Rapid method of determining factors limiting bacterial growth in soil

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A technique to determine which nutrients limit bacterial growth in soil was developed. The method was based on measuring the thymidine incorporation rate of bacteria after the addition of C, N, and P in different combinations to soil samples. First, the thymidine incorporation method was tested in two different soils: an agricultural soil and a forest humus soil. Carbon (as glucose) was found to be the limiting substance for bacterial growth in both of these soils. The effect of adding different amounts of nutrients was studied, and tests were performed to determine whether the additions affected the soil pH and subsequent bacterial activity. The incubation time required to detect bacterial growth after adding substrate to the soil was also evaluated.

Second, the method was used in experiments in which three different size fractions of straw (1 to 2, 0.25 to 1, and <0.25 mm) were mixed into the agricultural soil in order to induce N limitation for bacterial growth. When the straw fraction was small enough (<0.25 mm), N became the limiting nutrient for bacterial growth after about 3 weeks. After the addition of the larger straw fractions (1 to 2 and 0.25 to 1 mm), the soil bacteria were C limited throughout the incubation period (10 weeks), although an increase in the thymidine incorporation rate after the addition of C and N together compared with adding them separately was seen in the sample containing the size fraction from 0.25 to 1 mm. Third, soils from high-pH, limestone-rich areas were examined. P limitation was observed in one of these soils, while tendencies toward P limitation were seen in some of the other soils.

A faster way to study the limiting factors of bacterial growth is to measure the incorporation of radioactive thymidine of bacteria. The thymidine uptake technique, pioneered by Fuhrman and Azam (13) and subsequently modified by many workers (e.g., reference 39), has been the most widely used method to estimate bacterial activity and growth rate in aquatic systems. Recently, it has been replaced to some degree by the leucine incorporation technique (21), although these two methods usually give similar results (6, 32). Both of these techniques have been used to indicate which nutrient that limits bacterial growth in aquatic systems. Upon the addition of a limiting substance, bacteria will show an increased rate of incorporation of the radioactive compound compared with an unsupplemented control or the addition of a nonlimiting substance. Using one of these two techniques, it has been shown that the availability of carbon, nitrogen, or phosphorus can limit bacterial growth in aquatic systems depending on the season and habitat (7, 10, 15, 16, 20, 29, 36).
TABLE 1. Standard amounts of carbon, nitrogen, and phosphorus added to the soils before measurement of the bacterial activity

<table>
<thead>
<tr>
<th>Soil type</th>
<th>Amt of glucose-C (mg g [ww] of soil$^{-1}$)</th>
<th>Amt (µg [ww] g of soil$^{-1}$)</th>
<th>NH$_4$NO$_3$N</th>
<th>KH$_2$PO$_4$P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agricultural soil</td>
<td>0.27</td>
<td>14</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Forest humus</td>
<td>1.98</td>
<td>99</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Calcareous soils</td>
<td>0.27</td>
<td>27</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

The thymidine incorporation technique has been further developed for measurements of bacterial activity in soil (2, 3, 8, 14, 24), and the leucine incorporation technique has also been used in soils for a number of years (4, 24). The main differences in using these techniques in soil compared with water habitats are that the concentration of the labeled substrates added is higher and that the incubation time is usually longer. However, so far no one has used these two techniques to determine limiting factors for bacterial growth in soil in a way similar to that used in aquatic systems.

The aim of this work was to develop a technique based on thymidine and/or leucine incorporation to study nutrients limiting bacterial growth in soil. First, the thymidine incorporation technique was tested in two contrasting soils: an agricultural soil and a forest humus soil. A comparison with leucine incorporation measurements was also made. The effect of the amount of added substrates (carbon, nitrogen, and phosphorus in different combinations) on bacterial growth was evaluated, and tests were carried out to determine whether the substrate additions affected the soil pH. The incubation time required after substrate addition to detect increased bacterial growth in the soil was also determined. Second, the resulting method was then used in experiments, in which straw with a high C/N ratio was mixed into soil in order to induce nitrogen limitation. The importance of the particle size of the added straw was also investigated. Third, to ascertain whether the technique could detect phosphorus limitation, different soils from high-pH, limestone-rich areas were studied.

