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Colonization of contaminated soil by an introduced bacterium: effects of initial pentachlorophenol levels on the survival of *Sphingomonas chlorophenolica* strain RA2

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The survival of a *Sphingomonas* species that was introduced into pentachlorophenol (PCP)-contaminated soil was monitored with two complementary methods, a respiration-based assay and a most probable number (MPN) technique. *Sphingomonas chlorophenolica* strain RA2 is a PCP-mineralizing bacterium that was introduced into soil contaminated with a range of PCP concentrations (0–300 μg PCP g^{-1} soil). The population of introduced microorganisms was followed for 170 days using a substrate-induced growth-response method and a MPN assay that specifically targets PCP-mineralizing bacteria. Varying the initial PCP concentration resulted in the emergence of three distinct patterns of survival. In soil contaminated with 300 μg PCP g^{-1} the population of *S. chlorophenolica* strain RA2 immediately declined following introduction, increased by 200-fold and leveled off by the end of the 170-day incubation. In contrast, populations of *S. chlorophenolica* strain RA2 declined to levels below detection limits in uncontaminated soil by the end of the experiment. Intermediate PCP concentrations (10–100 μg PCP g^{-1} soil) resulted in the establishment of *S. chlorophenolica* strain RA2 that slowly declined in numbers. These results indicate that *Sphingomonas chlorophenolica* strain RA2 is an effective colonizer of PCP-contaminated soil but will not persist in the absence of PCP.

Keywords: *Sphingomonas*; pentachlorophenol; bioaugmentation; microbial survival

Introduction

The introduction of non-native species into environments has been occurring naturally over time with results ranging from no establishment to formidable change [25]. In cases where non-indigenous microorganisms are introduced into natural environments, the effects are typically benign with regard to microbially-mediated processes [2,8,10,27,28]. White *et al* [29] found that the introduction of a *lux*-modified *Pseudomonas fluorescens* to soil facilitated a transient decrease (up to 15 days) in soil microbial diversity. This decrease was due to a temporary suppression of a few underrepresented species immediately following inoculation with the *P. fluorescens*, a denitrifier. Senoo *et al* [24] compared the survival patterns of γ-hexachlorocyclohexane (HCH)-degrading *Sphingomonas paucimobilis* SS86 as an indigenous species in soil and as an introduced species in the same type of soil, but where it did not occur naturally. When the bacterium was inoculated into γ-HCH contaminated soil, its numbers declined rapidly after depletion of the contaminant. In contrast, in experiments where the microorganism was indigenous to the soil, the population remained constant regardless of the amount of contaminant. The mechanisms of survival were attributed to the indigenous species being able to inhabit soil micropores where it was less susceptible to predation, dessication and removal by leaching. Senoo *et al* [24] demonstrated that although the autecology of an indigenous species may be well characterized, it can not always be used to predict the behavior of the organism as an introduced species. Even so, the disappearance of a microorganism after a pollutant is removed is a desirable trait for a bacterium introduced to specifically remove a contaminant.

Sphingomonas chlorophenolica strain RA2 is a bacterium that degrades high concentrations of pentachlorophenol in soil [5]. Initially described as *Pseudomonas* sp strain RA2 [19], this organism was later shown to belong to the genus *Sphingomonas* [22] and its phylogenetic relation to other PCP-degrading *Sphingomonas* species has also been elucidated [9,12,17]. Experiments with *S. chlorophenolica* strain RA2 indicates that it survives for several months in PCP-contaminated soil [4,15,26] yet little is known about the population dynamics or the effects of varying PCP concentrations on the survival patterns of *S. chlorophenolica* strain RA2. This study was undertaken to investigate how the initial concentration of a toxic compound affects the survival of a bacterium specifically introduced to degrade that compound.

