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Investigating the mechanisms for the opposing pH relationships of fungal and bacterial growth in soil

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ABSTRACT

Soil pH is one of the most influential variables in soil, and is a powerful factor in influencing the size, activity and community structure of the soil microbial community. It was previously shown in a century old artificial pH gradient in an arable soil (pH 4.0–8.3) that bacterial growth is positively related to pH, while fungal growth increases with decreasing pH. In an attempt to elucidate some of the mechanisms for this, plant material that especially promotes fungal growth (straw) or bacterial growth (alfalfa) was added to soil samples of the pH gradient in 5-day laboratory incubation experiments. Also, bacterial growth was specifically inhibited by applying a selective bacterial growth inhibitor (bronopol) along the entire pH gradient to investigate if competitive interaction caused the shift in the decomposer community along the gradient. Straw benefited fungal growth relatively more than bacterial, and vice versa for alfalfa. The general pattern of a shift in fungal:bacterial growth with pH was, however, unaffected by substrate additions, indicating that lack of a suitable substrate was not the cause of the pH effect on the microbial community. In response to the bacterial growth inhibition by bronopol, there was stimulation of fungal growth up to pH 7, but not beyond, both for alfalfa and straw addition. However, the accumulation of ergosterol (an indicator of fungal biomass) during the incubation period after adding alfalfa increased at all pHs, indicating that fungal growth had been high at some time during the 5-day incubation following joint addition of alfalfa and bronopol. This was corroborated in a time-series experiment. In conclusion, the low fungal growth at high pH in an arable soil was caused to a large extent by bacterial competition, and not substrate limitation.

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1. Introduction

Fungi and bacteria dominate the decomposition of organic matter in soils. There are important differences between these microbial groups, however, and it has been shown that they are differently affected by such factors as nutrient status (De Vries et al., 2006, 2007; Demoling et al., 2008), metal toxicity (Rajapaksha et al., 2004) and substrate quality (Meidute et al., 2008; Rousk and Bååth, 2007b; Güsewell and Gessner, 2009; Strickland et al., 2009a,b). Changes in the relative importance of fungi and bacteria may have significant effects on the soil ecosystem. For instance, a fungal-dominated system has been suggested to contribute more to carbon (C) sequestration due to higher growth efficiency (Six et al., 2006), although this has been questioned (Thiet et al., 2006), and to increase biomass turnover time (Bardgett and McAlister, 1999; Van Groenigen et al., 2007).

Soil pH is one of the most influential factors in soil, and strongly influences the biomass, activity and composition of the microbial community (e.g. Matthies et al., 1997; Blagodatskaya and Anderson, 1998; Bååth and Anderson, 2003; Högberg et al., 2007; Nilsson et al., 2007; Lauber et al., 2008; Jones et al., 2009; Rousk et al., 2009). In a long-term experimental field at Rothamsted Research, UK, an artificial pH gradient was initiated in the mid-19th century that resulted in a pH gradient ranging from 4.0 to 8.3 within 200 m in the same agricultural field. No fertiliser amendments have been applied, and the same cropping history has been used since its establishment. This experiment, the Hoosfield acid strip, thus presented a soil where the variable soil pH was uniquely isolated from confounding variables (Aciego Pietri and Brookes, 2007a,b, 2009). Recently, fungal and bacterial growth was estimated along the Hoosfield acid strip in an attempt to estimate how soil pH influenced the relative importance of fungi and bacteria (Rousk et al., 2009). There was more than a five-fold increase in fungal growth between pH 8.3 and 4.5, while bacterial growth decreased more than five-fold in the same interval. This resulted in an almost 30-fold increase in the

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relative importance of fungi, indicated by the growth ratio, from the high pH soils to pH 4.5.

In this study we wanted to investigate potential mechanisms for the different pH relationships of fungal and bacterial growth, especially focusing on the competitive interaction between the decomposer groups. It was previously demonstrated that additions of straw especially promoted fungal growth, while additions of alfalfa especially promoted bacterial growth (Rousk and Bååth, 2007b). Thus, the addition of straw and alfalfa along the pH gradient would remove any confounding influence that different substrate availabilities may have on the pH influence on fungal and bacterial growth (Rousk et al., 2009), and also create a situation where either fungi (straw addition) or bacteria (alfalfa addition) would be relatively more benefited. Recently, a framework to estimate the competitive influence that bacteria exercise on fungi was developed using the combination of selective bacterial inhibitors and measurements of growth (Rousk et al., 2008). Unfortunately, the lack of efficient yet specific fungal inhibitors prevented the direct investigation of reciprocal relationship, the fungal competitive influence on bacteria. Thus, to partially test if competitive interaction between fungi and bacteria could explain their different pH relationships, we selectively inhibited bacterial growth along the entire pH gradient and monitored the fungal response to this in unamended soil, in soil with an added fungi-promoting substrate (straw), and in soil with an added bacteria-promoting substrate (alfalfa). In addition, we also investigated the functional consequence of the fungal:bacterial dynamics along the pH gradient by measuring basal respiration as well as the total microbial biomass. Consequently, our hypotheses were: (i) Addition of straw will especially promote fungal growth, while addition of alfalfa will especially promote bacterial growth, irrespective of pH. (ii) The bacterial growth inhibition (by bronopol) will stimulate fungal growth, and the stimulation will be proportional to the decline in bacteria, and will thus also be higher combined with bacteria-promoting alfalfa compared with fungi-promoting straw. (iii) When the increasing competitive pressure exerted by bacteria with increasing pH is removed (bacterial growth is inhibited), the negative correlation between fungal growth and increasing pH will cease.

