MICROBIAL POPULATIONS IN A BLACK SPRUCE HUMUS FERTILIZED WITH UREA

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FOREWORD

This report, based on material contained in the dissertation submitted by M. R. Roberge in partial fulfilment of the requirements of the Ph D degree at McGill University, forms part of a long term investigation of the effects of the accumulation of raw humus layers on the growth and regeneration of pulpwood stands in the Boreal forest. Earlier reports in this series include studies by Weetman of mor humus [Woodlands Research Index Nos. (wRi#) 121 and 126 (1961), 129 and 130 (1962), 139 (with Nykvist) and 141 (with Harland (1963)] and of this same black spruce stand [wRi #134 (1962), 156 (1964) and 165 (1965)].

The microbiological aspects of this problem are being studied at Macdonald College of McGill University in cooperation with the Institute. Dr. Knowles reported on a preliminary study of ammonium and urea by black spruce raw humus [wRi #155 (1964) and Reddy and Knowles on the fungal flora of a boreal forest raw humus [wRi #162 (1965)].

The present authors reported last year on a more detailed investigation of the nitrogen transformations [wRi #172 (1965)] and here are reporting on a complementary study of the microbial populations associated with those transformations under identical conditions. Numbers, development with time, and vertical distribution of bacteria and fungi in both the field-treated and field-untreated humus horizons of the soil supporting the black spruce stand were investigated by laboratory incubation experiments using the dilution plate method. It would appear that the microflora, and especially the fungi, are important in the decomposition of acid raw humus and in releasing nitrogen for tree growth.

Lowell Besley,
Chairman, Woodlands.

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KEYWORDS

Microorganisms, Bacteria, Fungi, Populations (Biological)*, Humus*, Mor Humus*, Soils (Material), Urea, Nitrogen, Fertilizers, Picea Mariana, Picea, Softwoods.

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MICROBIAL POPULATIONS IN A BLACK SPRUCE HUMUS FERTILIZED WITH UREA

by

M. R. Roberge[1] and R. Knowles[2]

ABSTRACT

Numbers, development with time, and vertical distribution of bacteria and fungi in the humus horizon of a soil supporting a black spruce (Picea mariana Mill.) stand were investigated by laboratory incubation experiments using the dilution plate method. Humus from untreated and from urea-treated (400 lbs N/ac) plots were incubated with urea for 42 days. Estimates were made of total viable bacteria and fungi as well as of numbers of ureolytic bacteria and fungi having high, medium, and low urease-producing activity. In the absence of added urea there were no significant changes in the observed populations. In the first 3 days following urea addition (3500 ppm N), large increases in numbers of bacteria and smaller increases in the fungi, both total and ureolytic occurred, whereas from the 3rd to the 42nd day the populations remained constant. Ureolytic organisms as a percentage of total organisms increased on the average by only 5 per cent. This suggests that the large increases in population during the first three days were not mainly the result of proliferation of specifically ureolytic organisms but that a basically non-specific increase in population occurred. This effect of urea addition was no doubt attributable to the liberation of available nitrogen and to the rise in pH accompanying hydrolysis. The results are consistent with the idea that after the initial response to urea addition, the final population is limited by some nutritional factor other than nitrogen.

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LA POPULATION MICROBIENNE D'UN HUMUS D'EPINETTE NOIRE FERTILISE AVEC DE L'UREE

par

M. R. Roberge [1] et R. Knowles [2]

RESUME

Le nombre, la multiplication avec le temps, el la distribution verticale des bactéries et des champignons dans un horizon d'humus d'épinette noire (Picea mariana Mill.) furent l'objet d'une recherche comportant des expériences d'incubation en laboratoire, par une technique de dilution sur vases de Petri.

L'humus d'une parcelle non-traitée et d'une parcelle traitée à l'urée (400 lb N/ac), furent soumises à l'incubation en laboratoire, avec addition d'urée pendant 42 jours.

On évalua le nombre total des bactéries et des champignons viables, ainsi que le nombre des bactéries et des champignons uréolytiques ayant une activité productrice d'uréase à un degré élevé, moyen ou faible.

Aucun changement appréciable des populations fut observé en l'absence d'addition d'urée.

Au cours des trois premiers jours, en présence de l'urée ajoutée (3500 ppm N), le nombre des organismes augmenta considérablement, soit dans leur ensemble, soit chez les champignons uréolytiques.

Toutefois, l'augmentation chez les champignons fut moindre que chez les bactéries. Du troisième au quarante-deuxième jour, les populations demeurèrent constantes.

Le pourcentage des organismes uréolytiques augmenta en moyenne de 5% seulement, par rapport au total des organismes. Ceci nous porte à croire que les grands accroissements de population durant les trois premiers jours, ne résultent pas principalement de la prolifération d'organismes spécifiquement uréolytiques, mais qu'il se produisit un accroissement de base non-spécifique de la population.

Un tel effet de l'addition d'urée doit être attribué, sans doute, à la libération de l'azote disponible et à la hausse en pH qui accompagne l'hydrolyse.

Les résultats concordent avec l'hypothèse qu'après une réaction initiale à l'addition d'urée, la population finale est limitée par quelque facteur de nutrition autre que l'azote.

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INTRODUCTION

In the maintenance of a high level of fertility in forest soils, their uppermost horizon appears to play a special role. It is in this horizon that the microorganisms are most active in carrying out a series of transformations upon which the higher plants are nutritionally highly dependent. Moreover, this horizon very often appears to control the establishment of higher plant seedlings. It is therefore important to study the microorganisms of this horizon and to study the effect of the addition of fertilizers on them.

The nitrogen transformations resulting from the addition of urea to black spruce raw humus have been described previously by Roberge and Knowles[19, 20], and the present report concerns a complementary study of the microbial populations under conditions identical to those of the earlier papers.

Reports on the total microbial populations of forest humus horizons are fairly numerous[1,2,4,8,9,10,11,12,18,21,22,24,27,30,32] but most are concerned with humus types or tree species different from those of the present study. Investigations of the ureolytic microflora of agricultural soils are rare[6] but as far as the authors know, similar studies of forest raw humus do not exist.