Materials and methods

Soil incubations

The soil used in these experiments was collected from around Boulder Creek near the University of Colorado campus. This soil is a sandy loam with 5% organic matter and a pH of 7.0. Additional soil characteristics have been given by Schmidt and Gier [23] and Colores *et al* [5,6]. Following collection, the soil was sieved (2 mm) and stored at 4°C until use. Prior to the beginning of this incubation

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20 or 30 g soil (dry weight) was added to 4 oz-polyethylene specimen cups (Fisher Scientific Co, Pittsburgh, PA, USA). The contaminated treatments had reagent grade PCP (Fluka Chemical Co, Ronkonkoma, NY, USA) added at concentrations of 0, 10, 50, 100 or 300 μg g^{-1} soil (ppm). Half of the contaminated treatments (remediated soil) had *S. chlorophenolica* strain RA2 added while the uninoculated treatments had an equivalent volume of sterile mineral salts medium added. For the remediated treatments, early stationary phase cells were added to PCP-contaminated soil at an inoculum level of approximately 2×10^7 cells g^{-1} soil. A working stock of *S. chlorophenolica* strain RA2 was maintained in a mineral salts medium containing 3.6 g Na_2HPO_4, 0.76 g KH_2PO_4, 0.1 g NH_4NO_3, 0.01 g $MgSO_4 \cdot 7\ H_2O$, 0.005 g $CaCl_2 \cdot 7\ H_2O$ and 0.2 g $FeSO_4 \cdot 7\ H_2O$ per liter and PCP at a concentration of 300 ppm. The medium was adjusted to a pH of 7.5. The inoculum, mineral salts medium and/or PCP were added in enough sterile water to obtain a final water content equivalent to 70% of gravimetric water-holding capacity at the onset of the incubation. Lids were loosely replaced to minimize water loss. Each treatment was weighed periodically throughout the incubation and any weight loss was compensated by addition of sterile water. All incubations were carried out at $22.5 \pm 1°$ C.

Enumeration with a Most Probable Number assay

A Most Probable Number (MPN) assay was used to quantify the population of *S. chlorophenolica* strain RA2. At each sampling time, individual replicates were sacrificed and 5 g soil from each of the 10 different treatments was serially diluted in a $MgSO_4$ solution (1 g L^{-1}). Fifty-microliter aliquots of each dilution were added to eight wells of a 96-well assay plate (Rainin Instrument Co, Woburn, MA, USA). Each well contained 150 μl of a low-buffered bromothymol blue medium containing 100 ppm PCP as the sole carbon source [20]. The sides of each plate were wrapped with Parafilm (American National Can, Chicago, IL, USA) and the plates were left to incubate in the dark for 30 days at 22.5 ± 1°C. After 30 days, the wells that changed from blue to yellow (due to the production of HCl) and had a pellet at the bottom were scored as positive for PCP mineralization. A BASIC computer program was used to calculate the MPN of PCP degraders and 95% confidence intervals. Wells along the outside edge of each plate were not scored due to evaporation of the medium causing false positive reactions.

Biomass determination using a substrate-induced growth-response method

Treatments that were contaminated with 10 and 300 ppm PCP were subjected to population estimates using a substrate-induced growth-response (SIGR) method [6,21] in addition to the MPN procedure. Two replicates were sacrificed at each sampling date and 20 g soil (dry weight) was used for SIGRs. The soil samples were added to a total of four 250-ml biometer flasks. In addition to the amended soil samples, uncontaminated soil was added to separate flasks to check for indigenous PCP mineralization. Both unlabeled and radiolabeled PCP (uniformly ^{14}C-labeled, 11.9 mCi $mmol^{-1}$, Sigma Chemical Co, St Louis, MO, USA) were added to achieve a final PCP concentration of 100 ppm and a target activity of 80 000 dpm per flask. Evolved $^{14}CO_2$ was captured in 1.0 ml of 0.5 N NaOH contained in the side arm of each biometer flask. The NaOH was added to 2.5 ml of ScintiVerse II scintillation cocktail (Fisher Scientific Co) in 4-ml Omni vials (Wheaton Industries, Millville, NJ, USA) and the radioactivity was counted with a liquid scintillation counter (LKB Wallac, 1209 RackBeta, Turku, Finland) with an average counting efficiency of 96%.

Population estimates in terms of biomass-C (X_a) were determined using the procedure outlined by Colores *et al* [6]. PCP mineralization data were fitted to the equation:

$$dp/dt = \mu(X_1 \exp(\mu t)) \quad (1)$$

in which dp/dt (change in product over change in time) is the rate of $^{14}CO_2$ production (μg C-CO_2 h^{-1}), μ is the specific growth rate (h^{-1}) at a given PCP concentration and X_1 is the active population of *S. chlorophenolica* strain RA2 with units μg C-CO_2. The parameter X_1 was converted to biomass of active PCP-degraders (X_a) with the expression:

