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Title: Reproducibility of a microbial river water community to self-organize upon perturbation with the natural chemicals enantiomers, *R*- and *S*-carvone.

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Abstract

A river water microbial community was studied in response to perturbation with the monoterpene enantiomers *R*- and *S*-carvone. The microbial community structure and function was also evaluated after enantiomers exposure was switched. Microbial communities were evaluated by length heterogeneity PCR. The addition of *R*- and *S*-carvone enriched for a range of functionally different communities: : enantiomer-selective, racemic and ones that contain both. After 5 days incubation, the *R*- and *S*-carvone treatments developed a range of dominant microbial communities, which were increasingly dissimilar from the ones in which no carvone degradation had taken part (R-values: *R*-carvone 0.743, *S*-carvone 0.5007).Upon carvone depletion, communities reverted to a less dominant community structure. After the cross-over, the rate of carvone utilization was significantly faster than after the first carvone addition ($p=0.008$) as demonstrated by concomitant carvone and oxygen depletion. The main *R*-degrading community (450 to 456 bp) appeared enantioselective and largely unable to degrade *S*-carvone, whereas the *S*-carvone degrading community (502 to 508 bp) appeared to have racemic catabolic capacity. In conclusion, chemical perturbations, such as enantiomers, might generate a significant shift in the river microbial ecology that can have implications for the function of a river in both a spatial and temporal context.

Introduction

The scientific method relies upon the premise that a response from a system can be replicated upon re-creation of the precise physical-chemical-biological conditions under which the response was generated. In the physical sciences, this can be easily demonstrated—as a projectile will precisely and accurately hit its target when all variables are reproduced. However, proving such a premise when it entails biological systems can be logistically impossible. Curiously, our inability to precisely reproduce physical-chemical-biological conditions in the natural sciences has not precluded genuine insights into the mechanics of natural systems. For example, the premise has enabled a deep understanding of the physiology of microorganisms despite the lack of control over many of its component parts, e.g. gene instability, mutations, gene regulation. The problems posed by microbial systems are considerably compounded when microbial ecology is interrogated, as each member of the community is uniquely responding to its individual physical-chemical-biological condition. Therefore, complex microbial systems are, arguably, impossible to replicate (Huisman & Weissing, 2001). Yet, despite their inherent complexity, the 'system' can function with a considerable degree of reliability—this is evident in as disparate operations as sewage treatment works and wineries. However, how natural systems self-organize to perform a function and why on occasion they don't, is largely unknown.

The current study aims to investigate the reproducibility of a river water system to self-organize upon perturbation with the natural chemical carvone. Carvone is one of over 400 different naturally occurring monoterpenes constituting the largest class of secondary metabolites in plants (van der Werf*, et al.*, 1999). *S*-(+)-Carvone is the principal constituent of the oils from caraway (50-70%) and dill (40-60%), while *R*-(–)-carvone is the principal constituent of the oil from spearmint (51%) (de Carvalho & da Fonseca, 2006). Monoterpenes play an important role in chemical ecology, where they act as attractants, repellents, sex pheromones, alerting pheromones, and defence secretions, as well as antifungals, and biofilm and enzyme inhibitors (Hartmans*, et al.*, 1995, Amaral & Knowles, 1998, Amaral*, et al.*, 1998, van der Werf & Boot, 2000, Niu & Gilbert, 2004). Emissions of monoterpene from trees are estimated at 127 x 10^{14} g carbon per year (Guenther*, et al.*, 1995) and are deemed central to a terpene-

based food chain in conifer-dominated locations (Button, 1984). Few studies have investigated the biotransformation of terpenoids. Aerobic biodegradation of limonene, the most widespread monoterpene in the world (van der Werf*, et al.*, 1999), has been among the best characterised (Trudgill, 1990, van der Werf*, et al.*, 1998, Duetz*, et al.*, 2001, Duetz*, et al.*, 2003). Cumate (Eaton, 1996), pinene (Trudgill, 1990, Singh*, et al.*, 2006, Smolander*, et al.*, 2006), cymene (DeFrank & Ribbons, 1977, DeFrank & Ribbons, 1977, Eaton, 1997, Harms*, et al.*, 1999, Nishio*, et al.*, 2001), and carveol (van der Werf*, et al.*, 1999, van der Werf & Boot, 2000) are among the few additional monoterpenes where microbial biodegradation studies have been conducted. A number of studies have investigated the role of carvone and other monoterpenes in the induction of xenobiotic degradation (Gilbert & Crowley, 1997, Hernandez*, et al.*, 1997, Singer*, et al.*, 2000, Dercova*, et al.*, 2003, Rhodes*, et al.*, 2007), implicating a fortuitous, and potentially important mechanism for the natural attenuation of contaminated aquatic and terrestrial systems (Singer*, et al.*, 2003, Singer, 2006).

The structural similarity of *R*- and *S*- carvone to many of the other 400+ naturally-occurring monoterpenes permitted its use as a model natural chemical for perturbing the river water ecosystem. By using optical isomers as the perturbation, the treatment effect can be isolated to the interaction between the chiral biomolecules within the river water microorganisms and the chiral nature of carvone—as all physical properties of enantiomers are identical (e.g., water solubility, log P, Henry's constant, etc). The ability to distinguish enantiomers plays an important role in fields such as drug effectiveness, insect chemical communication, taste perception and bioremediation (Laska*, et al.*, 1999, Singer*, et al.*, 2002). The varied physical activities and toxicological risks of enantiomers should be expected given that the biological receptors (e.g. proteins) are chiral and thus potentially enantioselective (Laska*, et al.*, 1999).

This study repeatedly monitored residual carvone, dissolved oxygen, protists, and community structure over a 22-day period to qualify the variability in the resulting community structure and functional response of a river water bacterial community after spiking with different carvone isomers. We hypothesized that despite every

104 effort having been made to replicate the physical-chemical-biological conditions
105 within and between treatments, the resulting community structure and function would
106 be highly variable. We predict one of three outcomes: 1) enrichment of enantiomer-
107 selective degraders; 2) enrichment of racemic degraders; or 3) enrichment of both
108 enantiomer-selective and racemic degraders. The study employed a cross-over design,
109 where communities exposed to one isomer of carvone were subsequently exposed to
110 the other isomer, raising the question: How rapidly can the function and structure of a
111 microbial community respond to fluctuating optical isomers, and how variable is the
112 response?
113
114 Materials and methods
115
116 Experimental design
117 Two experimental treatments, one spiked (*R*)-(+)-carvone and the other with (*S*)-(-)-
118 carvone and one control treatment were monitored over 12 days to investigate the
119 degradation process of the different carvone isomers. On day 13 the microbial
120 communities from each treatment were retrieved from their environment and re-
121 suspended in water spiked with the alternative carvone isomer (see Table 1). This
122 cross-over was employed to assess the functional diversity of the communities. After
123 the cross-over the degradation of the alternative isomer was also monitored. A final
124 sample was taken on day 22, after which the experiment was finished.
125
126 Sample preparation
127 Ten litres of water were collected from the River Cherwell at the weir in the
128 University Parks, Oxford, England. Details of this reach of the Thames catchment can
129 be found in a previous publication (Neal*, et al.*, 2006). The water was poured into a 50 L
130 plastic container through a 5 mm sieve to remove large particles. Half of the filtered
131 water was immediately autoclaved and stored at 4°C to be used in the second half of
132 the experiment. The remaining river water was incubated for four days in the dark to
133 equilibrate to room temperature and allow the microbial community to adjust to
134 laboratory conditions prior to initiating the study. The time span needed for the
135 microbial community to adjust to laboratory conditions had been determined in

136 preliminary unpublished studies, which utilised DNA recovery over time as a measure
137 of 'change' in the system.
138
139 The experimental treatments were generated from three 5 L conical flasks prepared
140 from the incubated river water. (*R*)-(+)-carvone (98% purity; Lancaster, Morecambe,
141 UK) and (*S*)-(-)-carvone (98%; Sigma-Aldrich, Gillingham, UK) were dissolved in
142 two of the flasks at 20 μg g^{-1}, whereas nothing was added to the third, control, flask.
143 The carvone treatments were divided into 24 pseudoreplicates by distributing 80 mL
144 into 125-mL wide-mouth amber glass bottles. Half of the prepared bottles (n = 12)
145 served to monitor carvone degradation while the remaining were used to monitor the
146 microbial community. Providing separate bottles for chemical and molecular analysis
147 ensured that the microbial community would be minimally disturbed during the study.
148 Only 12 replicate bottles were prepared for the control treatment, as there was no
149 carvone degradation to monitor. All bottles were loosely capped and stored at 19 ºC in
150 a temperature-controlled room in random order determined by true random number
151 generation (Haahr, 2006). A new order was established after each sampling occasion.
152
153 Sampling procedure
154 Samples for chemical analysis were taken on a daily basis for the first 7 days of the
155 study to record the rate and extent of carvone degradation (Table 1). Carvone analysis
156 was achieved by transferring 5 mL of water from each of the 12 replicate bottles into
157 a 20 mL glass vial with a PTFE-lined cap and frozen until further analysis (see
158 Chemical Analysis section). A minimum of 4 replicates from each of the bottles used
159 for chemical analysis from each treatment were also monitored for dissolved O_2
160 concentration with a Multiline P3 oxygen probe (WTW, Weilheim, Germany) to aid
161 in assessing the extent of microbial activity, as the concentration of dissolved oxygen
162 negatively correlates with the extent of aerobic bacterial activity in the bottles.
163
164 Microbial communities were sampled on 6 time points throughout the study. The first
165 four time points were located within the first 7 days and aimed to characterize the rate
166 of carvone degradation. The fifth sampling point (day 12) was set for 5 days after the
167 fourth sampling point (day 7) to allow the microbial communities to adapt to a

situation in which only residual organic carbon sources were available. The sixth sampling point (day 22) was set for one week after the cross-over, where the alternative carvone isomer was added so as to assess the extent of carvone degradation. The sampling regime in this study was determined fluidly, based on data generated from the previous sampling point to maximize the relevance of each sampling.

