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BACTERIA IN PHYTOREMEDIATION AT

THE C8 AND O13 SITES AT THE

GUADALUPE RESTORATION PROJECT

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Overview for Analysis of Data up to August 2005

The microbiology group at the Environmental Biotechnology Institute has been collecting 16S rRNA gene based Terminal Restriction Fragment (TRF) data from groundwater samples at C8 and O13 since 2000. We now have an accumulated 14 quarters of data spanning nearly six years. Last year's analysis showed some trends in the abundance of certain types of bacteria that were encouraging news on the functioning of phytoremediation efforts at both the C8 and O13 sites. This year we have incorporated an additional two quarters of data as well as expanding the scope of the analysis to include comparisons with wells outside planted zones both within the plume and outside the plume.

Previously identified research questions addressed in this report

1) Do tree roots enhance biodegradation of dissolved phase diluent (DPD) by encouraging specific types of bacteria that degrade DPD*?
    a. Do the roots increase both bacterial numbers and activity?
    b. Do the roots alter the type of bacteria in the groundwater and capillary fringe soil?
    c. Are the bacteria that increase in abundance in the presence of tree roots also degraders of DPD?

*Measurements made were using total petroleum hydrocarbons (TPH) as a surrogate for DPD and may not always reflect true DPD values.

Progress toward answering these questions

Question 1a was partially answered in previous phytoremediation reports. Using data from soil samples it was clear that the number aerobic heterotrophic bacteria increased in the presence of plant roots. We now have over 3 years of data on bacterial numbers in groundwater samples so a discussion of trends in the abundance of cultured aerobic groundwater bacteria is included in this report.

Questions 1b, c are also addressed in this year's report, although the focus remains on bacteria in groundwater samples. Temporal changes in hydrogeochemical data during the phytoremediation study are analyzed in tandem with temporal analyses of bacterial types (TRF data) – assuming that the effect of plant roots increased as their infiltration of the Dunes Aquifer progressed over time.

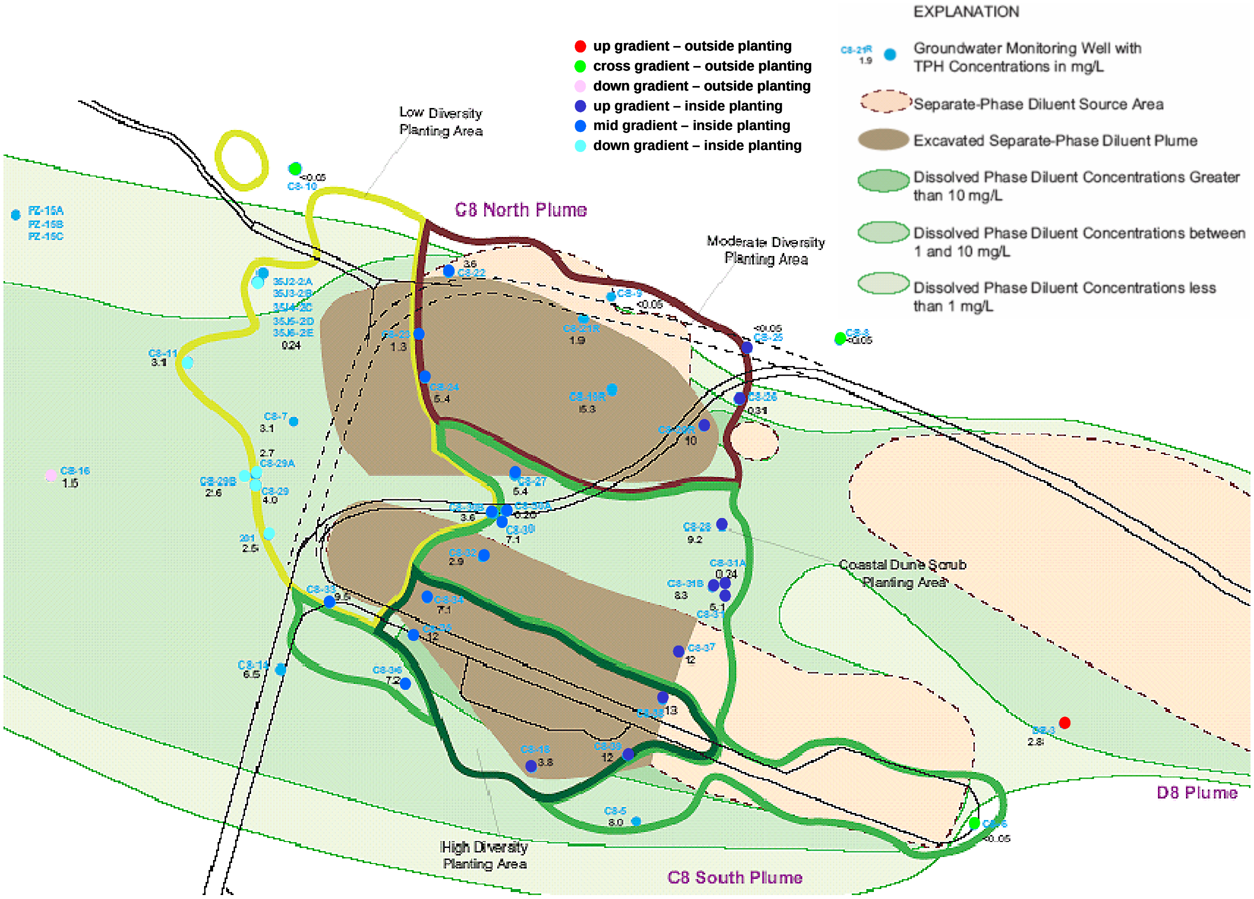
C8 Phytoremediation Study

Thirty four wells were sampled at C8 over 14 quarters, from October 2000 to August 2005. During the first two years of the study (10/2000 to 8/2002) samples were taken every quarter. This was reduced to the first and third quarters of the year for subsequent years. In last year's report, data from wells inside planted areas were averaged for each sampling time and then temporal trends were assessed. This year, the wells were put into 6 zones, depending on well position inside and outside planted areas as well as position in the groundwater gradient (Table 1 & Figure 1). TRF and bacterial count data were then averaged for each zone and temporal trends analyzed as before. The outside planting zones are represented by fewer wells than inside the planted areas. The "up gradient – outside planting" includes only a single well, D8-3. This is followed by the "down gradient – outside planting" (2 wells) and "cross gradient – outside planting" zone (3 wells). The inside planting zones (up, mid and down gradient) have 5, 12 and 11 wells respectively. This makes the data somewhat variable for zones outside the planted areas. However, it does allow for some comparisons to be made with areas presumably not influenced by plant roots so that the effect of plants on groundwater microbiology can be more accurately assessed. Because the number of wells above and below the planted zones was so small, some of these zones have no data for some physical variables measured because only a few wells were sampled. For example, all dissolved gasses were measured in only a few wells (indicated in bold on Table 1) thus the "up gradient – outside planting" zone has no data for dissolved carbon dioxide (Figure 3).