$$X_a = X_1(Y_c/1 - Y_c) \quad (2)$$

where X_a is an estimate of biomass of PCP-mineralizers with units μg C-biomass, Y_c is the yield coefficient with units of μg C-biomass μg C-substrate^{-1} and $1 - Y_c$ is the amount of product produced per unit of substrate consumed in μg C-CO_2 μg C-substrate^{-1}. The value used for Y_c was 0.2 μg C-CO_2 μg C-substrate^{-1} and was empirically determined from experiments done with pure cultures of *S. chlorophenolica* strain RA2 [19]. In order to directly compare population estimates derived by the SIGR and MPN methods, the biomass of *S. chlorophenolica* strain RA2 was converted to cell numbers using the conversion factor:

$$0.1\ \mu\text{g biomass-C} = 1.76 \times 10^6 \text{ cells of } \textit{S. chlorophenolica} \text{ strain RA2} \quad (3)$$

based on experiments with pure cultures of *S. chlorophenolica* strain RA2 [18]. All curve fitting and data analyses were carried out using Kaleidagraph 3.0.2 (Synergy Software, Reading, PA, USA).

Results

MPN-derived population estimates

The MPN assay for determining the number of PCP-degraders in soil was very sensitive to *S. chlorophenolica* strain RA2 population fluxes. The detection limit for this assay was 300 cells g^{-1} soil. MPN assays of the uninoculated samples never yielded a positive reading throughout the experiment.

The long-term survival of *S. chlorophenolica* strain RA2 was dependent on the initial PCP concentration (Figure 1). In soil contaminated with 300 ppm PCP, the population of *S. chlorophenolica* strain RA2 at day 0 was not different from day 170; however the population did experience an initial decline after inoculation that was followed by rapid

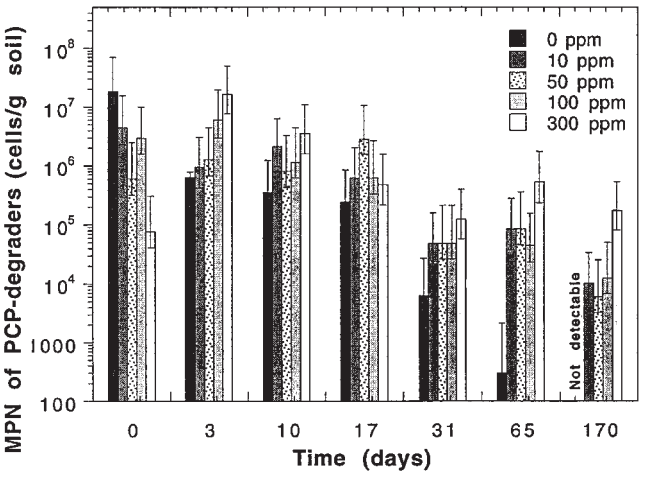

Figure 1 The most probable number of *S. chlorophenolica* strain RA2 cells in soil contaminated with a range of PCP concentrations. The day 0 measurements were taken immediately following PCP and/or inoculum additions. Error bars represent 95% confidence intervals.

growth. Conversely, in all of the treatments with lower initial PCP concentrations (0–100 ppm), the number of *S. chlorophenolica* strain RA2 cells experienced a decline that ranged from just over one order of magnitude to a drop to undetectable levels within 170 days. A greater than 200-fold increase in the number of *S. chlorophenolica* strain RA2 cells was observed over the first 3 days of the incubation in the 300 ppm treatment. In contrast, *S. chlorophenolica* strain RA2 decreased by over 10-fold in the uncontaminated soil over 3 days. None of the other initial concentrations resulted in a significant change in number of *S. chlorophenolica* strain RA2 cells over the first 3 days of the experiment.

After the first 3 days the population of *S. chlorophenolica* strain RA2 declined in three distinct patterns. In the absence of PCP a steep descent to undetectable levels after day 65 was observed (Figure 1). *S. chlorophenolica* strain RA2 populations were never significantly different among treatments that initially received 10, 50 and 100 ppm PCP. These initial PCP concentrations resulted in a steep decrease followed by a more gradual drop in the number of *S. chlorophenolica* strain RA2 cells over the 170 days. In soil containing 300 ppm PCP, the steep decrease was followed by a leveling-off of the population. At this initial PCP concentration, the number of *S. chlorophenolica* strain RA2 cells present in the soil never fluctuated significantly from day 17 through day 170.