In the present work the microbial population in the different layers of the humus horizon found under a highly productive black spruce stand was studied by laboratory incubation experiments. Humus samples were treated with 3,500 ppm of urea-nitrogen, and at several time intervals during a period of 42 days, estimates were made of the total, as well as of the ureolytic, bacterial and fungal populations.

MATERIALS AND METHODS

Humus Sampling and Storage

In June 1964, twenty samples of the L, F_1, and F_2 layers were collected from the A_o (01 and 02) horizon of a podzol under an uncut 54-year-old black spruce stand of fire origin. Ten were from an untreated plot, and ten from a plot treated in September 1961 with 400 pounds of urea-nitrogen per acre[28]. The properties of these samples are described briefly in Table 1 and were discussed more fully in a previous paper[19].

Within two weeks of storage in polythene bags at 5° C. in moist conditions in darkness, the samples were ground to 4 mm size and thoroughly mixed. They were stored again in the same way for ten months before the experiments to be described were carried out.

Humus Incubation

Aliquots of layers equivalent to 5.0 g oven-dry (105° C.) weight were incubated in 125-ml Erlenmeyer flasks at 60% of their maximum water holding capacity. Half of the samples were untreated and half received 3,500 ppm of urea-nitrogen. On a field basis, this is equivalent to 400 pounds of nitrogen per acre. Numbers of total and ureolytic bacteria and fungi were determined at the beginning of the incubation period and after 3, 7, 14 and 42 days of incubation in the dark at 20° C.

Estimation of the Microbial Population

Total viable count

The total numbers of viable bacteria and fungi were determined by the dilution plate method. The whole content of each Erlenmeyer flask was suspended in 120 ml of 0.1% Bacto-peptone and shaken mechanically for 30 minutes. Two series of tenfold dilutions were then prepared and one ml aliquots of the desired dilutions were plated with 10 ml aliquots of fluid agar (50° C.). For each medium, bacterial and fungal, two dilutions in each series were plated with 5 plates each. The forty plates thus prepared were incubated at 20° C. in the dark for 7 days for fungi and for 10 days for bacteria.

The bacterial medium was the nutrient agar described by the Society of American Bacteriologists[23], at a pH of 7.4. Actidione was added to the fluid agar (50° C.) just before plating. The actidione stock solution (1.0%) had been previously sterilized by autoclaving for 10 minutes at 121° C. and 20 pounds pressure. Preliminary experiments had shown that because of the large numbers of fungi in the humus the incorporation of actidione in the medium resulted in as much as a 40-fold increase in the count of bacteria. The fungal medium was the peptone-dextrose agar of Waksman and Fred[26], to which rose bengal and streptomycin were added according to Martin[14]. Its pH was 7.0.

Ureolytic organisms

After counts were recorded, the ureolytic microorganisms were determined by transferring, from the dilution plates, 40 randomly selected colonies each of

bacteria and fungi to slants of the same media but containing 2.0% urea and 0.002% indicator. Urea was sterilized by passage through a UF-sintered glass filter and was added aseptically to the fluid media (50° C.) just before slanting. Thymol blue was added to the bacterial medium and bromothymol blue to the fungal medium.

The microorganisms were considered ureolytic if they brought about a change in colour in the 7 days following their inoculation. They were subdivided into groups of high, medium, and low ureolytic activity according to the time required to change the colour (less than 2, between 2 and 4, and more than 4 days, respectively) and to the intensity of the colour change after 7 days (more than 1/2 of the butt, more than the slant part but less than 1/2 of the butt, and only on the slant part, respectively).

RESULTS AND DISCUSSION

All data are on an oven-dry basis (105° C., constant weight). The results from the duplicate dilution series of each of the duplicate aliquots of humus were within twice the standard deviation. For this reason, only the means of the 20 plates of the most appropriate dilution are reported.

Total Microbial Population

Before Incubation

The total microbial population was determined by the dilution-plate method. This, like other methods, leaves one uncertain as to the absolute numbers of organisms in the soil. However it has the advantages of being able to give more coherent results and of being able to supply, more easily than any other methods, a number of cultures of different (kinds of) organisms which can then be used in comparing different soils or in studying the variations of the microflora due to changes in ecological factors.

In the preparation of serial dilutions with undecomposed or partly decomposed organic residues such as the L and the F layers[13], respectively, satisfactory homogeneous suspensions are often difficult to obtain. Nevertheless, this technique has become the standard method for enumerating microbial populations in the organic layers of the humus horizon of forest soils[1, 2, 4, 8, 9, 10, 12, 21, 22, 24, 27, 30, 32]. As far as we have been able to ascertain, homogeneous suspensions for dilution could not be made with the unground L and F layers of humus used

in this study. However, by using ground material, standardized and homogeneous suspensions were easily achieved.

The question of the selectivity of the plating media used in microbial studies has often been debated. The most controversial point is the choice between soil extract media and dehydrated media. Many authors believe soil extract media undesirable because they lack standard composition. While recognizing the importance of standard composition when comparing several different soil types, other workers have felt that a soil extract prepared from the soil to be assayed would help determine the true microbial population of that soil. We did not find, however, any differences in the numbers of colonies between dehydrated media and unamended extract media prepared from the same layer samples on which counts were performed. Consequently in this study, dehydrated media, because they are more easily reproducible, were used in preference to soil extract media.

The total numbers of bacteria before incubation ranged between 46 and 247 millions per gram of oven-dry humus (Table 2). In every layer the greatest numbers were found in the humus from the treated plot. Whereas the numbers from the treated plot were not much greater in the L layer, they were 3 times greater in the F_1 and F_2 layers. In the samples from each plot there was a decrease from the L to the F_2 layer.

From a knowledge of the influence of acidity upon bacterial development and from some previous reports on mor humus horizons occurring mostly under different tree species, such large numbers of bacteria might not be expected[2, 4, 8, 9, 12, 27, 32]. It is well known that with this material the large number of fungal colonies frequently appearing on the bacterial plating medium makes the counting and isolation of the bacteria very difficult. Moreover many soil fungi produce inhibitory substances in laboratory media, and strains can be isolated which, when tested in pure culture, antagonize a variety of bacteria. Consequently, so as to obtain maximum counts of bacteria, the antibiotic actidione was added in order to reduce the competitive growth of fungi. Although a few fungal colonies still developed at the concentration of actidione used in this study, they did not occur in size and in numbers sufficient to interfere with the counting and isolation of the bacteria. It has been reported that actidione did not inhibit any of the bacteria tested, even at the concentration we have used[3, 17, 29]. In our own study, the L layer of the field-untreated humus showed only 5 millions of bacteria when no actidione was added to the bacterial medium and 222 millions when this antibiotic was added.

