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Fluorescent Methods to Study DNA, RNA, Proteins and Cytoplasmic Membrane Polarization in the Pentachlorophenol-Mineralizing Bacterium *Sphingomonas* sp. UG30 During Nutrient Starvation in Water

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The effect of sodium pentachlorophenolate (NaPCP) exposure on the nutrient-starved pentachlorophenol (PCP)-mineralizing bacterium *Sphingomonas* sp. UG30 was assessed using fluorescent methods to measure DNA, RNA, total cellular protein, and cytoplasmic membrane proteins. UG30 cells were inoculated into sterilized Speed River (Guelph, ON, Canada) water samples in the presence of 50, 100, and 250 ppm NaPCP. No marked changes were observed in the total cellular DNA, RNA or protein levels over 90 d, indicating the macromolecular composition of UG30 was not affected by both nutrient limitation and NaPCP. Total cell counts as determined by DAPI staining also did not change over 90 d. Over the same period, viable counts decreased with increasing concentrations of NaPCP. At 250 ppm NaPCP, viable cell counts decreased over 6 orders of magnitude after 1 hr exposure. Cell numbers partially recovered once NaPCP was degraded. The UG30 cytoplasmic membrane polarization ratio also decreased after NaPCP was depleted. The decreased polarization value at the end of the study period suggested the UG30 membrane was more fluid and that this increase in fluidity was due to nutrient starvation effects rather than exposure to NaPCP. These results indicated that UG30 is a robust organism that is able to degrade NaPCP even under adverse conditions and fluorescent methods are useful for determining macromolecular concentrations and cytoplasmic membrane polarization values.

KEY WORDS: Bacteria; DNA; fluorescence; macromolecular; membrane fluidity; methods; metabolism; nutrient-starvation; RNA; *Sphingomonas*; survival; water.

INTRODUCTION

Most aquatic and terrestrial environments are oligotrophic habitats that are chronically nutrient limited [1–4]. In these environments, microorganisms exist under nutrient-limited conditions where their responses have been studied in some bacterial species [3,5–10]. However, fluctuations in the amounts of available nutrients occurs, and when nutrients become available, bacteria can recover after being subjected to starvation for extended periods of time [3,11,12].

In response to nutrient starvation, bacteria may synthesize stress proteins such as the *Escherichia coli* sigma factor σ^s [6], which enhance the cells' tolerance to environmental changes [12–15]. During starvation, bulk protein synthesis and gene expression are reduced as shown in the starvation survival response of *Escherichia coli*, *Vibrio* spp., and *Salmonella typhimurium* [16,17]. The nature of

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the nutrient limitation (i.e., carbon vs. phosphate or amino acids starvation) and the bacterial strain determine the extent of protein synthesis during starvation [7]. The early production of stress proteins during starvation is crucial to continued metabolic activity for long-term starvation survival of the bacterial cells [7,12,16,18].

Starved bacteria may also undergo different macromolecular changes. For example, total cellular DNA may decrease [8,19], or remain constant following an initial increase [20,21]. As well, the appearance of long-lived (7 d) mRNA transcripts may occur, as a result of the formation of secondary structures on the 3′ end [12,22]. The cytoplasmic membrane fatty acid composition has been observed to change during starvation [23]. In *E. coli*, other changes such as condensation of the cytoplasm causing it to become smaller and denser, and an increase in the periplasm may occur [16]. During starvation some bacterial species exhibit reduced metabolic activity [9] and their cell size decreases to form smaller more spherical cells [9,10,12,15]. Some starved bacteria change to specialized adaptive forms such as spores and some enter a dormant or a viable but nonculturable (VBNC) state [24]. These adaptive responses to starvation also make bacteria more resistant to other adverse conditions such as low pH, heat, and oxidative stress [7,25].

Pentachlorophenol (PCP) and its sodium salt have been used in North America mainly to treat railway ties and hydro poles in the wood preservation industry [26,27]. PCP can accumulate in plant, animal, and human tissues [28–32]. It is embryotoxic, teratogenic, and a suspected carcinogen [30] and has been designated a priority pollutant by the USEPA [33] the European Community and Canada [25,34].