Five-mL aliquots of water from all treatments were passed through a 47-mm diameter, 0.2 μm pore filter (Millipore GSWP04700), which was frozen at -80 ºC until analysis. Particular attention was given not to disturb the samples, as the surface was sometimes, but not always covered in a biofilm while the bottom of the bottle occasionally collected precipitated organic matter and sediment. As not all pseudoreplicates developed a biofilm or bottom sediments all aliquots for molecular microbial community analysis were acquired from the middle zone (i.e., mid-depth) of each bottle. Negative controls were taken by filtering 5 mL sterile water in the same manner.

On day 13 (one day after the 5^{th} sampling point) the communities within each of the replicates were pelleted by centrifugation (14,000g, 40 min, 4°C, JA-20 rotor, Beckman J2-HS centrifuge). They were re-suspended in new bottles holding 85 mL of the autoclaved river water that was frozen at the beginning of the experiment to which 20 μg g^{-1} of the alternative carvone isomer was added (see Table 1). The cross-over was employed to assess the functional diversity of the communities. Chemical analysis was subsequently carried out on days 14, 17, 18 and 20, to measure the rate at which the carvone was removed. On day 22 final samples were taken for both chemical and community analysis. The control treatment was treated identically to the carvone treatments, less the addition of carvone.

Protist abundance

Samples from four randomly chosen replicate bottles from each treatment used for chemical analysis as well community analysis and from the controls were studied under the microscope on days 1, 4, 7, 13, 15, and 22. Protozoa were quantified on

200 days 13 and 15, and 22 from a 16 µL aliquot using a Nikon Phase Contrast
201 microscope 0.90 dry (40x magnification) with a counting chamber (Fuchs Rosenthal,
202 0.2mm deep).
203
204 Chemical analysis
205 A liquid-liquid extraction was employed for recovering carvone from the river water
206 samples. Briefly, 5 mL of ethyl acetate containing 20 µg g^{-1} α-pinene as internal
207 standard were added to each 5 mL sample in a 15 mL glass vial with a PTFE-lined
208 cap. The sample vials were shaken over night to extract carvone into the organic
209 phase. One mL of the organic phase was withdrawn into 1.8 mL vials and analyzed
210 using a Perkin-Elmer Autosystem XL gas chromatograph fitted with a flame
211 ionization detector (FID) and an Agilent DB-5 ms capillary column (30 m length ×
212 0.25 µm film thickness × 0.25 mm ID). A 3 µL injection was analysed with the
213 following instrument parameters: injector and detector temperature 280 °C, He carrier
214 at 1 mL min^{-1}, and a 40 mL min^{-1} split. The temperature program was, initially, 80 °C
215 held for 1 min, ramped at 10 °C min^{-1} to 110 °C followed by a ramp of 25 °C min^{-1} to
216 260 °C.
217
218 DNA preparation and LH-PCR analysis
219 The frozen filter papers were cut into 3 to 5 mm wide strips and placed in bead
220 beating tubes (Q-BIOgene lysing matrix E, BIO 101 systems) containing 1 mL of
221 CTAB DNA extraction buffer (consisting of 100mM Tris-Cl, 100mM NaEDTA, 100
222 mM phosphate buffer, 1.5M NaCl, 1% CTAB, pH 8.0). In addition to the negative
223 controls taken at the sampling stage, control blanks were produced by extracting from
224 an empty bead beating tube. The tubes were incubated for 30 min in a 65 °C water
225 bath. They were subsequently shaken for 30 sec at setting 5.5 in a FASTprep bead
226 beating machine (Bio 101, Vista, California), cooled and centrifuged for 10 min at
227 14,400 × g at 4 °C. The supernatant was transferred to new 1.5 mL microcentrifuge
228 tubes and 1 volume of 24:1 chloroform:isoamyl alcohol (IAA) added. The tubes were
229 centrifuged for 5 min at the same settings noted earlier. The aqueous phase was
230 transferred to new 1.5 mL microcentrifuge tubes, 0.6 volumes of isopropanol added to
231 precipitate DNA, and the tubes left over night at 4 °C. The next day the tubes were

centrifuged for 10 min as noted earlier, the supernatant was removed and the DNA pellet washed with 200 µL cold 70% ethanol. The tubes were centrifuged for a further 10 min as noted earlier, the supernatant removed and the remaining DNA pellet dried for 30 min in a laminar flow cabinet. Lastly, the DNA was re-suspended over 2 h in deionized water. The extraction product was analyzed by electrophoresis (ECPS 3000/150, Pharmacia, New Jersey, USA) in a 1% agarose gel containing ethidium bromide (Sigma-Aldrich, Gillingham, UK), photographed in a Geneflash image analyser (Syngene, Cambridge, UK) to determine if DNA was present and thereafter frozen at -20° C.

LH-PCR of 16S rDNA was carried out using a BF1 forward primer (nucleotide sequence TCA GA(A/T) (C/T)GA ACG CTG GCG G) labelled with Beckman D4 fluorescent dye (Proligo, France) and 530R reverse primer (GTA TTA CCG CGG CTG CTG) (Proligo, France). The thermal cycle consisted of 95 °C, 2 min; then 34 repeated cycles of 95 °C, 1 min; 60 °C, 2 min; 72 °C, 1.5 min, followed by 30 min at 72 °C. The PCR product was purified by filtration through Sephadex® (Sigma-Aldrich, Gillingham, UK) in a 96-well filter plate (Millipore, Watford, UK) as per manufacturer's instructions. Electrophoresis in a 1.2% agarose gel was used to confirm the presence of a PCR product and the absence of contamination. As the products were often weak, the magnesium ion concentration in the reaction mixture was further optimized (Ignatov*, et al.*, 2003). The optimized 50 µL reaction volumes contained 0.1 µL of each primer, 50 µM of each dNTP (Bioline), 1 unit Taq polymerase in 1 × PCR buffer (both Sigma) to which was added 2 µL of 50 mM MgCl and between 1 to 4 µL template DNA, depending on the strength of the PCR product. Materials, except for the active ingredients, were exposed to UV radiation for 2 min in a UV crosslinker (Hoefer, San Francisco, USA).

Both positive and negative controls were run alongside the DNA templates. Negative controls included those from the sampling stage, the DNA extraction and from the PCR stage, during which sterile deionized water was used instead of the DNA product. Analysis of fragment length was carried out with a Beckman Coulter CEQ2000 sequencer, using 1 µL of the PCR amplified 16S rDNA in 35 µL deionised

264 formamide and 0.5 μL of CEQ 600 bp DNA size standard (Beckman Coulter,
265 Fullerton, USA). Samples for which no DNA could be detected were re-analysed.
266
267 Statistical analysis
268 OTU detection and fragment size determination of the LH-PCR products were carried
269 out using CEQ8000 (Beckman Coulter, Fullerton, USA) and GeneMarker v 1.6
270 (Biogene Ltd, Kimbolton, UK) sequencing software. The fragments were
271 automatically aligned via the software's binning analysis function to remove
272 imprecision in the software's interpolation of size standards and fragment lengths
273 without introducing bias through manual alignment (Rees*, et al.*, 2004, Hewson &
274 Fuhrman, 2006). The negative controls which had been successfully analyzed showed
275 some bands (≤ 200 relative fluorescent units (RFU)) up to 374 bp but not beyond.
276 Most replicates showed bands between 450 bp and 520 bp which is consistent with
277 findings of Whiteley *et al.* (2003) using the same set of primers. Only fragments in
278 the range of 400 to 560 bp were used in defining microbial community structures
279 (Whiteley*, et al.*, 2003).
280
281 The band intensity and fragment length data thus obtained was imported into
282 Microsoft Excel where absolute band intensities were converted to show relative
283 abundance to standardize the data (Rees*, et al.*, 2004). Peaks lower than 1% were
284 subsequently removed to further reduce bias caused by the amount of PCR product
285 added (Rees*, et al.*, 2004). The resulting data set was used for the statistical analysis.
286
287 Analysis of variance including Tukey tests and correlations were performed in SPSS
288 (SPSS Inc.). Past v. 1.4.8 (Hammer, et al., 2001) was used for Analysis of similarity
289 (ANOSIM)—a non-parametric test for significant differences between two or more
290 groups (Clarke, 1993). ANOSIM produces a statistic, R, which indicates the
291 magnitude of difference among groups of samples. If $R>0.75$ the groups are well
292 separated, if $R>0.5$ they are overlapping, but clearly different, if $R<0.25$ the groups
293 are barely separable (Clarke & Gorley, 2001). As part of the analysis, the statistical
294 significance of R is tested by Monte Carlo randomization. PCA and cluster analysis
295 were done in Past v. 1.4.8 and MSVP v. 3.1.3 (Kovach Computing Services).

