CHAPTER FIVE

RESILIENCE OF THE 2,4-D DEGRADING BACTERIAL POPULATION IN THE FIELD FOLLOWING TERMINATION OF 2,4-D AMENDMENT

Abstract

The rebound of the soil microbial community was investigated over a three year period following termination of 2,4-D application on field plots that had received 2,4-D at various concentrations for 7 years. Methods used to analyze the microbial community included viable counts by MPN, 2,4-D degrader strain density by reverse sample genome probing (RSGP) and community analysis by PLFA . The number of culturable 2,4-D degraders declined by 1/2 log unit each year in each of the treatment plots. Two years after terminating 2,4-D application to the plots 2,4-D degraders in the 10X and 1X plots were close to the numbers of the control plot. This suggests that the magnitude of perturbation might determine the resilience of that ecosystem. Amplifying the *tfdA* gene in the terminated plots and probing with the *tfdA* of the pJP4 under high stringency showed high hybridization signal especially in the terminated 100X plots suggesting the residual effect of 2,4-D in these plots. Reverse sample genome probing (RSGP) also showed a decline in the hybridization intensity of the bacterial standards on the master filter. No signal was recorded for about 40 % of the standards in all treatments 3 years after terminating 2,4-D application. Microbial populations in a secondary succession seemed to decay at differential rates as the ecosystem reverts back to its normal condition.

Introduction

There are many kinds of perturbations such as the introduction of new substrates into ecosystems, volcanic eruptions, pesticide applications to soil etc. that may overwhelm the ecosystem's homeostatic control and disrupt the existing community. Once the disrupting factor is removed, homeostasis acts to restore the disturbed community through secondary succession (8). Some perturbations may involve changes in species abundance as observed by Peele et al (12) who noted a decline in the abundance of gram negative bacteria while gram positive bacteria increased at a marine site impacted by pharmaceutical dumping. Other perturbations may also involve the removal of some or all of the species requiring recoveries of a much longer duration (13).

The time taken for a perturbation to diminish to a given percentage of its initial value, however may be relatively independent of the size of the perturbation and also the systems resistance (13. Whether the system returns to normal i.e individual species abundance and number of species are restored after perturbation is not clear. Alternatively species composition may remain the same while densities of the species change, or vice versa. A rapid recovery after a stress is a measure of the resilience of the system. One measure of recovery is defined by O'Neill (11) as the square root of the sum of squares of the deviations between the perturbed transient behavior and the equilibrium. The response of soil trophic systems to stress and recovery following stress is highly variable with communities of similar structure.

Application of 2,4-D at higher than the normal field rate to soil can be a form of perturbation since it can be toxic for microbial populations. Lenhard (6) found that 100 to 1000 ppm of 2,4-D decreased dehydrogenase activity as well as

the total microbial population. Rates above 500 ppm caused autolysis of the bacteria and decreased nitrogen fixation by *Azotobacter*. Growing cells of *A. chroococuum* and *A. agile* were more resistant to the harmful effects of 300 ppm of 2,4-D triethanolamine salt than were resting cells (7). In vitro studies showed that the growth of *Aspergillus niger* and several other soil fungi were affected. Shifts in the microbial community from the more diverse to less diverse, primarily 2,4-D- degrading populations, was observed in soil microcosm and in field studies (3). High rates of 2,4-D application tend to disturb the ecosystem as was shown by an increase in the metabolic quotient (qCO_2) when 200 ug/g of 2,4-D was applied to the soil. An increase in qCO_2 indicates a disturbed ecosystem or those in early successional stages (8, 10). In such a disturbed state, a reduction in efficiency of the microbial biomass is observed. A higher percentage of substrate is respired as carbon dioxide than is incorporated into biomass production or maintenance (4).

Many studies focus on describing microbial communities but few study the rate and extent of return of the microbial community to its original state after a perturbation. This study attempts to characterize the recovery of soil microbial community shifted by long term 2,4-D application after that treatment had been terminated.

Materials and methods

Media and reagents The treatment plots were as described in Chapter 4.

