Chapter 6

Microbial growth, biomass, community structure and nutrient limitation in high pH soils from Pravaranagar area

(India)
6.1. Introduction

Modern agricultural practices have drastically altered soil physicochemical conditions and thus the environment for the soil microorganisms. This is not only due to mechanical practices, like plowing and tilling, shaping the physical soil environment or the use of monocrops of different kinds. Fertilization practices have also altered nutrient conditions and application of lime has increased pH. These changes are all made in order to improve plant productivity, but other changes can in the long time perspective have negative consequences. This includes salinization of soils due to artificial watering (Al-Ghobari, 2011). Worldwide more than 900 million hectares of the total agriculture land are affected by salt; this account to more than 6 % of the world’s total area (Zang et al., 2010). Some part of salinity is due to agricultural practices (Rousk et al., 2011).

In the present study investigated soils from Pravanagar, India. The Pravara area has a well developed agriculture sector. An intensive cropping pattern has been practiced since decades, specializing in sugarcane crops due to the sugar industry, which was established in 1953. Part of the agriculture practices includes the use of spent wash from the sugarcane production as a liquid fertilizer; an organic fertilizer rich in both nutrients and organic matter. Farmers also frequently use chemical fertilizers and other organic fertilizers. All the nutrients from these fertilizers are not taken up by the plants; hence they remain in the soil for several years. This has led not only to saturation of certain nutrients in the soil, but also in some cases to increased salinity. This, together with the initially rather high soil pH ($pH_W$ up to $> 8.5$), has led to soils with rather extreme physicochemical characteristics.
Initially 12 soils from the Pravaranagar area (district Ahmednagar) were characterized for the soil microbial community of estimating biomass, growth and community composition (using phospholipid fatty acid, PLFAs, as a proxy). Also the main and secondary nutrients limiting bacterial growth, the bacterial community response to pH and salt tolerance of the bacterial community was determined. Finally, since these high pH soils had very low fungal activity, then studied if more extensive fungal growth could be induced by adding different plant-derived substrate and to what extent these additives affected limiting nutrients for fungal and bacterial growth.

6.2. Materials and Methods

6.2.1 Soils and chemical analyses

12 soil samples from Pravaranagar (India), located between latitude 19°30’ to 19°34’ N and longitude 74°20’ to 74°25’E, were studied. Three soil samples were collected from each sampling site at 0-15 cm depth using a shovel. They were mixed and sieved to 2 mm mesh size, packed into polythene bags, brought to the laboratory and analyzed for chemical and biological parameters. Soil pH was determined using a glass electrode both in distilled water (pH_W; soil: water 1:5 w:v) and in 0.1 M KCl (pH_KCl; 1:1 w:v). Water content was gravimetrically determined after overnight heating at 105°C. Organic matter content was determined using loss on ignition (600°C for 4h). Electric conductivity (EC) was measured in 1:5 soil:H_2O extracts (EC_{1:5}).

6.2.2 Microbial characterization

6.2.2.1 Bacterial growth
Bacterial growth was estimated by using the leucine (Leu) incorporation technique (Bååth, 1994; Bååth et al., 2001). Briefly, 1 g of soil samples was mixed with 20 ml distilled water, vortexed for 3 minute, and then centrifuged for 10 minute at 3000 rpm to obtain a bacterial suspension (the supernatant). From the bacterial suspension, 1.5 mL was transferred into 2 mL micro-centrifugation tubes. Radiolabeled Leu (2µL L-4, 5-3H-Leucine, 37MBq mL\(^{-1}\), 1.48-2.22 TBq mmol\(^{-1}\), Perkin Elmer, USA) was added together with non-radioactive Leu (final concentration 275 nM). After a 2h incubation period at 22\(^o\)C, growth was terminated by adding 75 µL 100 % trichloroacetic acid (TCA). Washing and subsequent measurement of radioactivity of the bacteria were performed according to Bååth et al. (2001).