MATERIALS AND METHODS

**Addition of C, N, and P.** The soil to be studied was divided among eight jars, with 15 g (wet weight [ww]) of agricultural or calcarious soil or 7.5 g (ww) of humus in each. C, N, and P were added either alone or in the ratio 20:1:0.67 (by weight). Carbon was added as glucose, nitrogen as added as NH$_4$NO$_3$, and phosphorus as added as K$_2$HPO$_4$. The amount of substrate added was adjusted according to the organic matter content of the soil. It was estimated that 1.5% of the organic matter was microbial biomass (38), and the amount of carbon added was set to approximately half the weight of microorganism carbon. Nitrogen was added in amounts such that the pH should not be altered (but see below). The soil samples were incubated at 20°C in plastic bags, with one bag for each soil type. Measurements of thymidine incorporation and on soil pH was further evaluated by adding different nitrogen substrates: NH$_4$NO$_3$, (NH$_4$)$_2$SO$_4$, and KNO$_3$.

**Experiments with straw.** Straw was cut into 1-cm pieces and then further homogenized in an Omnimixer. The material was then sieved. Pieces that passed through a 2-mm-mesh sieve, but not a 1-mm-mesh sieve, were used (fraction 1 to 2 mm). In an experiment performed on a later occasion, straw was ball milled, and then the milled straw was separated into two fraction. One fraction passed through a 1-mm-mesh sieve but not through a 0.25-mm-mesh sieve (fraction 0.25 to 1 mm). The other passed through a 0.25-mm-mesh sieve (fraction <0.25 mm). Only the agricultural soil was used in this experiment. The straw addition (4% of the forest humus ww and 1% of the agricultural soil ww) amounted to approximately 20 and 25% of the organic matter content in humus and agricultural soil, respectively.

**Soil samples.** Seven calcarious soils originating from Alvaret on Öland, Sweden, were used. The soils had values of pH (H$_2$O) between 5.8 and 8.2 (most of them in the high range) and organic matter contents between 9.2 and 16.8%.

**Measurements of thymidine and leucine incorporation.** To measure the incorporation of thymidine, a modification of the homogenization-extraction technique (3) was used. A flow diagram of the technique is shown in Fig. 1.

**FIG. 1.** Flow diagram of the thymidine and leucine incorporation technique for determining limiting substances for bacterial growth in soil.
All soils were low in bicarbonate-soluble phosphate (<0.05 \( \mu \)mol g [dry weight] of soil \(^{-1} \)). Substrates were added as described above for the agricultural soil, except that the amounts of nitrogen and phosphorus added were doubled (Table 1). For each of the soils separate measurements were also made on two controls to which no substrates was added and three samples to which 10 times the standard amount of phosphorus had been added (0.17 mg g [ww] of soil \(^{-1} \)). The incubation period of the soils before measuring the thymidine incorporation was 64 h.

**Statistical analysis.** A full three-way factorial experimental design without replication was used for each measurement of nutrient limitation, and the data were analyzed by analysis of variance (ANOVA). When more than one experiment was performed, each experiment was treated as a replicate in the statistical analysis (as in Fig. 3). To facilitate comparisons between measurements on different dates and in different soils, the results were normalized by setting the value for the no-addition treatment to 1 in the graphs, although the statistical analyses were made on the original data.

In the experiments with different incubation times (see Fig. 2) and with straw addition (see Fig. 7), no significant effects were observed for phosphorus addition. These treatments were therefore considered to be replicates of similar treatments when no phosphorus was added. Thus, \( n = 2 \) for the different treatments (no addition and C, N, and CN addition). Separate ANOVAs were performed for each sampling date. Similarly, no effect of nitrogen was found in the calcareous soils, and the nitrogen treatments were therefore considered to be replicates of similar treatments when no nitrogen was added.