It is of interest to determine how nutrient starvation affects the ability of xenobiotic-degrading microorganisms to degrade pollutants, especially under toxic environmental conditions. *Sphingomonas* sp. UG30 is capable of mineralizing up to 250 ppm NaPCP in a minimal salts (MS) medium and up to 300 ppm NaPCP in MS medium supplemented with glutamate [35]. NaPCP and PCP have been shown to uncouple oxidative phosphorylation [36,37], and UG30 membranes become de-energized on contact with nonlethal concentrations (120 μg/mL or 120 ppm) of NaPCP [38]. In this study, we used a fluorescent method to examine the effect of nutrient starvation on cytoplasmic membrane polarization which is an estimate of membrane fluidity, and indicates the ability of the cell to withstand physiological changes while maintaining a fluid membrane [39]. We also assessed if nutrient starvation affected the macromolecular (total DNA, RNA, and protein) composition (using fluorescent methods) and the ability of UG30 to degrade NaPCP.

MATERIAL AND METHODS

Chemicals

Tetrahydrofuran (THF) was obtained from Fisher Scientific (Toronto, ON, Canada). Sodium pentachlorophenolate (NaPCP) and 2-nitrophenol were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). The fluorescent probes DPH (1,6 diphenyl-1,3,5-hexatriene) and DPH-PA (3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid) and the dyes NanoOrange, PicoGreen, RiboGreen, and DAPI (4′,6-diamidino-2-pheylindole, dihydrochloride) and the Antifade Reagent (Prolong®Antifade Kit) were obtained from Molecular Probes Inc. (Eugene, OR, USA).

Bacterial Growth Conditions

For the nutrient starvation experiments, *Sphingomonas* sp. UG30 cells were grown in a minimal salts plus glutamate (MSG) medium as described by Leung *et al*. [35] to mid-exponential phase (21 hr at 30°C) at 200 rpm from a 2% (v/v) inoculum of mid-exponentially growing cells. Cells were aseptically harvested by centrifugation (6,400 × *g* for 10 min at 21°C), washed three times with sterile 15 mM Tris-HCl buffer (pH 7.0) and resuspended to an $OD_{600\,nm}$ of 0.6 into 250-mL Erlenmeyer flasks containing 120 mL of sterile river water samples. Flasks were incubated in the dark with shaking at 200 rpm. River water was collected from the Speed River (Guelph, ON, Canada) during the month of January 2003 into a 10 L container and brought back to the lab immediately. Before use, river water was passed twice through sterile 0.2-μm filters, twice through sterile 0.1-μm filters and autoclaved at 121°C, 20 psi for 1 hr before use. Sterility of the water was checked by a 2% (v/v) inoculation of river water into 1/10 Luria–Bertani (LB) broth and incubated at 30°C with shaking at 200 rpm. No turbidity was observed in the LB broth over 21 days indicating sterility. Chemical analysis of the river water yielded 1.6, 18.8 and 16.7 mg/L of K^+, Mg^+ and Na^+, respectively. Total phosphorous, nitrogen, and organic carbon contents were <0.05, 2.4 and 8.2 mg/L, respectively. Chemical analyses were conducted at the Laboratory Services Division of the University of Guelph using standard methods. *Sphingomonas* sp. UG30 cells inoculated into river water samples were exposed to 50, 100, and 250 ppm of NaPCP at 15°C. At various times (1 hr, 2, 7, 15, 30, 60, and 90 days), aliquots (1 mL) were aseptically withdrawn for determination of total cellular DNA, RNA, and protein levels, total cell counts (DAPI staining) and viable cell counts.