Distance measures for the multivariate analyses are based on the Bray-Curtis similarity index (Clarke, et al., 2006). Diversity was measured in Past v. 1.4.8 with the Parker-Berger index showing a proportional representation of the most dominant species per sample.

Samples will be referred to as either [Treatment/Replicate] such as R1 to denote 'treatment *R*-carvone, replicate 1' or [Sampling day/Treatment/Replicate] such as D7R1 to denote 'sampling day 7, treatment *R*-carvone, replicate 1'. Samples used for chemical analysis will have the prefix 'Chem'.

Results and discussion

Dissolved oxygen concentration

The measurement of dissolved O_2 was used to determine the metabolic activity of samples, indicating when carvone degradation had begun, as a decline in the dissolved oxygen indicates metabolic activity suggestive of carvone degradation (Figure 1). Overall, the difference between the replicates which had carvone added to them and those which had not is clearly visible: the O_2 content in the replicates of the two carvone-supplemented groups decreased over the course of the experiment. By day 12 when all initial carvone had been degraded it returned to the baseline dissolved oxygen concentration (8.2 mg L^{-1}). After the cross-over (day >13), the dissolved oxygen concentration decreased again, only to return to baseline by day 22 after the carvone had been degraded (Figure 1). Notably, a number of replicates in both treatments reacted immediately upon the addition of the alternate isomer of carvone (day 14), as is shown in Figure 1. There was little change in the dissolved oxygen concentration in the control group up until the cross-over, at which point a number of the controls demonstrated a decline in dissolved oxygen. The observed decline in the controls was likely the result of residual labile dissolved organics in the archived river water used to resuspend the communities. When oxygen content measurements were correlated to the residual carvone data, significant results were obtained for both *R*-carvone (Kendall's tau, $p = 0.02$), and *S*-carvone (Pearson Correlation, $p = 0.01$)

328 Protists and grazing activity
329 Protist abundance was measured to gain insight into the potential effect of protozoan
330 grazing on microbial populations and the effect of microbial populations differentially
331 supporting the growth of protists. Protist numbers were estimated twice: 1) after
332 carvone had been degraded for 1 week (day 13 and 15), and 2) shortly after the cross-
333 over when carvone degradation had completed for the second time (day 22). On the
334 first count (day 13 and 15), the *R*-carvone samples yielded 551 protists mL^{-1}, the *S*-
335 carvone samples 504 protist mL^{-1} and the controls 104 protists mL^{-1}. On the second
336 count (day 22) the number of protists mL^{-1} in the *R*-carvone treatment was 3344, in
337 the *S*-carvone treatment 3328 and in the control 395.
338
339 Whilst there were no significant differences between the two carvone treatments, the
340 total number of protists in the carvone treatments was higher than in the control. The
341 second count showed not only a marked increase in protists, but also a significantly
342 wider margin between the number of protists in the carvone replicates and in the
343 control ($P < 0.01$, ANOVA). The greater abundance of protists in the carvone
344 treatments suggests that carvone served as a growth substrate for the indigenous
345 microorganisms, which in turn supported an increase in protists. The ability of the
346 protists to rapidly respond to an increase in bacterial abundance could have
347 contributed to the unusually low recovery of bacterial DNA from timepoints
348 immediately following the removal of carvone (days 7, 12, 22). Jaquet *et al.* (2005)
349 report that numbers of protozoa, comparable to that in the carvone treatments,
350 removed up to 7.8% of bacteria in 24 h whereas Domaizon *et al.* (2003) describe per
351 capita grazing rates from 1.2×10^3 to 5.1×10^6 bacteria l^{-1} h^{-1} for heterotrophic
352 flagellates and from 4.8×10^6 to 6.8×10^7 bacteria l^{-1} h^{-1} for mixotrophic flagellates.
353 Hence, grazing by protists might have masked the otherwise rapid increase in
354 microbial growth upon the addition of carvone.
355
356 The protozoa most often found in the samples were identified as typical freshwater
357 species such as *Amoeba sp.* (Sarcodina), *Actinosphaerium sp*. (Heliozoa), *Peranema*
358 *sp.* (Flagellates), *Vorticella sp*, *Coleps sp.* and *Colpoda maupasi* (Cilliates) (Patterson
359 & Hedley, 1996). Both flagellates and ciliates have been observed to significantly

360 reduce bacterial populations (Huws*, et al.*, 2005, Jacquet*, et al.*, 2005, Sonntag*, et al.*,
361 2006).

362

363 Chemical analysis

364 The rate of carvone utilization after the cross-over was significantly faster than after
365 the first carvone addition (*p=0.008*; days 4 to 5 versus days 16 to 17 (Figure 2)). This
366 was also supported by dissolved oxygen measurements for those days: on day 4 the
367 mean dissolved oxygen concentration for both carvone treatments was 8.08 mg L^{-1}, on
368 day 16 it was 7.3 mg L^{-1}. It must therefore be concluded that in the majority of
369 samples at least part of the bacterial community consisted of organisms that were able
370 to degrade both enantiomers (van der Werf, 2000). Two replicates, ChemS2 and
371 ChemR1, demonstrated very slow degradation of *R*-carvone and *S*-carvone,
372 respectively, before the cross-over. However, after the cross-over, these replicates
373 were among the first to degrade the alternative isomer, indicating that the carvone
374 degrader(s) within these treatments might have been highly enantioselective (Figure
375 3).

376

377 Community analysis – general patterns

378 To analyze and assess shifts in the phylogenetic structure of the bacterial community
379 in the two treatments and the control replicates, samples were taken both before the
380 addition of carvone (day 0) and at the time points described in Table 1. Analysis of
381 the microbial community by LH-PCR revealed a baseline community at day 0 (Figure
382 4) with several major bands representing OTUs ranging from 440 base pairs (bp) to
383 510 bp, suggesting a community of several comparably abundant organisms. A
384 similar community structure was found in the control replicates throughout the study.
385 On day 1 a comparable fingerprint could also be observed for *R*- and *S*-carvone
386 treatments. Over the course of the experiment, however, the carvone treated replicates
387 diverged from the community structure seen in day 0 as well as from the controls. The
388 majority of the *R*-carvone replicates showed a pattern dominated by an intense band
389 around 452 bp (Figure 6) and/or a broad band around 504 bp, whereas the *S*-carvone
390 replicates displayed three different pattern types: (I) band around 508 bp (Figure 4,
391 day 7a); (II) bands around 452 and 504 bp, similar to the majority of *R*-carvone

samples (Figure 4, day 7b), and (III) bands around 452, 500 and 506 bp, similar to the controls (Figure 4, day 7c).

A principal components analysis (PCA) for day 7 (explaining 30% of the variance in the data set) shows that in both treatments 7 of 12 replicates showed similar within-treatment fingerprints, but highly dissimilar fingerprints between treatments (Figure 5). The remaining *R*- and *S*-carvone replicates from day 7 clustered with the controls. The analysis of the three *R*-carvone samples clustering with the controls did not produce any discernible bands due to insufficient DNA yield, which potentially explains their 'outlier' status on the PCA plot.

The community development of a single replicate (R6) from the *R*-carvone treatment is shown in Figure 6, highlighting the dynamic nature of the river water system. Of particular interest was that after the cross-over, the newly developed intense bands (Figure 6, day 22) were more akin to the banding patters that could be seen in many of the *S*-carvone replicates (Figure 4, day 7b), indicating that the capacity to catabolise both enantiomers of carvone was not always harbored in the same OTU. This result suggests that the capacity to respond to subtly different chemical perturbations might require a significant shift in the dominant microorganisms in the river water.