Table 1. List of well zones used for data analysis by gradient and planting. Well names in **bold** denote wells used to measure dissolved gasses.

Gradient	Planting	Wells in each Zone
Down	Inside	C8-2E, **C8-11**, C8-29, C8-29A, C8-29B
Down	Outside	C8-13, **C8-16**
Up	Inside	C8-18, C8-20R, **C8-25**, C8-26, **C8-28**, **C8-31**, C8-31A, C8-31B, C8-37, C8-38, **C8-39**
Up	Outside	D8-03
Mid	Inside	C8-22, **C8-23**, C8-24, C8-27, **C8-30**, C8-30A, C8-30B, **C8-32**, C8-33, **C8-34**, C8-35, C8-36
Cross	Outside	C8-06, C8-08, **C8-10**

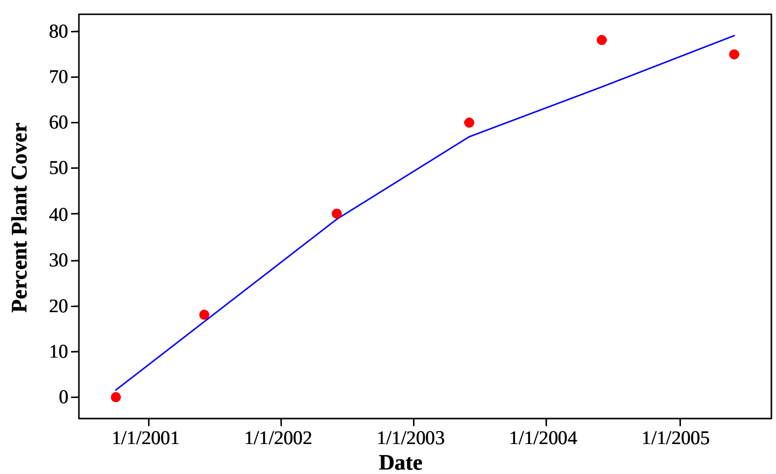
Figure 1. C8 site map showing planting areas and groundwater wells and zones (Table 1). Well C8-13 is off the map to the left, far down gradient of the planting areas.



Temporal Trends in Plant Growth and Hydrogeochemistry at C8

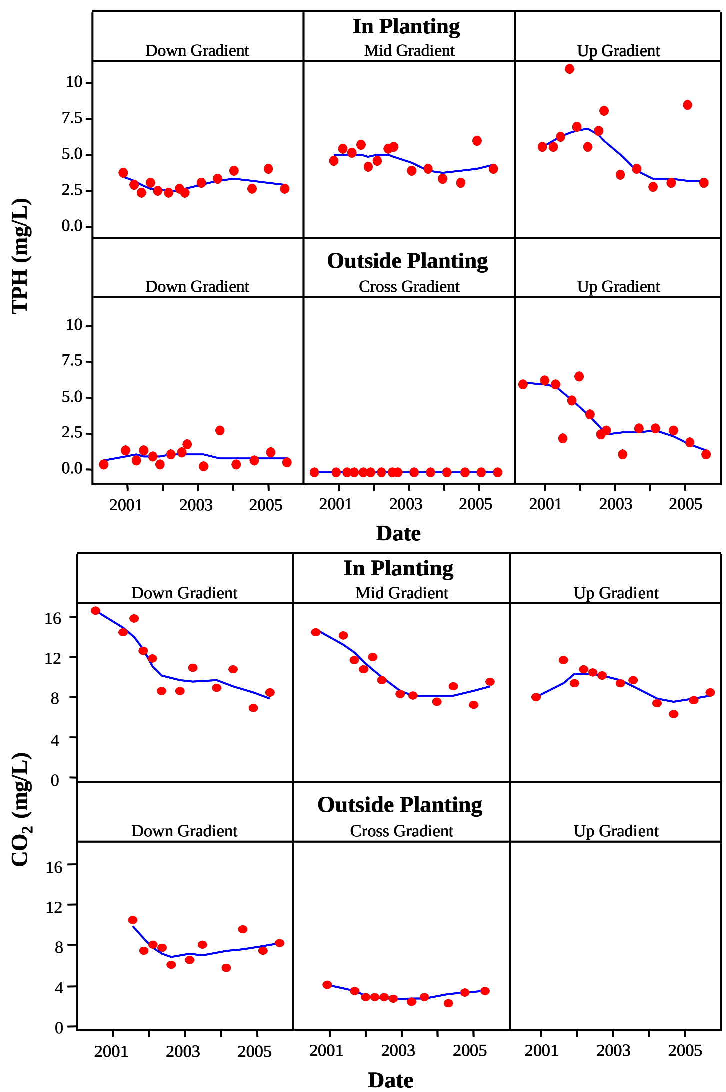
As before, the percentage of the ground surface covered by plants was used as a proxy for plant growth with the assumption that the amount of plant biomass above ground should be proportional to the amount of root mass below ground. Plant cover approached 50% in mid 2002 and leveled off by 2005.

Figure 2. Percent surface area covered by plants in the planted areas at C8 as a function of time.