2. Materials and methods

2.1. Soil

The soil pH gradient was previously described in greater detail by Aciego-Pietri and Brookes (2007a,b). In April 2008, 27 samples covering the pH gradient were sampled, after which they were stored frozen until September. The samples were subsequently thawed, sieved (<2.8 mm) and water content was determined (105 °C, 24 h). The variation in moisture content of the soils samples was low, and all were close to 40% of the water holding capacity, so moisture adjustment was not needed. The samples were then stored in the dark at 5 °C for 1–2 months, until used. Background data on soils from the same sampling has been presented previously (Rousk et al., 2009).

2.2. Experimentation

2.2.1. Main experiments

Soil subsamples (5 g) were added to 50 mL polyethene tubes. These soils samples were treated with two levels (with or without) of two factors (substrate and bacterial inhibitor) in a full-factorial design (totalling 4 treatments). Two different substrates were used in two separate experiments, each with the indicated full-factorial design. The substrates were dried and ball-milled (<250 μm) straw (C:N = 75) or alfalfa (C:N = 15) and were added at 1 mg C g^{-1} soil, thus increasing the soil C-content by about 10%. The bacterial inhibitor treatment was bronopol (40 μg g^{-1} soil added with 2 μl water g^{-1} soil; all treatments received the same amount of water). Pilot experiments were used to determine inhibitor concentrations and incubation periods; a concentration of bronopol was chosen with the criterion of almost completely reducing bacterial growth (>90% reduction) without affecting fungal growth across the entire pH gradient (we tested soils at pH 4.0, 5.1, 6.7 and 8.0). The two main experiments totaled 108 microcosms for each plant material. The straw-amended series was run in November and the alfalfa-amended series in December 2008. The microcosms were incubated in the dark for 5 days at 22 °C (cf. Rousk et al., 2008), and were subsequently analysed for fungal growth, bacterial growth, ergosterol concentration, respiration and SIR-biomass.

2.2.2. Time-series experiment

Microcosms (15 g) of four soil samples from the high end of the gradient (pH 8.1 ± 0.1), one sample at pH 5.1 and one sample at pH 4 were each treated in a factorial design of straw or alfalfa addition (1 mg C g^{-1} soil) and in the presence or absence of bronopol (40 μg g^{-1} soil in 2 μl water g^{-1} soil). Subsamples were analysed for fungal growth, ergosterol concentration and bacterial growth immediately following treatment application (0 days), and after 1, 2, 4, and 7 days incubation in the dark at 22 °C.

2.2.3. Bronopol tolerance

The bacterial community tolerance of one of the soils from the high end of the pH gradient (pH 8) was investigated for bronopol tolerance following the time-series experiment according to Aldén Demoling et al. (2009). Subsamples of the bacterial suspension from the four different treatments (straw and alfalfa addition with and without bronopol) were exposed to 12 bronopol concentrations of (0–1.5 mg ml^{-1}) to determine the concentration that inhibited bacterial growth by 50% (EC_{50}). A higher EC_{50} would indicate that the bacterial community was more tolerant to bronopol. Note that the units for bronopol additions, and subsequently also bacterial growth rates, are given per volume of bacterial suspension, which are not directly translatable to the bronopol concentration administered to the soil. However, this provides an effective index that can be used to screen for changes in tolerance in differently treated soils (Aldén Demoling et al., 2009).

2.3. Microbial analyses

2.3.1. Bacterial growth

The bacterial growth was estimated using leucine (Leu; Kirchman et al., 1985) incorporation in bacteria extracted from soil using the homogenization/centrifugation technique (Bååth, 1992, 1994) with modifications (Bååth et al., 2001). We added 2 μl radio-labelled Leu ([^{3}H]Leu 37 MBq ml^{-1}, 5.74 TBq $mmol^{-1}$, Amersham) combined with non-labelled Leu to each tube, resulting in 275 nM Leu in the bacterial suspensions. The amount of Leu incorporated into extracted bacteria per h and g soil was used as a measure of bacterial growth.

2.3.2. Fungal growth and biomass

Fungal growth was assessed using the acetate into ergosterol incorporation method (Newell and Fallon, 1991) adapted for soil (Pennanen et al., 1998; Bååth, 2001) with modifications (Rousk et al., 2009), adding 1-[^{14}C]acetic acid (sodium salt, 7.4 MBq ml^{-1}, 2.04 GBq $mmol^{-1}$, Amersham) combined with unlabelled sodium acetate resulting in a final acetate concentration of 220 μM in a soil slurry and having a 5 h incubation at 22 °C without light. Ergosterol was extracted, separated and quantified using HPLC equipped with

a UV detector (282 nm) (Rousk and Bååth, 2007a, b). The fungal biomass was estimated assuming 5 mg ergosterol g^{-1} fungal biomass (Joergensen, 2000; Ruzicka et al., 2000). The ergosterol peak was collected and the amount of incorporated radioactivity determined. The amount of acetate (Ac) incorporated into fungal ergosterol (pmol h^{-1} g^{-1} soil) was used as a measure of fungal growth. The initial concentration of ergosterol in the substrates was low, corresponding to an addition of about 10 (straw) and 20 (alfalfa) ng ergosterol g^{-1} soil.