The significance attributed to the changes in the observed community patterns was provided by ANOSIM (Clarke, 1993), which generates an R-value indicating dissimilarity if close to 1 and similarity if close to 0 (Clarke & Gorley, 2001). Comparing the treatment groups to each other at each time point often showed that differences between the groups were not very marked. On day 7 ANOSIM analysis produced the following R-values: 0.23 for *R*-carvone compared to control, 0.16 for *S*-carvone compared to control and 0.33 for *R*-carvone compared to *S*-carvone (all R-values were significant). These results mean a) that both the two carvone treatments are more similar to the control than they are to each other, and b) that the difference between the *R*-carvone and *S*-carvone samples was not very marked (Clarke, 2001). This similarity might be attributed to the high within-treatment variability or to the often low DNA yields in both carvone treatments and in the controls. A more

424 insightful use of ANOSIM was to analyze the replicates over time (Chapman and
425 Underwood, 1999). In this way it could be shown that the *R*-carvone treatment, in
426 particular, went through clear cycles of community structure divergence and
427 convergence.
428
429 The R-values displayed in Table 2 describe growing divergence during the process of
430 carvone degradation. On day 12 the communities had become more similar to the
431 initial (day 1) communities again, suggesting that a community reverts back to a
432 similar community structure when it becomes starved for carbon. After the cross-over,
433 the communities once again took on the structure of one of the carvone degrader
434 communities. The development is less pronounced in the *S*-carvone treatment
435 presumably because instead of one predominant community pattern at the peak
436 degradation activity there were three (Figure 4). The *S*-carvone treatment appeared to
437 have been predominantly degraded by at least two different organisms as opposed to
438 one (as shown by two bands at 454 bp and 502 bp respectively in Figure 4, day 7,
439 denoted 'b'). Many of the bands present in the day 7 replicates were also noted at day
440 1 and the statistical method used might have not been able to detect any further
441 differences.
442
443 To investigate diversity, the Parker-Berger index was calculated for all treatments on
444 sampling days 7 and 22. The index provides information about the most dominant
445 species and calculates the percentage an OTU is present in the data set. On day 7, the
446 dominant OTU made up 42, ~60 and 44% of the total microorganisms in the *S*-
447 carvone, *R*-carvone and control treatments, respectively. On day 22, the dominant
448 OTU made up 44, ~56 and 72% of the total microorganisms in the *S*-carvone, *R*-
449 carvone and control treatments, respectively. The low diversity of the control
450 treatment can be explained by weak PCR products which produced often just one or
451 two bands of a relatively low height. At days 7 and 22, the *R*-carvone treatment
452 exhibited lower diversity than the *S*-carvone treatments, suggestive of unequal
453 functional diversity within the *R*- and *S*-carvone degrader populations.
454
455 Functionality

Degradation rates for the *S*-carvone treatment were apparently not related to one particular community make-up but rather to the number of bacteria in the individual samples. Whilst a direct comparison of DNA mass between the different sample bottles was not possible in this study, matching of the measured dissolved O_2 content with the community analysis results showed that high relative fluorescence unit (RFU) values coincided with fast degradation. S1 and S3, the samples in which, according to dissolved O_2 content, degradation set in first, had distinct and intense bands around 508 bp (Type 1) and 454 bp (Type II), respectively. Samples S8 and S10, two *S*-carvone replicates whose dissolved O_2 content was not observed to change until late into the experiment, displayed a range of low intensity bands (up to 1500 RFU), suggestive of a more diverse community on days 5 and 7. However, on day 12 these had given way to the less diverse patterns and more intense bands (up to 25000 RFU) seen in the fast degrading replicates.

The same could be seen in the *R*-carvone treatment. The samples R10, and R3 in which rapid degradation took part showed community fingerprints with intense bands even on early sampling days, whereas replicates which had a persistently high dissolved O_2 content (such as R1 and R7) had markedly low intensity bands. It seems therefore likely that slow degradation was related to low bacterial counts in the sample bottles.

PCA of the treatments grouped by sampling day frequently showed clusters of rarely more than six replicates of the same treatment. Notably, little inference could be made into functionality (i.e., carvone degradation rate) based on these clusters. The replicates D7S1 and D7S3 on the PCA scatter plot (Figure 5) in which carvone was degraded fastest are a good example for this: though functionally very similar their microbial communities are among the least alike. D7S8 and D7S10 on the other hand are positioned close to D7S1 even though the carvone in S8 and S10 was degraded slowly, whereas S1 harbored a fast degrading community. Many similar examples suggested that PCA of the sampling days was of limited use to understand functionality.

After the cross-over (day 22), replicates S10 and S2 developed a community structure resembling that of *R*-carvone, while replicates R6 and R7 developed a community structure resembling that of *S*-carvone. Replicates R6 and R12 (which had a very pronounced R-community pattern) were unable to completely degrade the *S*-carvone by the termination of the study on day 22, whereas former *S*-carvone communities had all degraded the *R*-carvone shortly after the cross-over. In conclusion, the main *R*-carvone degrading microorganism(s) (450 to 454 bp) appeared enantioselective, whereas the *S*-carvone degrading microorganism(s) (502 to 508 bp) appeared to have racemic catabolic capacity. The ability to utilize both carvone isomers might explain why 11 of 12 replicates of *R*-carvone had at least a small band between 502 and 508 bp, indicative of the racemate-utilising OTU. It seems likely that the racemate utilizer might be less efficient at *R*-carvone utilization than *S*-carvone, explaining the dominance in one treatment versus another, yet its presence in both. To highlight the importance of the racemate-utilizing OTU, the gel image of R6 on day 22 (Figure 6, day 22) shows an abundant OTU between 450 to 454 bp (denoted by a bright white band) had been replaced with an abundant OTU at 502 bp. Similarly, the chromatograph of R12 on day 22 shows an abundant OTU between 502 and 508 bp and an abundant OTU at 450 bp, suggesting that the reason for the slow degradation had been insufficient numbers of *S*-carvone degraders.

Identification of possible degraders

The community structures that developed over the course of the experiment as described in the previous sections suggest that the dominant likely degrader in the *R*-carvone treatment, found between 450 and 456 bp, was enantioselective and could not degrade *S*-carvone. Therefore replicates that had developed a structure in which this likely degrader was very dominant were slow to re-adjust when the isomers were reversed.

The dominant likely degrader in the *S*-carvone treatment, found between 500 bp and 508 bp, could degrade both *S*- and *R*-carvone. This seems evident because bands at 508 bp were prominent in many replicates of both treatments, and they appeared in former *R*-carvone treatment replicates in which they were not detectable at earlier timepoints but they never disappeared totally in the former *S*-carvone treatment. The

presence of this likely degrader allowed for a less hierarchical structure in as much as other OTU bands were also prevalent in *S*-carvone (before and after the cross-over), thus indicating that this organism might in fact have facilitated carvone degradation for other bacteria. One possible bacterium that fits this profile would be *Rhodococcus erythropolis* DCL14 which can degrade both carvone enantiomers by cleaving the ring-structure allowing other bacteria to process the product (van der Werf, 2000). However, this hypothesis was not tested experimentally.

The initial microbial communities at day 1 and in the controls changed to a more hierarchical community structure with fewer and more dominating OTU in the presence of carvone. There was considerable within-treatment variability between replicates. No significant differences exist between the rates of degradation of carvone in the different treatments before the cross-over, however, after the cross-over the *S*-degrading communities generally degraded *R*-carvone more efficiently than *R*-carvone utilisers degraded *S*-carvone. Therefore, whilst the fast on-set of degradation in most of the replicates after isomer-reversal shows that the initial addition of carvone induced the development of bacterial communities which readily utilized carvone of both types when it was added again, there was nevertheless diversity in function between the two treatment-groups.

It is likely that the observed variability in the extracted DNA and PCR products during the study is a reflection of grazing activity by protists. Predation brings about considerable changes to bacterial population size and structure (Boenigk & Arndt, 2002, Sherr & Sherr, 2002, Corno & Jürgens, 2006). Corno and Jürgens (2006) describe how in chemostats including grazers and sufficient carbon substrate, filamentous bacteria, too long to be grazed, were dominant. In carbon starved environments containing grazers (as in the current experiment towards the later sampling days), the bacteria found when samples were observed under the microscope were, however, not filamentous. The predominant bacterial morphology of the carvone degrader might have been easily bioaccessible to the protist grazers, leading to a rapid decline in bacterial abundance. Corno and Jürgens (2006) also observed the lack of freely suspended bacteria, a fact equally noted in other studies (Jürgens &