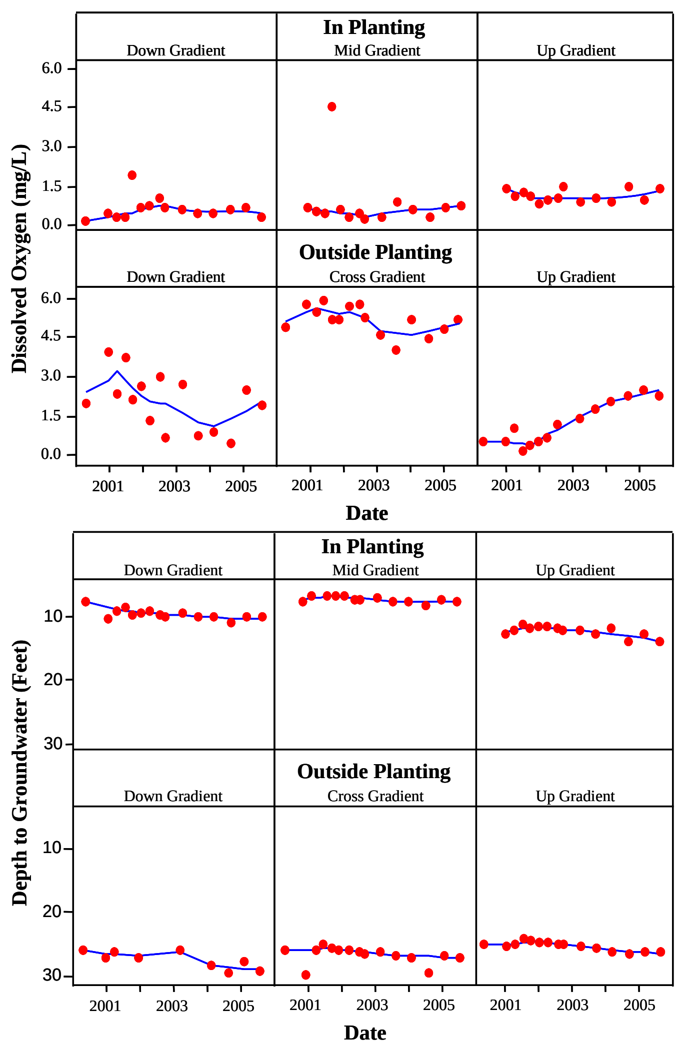
TPH concentrations did not change significantly in down gradient or cross gradient zones (Figure 3). However, there was a significant negative correlation with sampling date for the “up gradient – outside planting” zone ($p = 0.005$). A decrease in the “up gradient – inside planting” zone was not significant ($p = 0.124$) but became significant when the outlier time point at Feb 2005 was discarded ($p = 0.014$). The same was true for an apparent decrease in TPH in the “up gradient – inside planting” zone; not quite a significant decrease over time ($p = .168$) unless the outlier time point at Feb 2005 was discarded ($p = 0.011$). The decreases in TPH concentration in the planted areas are encouraging but would be strengthened if an explanation could be found for the anomalous results from Feb 2005. Perhaps next year’s data will clarify this issue. The drop in TPH measurements in the “up gradient – outside planting” zone (a single well D8-3) may indicate a shrinking of the D8 plume over time that is not related to plant growth. Carbon dioxide (CO_2) concentrations decreased somewhat in all zones within the DPD plume (Figure 3). The rate of decrease declined after plant cover had reached 50% in mid 2002, indicating a possible correlation with plant roots infiltrating into the groundwater.

Figure 3. TPH concentration (top) and dissolved carbon dioxide concentration (bottom) over time averaged by planting and gradient zone. Lowess smoothing lines are included.



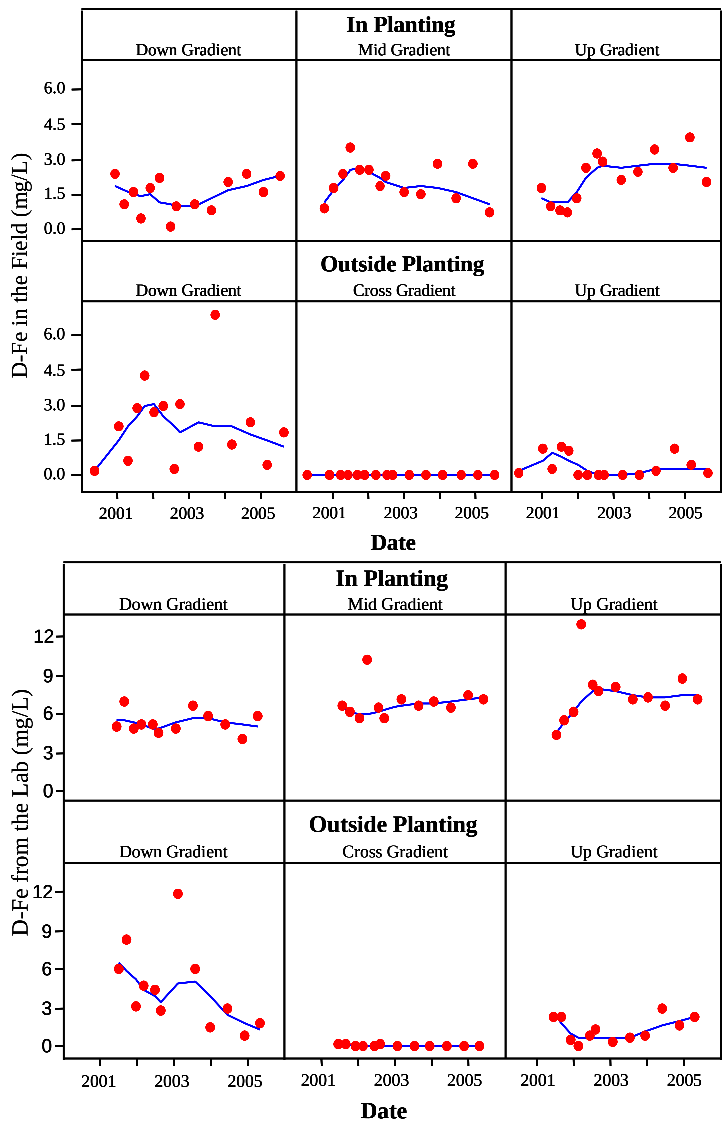
Dissolved oxygen (DO) was measured in two different ways during the study. An in-line DO probe was used while sampling groundwater and these data remained mostly constant throughout the study (Figure 4, top). As expected, in-line measured DO levels were highest in the cross gradient wells. A slight decrease in DO can be seen in the down and cross gradient zones. The "up gradient – outside planting" zone showed a significant increase in DO, corresponding to the decreased TPH levels in that well (Figure 3). A second method for measuring DO involved sending out samples for gas chromatographic (GC) analysis (data not shown). A subset of 11 wells (Table 1) was used to collect these data. The GC data showed an anomalous upward trend in DO that suddenly returned to baseline in late 2004. This did not correspond to trends in TPH or other indicators of redox conditions, such as dissolved iron. Therefore these data could have been influenced by sampling artifacts and were not considered in this report. An increase in the depth to groundwater beginning in May 2001 and continuing through 2005 was apparent in all zones (Figure 4, bottom). This corresponds to low precipitation conditions throughout the Central Coast during those years and may account for the changes in TPH and DO in the "up gradient – outside planting" zone (Well D8-3). We might expect to see this trend reverse if rainfall returns to normal.

Figure 4. Dissolved oxygen over time at C8 as measured in the field by an in-line DO probe during sampling (top) and depth to groundwater (bottom). Lowess smoothing lines are included.