6.2.2.2 Determination of Fungal growth and biomass using ergosterol

Fungal growth was estimated by acetate incorporation into ergosterol (Ac-in-erg) (Newell and Fallon, 1991; Bååth, 2001; Rousk et al., 2009). 0.5 g of soil was put into 10 ml test tubes with 1.5 mL water, 20µl \([1^{14}\text{C}]\) acetic acid (sodium salt; 7.4 MBq mL\(^{-1}\) and 2.04 GBq mmol\(^{-1}\); Perkin Elmer, USA), and 480 µL 1 mM non labeled sodium acetate, resulting in a final acetate concentration of 220 µM. The test tubes containing the soil slurry was then incubated at 22\(^o\)C for 4 h. One milliliter of 5 % formalin was used to terminate the incorporation of acetate, after which the tubes were centrifuged and the supernatant discarded. Ergosterol in the soil was then extracted in 5 mL 10% KOH in methanol, separated and quantified using HPLC with a UV detector (282 nm) according to Rousk et al. (2009) The ergosterol peak was collected and the amount of incorporated radioactivity was determined using a scintillator counter. The amount of ergosterol was used as a proxy of fungal biomass in soil.
6.2.2.3 Respiration and total biomass using substrate induced respiration (SIR)

Respiration was measured by transferring 1 g of soil to a 20 mL glass vial. The vial was sealed with a crimp cap and incubated for 24 h, after which the CO₂ concentration was determined using a gas chromatography.

Substrate induced respiration (SIR) was measured according to Anderson and Domsch (1978) using the same vials as for respiration. After removing the caps, 10 mg glucose g⁻¹ soil was added together with talcum (4:1) and respiration measured during 0.5-2.5 h after glucose addition at 22°C. Microbial biomass was calculated assuming that 1 µg CO₂ h⁻¹ corresponded to 20 µg biomass-C (recalculated from Anderson and Domsch, 1978).

6.2.2.4 Phospholipid fatty acid (PLFA) analysis

The phospholipid fatty acid (PLFA) pattern was determined according to Frostegård et al. (1993) using duplicate samples from each soil. Briefly, 0.25 g of soil samples were extracted for 2 h in a one phase mixture of chloroform, methanol and citrate buffer (1:2:0.8, v:v:v). After splitting the extract into two phases by adding chloroform and buffer, the lipid-containing lower phase was collected and evaporated under N₂. The lipid material was fractionated on columns containing silicic acid into neutral, glycolipids and polar lipids. The polar fractions containing phospholipids was collected for further analysis. Methyl nonadecanoate (19:0) was then added as an internal standard. The phospholipids were methylated by mild alkaline methylation before being analyzed on a GC. 28 different PLFAs were detected. The sum of these (PLFAₜₒₜ) were used as indicator of microbial biomass, the PLFA 18:2ω6,9 was used as an indicator of fungal biomass and the ratio of 18:2ω6,9 to the sum of bacterial PLFAs (Frostegård and Bååth, 1996) was used as an index for fungal: bacterial
biomass ratio. The PLFA 18:1ω9, although not only emanating from fungi, was used as additional evidence for changes in fungal biomass (Frostegård et al., 2011). A principal component analysis (PCA) was performed on mol percent of the PLFAs after standardizing to unit variance.

6.2.2.5 Nutrient limitation of bacterial growth

In order to study nutrient limitation, carbon (C as glucose), N (N) and phosphorus (P) was added to soil in a full factorial design and bacterial growth was measured using Leu incorporation (Aldén et al., 2001; Demoling et al., 2007). The optimal amounts of nutrients were initially tested to find suitable concentrations of glucose, NH$_4$NO$_3$ and K$_2$HPO$_4$ that together gave a strong bacterial growth response. The different nutrient were choose - 5 mg g$^{-1}$ glucose (equivalent to 2 mg g$^{-1}$ glucose-C), 0.142 mg g$^{-1}$ NH$_4$NO$_3$ (equivalent to 0.05 mg g$^{-1}$ NH$_4$NO$_3$-N) and 0.112 mg g$^{-1}$ K$_2$HPO$_4$ (equivalent to 0.02 mg g$^{-1}$ K$_2$HPO$_4$-P), which gave a growth response in all soils except no. 6 and 8, which became anaerobic. These soils were not used. For soil no. 5 the double amounts of glucose was used in order to achieve a growth response.

In the final test, 1 g of soil was placed in 50 mL of centrifuge tubes, the different combinations of C, N and P were added in a small amount of water, and tubes were sealed with lids to avoid drying. The soils were then incubated for 72 hr at 22°C and then bacterial growth was estimated by the Leu incorporation technique (see 2.2.1).

6.2.2.6 Bacterial community growth response to pH

The response of the bacterial community to pH was determined according to Fernández-Calviño and Bååth (2010). Briefly, a soil suspension was prepared (see
2.2.1) and aliquots (1.35 mL) of this suspension were transferred into 2 mL micro-centrifugation tubes. Then 0.15 mL of different pH buffers, or deionized water as a control, were added. Buffers solution with pH values between 4.0 and 9.0 were used. Three different types of buffers were used to cover a large range of pH: citrate-phosphate (pH: 4 to 7; citrate 10.33-3.2 mM), phosphate buffer (pH: 6 to 8; 66.6 mM) and borate-HCl buffer (pH: 9; 2.1 mM). Bacterial growth was then determined by the Leu incorporation technique (see 2.2.1).