Membrane Polarization Measurements

Three mL aliquots from UG30 cultures subjected to the various NaPCP treatments were aseptically removed and harvested by centrifugation (6,400 $\times$ g for 10 min at 21°C) and washed three times with sterile 15 mM Tris-HCl buffer, pH 7.0. The resuspended cultures were adjusted to an $OD_{600\,nm}$ of 0.4 and used immediately for fluorescence polarization experiments. Fluorescence polarization measurements were carried out using the membrane lipid probes DPH or DPH-PA as described in Denich *et al*. [40]. The fluorescence polarization ratio (P) was determined according to Shinitzky and Barenholz [41]: $P = (I_{vv} - I_{hv}G)/(I_{vv} + I_{hv}G)$, where I_{vv} and I_{hv} are the intensities of emitted light polarized in parallel and perpendicular directions, respectively, with respect to the exciting beam of light [42]. The grating factor (G) is the correction factor for the photomultiplier tubes [43] and is the ratio of vertically to horizontally polarized light when the exciting beam of light is polarized horizontally [42,44]. The rotational constraint of the probe was determined by depolarization of the exciting beam as the membrane probes rotated [45]. Membrane fluidity decreases when the membrane lipids become more rigid or gel-like causing the polarization ratio to increase due to the constraint exerted on the membrane probe [46,47]. When membrane lipids are in the liquid-crystalline phase, a greater degree of fluidity prevails and increased rotation of the membrane probe can occur, resulting in a lower polarization ratio.

Total Cellular Fatty Acid Compositional Analysis

Total cellular fatty acids were extracted according to the protocol recommended by Microbial ID, Inc., Newark, DE, USA [48]. Fatty acids were saponified, methyl esterified and the fatty acid methyl esters analyzed by GC-FID using the MIDI microbial identification system [49].

Total and Viable Counts

Total counts of *Sphingomonas* sp. UG30 cells were obtained after staining with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). A 30 μM stock solution of DAPI was prepared in filter sterilized, distilled water and diluted into PBS buffer (pH 7.4) according to the manufacturers' instructions. The DAPI stock solution (100 μL) was added to UG30 cells that had been serially diluted in 1 mL of sterile PBS buffer to obtain a final DAPI concentration of 2.9 μM. Cell suspensions were incubated in the dark for 15 min and filtered through 0.2 μm-black Millipore polycarbonate filters, washed three times with 1 mL aliquots of PBS buffer, placed on slides with 10 μL of Antifade Reagent and a cover slip was applied. Cells were counted after placing the slides under immersion oil at 1000× magnification using a Nikon Eclipse E600 Fluorescent Microscope (Nikon, Mississauga, ON, Canada) with the appropriate filter (330–380 nm). Cell numbers were obtained from an average count taken from 30 fields. Viable counts were estimated by the drop plate method of Cassidy *et al*. [50] from UG30 cultures serially diluted in PBS buffer (pH 7.4) and plated on MSG agar and incubated at 21°C for 2 days.

Extraction and Fluorescent Quantification of Total Cellular DNA, RNA, and Protein

DNA and RNA from UG30 cells incubated in Speed River water samples were extracted using QIAmp DNA Mini Kit and RNeasy Mini Kit, respectively (Qiagen Inc. Mississauga, ON, Canada). The only modification from the standard protocol was that aliquots were centrifuged for 30 min at 14,000 $\times$ g at 4°C to ensure all cells were harvested. Total protein was extracted using a modified method of Gallant and Suskind [51]. One milliliter aliquots from the cultures treated with different concentrations of NaPCP were withdrawn and centrifuged at 14,000 $\times$ g for 30 min at 4°C. The pellet was resuspended in 1.5 mL cold (4°C) 5% trichloroacetic acid and incubated for 1 hr at 4°C. Cellular materials were centrifuged again (14,000 $\times$ g for 30 min at 4°C) and digested with 100 μL of 1 M NaOH for 1 hr. The volume adjusted to 1 mL by adding 900 μL of sterile distilled water. Samples were removed for protein determinations. Extractions were performed on control samples (water only) and no detectable free DNA, RNA, or protein was found.