Dissolved or ferrous iron (d-Fe) is another good parameter indicating anaerobic bacterial degradation of petroleum and should reflect plant root influences on bacterial activity. Two methods were also used to measure d-Fe in this study, beginning in May 2001. The first method involved the use of a field kit and was performed immediately after filtration of a groundwater sample at the time of sample collection and measured ferrous iron specifically (Figure 5, top). The second method was performed on a sample that was sent to B-C Labs and measured total dissolved iron (Figure 5, bottom). Although the dissolved iron method consistently gave higher numbers, the trend in d-Fe was generally the same with both methods. Essentially no d-Fe was observed in the cross gradient wells and the concentration of d-Fe remained mostly stable in all zones, with the exception of an increase in d-Fe early on in the up gradient zone. The increase in up gradient zone d-Fe corresponds to an increase in up gradient TPH concentrations through early 2001 (Figure 3). A downward trend in mid gradient d-Fe is visible in the Field data (top) but it is not corroborated by the Lab data.

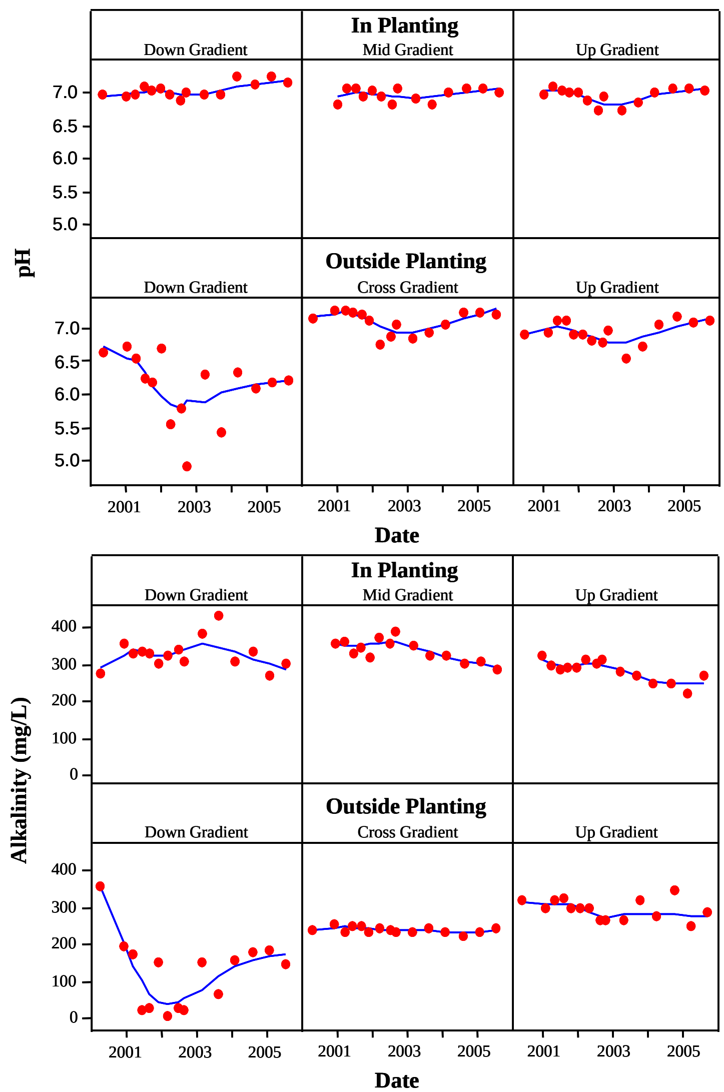
Figure 5. Dissolved iron (d-Fe)over time at C8 as measured in the field immediately after sampling (top) or in a sample sent to B-C labs (bottom). Lowess smoothing lines are included.



Methane concentrations are also a good indicator of anaerobic bacterial activity and dissolved methane was measured in this study. However, with the exception of well C-39, the average methane concentration in the wells selected for measuring dissolved gasses (Table 1) remained below 0.5 mg/L without a temporal trend (data not shown) indicating that the C8 DPD plume was not anaerobic enough to sustain methanogenic conditions.

Two other measured parameters showed interesting trends with time, although their significance for bacterial activity is not clear. The average pH of groundwater in many zones experienced a dip of between 0.2 and 1 units over the 1.5 year period from mid 2001 to early 2003 (Figure 6). The pH in these zones returned to levels seen in 2001 by the mid 2004, a further 1.5 years. Total alkalinity showed a similar dip and rise in the “down gradient – outside planting” zone but showed no rebounding increase in the planted zones. Although the initial decreases in pH and alkalinity roughly correspond to decreasing CO_2 levels (Figure 3) in most zones, this cannot explain the change in pH for the cross gradient wells and does not address the recovery of pH values later in the study. Both pH and alkalinity were much lower in the “cross gradient – outside planting” zone, mostly due to very low results from well C8-16.

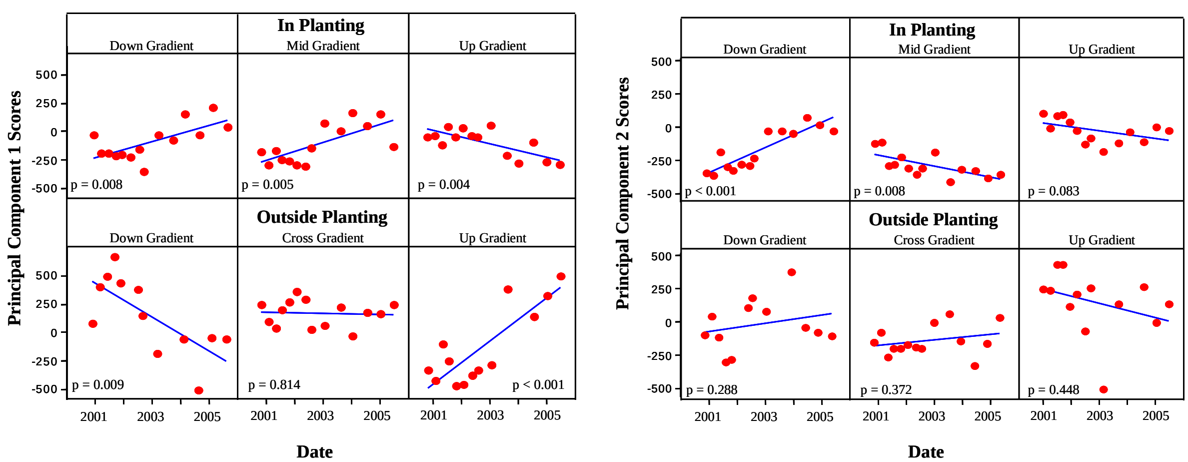
Figure 6. Groundwater pH (top) and total alkalinity (bottom). Lowess smoothing lines are included.