2.3.3. Basal respiration and SIR-biomass

Basal respiration was determined from CO_2 evolved from 3 g soil in 20 ml glass vials closed with crimp caps and incubated in dark conditions at 22 °C for 22–24 h. The CO_2 was determined using gas chromatography. Microbial biomass was estimated using the substrate-induced-respiration (SIR) method (Anderson and Domsch, 1978). Glucose:talcum (4:1; 6 mg g^{-1}) was added to the soil samples following the gas chromatography analyses. After 20 min, the atmosphere was purged of CO_2 with pressurised air, after which the vials were again closed with crimp caps, and incubated for 2–3 h at 22 °C. The CO_2 evolved was then determined. SIR-respiration was converted to biomass using the relationship: 1 mg CO_2 h^{-1} at 22 °C corresponds to 20 mg biomass C (recalculated from Anderson and Domsch, 1978), and that microbial biomass contains 45% C.

2.4. Statistics

The experimental design most closely resembles a regression in its range of single soil samples covering a wide span in pH. However, it was not possible to unequivocally fit a single function to describe the microbial variables' response to soil pH. Instead a smoothing function was used to illustrate the relationships between the microbial variables and pH (Figs. 1 and 2). These curves were drawn using the locally weighted least squared error (Lowess) method with a 33% smoothing factor.

To evaluate the effect of the treatment factors and that of soil pH on the measured variables three-way ANOVAs were used. Eleven soil samples at low pH (pH 4.0–4.4) were used to represent the low-pH end, and 7 samples (pH 7.9–8.3) were used to represent the high end of the pH gradient. The soil pH (4 or 8), the presence of substrate (with or without) and the presence of bronopol (with or without) were used as fixed factors. The different substrates, straw and alfalfa, were evaluated with different ANOVAs, since they were tested in separate experiments. The ANOVA analyses were performed on log-transformed data to stabilize variation. Significance was determined by comparing the means using the estimate of variation obtained from the ANOVA analyses.

This analysis categorised the pH-scale, and only compared high and low pH, thus disregarding the values between pH 4.5 and 7.8 from the formal statistical analysis. In all cases, however, values at intermediate pH corroborated the results of the categorical contrast, and can thus be seen as additional evidence. The intermediate pH levels are consequently included in all the figures (Figs. 1 and 2).

The dependence between the ratio of fungal and bacterial growth to soil pH was described with an exponential function (Fig. 3), while the dose–response curves of bacterial growth to bronopol concentration were fitted with a sigmoid function (Fig. 5).

3. Results

3.1. Main experiments

3.1.1. Unamended soils

Unamended soils were analysed twice, in both the straw and the alfalfa addition experiments, with similar results. Bacterial growth was highest at pH 6.5–8.0 (Fig. 1C, D), and gradually declined to the low-pH end, by more than a factor of 3 ($p < 0.0001$). Fungal growth

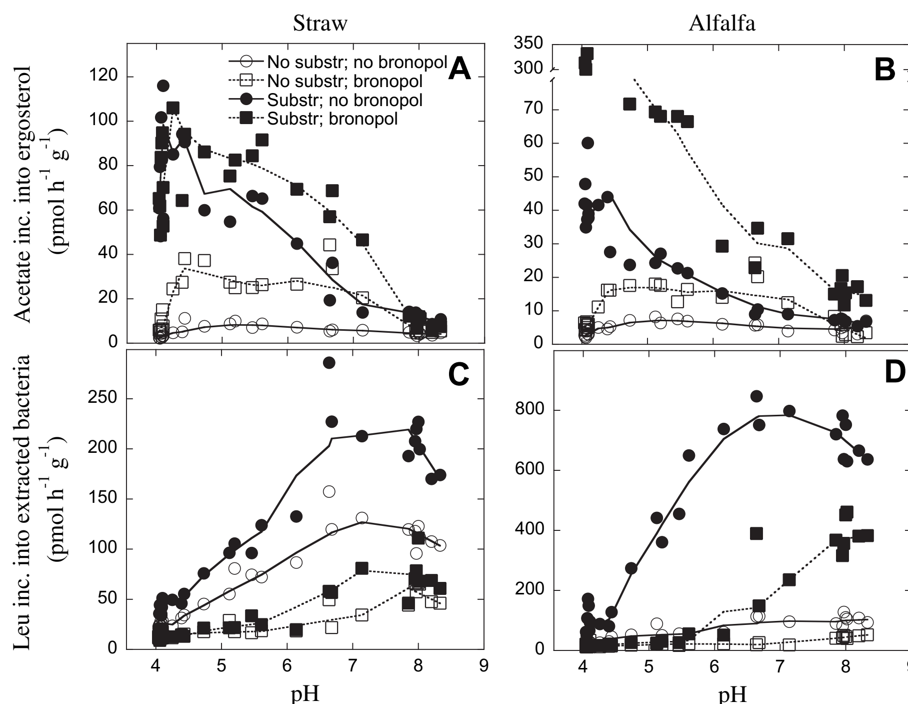

Fig. 1. Fungal growth (acetate incorporation into ergosterol, A and B) and bacterial growth (leucine incorporation, C and D) along the pH gradient of Hoosfield acid strip following the addition of substrate (1 mg C g^{-1} soil) in the form of straw (A and C) or alfalfa (C and D) and the bacterial inhibitor bronopol (40 µg g^{-1} soil). The curves are fitted using the locally weighted least squared error (Lowess) method with a 33% smoothing factor.