RNA, DNA, and protein contents were quantified in accordance with the manufacturers' protocols using the fluorescent probes RiboGreen, PicoGreen and NanoOrange (Molecular Probes, Eugene, OR, USA), respectively. Samples were serially diluted and the fluorescent intensity of the macromolecular-fluorescent probe complex was quantified using a Fl_x800 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Standards for RNA, DNA and protein were prepared from stock solutions included in the kits of 16S and 23S *E. coli* rRNA (100 μg/mL), bacteriophage lambda DNA (100 μg/mL) and bovine serum albumin (2 mg/mL), respectively.

Determination of NaPCP Degradation by HPLC

Degradation of NaPCP by UG30 cells starved in river water samples was monitored. Samples (1 mL) were periodically withdrawn and centrifuged at 14,000 × *g* for 30 min to remove the bacterial cells. An internal standard, 2-nitrophenol (12 ppm) was added to 800 μL of the supernatant. Samples were analyzed by HPLC equipped with a UV detector (Gilson, Mandel Scientific Co. Inc., Guelph, ON, Canada) and NaPCP concentrations were determined by comparison to a standard curve prepared with authentic NaPCP standards. The flow rate was maintained at 0.75 mL/min using a 50% acetonitrile mobile phase on a Hypersil® Green ENV column (length × ID, 150 × 4.6 mm, particle size, 5 μm) (Mandel Scientific Co. Inc., Guelph, ON, Canada).

Confirmation of the Presence of the *pcpC* Gene in UG30

After long-term incubation, the identity of UG30 cells was confirmed in two ways. First, UG30 was inoculated into MSG media containing 100 ppm NaPCP. Degradation of NaPCP was assessed by monitoring decreases in $OD_{325\,nm}$ readings. Cultures were found to degrade NaPCP (data not shown) in agreement with the previous results from our laboratory [35]. Second, total DNA was extracted with the QIAmp DNA Mini Kit from UG30 cells starved in river water in the absence or presence of NaPCP. Samples were probed for the presence of the UG30 *pcpC* gene (GenBank Accession No. AY057901), which codes for tetrachlorohydroquinone reductive dehalogenase [52] by PCR using genomic DNA as the template. A 780 bp DNA fragment was amplified using a pair of primers targeting the UG30 *pcpC* gene. The forward primer (PCPC Nde1) was 5′-TTCCGGGAATTCCATATGTCTGAAGTCAGTCTC TATA, and the reverse primer (PCPC rev Sap1B) was 5′-GGTGGTTGCTCTTCCGCAGATGCCGCCCTTCC AGTT. The PCR mixture contained 1 μL of DNA extract, 10.5 μL of deionized water, 0.5 μL of each primer (0.1 μg/μL) and 12.5 μL of 2× Master Mix (0.05 U/μL *Taq* DNA polymerase in reaction buffer, 4 mM $MgCl_2$ and 0.4 mM of dNTPs) (MBI Fermentas, Burlington, ON, Canada). The PCR cycle included an initial denaturation for 3 min at 94°C, followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C and 1.5 min of extension at 72°C. Final extension was done for 10 min at 72°C. PCR products were analyzed by agarose gel electrophoresis.

Statistical and Data Analysis

All experiments were conducted in triplicate. Values presented are the averages ± S.D. Statistical analysis was carried out using SigmaStat 2.0 (Jandel Scientific Software).