**RESULTS**

**Initial experiments.** In order to establish the time of maximum response after the addition of carbon, nitrogen, and phosphorus, thymidine incorporation was monitored for up to 7 days after the addition of substrate to the soil. Carbon addition increased the bacterial activity during the whole incubation period in the agricultural soil, while nitrogen and phosphorus (data not shown) did not have any effect (Fig. 2A). A maximum response was observed after 1 to 4 days. Carbon addition also increased bacterial activity in the forest humus (Fig. 2B). However, thymidine incorporation rates after 24 h were no higher than that in the unamended control sample. After 48 h of incubation, the incorporation rate increased due to carbon addition, with a maximum appearing 3 days after the addition. Nitrogen addition appeared to have no, or even a negative, effect on the activity (especially when added with C, indicated by significant CxN interaction), while phosphorus did not affect the thymidine incorporation rate at all (data not shown). It was thus deemed practical to measure the bacterial activity after 48 h of incubation of both soils.

Limiting factors for bacterial growth were then determined on several occasions to study the reproducibility of the results. In the agricultural soil the availability of carbon was found to be limiting for bacterial growth (\( P < 0.001 \)), since the thymidine incorporation rate increased in all samples when carbon was added compared with the unamended control sample (Fig. 3A). Nitrogen and phosphorus did not have any effect on the thymidine incorporation rate, indicating that there was no nitrogen or phosphorus limitation for bacterial growth. The mean effect of carbon addition was to increase thymidine incorporation rates by about 2.4 times 48 h after glucose addition.

The availability of carbon was also the limiting factor in the humus (Fig. 3B, \( P < 0.001 \)). Nitrogen appeared to have a negative effect on the bacterial activity, especially in the samples to which carbon had been added. Phosphorus addition did not affect the bacterial activity. The mean effect of carbon addition (not including samples to which nitrogen had been added) was to increase the bacterial activity by about 4.8 times.

Changes in the incorporation rate of leucine due to nutrient additions were similar to those in the thymidine incorporation rates in the agricultural soil (Fig. 4A). Carbon had a positive effect, while nitrogen and phosphorus did not appear to have any effect. Leucine incorporation rates increased more than thymidine incorporation rates after carbon addition to the forest humus (Fig. 4B). However, the conclusions were the same, irrespective of the analysis technique used. The availability of carbon was the limiting factor for bacterial growth in the humus, nitrogen addition appeared to have a negative effect, while phosphorus did not affect the bacterial activity.
In order to investigate whether too-low concentrations of nitrogen and phosphorus were used, the effect of a 10-fold increase in the concentrations was studied. Phosphorus addition still did not affect the thymidine incorporation rates in either soil, as was the case for nitrogen addition to the agricultural soil. However, in the forest humus the negative effect of nitrogen addition was even more evident than before. The addition of nitrogen together with carbon totally inhibited the positive effect of carbon, while nitrogen alone decreased bacterial growth to less than half of that in the unamended control sample. The effect of nitrogen addition was therefore studied further.

Different nitrogen substrates added at different concentrations to the agricultural soil did not appear to change the relative bacterial activity until a level of 1.4 mg N g (ww)⁻¹ (100 times the standard amount of nitrogen) was added, when the activity appeared to increase (Fig. 5A). This was especially evident using NH₄NO₃, whereas adding KNO₃ had no effect. The activities in the humus, on the other hand, were almost in the same range until 0.99 mg of N g (ww)⁻¹ (10 times the standard addition) was added. Then, the thymidine incorporation decreased with increasing amounts of nitrogen compared with the unamended control (Fig. 5B). There were no major differences between bacterial activities when different nitrogen substances were used. It was decided to use a standard nitrogen concentration of ammonium nitrate equivalent to 14 mg of nitrogen g (ww)⁻¹ and 99 mg of nitrogen g (ww)⁻¹ in further studies with these soils.

The pH decreased with increasing amounts of nitrogen added to the humus. There appeared to be a correlation between the decrease in pH and the decrease in thymidine incorporation rates after nitrogen addition (Fig. 6). The pH was only affected to a minor degree by adding different concentrations of nitrogen to the agricultural soil (data not shown).