Temporal Trends in Bacterial Populations at C8

TRF data from the C8 groundwater wells included relative abundances from over 250 different types of bacteria. Therefore, principal component analysis (PCA) was used to look for significant trends in the entire assemblage of bacteria in each of the different zones (Table 1). The first 2 principle component scores (PC1 and PC2) convey the most variation in the data and correlation to time indicates that a significant fraction of the bacterial population was changing over time. All of the zones in the planted area showed significant correlation to date of sampling for PC1 and PC2 (Figure 7). In addition, the trends in PC1 for the planted zones became more apparent after May 2002, once plants at C8 became well established. By comparison, the “cross gradient – outside planting” zone showed no significant change over time, indicating that bacteria in groundwater not influenced by petroleum or plants did not change with any significant trend over this time period. Both “up gradient – outside planting” and “down gradient – outside planting” showed as significant change with time for PC1 but not PC2. This may indicate that some temporal changes in groundwater bacterial populations are due to changing petroleum influences rather than the influence of plants in the phytoremediation plot. However, because groundwater sampled in the “down gradient – outside planting” zone has passed by the roots of plants in the phytoremediation plot, a plant influence cannot be ruled out for this zone. The response seen in the “up gradient – outside planting” zone is clearly related to changes in TPH levels but since PC2 is significant for all inside planting zones and none of the outside planting zones, a plant related influence on bacterial communities in the groundwater is still likely.

Figure 7. Principle Components Scores from TRF data averaged for each zone and plotted against date, PC1 on the left and PC2 on the right. A linear regression line is included for each panel; along with a p-value ($p < 0.05$ indicates a significant correlation). The direction of the correlation (negative or positive) is random for each PC calculation.



Once consistent temporal trends in the bacterial community were apparent, individual TRF peaks that varied consistently with time were identified. As outlined in previous reports, TRF data was analyzed first by calculating the average relative abundance of a TRF peak only when it was present, not including samples for which that TRF peak was not present. As a counter check to this approach, the number of wells where a TRF peak was present was also compared with sampling time to determine if the bacteria represented by the TRF peak were indeed changing in relative abundance with time across the entire site. Because each zone had a different number of wells in it, the number of positive wells was converted to a percentage of the total number of wells in the zone.

During this analysis it became clear that clusters of similar TRF peaks responded in a linked manner, thus indicating the response of a cluster of similar organisms. For example, TRF 153 consistently decreased over time in the planting zones, as did TRFs 151, 152, 154, 155 and 156. Thus the relative abundance and presence of TRFs in a cluster from 151 to 156 was summed together and tracked as representing one closely related group of organisms. A total of 7 TRF peak clusters were chosen as being the clearest examples of time dependant changes in bacterial populations.

Five TRF clusters were at high relative abundance when present early in the project (2000 to mid 2002) and then began to drop off later on (Figures 8-12). In many zones these TRFs were present in a larger number of C8 groundwater wells at first and then fewer wells as time went on. Two additional TRF clusters increased in relative abundance as the study progressed (Figures 14 & 14). The bacteria most likely represented by these TRF clusters were identified by matching to a library of 16S rRNA gene sequences taken from wells C8-2E (Feb 2001), C8-23 (Sept 2003) and C8-36 (Aug 2005). TRF clusters were also matched to 16S rRNA gene sequences from the Land Treatment and Natural Attenuation studies at Guadalupe and by reference to a database of TRFs predicted from public archives of 16S rRNA gene sequences (Table 2).

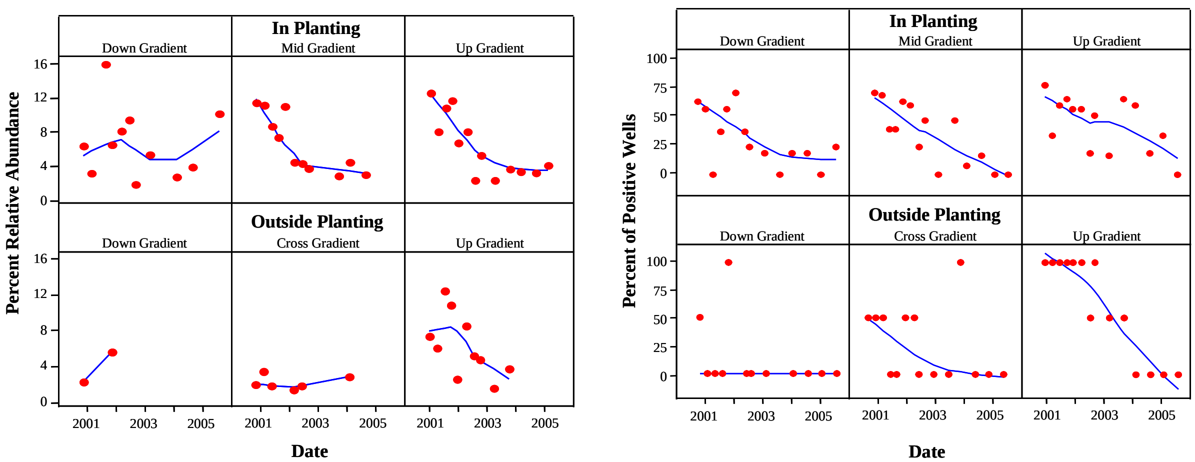
Table 2. Bacteria associated with TRF clusters from C8 (and O13) samples as confirmed by matching to 16S rRNA gene sequences isolated from three different wells at different times (see text).

TRF Cluster	Matching Sequences	Best Identification
73-75	None (2*)	Chloroflexi*
85-87	1	*Acidovorax*, (*Aeromonas*† or *Shewanella*†)
151-156	14	beta-Proteobacteria, *Methylobacter*, *Dechloromonas*, *Pseudomonas*
229-230	7 (6**)	*Pseudomonas***
253-254	7 (23**)	*Flavobacterium***
272	1	delta-Proteobacteria
469-471	1 (7*)	*Nitrospirae* and Chloroflexi*

* match to TRF found in the Guadalupe Natural Attenuation clone sequence library
** match to TRF found in the Guadalupe Land Treatment Unit clone sequence library
† Predicted from TRF database

The TRF cluster at 85-87 is interesting because of the clear response over time in the mid gradient and up gradient zones (Figure 8). These TRFs most likely represent iron reducing bacteria in the genera *Acidovorax*, *Aeromonas* and *Shewanella* (Table 2). The clear decrease in abundance and presence of this TRF cluster is most likely not due to plant root influences since the "up gradient – outside planting" zone shows the same downward trend. However, it could be related to the decreasing trend in TPH, especially in the up gradient well D8-3 (Figure 3). Down gradient and cross gradient zones showed much weaker trends for this TRF cluster, regardless of planting. This is consistent with an organism whose presence is tied to high concentrations of TPH and more anaerobic conditions.

Figure 8. Average relative abundance of TRF cluster 85-87 when present (left) and percent of positive wells for these TRFs (right). A Lowess smoothing line is included for each graph.



