Statens
Planteavlsforsøg

Report no. S 1777

Determination of adenosine triphosphate (ATP) and adenylate energy charge (AEC) in soil and use of adenine nucleotides as measures of soil microbial biomass and activity

Bestemmelse af adenosin trifosfat (ATP) og adenylat energiladning (AEC) i jord og anvendelse af adenin nukleotider som mål for mikroorganismernes biomasse og aktivitet i jorden

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Tidsskrift for Planteavls Specialserie

Copenhagen 1985

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I § 19 i undervisningsministeriets bekendtgørelse af 31. januar 1977 om erhvervelse af doktorgraden er fastsat følgende bestemmelser vedrørende det offentlige forsvar af doktorafhandlinger:

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6. Forsvarshandlingen skal foregå på dansk, norsk eller svensk, medmindre der af det kollegiale organ gives tilladelse til at anvende et andet sprog.

Den kgl. Veterinær- og Landbohøjskole.

Centerleder E. Hennings Jensen
Med venlig hilsen
Finn Eiland

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Forsvaret finder sted fredag den 10. januar 1986 kl. 14.00 i auditorium 1-01, Bülowsvej 13, Den kgl. Veterinær- og Landbohøjskole.

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Denne afhandling samt 10 tilsluttende arbejder er af Den kgl. Veterinær- og Landbohøjskoles fagråd for grundvidenskab antaget til offentligt at forsvares for den jordbrugsvidenskabelige doktorgrad.

København, den 22. oktober 1985.

Uwe Kaufmann

Formand for fagrådet for grundvidenskab.

DETERMINATION OF ADENOSINE TRIPHOSPHATE (ATP) AND ADENYLATE ENERGY CHARGE (AEC) IN SOIL AND USE OF ADENINE NUCLEOTIDES AS MEASURES OF SOIL MICROBIAL BIOMASS AND ACTIVITY

by FINN EILAND

CONTENTS

PREFACE

In this thesis are summarized investigations on the analyses of adenosine triphosphate (ATP) and adenylate energy charge (AEC) in soil, together with work dealing with the use of adenine nucleotides (ATP, ADP and AMP) as measures of soil microbial biomass and activity. Some of the results have been published in papers as listed below. The papers are arranged chronologically after years of publication, and will be referred to by Roman numerals. Other literature will be cited by name of authors and publishing year and listed under references.

I. Eiland, F. (1979): An improved method for determination of adenosine triphosphate (ATP) in soil. Soil Biol. Biochem., 11, 31-35.

II. Eiland, F. & Nielsen, B.S. (1979): Influence of cation content on adenosine triphosphate determinations in soil. Microbial Ecology, 5, 129-137.

III. Nielsen, J.D. & Eiland, F. (1980): Investigations on the relationship between P-fertility, phosphatase activity and ATP content in soil. Plant and Soil, 57, 95-103.

IV. Eiland, F. (1980): The effects of manure and NPK fertilizers on the soil microorganisms in a Danish long-term field experiment. Danish J. Plant and Soil Sci., 84, 447-454.

V. Eiland, F. (1981): The effects of application of sewage sludge on microorganisms in soil. Danish J. Plant and Soil Sci. 85, 39-46.

VI. Eiland, F. (1981): The effects of high doses of slurry and farmyard manure on microorganisms in soil. Danish J. Plant and Soil Sci., 85, 145-152.

VII. Eiland, F. (1981): Organic manure in relation to microbiological activity in soil. In Agricultural Yield Potentials in Continental Climates. Proceedings of the 16th Colloquium of the International Potash Institute held in Warsaw/Poland. 147-156.

VIII. Sparling, G. & Eiland, F. (1983): A comparison of methods for measuring ATP and microbial biomass in soils. Soil Biol. Biochem. 15, 227-229.

IX. Andersen, C., Eiland, F. & Vinther, F.P. (1983): Undersøgelser af landbrugsjordens økologi med henblik på jordbundens mikroflora og fauna i dyrkninssystemer med vårbyg, reduceret jordbehandling og en efterafgrøde. (In Danish; Summary in English) Tidsskr. Planteavl 87, 257-296.