552 Sala, 2000). As samples for the current experiment were taken from the middle depth
553 of the water column, a lack of freely suspended bacteria would have led to very weak
554 DNA extraction products as there would have been relatively low DNA recovery.
555 Preferential grazing or the absence thereof might have also brought about differences
556 in community composition. Some have described how the absence of protists allowed
557 for the dominance of previously rare species (Suzuki, 1999) and it is assumed by
558 some that protist grazing pressure contributes to bacterial diversity (Boenigk & Arndt,
559 2002) in as much as the most active bacteria are preferentially grazed thus allowing
560 less dominating groups to coexist (Kent*, et al.*, 2006). Kent *et al.* (2006) in an
561 experiment to explore mechanisms causing community shifts in a humic lake, name
562 top-down pressure as the most important factor to structure bacterial communities.
563 Hence, protistan grazing could have controlled the absolute population size of the
564 most dominant species, thereby increasing the likelihood of recovering less dominant
565 community members.
566
567 As a majority of monoterpenes are released from plants and plant detritus between
568 spring and autumn (Llusia & Penuelas, 2000, Hellen*, et al.*, 2006), it is likely that the
569 river water in this study, acquired in June, might contain a microbial community more
570 readily able to perform carvone degradation than a community extracted from river
571 water in the winter. This might explain why some OTUs present on day 0 are
572 consistent with OTUs present in the fully developed carvone degrading communities.
573 Jaspers et al (2001) found that bacterial strains which were abundant one month could
574 not be isolated only one month later, suggesting the same study presented here might
575 yield considerably different results if initiated in a different season, when secondary
576 plant metabolites are less abundant.
577
578 Conclusions
579 To return to our original hypotheses, there was: 1) enrichment of enantiomer-selective
580 degraders; 2) enrichment of racemic degraders; and 3) enrichment of both
581 enantiomer-selective and racemic degraders. Recognition of the range of outcomes
582 possible from a homogenized river water sample was clearly great and would not
583 have been evident without the many replicate vials employed in this study. The study

584 has shown great within-treatment variability and the presence of a dynamic bacterial
585 community system changing rapidly between structures dominated by very few
586 species in the presence of carvone to more diverse community structures when
587 carvone was depleted. Functional diversity was evident during the first carvone
588 amendment, but became more evident after the cross-over, largely manifested as the
589 immediate removal of carvone in non-enantioselective populations, whereas carvone
590 removal was less rapid in highly-enantioselective populations. Notably, despite both
591 degrader phenotypes being present in both treatment groups, the overall rate of
592 carvone degradation varied considerably after the cross-over. Protist grazing likely
593 impacted the abundance of carvone utilisers, but it remains unclear as to the role
594 protists play in parameterizing microbial community function. Further study on
595 protist-free river water using a similarly designed study as this one, may well lend
596 some insight into these important trophic interactions.

References

[1] Amaral JA & Knowles R (1998) Inhibition of methane consumption in forest soils by monoterpenes. *Journal of Chemical Ecology* **24**: 723-734.

[2] Amaral JA, Ren T & Knowles R (1998) Atmospheric methane consumption by forest soils and extracted bacteria at different pH values. *Applied and Environmental Microbiology* **64**: 2397-2402.

[3] Boenigk J & Arndt H (2002) Bacterivory by heterotrophic flagellates: community structure and feeding strategies. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **81**: 465-480.

[4] Button DK (1984) Evidence for a terpene-based food chain in the Gulf of Alaska. *Applied and Environmental Microbiology* **48**: 1004-1011.

[5] Clarke K & Gorley R (2001) *PRIMER v5: User manual*. Primer-E, Plymouth, UK.

[6] Clarke KR (1993) Nonparametric Multivariate Analyses of Changes in Community Structure. *Australian Journal of Ecology* **18**: 117-143.

[7] Clarke KR, Somerfield PJ & Chapman MG (2006) On resemblance measures for ecological studies, including taxonomic dissimilarities and a zero-adjusted Bray-Curtis coefficient for denuded assemblages. *Journal of Experimental Marine Biology and Ecology* **330**: 55-80.

[8] Corno G & Jürgens K (2006) Direct and indirect effects of protist predation on population size structure of a bacterial strain with high phenotypic plasticity. *Applied and Environmental Microbiology* **72**: 78-86.

[9] de Carvalho CCCR & da Fonseca MMR (2006) Carvone: Why and how should one bother to produce this terpene. *Food Chemistry* **95**: 413-422.

[10] DeFrank JJ & Ribbons DW (1977) p-Cymene pathway in *Pseudomonas putida*: ring cleavage of 2,3-dihydroxy-p-cumate and subsequent reactions. *Journal of Bacteriology* **129**: 1365-1374.

[11] DeFrank JJ & Ribbons DW (1977) p-cymene pathway in *Pseudomonas putida*: initial reactions. *Journal of Bacteriology* **129**: 1356-1364.

[12] Dercova K, Tandlich R & Brezna B (2003) Application of terpenes as possible inducers of biodegradation of PCBs. *Fresenius Environmental Bulletin* **12**: 286-290.

[13] Domaizon I, Viboud S & Fontvieille D (2003) Taxon-specific and seasonal variations in flagellates grazing on heterotrophic bacteria in the oligotrophic Lake Annecy - importance of mixotrophy. *Fems Microbiology Ecology* **46**: 317-329.

[14] Duetz WA, Bouwmeester H, van Beilen JB & Witholt B (2003) Biotransformation of limonene by bacteria, fungi, yeasts, and plants. *Applied Microbiology and Biotechnology* **61**: 269-277.

[15] Duetz WA, Fjallman AHM, Ren SY, Jourdat C & Witholt B (2001) Biotransformation of D-limonene to (+) trans-carveol by toluene-grown Rhodococcus opacus PWD4 cells. *Applied and Environmental Microbiology* **67**: 2829-2832.

637 [16] Eaton R (1997) p-Cymene catabolic pathway in *Pseudomonas putida* F1: cloning
638 and characterization of DNA encoding conversion of p-cymene to p-cumate. *J.*
639 *Bacteriol.* **179**: 3171-3180.

640 [17] Eaton RW (1996) p-Cumate catabolic pathway in Pseudomonas putida Fl:
641 cloning and characterization of DNA carrying the cmt operon. *Journal of*
642 *Bacteriology* **178**: 1351-1362.

643 [18] Gilbert ES & Crowley DE (1997) Plant compounds that induce polychlorinated
644 biphenyl biodegradation by Arthrobacter sp. strain B1B. *Applied and Environmental*
645 *Microbiology* **63**: 1933-1938.

646 [19] Guenther A, Hewitt CN, Erickson D*, et al.* (1995) A global model of natural
647 volatile organic compound emissions. *Journal of Geophysical Research-Solid Earth*
648 **100**: 8873-8892.

649 [20] Haahr M (2006) Random.org true random number service.
650 http://www.random.org.

651 [21] Hammer Ø, Harper DAT & Ryan PD (2001) PAST: Paleontological Statistics
652 Software Package for Education and Data Analysis. . *Palaeontologia Electronica* **4**.

653 [22] Harms G, Rabus R & Widdel F (1999) Anaerobic oxidation of the aromatic plant
654 hydrocarbon *p*-cymene by newly isolated denitrifying bacteria. *Archives of*
655 *Microbiology* **172**: 303-312.

656 [23] Hartmans KJ, Diepenhorst P, Bakker W & Gorris LGM (1995) The use of
657 carvone in agriculture: sprout suppression of potatoes and antifungal activity against
658 potato tuber and other plant diseases. *Industrial Crops and Products* **4**: 3-13.

659 [24] Hellen H, Hakola H, Pystynen KH, Rinne J & Haapanala S (2006) C-2-C-10
660 hydrocarbon emissions from a boreal wetland and forest floor. *Biogeosciences* **3**: 167-
661 174.

662 [25] Hernandez BS, Koh SC, Chial M & Focht DD (1997) Terpene-utilizing isolates
663 and their relevance to enhanced biotransformation of polychlorinated biphenyls in
664 soil. *Biodegradation* **8**: 153-158.

665 [26] Hewson I & Fuhrman JA (2006) Improved strategy for comparing microbial
666 assemblage fingerprints. *Microbial Ecology* **51**: 147-153.

667 [27] Huisman J & Weissing FJ (2001) Fundamental unpredictability in multispecies
668 competition. *American Naturalist* **157**: 488-494.

669 [28] Huws SA, McBain AJ & Gilbert P (2005) Protozoan grazing and its impact upon
670 population dynamics in biofilm communities. *Journal of Applied Microbiology* **98**:
671 238-244.

672 [29] Ignatov KB, Miroshnikov AI & Kramarov VM (2003) A new approach to
673 enhanced PCR specificity. *Russian Journal of Bioorganic Chemistry* **29**: 368-371.

674 [30] Jacquet S, Domaizon I, Personnic S, Ram ASP, Hedal M, Duhamal S & Sime-
675 Ngando T (2005) Estimates of protozoan- and viral-mediated mortality of
676 bacterioplankton in Lake Bourget (France). *Freshwater Biology* **50**: 627-645.

[31] Jacquet W, Lair N, Hoffmann L & Cauchie HM (2005) Spatio-temporal patterns of protozoan communities in a meso-eutrophic reservoir (Esch-sur-Sure, Luxembourg). *Hydrobiologia* **551**: 49-60.

[32] Jaspers E, Nauhaus K, Cypionka H & Overmann J (2001) Multitude and temporal variability of ecological niches as indicated by the diversity of cultivated bacterioplankton. *Fems Microbiology Ecology* **36**: 153-164.

[33] Jürgens K & Sala MM (2000) Predation-mediated shifts in size distribution of microbial biomass and activity during detritus decomposition. *Oikos* **91**: 29-40.