Leu incorporation was standardized to one in the sample with deionized water (having pH of the soil). Then two models were applied. A second degree equation as a simple symmetrical, unimodal model was used (Fernández-Calviño and Bååth, 2010). The nonsymmetrical, unimodal cardinal pH model (CPM) for pure culture bacterial growth at different pH was also used (Rosso et al., 1995). These models were fitted with to the data by nonlinear regression using KALEIDAGRAPH 4.0 (Synergy Software).

6.2.2.7 Bacterial community tolerance to NaCl

Bacterial community tolerance to NaCl (sodium chloride) were estimated after challenging the bacterial community with different concentrations of NaCl (1.5 to 800 mM) according to Bååth et al. (2001), as modified by Rousk et al. (2011). Briefly, a soil suspension was prepared (see 2.2.1) and aliquots (1.35mL) of this suspension were transferred to 2 mL micro-centrifugation tubes. Then 0.15 ml of different concentrations of NaCl (1.5 to 800 mM) and deionized water as a control, was added to the bacterial suspension in the tubes. Bacterial growth was then determined by the Leu incorporation technique (see 2.2.1).

Leu incorporation was standardized to one in the control with deionized water. A logistic equation (Rousk et al., 2011) was then fitted to the data and IC$_{50}$-values
(inhibition concentration of NaCl giving 50% of the Leu incorporation in to the control) were calculated.

6.2.3 Experimental

6.2.3.1 Soil and experimental setup for induced N limitation

Soils samples No. 3, 7, 10 and 11 having high pH (>8) were used as replicates for this experiment. Straw (C/N= 75) and starch were used; straw was cut, milled, sieved, and the fraction < 0.25 mm size was used. Straw (80 mg g$^{-1}$ of soil) and starch (40 mg g$^{-1}$ of soil) were added to all four soil saples. A treatment with no substrate addition was used as a control. The 12 jars containing 20 g of soil samples were then preincubated at 22°C.

After four weeks preincubation, nutrients limiting bacterial and fungal growth, as well as respiration, were estimated. Since earlier results (2.2.5) had shown that C was the primary and N the secondary limiting nutrient for bacterial growth in these soil, only these two nutrients were tested; glucose (5mg g$^{-1}$ equivalent to 2 mg g$^{-1}$ glucose-C) and nitrogen (0.142 mg g$^{-1}$ NH$_4$NO$_3$ equivalent to 0.05 mg g$^{-1}$ NH$_4$NO$_3$-N) were used in a full factorial experiment. Incubations were according to 2.2.5, and bacterial growth (Leu incorporation), fungal growth (Ac-in-erg), fungal biomass (ergosterol content) and respiration were measured after four days.

6.2.3.2 Effect of substrate addition

Due to lack of soil material, soil samples No. 3, 7, 10 and 11 having high pH (>8) were combined in order to study if fungal growth could be induced by substrate addition in these high pH soil samples. Starch, straw (milled using the fraction <250 µm) and alfalfa (also milled using the fraction <250 µm) were added into soil (10 mg g$^{-1}$ soil) in duplicate together with a no amendment control. The eight jars containing
30 g of soil samples were then incubated at 22°C and the soils were sampled after 1, 2, 4, 7, 14 and 28 days. Bacterial growth, fungal growth and biomass, and respiration were measured.

6.2.4 Statistics

ANOVA were used to analyze data of nutrient and substrate addition. Data were log transformed to stabilize the variance. Tukey’s post-hoc test (p<0.05) was used to differentiated between treatments.

6.3. Results

6.3.1 Soil chemistry

Soil samples pHW (pH measured in 1:5, Soil:H₂O) ranged between 7.4 and 8.8 and pH_KCl between 7.0 and 7.9 (Table 1 and 3). The highest value was observed in soil 8, while the lowest was in soil 12. EC_{1:5} values were low (9-29 mS m⁻¹) in most soils, but high (89, 154 and 229 mS m⁻¹) in soil samples 12, 5 and 6. Water content ranged between 3.5 to 15.3 %, with highest values in soil 8 and lowest in soil 4. Organic matter content varied between 3.7 to 12.8 %, with highest values in soil 1 and lowest in soil 4 (Table 6.1).