Effect of straw addition. Directly after adding straw (fraction 1 to 2 mm), significant carbon limitation for bacterial growth was observed, as before (P < 0.05; Fig. 7A). During the 10
weeks after addition of the straw, the carbon, and especially the carbon combined with nitrogen, increased the thymidine incorporation rate in most cases compared with the unamended control sample, while nitrogen addition alone had no effect on thymidine incorporation. Phosphorus addition had no effect on the bacterial activity (data not shown) during the incubation period studied.

Carbon was also the limiting factor for bacterial growth in the humus during the 10 weeks after straw addition, while nitrogen had a slightly negative effect on the activity (data not shown) during the incubation period studied.

In the agricultural soil to which the straw fraction from 0.25 to 1 mm was added, carbon initially had a significant positive effect on the bacterial activity (P < 0.01, Fig. 7B), although the stimulating effect of carbon on thymidine incorporation was lower than it was previously. Phosphorus application did not affect the bacterial growth rate during the 10-week period of study (data not shown). During this incubation period with straw, carbon addition usually increased the thymidine incorporation rate more than nitrogen addition did. However, adding carbon and nitrogen together increased the bacterial activity much more than separate additions, which may indicate combined carbon and nitrogen limitation.

In the agricultural soil containing the straw fraction smaller than 0.25 mm, the bacterial activity was initially limited by carbon availability (P < 0.05). Between the third and fifth weeks, nitrogen became the limiting factor (P < 0.05; Fig. 7C). After 10 weeks, the availability of carbon was again the limiting factor for bacterial growth (P < 0.05). Phosphorus addition did not have any effect on the activity (data not shown). The activity increased more when carbon was added together with nitrogen than when carbon was added alone after the first week.

The pH was in the same range, ca. 7.6 to 7.7 during the whole period studied for all substrate combinations in the three experiments on the agricultural soil and ca. pH 5.0 in the single experiment on forest humus.

**Calcarius soils.** There was no evidence of nitrogen limitation in any of the calcarius soils. Therefore, nitrogen addition experiments were treated as replicates. Carbon limitation was observed in soils A (P < 0.001), B (P < 0.001), and E (P < 0.05) (Fig. 8A, B, and E). There was an indication of phosphorus limitation in soil G (P < 0.14). This was further investigated by adding 10-fold amounts of phosphorus in a separate experiment. This stimulated the bacterial activities in four soils, C, D, F, and G (with a significance of P < 0.05 for the two latter soils; Fig. 8C, D, F, and G). No such stimulation was observed in the soils exhibiting carbon limitation (Fig. 8B and E).

**DISCUSSION**

This method of detecting substances limiting bacterial growth in soil using thymidine incorporation appeared to work well. The method was rapid and gave reproducible results, and it was possible to detect cases in which the availability of...
carbon, nitrogen, or phosphorus was the main factor limiting bacterial growth in soil. One must bear in mind, however, that absolute growth rates of bacteria in soil were not measured, since this would have involved measuring isotope dilution and the fraction of thymidine incorporated into DNA, as well as applying a conversion factor to recalculate the thymidine incorporation into bacterial production (6). However, Kirchman (20) stated that although the determination of actual in situ bacterial growth rates using this radioisotope incorporation technique is problematic, the technique is useful for relative monitoring in natural bacterial communities. Since, in our case, we always compared the thymidine or leucine incorporation after adding C, N, or P with a nonamended control, there was no need to calculate actual growth rates.

Incorporation of both leucine and thymidine into bacteria gave the same results as in aquatic habitats (7), that is, the detection of increased activity after adding a limiting nutrient was the same, regardless of whether leucine or thymidine incorporation was measured (Fig. 4). Thus, either of the two techniques could be used. However, the similar results for leucine and thymidine incorporation indicated that the results based on thymidine incorporation were not due to the labeled substrate being taken up differently by an altered bacterial community after, e.g., glucose addition, but really were due to increased activity and growth.

It is widely assumed that carbon availability is the most common limiting factor for microbial growth in soil (22, 33). We found this to be the case for bacterial growth in most of the soils studied (Fig. 3A and B and 8A, B, and E). Phosphorus availability, however, appeared to be the limiting factor for bacterial growth in certain calcareous soils (Fig. 8F and G). Similar results were found in this type of soil by Scheu (30) in a beech tree forest growing on limestone and by Duah-Yentumti et al. (12) in a tropical forest soil. Possible phosphorus limitation was also observed by Demetz and Insam (11) in a beech-spruce forest in the calcareous Alps.

