

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

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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
25 the diversity of active PNP- degrading bacterial populations in river water using a novel functional marker approach coupled with [¹³C₆]-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 stable isotope
30 probing-pyrosequencing. Similarly, analysis of functional genes involved in PNP degradation demonstrated that *Pseudomonas* spp. were the key PNP-degraders, with biodegradation following the Gram-negative pathway involving *pnpA*. Further analysis based on the gene encoding maleylacetate reductase (*mar*), an enzyme common to both Gram negative and Gram positive bacterial PNP degradation pathways,, identified a diverse assemblage of
35 bacteria which were potentially associated with PNP degradation. 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.

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

Introduction

45 *Para*-nitrophenol (PNP) is the most common and important mononitrophenol, in
terms of quantities used by industry and extent of environmental contamination (Wan *et al.*,
2007; Yi *et al.*, 2006). PNP is used for synthesis of medicines, dyes, explosives and wood
preservatives (Wan *et al.*, 2007; ATSDR, 1992), and is also generated in the environment by
the degradation of pesticides that contain the nitrophenol moiety (US EPA, 1980). As a
50 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 (Japan Environment
Agency, 1995).

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
55 and validate regulatory tests of chemical persistence (Comber and Holt, 2012). 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
60 unclear (Davenport *et al.*, 2013).

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.
65 Culturability in nearly all environments varies from 1 to 10% (Amann *et al.*, 1995), 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 (Eilers *et al.*, 2000).

70 PNP degrading isolates belong to several different genera including *Flavobacterium*,
Bacillus, *Pseudomonas*, *Moraxella*, *Nocardia*, and *Arthrobacter* (Spain and Gibson, 1991;
Malgoza and Buitrón, 2001; Kulkarni and Chaudhari, 2006; Qiu *et al.*, 2007). The enzymes
and genes associated with PNP degradation pathways in bacterial isolates from soil and
sludge have been well characterized (Kitagawa *et al.*, 2004; Perry and Zylstra, 2007; Takeo *et*
75 *al.*, 2008; Zhang *et al.*, 2009). According to Chauhan *et al.* (2010) 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* (Zhang *et al.*, 2009). The resulting 1,4-
benzoquinone is further degraded via 4-hydroxymuconic semialdehyde to maleylacetate. In
80 Gram-positive bacteria such as *Arthrobacter* spp., PNP biodegradation proceeds via 4-
nitrocatechol and involves 4-nitrophenol 2-monooxygenase which is encoded by the *npdA2*
gene (Zhang *et al.*, 2009). 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
85 degradation. This is degraded by maleylacetate reductase (MAR) to keto adipate and further
to acetyl-CoA, which is channelled into the citric acid cycle (Chauhan *et al.*, 2010). MAR is
involved in various bacterial catabolic pathways involving hydroquinone or hydroxyquinol as
ring cleavage substrates (Supporting Fig. 2) (Seibert *et al.*, 1993), and a variety of genes
encoding this enzyme have been identified in different bacterial strains e.g. the *pnpF* gene
90 from *Pseudomonas* sp. WBC3 (Zhang *et al.*, 2009), the *clcE* gene from *Pseudomonas* sp.
Strain B13 (Kaschabek and Reineke, 1992), the *macA* gene from *Rhodococcus opacus* 1CP
(Seibert *et al.*, 1998) and the *tftE* gene from *Burkholderia cepacia* AC1100 (Kasberg *et al.*,

1995). In the present paper we refer to these maleylacetate reductase genes collectively as *mar*.