SIGR-derived population estimates

With the exception of day 0, parallel SIGR incubations were carried out on the same sampling dates as the MPN measurements. Treatments receiving the lowest amount of PCP (10 ppm) and the highest amount of PCP (300 ppm) were used in these experiments. Respiking inoculated flasks with PCP at each sampling point resulted in the detection of a population capable of mineralizing the added PCP (Figure 2). An increased lag period prior to the onset of PCP mineralization was observed at each successive sampling date. Biomass estimates were generated with the SIGR method by using the mineralization curves shown in

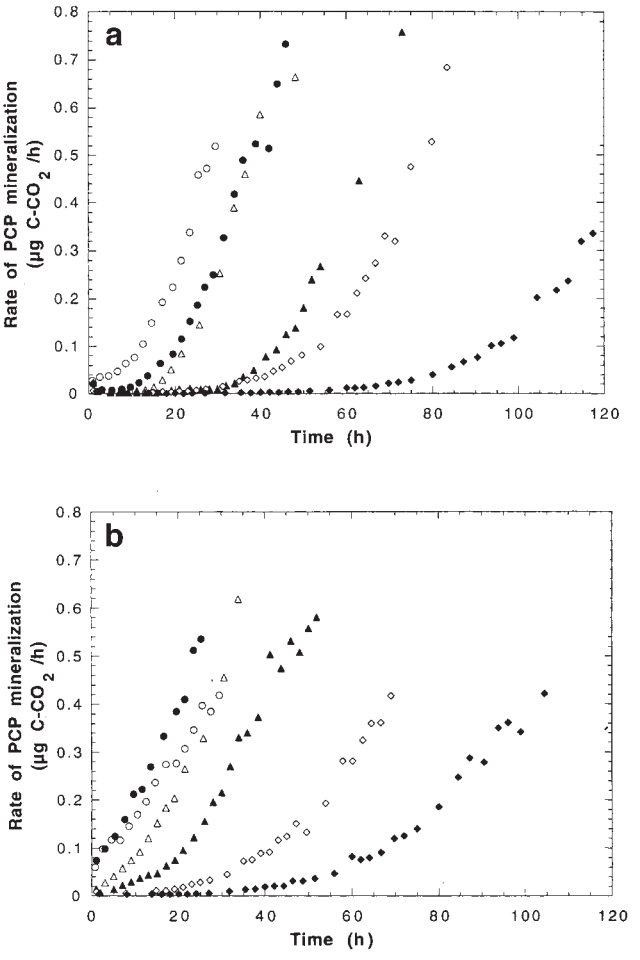

Figure 2 Rates of PCP mineralization when radio-labeled PCP (100 ppm) was reapplied to previously contaminated soils inoculated with *S. chlorophenolica* strain RA2. The initial PCP concentrations were (a) 10 ppm or (b) 300 ppm. Each point represents the mean of two replicate samples. ○ Day 13; ● day 7; △ day 14; ▲ day 28; ◇ day 62; ◆ day 167.

Figure 2. The estimates revealed survival patterns similar to those observed with the MPN assay (Figure 3). There was an early decline in the population of *S. chlorophenolica* strain RA2 followed by a steady biomass level that was detectable through 170 days. As with the MPN assay, the treatment with the higher initial PCP concentration resulted in a greater biomass of *S. chlorophenolica* strain RA2 throughout the experiment. Flasks not inoculated with *S. chlorophenolica* strain RA2 resulted in no $^{14}CO_2$ evolution during the incubation period (data not shown).

To directly compare population estimates between the two methods, the biomass of *S. chlorophenolica* strain RA2 was multiplied by a conversion factor (see Eqn 3) to estimate cell number. The estimates from the two methods never differed by more than one order of magnitude and the errors for the two estimates usually overlapped (Figure 3).