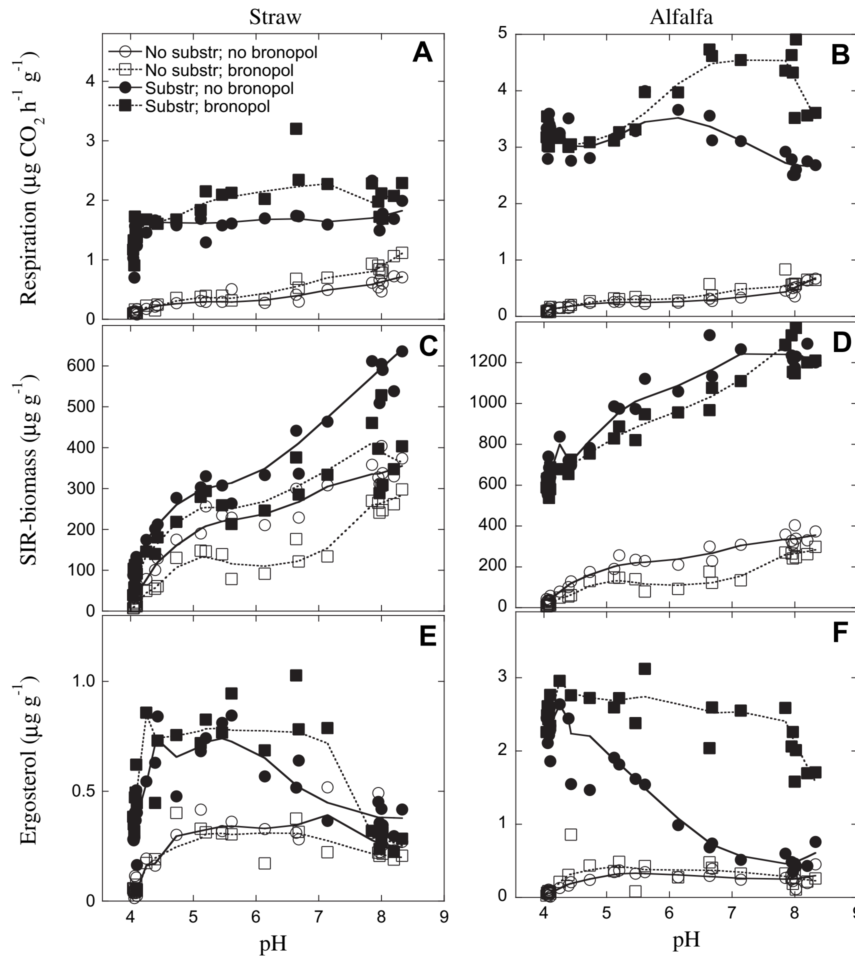

Fig. 2. The respiration rate (A, B) the SIR-biomass (C, D) and the ergosterol concentration (E, F) along the pH gradient of Hoosfield acid strip following the addition of substrate (1 mg C g^{-1} soil) in the form of straw (A, C and E) or alfalfa (B, D and F) and the bacterial inhibitor bronopol (40 μg g^{-1} soil). The curves are fitted using the locally weighted least squared error (Lowess) method with a 33% smoothing factor.

was highest at pH 4.5–5.5 (Fig. 1A, B), and decreased by a factor of about 2–3 toward both the low- and high pH ends of the gradient, reaching minimum values at both pH 4 and 8. Respiration decreased gradually with lower pH by about 90% from pH 8.3 to 4.0 (Fig. 2A, B; $p < 0.0001$). The SIR-biomass decreased with pH ($p < 0.0001$) from about 350 μg g^{-1} at pH 8.3–150 μg g^{-1} at pH 4.5; below pH 4.5 there was a rapid decline to less than 50 μg g^{-1} (Fig. 2C, D). The ergosterol concentration was mostly unchanged between pH 4.5 and pH 8.3. Below pH 4.5, there was a tendency for ergosterol to be lower (Fig. 2E, F).

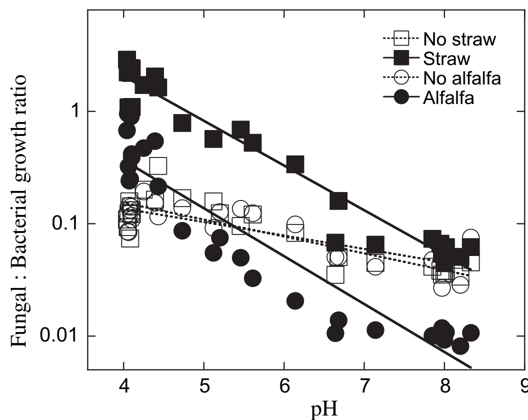

Fig. 3. The ratio between fungal growth and bacterial growth along the pH gradient of Hoosfield acid strip following the addition of substrate (1 mg C g^{-1} soil) in the form of straw (closed squares) or alfalfa (closed circles). Open symbols and broken lines represent no substrate additions in the straw and alfalfa addition experiment, respectively. The curves are fitted using an exponential function. Note the logarithmic *y*-axis scale.

3.1.2. Microbial growth in experimental treatments

Bacterial growth was stimulated by both straw (Fig. 1C) and alfalfa (Fig. 1D) additions ($p < 0.0001$ in both experiments). Maximal bacterial growth was achieved around pH 7 following both substrate amendments, while the lowest bacterial growth occurred at the low pH end of the gradient (Fig. 1C, D). There was a strong interaction between pH and substrate application for the alfalfa application ($p < 0.0001$) but not for the straw application. This indicated that bacterial growth responded more strongly to alfalfa at high pH compared to low, while straw stimulated bacterial growth proportionally across the pH gradient. The bronopol application decreased bacterial growth following both substrate applications ($p < 0.0001$ in both experiments). There were strong interactions between pH and the bronopol effect for both substrate added ($p < 0.001$ in both experiments), indicating that bronopol was proportionally more effective at high pHs for the straw application and vice versa for the alfalfa application.

Fungal growth was stimulated by both straw (Fig. 1A) and alfalfa (Fig. 1B) additions ($p < 0.0001$ in both experiments).