As has been found to be generally true in mor humus horizons [1, 2, 4, 8, 10, 12, 27, 32], fungi occurred in large numbers. The total numbers of fungi before incubation ranged between 6 and 46 millions per gram of oven-dry humus (Table 2). The numbers were much greater in all the layers of the humus from the treated plot including the L layer (compare with the bacterial count in this layer where the difference between untreated and treated plots was very small). In the samples from each plot, there was also a decrease from the L to the F_2 layer.

Thus the present results indicate that the microbial population is composed of a large number of both bacteria and fungi and does not consist predominantly of the acid-tolerant fungi. This population was increased by nitrogen fertilization with urea, indicating that the profuse growth of bacterial cells and of fungal mycelia was controlled by the availability of mineral nitrogen. It is likely that the availability of nitrogen for tree growth is closely related to the nitrogen nutrition of microbes in the humus horizon. Therefore, it seems reasonable to assume that a nitrogen fertilization would increase tree growth.

The small increase in the bacterial population of the L layer compared to the large increase in the fungal population of the same layer following the field addition of urea might be explained in the following way. From Table 1 it may be seen that there was almost no mineral nitrogen in the L layer of humus from both plots and in the F layers from the untreated plots, but that there was a large amount in the F layers of the field-treated humus. Thus the shortage of mineral nitrogen may have limited bacterial growth in the L layer from both plots, but the fungi in the L layer of the field-treated plot could no doubt extend their mycelia downwards into the F layers where mineral nitrogen was available. Such translocation of nutrients through fungal hyphae has been demonstrated by Melin and Nilsson[15], amongst others.

The decrease in the microbial populations from the L to the F_2 layer is doubtless to a large extent the result of the decrease of readily available organic matter from the L to the F_2 layer. This observation refers to the development of the humus horizon which receives continuous additions of organic matter and mineral nutrients from above and so grows upwards. Correspondingly, decomposition and decrease of organic matter take place in the lower humus layers.

Table 1 shows further that pH values decrease from the L to the F_2 layer. A decrease in the numbers of bacteria and fungi from the uppermost to the lowermost layer would thus be expected a priori. There is, however, no strict correlation between the reaction and the microbial densities, evidently because these are affected by many other factors besides the reaction. Differences in microbial populations between the layers of mor humus horizons have not been studied previously. However, from respiratory measurements made on the L, F_1, and F_2 layers of the A_o horizon of a podzol supporting a Pinus sylvestris stand[16], a decrease in the numbers of microorganisms with depth would be expected.

Changes During Incubation

Environmental variables affecting the density and composition of the microflora and its biochemical potential include moisture, aeration, temperature, organic matter, acidity, and inorganic nutrient supply. Many lesser variables such as cultivation, season, and depth have been described and are of undoubted importance. If quantitative and qualitative microbial studies are to have any significance, then, there must be repeated determinations over an extended period.

In order to study the effect of urea on the density and composition of the microbial floras of both the field-untreated and the field-treated humus, samples of each were incubated under controlled conditions in the laboratory. The changes in the microbial populations were examined by carrying out several counts during an incubation period of 42 days. This study was necessary in order to judge to what extent the microorganisms were capable of multiplying when urea decomposition was going on in the humus and when an ample supply of mineral nitrogen was present.

Table 3 shows that when no urea was added there was very little change in the total numbers of bacteria during the incubation, whatever the humus or layer. However, when urea was added, there was an appreciable increase of bacteria in both humus samples and in all layers. This increase on a percentage basis was almost equal for the F_1 and F_2 layers of both humus samples (218% and 187% for the F_1 layers and 37% and 31% for the F_2 layers), but was nearly 4 times as large in the L layer of the field-untreated humus as it was in the L layer of the field-treated humus (513 and 139%, respectively). The increase in numbers was greatest in the L layer and lowest in the F_2 layer in the humus samples from each plot.

Table 4 shows that when no urea was added there was also not much change in the total numbers of fungi during the incubation whatever the humus sample or layer. When urea was added, only a slight increase was found in both humus samples in all layers.

Due to the variation in the results, little importance can be attached to the differences observed in the counts of both bacteria and fungi between 3 and 42 days. It might indeed be considered that the increase in microbial densities following the addition of urea was completed during the first 3 days of incubation. The death of individual cells roughly equalled the rate of cell multiplication during the later incubation period. Presumably the factors responsible for this dynamic equilibrium include nutrient deficiencies and/or staling products.

The F layers of the humus from the treated plot, when no urea was added in the laboratory, did not show any increase in microbial densities (Tables 3 and 4). Mineral nitrogen present at the start of the incubation was shown earlier[19], not to increase or decrease during incubation. Thus if cell multiplication occurred it was presumably balanced by cell death without any net mineralization or immobilization of nitrogen in these humus layers.

When urea was added, however, the increases in bacterial population after 3 days (Table 3) paralleled the amounts of mineral nitrogen not recoverable by extraction after the same interval[19]. That is, both population increase and non-recovery (i.e. immobilization) of mineral nitrogen were greater in the field-untreated than in the field-treated samples and in the upper than in the lower layers.

However, if one considers that the biological immobilization of one ppm of nitrogen would require the production of 30 million bacterial cells per gram of humus, it is clear that even the largest increase observed (1230 millions in the L layer, Table 3) is not sufficient to account for the 1000 - 2000 ppm of mineral nitrogen unrecovered or immobilized under the same conditions[19]. Decomposition of cells and polymerization of nitrogenous decomposition products into humic compounds could explain why the immobilization was higher than would have been expected from the population increase alone.

The counts clearly show that free ammonia is not likely to have been present at any time during the incubation. The presence of any free ammonia would be likely to have reduced the numbers of both bacteria and fungi[5].