Discussion

The survival of *S. chlorophenolica* strain RA2 in soil was dependent on the initial PCP concentration. Soil contaminated with the highest concentration of PCP tested (300

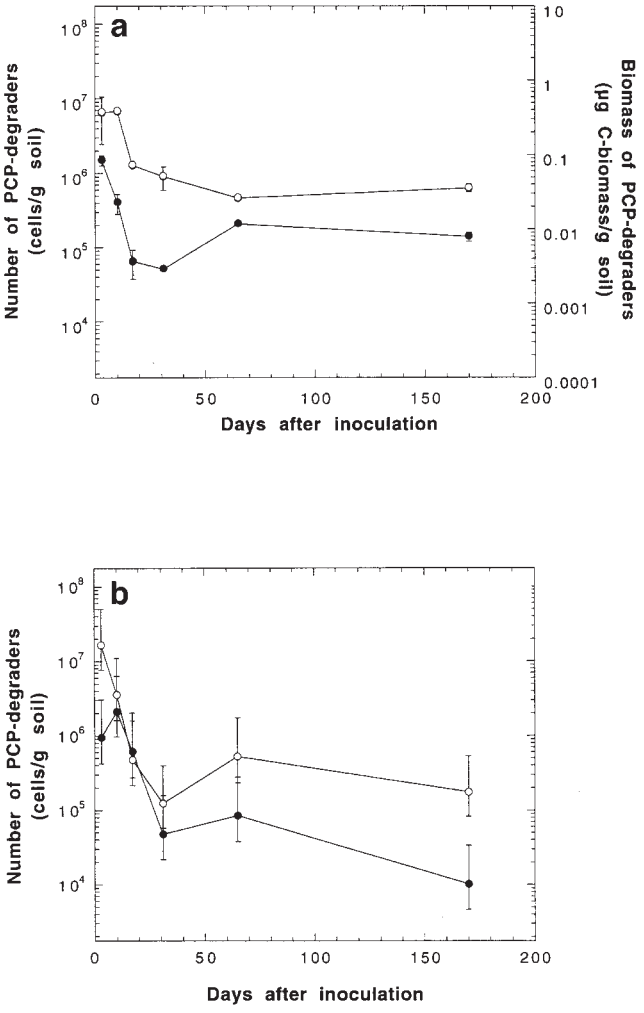

Figure 3 (a) SIGR-determined biomass of *S. chlorophenolica* strain RA2 (in terms of X_a) and the corresponding population size (derived from Eqn 3) in soil contaminated with 10 (–●–) and 300 (–○–) ppm PCP. Error bars are standard errors of the mean. (b) The MPN of *S. chorophenolica* strain RA2 cells in soil contaminated with 10 and 300 ppm PCP. Error bars represent 95% confidence intervals.

ppm) resulted in the greatest number of *S. chlorophenolica* strain RA2 cells at the end of the 170-day experiment. *S. chlorophenolica* strain RA2 was not as successful at colonizing soil in the absence of PCP. This was especially clear after day 65 of the experiment (Figure 1). Intermediate concentrations of PCP supported the long-term survival of *S. chlorophenolica* strain RA2; however, not at the level that 300 ppm supported. These findings are similar to what Jacobsen and Pedersen [11] observed after inoculating a 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading *Pseudomonas cepacia* strain into contaminated soil. The addition of 500 ppm 2,4-D to soil led to a stable population of *P. cepacia*, whereas in soil with no 2,4-D the population declined rapidly over the 44-day experiment. In contrast to our findings, however, they found that the presence of a low amount of 2,4-D (5 ppm) did not support the survival of the introduced bacterium. Leung *et al* [13] observed a similar pattern of decline over a 50-day incubation after inoculating a PCP-degrading *Sphingomonas* sp into uncontaminated soil. However, it is impossible to tell if the population of this microorganism would also have fallen below their detection limits after 170 days since their experiment concluded at 50 days.

Studies with other PCP-degrading organisms have yielded markedly different results from those of this study. Crawford and Mohn [7] reported having to inoculate a contaminated soil sample four times with a PCP-mineralizing *Flavobacterium* species (since redescribed as a *Sphingomonas* species [9,12,17]) before observing any appreciable (80%) PCP mineralization. In pure culture this organism tolerates moderate levels of PCP (100 ppm) but was unable to colonize the contaminated soil they used. Briglia *et al* [3] compared the performance of a *Sphingomonas* species in PCP-contaminated soil to that of another PCP-degrading bacterium, *Rhodococcus chlorophenolicus* PCP-1. They also observed a rapid decline in the number of *Sphingomonas* cells but a notably different pattern for *R. chlorophenolicus* PCP-1. *R. chlorophenolicus* PCP-1 cells were detected for at least 200 days in both uncontaminated and PCP-contaminated soil. Two hundred days was also the length of time required for complete PCP removal (750 ppm) by *R. chlorophenolicus* PCP-1. Additionally, the population of *R. chlorophenolicus* PCP-1 remained relatively constant throughout the incubation whether or not PCP was added to soil. One distinction however, is that Briglia *et al* [3] immobilized the cells on polyurethane foam which may have contributed to the survival and stability of *R. chlorophenolicus* PCP-1 in soil. Miethling and Karlson [15] added this same organism without the polyurethane foam carrier to soil contaminated with a much lower amount of PCP, 100 ppm. In the absence of the carrier this organism was an ineffective inoculum since the rate of PCP mineralization was not different from that of the uninoculated soil.