However, the stimulation was largest at the low pH end, with little (straw addition) or no (alfalfa addition) growth stimulation at the high pH end of the gradient, as indicated by a significant ($p < 0.001$) pH and substrate interaction in both experiments. Bronopol application stimulated fungal growth more clearly following alfalfa ($p < 0.0001$; Fig. 1B) than straw ($p = 0.01$; Fig. 1A). Fungal growth was stimulated by straw between pH 4.5 and 7.0, but not at lower or higher pH (Fig. 1A). Conversely, the additional fungal growth stimulation due to bronopol was highest at the low pH end in the alfalfa treatment, and decreased continually to a minimum at the high pH end (Fig. 1B). These patterns were confirmed by highly significant interactions between both substrates and bronopol ($p < 0.0001$ in both experiments).

The ratio between fungal and bacterial growth was consistently higher following straw application, and consistently lower following alfalfa application (Fig. 3). Additionally, between pH 5 and the high pH end, the no substrate treatments were intermediate between the substrate treatments, with no major difference depending on measurement occasion (i.e. between the no substrate controls of the straw and alfalfa experiments, indicating no differences between the two runs). The ratio decreased about 40-fold, from 0.4 to 0.01, between pH 4.0 and 8.3 following alfalfa, and 50-fold, from 2.0 to 0.04 between pH 4.0 and 8.3 with straw. The increase of the ratio was smaller without substrate addition, decreasing only 3–4 times from low to high pH.

3.1.3. Respiration and SIR-biomass in experimental treatments

Both straw and alfalfa additions affected the respiration rate similarly, but to different degrees. Following straw application, the respiration rate increased to about 1.5 μg CO_2 h^{-1} g^{-1} over the entire gradient (Fig. 2A). Alfalfa application resulted in a larger increase in respiration rate (Fig. 2B), to about 3 μg CO_2 h^{-1} g^{-1} over most of the soil pH gradient. Thus the application of substrate removed the influence of pH on soil respiration, as indicated by a significant interaction between substrate application and pH ($p < 0.0001$ in both experiments). Combined bronopol and substrate addition tended to marginally increase the respiration rate at intermediate pH following straw ($p = 0.09$) and resulted in increasing stimulation with higher pH starting at pH 6 following alfalfa addition ($p < 0.001$).

The gradual increase in SIR-biomass with higher pH along the unamended gradient was, unlike respiration, largely maintained following the substrate amendments. The SIR-biomass increased by about 100 μg g^{-1} along most of the gradient as a result of straw addition ($p < 0.0001$; Fig. 2C). Alfalfa addition increased SIR-biomass ($p < 0.0001$) by about 700 μg g^{-1} (Fig. 2D).

3.1.4. Ergosterol concentration in experimental treatments

Both substrate applications stimulated the accumulation of ergosterol ($p < 0.0001$ in both experiments), but the stimulation was more amplified by alfalfa (Fig. 2F) than straw (Fig. 2E). The stimulation of ergosterol accumulation was highly pH dependent following addition of both substrates, being more stimulated at lower than at higher pH, as indicated by strong interactions between substrate and pH ($p < 0.0001$ in both experiments). There was a weak but non-significant tendency for further stimulation of ergosterol accumulation when straw was combined with bronopol, starting low at low pH, increasing gradually until pH 7.5, after which it declined toward the high end of the gradient. Combining alfalfa with bronopol resulted in a highly significant increase in ergosterol ($p < 0.0001$). Bronopol application especially increased ergosterol content at high pH after adding alfalfa, as indicated by the three-way interaction between pH, bronopol and substrate ($p = 0.002$).

3.2. Time-series experiment

Fungal growth measured with acetate incorporation (Fig. 1A) and the fungal biomass measured as ergosterol concentration (Fig. 2E) correlated strongly in the straw treated soils ($p < 0.0001$, $R = 0.90$). However, there was a discrepancy in the bronopol treatment in the alfalfa-amended soil. Although the fungal biomass was high at all pHs of the gradient (Fig. 2F), the fungal growth was significantly lower at high pHs (Fig. 1B). This fungal growth decrease coincided with an elevation of the bacterial growth, however (Fig. 1D). To investigate if the discrepancy between fungal biomass and growth could be reconciled by a cessation of bacterial inhibition late in the incubation period (with a subsequent inhibition of fungal growth), we studied the development of the microbial variables over time.

Bacterial growth increased rapidly in straw-amended soils at pH 8.1, reaching 60 pmol Leu h^{-1} g^{-1} after 2 days and 80 pmol Leu h^{-1} g^{-1} after 7 days. At pH 5.1 the absolute levels were lower, and rates of 20 pmol Leu h^{-1} g^{-1} and 40 pmol Leu h^{-1} g^{-1} were reached after 2 and 7 days, respectively. At pH 4 the rates were lowest, at 6 pmol Leu h^{-1} g^{-1} day 2 and 15 pmol Leu h^{-1} g^{-1} day 7 (Fig. 4C), and the increase was slower than at higher pHs. Bronopol application suppressed virtually all bacterial growth until day four in all soils, but then the bacterial growth in the pH 8.1 soils started increasing.

The fungal growth rate following straw addition was very low, but increased from 6 to 14 pmol Ac h^{-1} g^{-1} over the course of the experiment at pH 8.1 (Fig. 4A). The fungal growth at pH 4 increased more rapidly, starting at about 4 and increasing to 25 pmol Ac h^{-1} g^{-1} by day 7. At pH 5.1, fungal growth started at 9 pmol Ac h^{-1} g^{-1}, increased to a maximum of about 25, and then slowly declined to about 20 pmol Ac h^{-1} g^{-1} by day 7. Bronopol application resulted in an additionally increased fungal growth rate at both pH 4.0 and especially at pH 5.1, but not at pH 8.1.

