
Kowalczyk, Agnieszka; Price, Oliver R.; van der Gast, Christopher J.; Finnegan, Christopher J.; van Egmond, Roger A.; Schäfer, Hendrik; Bending, Gary D.. 2016. **Spatial and temporal variability in the potential of river water biofilms to degrade p-nitrophenol.** *Chemosphere*, 164. 355-362.
10.1016/j.chemosphere.2016.08.095

Spatial and temporal variability in the potential of river water biofilms to degrade p-nitrophenol

Agnieszka Kowalczyk*[1], Oliver R. Price[2], Christopher J. van der Gast[1,3], Christopher J. Finnegan[2], Roger A. van Egmond[2], Hendrik Schäfer[1] 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]NERC Centre for Ecology and Hydrology, Wallingford, OX10 8BB, 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

Abstract

In order to predict the fate of chemicals in the environment, a range of regulatory tests are performed with microbial inocula collected from environmental compartments to investigate the potential for biodegradation. The abundance and distribution of microbes in the environment is affected by a range of variables, hence diversity and biomass of inocula used in biodegradation tests can be highly variable in space and time. The use of artificial or natural biofilms in regulatory tests could enable more consistent microbial communities be used as inocula, in order to increase test consistency. We investigated spatial and temporal variation in composition, biomass and chemical biodegradation potential of bacterial biofilms formed in river water. Sampling time and sampling location impacted the capacity of biofilms to degrade p-nitrophenol (PNP). Biofilm bacterial community structure varied across sampling times, but was not affected by sampling location. Degradation of PNP was associated with increased relative abundance of *Pseudomonas syringae.* Partitioning of the bacterial metacommunity into core and satellite taxa revealed that the *P. syringae* could be either a satellite or core member of the community across sampling times, but this had no impact on PNP degradation. Quantitative PCR analysis of the *pnpA* gene showed that it was present in all samples irrespective of their ability to degrade PNP. River biofilms showed seasonal variation in biomass, microbial community composition and PNP biodegradation potential, which resulted in inconsistent biodegradation test results. We discuss the results in the context of the mechanisms underlying variation in regulatory chemical degradation tests.

Key words

Biofilm; Metacommunity; Seasonality, Biodegradation; OECD tests; *Pseudomonas*; Para-nitrophenol

1. Introduction

There is a vast diversity of chemicals in the environment, which arrive from industry, agriculture, medical treatment and common household products. Many of these chemicals have the potential to exert adverse human and ecological health effects (Mnif et al. 2011, Jones et al. 2003, Lapertot and Pulgarin, 2006). The persistence of chemicals entering the environment is controlled by a range of biotic and abiotic processes, which together determine environmental concentrations of the chemical and the extent to which it is transformed to metabolites (Kowalczyk et al. 2014). For most chemicals, biodegradation, mediated by microorganisms, is the main factor controlling persistence. In order to predict chemical persistence the Organisation for Economic Cooperation and Development (OECD) has established a tiered series of biodegradation tests (Kowalczyk et al. 2014).

The first tier ready biodegradability tests are intended as screening approaches to gauge if a chemical is rapidly degraded, or has potential to degrade, in the environment. These tests utilize a variety of environmental materials, such as water and sediments as inocula (Doi et al. 1996; Reuschenbach et al. 2003; Yuan et al. 2004). Guidelines provide flexibility for the inoculum that can be used and its method and time of collection. However, it is known that inoculum (i.e. microbial biomass) density is an important factor affecting the outcome of chemical biodegradation tests (Thouand et al. 1995; Godhead et al. 2014) and in particular, inconsistent test results may reflect differences in inoculum quality because of temporal and spatial variability of microbial community density and diversity within environmental compartments (Courtes et al. 1995; Mezzanotte et al. 2005).

Microbial communities are subject to seasonal variability due to changes in environmental conditions, which shape natural ecosystems and affect processes occurring in environmental compartments (Bertram, et al. 2001; Yunus and Nakagoshi, 2004). In particular, changes in river water volume and flow rates (Naudin et al. 2001), temperature (LaPara et al. 2000), light penetration (Romaní and Sabater, 1999) and suspended matter (Cébron et al. 2007) can impact the composition of microbial communities inhabiting river water and sediment (Hudon, 1997; Hunt and Parry, 1998; Midwood and Chow-Fraser, 2012, LaPara et al. 2000). This can affect chemical bioavailability and biodegradation *in situ*. For example, seasonal variations in biodegradation of 2,4-dichlorophenoxyacetic acid (2,4-D) were reported by Watson (1977) in river water and sediment, with maximum breakdown observed during winter flood conditions, probably because of adsorption of the chemical onto suspended sediment, where microbial activity was high (Nesbit and Watson, 1980). Similarly, seasonal variation in degradation of hexadecane, associated with changes in nutrient availability and biofilm composition, was reported by Chénier et al. (2003).

As an alternative to the use of natural inoculum, there has been interest in generating standardised biofilms for use in chemical biodegradation tests (Kowalczyk et al. 2014). Currently, biofilms are used as inocula in some OECD tests, such as the biodegradation simulation test guideline OECD 303 b (OECD, 2005), and bioreactors with biofilms comprising of defined bacterial strains and mixtures have been applied to study the bioremediation of a variety of chemicals, including chlorophenols (Puhakka et al. 1995; Kargi and Eker, 2005), herbicides (Oh and Tuovinen, 1994) and azo dyes (Zhang et al. 1995).

In natural habitats, biofilms typically have high species richness (Sabater et al. 2007) and density (Singh et al. 2006), show resistance to toxic chemicals e.g. antimicrobial agents (Mah and O'Toole, 2001) and recover quickly from perturbation (Morin et al. 2007; Proia et al. 2010). Furthermore, biofilms support synergistic interactions within communities (Elias and Banin, 2012), provide microhabitat heterogeneity (Donlan, 2002), and the potential for genetic exchange (Schwartz et al. 2003), all of which can promote chemical biodegradation.

For these reasons, use of biofilms in biodegradation tests could provide a means of reducing the chance of common test failures described by Thouand et al. (1995) and Godhead et al. (2014), by eliminating the 'biodegradation lottery' associated with low biomass in regulatory tests (Kowalczyk et al., 2014). However, to date, investigations of the potential to use biofilm inoculum in regulatory tests has centered on laboratory generated inoculum.

In recent studies we have used a variety of culture dependent and independent approaches, including DNA-stable isotope probing and functional gene markers to identify para-nitrophenol (PNP) degrading communities in a UK river, establishing that species of *Pseudomonas* were associated with degradation in samples taken between 2010 and 2013 (Kowalczyk et al. 2015). In the current study, we investigated temporal and spatial assembly of *in situ* river biofilm communities and relationships between community composition and biodegradation of PNP, addressing the following questions: (1) how does time affect the composition and biodegradation potential of river water biofilms? (2) does spatial location affect biofilm composition and degradation potential? and (3) does temporal and spatial variation in chemical biodegradation kinetics relate to dynamics of specific degraders within the biofilm community?

2. Materials and methods

2.1 Biofilm preparation

Biofilms were prepared in the River Dene, in Warwickshire, UK (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. At each sampling time and location, Biofilms were generated on polysine glass slides (5.7 x 2.5 cm) (VWR, International) attached to a Hole Airbrick (21.0 x 13.5 x 5.0 cm) (Travis Perkins Trading Co. Ltd, UK) with cable ties (165 x 2.6 mm) (BHGS Ltd, UK). Each slide had a total surface area of 28.5 cm^2.11 slides were attached to each brick.