95 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.* (2006)
determined microbial dioxygenase gene shifts during polycyclic aromatic hydrocarbon
biodegradation while Zhang *et al.* (2008) studied the changes of key catabolic genes and
microbial community structure during the degradation of nonylphenol ethoxylates and
100 nitrophenol in natural water microcosms. Functional gene based methods are advantageous
over 16S rRNA-based methods in that they can reveal the biodegradation potential of an
environment. However, these methods cannot identify the actual microbial populations
responsible for biodegradation *in situ*. Stable-isotope probing (SIP) overcomes this limitation
by allowing the identification of active microorganisms that utilize enriched compounds
105 (Radajewski *et al.*, 2000). To date, SIP has been applied with several ¹³C-labelled organic
compounds such as methane, naphthalene, polycyclic aromatic hydrocarbons, 2,4-
dichlorophenoxyacetic acid, phenol, benzene and pentachlorophenol (Jones *et al.*, 2011;
Neufeld *et al.*, 2007; Cupples and Sims, 2007; Kasai *et al.*, 2006; Mahmood *et al.*, 2005;
DeRito *et al.*, 2005). These studies revealed that microbial populations actively utilising
110 chemicals in the environment are distinct from those found by 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
115 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 and late 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 [¹³C₆]-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

130 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 (Goodhead et al., 2013). 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.

160

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 (Wood and Kelly, 1977) 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.

170 **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 (Marchesi et al., 1998) and 1087R (Hauben et al., 1997), and *MspI* and *HhaI* used for restriction digestion. Capillary electrophoresis and analysis of TRFLP data were performed as described by Hilton *et al.* (2013).

180 **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.

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Pyrosequencing of *pnpA* and *mar* genes

DNA aliquots (25 μ l) 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 Biosystems, 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 amplification 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 T₀ 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.* (2010a). The representative sequences from operational taxonomic units (OTUs) were analysed using an NCBI BLAST search (Altschul *et al.*, 1990) 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.* (2007). Representative DNA sequences from OTUs were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) at 95% similarity according to Caporaso *et al.* (2010 b). 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.

220

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.* (2013). 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.

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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 (Tamura *et al.*, 2011). The evolutionary history was inferred using the neighbour-joining method (Saitou and Nei, 1987). Details about the phylogenetic analysis can be found in the Supporting Information.

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Stable Isotope Probing

Stable-isotope probing (SIP) of PNP-degrading bacteria was carried out according to
245 Neufeld *et al.* (2007) using 99% pure [$^{13}\text{C}_6$]-PNP (Sigma Aldrich, UK). An initial study was
done in order to check [$^{12}\text{C}_6$]- or [$^{13}\text{C}_6$]-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 [$^{13}\text{C}_6$]-PNP (24 μmoles of carbon) as described
above. [$^{12}\text{C}_6$]-PNP incubations were also set up as controls in order to confirm the stable
250 isotope enrichments in the [$^{13}\text{C}_6$]-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 [$^{12}\text{C}_6$]- or [$^{13}\text{C}_6$]-PNP to verify $^{13}\text{C}_6$ -PNP
utilization by bacteria while negative controls had only [$^{12}\text{C}_6$]- or [$^{13}\text{C}_6$]-PNP. The amount of
PNP in incubation bottles was monitored by HPLC as described above.

255 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 [$^{12}\text{C}_6$]- or [$^{13}\text{C}_6$]-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
260 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
265 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/ μ l. Approximately 2.7 μ g of DNA was added to the caesium chloride/gradient buffer and
270 centrifuged (Neufeld *et al.*, 2007). 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 μ l of nuclease-free water as described by Neufeld *et al.* (2007). In order to investigate whether differences in labelling patterns in ^{12}C
275 and ^{13}C amended microcosms occurred as a result of stable isotope incorporation into PNP degraders in the [$^{13}\text{C}_6$]-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

280 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 (Bragg *et al.*, 2012). QIIME was used for downstream analyses (Caporaso *et al.*,
285 2010a). OTUs were determined against the Greengenes database at $\geq 97\%$ identity (Edgar, 2010; DeSantis *et al.*, 2006) and alignment of the sequences to the Greengenes Core reference set was achieved using PyNAST (Caporaso *et al.*, 2010b). Chimeras were detected using BLAST (Altschul *et al.*, 1990) and removed before further analysis. The RDP Classifier 2.2 (Wang *et al.*, 2007) was used for taxonomy assignment of the OTUs against the
290 Greengenes reference database (McDonald *et al.*, 2012). Sequences have been submitted to the NCBI read archive (PRJNA271567).