The TRF clusters at 73-75 and 469-471 also show a downward trend over time in the planting zones (Figures 9 & 10). Both these TRF clusters most likely represent anaerobic fermenting bacteria in the Spirochete and Chloroflexi families (Table 2). These TRF peaks were previously identified as associated with highly anaerobic TPH affected groundwater and methanogenic conditions in a natural attenuation study at Guadalupe. Neither TRF cluster shows up very often outside the planting zones or in down gradient wells. In fact, the 469-471 TRF set was never detected in the "down gradient – outside planting" or "cross gradient – outside planting" zones. The decreasing trend in these TRF sets seems to indicate a shift away from anaerobic TPH degradation.

Figure 9. Average relative abundance of TRF cluster 73-75 when present (left) and percent of positive wells for these TRFs (right). A Lowess smoothing line is included for each graph.

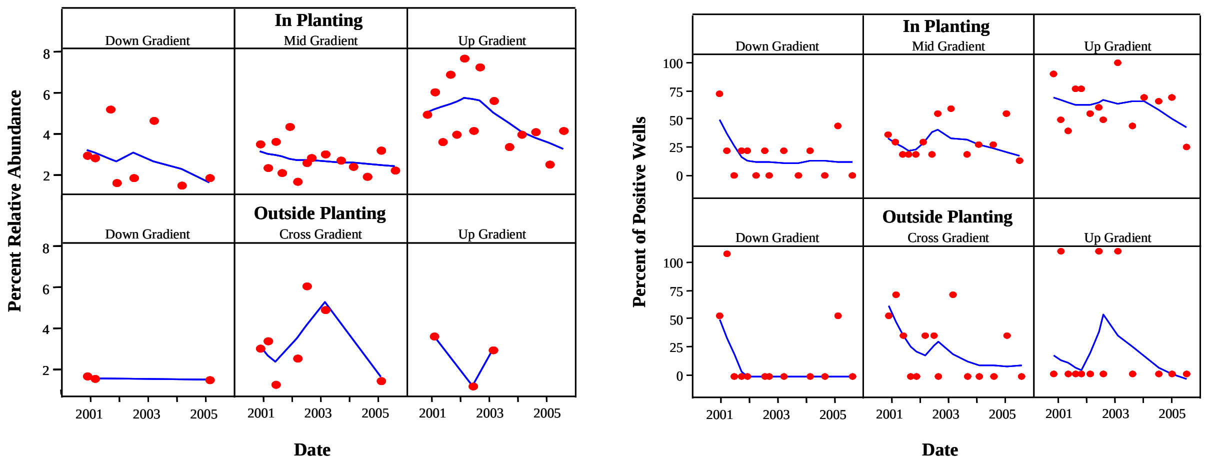
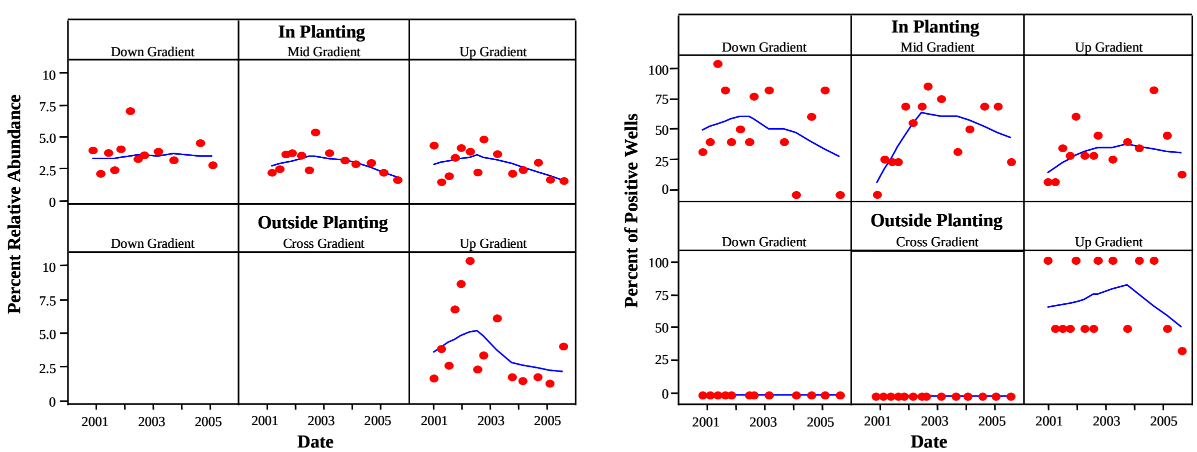
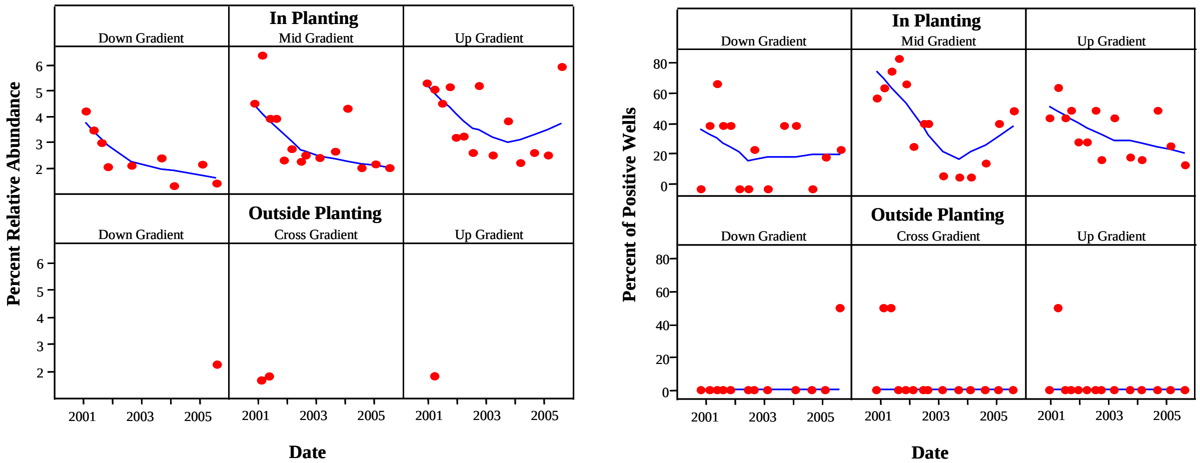


Figure 10. Average relative abundance of TRF cluster 469-471 when present (left) and percent of positive wells for these TRFs (right). A Lowess smoothing line is included for each graph.