Alfalfa induced a 40-fold increase in bacterial growth within one day, from less than 10–400 pmol Leu h^{-1} g^{-1} at pH 8.1 (Fig. 4D). The increase was gradually slower and less marked at pH 5.1 and 4.0. Bronopol efficiently decreased the bacterial growth at all pHs until day 4, when the bacterial growth rate at pH 8.1 started to increase rapidly (from less than 10 to more than 100 pmol Leu h^{-1} g^{-1} by day 7).

The fungal growth progressed slowly with alfalfa addition, increasing from initially 4–8 pmol Ac h^{-1} g^{-1} by day 1 and never increased markedly after, at pH 8.1 (Fig. 4B). In combination with bronopol, however, the fungal growth increased to almost 20-fold by day 4, from about 2 to 35 pmol Ac h^{-1} g^{-1}, after which it decreased to less than 15 by day 7. Fungal growth at pH 5.1 and 4.0 increased from about 6 to almost 50 pmol Ac h^{-1} g^{-1} within one day, and from 3 to 50 pmol Ac h^{-1} g^{-1} within 2 days, respectively, following alfalfa application. The increase was even larger when alfalfa was combined with bronopol. At pH 5.1, fungal growth increased from 8 to 60 pmol Ac h^{-1} g^{-1} within one day, which was sustained for the 7 days. At pH 4.0, the increase was slower, but after 2 days it increased from about 4 to 120 pmol Ac h^{-1} g^{-1}, after which it reached a maximum rate at day 4 just over 150 pmol Ac h^{-1} g^{-1}, and then declined at day 7 to about 60 pmol Ac h^{-1} g^{-1}.

3.3. Bronopol tolerance

The increase in the bacterial growth toward the end of the incubation time in the high pH soils (Fig. 4C, D) could be explained either by an emerging bacterial community more tolerant to bronopol, or decreased availability of bronopol due to degradation and inactivation especially in soil with added substrate where the microbial activity increased. The former

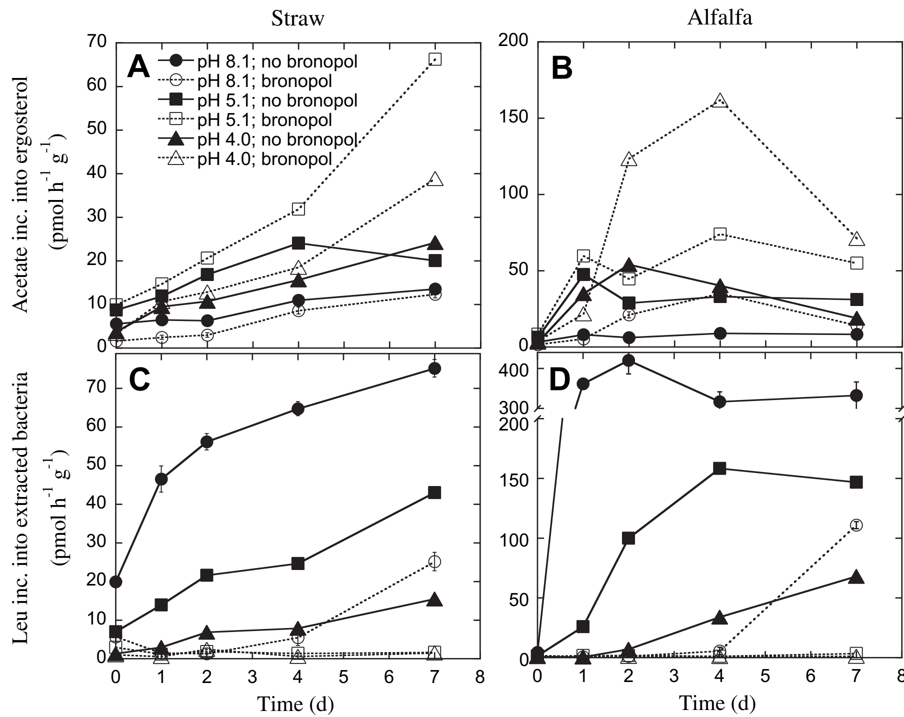

Fig. 4. Fungal (A and B) and bacterial growth (C and D) following the addition of substrate (1 mg C g^{-1} soil) in the form of straw (A and C) or alfalfa (B and D) (1 mg C g^{-1} soil) and the bacterial inhibitor bronopol (40 μg g^{-1} soil) over a 7 days time-series. Circles (with errors bars denoting SE, $n = 4$) represent soils samples of pH 8.1, squares represent a soil sample of pH 5.1, and triangles represent a soil sample of pH 4.0. The curves are fitted using the locally weighted least squared error (Lowess) method with a 33% smoothing factor.

explanation implies increased bronopol tolerance of the bacterial community. There was, however, only a weak, non-significant, tendency for increased tolerance to bronopol in the bronopol treated soils. The EC_{50} value for the straw application treatment without bronopol was 2.6 μg ml^{-1}, while with bronopol it was 3.5 μg ml^{-1} bacterial suspension (Fig. 5A). The untreated alfalfa soil had an EC_{50} of 3.2 μg ml^{-1}, while the bronopol treated alfalfa soil had an EC_{50} value of 4.7 μg ml^{-1} bacterial suspension (Fig. 5B). Thus, it was likely that bronopol concentration decreased with time due to degradation, eventually becoming exhausted allowing for bacterial growth at the end of the incubation period (Fig. 4C, D).