6.3.2 Microbial biomass and activity

Basal respiration was ranges between 1.6 to 7.7 µg CO₂-C h⁻¹ g⁻¹ soil organic C (mean 2.6) and the microbial biomass was estimated by using SIR between 5.7 and 30.0 mg biomass C g⁻¹ soil organic C (mean 11.6) (Table 6.2). TotPLFA had a mean of 3.0 µmol g⁻¹ soil organic C, with a fungal/bacterial PLFA index of 0.11. The fungal/bacterial growth ratio was low, ranging from 0.035 to 0.315, with a mean of 0.124 pmol h⁻¹ log.
Table 6.1. Chemical data of the 12 soil samples from Pravaranagar area.

<table>
<thead>
<tr>
<th>Soil No.</th>
<th>pH&lt;sub&gt;W&lt;/sub&gt;</th>
<th>EC&lt;sub&gt;(1:5)&lt;/sub&gt; (mS m&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Water content (%)</th>
<th>Organic matter (%)</th>
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<tbody>
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<td>7.5</td>
<td>29</td>
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<td>89</td>
<td>6.4</td>
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Table 6.2. Biomass and activity in the 12 soils. All data are expressed per g of soil organic C.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
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<tr>
<td>Basal resp. (µg CO₂-C h⁻¹ at 22°C)</td>
<td>2.6</td>
<td>1.8</td>
<td>1.6 - 7.7</td>
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<tr>
<td>Microbial biomass (SIR, mg biomass-C)</td>
<td>11.6</td>
<td>7.2</td>
<td>5.7 - 30.0</td>
</tr>
<tr>
<td>TotPLFA (µmol)</td>
<td>3.0</td>
<td>2.1</td>
<td>1.3 - 7.5</td>
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<td>Fungal/bacterial PLFA</td>
<td>0.11</td>
<td>0.05</td>
<td>0.05 - 0.18</td>
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<tr>
<td>Bacterial growth as Leu incorporation into extracted bacteria (pmol Leu h⁻¹)</td>
<td>3140</td>
<td>3310</td>
<td>961 – 12600</td>
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<tr>
<td>Fungal growth as Ac-in-erg incorporation (pmol h⁻¹)</td>
<td>282</td>
<td>188</td>
<td>96 – 769</td>
</tr>
<tr>
<td>Fungal/bacterial growth ratio (log)</td>
<td>0.124</td>
<td>0.078</td>
<td>0.035 – 0.315</td>
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Table 6.3. Soil pH (in water and KCl) and optimum pH ($pH_{opt}$) for bacterial growth calculated by 2nd degree polynomial and CPM model (see Fig. 7).

<table>
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<tr>
<th>Soil No.</th>
<th>$pH_W$</th>
<th>$pH_{KCl}$</th>
<th>$pH_{opt}$ CPM</th>
<th>$pH_{opt}$ 2nd degree</th>
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Fig. 1. Principal component analysis of the phospholipid fatty acid (PLFA) pattern of the 12 soils. A) Scores of replicate soil samples, B) Loadings of the individual PLFAs.
6.3.3 Community composition (PLFA)

There was good similarity between replicate soil samples. The first principal component (PC1, explaining 26.0% of the variation) was partly reflected salinity (EC$_{1:5}$), with two of the most saline soil samples, 6 and 12, having the most negative values for PC1 (Fig. 1A). There was also a significant correlation between PC1 and log EC$_{1:5}$ ($r=0.57$, $p<0.05$). Saline soils and especially soil 6 was relatively high in fungi, suggested by high relative values of the fungal PLFAs 18:2ω6,9 and 18:1ω9 (Fig. 1B).

PC2, explaining 18.8% of the variation, was positively correlated with pH$_W$ ($r=0.69$, $p<0.05$).
Fig 2. The effect of substrate amendments (control = no addition, starch, alfalfa and straw amendment) on A) relative respiration rate, B) relative bacterial growth as leucine incorporation and C) relative fungal growth as acetate in ergosterol incorporation. Data were standardized to that in the control with no addition.
Fig 3. The effect of substrate amendments (control = no addition, starch, alfalfa and straw amendment) on A) cumulative respiration rate, B) cumulative bacterial growth as leucine incorporation, C) cumulative fungal growth as acetate in ergosterol incorporation and D) relative fungal biomass as ergosterol.
6.3.4 Effect of substrate addition

6.3.4.1 Respiration

The respiration rate following alfalfa application was increased more than 100 times compared to the control at day 1 and 2 after which it was decreased rapidly (Fig. 2A). However, it was still 2 times higher than the control after 28 days. The respiration rate after straw addition was also increased initially and reached a maximum about 50 times compared to the control after 2 days. Around 15 times higher respiration rate was found after 7 and 14 days converging with the control value after 28 days. Starch addition showed around 10 to 15 time higher respiration rate than in the control during the first 14 days and then it was declined to 4 times the control after 28 days.