[34] Kent AD, Jones SE, Lauster GH, Graham JM, Newton RJ & McMahon KD (2006) Experimental manipulations of microbial food web interactions in a humic lake: shifting biological drivers of bacterial community structure. *Environmental Microbiology* **8**: 1448-1459.

[35] Laska M, Liesen A & Teubner P (1999) Enantioselectivity of odor perception in squirrel monkeys and humans. *Am J Physiol Regul Integr Comp Physiol* **277**: R1098-1103.

[36] Llusia J & Penuelas J (2000) Seasonal patterns of terpene content and emission from seven Mediterranean woody species in field conditions. *Am. J. Bot.* **87**: 133-140.

[37] Neal C, Hilton J, Wade AJ, Neal M & Wickham H (2006) Chlorophyll-a in the rivers of eastern England. *Science of The Total Environment* **365**: 84-104.

[38] Nishio T, Patel A, Wang Y & Lau PCK (2001) Biotransformations catalyzed by cloned *p*-cymene monooxygenase from *Pseudomonas putida* F1. *Applied Microbiology and Biotechnology* **55**: 321-325.

[39] Niu C & Gilbert ES (2004) Colorimetric Method for Identifying Plant Essential Oil Components That Affect Biofilm Formation and Structure. *Appl. Environ. Microbiol.* **70**: 6951-6956.

[40] Patterson DJ & Hedley S (1996) *Free-living freshwater protozoa : a colour guide*. Wiley ; Manson ; UNSW Press, New York, London, Sydney.

[41] Rees GN, Baldwin DS, Watson GO, Perryman S & Nielsen DL (2004) Ordination and significance testing of microbial community composition derived from terminal restriction fragment length polymorphisms: application of multivariate statistics. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **86**: 339-347.

[42] Rhodes AH, Owen SM & Semple KT (2007) Biodegradation of 2,4-dichlorophenol in the presence of volatile organic compounds in soils under different vegetation types. *FEMS Microbiology Letters* **269**: 323-330.

[43] Sherr EB & Sherr BF (2002) Significance of predation by protists in aquatic microbial food webs. *Antonie Van Leeuwenhoek International Journal Of General And Molecular Microbiology* **81**: 293-308.

[44] Singer AC (2006) The chemical ecology of pollutant biodegradation. *Focus on biotechnology: Phytoremediation and rhizoremediation: theoretical background,* Vol. 9A (Macková M, Dowling DN & Macek T, ed.^eds.), p.^pp. Springer.

718 [45] Singer AC, Wong CS & Crowley DE (2002) Differential enantioselective
719 transformation of atropisomeric polychlorinated biphenyls by multiple bacterial
720 strains with different inducing compounds. *Applied and Environmental Microbiology*
721 **68**: 5756-5759.

722 [46] Singer AC, Crowley DE & Thompson IP (2003) Secondary plant metabolites in
723 phytoremediation and biotransformation. *Trends in Biotechnology* **21**: 123-130.

724 [47] Singer AC, Gilbert ES, Luepromchai E & Crowley DE (2000) Bioremediation of
725 polychlorinated biphenyl-contaminated soil using carvone and surfactant-grown
726 bacteria. *Applied Microbiology and Biotechnology* **54**: 838-843.

727 [48] Singh HP, Batish DR, Kaur S, Arora K & Kohli RK (2006) alpha-Pinene inhibits
728 growth and induces oxidative stress in roots. *Ann Bot* **98**: 1261-1269.

729 [49] Smolander A, Ketola RA, Kotiaho T, Kanerva S, Suominen K & Kitunen V
730 (2006) Volatile monoterpenes in soil atmosphere under birch and conifers: Effects on
731 soil N transformations. *Soil Biology and Biochemistry* **38**: 3436-3442.

732 [50] Sonntag B, Posch T, Klammer S, Teubner K & Psenner R (2006) Phagotrophic
733 ciliates and flagellates in an oligotrophic, deep, alpine lake: contrasting variability
734 with seasons and depths. *Aquatic Microbial Ecology* **43**: 193-207.

735 [51] Suzuki M (1999) Effect of protistan bacterivory on coastal bacterioplankton
736 diversity. *Aquatic Microbial Ecology* **20**: pp. 261-272.

737 [52] Trudgill PW (1990) Microbial metabolism of monoterpenes — recent
738 developments. *Biodegradation* **1**: 93-105.

739 [53] van der Werf MJ (2000) Purification and characterization of a Baeyer-Villiger
740 mono-oxygenase from Rhodococcus erythropolis DCL14 involved in three different
741 monocyclic monoterpene degradation pathways. *Biochemical Journal* **347**: 693-701.

742 [54] van der Werf MJ & Boot AM (2000) Metabolism of carveol and dihydrocarveol
743 in Rhodococcus erythropolis DCL14. *Microbiology* **146**: 1129-1141.

744 [55] van der Werf MJ, Overkamp KM & de Bont JAM (1998) Limonene-1,2-Epoxide
745 Hydrolase from Rhodococcus erythropolis DCL14 Belongs to a Novel Class of
746 Epoxide Hydrolases. *J. Bacteriol.* **180**: 5052-5057.

747 [56] van der Werf MJ, Swarts HJ & de Bont JAM (1999) Rhodococcus erythropolis
748 DCL14 Contains a Novel Degradation Pathway for Limonene. *Appl. Environ.*
749 *Microbiol.* **65**: 2092-2102.

750 [57] van der Werf MJ, van der Ven C, Barbirato F, Eppink MHM, de Bont JAM &
751 van Berkel WJH (1999) Stereoselective Carveol Dehydrogenase from Rhodococcus
752 erythropolis DCL14. A novel nicotinoprotein belonging to the short chain
753 dehydrogenase/reductase superfamily. *J. Biol. Chem.* **274**: 26296-26304.

754 [58] Whiteley AS, Griffiths RI & Bailey MJ (2003) Analysis of the microbial
755 functional diversity within water-stressed soil communities by flow cytometric
756 analysis and CTC plus cell sorting. *Journal of Microbiological Methods* **54**: 257-267.

757

758 Table 1: Overview over the sampling regime for both chemical and
759 community analysis.

Day 0	Addition of Carvone enantiomers and division of water into replicates (12)
Day 1	Chemical and community structure sampling
Days 2/3	Chemical sampling
Day 4	Chemical and community structure sampling
Day 5	Chemical and community structure sampling
Day 6	Chemical sampling
Day 7	Chemical and community structure sampling
Day 8/9/10	Chemical sampling
Day 11	Chemical and community structure sampling
Day 13	Extraction and resuspension of cells in sterile river water containing the carvone isomer that had not been degraded before. Controls were resuspended in sterile river water only.
Day 16, 17, 19	Chemical sampling
Day 22	Chemical and community structure sampling

760

761 Table 2. R-values generated by ANOSIM from comparing microbial communities
762 over time from each treatment to the Day 1 community.
763

	Day 1	
Sampling Day	R-carvone	S-carvone
4	0.514[a]	0.509[a]
5	0.743[a]	0.501[a]
7	0.781[a]	0.188[c]
13	0.424[b]	0.339[b]
22	0.686[a]	0.595[a]

764 [a] Represents clear differences between treatment-communities analysed at sampling day noted and
765 treatment-communities at day 1

766 [b] Represents some dissimilarities between treatment-communities analysed at sampling day noted and
767 treatment-communities at day 1

768 [c] Represents no differences between treatment-communities analysed at sampling day noted and
769 treatment-communities at day 1

770 Figure Legends

771

772 Fig. 1. Mean dissolved O_2 concentrations in the microbial community analysis bottles.
773 Four replicates per treatment were measured. Error bars mark 95% confidence
774 intervals. *S*-carvone (gray squares) and R-carvone (dark gray triangles) vary
775 considerably as the O_2 concentration responds to the microbial activity, whereas the
776 controls (light gray diamonds) stay constant, except after cross-over when residual
777 dissolved carbon sources in the freshly added river water are digested.

778

779 Fig. 2: Carvone degraded faster after the cross-over (light gray rombus/ dark gray
780 squares) than at the outset of the study (black triangles/ light gray dots) . Some *S*-
781 carvone (dark gray squares) remains unprocessed at the end of the experiment, but R-
782 carvone (light gray dots) has been removed. The y-axis denotes the amount of carvone
783 relative to what was initially added, the error bars mark 95% confidence intervals.

784

785 Fig. 3. Cavone degradation in replicates ChemS2 and ChemR1, showing rapid
786 degradation after carvone isomer reversal. The fast removal after cross-over
787 (degradation curves starting on day 13) of *S*-carvone (black) and *R*-carvone (gray)
788 respectively may show enantioselective degraders.