4. Discussion

4.1. Alfalfa and straw effects

Alfalfa addition increased bacterial growth more than straw while straw addition increased fungal growth more than alfalfa irrespective of pH (Fig. 3), supporting the first hypothesis. This corroborated previous results concerning the selective effect of these substrates in one soil (Rousk and Bååth, 2007b), and also generalises these findings to be applicable to soils with different pH. However, the differential pH effect on fungal and bacterial growth strongly modified the substrate effects. Below pH 5 the

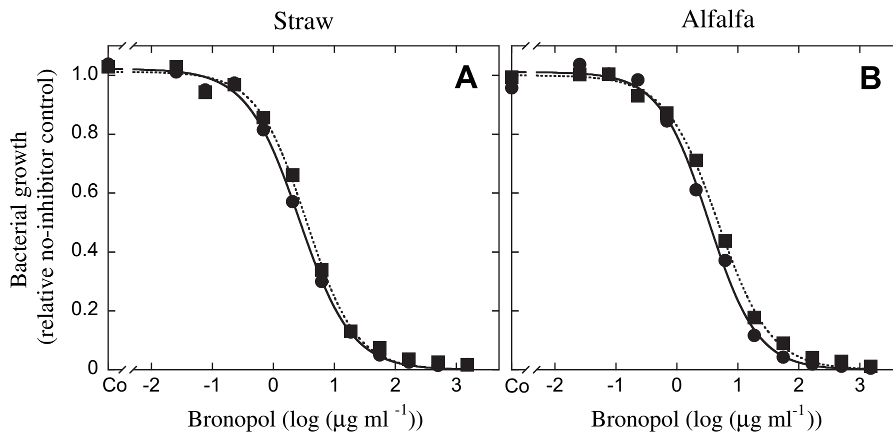

Fig. 5. The dose–response curves of bronopol toxicity of the bacterial community extracted from one of the pH 8 soils after the time-series experiment treated with the substrates straw (A) or alfalfa (B) (1 mg C g^{-1} soil) and bronopol (40 μg g^{-1} soil) 7 days prior to the depicted short-term exposure. The bronopol concentrations on the x-axis relates to the concentration in the bacterial suspension during the short-term assay (see Section 2.2.3), with Co indicating the control with no added bronopol. Square symbols and broken lines represent soil with bronopol additions and circles and unbroken lines represent soils without bronopol. The inhibition curves were fitted using a sigmoid function.

addition of both substrates benefited fungi, showing that only pH constrained the bacterial growth there. Similarly, fungal growth could not be stimulated to a high degree at high pH. Consequently, the slow bacterial growth rate in the low pH soils, and low fungal growth rate in the high pH soils, did not seem to be limited by lack of a suitable substrate for fungal or bacterial growth.

An alternative explanation could be limitations by resources other than C along the pH gradient. Previous work showed, however, that the soil C:N ratio is highly constant at around 10 (Rousk et al., 2009), indicating small differences in N availability along the gradient. The general pattern due to pH was also unaffected by the substrate, despite very different C:N ratios for straw and alfalfa, suggesting that that the different patterns of fungal and bacterial growth with pH was unrelated to soil N availability. To test if P limitation could influence the growth rate patterns along the gradient, straw addition was also combined with K_2HPO_4 (at a substrate C:P ratio of 20:1). There were no measurable changes in the fungal or bacterial growth rates compared to no addition of P (data not shown), indicating that P limitations did not affect the results.

4.2. Mechanisms for the fungal pH relationship

The application of bronopol indicated that the suppression of bacterial growth stimulated fungal growth, and thus that the dynamics in the fungal:bacterial growth ratio along the pH gradient was a consequence of bacterial competitive control of fungal growth. However, the increase in fungal growth following bacterial inhibition was more evident below pH 7 than above in the five-day incubation experiments, especially after adding alfalfa. This would suggest that the fungal growth was competitively limited by bacteria up to about pH 7, but that another factor may have constrained fungal growth above this pH. However, there was a discrepancy at the highest soil pH between no increased fungal growth at day 5 (measured as incorporation of acetate into ergosterol) and the increased cumulative fungal growth (measured as changes in ergosterol concentration) over the 5-day incubation times in the alfalfa treatment with bronopol. If bacterial growth increased only toward the end of the incubation time, and this inhibited fungal growth, this would permit accumulation of fungal biomass (until day 4), and still produce low fungal growth rates at the end of the incubation period (at day 5). The time-series experiment corroborated this explanation, showing that bacterial growth was suppressed for about 4 days at high pH following bronopol treatment in the presence of substrate, after which it started to increase. Also, when bacterial growth started to increase there were simultaneous indications of decreased, or at least not increased, fungal growth.

Our results thus showed that high fungal growth was possible along the entire pH gradient in the presence of substrate, when the competitive influence of bacteria was removed, supporting the third hypothesis. This indicated that the competitive pressure exerted by bacteria inhibited fungal growth at high pH, causing the increased fungal:bacterial growth ratio with decreasing pH. In a study of the effects of bacterial and fungal inhibitors in soil, increased fungal biomass production following the application of a bacterial inhibitor has been shown (Feeney et al., 2006). Romani et al. (2006) showed that bacteria suppressed fungal growth on submerged plant material, and De Boer et al. (2003) demonstrated that bacteria controlled fungal growth (fungistasis) in soil. In addition, increasing inhibition of bacterial growth using a range of concentrations of different bacterial inhibitors has been shown to cause a positive growth response in fungi (Rousk et al., 2008). The surplus of added substrate did not appear to affect the intensity of this competitive interaction in the present study, suggesting that the mode of competition was not solely explained by exploitive competition as previously indicated in a similar experiment (Rousk et al., 2008), but that competition by direct interference of one group by another was also probably an important element (De Boer et al., 2003; Mille-Lindblom and Tranvik, 2003; Mille-Lindblom et al., 2006).