X. Eiland, F. (1983): A simple method for quantitative determination of ATP in soil. Soil Biol. Biochem. 15, 665-670.

A. INTRODUCTION

Microorganisms are essential in the circulation of nutrients between the living and nonliving components in the soil. In order to understand these processes it is important to be able to determine the amount of living biomass and the activity of the microorganisms.

During the last decade much effort has been spent on the development of new methods in soil microbiology, which could replace the classic procedures, e.g. the plate count technique and the direct microscopic count technique with all their inherent limitations. One of the most promising approaches in this direction both from a theoretical and a practical view point is the ATP analysis. It is suitable as a routine procedure in ecological studies, which can be carried out on a large number of samples within a short time.

The ATP analysis has already been used to a considerable extent as an estimate of microbial biomass or activity in aquatic ecosystems (Bowie & Gillespie, 1976; Hodson et al., 1976; Paerl & Williams, 1976) as well as in soils (MacLeod et al., 1969; Lee et al., 1971a; Ausmus, 1973; Paul & Johnson, 1977; Jenkinson et al., 1979; Oades & Jenkinson, 1979; refs I, III, IV, V, VI & VII; Ahmed et al., 1982; refs VIII & IX). But because of methodological problems (e.g. ATP extraction efficiency, ionic interferences, ATP adsorption onto soil particles, use of crude or purified luciferin-luciferase enzymes and effects of environmental factors on ATP content in soil samples), the main part of the above mentioned papers have dealt with ATP methods and only a few studies (e.g. refs IV, V, VI, VII & IX) have been carried out to elucidate seasonal changes in microbial biomass or activity in terrestrial ecosystems by use of the ATP method.

ATP concentration used as an estimate of living biomass requires a fairly constant ratio of ATP to biomass in different

species and during different conditions of growth, while ATP concentrations used as an estimate of the microbial activity require a correlation with the growth rate. Some investigators have found that ATP content in bacteria is independent of the cellular growth rate (Smith & Maaløe, 1964; Forrest, 1965; Knowles & Smith, 1970; Hobson & Summers, 1972), whereas others have indicated a positive correlation between ATP content and the growth rate (Neidhardt & Fraenkel, 1961; Bagnara & Finch, 1973).

The ATP content of a soil sample, probably will reflect the amount of living cytoplasm and the state of activity (the adenylate energy charge value) of the living cells present in the soil but the interpretation of the analytical data has been difficult.

Determination of an adenylate energy charge value based on cellular adenine nucleotide concentrations (ATP, ADP and AMP) is proposed to be a measurement of the energetic state of microbial populations in vivo. The ratio of the three mentioned adenine nucleotides is depending on the metabolic activity of the cells. Therefore, it seems quite reasonable that the activity of the total microbial community in soil could be determined more exactly by the adenylate energy charge value rather than by ATP determinations alone.

In addition to a discussion of the mentioned subjects the aim of the present study has been: (1) To work out a simple method for quantitative extraction and determination of ATP in soil; (2) to develop a method for determination of adenylate energy charge (AEC) in soil; (3) to determine the concentrations of the adenine nucleotides and AEC in soil; (4) to examine different environmental factors which might affect the ATP content in soil and (5) to examine the relationships between ATP content and other methods which reflect either microbial activity or microbial biomass in order to evaluate the suitability of ATP measurements for detection of changes in soil microbial biomass and activity in terrestrial ecosystems.