At each sampling time, two bricks with slides were placed on the bed of the River 200 m upstream, 200 m downstream and at the effluent discharge point of the Wellesbourne Wastewater Treatment Plant (WWTP) (52˚11'58.54''N and 1˚36'44.94''W). Bricks were left in the river for eight days to allow colonisation of the slides by microorganisms and development of natural river water biofilms. Biofilms grown in this way were collected in November 2011, February 2012 and May 2012.

2.2 The biodegradation of PNP by river biofilms

At each sampling time, biofilms collected from each site were used to determine potential for PNP biodegradation. For each site, triplicate tests were set up, consisting of a sterile amber Duran Schott bottle (500 mL) (Fisher Scientific, UK) with 300 mL of sterile river water with 2 mg L^{-1} PNP, to which were added three of the Polysine glass slides supporting river water biofilms.

Sterile river water was prepared by autoclaving water samples collected downstream of the River Dene STP at 121˚C, for 15 minutes, at 1.1 atm. A positive control consisted of sterile river water inoculated with the PNP-degrading isolate *Pseudomonas syringae* AKHD2 (Kowalczyk et al., 2015), grown on LB medium with PNP (56 mg/L) for 48 hours at 25˚C. Sterile river water was used as a negative control. Additionally for the February and May 2012 time points, non-sterile river water collected from the effluent location was used to compare degradation rates with the biofilm materials. PNP (dissolved in water) was added to biodegradation test bottles as a sole source of carbon to a concentration of 2 mg L^{-1}. Bottles were incubated in a controlled environment room with an 18 hour light and six hour dark cycle at 20±2 °.

Every two days, a 1 mL aliquot of liquid was taken from each bottle and used to determine PNP concentration using HPLC, which comprised 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 the flow rate was 0.50 mL/minute. Analysis was performed with UV detection at 254 nm, with column pressure of 115 bar and column temperature at 25 ˚C. The volume of the injected sample was 20 µL and the run time was 13 minutes. HPLC calibration was carried out using concentrations of 2-2000 µg/L of PNP standards.

Time to 50 % PNP-degradation (DT50) was calculated using the model of best fit to the biodegradation kinetics for each sample, as described by Rodriguez-Cruz et al, (2006). Least significant difference (LSD) was used to determine significant differences in DT50 between treatments using GenStat (13th edition, VSN International Ltd.).

2.3 Biofilm biomass

Extracellular polysaccharide (EPS) was measured as a proxy for biofilm biomass (Flemming et al. 2007; Underwood and Paterson, 1995). At each sampling time and location, biofilm was removed from 3 glass slides using a sterile nylon brush and pooled, as described by Deines et al. (2010). Soluble carbohydrate was determined by reaction with phenol and H_2SO_4 (Dubois et al. 1956) using glucose as a standard. LSD was used to determine significant differences between treatments.

2.4 DNA extraction from biofilms

Before incubation (T_0), after complete PNP biodegradation or when there was no biodegradation at the end of the incubation (T_{end}), biofilms were harvested from slides in each bottle as described above. The suspended biofilm biomass was collected on a Millipore GVWP04700, (hydrophilic Durapore) polyvinylidene fluoride membrane filter (0.22 μm mesh, 47 mm diameter) using a vacuum filtration system. Membrane filters with concentrated biofilm biomass were folded and placed in the bead tubes of the Power Water DNA extraction kit (MoBio, UK), before DNA extraction according to the manufacturer’s protocol. DNA was extracted from effluent location water samples using procedures described in Kowalczyk et al. (2015). The DNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific Fisher, UK).

2.5 Quantitative PCR analysis of *pnpA* gene.

Our earlier work (Kowalczyk et al. 2015) showed that p-nitrophenol degradation in the River Dene is mediated through the Gram-negative degradation pathway, involving the gene *pnpA*. Quantitative PCR was used to determine the number of *pnpA* genes in DNA extracted from biofilm and effluent water samples prior to and following degradation of p-nitrophenol, using approaches described in Kowalczyk et al. (2015). LSD was used to determine significant differences between treatments.

2.6. Microbial community structure

16S rRNA Terminal Restriction Fragment Length Polymorphism (TRFLP) was performed to determine bacterial community structure in T_0 and T_{end} biofilms and effluent water samples. Procedures described in Kowalczyk et al. (2015) were used for PCR, restriction digestion and analysis.

2.7 Bacterial community analyses

Bray Curtis dissimilarity matrices of bacterial communities were generated from TRFLP data, and visualized using non-metric multidimensional scaling (NMDS), with Analysis of Similarities (ANOSIM) used to determine significance of differences between samples (Hilton et al. 2013). These analyses were performed using Primer 6 software V10.1.12 (PRIMER-E Ltd. UK). TRF 245 nt was identified in previous work as a specific marker for *Pseudomonas syringae,* which was linked to degradation of PNP in parallel studies over the same sampling period (Kowalczyk et al. 2015). Relative abundance of TRF 245 nt as a percentage of total fluorescence intensity was determined, and Analysis of Variance used to compare differences across sampling locations and times.

2.8 Analyses of core and satellite taxa

2.8.1 Analysis of the local community

Each biofilm bacterial TRFLP profile was treated as a local community. Rank-abundance plots were used to determine differences in bacterial local community structure for each sampling site and incubation time point (T_0 and T_{end}). Rank-abundance plots were constructed using the relative abundance of each TRF (taxon) The rank-abundance plots were visualized by plotting the taxa rank order on the x-axis against relative abundance ($\log_{10}$ transformed) on the y-axis. For each plot a linear regression model was fitted, represented by the equation, $\log_{10}y = a + bx$, where a is the intercept and b is the slope of the plot. The slope (b) was used as a descriptive statistic to compare community structure (Ager et al. 2010). Linear regressions (r^2) were calculated using Microsoft Excel, and one-way ANOVA, using GenStat13th edition, was used to assess significance between sampling times and locations.

2.8.2 Analysis of the metacommunity

The two-group core-satellite modeling approach (van der Gast et al. 2011) was applied to the bacterial metacommunities to (1) establish whether the PNP-degrading *Pseudomonas syringae* (i.e. TRF 245 nt) was a core or satellite component, (2) determine whether distribution and persistence of *P. syringae* was affected by sampling date, and (3) investigate how relative abundance and distribution of *P. syringae* correlated with PNP biodegradation.