Results

Biodegradation of PNP and isolation of PNP-degraders

295 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: $DT_{50} = 5.19 (\pm 0.54)$, and $DT_{90} = 5.90 (\pm 0.08)$ days.

300 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 (Altschul et al, 1990) revealed that all three strains were related ($\geq 98\%$ 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 305 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}) 310 (Fig. 2). The 245 nt TRF was present in the river water before incubation (mean relative abundance $8.51\% \pm 2.87\%$) and increased significantly ($P < 0.01$) after complete degradation of PNP (mean relative abundance $51.33\% \pm 8.22\%$).

TRFLP analysis was performed for the 16S rRNA genes of the PNP-degrading river isolates *Pseudomonas syringae* (AKHD2 and AKHD3), *Pseudomonas fluorescens* (AKHD1) 315 (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
320 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
325 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*
330 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
335 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 ($P=0.066$).

340 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 T₀ 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 T₀ 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 T₀ and following PNP degradation.

355 **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 (\pm 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 $27.9 \pm$

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 [$^{12}\text{C}_6$]- or [$^{13}\text{C}_6$]-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 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 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 (2007).

Quality and chimera filtering of the pyrosequencing reads yielded 57 444 sequences 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 ¹³C-labelled 'heavy' fractions (F7). At the family level, the 'heavy' fractions from the three time points were dominated by *Pseudomonadaceae*, which accounted for between 51.4 and 99.8% of the sequences. Similarly sequencing of the DGGE band which became enriched in ¹³C and ¹²C 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 ¹³C-labelled 'heavy' fractions (Fig. 6). It constituted 51.4% and 62.2% of sequences in the two first 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 [¹³C₆]-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 ¹³C-PNP, coupled with those from qPCR quantification of functional genetic markers (*pnpA* and *mar*), 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 (Torang and Nyholm, 2005).

445 *Pseudomonas* spp. capable of degrading PNP have previously been isolated from soil and sludge (Munnecke and Hsieh, 1974; Kulkarni and Chaudhari, 2006; Yongliang *et al.*, 2009). However, a range of other PNP strains have been isolated from soil and sludge, including *Sphingomonas* spp. (Leung *et al.*, 1997), *Serratia* sp. (Pakala *et al.*, 2007), *Stenotrophomonas* sp. (Liu *et al.*, 2007) and there is a lack of knowledge of how complex
450 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 (Seeger *et al.*, 2001; Haritash and Kaushik, 2009). This study
455 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
460 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
465 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* and *mar* 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 [¹³C₆]-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 (Neufeld *et al.*, 2007). 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
495 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.* (2002) demonstrated that *Thauera* dominated an industrial bioreactor degrading phenol while an earlier study using culture-dependent methods revealed the presence of only pseudomonads
500 in the same bioreactor. Similarly, Jeon *et al.* (2003) 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 (2007) found that specific profiling of Sphingomonad communities revealed much greater complexity of
505 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
510 pathways, with both pathways present in some strains (Arora *et al.*, 2014). 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
515 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.

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750 **Figure legends**

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

755 **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 *MspI* and *HhaI*.

760 **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 (Ludwig *et al.*, 2004), 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 (Felsenstein, 1985).

765 **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 (Ludwig *et al.*, 2004) 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 (Felsenstein, 1985).

770 **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.

775 **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.

780 **Tables legend**

Table 1 Diversity of *mar* clones from T₀ 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.