RESULTS AND DISCUSSION

Viable and Direct Counts of *Sphingomonas* sp. UG30 Cells Starved in River Water

One objective of this research was to determine if UG30 cells starved for a period of 90 d in the presence of NaPCP in river water would remain viable. Flasks were initially inoculated with 1.0×10^8 cells/mL. At the highest concentration of NaPCP tested (250 ppm), viable cell counts decreased by 6 orders of magnitude from the initial inoculum concentration after 1 hr exposure (Table I). A less marked decrease was observed when UG30 cells were exposed to lower concentrations (50 and 100 ppm) of NaPCP for 1 hr in the river water. On further incubation, viable cell counts in the presence of NaPCP recovered. For UG30 cells exposed to 100 ppm NaPCP, the viable cell count increased to 9.0×10^5 after 7 d and for UG30 cells exposed to 250 ppm NaPCP the viable count increased to 2.8×10^5 after 15 d. At the end of the 90 d incubation, the viable cell count remained between 1×10^5 and 1×10^6 in all water samples inoculated with UG30. Cell growth occurred after NaPCP was degraded, as the growth of UG30 (verified by increased colony counts) coincided with degradation of NaPCP as determined by HPLC (Fig. 1a). This is consistent with the finding of Leung *et al*. [35], who observed that growth of UG30 does not occur until after the concentration of NaPCP decreases below a threshold of about 16 μg/mL (16 ppm). Complete degradation of NaPCP was observed only in the cultures containing viable UG30 cells, as the controls (flasks without UG30 cells added or the flasks containing heat-killed UG30 cells and NaPCP), did not show complete loss of NaPCP (Fig. 1b). Throughout the 90 d period, total cell counts measured by DAPI staining did not change (data not shown). This suggests some of the cells remained structurally intact but not necessarily viable [5]. Due to the length of the study and the possibility for contamination, especially in the uninoculated controls (flasks to which UG30 was not added), each test culture was probed for the presence of the *pcpC* gene. All flasks inoculated with UG30 yielded a strong PCR signal of the correct DNA fragment size (780 bp) for the *pcpC* gene target (data not shown). No PCR product was detected in samples from the uninoculated control flasks.

Table I. Viable Cells (per ml) of *Sphingomonas* sp. UG30 Inoculated Into Sterile River Water in the Presence of 0, 50, 100, and 250 ppm NaPCP Observed Over a 90 d Period at 15°C

	Treatments			
Days	(0 ppm) NaPCP	NaPCP (50 ppm)	NaPCP (100 ppm)	NaPCP (250 ppm)
1	$9.9 \times 10^7 \pm 1.7 \times 10^7$	$8.7 \times 10^6 \pm 4.1 \times 10^6$	$4.1 \times 10^4 \pm 4.0 \times 10^4$	$4.4 \times 10^1 \pm 5.1 \times 10^1$
2	$3.4 \times 10^7 \pm 1.0 \times 10^7$	$7.1 \times 10^6 \pm 1.8 \times 10^6$	$5.6 \times 10^4 \pm 5.8 \times 10^4$	$2.2 \times 10^1 \pm 1.9 \times 10^1$
7	$2.6 \times 10^7 \pm 6.2 \times 10^6$	$6.0 \times 10^6 \pm 6.7 \times 10^5$	$9.0 \times 10^5 \pm 6.5 \times 10^5$	$1.1 \times 10^1 \pm 8.4 \times 10^1$
15	$3.3 \times 10^7 \pm 1.1 \times 10^7$	$8.4 \times 10^6 \pm 5.1 \times 10^5$	$4.1 \times 10^5 \pm 2.2 \times 10^5$	$2.8 \times 10^5 \pm 1.9 \times 10^5$
28	$1.9 \times 10^7 \pm 7.9 \times 10^6$	$4.5 \times 10^6 \pm 1.1 \times 10^6$	$3.0 \times 10^5 \pm 8.5 \times 10^4$	$3.9 \times 10^4 \pm 3.4 \times 10^4$
56	$6.1 \times 10^6 \pm 4.2 \times 10^6$	$7.6 \times 10^6 \pm 6.9 \times 10^5$	$3.7 \times 10^5 \pm 2.9 \times 10^5$	$4.8 \times 10^4 \pm 2.1 \times 10^4$
90	$1.5 \times 10^6 \pm 1.2 \times 10^6$	$4.4 \times 10^6 \pm 1.4 \times 10^6$	$4.4 \times 10^5 \pm 8.4 \times 10^4$	$1.2 \times 10^5 \pm 1.1 \times 10^5$

Note. Cells/ml are means ± SD ($n = 3$). An uninoculated control was also included, no growth was observed over the 90 d period.