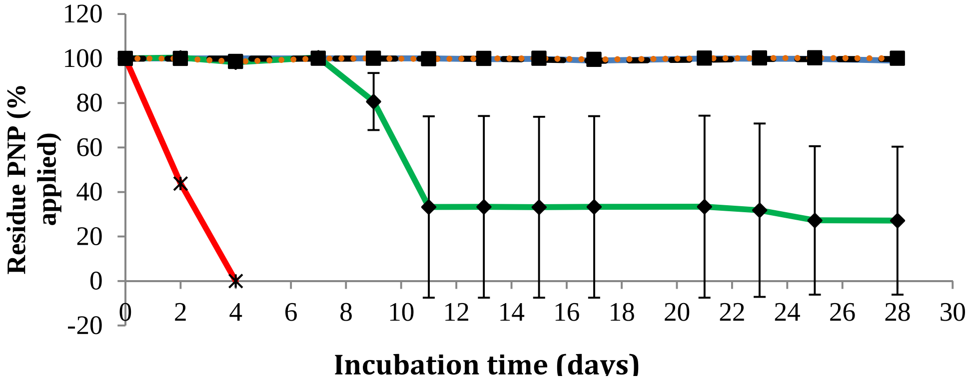
The TRFs in each biofilm metacommunity were divided into core and satellite groups by decomposing the overall distribution using the ratio of variance to the mean abundance for each bacterial taxon (van der Gast et al. 2011). The variance to mean ratio, or index of dispersion, is an index used to model whether species follow a Poisson distribution, falling between 2.5% and 97.5% confidence limits of the χ^2 distribution. Bacterial TRFs that occurred only once in TRFLP profiles were excluded from this analysis, as their dispersion in space would have no variance. Poisson distribution tests were carried out according to the method described by Krebs (1999). Rank-abundance plots with core and satellite taxa were visualized by plotting the taxa rank order on the x-axis against mean % relative abundance on the y-axis with $\log_{10}$ scale (Rogers et al. 2013). An Excel macroprogram was applied to compare the slopes of rank-abundance plots with core and satellite species between metacommunities to determine the significance of differences in metacommunity structure between sampling dates and incubation time points. Regression analysis, coefficients of determination (r^2), residuals and significance (p) were calculated using Minitab software (version 15, Minitab, University Park, PA, USA).

3. Results

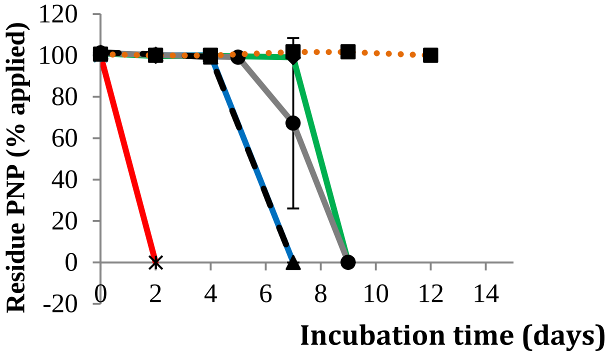
3.1 The biodegradation of PNP

DT50 ranged between 5.5 and 16 days across sampling times and locations. No PNP biodegradation (Figure 1) was observed for upstream and downstream river water biofilms collected in November 2011, while two out of three replicates of effluent biofilm completed PNP biodegradation within nine days (Figure 1). There was a significant difference in DT_{50} between effluent biofilms collected in November 2011 (16.11 days) and February 2012 (8.2 days) (Table 1). There were no significant differences in DT50 between sampling locations in February or May 2012, except effluent water, for which DT50 was significantly faster (6.2 days) in May relative to February 2012 (7.3 days).

a. November 2011



b. February 2012



c. May 2012

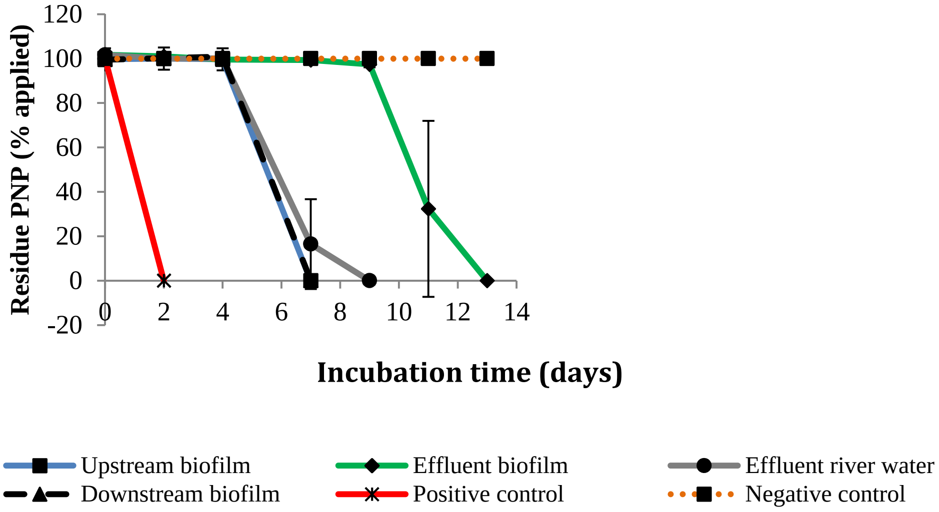


Fig. 1. The biodegradation of PNP in biofilms and river water.

Inoculum collected in a, November 2011; b, February 2012; c, May 2012. Average values from triplicate samples. Error bars show standard error of the mean (S.E.M.), n=3.

Table 1. Time to 50 % biodegradation (DT50) of PNP using biofilm and river water inoculum.

Date	Location	DT_{50}
November 2011	Upstream	ND
	Effluent	16.11(±5.95)^{a}
	Downstream	ND
February 2012	Upstream	5.50(±0.00)^{bc}
	Effluent	8.20(±0.00)^{bc}
	Downstream	5.50(±0.00)^{bc}
	Effluent water	7.30(±0.89)^{c}
May 2012	Upstream	5.63(±0.03)^{bc}
	Effluent	10.83(±0.63)^{ba}
	Downstream	5.70(±0.03)^{bc}
	Effluent water	6.20(±0.40)^{b}

Treatments with different letters are significantly different ($P<0.05$); ± standard error of the mean (S.E.M.); n=3.

3.2 Biofilm biomass

Sampling date and location were found to significantly affect the amount of biofilm that had developed on glass slides (Table 2). Effluent biofilm had significantly lower biomass than upstream and downstream biofilms at all-time points. Biofilms collected in May 2012 had up to twice as much biomass as those collected in November 2011 and February 2012. There was no significant correlation between DT50 for PNP biodegradation and the amount of biofilm biomass on the glass slides at T_0.

Table 2. Biofilm biomass concentrations.

Date	Sampling site	EPS* as glucose equivalent (µg/L)
November 2011	upstream	251[a] (± 3.80)
	effluent	73.2[b] (± 0.63)
	downstream	252[a] (± 5.70)
February 2012	upstream	227[c] (± 2.21)
	effluent	173[d] (± 9.17)
	downstream	134[e] (± 1.90)
May 2012	upstream	668[f] (± 1.26)
	effluent	261[a] (± 0.95)
	downstream	635[g] (± 0.63)

Treatments with different letters are significantly different ($P<0.05$); ± standard error of the mean (S.E.M.), n=3, *EPS: extracellular polysaccharide.

3.3 Biofilm and river water bacterial community

3.3.1 NMDS analysis of TRFLP data

Bacterial TRFLP profiles of river water and river biofilm bacterial profiles collected on different sampling dates and locations were compared before and following degradation of PNP. NMSDS and ANOSIM (Figure 2) revealed that the bacterial community structure of river water and biofilms clustered by sampling date with significantly different (20 % similarity) profiles for each season. There was a significant shift ($p<0.001$) in the community from T_0 to post-PNP degradation (T_{end}), the nature of which was specific to each sampling time. Location of biofilm sampling site had no significant effect on bacterial community profiles and upstream, downstream and effluent biofilms clustered together by sampling time.