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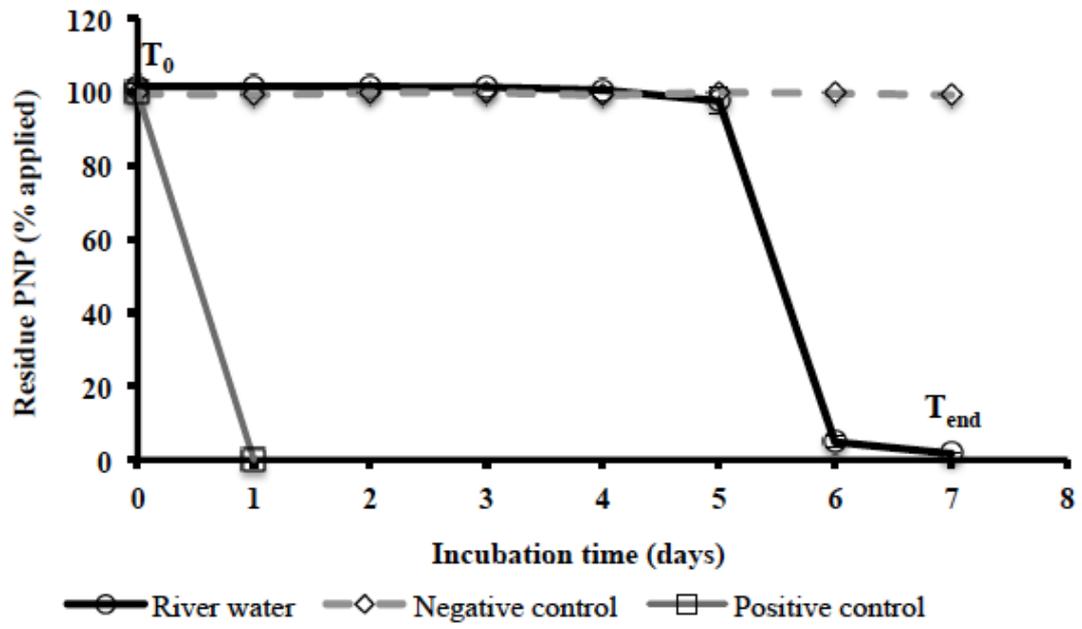