The cumulative respiration showed that there were significant substrate addition effects following alfalfa, straw and starch addition (p<0.001 in all cases), with around 35, 18 and 11 times higher cumulative respiration as compared with the control (Fig. 3A).

6.3.4.2 Bacterial growth

Alfalfa addition showed a more than 10 times higher maximum bacterial growth rate than that of the control after a few days, while an increase of around 3 times that of the control resulted from straw and starch addition (Fig. 2B). Maximum growth rate were reached later for straw then for alfalfa and starch addition (Fig. 2B).

Cumulative bacterial growth was about 10 times higher than that of the control sample after alfalfa addition (p<0.001). Straw addition showed only a 2 times increase in bacterial growth and starch addition only 1.5 times higher bacterial growth than that of the control (in both cases p<0.001; Fig. 3B).
6.3.4.3 Fungal growth

Straw addition showed a 3 times increase in fungal growth at day 1, 12 times at day 2, 9 times at day 4, and decreasing to about 5 times at day 28 as compared to the control. Alfalfa addition increased fungal growth 4 times at day 1, 7 times at day 2 and around 4 times at day 4 to day 28 as compared to the control. Starch addition did not affect fungal growth (Fig. 2C).

Cumulative fungal growth showed 5 times higher fungal growth than controls following straw (p<0.001) addition. Alfalfa (p<0.001) addition resulted in almost 5 times higher fungal growth than that of control. Starch addition had no effect on cumulative fungal growth (Fig. 3C).

6.3.4.4 Fungal biomass

The fungal biomass was stimulated by all three substrate. After 28 days, starch addition was around 4 times the control (p<0.001) and straw and alfalfa addition around 6 times the control (p<0.001 for both). Fungal biomass started to increase immediately following straw and alfalfa addition, reaching maximum values after 7 days, while after starch addition fungal biomass only started to increase after around one week, having highest values at the end of the incubation period.
Fig. 4. Effect of adding nutrients to determine limiting factors of bacterial growth estimated as leucine incorporation in the 10 soil samples. Nutrients were added in full factorial design and bacterial growth measured after 72 h. All data were standardized to that in the control with no nutrients added.
6.3.5 Limiting nutrients for bacterial growth

P addition had no effect on bacterial growth as compared to the similar treatment without P addition. The mean P/No addition ratio for the 10 soils tested were 1.04±0.03, the NP/P ratio 0.95±0.04 and the CP/C ratio 0.95±0.04. Thus, the ANOVA was made using the P treatments as replicates, and only having a full factorial C and N treatment design (Fig. 4).

All soils were primarily limited by C, while the addition of only N had no effect (the mean N/No addition ratio was 1.00±0.05). Soil 1, 5, and 12 was only limited by C (p<0.001 for the C effect in all cases, with no significant interaction effect). For the other soils a significant CxN interaction showed that adding N together with C resulted in additional bacterial growth, indicating that N was the secondary limiting nutrient (Fig. 4).
Fig. 5. Bacterial (leucine incorporation) and fungal (acetate in ergosterol incorporation) growth in four of the soils soil amended with starch, straw or kept unamended. Growth was measured 4 weeks after amendments. Direct effect of adding starch and straw in the four soils used for A) relative respiration rate, B) relative bacterial growth as leucine incorporation, C) relative fungal growth as acetate in ergosterol incorporation and D) relative fungal biomass as ergosterol. Data were standardized to that in the control with no addition.
Fig. 6. Limiting nutrients for bacterial (leucine incorporation) and fungal (acetate in ergosterol incorporation) growth in four of the soil samples amended with starch, straw or kept unamended for 4 weeks. Growth was measured 4 weeks after amendments and four days after adding C and N in a full factorial design. Relative growth (standardized to that without any nutrient addition) for A) respiration rate, B) relative Leu incorporation, C) relative Ac-in-erg incorporation and D) relative fungal biomass.

6.3.6 Induced N limitation

6.3.6.1 Respiration rate

The respiration rate was increased with both straw (p<0.001) and starch (p<0.001) addition as compared to the no substrate control (Fig. 5A). When adding C and N to test for limiting nutrients, the control soil without substrate addition was only showed a significant C effect (p<0.001), with around 6 time higher respiration rate than
without C addition (Fig. 6A). Soils amended with starch had higher respiration both after C (p<0.010) and N (p<0.001) addition. Soils amended with straw had a significant statistical CxN interaction (p<0.01) due to both C (p<0.001) and N (p<0.001) addition having a positive effect on respiration, with no additional effect of adding both (Fig. 6A).