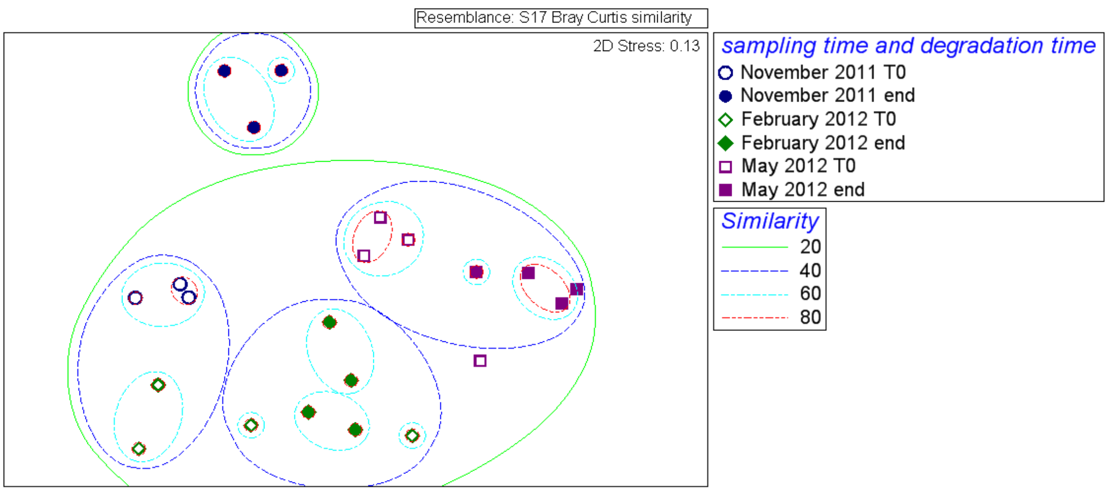


Fig 2. Non-metric multidimensional scaling (NMDS) of bacterial community structure prior to and following degradation of PNP across sampling times Each symbol shows the mean of 3 replicates. T_0, freshly collected biofilm/river water; T_{end}, biofilm/river water after incubation/complete PNP biodegradation.

3.3.2. Quantitative PCR analysis of the *pnpA* gene

There were no significant differences in the copy number of *pnpA* at T_0 or T_{end} between biofilms collected from different sampling points in November (Figure 3), in which degradation occurred only in which 2 of the 3 effluent replicates. However, with the exception of the upstream location in February 2012, there were significant differences in the number of *pnpA* genes between T_0 and T_{end} biofilms and effluent river water collected in February and May, with a 10-100 fold increase following complete PNP degradation. No correlation was found between *pnpA* gene copy numbers and the number of TRFs detected in biofilms.

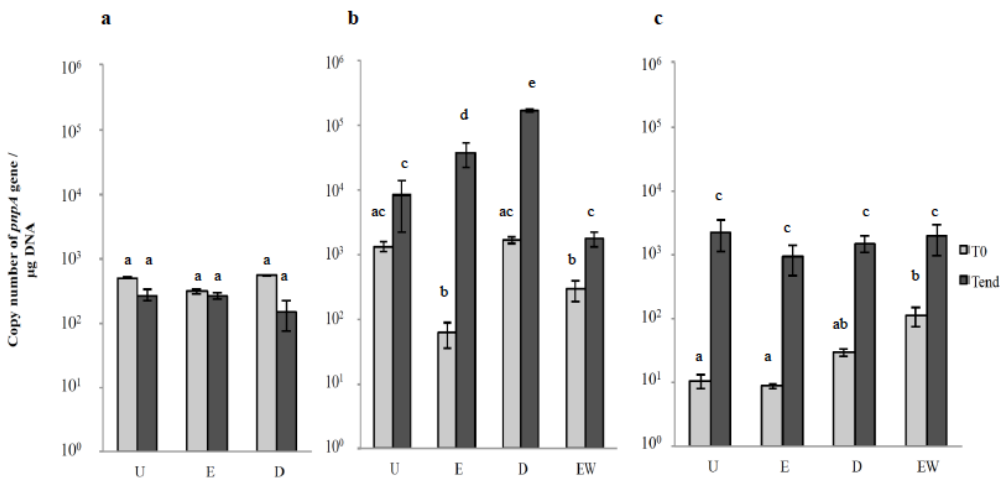


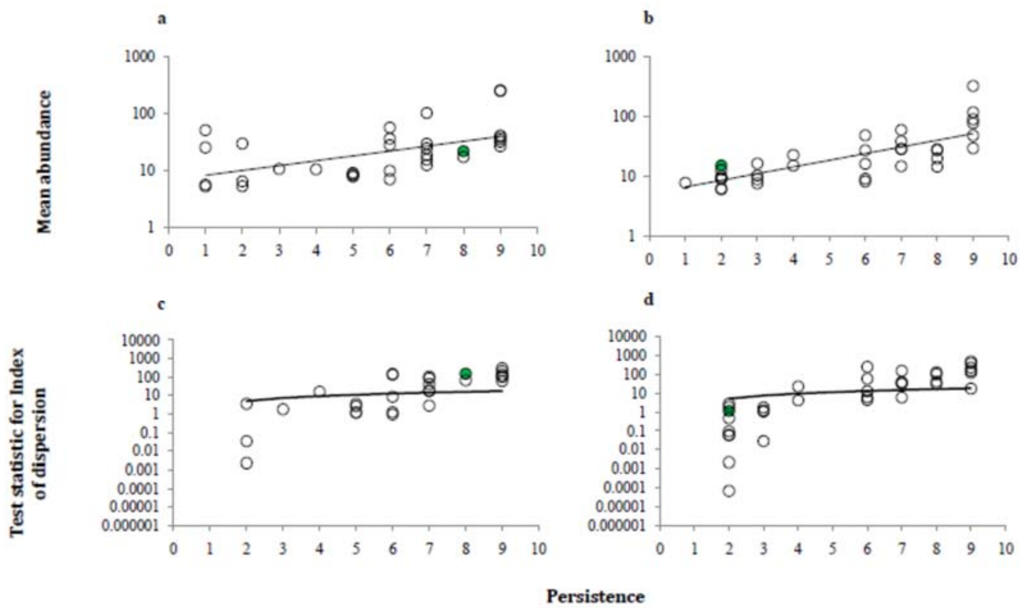
Fig 3. Copy number of *pnpA* genes prior to and following PNP degradation, across sampling locations and times.

Figure legend: a, November 2011; b, February 2012; c, May 2012,U, biofilm upstream; E, effluent biofilm; D, downstream biofilm; EW, effluent river water. Treatments with different letters are significantly different ($P<0.05$); error bars show ± standard error of the mean (S.E.M.), n=3.