However, some results still remain to be explained: Why did straw, a better stimulator of fungal growth than alfalfa, not result in greater fungal growth when bacteria were inhibited in high pH soils? And why did fungal growth at the low pH end in the alfalfa treatment increase so much when combined with bronopol, even though the bacterial establishment in the low pH soil was low?

Since straw addition benefited fungal more than bacterial growth, straw as a C resource was less exploited by bacteria than by fungi. The exclusion of bacteria from using straw in the bronopol application may thus present fungi with relatively little new niche-space to exploit, compared with the previously unused resource alfalfa. In line with this rationale, the fungal growth stimulation following bronopol was smaller in straw-amended soils compared to alfalfa-amended soils, supporting the second hypothesis.

The substantially higher fungal growth stimulation in the alfalfa treatment compared with straw following bacterial growth inhibition in the low pH soils could have a similar explanation. The bacterial growth at the low pH end in the alfalfa treatment was very low compared to the high pH end soils of the same treatment. However, the level of bacterial growth in the low pH end following alfalfa was still substantial, and similar in magnitude to the non-amended high pH soils, indicating that bacteria could still have dominated the utilisation of alfalfa. This would, as discussed previously, present fungi with a newly unoccupied resource in the absence of bacteria (following bronopol). The suppression of bacterial influence in the already fungi-dominated straw addition would thus present the fungi with minor additional resources to exploit, while the suppression of bacteria in the bacteria-dominated alfalfa would yield significant increases in available resources to exploit.

4.3. Mechanisms for the bacterial pH relationship

Assuming that it is bacteria that inhibit fungal growth at high pH, the most fundamental aspect remains unresolved: What mechanism caused the strong dependence of bacterial growth on pH along the gradient? One candidate mechanism is interaction between the decomposer groups. The presence of fungi has, however, been shown to both inhibit the growth of bacteria during the colonization of plant material (Mille-Lindblom and Tranvik, 2003; Mille-Lindblom et al., 2006), and facilitate it (Bengtsson, 1992; Romani et al., 2006; Meidute et al., 2008) indicating that competitive interactions between the decomposer groups presently is an equivocal explanation for the low bacterial growth in the low pH soils. Another candidate mechanism could be related to the disparate physiologies of the decomposer groups. Both groups need to maintain a homeostatic intracellular pH at low pH, which is energy-demanding. In addition to this, bacterial cells will also face the problem of maintaining a proton motive force across the cell membrane (Garland, 1977; Russell et al., 1979), while this function is intracellular in the mitochondria of fungal cells. Consequently, it is possible that the bioenergetics of bacteria may be more susceptible to ambient chemical conditions than fungi. On the other hand, both culture-based studies (Sundman, 1970; Bååth et al., 1992) and measurements of instantaneous growth at different pH (Bååth, 1996; Pettersson and Bååth, 2003,2004) have indicated that bacteria are adapted to the prevailing soil pH, even at low pH soils, where the bacterial growth rate is low. Tolerance to low pH does not rule out, however, that the metabolism of bacteria in low pH soils still is impeded.

4.4. Substrate effects and aluminium toxicity below pH 4.5

It was previously concluded that the microbial variables were differently related to pH below 4.5 in the Hoosfield gradient (Rousk et al., 2009), which was corroborated in the present study. Especially fungal growth, which increased with decreasing pH in unamended soils, decreased again at pH below 4.5. Two explanations for this have been put forward; Al toxicity at low pH or lack of substrates due to impaired plant growth (Aciego Pietri and Brookes, 2007b; Rousk et al., 2009). The addition of plant material at pH 4.0–4.5 stimulated both bacterial and fungal growth, as well as other microbial variables including respiration rate, SIR-biomass, and ergosterol. An increased respiration rate was also found after adding straw to a low pH soil from this gradient (Aciego Pietri and Brookes, 2009). The large increase in fungal growth after substrate amendments suggested that fungal growth in unamended soil were low due to lack of easily available substrate and not due to Al toxicity (Aciego Pietri and Brookes, 2007b; Rousk et al., 2009). The increase in the bacterial growth was, however, rather small following addition of substrate, which may be interpreted as bacteria being more susceptible to Al toxicity. However, available Al was highly pH dependent, increasing from undetectable levels to more than 500 mg kg^{-1} between pH 5.5 and 4.0 (Aciego Pietri and Brookes, 2007b). In contrast, the decrease in bacterial growth started at about pH 7, and continued gradually all the way to pH 4 (Fig. 1C, D), with no apparent additional effect between pH 5 and 4. Hence, the contribution to bacterial growth inhibition by Al appeared minimal. That bacterial growth only increased marginally after adding substrate below pH 4.5 was thus most likely due to direct negative effects of pH or competition by fungi at low soil pH.

4.5. Conclusion

Fungal growth was especially promoted by straw, while alfalfa especially promoted the bacterial growth, irrespective of soil pH. Inhibition of bacterial growth using bronopol consistently stimulated fungal growth, indicating a strong competitive interaction. When the positive relationship between bacterial growth and increasing pH was terminated with bacterial inhibitor application, the negative relationship between fungal growth and increasing pH was alleviated. Thus, a negative interaction where bacteria out competed fungi at high pH was a major mechanism for the negative relationship between the fungal growth rate and increasing pH in the studied soil.

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