The application of urea in the field or in the laboratory has thus modified the abundance of the microorganisms of the humus. Such alterations might be the result of alkalinization (Table 1) or of nutrient addition or, more likely, of both together. As the relative degree of availability of many essential elements increases with humus reaction and as there were already large quantities of mineral nitrogen in the F layers of the field-treated humus (Table 1) before the laboratory addition of urea, it is certain that the increase in the humus reaction cannot be ignored. Moreover, it is important to remember that the pH increase was completed after 3 days as was the microbial increase (Tables 3 and 4), and that the pH increase was the greatest in the L layer as was the microbial increase. It might also be well to recall that the recovery of mineral nitrogen was greater when urea instead of ammonium carbonate was applied and that this finding might well be the result of the smaller rise in pH brought about by the addition of urea[19].

Ureolytic Microbial Population

Before Incubation

Microorganisms capable of converting urea to ammonia are simple to demonstrate by the testing of isolated cultures. Forty colonies of bacteria and 40 of fungi were randomly selected from soil dilution plates from each of the humus treatments at each sampling time, making a total of 2,400 isolations each of bacteria and of fungi.

Urease production was detected by the change in indicator colour as the pH increased with ammonia production. Thymol blue and bromothymol blue at a concentration of 0.002 per cent were selected after testing several indicators at different concentrations. As it was possible that the alkali production responsible for the colour change in the indicator may have been due to factors other than ammonia production from urea, at the first sampling time 40, and at each subsequent sampling time 5, randomly selected colonies of bacteria and of fungi were also transferred to slants containing no urea. In the urea-free cultures, no colour changes were observed; hence, this method of studying urease production was considered to be satisfactory.

The numbers of ureolytic bacteria before incubation ranged between 15 and 156 millions per gram of oven-dry humus (Table 2). In all layers, the greatest numbers were found in the humus from the treated plot. Whereas the numbers were not quite 2 times greater in the L layer, they were 5 times greater in the F_2. In the samples from each plot there was a decrease from the L to the F_2 layer.

In all layers the numbers of bacteria having high, medium, and low urease-producing activity were greater in the humus from the treated plot (Table 5). In the untreated humus, the numbers of bacteria in all groups decreased from the L to the F_2 layer. In the treated humus, the number of bacteria having high urease-producing activity was the greatest in the F_1 layer. The two other groups decreased from the L to the F_2 layer.

The numbers of ureolytic fungi before incubation ranged between 5 and 45 million (Table 2). They resembled the bacteria in that they were much more numerous in the treated than in the untreated humus in all layers and they decreased from the L to the F_2 layer in each humus.

The numbers of fungi having high urease-producing activity were much more numerous in the treated than in the untreated humus in all layers (Table 5). They decreased from the L to the F_2 layer. The numbers of the other two groups showed much smaller differences between plots and between layers.

The present data are consistent with observations on the rate of urea hydrolysis[19]. The rate of urea breakdown was higher in the field-treated than in the field-untreated humus whatever the layer and decreased from the L to the F_2 layer in each humus. It thus roughly paralleled the numbers of ureolytic microorganisms present in the humus samples before the addition of urea in the laboratory.

Since the production of urease was undoubtedly influenced markedly by the composition of the growth media, particularly with respect to the nature and amount of organic nitrogen sources and to the availability and interactions of minerals present, it cannot be said that the microorganisms which did not show urease activity were not urease producers. Nor is it known if all the microorganisms treated are, or are not, ureolytic in natural conditions. However, the correspondingly larger number of ureolytic microorganisms in the samples where a large urea breakdown was measured indicates clearly the validity of the media and other conditions of the urease test.

It is difficult to state whether the bacteria or the fungi had the larger share of the urease activity in the humus. However, if one considers the general activity of 140 bacterial counts to be roughly equal to that of one fungal count[30], it could be said that urease produced by bacteria was negligible in comparison to that produced by fungi.

Organisms capable of decomposing urea have been reported from most families of bacteria and fungi[7, 25, 31] and have been observed in soils[6]. It was thus not surprising that many ureolytic microorganisms were found in this raw humus.

There were differences both in the numbers of ureolytic microorganisms and in their percentages of the total microorganisms, before incubation, between the humus samples and between each layer of the samples, (Table 2). The percentages of urease-producing bacteria ranged between 33 and 63 (Table 2), and in all layers they were much larger in the humus from the treated plot than in that from the untreated plot. The changes in percentage of ureolytic bacteria following the field application of urea were mainly made up of an increase of bacteria having high urease-producing activity (Table 6, and Fig. 1). This increase was much larger in the F_1 and F_2 than in the L layers.

The percentages of urease-producing fungi ranged between 78 and 100 (Table 2). They were slightly larger in the treated than in the untreated humus, whatever the layer. The changes in percentage of ureolytic fungi following the fertilization with urea were made up entirely of an increase in fungi having high urease-producing activity (Table 6, and Fig. 1). Thus the percentages of the groups having medium and low urease-producing activity decreased strikingly.

These results indicate clearly that the microfloras of the two humus samples were qualitatively different before incubation. There was also a qualitative difference between the layers of the humus of the two plots. These qualitative differences could be at the generic, specific, or strain levels.

Only two qualitative studies have been reported on the microbial population of the different layers of raw humus. Both were concerned only with fungi and employed the washing technique instead of the standard dilution plate technique. Kendrick and Burges[11] found a qualitative difference between the layers of a raw humus developed under a Pinus sylvestris stand, whereas Reddy and Knowles[18] reported that there was no consistent difference between the layers of the same humus samples studied in this work. The latter authors were reluctant to attribute

significance to some of the differences noted in their data because of the inherent variation of the soil washing technique. However, their results did show some qualitative differences between the humus samples and between the layers. The field application of urea had caused a decrease in the occurrence of the Mucorales and of Pullularia spp. in all the layers, and of the fusaria in the L layer, whereas it had caused an increase in the occurrence of the yeasts in the L and F_2 layers, of the penicillia in the L layer, and of the fusaria in the F_2 layer. Also, in the humus from the untreated plot, the occurrence of the penicillia increased from the L to the F_2 layer, whereas the occurrence of the Pullularia, Mucorales, and fusaria decreased in the same direction.

Changes during incubation

In the present study of both the field-untreated and the field-treated humus, samples were incubated under controlled conditions in the laboratory with and without urea so as to study their quantitative and qualitative changes in the microbial ureolytic population.