3.4 Core and satellite taxa

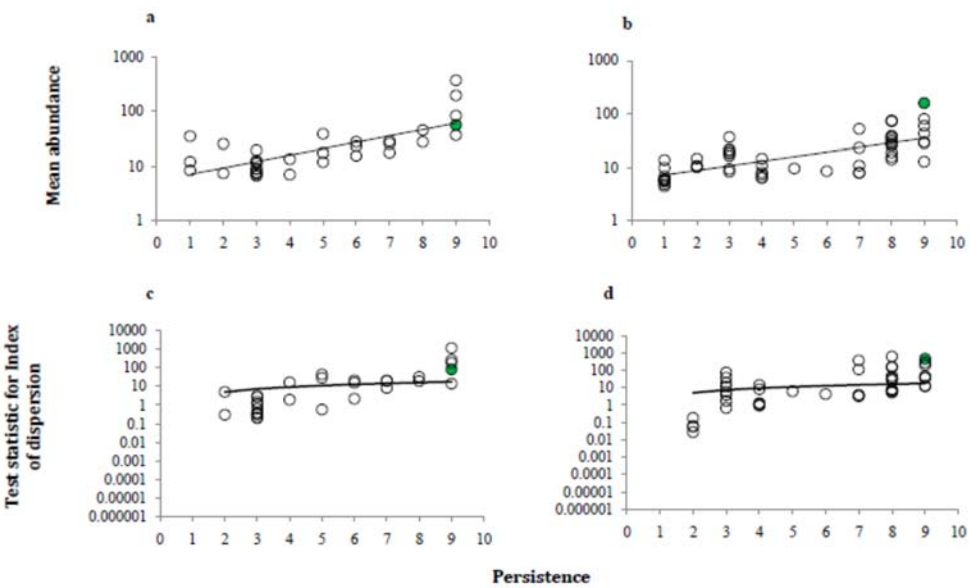
3.4.1 Biofilm metacommunity structure, distribution and persistence of *Pseudomonas syringae*

After dispersion was plotted against the persistence of taxa for biofilm metacommunities before and after degradation (SI Figure 1-3 a and b), Poisson distributions were fitted to identify core and satellite taxa (SI Figure 1-3 c and d). Since there was no significant difference in community composition by sampling location, upstream, downstream and effluent T_0 or T_{end} biofilms were pooled together by sampling time for this analysis. The taxa which fell below the 2.5% confidence limit line were identified as randomly distributed satellite species. Taxa above the 2.5% confidence limit line were considered non-randomly distributed core species. It appeared that *P. syringae* was a core taxon at T_0 in the November and February biofilm metacommunities but was a satellite taxon in the May T_0 metacommunity (Fig 4). After the biodegradation of PNP (T_{end}) *P. syringae* remained a core taxon in February 2012, but became the most abundant taxon, and similarly in May it increased enormously in relative abundance following biodegradation, to become the second highest taxon by relative abundance. In November 2011, the persistence of *P. syringae* decreased after incubation with PNP and shifted from being a core to a satellite taxon.



Supplement Fig. 1. Distribution and dispersal of bacterial taxa in biofilm communities in November 2011.

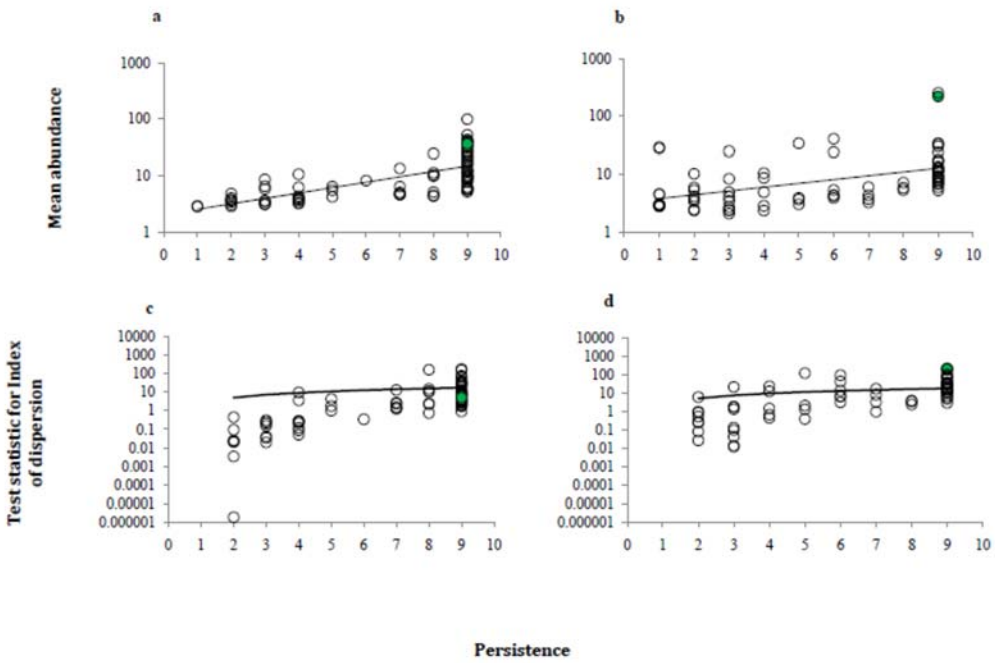
The number of biofilm TRFLP profiles for which each bacterial TRF (taxa) was observed, plotted against the mean abundance ($\log_{10}$ scale) across all TRFLP profiles a, before incubation (T_0) (r^2= 0.29; $F_{1,32}$=12.57 ; P<0.0001), and b, after complete PNP biodegradation (T_{end}) (r^2= 0.60; $F_{1,35}$=51.57 ; P<0.0001). c, d random and non-random dispersal of TRFs from biofilm TRFLP profiles before and following PNP degradation respectively (*P. syringae* highlighted in green). Dispersal visualised by decomposing the overall distribution using an index of dispersion based on the ratio of variance to the mean abundance for each bacterial TRF from analysed 9 TRFLP profiles. The line depicts the 2.5% confidence limit for the χ^2 distribution. The 97.5% confidence limit was not plotted, as no taxon fell below that line.



Supplement Fig 2. Distribution and dispersal of bacterial taxa in biofilm communities in February 2012.

The number of biofilm TRFLP profiles for which each bacterial TRF (taxa) was observed, plotted against the mean abundance (log_{10} scale) across all TRFLP profiles a, before incubation (T_0) (r^2= 0.55; $F_{1,32}$=37.52 ; P<0.0001), and b, after complete PNP biodegradation (T_{end}) (r^2= 0.51; $F_{1,54}$=54.91;

P<0.0001). c, d random and non-random dispersal of TRFs from biofilm TRFLP profiles before and following PNP degradation respectively (*P. syringae* highlighted in green). Dispersal visualised by decomposing the overall distribution using an index of dispersion based on the ratio of variance to the mean abundance for each bacterial TRF from analysed 9 TRFLP profiles. The line depicts the 2.5% confidence limit for the χ^2 distribution. The 97.5% confidence limit was not plotted, as no taxon fell below that line.



Supplement Fig 3. Distribution and dispersal of bacterial taxa in biofilm communities in May 2012.

The number of biofilm TRFLP profiles for which each bacterial TRF (taxa) was observed, plotted against the mean abundance ($\log_{10}$ scale) across all TRFLP profiles a, before incubation (T_0) (r^2= 0.50; $F_{1,80}$=78.10 ; P<0.0001), and b, after complete PNP biodegradation (T_{end}) (r^2= 0.23; $F_{1,76}$=22.04 ; P<0.0001). c, d random and non-random dispersal of TRFs from biofilm TRFLP profiles before and following PNP degradation respectively (*P. syringae* highlighted in green). Dispersal visualised by decomposing the overall distribution using an index of dispersion based on the ratio of variance to the mean abundance for each bacterial TRF from analysed 9 TRFLP profiles. The line depicts the 2.5% confidence limit for the χ^2 distribution. The 97.5% confidence limit was not plotted, as no taxon fell below that line.