Another experiment was conducted to determine if *Sphingomonas* sp. UG30 retained the ability to degrade NaPCP after being starved in river water samples for 30 days in the absence of NaPCP. UG30 was able to degrade NaPCP (50, 100, and 250 ppm) even after starvation in river water (data now shown) although at a slower rate (5, 9, and 27 d to degrade 50, 100, and 250 ppm, respectively) than that observed in cells not previously starved (Fig. 1a). This may be due in part to the decreased number of cells in the cultures starved for 30 d in river water prior to the addition of NaPCP. After the 30 d, cell counts decreased by an order of magnitude in starved cultures and exposed to NaPCP compared to cells that had not been starved, but were transferred to river water with NaPCP added (data not shown).

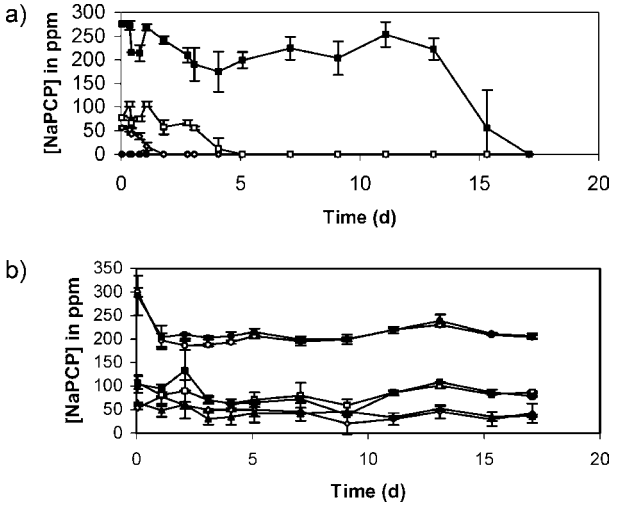

Fig. 1. (a) HPLC analysis of the degradation of NaPCP by *Sphingomonas* sp. UG30 inoculated into sterile river water in the absence 0 (●) or presence of 50 (◇), 100 (□), and 250 (■) ppm NaPCP monitored over 16 d. (b) HPLC analysis of NaPCP disappearance by heat-killed *Sphingomonas* sp. UG30 cells inoculated into sterile river water with 50 (▲), 100 (■) and 250 (●) ppm NaPCP and 50 (◇), 100 (□) and 250 (○) ppm NaPCP added to sterile river water, monitored over 16 d. All flasks were incubated at 15°C. Values are means ± SD ($n = 3$).

Macromolecular Changes in *Sphingomonas* sp. UG30 Cells Starved in River Water

During starvation survival, bacterial RNA and protein synthesis usually declines [10,12]. Some research has indicated that unlike RNA and protein, the DNA content remains more stable in most bacteria during prolonged starvation to minimize genome degradation [53]. Over the 90-d period, total cellular DNA and RNA levels of the UG30 cultures exposed to NaPCP in sterile river water remained unchanged (at a value about 1×10^6 ng/mL) relative to the control (Fig. 2a, b, and c, respectively). The total protein levels also remained unchanged at about 100 μg/mL.

Effect of Starvation on the Cell Membrane of *Sphingomonas* sp. UG30

The cytoplasmic membrane polarization of UG30 cells was monitored over the 16 days when NaPCP was degraded during starvation in river water. Membranes of UG30 cells exposed to 50, 100, and 250 ppm NaPCP exhibited higher polarization values (less fluid membrane) at the end of 16 days compared to membranes of the control cells (Fig. 3). No significant difference in the polarization values of the UG30 cultures exposed to NaPCP was observed during the first 3 d of the study. In response to starvation and exposure to NaPCP, there was a decrease in total branched and cyclopropane fatty acids (fatty acids containing a ring structure) compared to the control cells