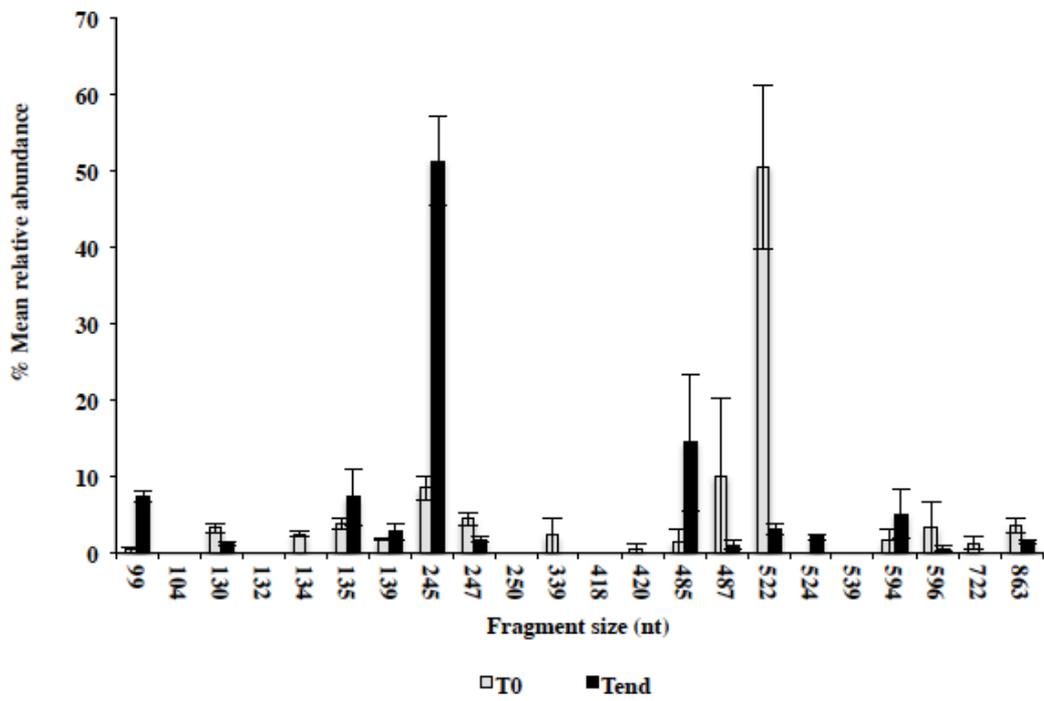
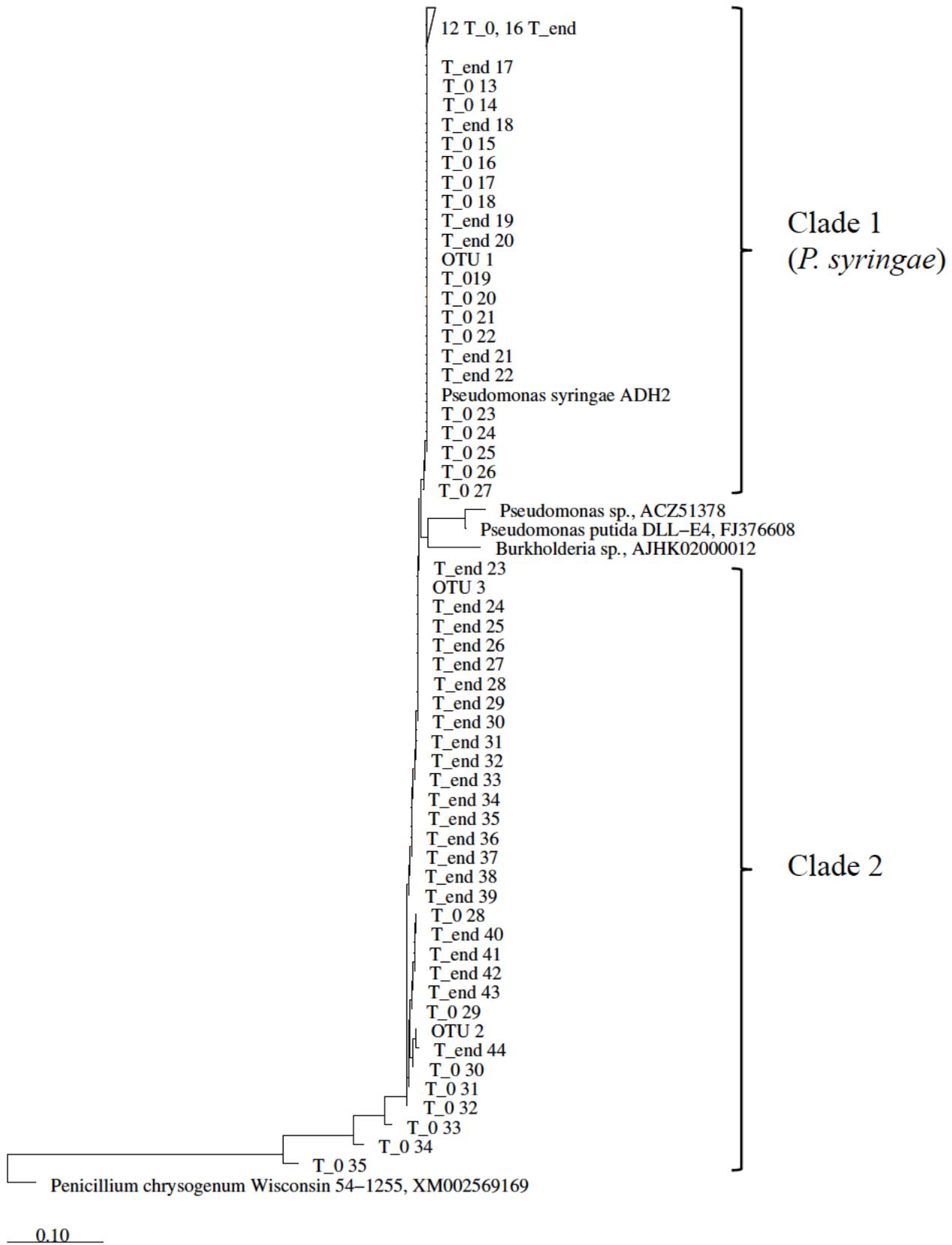