We added straw to soil samples in order to ascertain whether the bacterial growth was also limited by nitrogen availability, since it has been found that microorganisms decomposing straw with a high C/N ratio immobilize available nitrogen in soil (26). Similar conclusions were drawn from another experiment using 15N-labeled straw (27). We also studied different size fractions of straw to investigate whether the size had any effect on limiting factors for bacterial growth. Smaller straw fractions have been shown to immobilize nitrogen faster and to a greater extent than larger ones (1, 7). The hypothesis was thus that the smaller the straw fraction, the faster and more evident the nitrogen limitation for microbial growth. This was also found to be the case. When straw <0.25 mm was added to agricultural soil which was initially carbon limited, it became

![Graph A](image1)

**FIG. 7.** Relative bacterial activity (thymidine incorporation) 48 h after C and N addition to the agricultural soil. The activity of the nonamended control sample was set to 1. Straw was added at the beginning of the experiments. (A) Straw size fraction 1 to 2 mm. (B) Straw size fraction 0.25 to 1 mm. (C) Straw size fraction <0.25 mm. The bars indicate SEs (n = 2) obtained from ANOVA for each separate sampling date. C, N, or CxN over the graph indicates significant effects of carbon, nitrogen, or the interaction of carbon and nitrogen. *, P < 0.05; **, P < 0.01.
nitrogen limited after 3 weeks (Fig. 7C). On the other hand, in the experiment where straw 1 to 2 mm in size was added, the soil bacteria were carbon limited during all 10 weeks (Fig. 7A). In the soil sample initially carbon limited and then nitrogen limited after 3 weeks (straw fraction size of <0.25 mm, Fig. 7C), carbon became the limiting factor again after 10 weeks, which could be explained by the easily available carbon in the straw being exhausted.

When the intermediate straw fraction (0.25 to 1 mm) was added, the soil bacteria were also mainly carbon limited, but adding a combination of carbon and nitrogen led to a high relative thymidine incorporation (Fig. 7B). This might be an indication that the bacterial growth in this experiment was close to becoming nitrogen limited. Similar findings have been reported when limiting factors were measured by the respiration technique. In some cases, glucose addition increased microbial growth, but the addition of nitrogen or phosphorus increased growth even more, although adding them without carbon did not affect respiration (e.g., reference 12). This could be interpreted as evidence that carbon is the main limi-
iting substance for bacterial growth, but the situation is close to other nutrients also being limiting.

The most common technique for determining limiting factors for microbial growth in soil has hitherto been the measurement of microbial respiration after the addition of glucose and a nutrient together (9, 12, 23, 30, 31, 34, 35). There are several differences between this technique and the one described here involving thymidine and leucine incorporation. The respiration technique provides information on limiting factors for the total microbial community. Our method of determining the limiting factors only involves the bacterial part of the microbial community. However, it should be possible to determine the bacterial and fungal growth separately, since fungal activity after substrate addition could be measured using acetate incorporation into ergosterol (28). Thus, it should be possible to determine whether limiting factors in soil differ for bacterial and fungal communities. In the agricultural soil and the forest humus studied here, both bacterial and fungal growth were limited by carbon (unpublished results).

To be able to use the respiration technique it is necessary to monitor the respiration rate after glucose addition more or less continuously for at least 24 h to be able to determine the additional microbial respiration with sufficient precision. With the technique developed here, no special equipment is needed, and the measurements are only made once. However, it must be emphasized that the kinetics of thymidine or leucine incorporation are not made before the bacteria have increased their growth rate due to the addition of a limiting substance or after this effect has disappeared. This probably has to be determined for each soil studied. The incubation temperature is also important in this aspect, since at a lower incubation temperature after addition of nutrients to the soil, it will take more time for the bacteria to start growing. On the other hand, increasing the temperature from ca. 20°C, as used in the present study, to 25°C would decrease the lag time, and thus thymidine incorporation measurements could probably be made only 1 day after adding nutrients in certain soils.