In June 1996 the subplots were divided into two, a northern side and a southern side. 2,4-D treatment was continued on the northern side and discontinued on the southern side. Thus for the northern side 2,4-D had been applied for 10 yr as of August 1998 whilst on the southern side, 2,4-D was applied for 7 yr, through

1995. MPN counts, soil DNA extraction, reverse sample genome probing, *tfdA* amplification were as described in chapter 4. PLFA analysis was described in Chapter 3. Sampling of soil from the various plots was usually done in September but in 1997, the sampling was done in early October.

Results.

Enumeration of bacteria. Total viable counts remained constant in 1996 at approximately 5x 10^8 cells / g whilst in 1997 they were slightly higher at 10^9 cells/g irrespective of the level of 2,4-D application (Fig 1). MPN counts of 2,4-D degraders was generally 1/2 log higher in 1996 than in 1997 but they had already dropped 0.4- 0.8 log in 1997 from the counts found in the comparable treated plots (Chapter 4). Two years after terminating 2,4-D application, the number of 2,4-D degraders in all the treatments were higher than that of the control indicating that it takes at least 2 years for the enriched microbial population to die back to its normal carrying capacity.

Reverse sample genome probing. Relative hybridization intensity was generally low, but plots of higher 2,4-D treatments had a slightly higher hybridization signal than plots that had lower 2,4-D treatment (Fig. 2). Hybridization signals for about 40 % of the standard strains were non- detectable over all treatments. The detection limit for the method was determined to be 10 pg of target DNA in a genomic Southern blot (ECL Kit detection). This corresponds to a relative hybridization intensity of approximately 0.1 %. *Sphingomonas* (standard 19) was not detected. Strains which were detectable were mostly *Burkholderia* sp, *Variovorax* sp. and *Alcaligenes* sp and an unidentified standard 23 which maintained a high hybridization intensity in almost all the treatments. Bacterial

standards which were non- detectable were mostly non- *tfdA* containing strains already noted to be rare members of the community (Chapter 4).

PLFA Analysis. The total PLFA in the 100X plots was higher than that in the control plots by 6 % whilst the total PLFA in 1X plot was the highest for the all treatment plots (Table 1). The percent monoenoics and the percent eukaryotes in the 100X plots were slightly higher than that in the control plots. Microorganisms in all treatment plots were in the active phase of growth as indicated by the cy 17:0/16:1w7c +cy19:/18:1w7c which ranged from 1.0 to 1.2 in 100X plots. The health status of the gram negative community as measured by 16:1w7t/16:1w7c + 18;1w7t/18:1w7c, ranged from 0.9 in the control plots to 0.11 in the 100X plots. The slightly higher ratio for the 100X plots indicates that the gram negative community in the 100X plots had not fully recovered from the 2,4-D application.

***tfdA* analysis.** The first year after terminating 2,4-D application (1996), *tfdA* hybridization signal was detected in the 100X plot and one of the control plots. In 1997, hybridization signal was detected in 100X plots and one of the 10X plots (Fig. 3). Increased *tfdA* signal was obtained for 1998 which I suspected was due to runoff from north to south following heavy rainfall. Cross contamination was confirmed by finding *tfdA* amplification from the border areas. Hence 2,4-D and / or 2,4-D degraders on the south plots might have come from the north plots, especially in 1998.

Discussion

The higher total viable counts for the 1997 plot versus 1996 might be due to the differences in the time of sampling. The 1997 sampling was early October versus September for the 1996 sample. In October the maize had been harvested and the stover left on the field likely created a conducive microenvironment for

microorganisms to flourish. MPN counts of 2,4-D degraders declined by 1/2 log /year suggesting that it will take 2 more years for the MPN of 2,4-D degraders in the 100X plots to reach the level of the control plots. Thus to some extent the magnitude of the perturbation determines the resilience of the system. PLFA analysis also confirmed that the microorganisms in the 100X plots needed time to recover. The MPN values for the terminated side of the plots in 1998 could not be included because of the suspected contamination of these subplots from the treated plots.

The presence of the *tfdA* gene was monitored in the plots because it is the only gene in the 2,4-D pathway known to be used exclusively in 2,4-D degradation, unlike *tfdB* and *tfdC*, which have homologs found in other degradative pathways. The *tfdA* gene encodes an alpha-ketoglutarate-dependent 2,4-D dioxygenase (3) and is the first enzyme in the pathway which converts 2,4-D into 2,4-dichlorophenol and glyoxylate. Detecting the presence of the gene in terminated field plots one or two years after 2,4-D application, especially in the 100X plots, suggests either that there might be some residual effect of 2,4-D, or that the 2,4-D populations persistent for several years without substrate.