B. THE FIREFLY BIOLUMINESCENCE ASSAY FOR MEASURING ATP CONTENT

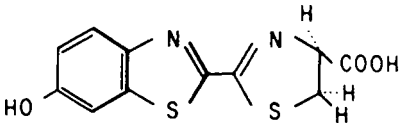
Introduction

It is generally agreed that there is an association between adenine 5'-triphosphate and living cells and therefore it can be a useful indicator of life (Huennekens & Whiteley, 1960). All living cells contain molecules, referred to as nucleotides, which are essential for viability and growth. Adenine-containing nucleotides, adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), and adenosine 5'-monophosphate (AMP) have several functions. They are thus responsible for coupling intracellular energy-producing and energy-requiring metabolic reactions, e.g. for 1) storage and transport of cellular metabolic energy, 2) synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), 3) activation and transfer of precursors for cellular biosynthesis, and 4) control and regulation of cellular metabolism (Karl, 1980). Studies on the regulation of enzyme function and the control of biosynthetic processes have resulted in the adenylate energy charge concept (AEC) as defined by Atkinson & Walton (1967): $AEC = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$.
AEC provides a measure of the total amount of metabolic energy momentarily stored in the adenine nucleotide pool (see Chapter C). For the determination of AEC, ADP and AMP are enzymatically converted to ATP and measured. The turnover times for the intracellular ATP pool in growing bacteria have been found to be as short as 0.1 to 1.0 s in growing bacteria (Harrison & Maitra, 1969; Holms et al., 1972; Chapman & Atkinson, 1977).

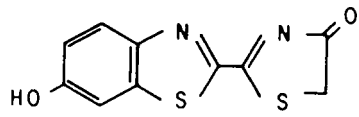
The emission of light catalysed by firefly luciferin-luciferase for determination of adenosine triphosphate (ATP), has been studied extensively since the work by McElroy (1947), which first recognized that light emission from a firefly lantern extract could be induced by the addition of ATP and that the duration of light emission was proportional to the amount of ATP added.

The luciferin-luciferase system had in most cases been obtained from the firefly Photinus pyralis, although many other luminescent organisms are known.

Luciferin is a heterocyclic compound which is activated by adenylation in an initial reaction step (Fig. 1).



D(-) LUCIFERIN



OXYLUCIFERIN

Fig. 1. Structures of D(-)luciferin and oxyluciferin.
(From DeLuca, 1977)

Oxyluciferin is produced by oxidation (Fig. 1). An exited anionic form of the oxyluciferin molecule is probably the light emittor (White et al., 1969, 1971).

The firefly enzyme luciferase consists of two subunits each with a molecular weight of approximately 50.000 (Travis & McElroy, 1966). Sulfhydryl groups are essential for the catalytic action (DeLuca, 1969). The method is based on measurement of the light emitted from the interaction of ATP with luciferin-luciferase and atmospheric oxygen. The reaction requires a pH of about 7.5, the presence of Mg^{2+} ions and a temperature of 20-25^{o}C. The total light output is directly proportional to the amount of ATP present, when all the other components of the system are in excess (Seliger & McElroy, 1959; Strehler, 1968).

The reactions involved are simplified in Fig. 2 as follows:

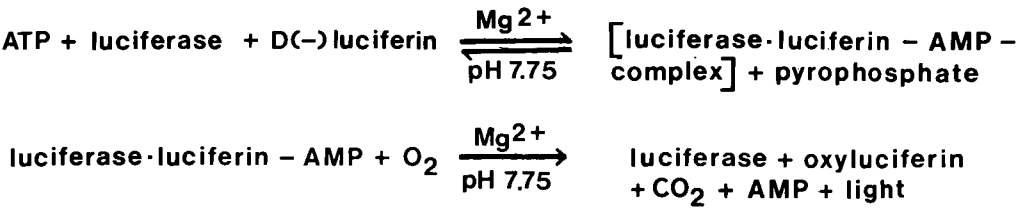


Fig. 2. Scematic presentation of the firefly luciferin-luciferase reaction.