6.3.6.2 Bacterial growth

The straw (p<0.001) addition had higher bacterial growth as compared to no addition control, while starch addition had no effect on bacterial growth (Fig. 5B). After adding C and N to test for limiting nutrients, the control soil had a significant CxN interaction (p<0.001), due to C but not N increasing bacterial growth as compared to control, while the combined addition of CN increased growth even further. Soil amended with starch had a significant increased bacterial growth with both C (p<0.01) and N (p<0.001) addition. In soil amended with straw, only the N (p<0.001) addition had a positive significant impact on bacterial growth (Fig. 6B). Thus, straw addition shifted the soil bacteria from being C to being N limited.

6.3.6.3 Fungal growth

Straw addition resulted in higher fungal growth (p<0.001) compared to the no addition control, while starch addition had no effect (Fig. 5C). After nutrient addition, the control soil had slightly lower fungal growth after adding N (p<0.05). Soils amended with starch addition had no significant effect of adding either C or N, while soils amended with straw addition had shifted to become N limited for fungal growth, since N addition increased fungal growth (p<0.001, Fig. 6C).

6.3.6.4 Fungal biomass
Straw (p<0.001) addition had increased fungal biomass compared with the control after four weeks of incubation, while starch addition had no effect on fungal biomass (Fig. 5D). Only the straw amended soil was affected by C and N addition in that the fungal biomass increased both after C (p<0.01) and N (p<0.01) addition as compared to control. Adding both C and N increased fungal biomass even more (Fig. 6D).
Fig. 7. Bacterial community response to pH. Growth of the bacterial community extracted from the 12 soil samples was estimated after changing the solution pH by adding buffers. The data were standardized to one for the control with no buffer added (the natural pH). Lines were fitted using the CPM model (see Materials and Methods).
Fig. 8. Bacterial community tolerance to NaCl. Growth of the bacterial community extracted from the 12 soil samples was estimated after changing the salt concentration. The data were standardized to one for the control with no salt added. Lines were fitted using a logistic equation.
Fig. 9. Correlation between soil salinity (EC₁:₅) and bacterial community tolerance to NaCl expressed as IC₅₀ values.
Fig. 10. Comparison of the phospholipid fatty acid (PLFA) pattern of the 12 soils (mean±SE) with a pH gradient of English soils (Hoosefield, Rousk et al., 2011). A PCA of the combined data were made and the scores along the first PC are plotted. Mean and SE for the Indian soils are given.
6.3.7 Bacterial community pH tolerance

Bacterial growth was strongly influenced by pH (Fig. 7), being close to zero at low pH values (pH 4), and having a maximum value around pH 7 to 8 in all soil samples. The data for bacterial growth were fitted both to a 2nd degree polynomial function (all $R^2 \geq 0.95$, $p<0.001$) and the cardinal pH model (CPM; all $R^2 \geq 0.98$, $p<0.001$) to calculate optimum pH for the bacterial growth (Table 3, fitted lines to the CPM model in Fig. 7). The optimum pH for bacterial growth varied between 6.9 and 8.0 for the CPM model and between 7.0 and 7.8 for the 2nd degree polynomial function (Table 6.3). This is similar to the soil pH measured with KCl, but slightly lower than that measured in water (Table 6.3). Mean value for pH$_W$ was 8.1 and for pH$_{KCl}$ 7.4, while pH$_{opt}$, calculated using the CPM model was 7.5 and pH$_{opt}$, calculated using a 2nd degree polynomial, was 7.4.

3.8 Bacterial community tolerance to NaCl

In all 12 soil samples NaCl was inhibiting bacterial growth at high concentrations, resulting in clear dose-response effects (Fig. 8). In case of soil samples 1 to 4 and 8 to 11, IC$_{50}$ values (inhibition concentration giving 50% of growth in soil without any salt addition) for salt tolerance were 1.8 to 1.9 log mM NaCl, while soil samples 5, 6 and 12 had the highest IC$_{50}$ values, 3.5, 1.9 and 2.2 log mM NaCl. A significant positive linear regression was found between electrical conductivity and IC$_{50}$ values ($R = 0.62$, $p<0.001$) (Fig. 9), indicating higher salt tolerance of the bacterial community in soils with high salt concentration.

