, Schlömann M.** (1998). Characterization of the maleylacetate reductase MacA of *Rhodococcus opacus* 1CP and evidence for the presence of an isofunctional enzyme. J. Bacteriol. **180**, 3503-3508.

22. **Kasberg T, Daubaras DL, Chakrabarty AM, Kinzelt D, Reineke W.** 1995. Evidence that operons *tcb*, *tfd*, and *clc* encode maleylacetate reductase, the fourth enzyme of the modified ortho pathway. J. Bacteriol. **177:** 3885-3889.

23. **Ní Chadhain SM, Norman RS, Pesce KV, Kukor JJ, Zylstra JJ.** 2006. Microbial dioxygenase gene population shifts during polycyclic aromatic hydrocarbon biodegradation. Appl. Environ. Microbiol. **72:** 4078-4087.

24. **Zhang Y, Sei K, Toyama T, Ike M, Zhang J, Yang M, Kamagata Y.** 2008. Changes of catabolic genes and microbial community structures during biodegradation of nonylphenol ethoxylates and nonylphenol in natural water microcosms. Biochem. Eng. J. **39:** 288-296.

25. **Copley SJ.** 2009. Evolution of efficient pathways for degradation of anthropogenic chemicals. Nat Chem Biol **5:** 559-566

26. **Radajewski S, Ineson P, Parekh NR, Murrell JC.** 2000. Stable isotope probing as a tool in microbial ecology. Nature **403:** 646-649.

27. **Jones MD, Crandell DW, Singleton DR, Aitken MD.** 2011. Stable-isotope probing of the polycyclic aromatic hydrocarbon-degrading bacterial guild in a contaminated soil. Environ. Microbiol. **13:** 2623-2632.

28. **Neufeld JD, Vohra J, Dumont MG, Lueders T, Manefield M, Friedrich MW, Murrell JC.** 2007. DNA stable-isotope probing. Nat. Protocols **2:** 860-866.

29. **Cupples AM, Sims GK.** 2007. Identification of in situ 2,4-dichlorophenoxyacetic acid-degrading soil microorganisms using DNA-stable isotope probing. Soil. Biol. Biochem. **39**: 232-238.

30. **Kasai Y, Takahata Y, Manefield M, Watanabe K.** 2006. RNA-based stable isotope probing and isolation of anaerobic benzene-degrading bacteria from gasoline-contaminated groundwater. Appl. Environ. Microbiol. **72:** 3586-3592.

31. **Mahmood S, Paton GI, Prosser JI.** 2005. Cultivation-independent in situ molecular analysis of bacteria involved in degradation of pentachlorophenol in soil. Environ. Microbiol. **7:** 1349-1360.

32. **DeRito CM, Pumphrey GM, Madsen EL.** 2005. Use of field-based stable isotope probing to identify adapted populations and track carbon flow through a phenol-degrading soil microbial community. Appl. Environ. Microbiol. **71:** 7858-7865.

33. **Wood AP, Kelly DP.** 1977. Heterotrophic growth of *Thiobacillus* A2 on sugars and organic acids. Arch. Microbiol. **113**: 257-264.

34. **Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Dymock D, Wade WG.** (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol. **64:** 795–799.

35. **Hauben L, Vauterin L, Swings J, Moore ERB.** (1997). Comparison of 16S ribosomal DNA sequences of all Xanthomonas species. Int. J. Syst. Bacteriol. **47:** 328–335.

36. **Hilton S, Bennett AJ, Keane G, Bending GD, Chandler D, Stobart R, Mills P.** 2013. Impact of shortened crop rotation of oilseed rape on soil and rhizosphere microbial diversity in relation to yield decline. PLoS ONE **8:** 1-12.

37. **Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R.** 2010. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics **26:** 266-267.

38. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:** 403-410.

39. **Wang Q, Garrity GM, Tiedje JM, Cole JR.** 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. **73**: 5261-5267.

40 **Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R.** 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods **7:** 335-336.

41. **Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S.** 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. **28:** 2731-2739.

42. **Saitou N, Nei M.** 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:** 406-425.

43. **Bragg L, Stone G, Imelfort M, Hugenholtz P, Tyson GW.** 2012. Fast, accurate error-correction of amplicon pyrosequences using Acacia. Nat. Methods **9**: 425-426.

44. **Edgar RC.** 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics **26**: 2460-2461.

45. **DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Huber T, Hu P, Andersen GL.** 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. **72**: 5069-5072.

46. **McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, Knight R, Hugenholtz P.** 2012. An improved Greengenes taxonomy with explicit

ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. **6**: 610-618.

47. **Ludwig W, Strunk O, Westram R, Richter L, Meier H, Buchner A, Lai, T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann R, Jost A.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:** 1363-1371.

48. **Felsenstein J.** 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution **39:** 783-791.