When no urea was added, there was very little consistent change of any kind, either in the numbers of urease-producing bacteria (Table 7) or in the numbers of urease-producing fungi (Table 8). The variation noted between the sampling times may reflect actual fluctuations in population but they may also be due to the error involved in testing only 40 bacteria or 40 fungi for urease activity. However, following the addition of urea in the laboratory, an increase in the numbers of ureolytic bacteria and of ureolytic fungi occurred during incubation in both humus samples in all layers (Tables 7 and 8), the largest increases being in bacteria of the L and F layers.

Consider now the ureolytic organisms as a percentage of the total number. When no urea was added (data not shown), the results reflected the total counts, in that there was very little change in the percentages of either urease-producing bacteria or urease-producing fungi whatever the humus, the layer, or the group of ureolytic microorganisms.

Following the addition of urea in the laboratory there was a slight increase in the percentages of ureolytic bacteria whatever the humus, the layer and the group of urease-producers (Figure 1).

In the field-untreated humus, after the addition of urea the percentages of urease-producing fungi increased in all the layers. When considered by urease-producing activity groups, a similar consistent increase was found only in the high group. The medium group increased in percentage only in the L layer whereas the low-activity group increased only in the two F layers (Figure 1).

In the field-treated humus, there was not much change in the percentages of urease-producing fungi whatever the layer. However, there was a decrease in the percentages of the high group, and a corresponding increase in the percentages of the medium group. There was not much change in the percentages of the low group (Figure 1).

It is important to note the similarity of the results obtained after the laboratory application to those obtained after the field application of urea. In both cases the urea fertilization brought about a qualitative change in the microbial population, but it is clear that the differences between untreated and treated field plots are greater than those between samples incubated in the laboratory in the absence and in the presence of urea (Figure 1). Apparently the changes occurring in the field are similar to, but of much longer duration and eventually of greater magnitude than, those occurring during the 42 days of laboratory incubation.

The urease activity tests indicate that no qualitative change in ureolytic population occurred after 3 days. It is evident, however, that other qualitative changes in the whole microbial population may well have occurred. For example, the increase in pH brought about by urea application probably permitted the growth of some autotrophic and/or heterotrophic nitrifiers as indicated by the nitrates which were previously observed in the field-treated humus after 28 days of incubation[19].

SUMMARY AND CONCLUSIONS

As part of a long-term investigation of the effects of the accumulation of raw humus layers on the growth and regeneration of pulpwood stands in the Boreal forest, number, development with time, and vertical distribution of the total and ureolytic microfloras of an unfertilized and of a urea-fertilized black spruce humus were investigated under controlled conditions in the laboratory by means of

the dilution plate method. Although only a limited segment of the bacterial and fungal floras of the humus could be isolated by this method, these represent some of the most prominent members of the humus microbial population in the forest studied.

The total bacterial and fungal populations were greater after an application at the rate of 400 pounds per acre of urea-nitrogen either in the field or in the laboratory. The increase in numbers of fungi in all layers and in numbers of bacteria in the F_2 layer was much larger in the humus collected two years after the field application than in the humus studied 42 days after the laboratory application, but in the L layer the increase in bacteria was much larger after the laboratory incubation. It seems likely that immediately following the field application of urea a much larger increase of both bacteria and fungi would have been found in the L than in the F_1 layer and in the F_1 than in the F_2 layer, but that there was a diminution in the urea effect between 1961 and 1964. The ability of the fungi in the L layer to extend their mycelia into the layers below has prevented a similar reduction in the urea effect on them. The urea effect varied among humus layers. The magnitude of the increase in bacterial and fungal populations observed in any particular layer after urea addition no doubt depended upon factors such as the amount of readily available organic matter, the amount of available nitrogen present initially or liberated by urea hydrolysis, the initial pH of the humus and the extent of the rise in pH which accompanied urea hydrolysis. The relative importance of these factors was not demonstrated in this experiment.

The large increase in total bacterial numbers and the slight increase in fungal numbers following the addition of urea appeared to be related to the amount of mineral nitrogen assimilated as reported earlier[19], where the uptake of available nitrogen was the greatest in the field untreated humus and in the L layer in each humus.

The numbers of ureolytic microorganisms also were found to increase after the application of urea either in the field or in the laboratory. The increase was much larger after the field application than after the laboratory application and increased in magnitude from the F_2 to the F_1 to the L layers. When the numbers of ureolytic microorganisms were expressed as percentages of the total numbers of all microorganisms, it was apparent that a qualitative change resulted

from either the field or laboratory application of this fertilizer. However, the increase in the percentages of ureolytic bacteria and fungi averaged only about 5%. Thus the often very large increases in total numbers observed following the addition of urea in the laboratory resulted, not from proliferation of specifically ureolytic organisms, but rather from a general increase in population, with only a modest rise in the proportion of urea hydrolysers. Nevertheless, a good relation was obtained between the content of ureolytic microorganisms of the humus and its urea-decomposing activity as reported earlier[19]. The practice of estimating the numbers of ureolytic microorganisms as a means of characterizing the humus from the biological point of view can be considered valid.

The application of urea in the laboratory to the humus which had already been fertilized in the field caused a further increase in the total numbers of microorganisms and in the ureolytic microbial population. It also resulted in some qualitative changes in the bacterial and fungal microflora.

The large fungal population and its relatively large biomass compared to that of the bacterial population, and the high percentage or ureolytic fungi, indicate that fungi are likely to play an important part in decomposing urea in acid raw humus.

It is not claimed that the results obtained here can be applied directly to other locations even under the same tree cover. Climatic and site differences may lead to variations in the form of the humus. Furthermore, where different species of plants form the litter, it would be logical to expect differences in the microflora. Nevertheless, there can be no question that the microflora, and especially the fungi, are important in the decomposition of acid raw humus and in releasing nitrogen for tree growth. The microbiological processes occurring in the humus of other northern forest soils are not likely to differ markedly from those reported here.

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Table 1. Some Properties of the Humus Samples Used in the Present Microbiological Study.