6.4. Discussion

4.1. Microbial biomass and activity
Microbial biomass-C usually ranges between 1 and 2.5 % of the soil organic-C (Wardle, 1992), and thus the soils studied here were within this normal range, indicating no negative effect of salinization even in the three high salinity soils. TotPLFA also indicated a similar microbial biomass, amounting to around 0.9 % of soil organic C using a conversion factor of 1 mg biomass-C = 340 nmol totPLFA (Frostegård et al., 1991) and around 2.3 % using 1 mg biomass-C = 130 nmol totPLFA (Bååth and Anderson, 2003).

Bacterial growth was fairly high and fungal growth was fairly low as compared to studies in other soils (Fernandez-Calvino et al., 2011), making the fungal/bacterial growth ratio very low, around 0.1 (Table 2). However, this is the same ratio found earlier for soils with pH around 7 and above (Rousk et al., 2009; Fernandez-Calvino et al., 2011), indicating that the high pH in the studied soils was the reason for the relative dominance of bacterial growth over fungal growth.

6.4.2. Community composition (PLFA)

There was a tendency for relatively higher fungal biomass, estimated using the fungal indicator PLFA 18:2ω6,9, in some of the high saline soils, especially soil sample no 6 (Fig. 1B). This soil also had the highest fungal growth rate, estimated with the Ac-in-erg method. This suggests that fungi would be more important in saline soils. Fungi as a group are usually considered to be more adapted to grow at lower soil water potentials than bacteria (Griffin, 1972). However, more saline soils have to be studied in order to establish how salinity affects the balance of fungi and bacteria in soil.

The most important environmental factor determining the PLFA composition of the studied soils was the high pH. This was not directly evident when comparing the 12 different soil samples (Fig. 1A), since these had a rather narrow pH gradient.
However, using data from a broad pH gradient (from an agricultural soils in England; Rousk et al., 2010) in a PCA plot together with soils from the present study, the PLFA pattern of the Indian soil was very similar to the high pH soils from England (pH effect along PC1; Fig. 10). The Indian soil samples also were high in several unsaturated PLFAs, like 18:ω7, and relatively low in e.g. cy19:0, a pattern that has been suggested to be indicative of high pH conditions in soil (Rousk et al., 2010)

4.3. Effect of substrate addition

In order to study if fungal growth could be induced even in the high pH soils studied here different substrates were added. Both straw and alfalfa addition increased fungal growth, showing that when substrate limitation was alleviated, fungi could grow well in these high pH soils. Previous studies have shown that straw addition would favor fungal growth more than alfalfa addition and vice versa for bacteria (Rousk and Bååth, 2007). This was also found here for bacteria (more than 3 times higher with alfalfa than straw, Fig. 3B), while both substrates were favored by fungi (Fig. 3C and D), suggesting that these high pH soils affects fungi in a different way from soils with more neutral pH.

6.4.4. Limiting nutrients

As expected, bacterial growth was C limited in all soil samples tested (Fig. 4), since due to frequent addition of organic and inorganic fertilizers to these soils, they were expected to be high in available nutrients, including N and P (Jain and Srivastava, 2011). Bacterial growth in soil has most commonly being shown to be limited by lack of easily available C (Demoling et al., 2007), even in soils low in N and were plant growth is N-limited (Kamble et al., chapter III).

When comparing N and P, N appeared to be the secondary limiting substance, since no effect of adding P in combination with any other nutrient was found. In three
soils, however, there were no extra growth when adding N in combination with C (Fig. 4, soil samples 1, 5 and 12). The most likely explanation for this is not that another nutrient was the secondary limiting one, but instead of that N availability was very high, and the amount of C added was too low to induce limitation of a secondary limiting substance. A similar situation was found in N fertilized forest soils (Kamble et al., unpublished). A further indication of this is that these 3 soil samples had the largest increase in bacterial growth after adding only C (>5 times). The growth increase after adding only C has earlier been suggested to indicate the availability of the secondary limiting substance (Kamble et al., unpublished).

6.4.5. Induced N limitation

Both starch and straw (C/N= 75) were added to study if these high nutrient soils could be changed from being C to N limited for microbial growth. Straw clearly had this effect, with both fungal and bacterial growth increasing in straw-amended soil by adding N (Fig. 6B, C). Also starch induced N limitation, although this was only seen in the bacterial growth (Fig. 6B). Using only respiration it was more difficult to elucidate C and N limitation, since adding C as glucose always resulted in increased respiration (Fig. 6A). Thus, in starch amended soils, respiration was only affected by C addition, while the bacterial growth clearly showed that N had become the primary limiting substance. For straw amended soil, both C and N addition increased respiration, while fungal and bacterial growth was only increased by N addition. Thus, respiration measurements cannot be used to differentiate clearly between C limitation and limitation by other nutrients, while the direct measurements of growth are more efficient in this respect. Earlier results after straw-addition (Cochran et al., 1988), where increased respiration was found both after C- (glucose) and N-addition,
and explained by different pools of microorganisms being limited by C and N, respectively, is therefore most likely not correct.