789

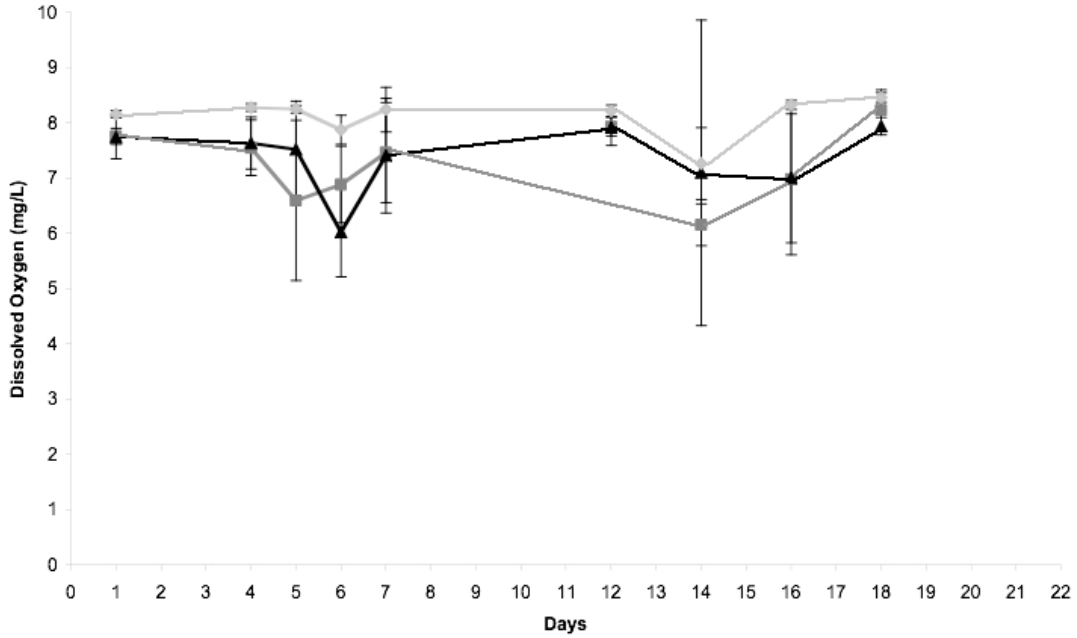
790 Fig. 4. Typical LH-PCR microbial community fingerprints as seen before the addition
791 of carvone on day 0, followed by the three different patterns of *S*-carvone
792 communities at D7. Pattern 1 (day 7a) has an abundant OTU around 508 bp, (day 7b)
793 is similar to the *R*-carvone structure and (day 7c) resembles more the diversity of day
794 0. Lighter coloured bands correspond to fluorescence intensity, representing fragment
795 abundance

796

797 Fig. 5. Principal component analysis of binary encoded bacterial communities
798 acquired from the LH-PCR profile for the *R*-carvone (triangle), *S*-carvone (square)
799 and control (diamond) treatments on day 7 after most of the carvone had been

consumed. Only 30% of the variance in the data set can be explained by the PCA1 and PCA2.

Fig. 6. Time series of bacterial community LH-PCR in sample R6 on day 1, day 4, day 5, day 7, day 12, and day 22 of the experiment. The replicate had received *R*-carvone first, and had *S*-carvone added after the cross-over. The subsequently developing pattern (day 22) is akin to earlier observed *S*-carvone patterns. The brighter the band, the more abundant is the OTU.


Dissolved Oxygen (mg/L)
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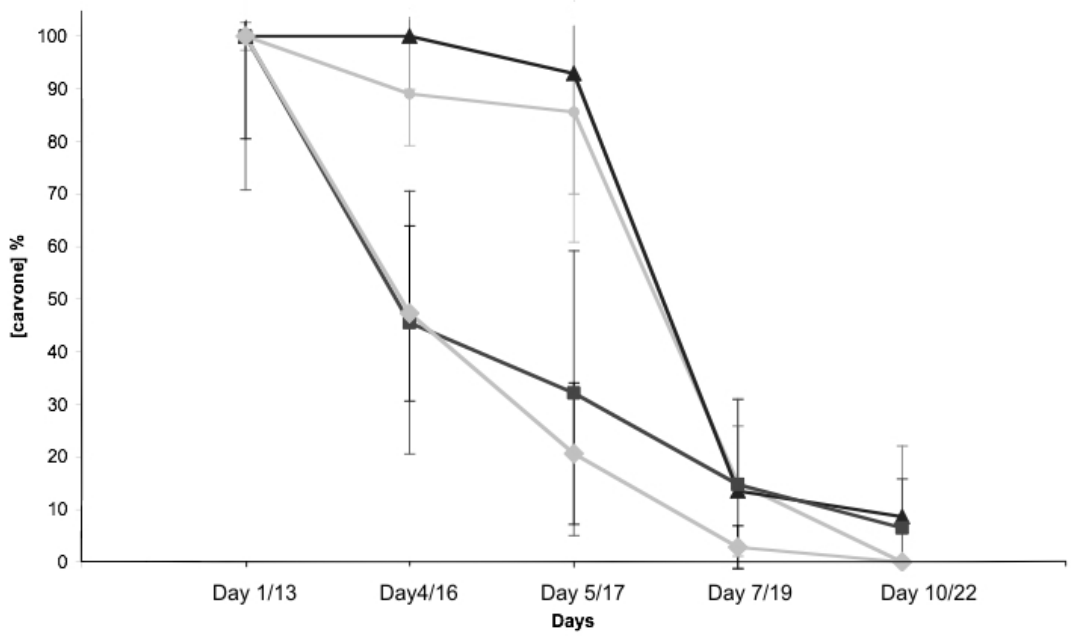
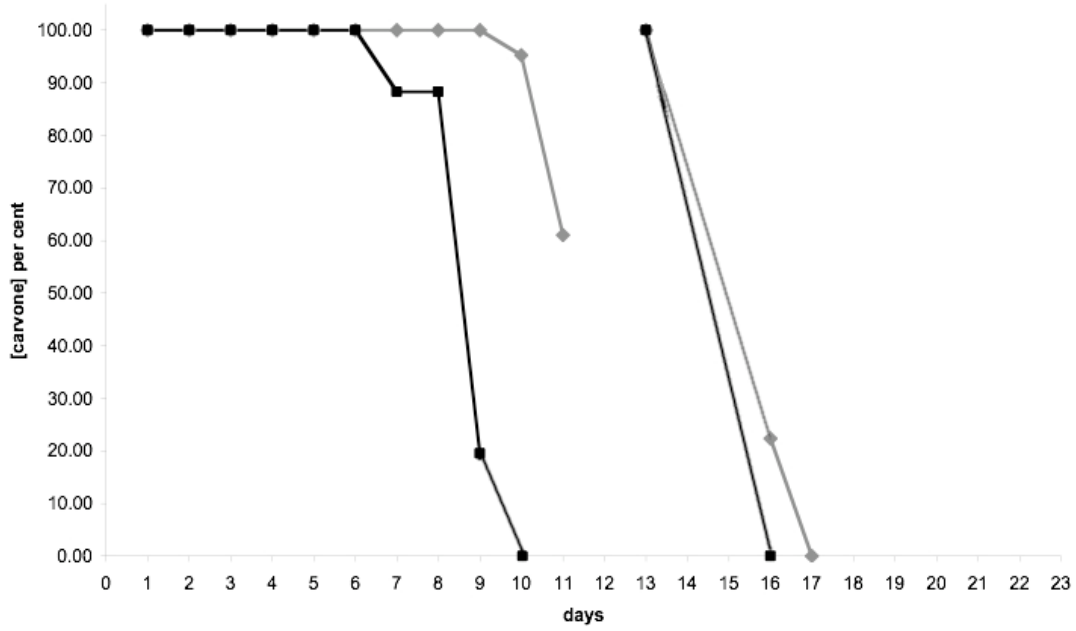
810