Humus layer	O.M. %	Total N %	C/N	pH	NH_4-N ppm
	Untreated plot - Sampled 1964				
L	95.2	0.86	58	4.8	17
F_1	95.0	0.88	58	3.3	13
F_2	94.6	0.90	55	3.3	8
	Plot treated with 400 lbs n/ac 1961 - Sampled 1964				
L	95.0	1.20	42	4.7	31
F_1	94.0	1.18	43	4.4	459
F_2	93.1	1.22	41	3.9	823

Table 2. Microbial Population in the Humus Samples Used in the Incubation Experiments.

Humus layer	Bacteria			Fungi		
	Number* Total	Number* Ureolytic	Per cent Ureolytic	Number* Total	Number* Ureolytic	Per cent Ureolytic
	Untreated plot - Sampled 1964					
L	228	86.6	38	10.2	9.2	90
F_1	50.0	23.0	46	6.8	5.3	78
F_2	46.1	15.2	33	5.8	4.6	79
	Plot treated with 400 lbs N/ac 1961 - Sampled 1964					
L	247	156	63	45.7	44.8	98
F_1	164	100	61	23.8	23.1	97
F_2	148	74.0	50	10.7	10.7	100

* Millions per g of oven-dry humus (105°C., constant weight).

Table 3. Numbers of Bacteria (millions per gram) in the Humus Samples Incubated in the Laboratory.

Humus Layer	Treatment	Incubation time (days) 0	3	7	14	42	Ave.*3-42	Increase %**
		Untreated plot - Sampled 1964						
L	None	228	230	253	235.	222	235	+ 3
F_1	"	50.0	60.1	64.3	64.0	67.2	63.9	+ 27
F_2	"	46.1	47.0	51.6	49.7	43.5	47.9	+ 4
L	Urea	240	1380	1520	1440	1540	1470	+513
F_1	"	52.7	114	167	171	222	168	+218
F_2	"	47.2	64.0	64.6	67.4	62.7	64.7	+ 37
		Plot treated with 400 lbs N/ac 1961 - Sampled 1964						
L	None	247	210	237	216	225	222	- 10
F_1	"	164	158	188	168	151	166	+ 1
F_2	"	148	158	152	145	175	157	+ 6
L	Urea	253	552	587	561	717	604	+139
F_1	"	170	470	420	448	617	489	+187
F_2	"	150	217	186	185	199	197	+ 31

* Ave. Number at 3, 7, 14 and 42 Days

** $\frac{\text{(Ave. Number) - Number at 0 Days)}}{\text{(Number at 0 Days)}} \times 100\%$

Table 4. Numbers of Fungi (millions per gram) in the Humus Samples Incubated in the Laboratory.

Humus Layer	Treatment	Incubation time (days) 0	3	7	14	42	Ave.*3-42	Increase % **
	Untreated plot - Sampled 1964							
L	None	10.2	9.7	10.6	10.9	10.0	10.3	1
F_1	"	6.8	6.9	7.5	6.5	6.7	6.9	1
F_2^1	"	5.8	6.5	6.5	6.0	6.4	6.3	8
L	Urea	10.0	12.5	12.3	11.9	11.7	12.1	21
F_1	"	6.8	7.4	7.9	7.5	7.5	7.6	12
F_2^1	"	5.3	5.8	5.5	6.0	5.7	5.7	7
	Plot treated with 400 lbs N/ac 1961 - Sampled 1964							
L	None	45.7	45.2	45.7	48.2	46.0	46.3	1
F_1	"	23.8	24.9	24.1	23.7	25.4	24.5	3
F_2^1	"	10.7	10.9	11.5	9.1	12.4	11.0	3
L	Urea	45.0	46.8	45.3	46.0	47.0	46.3	3
F_1	"	25.3	27.1	28.1	27.2	26.8	27.3	8
F_2^1	"	10.5	11.9	11.7	13.4	12.0	12.2	16

* Ave. Number at 3, 7, 14 and 42 Days.

** $\frac{\text{(Ave. Number*)} - \text{(Number at 0 Days)}}{\text{(Number at 0 Days)}} \times 100\%$

Table 5. Numbers of Ureolytic Organisms in the Humus before Incubation.

Humus layer	Bacteria*			Fungi*		
	Urease-producing activity			Urease-producing activity		
	High	Medium	Low	High	Medium	Low
	Untreated plot - Sampled 1964					
L	13.7	27.4	45.6	2.8	3.0	3.4
F_1	2.5	10.0	10.5	1.6	1.8	1.9
F_2	2.2	3.8	9.2	1.4	1.9	1.3
	Plot treated with 400 lbs N/ac 1961 - Sampled 1964					
L	39.5	39.5	76.6	34.4	8.2	2.2
F_1	45.9	23.0	31.2	20.5	1.4	1.2
F_2	37.0	21.7	15.3	7.6	2.0	1.1

* Millions per g of oven-dry humus (105° C., constant weight).

Table 6. Percentages of Ureolytic Organisms in the Humus before Incubation.

Humus layer	Bacteria*			Fungi*		
	Urease-producing activity			Urease-producing activity		
	High	Medium	Low	High	Medium	Low
	Untreated plot - Sampled 1964					
L	6	12	20	28	29	33
F_1	5	20	21	24	26	28
F_2	7	6	20	28	32	19
	Plot treated with 400 lbs N/ac 1961 - Sampled 1964					
L	16	16	31	75	18	5
F_1	28	14	19	86	6	5
F_2	25	16	9	72	18	10

*Percentage of the total numbers of bacteria or fungi.

Table 7. Numbers of Ureolytic Bacteria (millions per gram) in the Humus Samples Incubated in the Laboratory.

Humus Layer	Treatment	Incubation time (days) 0	3	7	14	42	Ave*3-42	Increase %**
	Untreated plot - Sampled 1964							
L	None	86.6	138.0	137.0	124.0	88.8	122.0	41
F_1	"	23.0	30.0	40.5	38.4	37.6	36.6	59
F_2^1	"	15.2	20.2	19.1	24.3	13.9	19.4	28
L	Urea	142.0	787.0	821.0	850.0	693.0	788.0	455
F_1	"	26.3	54.4	83.5	92.3	129.0	89.8	242
F_2^1	"	20.3	30.5	25.8	25.6	27.6	27.4	35
	Plot treated with 400 lbs N/ac 1961 - Sampled 1964							
L	None	156.0	130.0	102.0	117.0	106.0	114.0	- 27
F_1	"	100.0	96.4	103.0	116.0	94.9	103.0	3
F_2^1	"	74.0	98.0	95.8	72.5	114.0	95.1	28
L	Urea	104.0	343.0	370.0	308.0	359.0	345.0	232
F_1	"	107.0	297.0	273.0	300.0	382.0	313.0	192
F_2^1	"	73.5	141.0	128.0	131.0	121.0	130.0	77

* Ave. Number at 3, 7, 14, and 42 Days.