Furthermore, our results have emphasized the importance of studying both fungal and bacterial growth when elucidating nutrient limitations in a soil. In non-amended soils, the C limitation was clearly evident for bacteria, while fungal growth was not affected. In straw amended soil, the N limitation was most evident for fungal growth, while in straw-amended soil, both groups responded to N addition. Thus, if only one group is studied one can wrongly draw the conclusions that another nutrient than those added is limiting growth, when actually it is the other group of microorganism that is responding.

6.4.6. Bacterial community pH tolerance

Earlier studies have shown that optimum pH for bacterial growth was approximately similar to the soil pH measured in water (Bååth, 1996, Fernandez-Calvino and Bååth, 2010, Rousk, et al., 2010 and Fernandez-Calvino et al., 2011). This was also found for the soil samples studied here (Fig. 7). However, the optimal pH for growth was more similar to that determined with KCl then with soil pH extracted in water. Thus, compared to pH<sub>W</sub>, the optimum pH for bacterial growth was slightly lower. This differed from earlier studies (see refs. above). One reason could be that this is the first time bacterial pH tolerance in soils with pH >8 have been studied, and at high soil pH the bacterial community are more adapted towards neutral pH. However, in water a close correlation between water pH and optimum pH for bacterial growth was found even at pH >8 (E. Kritzberg and E. Bååth, unpublished). Another explanation is the problems to achieve buffering conditions above pH 9, since the only buffer used was sodium borate buffer of pH 9, which only resulted in around 15 % decrease in
bacterial growth (Fig. 6), and thus some problems in fitting the models, which are symmetrical, were encountered.

6.4.7. Bacterial community tolerance to NaCl

Soil salinity has been shown to affect soil microorganisms, often resulting in decreased CO$_2$-evolution, enzymatic activity and microbial biomass (Muhammad et al., 2006, 2008; Wichern et al., 2006; Setia et al., 2011b; Chowdhury et al., 2011; Setia et al., 2011a). Soil bacterial communities have, however, an ability to change in order to tolerate osmotic stress caused by salinity (Wichern et al., 2006, Bååth et al., 2001) We partly detected an altered community composition in high salinity soils (along PC 1, Fig. 1). We also observed increasing bacterial community tolerance to NaCl in the 3 soil with highest salinity (Figs 6 and 7, soil samples 5, 6 and 12), indicating that the increased salinity had selected for bacterial communities more tolerant to high salt concentration, as found earlier by Bååth et al. (2001). Rousk et al. (2011) studied bacterial community tolerance to NaCl in 4 soils from an arid agro-ecosystem salinity gradient with the same technique as in the present study, but found no increased tolerance to NaCl. However, the most saline of their 4 soil samples had an EC$_{1:1}$ of around 400 mS m$^{-1}$, which would be around 4 times less as EC$_{1:5}$ (Sonmez et al., 2008), and thus similar to the lowest value of our 3 most saline soils. The difference between our studies was thus most likely that our most saline soil was > 2 times more saline then their most saline soil.

6.5 Conclusions

In conclusion, I have demonstrated that both the high pH and the high salinity had affected the community composition (PLFA patter) and the tolerance spectrum to pH and salinity, respectively. However, biomass and total activity (respiration) appeared
not to be affected. Despite the high pH, though to disfavor fungi, adding straw (and to some extent alfalfa) resulted in a significant increased fungal growth, stressing the importance of both fungi and bacteria for the functioning also of these high pH soils. In all soil samples bacterial growth was limited by C. Alleviating N limitation favoured fungal and bacterial growth depending on the C-source, starch favouring bacteria and straw fungi, stressing the importance of studying both bacterial and fungal growth limitations simultaneously.

This study thus emphasize the use of methods indicating growth of bacteria and fungi, both concerning effects of limiting nutrients, as well as effects of environmental factors. The growth based methods are easy to perform, reproducible and thus a rapid way of studying microbial nutrient limitation in soils. These methods are therefore suggested to be included as standard characterizations of the microbial community in soil.
References


Chapter 7

Summary and Conclusion