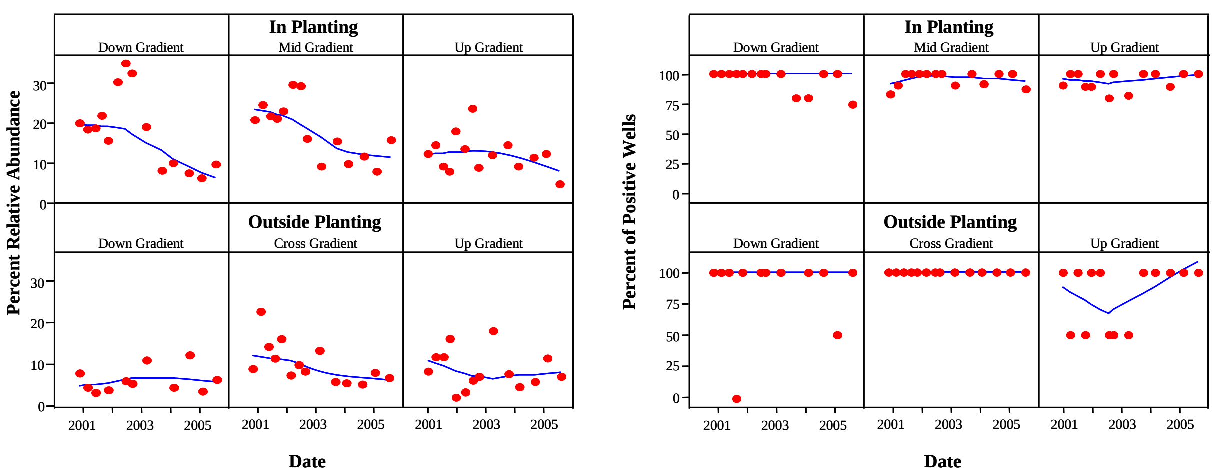
The TRF peak at 272 was not associated with other peaks to form a cluster, but also showed a downward trend over time in the planting zones (Figure 11). In fact, the TRF 272 was rarely detected in the outside planting zones. This TRF peak could not be identified with confidence but most likely represents bacteria in the delta-Proteobacteria group (Table 2). Many sulfate reducing bacteria are in this group but we cannot be sure of the significance of this TRF peak.

Figure 11. Average relative abundance of TRF 272 when present (left) and percent of positive wells for this TRF (right). A Lowess smoothing line is included for each graph.



The large TRF cluster at 151-156 was very common in all zones, but also showed a downward trend over time in the planting zones (Figure 12). This downward trend was most apparent in the "down gradient – inside planting" zone, although all three planting zones showed an increased downward trend beginning in mid 2002, when plants began to establish themselves at the site. This TRF cluster most likely represents a broad range of bacteria in the beta-Proteobacteria family (Table 2). This TRF cluster could represent TPH degraders in the genera *Pseudomonas* or methane oxidizers in the genus *Methylobacterium*. Bacteria in this group have a wide range of both aerobic and anaerobic metabolic adaptations so no clear relevance to petroleum degradation could be gleaned from this data.

Figure 12. Average relative abundance of TRF cluster 151-156 when present (left) and percent of positive wells for these TRFs (right). A Lowess smoothing line is included for each graph.



The most intriguing TRF clusters were at 229-30 and 253-4 because they showed an upward trend over time in the planting zones (Figures 13 & 14). TRF cluster 229-230 was common in all zones but showed a clear increase in relative abundance in the planting zones after mid 2002. This TRF cluster most likely represents a broad range of bacteria in the gamma-proteobacteria family but also corresponds to TRF peaks previously identified as *Pseudomonas* spp. in an aerobic TPH degrading land treatment unit (LTU) experiment at Guadalupe (Table 2). TRF cluster 253-4 increased in relative abundance and presence after mid 2002 and most likely represents bacteria in the genus *Flavobacterium* (Table 2). Once again this TRF cluster corresponds to bacteria seen in the aerobic LTU experiment during the rapid TPH degradation phase. Both TRF clusters showed little or no increasing trend in the outside planting zones, indicating a plant related effect.

Figure 13. Average relative abundance of TRF cluster 229-230 when present (left) and percent of positive wells for these TRFs (right). A Lowess smoothing line is included for each graph.

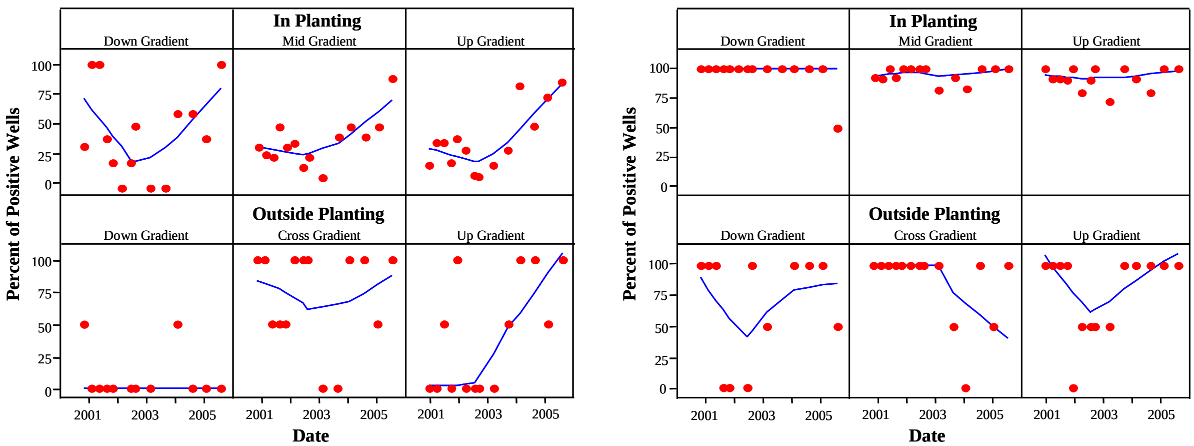
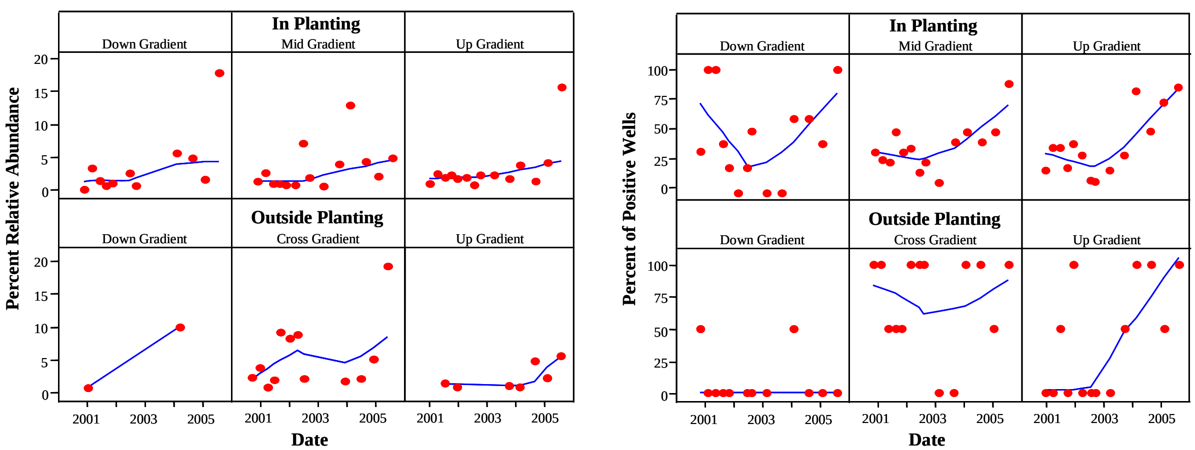


Figure 14. Average relative abundance of TRF cluster 253-254 when present (left) and percent of positive wells for these TRFs (right). A Lowess smoothing line is included for each graph.