** $\frac{\text{(Ave. Number)} - \text{(Number at 0 Days)}}{\text{(Number at 0 Days)}} \times 100\%$

Table 8. Numbers of Ureolytic Fungi (millions per gram) in the Humus Samples Incubated in the Laboratory.

Humus Layer	Treatment	Incubation time (days) 0	3	7	14	42	Ave*3-42	Increase %**
	Untreated plot - Sampled 1964							
L	None	9.2	8.6	10.4	10.4	9.2	9.6	4
F_1	"	5.3	5.0	6.6	5.9	5.0	5.6	6
F_2	"	4.6	3.8	4.3	4.7	4.0	4.2	- 9
L	Urea	9.3	11.7	11.1	11.3	11.1	11.3	21
F_1	"	5.4	6.5	6.3	7.1	6.8	6.7	24
F_2	"	4.0	4.6	4.3	5.2	4.6	4.7	17
	Plot treated with 400 lbs N/ac 1961 - Sampled 1964							
L	None	44.8	44.3	43.4	48.2	44.2	45.0	0
F_1	"	23.1	23.6	23.6	23.2	23.9	23.6	2
F_2	"	10.7	10.3	10.1	8.6	9.9	9.7	- 9
L	Urea	44.1	45.9	44.3	45.7	46.2	45.5	3
F_1	"	23.0	25.3	26.7	27.2	24.1	25.8	12
F_2	"	8.9	11.3	11.2	12.9	11.8	11.8	33

* Ave. Number at 3, 7, 14, and 42 Days.

** $\frac{\text{(Ave. Number*) - (Number at 0 Days)}}{\text{(Number at 0 Days)}} \times 100\%$

APPENDIX TABLE 1. Numbers of Bacteria Having High Urease-producing Activity (millions per gram) in the Humus Samples Incubated in the Laboratory.

Humus Layer	Treatment	Incubation time (days) 0	3	7	14	42	Ave*3-42	Increase %**
	Untreated plot - Sampled 1964							
L	None	13.7	13.8	32.9	14.1	31.1	23.0	68
F_1	"	2.5	3.0	6.4	3.2	3.4	4.0	60
F_2^1	"	3.2	6.6	2.6	2.5	2.2	3.5	9
L	Urea	26.4	166	122	144	154	146	453
F_1	"	2.6	13.7	10.0	10.3	17.8	12.9	396
F_2^1	"	5.2	7.8	3.2	6.7	2.5	5.1	- 2
	Plot treated with 400 lbs N/ac 1961 - Sampled 1964							
L	None	39.5	35.7	16.6	28.1	18.0	24.6	-38
F_1	"	45.9	39.5	47.0	25.2	40.8	38.1	-17
F_2^1	"	37.0	37.9	44.1	31.9	52.5	41.6	12
L	Urea	20.2	60.7	51.8	44.9	65.4	55.7	176
F_1	"	51.0	99.3	96.6	130.0	167.0	123.0	141
F_2^1	"	24.0	54.2	40.9	61.0	49.7	51.4	114

* Average Number at 3, 7, 14, and 42 Days.

** $\frac{\text{(Ave. Number*) - (Number at 0 Days)}}{\text{(Number at 0 Days)}} \times 100\%$

APPENDIX TABLE 2. Numbers of Bacteria Having Medium Urease-producing Activity (millions per gram) in the Humus Samples Incubated in the Laboratory.

Humus Layer	Treatment	Incubation time (days) 0	3	7	14	42	Ave*3-42	Increase %**
	Untreated plot - Sampled 1964							
L	None	27.4	39.1	17.7	37.6	15.5	27.5	0
F_1	"	10.0	15.0	6.4	12.8	18.9	13.3	33
F_2	"	2.8	6.1	8.3	9.9	4.3	7.1	154
L	Urea	40.8	235.0	182	230.0	154.0	200.0	390
F_1	"	7.9	18.0	33.4	22.2	55.5	32.3	309
F_2	"	6.1	10.4	5.8	11.5	9.4	9.3	52
	Plot treated with 400 lbs N/ac 1961 - Sampled 1964							
L	None	39.5	31.5	19.0	32.4	18.0	25.2	- 36
F_1	"	23.0	41.1	37.6	31.9	34.7	36.3	58
F_2	"	23.7	19.0	27.4	17.4	21.0	21.2	- 11
L	Urea	35.4	144.0	90.4	101.0	81.7	104.0	194
F_1	"	40.8	103.0	67.2	89.6	123.0	95.7	134
F_2	"	15.0	43.4	33.5	25.9	33.8	34.1	127

* Average Number at 3, 7, 14, and 42 Days.

** $\frac{\text{(Ave. Number) - (Number at 0 Days)}}{\text{(Number at 0 Days)}} \times 100\%$

APPENDIX TABLE 3

Numbers of Fungi Having High Urease-producing Activity (millions per gram) in the Humus Samples Incubated in the Laboratory.

Humus Layer	Treatment	Incubation time (days)						Increase %**
		0	3	7	14	42	Ave. *3-42	
	Untreated plot - Sampled 1964							
L	None	2.8	3.3	3.4	3.2	3.5	3.3	18
F_1	"	1.6	2.0	1.7	2.2	1.4	1.8	12
F_2^1	"	1.6	1.2	1.7	1.8	1.3	1.5	- 6
L	Urea	2.8	3.6	3.5	4.8	4.9	4.2	50
F_1	"	2.1	2.7	2.5	3.0	2.2	2.6	24
F_2^1	"	1.6	1.7	1.4	2.0	1.2	1.6	0
	Plot treated with 400 lbs N/ac 1961 - Sampled 1964							
L	None	34.4	37.0	33.3	36.2	36.8	35.8	4
F_1	"	20.5	18.6	18.0	20.4	18.6	18.9	- 8
F_2^1	"	6.6	5.1	6.1	5.3	5.2	5.4	-18
L	Urea	36.5	29.9	24.8	30.8	27.3	28.2	-23
F_1	"	17.6	18.3	19.7	19.0	16.1	18.3	4
F_2^1	"	6.0	4.2	6.0	7.1	4.0	5.3	-12

* Average Number at 3, 7, 14 and 42 Days

** $\frac{\text{(Ave. Number *)} - \text{(Number at 0 Days)}}{\text{Number at 0 Days}} \times 100\%$

APPENDIX TABLE 4

Numbers of Fungi Having Medium Urease-producing Activity (millions per gram) in the Humus Samples Incubated in the Laboratory.