49. **Toräng L, Nyholm N.** (2005). Biodegradation rates in adapted surface water can be assessed following a preadaptation period with semi-continuous operation. Chemosphere **61**: 1-10.

50. **Munnecke DM, Hsieh DPH.** 1974. Microbial decontamination of parathion and *p*-nitrophenol in aqueous media. Appl. Microbiol. **28:** 212-217.

51. **Yongliang Z, Deli L, Shiwang L, Shangying X, Yongze Y, Li X.** 2009. Kinetics and mechanisms of *p*-nitrophenol biodegradation by *Pseudomonas aeruginosa* HS-D38. J. Environ. Sci. **21:** 1194-1199.

52. **Leung KT, Tresse O, Errampalli D, Lee H, Trevors JT.** 1997. Mineralization of *p*-nitrophenol by pentachlorophenol-degrading *Sphingomonas* spp. FEMS Micro. Lett. **155:** 107-114.

53. **Pakala SB, Gorla P, Pinjari AB, Krovidi RK, Baru R, Yanamandra M, Merrick M, Siddavattam D.** 2007. Biodegradation of methyl parathion and *p*-nitrophenol: evidence for the presence of a *p*-nitrophenol 2-hydroxylase in a Gram-negative *Serratia* sp. Strain DS001. Appl. Microbiol. Biotechnol. **73:** 1452-1462.

54. **Liu YY, Yang C, Qiao C.** 2007. Biodegradation of *p*-nitrophenol and 4-chlorophenol by *Stenotrophomonas* sp. FEMS Microbiol. Lett. **277:** 150-156.

55. **Seeger M, Cámara B, Hofer B.** 2001. Dehalogenation, denitration, dehydroxylation, and angular attack on substituted biphenyls and related compounds by a biphenyl dioxygenase. J. Bacteriol. **183**: 3548-3555.

56. **Haritash AK, Kaushik CP.** 2009. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. J. Hazard Mat. **169:** 1-15.

57. **Manefield M, Whiteley AS, Griffiths RI, Bailey MJ.** 2002. RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. Appl. Environ. Microbiol. **63:** 5367-5373.

58. **Jeon CO, Park W, Padmanabhan P, DeRito C, Snape JR, Madsen EL.** 2003. Discovery of a bacterium, with distinctive dioxygenase, that is responsible for in situ biodegradation in contaminated sediment. PNAS. **100**: 13591-13596.

59. **Shi S, Bending GD.** 2007. Changes to the structure of *Sphingomonas* spp. associated with biodegradation of the herbicide isoproturon in soil. FEMS Microbiol. Lett. **269**: 110-116.

60. **Arora PK, Srivastava A, Singh VP.** (2014) Bacterial degradation of nitrophenols and their derivatives. J. Hazard. Mat. **266**, 42-59.

Figure legends

Fig. 1. The biodegradation of PNP at a concentration of 14 μM with river water. Error bars show standard error of the mean (S.E.M.), n=3.

Fig. 2. TRFLP profiles obtained from DNA extracted from river water before incubation (T_0) and river water after complete PNP biodegradation (T_{end}) presented as a mean (n=3) relative abundance of TRFs against TRF size. Error bars are showing standard error of the mean (S.E.M.) Profiles were generated by the bacterial primer 1087R-VIC fluorescently labelled amplifying 16S rRNA from the bacterial domain. Restriction digest was performed with enzymes *Msp*I and *Hha*I.

Fig. 3. Neighbour-joining tree showing diversity of *pnpA* gene sequences obtained from cloning and 454 sequencing. Tree created based on nucleotide sequences translated into amino acid sequences using ARB (47), and the position 89-155, 67 valid columns in the *pnpA* sequence from *Pseudomonas* sp. WBC-3 as a filter. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (48).

Fig. 4. Neighbour-joining tree showing diversity of mar gene sequences obtained from cloning and 454 sequencing. Tree created based on nucleotide sequences translated into amino acid sequences using ARB (47) and the position 133-242, 110 valid columns in the *mar* sequence from *Rhodococcus opacus* B4 as a filter. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (48).

Fig. 5. The relative abundances of functional gene sequences, *pnpA* and *mar*, in the river water replicate samples after PNP degradation (T_{end}). Results were obtained from pyrosequencing datasets and given as percentages. Affiliations of the OTUs are given in Supporting Table 3.

Fig. 6. Genus-level taxonomic profiling of the pyrosequence datasets. Bar names include the time-point, replicate number, SIP fraction number (F7 or F12) and carbon isotope used (^{12}C or ^{13}C). *i.e.* 2.1.C12F7: second time-point, replicate 1, C^{12} incubation, fraction 7.

Tables legend

705

Table 1 Diversity of *mar* clones from T_0 and PNP degrading river water (T_{end}) based on *mar* neighbour-joining tree. 86% similarity at amino acid level was used as a cutoff to distinguish clades within a preexisting tree.