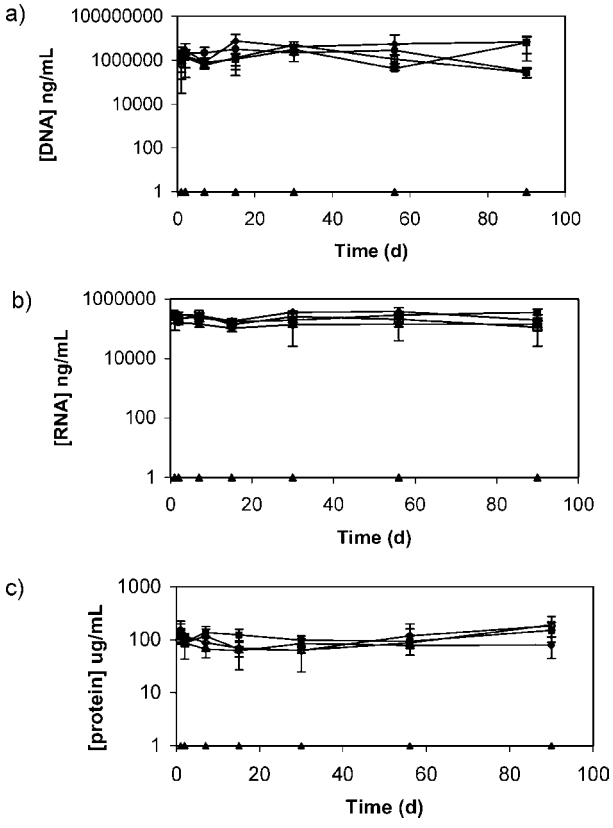

Fig. 2. (a) Total cellular DNA (ng/mL), (b) RNA ng/mL) and (d) protein (μg/mL) concentration of *Sphingomonas* sp. UG30 inoculated into sterile river water in the absence 0 (•) or presence of 50 (◇), 100 (□), and 250 (■) ppm NaPCP compared to the uninoculated control (▲). All flasks were incubated at 15°C and monitored over 90 d. Values are means ± SD ($n = 3$).

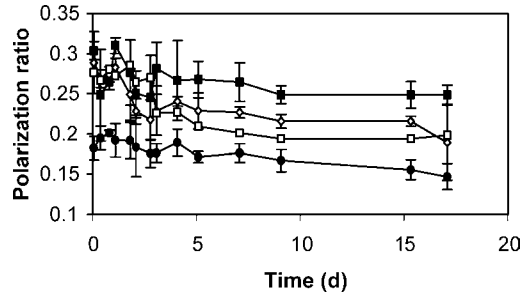

Fig. 3. DPH fluorescence polarization ratio membranes of *Sphingomonas* sp. UG30 cells inoculated into sterile river water in the absence 0 (•) or presence of 50 (◇), 100 (□), and 250 (■) ppm NaPCP. Cells were incubated at 15°C and monitored over 16 d. Values are means ± SD ($n = 3$).

(Table II). The percentage of cyclopropane fatty acids decreased from 4.2 ± 1.1% in the control culture, to zero in all samples exposed to NaPCP. The percent of branched fatty acids decreased from 32.9 ± 6.4% in control cells to 16.7 ± 1.1%, 15.1 ± 0.9% and 12.6 ± 1.0% in UG30 cultures exposed to 50, 100, 250 ppm, respectively. However, the saturated to unsaturated fatty acid ratio (which is a strong indicator of membrane fluidity changes) and the fluorescence polarization ratio were not significantly changed by nutrient starvation.

Understanding starvation-survival responses is important with regard to pollutant-degrading microorganisms. During starvation in river water, UG30 cells were able to resume growth after NaPCP was degraded. The trends of declining viable counts but stable total direct counts observed in UG30 in this study has been reported in other starvation studies for other microorganisms conducted in water samples without NaPCP [5,8,10]. This has been attributed to cells entering a VBNC state. However, in this study the declining viable count was likely caused by the toxicity of NaPCP. Although the number of viable UG30 cells decreased during starvation in river water, UG30 retained its ability to degrade NaPCP and it may be that the lower number of viable cells is the reason why the rate of PCP degradation decreased.