Humus Layer	Treatment	Incubation time (days) 0	3	7	14	42	Ave.* 3-42	Increase %**
	Untreated plot - Sampled 1964							
L	None	3.0	3.3	4.0	3.9	3.0	3.5	17
F_1	"	1.8	1.8	2.5	1.7	1.7	1.9	5
F_2	"	1.9	1.4	1.8	1.2	1.3	1.4	- 26
L	Urea	2.9	5.1	5.5	5.3	4.1	5.0	72
F_1	"	2.0	2.1	1.5	2.2	1.6	1.8	- 10
F_2	"	1.1	1.1	0.8	1.9	1.6	1.3	18
	Plot treated with 400 lbs N/ac 1961 - Sampled 1964.							
L	None	8.2	5.4	9.6	9.6	4.6	7.3	- 11
F_1	"	1.4	2.5	1.4	0.9	1.0	1.4	0
F_2	"	2.0	2.0	2.5	2.2	1.9	2.1	5
L	Urea	6.3	15.4	17.7	12.4	13.6	14.8	135
F_1	"	3.5	3.4	2.8	4.1	4.8	3.8	9
F_2	"	1.3	4.5	3.7	4.7	4.6	4.4	238

* Average Number at 3, 7, 14 and 42 Days

** $\frac{\text{(Ave. Number*)} - \text{(Number at 0 Days)}}{\text{(Number at 0 Days)}} \times 100\%$

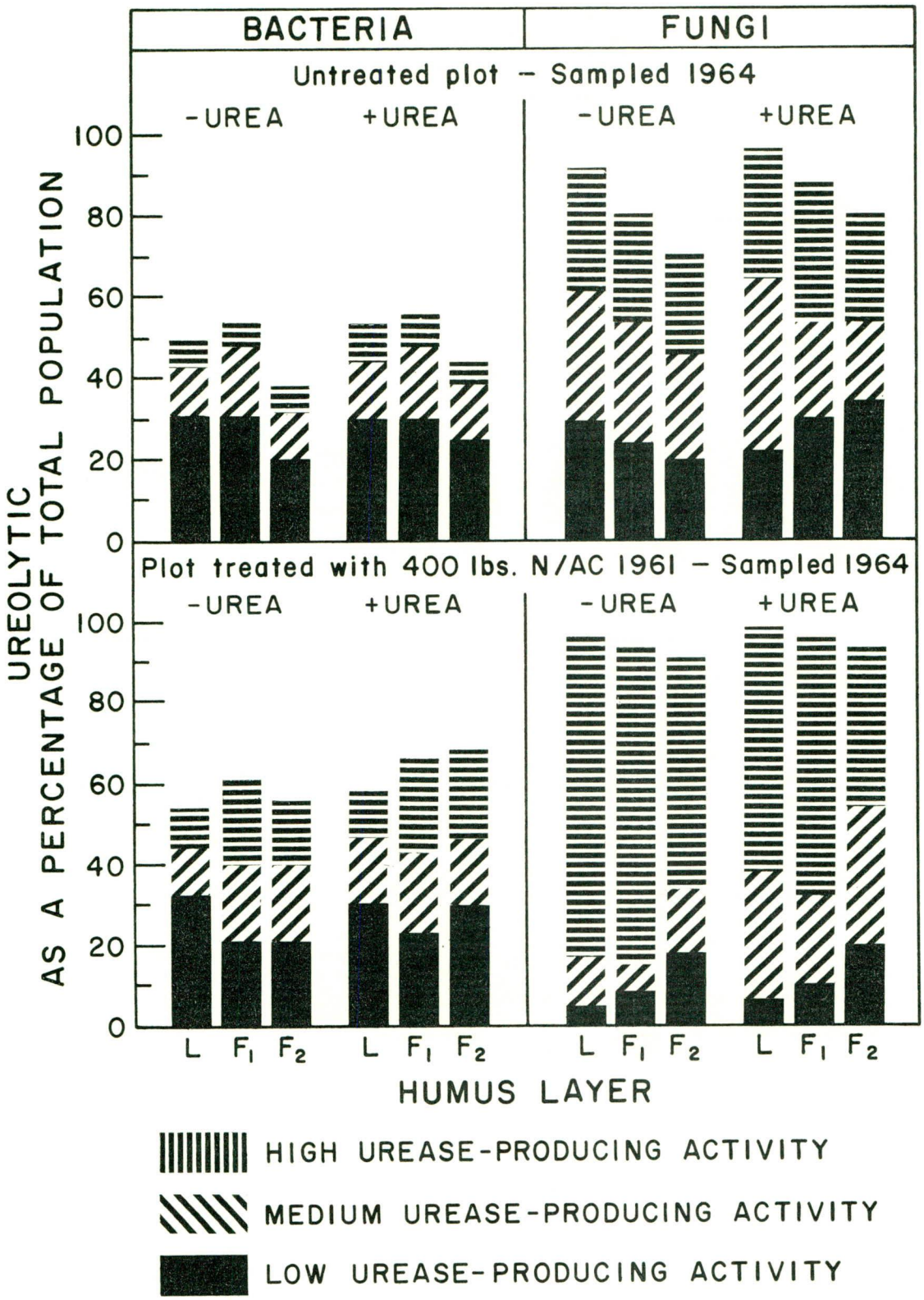

Fig. 1. Mean percentages of urease-producing bacteria and fungi found during 42 days of incubation in the laboratory in the absence and in the presence of 3500 ppm urea-N added at zero time.