Analysis of slopes of rank-abundance plots (SI Table 1, Figure 4) revealed significant differences (p<0.001) between the T_0 and T_{end} metacommunities. Shifts in the November and February metacommunities were observed with increasing slopes between T_0 and T_{end} from b=-0.0426 to b=-0.0356 (p<0.0001) in November, and from b=-0.0386 to b=-0.0221 (p<0.0001) in February. This indicates a more even community structure after PNP biodegradation. However, in May the biofilm metacommunity became more heterogeneous since the slope changed between T_0 and T_{end} from b=-0.0155 to b=-0.0177 (p<0.0001). The data indicate increased relative abundance of *P. syringae* following degradation of PNP in February and May biofilms, but not in those from November.

Supplement Table 1. Comparison of slopes of metacommunity rank-abundance plots prior to and following PNP degradation.

Rank-abundance plot	*F*-value	Slope (*b*)	*P*-value	r^2
November T_0	$F_{1,31}$=315.78	-0.0426[a]	<0.0001	0.91
November T_{end}	$F_{1,34}$=266.61	-0.0356[b]	<0.0001	0.89
February T_0	$F_{1,31}$= 165.96	-0.0386[ac]	<0.0001	0.84
February T_{end}	$F_{1,53}$=1176.32	-0.0221[d]	<0.0001	0.96
May T_0	$F_{1,79}$=1362.73	-0.0155[e]	<0.0001	0.95
May T_{end}	$F_{1,75}$ =452.11	-0.0177[f]	<0.0001	0.86

Treatments with different letters are significantly different (P<0.05); n=3.

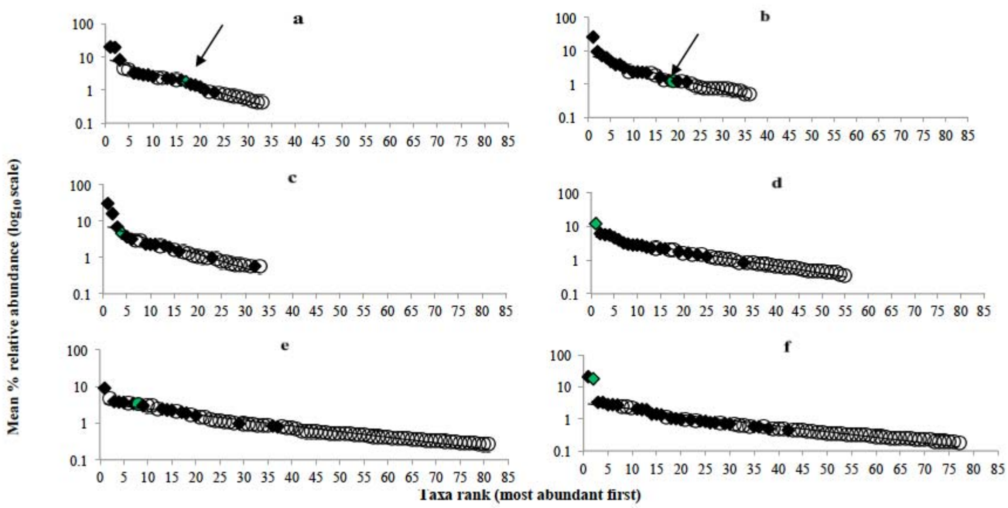


Fig 4. Rank-abundance plots of core and satellite taxa in biofilms at prior to and following PNP degradation at different sampling times

Figure legend: a, and b are November T_0 and T_{end}; c, d are February T_0 and T_{end}; e and f are May T_0 and T_{end}. T_0, freshly collected biofilms; T_{end}, biofilms after complete PNP biodegradation. The relative rank positions of core group (closed diamonds), satellite group (open circles) and *Pseudomonas syringae* (highlighted in green) are given. Each plot has been fitted with a slope.

4. Discussion

In the current study we have demonstrated that the major factor determining microbial community composition of *in situ* biofilms and river water was sampling time, with minor differences between sampling locations. Biofilms taken at different sampling times had different potential for chemical biodegradation, with higher biodegradability potential observed for inocula collected in February and May 2012 in comparison with inocula sampled in November 2011. Significantly, differences in PNP degradation between sampling locations were identified in November 2011, with 2 of the 3 locations showing no potential for biodegradation.

River biofilm communities are known to show considerable spatial and temporal variation associated with seasonal variation in water characteristics (Anderson-Glenna et al., 2008). Brümmer et al. (2000) reported clear seasonal peaks of abundance among major phylogenetic groups of bacteria in a polluted river over an annual cycle. Similarly, Böckelmann et al. (2000) described a reduction of metabolic activity of microbial communities of aggregates of phototrophic and heterotrophic microorganisms in the Elbe

River, from summer (August) to winter (December) for both, free-living and surface associated bacterial populations. Seasonal changes in biofilm community structure and function may have implications for chemical biodegradation. Palmisano et al. (1991) found lower first-order rate constants for 2,4D mineralization in river biofilms collected in the winter relative to the summer. Furthermore, Chénier et al. (2003) showed that hexadecane biodegradation potential of biofilm grown in bioreactors from river water inoculum depended on season. River water collected in autumn generated biofilm with greater mineralization potential than material from other seasons.

Variability in the biomass and diversity of inocula used in OECD biodegradability tests has been identified as the main reason for test failures and the inconsistency of test results (Courtes et al. 1995; Mezzanotte et al. 2005). Efforts have been made to standardize microbial inocula prior to biodegradation testing (e.g. increasing microbial biomass via filtration or centrifugation) and several pre-adaptation strategies have been developed (e.g. semi-continuous pre-exposure procedure) (Toräng and Nyholm, 2005). Although increases in inoculum biomass and pre-adaptation of inocula is known to increase the probability of chemical degradation, inoculation with high amounts of microbial biomass may still fail to degrade the chemical (Kool, 1984; Szabó et al. 2007; Thouand et al. 2011).

Com

A ‘biodegradation lottery’ has been suggested by several authors as the main reason for test failures, due to uneven distribution of specific degraders within aliquots of inoculum used in biodegradation tests (Thouand et al. 2011). Alternatively, microbe-microbe interactions which prevent proliferation of a degrader could also contribute to test failures. In our study, *pnpA* genes, which encode 4-nitrophenol 4 monooxygenase, the first enzyme in the Gram-negative pathway for PNP degradation, were found in all samples, including those from November 2011, in which no degradation of PNP was detected. P. syringae was previously reported by (Kowalczyk et al. 2015) as a PNP degrader. Sequencing of both 16S rRNA genes and *pnpA* genes indicated that PNP degraders present in river water samples belong to *Pseudomonas* spp. Similarly, community analysis indicated that a ribotype indicative of *P. syringae,* identified as the key PNP degrader in samples from this river in earlier work (Kowalczyk et al. 2015) was present in all samples irrespective of whether they subsequently degraded PNP.

Test failure arising from a biodegradation lottery because of an uneven distribution of degraders between biodegradation tests would be indicative of degraders showing low abundance and persistence, and thereby being members of the satellite community, However, our results show that the *P. syringae* ribotype was a satellite taxon in February 2012, yet proliferated across all samples following PNP degradation to become the most abundant taxon. In contrast, in November 2011 and May 2012, the *P. syringae* ribotype was a core component of the community, yet it only proliferated in the May samples, with degradation rates across all locations similar to the February samples. However, despite being a core community component in the November 2011 samples, it's taxa rank was 18, compared to 1 and 2 in the February and May 2012 samples, respectively.