Many possible reasons contribute to the higher total PLFA observed for the 100X plots than in the control plots. One reason might be the release of nutrients from microorganisms which were killed when high 2,4-D concentrations were applied. The surviving microorganisms make use of the released microbial nitrogen. Van Veen and Paul (17) noted that with a field population of $5x10^8$ cells/g soil, death by lysis releases about 9 ug nitrogen g^{-1} soil. A similar observation was made by Allen-Morley et al., (1) who noted an increase in enumerated bacteria following freezing.

RSGP showed that bacterial populations as represented on the master filter declined at different rates, some at fast rates and some at slow rates. This is because there is less substrate available to maintain their growth after 2,4-D application had been terminated and some of the populations enter into a stable stationary phase whilst others die. Released plasmids to such as those carrying *tfd* genes might be taken up by indigenous strains before they are mineralized. Standard 23 is one of isolate that maintains relatively high population levels in all the treatment plots and its decline rate appears to be slower than that of the other isolates even though it does not contain *tfdA*. The exact reason for the observed differences in decline rates of the microbial populations is not known and would need further investigation.

This study demonstrates that three years after terminating 2,4-D application, microbial populations decay at differential rates and that the magnitude of the perturbation determined the resilience of the ecosystem.

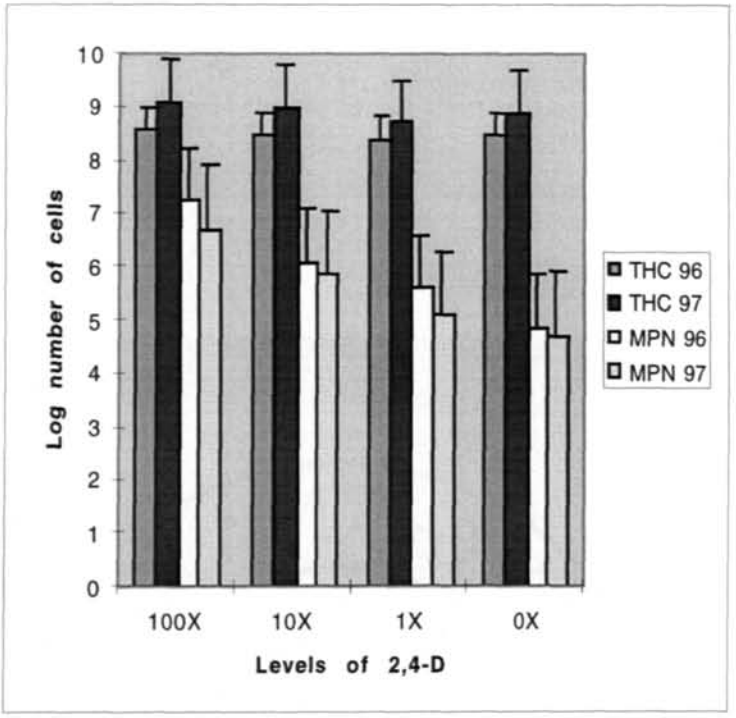


Fig.1. MPN of 2,4-D degraders and total heterotroph count (THC) for field soil samples in 1996 & 1997. Amount of 2,4-D applied shown relative to normal agricultural practice = X. The bars are from 2 MPNs, one of each field plot replicates.