In *E. coli* cells previously grown on minimal medium at 30°C and starved in sterile seawater (20°C) for 30 days, total cellular DNA concentrations were maintained at a constant level after an initial increase in the first 5 days [21]. However, Muela *et al.* [8] reported that chromosomal DNA levels of *E. coli* were constant even after 54 d of starvation in river water before decreasing slowly to 13.9% of the initial value after 95 d. Rates of RNA and protein synthesis declined under starvation conditions in a *Sphingomonas* ultramicrobacterium strain until constant levels were maintained [54] In *Vibrio cholerae*, the total RNA decreased 2% in the first 14 d of starvation, and then decreased by 20% after 30 d, whereas total DNA decreased by 30% after 7 d and 80% after 30 d [55]. Cellular lipids, proteins and DNA decreased by 5–25% in *Salmonella tymphimurium* after 32 d of starvation in sterile seawater at 22°C, although RNA did not decrease significantly in that time [56]. In *Brevibacterium linens*, cellular RNA content decreased in the first 5 d, and stabilized at one quarter of its initial value while cellular DNA increased by 15% within the first 24 hr and then remained constant [20]. In addition, the protein content and intracellular amino acid pool decreased to 60% of their initial values after 30 days [20]. In *Vibrio cholerae*, the protein level was found to decrease by 20% after 7 d, and by 80% after 30 days [55].

Environmental factors such as temperature, nutrients, chemicals, ions, age of culture, pressure, pH, water activity, and enzyme activity can alter the cytoplasmic

Table II. Fatty Acid Composition in *Sphingomonas* sp. UG30 Membranes After Starvation in River Water for 90 days at 15°C and Exposure to NaPCP

Fatty acid	Treatments			
	NaPCP (0 ppm)	NaPCP (50 ppm)	NaPCP (100 ppm)	NaPCP (250 ppm)
	Percentage of total fatty acids			
Total straight	8.2 ± 1.5	8.8 ± 1.7	7.2 ± 0.2	8.1 ± 0.6
Total *cis*-unsaturated	58.8 ± 12.0	66.9 ± 2.1	67.8 ± 1.6	68.8 ± 0.9
Total branched	32.9 ± 6.5	16.7 ± 1.1	15.1 ± 0.9	12.6 ± 1.0
Total cyclopropane	4.2 ± 1.1	0	0	0
Total *iso*-branched	5.8 ± 10.1	0	0.9 ± 0.9	3.2 ± 0.6
[a]Total saturated	8.3 ± 1.5	8.8 ± 1.7	7.2 ± 0.2	8.1 ± 0.6
[b]Total unsaturated	91.7 ± 1.5	91.2 ± 1.7	92.8 ± 0.2	91.9 ± 0.6
Saturated/unsaturated	9.0 ± 1.8	9.7 ± 2.0	7.8 ± 0.3	8.9 ± 0.7

Note. Values are means ± SD ($n = 3$).
[a]Total saturated fatty acids including straight- and cyclopropane chains.
[b]Total unsaturated fatty acid including hydroxylated chains.

membrane structure [57]. Bacterial cytoplasmic membranes can compensate for altered growth conditions through homeoviscous adaptation which maintains the membrane at a optimal level of fluidity for function [39]. As the membrane is the first point of contact between the microorganism and its environment, it is significant to determine if this was a site affected by exposure to NaPCP. The membrane fluidity of *Sphingomonas* sp. UG30 cells starved in distilled water for 30 d showed some deviations from the control. However, the membranes of UG30 cells monitored over a 16 d period of NaPCP degradation during starvation in river water showed a slow but discernible decrease in the polarization ratio (membrane became more fluid) after the NaPCP was depleted. The combined stresses of starvation and NaPCP exposure appeared to be more significant than each stress acting alone. Overall, nutrient depletion and exposure to NaPCP had short-term effects on the viability of the UG30, and this did not hinder its ability to degrade NaPCP, nor its macromolecular composition or membrane polarization. The results of this study suggest that UG30 is a robust bacterial strain able to cope with a range of nutrient stresses and the presence of NaPCP. The fluorescent methods used for cells staining, DNA, RNA, and protein determination and cytoplasmic membrane polarization were highly suitable for researching bacterial cells under nutrient starvation and in the presence of the pollutant, NaPCP.

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