Differences in biodegradation of PNP by biofilms at different time points could be due to a variety of mechanisms. Failure of PNP degraders to proliferate could result from microbe-microbe interactions, which may be competitive or synergistic. For example, the persistence of specific degraders in the community may require the presence of other bacterial species to provide cofactors required for growth (Bending et al., 2003). Therefore, microbial interactions within inoculum could determine the proliferation of specific degraders in microbial populations thereby impacting the outcome of biodegradation tests. McGenity et al. (2012) reported that a network of direct and indirect interactions within and between species is observed in the presence of hydrocarbons in marine environment. Some of the key cooperative interactions may include consumption of metabolites, biosurfactant production, provision of oxygen and fixed nitrogen. Microbial consortia are known to have better ability to degrade complex pollutants than single strains (Hoskeri et al. 2014; Rahman et al. 2002). Greater resolution of the biofilm composition and organisation such as studying matrix composition, viable cell number and localization of degraders (Pantanella et al. 2013) could help explain seasonal differences in biodegradation potential.

However, in the case of degradation of PNP, which is typically a readily degraded chemical, no such synergisms have been identified (Kowalczyk et al. 2015), suggesting that competitive interactions could have contributed to test failures.

Approaches to profile complex microbial communities continue to evolve, and metagenomic, metaproteomic and metatranscriptomic tools are becoming increasingly accessible. They allow detection of unculturable microbes and link their presence to environmental processes, including chemical degradation, without biases associated with PCR primers (Fang et al. 2013; Kowalczyk et al. 2015).

In conclusion, river collected biofilms showed considerable seasonal variation in biomass, microbial community composition and PNP biodegradation potential, and there was also evidence that location at which the biofilm was grown affected biodegradation outcome. These characteristics may limit the usefulness of field grown biofilms as inocula for biodegradation tests. However, our data show that biodegradation test failures could arise from microbe-microbe interactions rather than a failure of inocula to contain specific degraders or genetic potential for biodegradation.

Acknowledgements

We thank the Safety and Environmental Assurance Centre, UK, Unilever for the financial support of this study.

References

Ager, D., Evans, S., Li, H., Lilley, A.K., van der Gast, C.J., 2010. Anthropogenic disturbance affects the structure of bacterial communities. Environ. Microbiol., 12, 670-678.

Bending, G. D., Lincoln, S.D., Sørensen, S.R., Morgan, J.A.W., Aamand, J. Walker, A. 2003. In-field spatial variability in the degradation of the phenyl-urea herbicide isoproturon is the result of interactions between degradative *Sphingomonas* spp. and soil pH. Appl. Environ. Microbiol. 69**,** 827-834.

Bertram, D.F., Mackas, D.L., McKinnell, S.M., 2001. The seasonal cycle revisited: interannual variation and ecosystem consequences. Prog. Oceanogr. 49: 283-307.

Böckelmann, U., Manz, W., Neu, T.R., Szewzyk, U., 2000. Characterization of the microbial community of lotic organic aggregates ('river snow') in the Elbe River of Germany by cultivation and molecular methods. FEMS Microbiol. Ecol. 33, 157-170.

Brümmer, I.H.M., Fehr, W., Wagner-Döbler, I., 2000. Biofilm community structure in polluted rivers: abundance of dominant phylogenetic groups over a complete annual cycle. Appl. Environ. Microbiol. 66, 3078-3082.

Cébron, A., Coci, M., Garnier, J., Laanbroek, H.J., 2007. Denaturing gradient gel electrophoretic analysis of ammonia-oxidizing bacterial community structure in the lower Seine River: impact of Paris wastewater effluents. Appl. Environ. Microbiol. 70, 6726-6737..

Chénier, M.R., Beaumier, D., Roy, R., Driscoll, B.T., Lawrence, J.R., Greer, C.W., 2003. Impact of seasonal variations and nutrient inputs on nitrogen cycling and degradation of hexadecane by replicated river biofilms. Appl. Environ. Microbiol. 69, 5170-5177.

Courtes, R., Bahlaoui, A., Rambaud, A., Deschamps, F., Sunde, E., Dutrieux, E., 1995. Ready biodegradability test in seawater: a new methodological approach. Ecotox. Environ. Safe. 31, 142-148.

Deines, P., Sekar, R., Husband, P.S., Boxall, J.B., Osborn, A.M. and Biggs, C.A., 2010. A new coupon design for simultaneous analysis of in situ microbial biofilm formation and community structure in drinking water distribution systems. Appl. Microbiol. Biotechnol., 87, 749-756.

Doi, Y., Kasuya, K., Abe, H., Koyama, N., Ishiwatari, S., Takagi, K., Yoshida, Y., 1996. Evaluation of biodegradabilities of biosynthetic and chemosynthetic polyesters in river water. Pol. Deg. Stab. 51, 281-286.

Donlan, R.M. 2002. Biofilms: microbial life on surfaces. Emerg Infect Dis. 8, 881-890.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356.

Elias, S., Banin, E., 2012. Multi-species biofilms: living with friendly neighbors. FEMS Microbiol. Rev. 35, 990-1004.

Fang, H., Cai, L., Yu, Y., Zhang, T., 2013. Metagenomic analysis reveals the prevalence of biodegradation genes for organic pollutants in activated sludge. Biores. Technol. 129, 209-218.

Flemming H.-C., Neu, T.R., Wozniak, D.J., 2007. The EPS matrix: The “House of Biofilm Cells”. J. Bacteriol. 189, 7945-7947.

Godhead, A.K., Head, I.M., Snape, J.R., Davenport, R.J., 2014. Standard inocula preparations reduce the bacterial diversity and reliability of regulatory biodegradation tests. Environ. Sci. Pollut. Res. 21, 9511-9521.

Hilton, S., Bennett, A.J., Keane, G., Bending, G.D., 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.

Hoskeri, R.S., Mulla, S.I., Ninnekar, H.Z., 2014. Biodegradation of chloroaromatic pollutants by bacterial consortium immobilized in polyurethane foam and other matrices. Biocat. Agri. Biotechnol. 3, 390-396.

Hudon, C., 1997. Impact of water level fluctuations on St. Lawrence River aquatic vegetation. Can. J. Fish. Aquat. Sci. 54, 2853-2865.

Hunt, A.P., Parry, J.D., 1998. The effect of substratum roughness and river flow rate on the development of a freshwater biofilm community. Biofouling 12, 287-303.

Jones, O.A.H., Voulvoulis, N., Lester, J.N., 2003. Potential impact of pharmaceuticals on environmental health. Bull. World. Health. Organ. 81.
http://dx.doi.org/10.1590/S0042-96862003001000015

Kargi, F., Eker, S., 2005. Removal of 2,4-dichlorophenol and toxicity from synthetic wastewater in a rotating perforated tube biofilm reactor. Proc. Biochem. 40, 2105-2111.

Kool, H.J. 1984. Influence of microbial biomass on the biodegradability of organic compounds. Chemosphere 7:751-761.

Kowalczyk, A., Martin, T.J., Price, O.R., Snape, J.R., van Egmond, R.A., Finnegan, C.J., Schäfer, H., Devenport, R.J., Bending, G.D., 2014.

Refinement of biodegradation tests methodologies and the proposed utility of new microbial ecology techniques. Ecotox. Environ. Safety. 111, 9-22.

Kowalczyk, A., Eyice, Ö., Schäfer, H., Price, O.R., Finnegan, C.J., van Egmond, R.A., Shaw, L.J., Barrett, G. and Bending, G.D. 2015. Characterization of *para*-nitrophenol degrading bacterial communities in river water using functional markers and stable isotope probing. Appl. Environ. Microbiol. doi: 10.1128/AEM.01794-15.