Fig. 1.



795

Fig. 2.



800 Fig. 3.

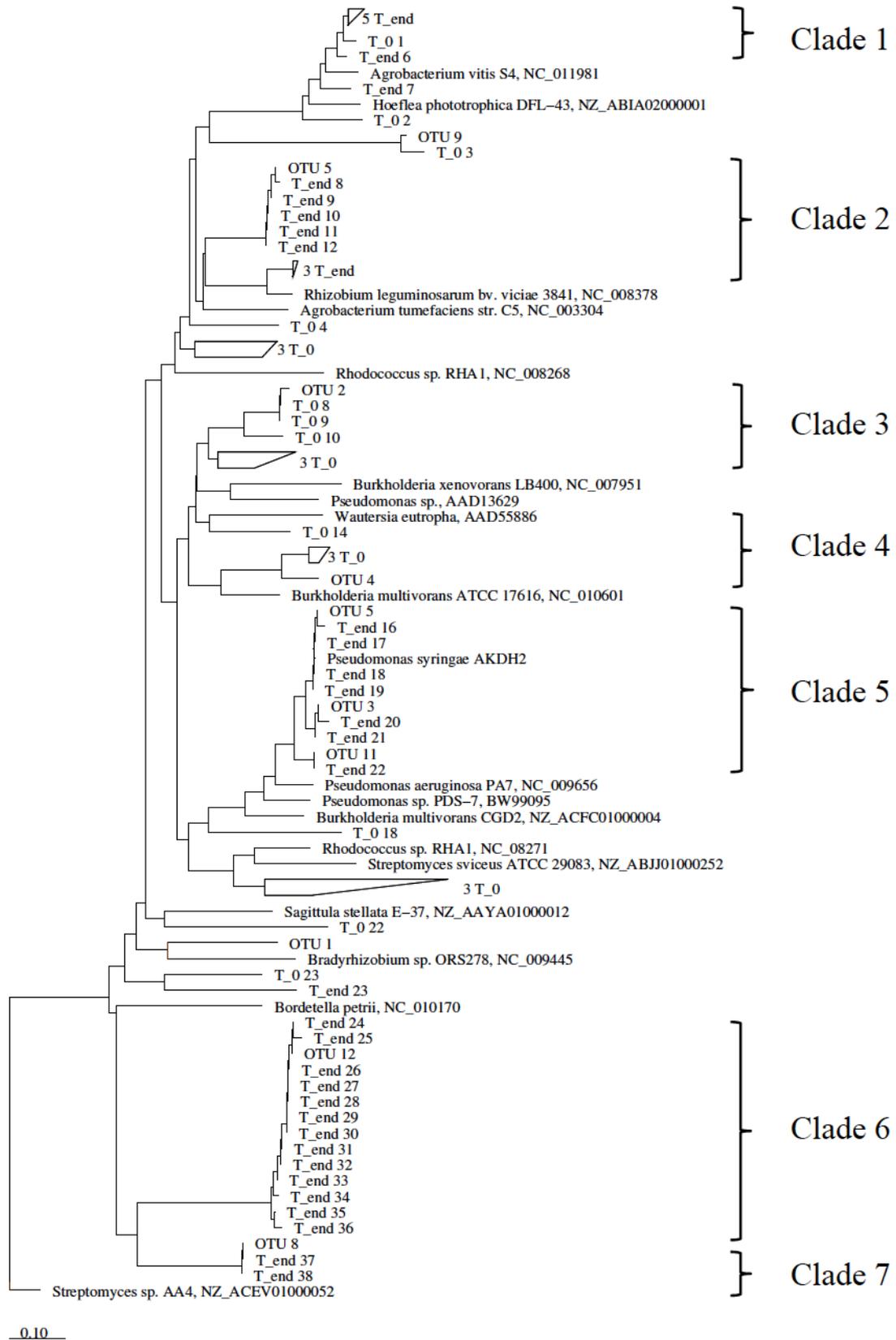