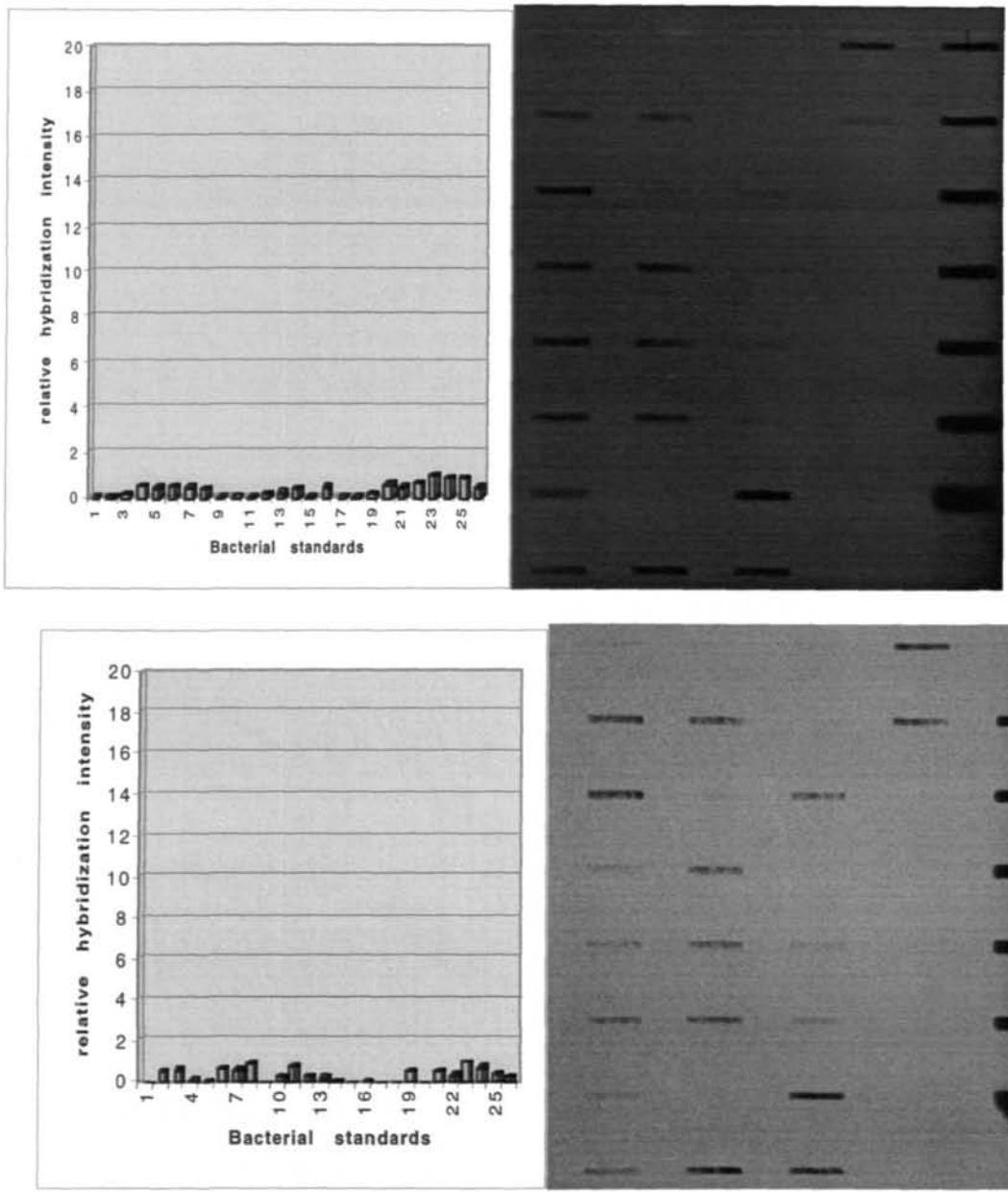
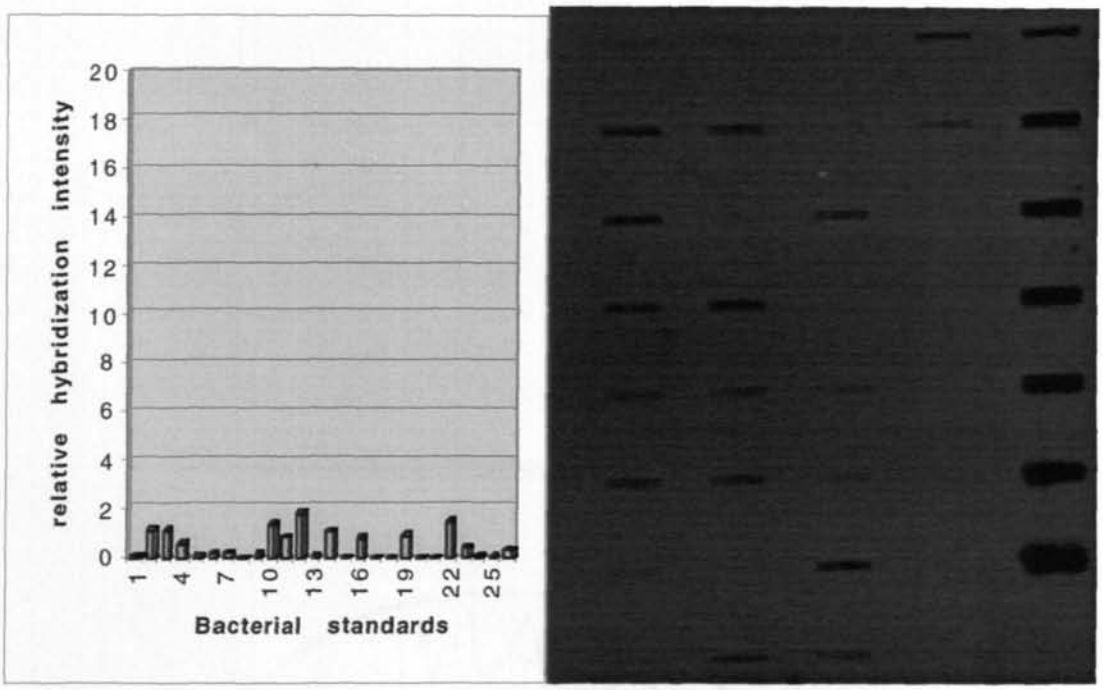