Another problem with the respiration technique is that glucose must usually be added first in order to detect any limitation due to other nutrients. In fact, if the nutrient is added alone there is often no increase in respiration rate (12). This was also the case with the soils used here, where neither N nor P addition increased soil respiration rate 1 or 2 days after the addition (unpublished results). This might be interpreted as evidence that the respiration technique does not measure nutrient limitation in the natural soil but in a glucose-amended soil.

The amount of nutrients added in the respiration technique is usually higher than the amount used in our experiments. For example, Christensen et al. (9) added 2 mg glucose g (ww) of soil⁻¹ (a sandy loam), compared with 0.68 mg g (ww) of agricultural soil⁻¹ in our study. The addition of nitrogen and phosphorus in respiration experiments is also usually higher, 0.48 mg of NH₄NO₃-N and 0.49 mg of KH₂PO₄-Na₂HPO₄-P g (ww) of soil⁻¹ (9), compared with our additions of 0.014 mg of NH₄NO₃-N and 0.009 mg of KH₂PO₄-P g (ww) of agricultural soil⁻¹. Tiunov and Scheu (35) used 6.4 mg of C, 1.28 mg of N, and 0.64 mg of P g (dry weight) of soil⁻¹ in soils with 7 to 13% soil C, which are a similar to or higher additions than the amounts we used in the humus soil with ca. 35% soil C. Adding high amounts of carbon, nitrogen, and phosphorus could affect the soil microbial community. Too high a concentration of glucose may lead to the selection of fast-growing bacteria or fungi able to cope with the altered osmotic potential. Thus, the response measured after high glucose addition may be that of only a fraction of the soil microorganisms.

Microorganisms may also be killed by osmotic changes when the amounts of nutrients added are high. The dead microorganisms might then release nutrients which will affect the conditions in the soil. This might be the reason for the increase in the bacterial activity in the present study at the highest addition of nitrogen, when different concentrations of nitrogen were added to the agricultural soil (Fig. 5A). The high nitrogen concentration might have had a toxic effect on some bacteria. The carbon which then became available from the dead bacteria might be assimilated by the remaining bacteria, resulting in increased growth after 48 h and thus increased thymidine incorporation. The increased growth rate would then not have been due to the nitrogen limitation being relieved by the addition of nitrogen but to carbon limitation being relieved by carbon made available in dead microorganisms.

The addition of nitrogen may also alter the soil pH, which could negatively affect bacterial activity (5, 19). This was seen in the humus (Fig. 5B and 6). The effect on pH is probably due to a general effect of adding salt to this soil type and not specifically to nitrogen addition, since adding, e.g., NaCl to humus decreases pH (unpublished results). Thus, adding too high a concentration of the substrate might make the interpretation of the results difficult. On the other hand, initially too few nutrients were added to the calcareous soils, since phosphorus limitation was not observed until 10 times the standard amount was added (Fig. 8C, D, F, and G). The probable explanation is that these calcareous soils have a high capacity to bind phosphorus, and thus the active concentration was lower than that originally added. It is thus important to add suitable amounts of C, N, and P to each soil type. It will not always be possible to add the same amounts to different kinds of soils. If none of the added nutrients give an increased growth rate, one could suspect that either another nutrient is limiting growth or that one of the added nutrients has been used at a too-low concentration. However, if the addition of two different nutrients both give an increased growth rate, one has to ascertain that the addition of one of them does not kill part of the microbial community.

The way in which we measured incorporation rates, after the extraction of bacteria by homogenization-centrifugation is, of course, only one way of measuring bacterial activity. Any of the modifications of the thymidine or leucine incorporation techniques for a soil habitat mentioned in the introduction could be used. However, irrespective of the technique used, one must bear in mind that the method provides information on the instantaneous limitations for bacterial growth. In this respect, it is similar to the respiration technique. The effect of the addition of nutrients on microbial biomass and activity on a longer time-scale might be different and must be studied with other methods.
ACKNOWLEDGMENT
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REFERENCES