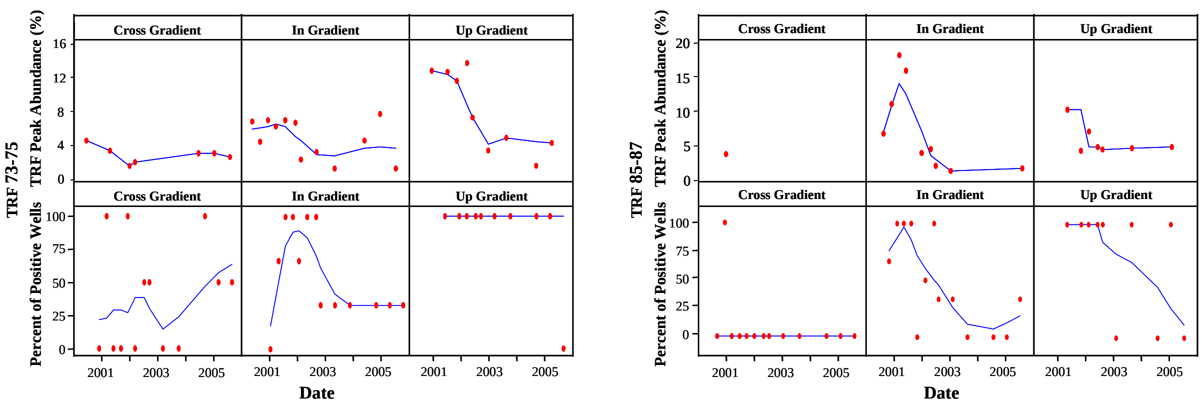
Counts of aerobic heterotrophic bacteria in groundwater.

Beginning with samples taken in June 2002, aerobic bacteria in groundwater were counted via plating on R2A agar – a medium that should allow most aerobic heterotrophic bacteria, including TPH degraders, to grow. Most samples had approximately 10^5 colony forming units per mL (CFU/mL) of groundwater. No significant correlation to sampling date was visible in any of the zones (Table 1) or in the planted area in general (data not shown). The same results were found in the groundwater from the O13 site. This is in contrast to a positive trend in counts of bacteria in soil taken from the capillary fringe soil samples from 2000 to 2002. More capillary fringe soil samples are planned next year to confirm this trend.

O13 Temporal Study

Six wells were consistently sampled at O13 from October 2000 to August 2005. Sampling times were consistent with sampling at the C8 site. Because of the paucity of wells, only three zones were investigated at O13: "cross gradient" (wells N13-29 and N13-21), "in gradient" (wells N13-15, N13-28, and N13-30) and "up gradient" (well N13-8R). The same analysis performed with the C8 TRF data was attempted at O13 and only two TRF clusters showed clear trends with sampling date at O13 (Figure 15). These same TRF clusters demonstrate the same trends in the C8 data, a good sign that the trends are not artifacts.

Figure 15. Average abundance (top panels) and positive wells (bottom panels) for TRF cluster 73-75 (left) and TRF cluster 85-87 (right). A Lowess smoothing line is included for each TRF.



Correspondence Between C8 and O13 Results

The C8 and O13 phytoremediation projects are about a mile apart and the groundwater characteristics are quite different, C8 being near the ocean and O13 being near the Santa Maria River. It would not be surprising to see different types of bacteria responding to plant growth at the different sites. In addition, C8 has a large number of lupines at the site and other plants whose roots have been shown to reach groundwater. Consequently, it's gratifying to see a close correspondence between the TRFs that were decreasing at both sites (Table 2, Figure 15). Because these bacterial types have been associated with anaerobic TPH degradation at Guadalupe this is a good indication that the result of plant root intrusion into TPH containing groundwater is to tip the bacterial community away from anaerobic TPH degraders. The C8 TRF data also illuminated the rise of aerobic TPH degraders (Figures 13 & 14) though this data could not be corroborated with the low number of wells available at O13.

CONCLUSIONS

The original questions 1b) and 1c) are best addressed by this report:
Do the plant roots alter the type of bacteria in the groundwater and capillary fringe soil?
Are the bacteria that increase in abundance in the presence of tree roots also degraders of TPH?

1) The average composition of bacteria in the groundwater at C8 and O13 developed over time in tandem with the growth of the plants at the site.
    a. At the same time that plant cover reached a point where it was clear that roots should begin to have an impact on groundwater (mid 2002) changes in the bacterial community were accelerated (Figures 7-15).
    b. The changes in bacterial community structure could be due to changes in TPH concentration in the up gradient zones. However, many changes in the presence and relative abundance of specific bacterial types occurred in the mid and down gradient planted zones, indicating a plant influence.
    c. TRF clusters representing several types of anaerobic bacteria decreased in presence and relative abundance (Figures 8-12).
    d. TRF clusters representing aerobic TPH degrading bacteria increased in presence and relative abundance (Figures 13 & 14).
    e. These changes indicate a shift in the bacterial community toward more aerobic community members that might therefore be expected to degrade TPH at a faster rate, although evidence of such an effect was limited to the "up gradient – inside planting zone".

Other conclusions:

2) TPH attenuation may be increased in the up gradient and possibly the mid gradient planting zones although the evidence for this is diluted by a similar decrease in TPH concentrations further up gradient of the plantings (Figure 3).

3) The dissolved carbon dioxide levels appear to have declined in all portions of the planted area, which is an interesting observation considering the apparent increase with time in the presence of aerobic bacteria at the site (Figure 3, Figures 13 & 14). Are the plants utilizing dissolved carbon dioxide?

4) Low dissolved oxygen/iron reducing/essentially anaerobic conditions appear to characterize the groundwater in the planted area (Figures 4-5), although conditions did not seem anaerobic enough to support methane generation.

5) Approximately neutral pH conditions characterize groundwater within the planted area (Figure 4). The groundwater in the "down gradient" wells was more acidic, mostly due to a consistently low pH in well C8-16.