The relative success of *S. chlorophenolica* strain RA2 in degrading PCP in soil and surviving in PCP-contaminated soil may be due to the fact that the strain used in our studies was never sub-cultured on rich laboratory media. As a result, *S. chlorophenolica* strain RA2 is a PCP specialist that mineralizes PCP at a faster rate than glucose when presented with both substrates simultaneously [19]. The specialized nature of this organism may also help to explain why *S. chlorophenolica* strain RA2 did not persist in uncontaminated Boulder Creek soil. It is unlikely that a PCP-specialist would be able to compete with indigenous microorganisms in non-contaminated soil.

We found that there were advantages and disadvantages unique to the MPN and SIGR assays. The MPN is an extractive method that is highly dependent on the ability to remove cells within and bound to the soil matrix. The release of the target cells is often a limiting step in this and other extraction-based procedures [30,31]. Accordingly, we observed that as the number of *S. chlorophenolica* strain RA2 cells decreased, the sensitivity of the assay did so as well (data not shown). The MPN method is based on the ability of one cell to produce a positive reaction, yet because of the toxicity of PCP towards *S. chlorophenolica* strain RA2 it is likely that a single cell will not always survive addition to a liquid medium containing PCP [18]. However, this assay was very specific for *S. chlorophenol-*

ica strain RA2 and did not produce any false positives in uninoculated soil.

The main advantage to using the SIGR method is that it is truly an *in situ* measure of the microbial biomass capable of growth on a specific substrate. Unlike the MPN assay this method is not dependent on the ability to culture the target organism(s). This method was specific for *S. chlorophenolica* strain RA2 since uninoculated soil showed in significant $^{14}CO_2$ production. In addition to its specificity, another advantage of this method is that in cases where the organism being monitored is well characterized, the biomass estimates obtained can be converted to number of cells and compared to values obtained by other methods.

One of the goals of this research was to validate the SIGR approach as a means of monitoring an introduced bacterium in soil. The premise of the SIGR approach is that the rate of substrate consumption or product formation is proportional to the microbial population that is carrying out the process [21]. However, accelerated removal of pesticides from the environment has been observed without a concomitant increase in the pesticide-degrading population [1,16]. Therefore, an increase in respiration rates (product formation) may not reflect a population increase of the functional group being monitored. Moorman [16] observed increased rates of EPTC (S-ethyl dipropyl carbamothioate) degradation after subsequent applications, but detected no increase in the numbers of culturable microorganisms that could grow at the expense of EPTC. The author postulated that the increased rate of mineralization was due to a physiological adaptation rather than growth. Our finding that these two disparate approaches resulted in similar population estimates and the same survival patterns is evidence that SIGR biomass estimates are valid for *S. chlorophenolica* strain RA2 in this soil. Recent studies have validated the SIGR method for quantifying amino acid- and phenolic-mineralizing microorganisms in natural soils [14].

Overall, we found *Sphingomonas chlorophenolica* strain RA2 to be effective at colonizing PCP-contaminated soil. Using two complementary methods, inoculated *S. chlorophenolica* strain RA2 cells were detectable for at least 170 days in PCP-contaminated soil. We found that the initial concentration of PCP in soil dictated the survival patterns we observed for *S. chlorophenolica* strain RA2. Higher PCP concentrations resulted in more cells of *S. chlorophenolica* strain RA2 surviving for a longer period while the population rapidly declined and eventually was undetectable in uncontaminated soil. This conclusion was reached using two separate approaches that are based on different principles. The MPN method is an extract and culture method whereas the SIGR method is an *in situ* procedure that generates biomass estimates based on the microbial transformation of a target compound. Both methods were very specific and sensitive to population fluctuations of *S. chlorophenolica* strain RA2 in soil.

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