811 Fig.1.

812

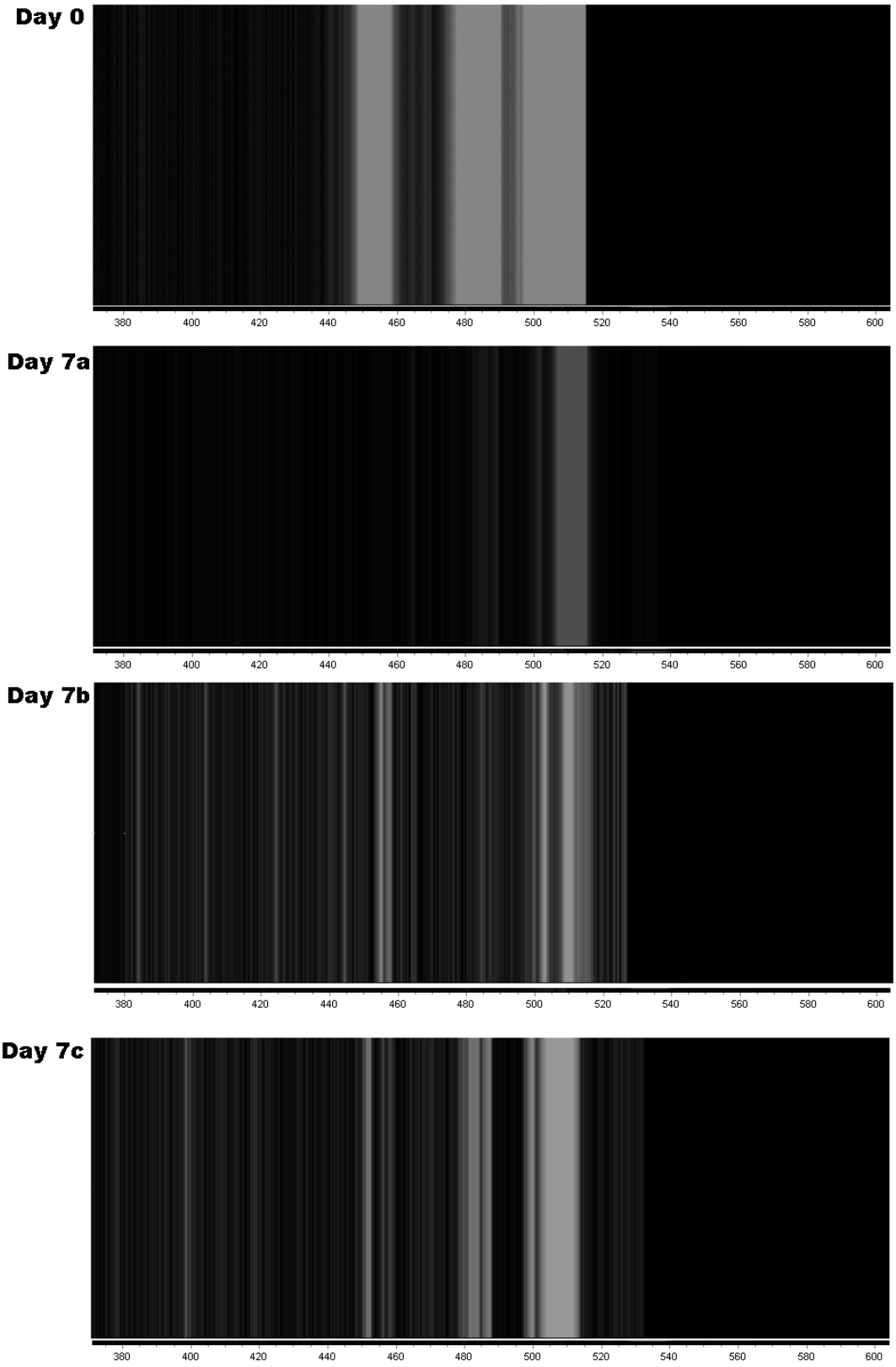


813
814 Fig. 2.



815
816 Fig. 3.

817


Day 0
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Day 7a
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Day 7b
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Day 7c
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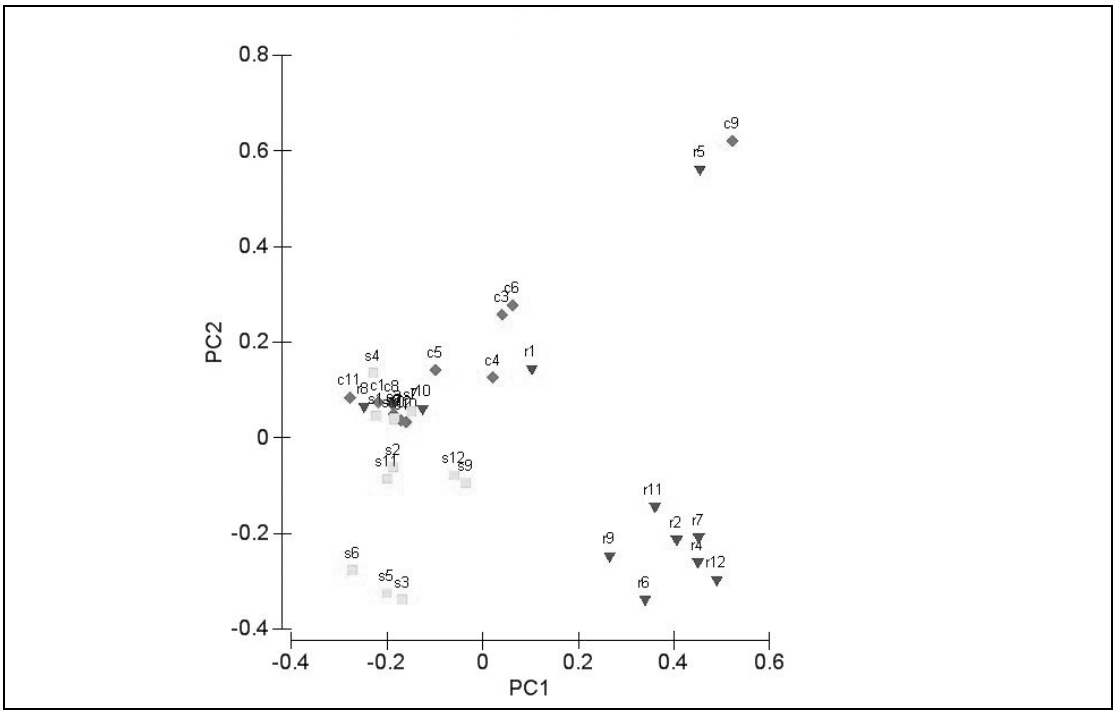
818

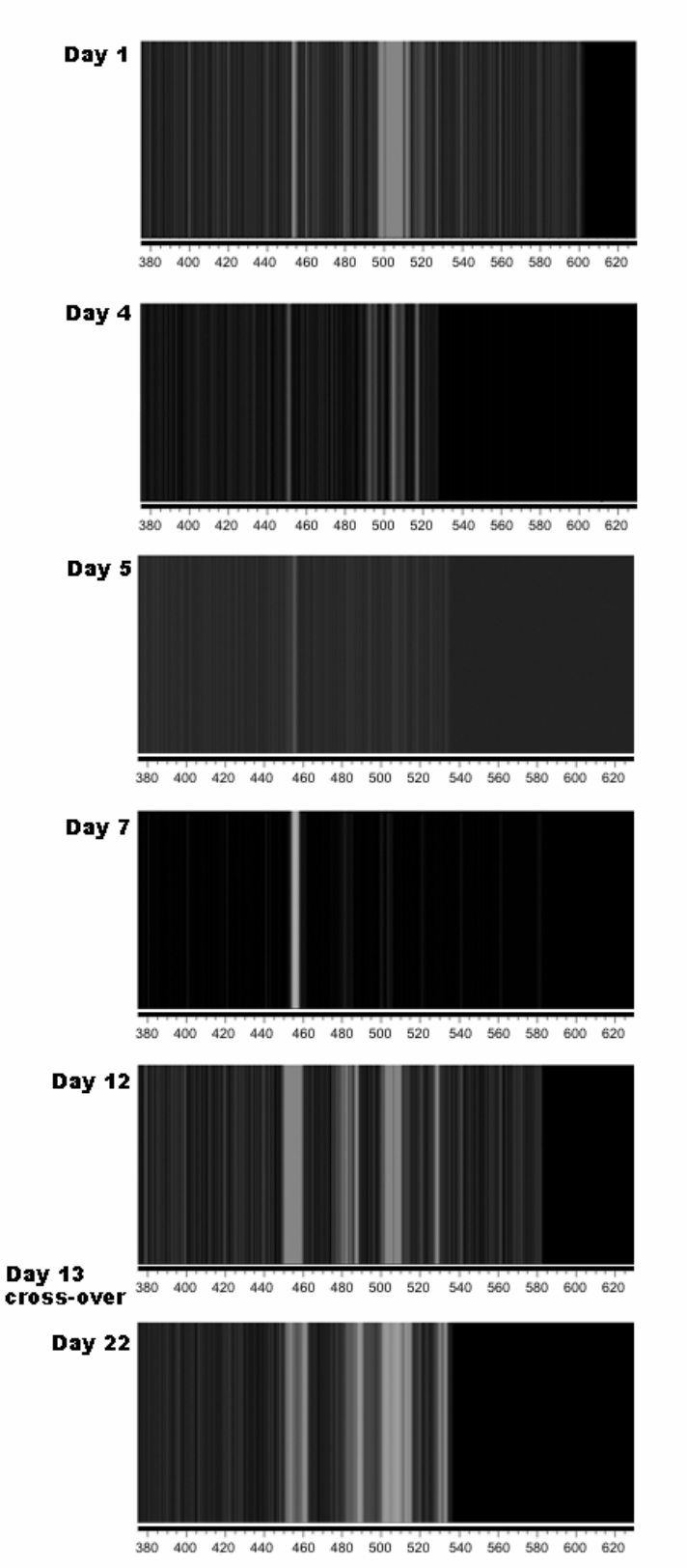
819 **Fig. 4.**

820



821

822 Fig. 5.



823

824 Fig. 6.

825