Krebs, C.J., 1999. Ecological Methodology 2nd edn. Harper and Row: New York, NY.

LaPara, T.M., Konopka, A., Nakatsu, C.H., Alleman, J.E., 2000. Effects of elevated temperature on bacterial community structure and function in bioreactors treating a synthetic wastewater. J. Ind. Microbiol. Biotech. 24, 140-145.

Lapertot, M.E., Pulgarin, C., 2006. Biodegradability assessment of several priority hazardous substances: Choice, application and relevance regarding toxicity and bacterial activity. Chemosphere, 65, 682-690.

Mah, T.F.C., O'Toole, G.A., 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 9, 34-39.

McGenity, T., Folwell, D., McKew, B.A., Sanni, G.O., 2012. Marine crude-oil biodegradation: a central role for interspecies interactions. Aquat. Biosys. 8,10.

Mezzanotte, V., Bertani, R., Innocenti, F.D., Tosin, M., 2005. Influence of inocula on the results of biodegradation tests. Polym. Deg. Stab. 87, 51-56.

Midwood, J.D., Chow-Fraser, P., 2012. Changes in aquatic vegetation and fish communities following 5 years of sustained low water levels in coastal marshes of eastern Georgian Bay, Lake Huron. Global Change Biol. 18, 93-105.

Mnif, W., Hassine, A.I.H., Bouaziz, A., Bartegi, A., Thomas, O., Roig, B., 2011. Effect of endocrine disruptor pesticides: a review. Int. J. Environ. Res. Public Health. 8, 2265–2303.

Morin, S., Pesce, S., Tlili, A., Coste, M., Montuelle, B., 2007. Recovery potential of periphytic communities in a river impacted by a vineyard watershed. Ecol. Indicators 10, 419-426.

Naudin, J.J., Cauwet, G., Fajon, C., Oriol, L., Terzić, S., Devenon, J.L., Broche, P., 2001. Effect of mixing on microbial communities in the Rhone River plume. J. Marine Sys. 28, 203-227.

Nesbit, H.J., Watson, J.R., 1980. Degradation of the herbicide 2,4-D in river water-II. The role of suspended sediment, nutrients and water temperature. Wat. Res. 14, 1689-1694.

OECD Guideline for testing of chemicals, 2005. Proposal for revised introduction to the OECD Guidelines for testing of chemicals, section 3. Annex 1. ENV/JM/TG(2005)5/REV1.

Oh, K.H., Tuovinen, O.H., 1994. Biodegradation of the phenoxy herbicides MCPP and 2,4-D in fixed-film column reactors. Int. Biodet. Biodeg. 33, 93-99.

Palmisano, A.C., Schwab, B.S., Maruscik, D.A., Ventullo, M.R., 1991. Seasonal changes in mineralization of xenobiotics by stream microbial communities. Can. J. Microbiol. 37, 939-948.

Pantanella, F., Valenti, P., Natalizi, T., Passeri, D., Berlutti, F., 2013.

Analytical techniques to study microbial biofilm on abiotic surfaces: pros and cons of the main techniques currently in use. Ann. Ig. 25, 31-42.

Proia, L., Morin, S, Peipoch, M., Romaní, A.M., Sabater, S., 2010. Resistance and recovery of river biofilms receiving short pulses of triclosan and diuron. Sci. Tot. Environ. 409, 3129-3137.

Puhakka, J.A., Herwig, R.P., Koro, P.M., Wolfe, G.V., Ferguson, J.F., 1995. Biodegradation of chlorophenols by mixed and pure cultures from a fluidized-bed reactor. Appl. Micro. Biotech. 42, 951-957.

Rahman, K.S.M., Thahira-Rahman, J., Lakshmanaperumalsamy, P., Banat, I.M., 2002. Towards efficient crude oil degradation by a mixed bacterial consortium. Biores. Technol. 85, 257-261.

Reuschenbach, P., Pagga, U., Strotmann, U., 2003. A critical comparison of respirometric biodegradation tests based on OECD 301 and related test methods. Wat. Res. 37, 1571-1582.

Rodriguez-Cruz, M.S., Jones, J.E., Bending, G.D., 2006. Field-scale study of the variability in pesticide biodegradation with soil depth and its relationship with soil characteristics. Soil. Biol. Biochem. 38, 2910-2918.

Rogers, G.B., Cuthbertson, L., Hoffman, L.R., Wing, P.A.C., Pope, C., Hooftman, D.A.P., Lilley, A.K., Oliver, A., Carroll, M.P., Bruce, K.D. and van der Gast, C.J., 2013. Reducing bias in bacterial community analysis of lower respiratory infections. The ISME Journal, 7, 697-706.

Romaní, A.M., Sabater, S., 1999. Effect of primary producers on the heterotrophic metabolism of a stream biofilm. Freshw. Biol. 41, 729-736.

Sabater, S., Guasch, H., Ricart, M., Romaní, A., Vidal, G., Klünder, C., Schmitt-Jansen, M., 2007. Monitoring the effect of chemicals on biological communities. The biofilm as an interface. Anal. Bioanal. Chem. 387, 1425-1434.

Schwartz, T., Kohnen, W., Jansen, B., Obst, U., 2003. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. FEMS Microbiol. Ecol. DOI: http://dx.doi.org/10.1111/j.1574-6941.2003.tb01073.x 325-335.

Singh, R., Paul, D., Jain, R.K., 2006. Biofilms: implications in bioremendiation. Trends Microbiol. 14, 389-397.

Szabó, K.E., Itor, P.O.B., Bertilsson, S., Tranvik, L., Eiler, A., 2007. Importance of rare and abundant population for the structure and functional potential of freshwater bacterial communities. Aquatic Microbial Ecol. 47,1-10.

Thouand, G., Durand, M.J., Gancet, C., Blok, H., 2011. New concepts in the evaluation of biodegradation/persistence of chemical substances using a microbial inoculum. Front. Microbiol. 2, 1-6.

Thouand, G., Friant, P., Bois, F., Cartier, A., Maul, A., and Block, J.C., 1995. Bacterial inoculum density and probability of para-nitrophenol biodegradability test response. Ecotox. Environ. Safe. 30, 274-282.

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

Underwood, G.J.C., Paterson, D.M., 1995. The measurement of microbial carbohydrate exopolymers from intertidal sediments. Limnol. Oceanogr. 40, 1243-1253.

van der Gast, C.J., Walker, A.W., Stressmann, F.A., Rogers, G.B., Scott, P., Daniels, T.W., Carroll, M.P., Parkhill, J., Bruce, K.D., 2011. Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. The ISME J. 5, 780-791.

Watson, J.R., 1977. Seasonal-variation in biodegradation of 2,4-D in river water. Wat. Res., 11, 153-157.

Yuan, S.Y., Yu, C.H., Chang, B.V., 2004. Biodegradation of nonylphenol in river sediment. Environ. Poll. 127, 425-430.

Yunus, A.J.M., Nakagoshi, N., 2004. Effects of seasonality on streamflow and water quality of the Pinang River in Penang Island, Malaysia. Chinese Geogr. Sci. 14, 153-161.

Zhang, T.C., Fu, Y.C., Bishop, P.L., Kupferle, M., FitzGerald, S., Jiang, H.H., Harmer, C., 1995. Transport and biodegradation of toxic organics in