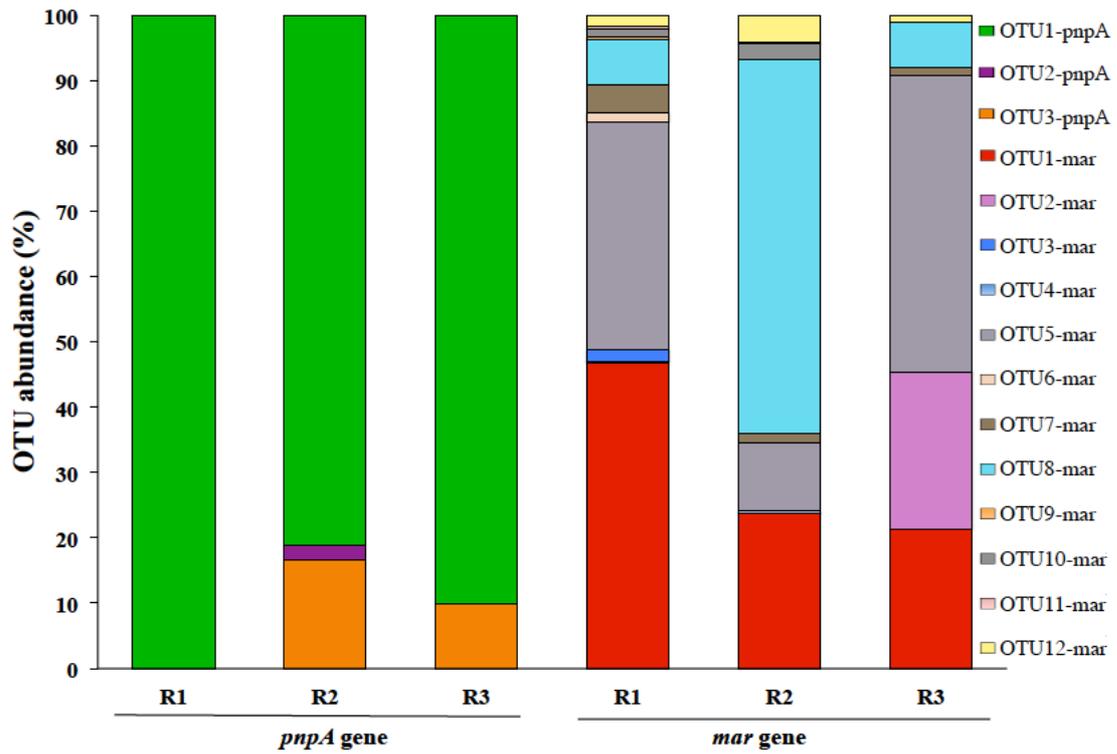