Fig. 2a and 2b. top and bottom. The effect of terminating 2,4-D application on the microbial community in field plots (100X and 10X where 2,4-D application was terminated for 3 years) as measured by RSGP. The community DNA from the plot was labeled and hybridized with master filter. Hybridization intensity in integrated optical density (10^5 on the vertical axis). The standard number on the horizontal axis: data not corrected for cross-hybridization.



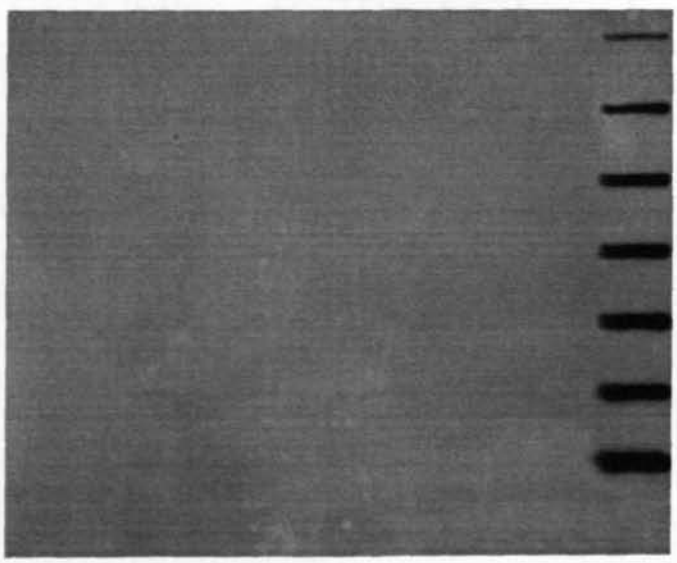


Fig. 2c and 2d. top and bottom. The effect of terminating 2,4-D application on the microbial community in field plots (1X and 0X i.e the control plot where 2,4-D application was terminated for 3 years) as measured by RSGP. The community DNA from the plot was labeled and hybridized with master filter. Hybridization intensity in integrated optical density (10^5 on the vertical axis). The standard number on the horizontal axis: data not corrected for cross-hybridization.