Fig. 4.



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Fig. 5.

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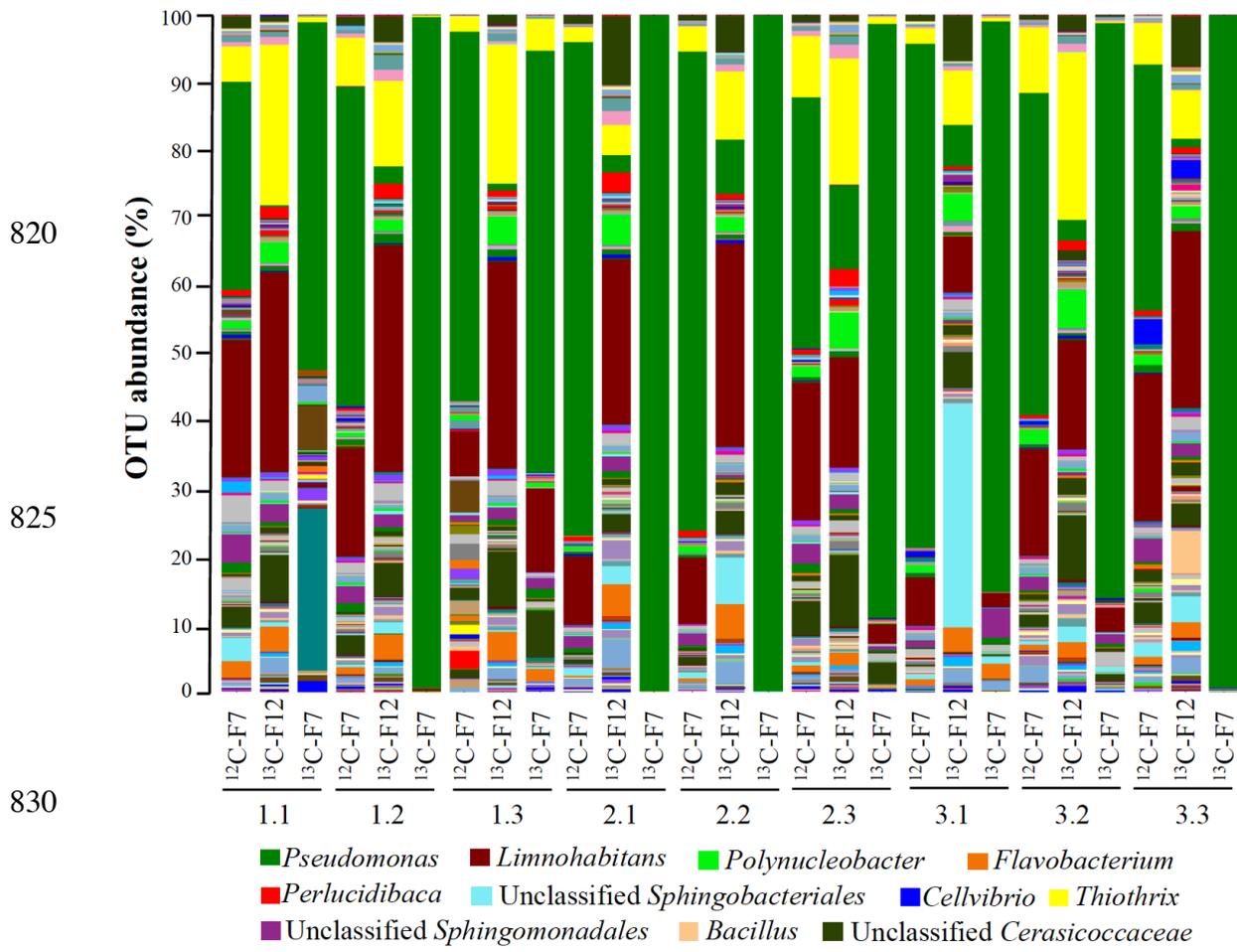


Fig. 6.

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Table 1

Closest related clade in phylogenetic tree	Lineage number	% <i>mar</i> clones	
		T ₀	T _{end}
<i>Pseudomonas syringae</i> AKHD2	1	0	18.4
<i>Burkholderia cenocepacia</i> J215	single <i>mar</i> sequence	4.2	0
<i>Pimelobacter simplex</i> strain 3E	single <i>mar</i> sequence	4.2	0
<i>Mycobacterium smegmatis</i> strain MC2	single <i>mar</i> sequence	8.3	0
<i>Achromobacter xylosoxidans</i>	2	29.2	0
<i>Wautersia eutropha</i>	single <i>mar</i> sequence	4.2	0
<i>Burkholderia multivorans</i>	3	12.5	0
<i>Agrobacterium vitis</i> S4	4	4.2	18.4
<i>Hoeflea phototrophica</i> DFL-43	single <i>mar</i> sequences	8.3	0
<i>Rhizobium leguminosarum</i> <i>bv.</i> <i>vitiae</i> 3841	5	4.2	21.1
<i>Rhodococcus</i> sp. RHA1	6	12.5	0
<i>Sagittula stellata</i> B-37	single <i>mar</i> sequence	4.2	0
<i>Azospirillum</i> sp. B510	single <i>mar</i> sequences	4.2	2.6
<i>Bordetella petrii</i>	7	0	39.5