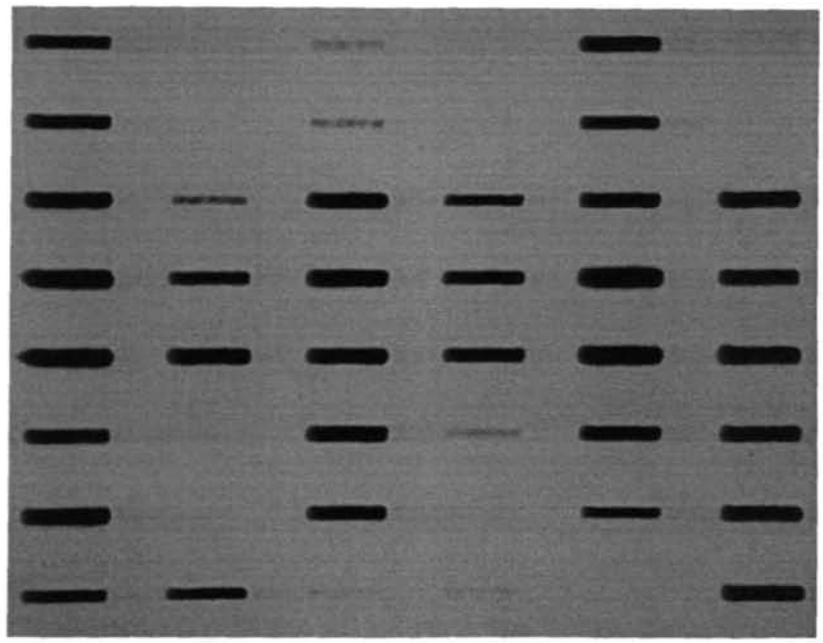
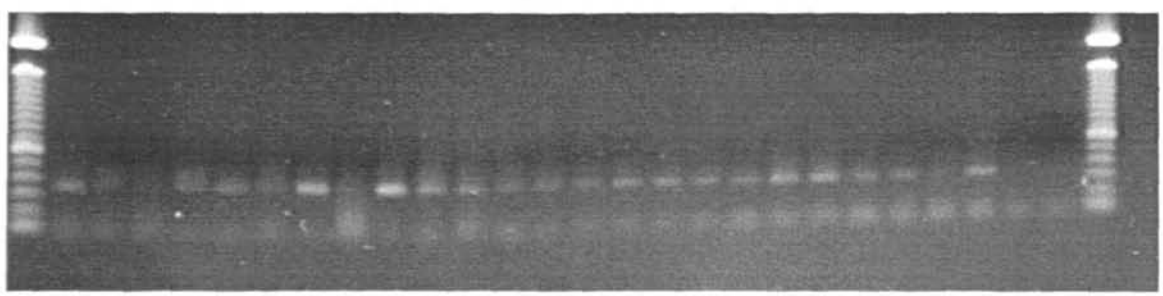


Fig3 Community DNA from field subplots (0X, 1X, 10X and 100X) probed with *tfdA* of the pJP4 under high stringency. Lanes 1 & 2 are for 1996, lanes 3 ,4 are for 1997 and lanes 5 and 6 are for 1998. Lanes 2, 4 and 6 are the terminated side. For a particular lane, eg. Lane 1 from top the treatments are 0X, 1X, 10X, 100X, 100X, 10X, 1X, and 0X.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

Fig.4. Soil community DNA from all treatment plots from both the terminated and the unterminated side amplified with the conserved *tfdA* primers (size of fragment is 360 bp) to demonstrate the leaching of 2,4-D to the southern section of terminated plots.Lanes 1 to 15 are Soil DNA from the northern side , 1,3,5,7,9,11,13,and 15 are from 0X, 1X, 10X, 100X, 100X, 10X, 1X, 0X plots, whist 2,4,6,8,10,12,and 14 are the border plots in between the 2,4-D plots, thus lane 2 is a border plot between plot 1 and plot 3.Lanes 16 to 26 are soil DNA from southern side. Lanes 16,17,18,20, 22, 24, 25 and 26 are)X, 1X, 10X, 100X, 100X, 10X, 1X, 0X, whilst 19, 21, 23, are border plots. Marker DNA 100 bp ladder are at the sides of the PCR products.

Table 1. Changes in microbiable variables with the termination of 2,4-D application for three years

	0X	1X	10X	100X
Total PFLA pM/ g dry soil	26,023±2305	29,841±2817	24839±1740	29532±2805
Monoenoics %	33.7±0.49	34±1.2	33.15±0.64	34.25±0.78
Branched Monoenoic %	5.35±0.07	5.3±1.06	4.9±0.14	5.7
MidBrSats %	11±0.56	11±0.21	11±1.27	10.5±0.57
Eukaryotes %	17.8±0.85	16.7±1.33	17.6±1.4	17.9±1.22
		Metabolic status		
cy:0/16:1w7c +cy19:0/18:1 w7c	1.1±0.34	1.2±0.03	1.0±0.02	1.2±0.25
16:1w7t/16:1 w7c + 18:1w7t/18:1 w7c	0.9±0.04	0.11±0.04	0.08	0.11±0.03

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