The first step is the initial activation resulting in formation of enzyme bound luciferyladenylate from reduced luciferin, luciferase and ATP in the presence of Mg^{2+}ions. The luciferyladenylate remains tightly bound to the luciferase and pyrophosphate is released. In a second step, the luciferyladenylate enzyme complex is oxidized by molecular oxygen, resulting in CO_2, AMP, luciferase and electronically excited oxyluciferin. On returning to the ground state the excited complex emits light. The stoichiometry is one D(-)luciferin oxidized per ATP and O_2 utilized. The quantum yield is very high, about 0.88 (Seliger & McElroy, 1960). Details of the reaction mechanism are presented in the review by DeLuca (1976). The normal light emission has a yellow-green light with a maximum at 562 nm (White et al., 1971). However, temperature, pH and certain metal ions can affect the colour, resulting in a red light emission with a maximum about 618 nm (Seliger & McElroy, 1964).

The method is very sensitive, 10^{-14} mol ATP can be measured under optimum conditions (Karl & Holm-Hansen, 1976).

Survey of different methods used for extraction of ATP from soil

Originally ATP extraction methods were developed for extracting ATP from microbial cells in pure cultures. A range of such methods are discussed by Lundin & Thore (1975). These methods are not equally effective in extracting ATP from such a complex medium as soil. A good extraction procedure for soils must quickly inactivate the enzyme reactions in the soil suspension, so that neither synthesis nor degradation of ATP can occur. Furthermore, ATP present in all the microbial cells should be quantitatively extracted, and the extracts should not contain ions which strongly depress the light emission.

The extraction of ATP from soil has been found to present a number of special problems, arising from; 1) adsorption of ATP molecules to inorganic and organic particles in soil, e.g. clay (Anderson & Davies, 1973), aluminum- and ironsesquioxides (ref. II) and acid-soluble organic compounds (fulvic acid) (Cunningham & Wetzel, 1978); 2) ionic inhibition of ATP light emission by organic and inorganic material in soil (ref. II); 3) poor extraction efficiency of ATP from microorganisms associated with soil substances (ref. VIII), and 4) co-precipitation of ATP with other substances upon neutralization of acid extracts (refs I & II).

Numerous methods have been suggested during the last decade for extraction of ATP from microbial cells using many different solvents. However, none of these methods fulfil the requirements for a simple, rapid and quantitative extraction using non-toxic reagents, except the method suggested in ref. X.

Soil ATP extraction has been performed with cold or boiling extractants having a pH in the neutral range (Table 1). Other soil ATP extraction methods are based on cold acid extractants with a pH below 2 (Table 1).

Table 1. Methods available for extraction of ATP from soil

Menstruum	References
Cold extractants with a pH in the neutral range:	
Bicarbonate-chloroform ($NaHCO_3$-$CHCl_3$)	Paul & Johnson, 1977
N-butanol-octanol	Conklin & Macgregor, 1972
NRB® (Lumac) nucleotide-releasing agent	Van de Werf & Verstraete, 1979
	Nielsen & Eiland, ref. III
	Verstraete et al., 1983
N-bromosuccinimide-EDTA-aresenate	MacLeod et al., 1969
Boiling extractants with a pH in the neutral range:	
Bicarbonate ($NaHCO_3$)	Christian et al., 1975
	Paul & Johnson, 1977
[Tris (hydroxymethyl) aminomethane] -Ethylenediaminetetraacetic acid buffer (Tris-EDTA buffer)	Hamilton & Holm-Hansen, 1967
	Lee et al., 1971a
	Conklin & Macgregor, 1972
	Aysmus, 1973
	Karl & LaRock, 1975
	Kaczmarek et al., 1976
	Eiland, ref. I

Cold acid extractants with a pH below 2:

Dimethylsulphoxide-acid (DMSO-acid)	Lee et al., 1971a
	Ausmus, 1973
	Eiland, ref. I
Formic acid (HCOOH)	Klofat et al., 1969
Perchloric acid ($HClO_4$)	Ausmus, 1973
	Eiland, ref. I
Sulphuric acid (H_2SO_4)	Lee et al., 1971a
	Ausmus, 1973
	Eiland, ref. I
Sulphuric acid-phosphate and NRB® (H_2SO_4-PO_4 and NRB®)	Eiland, ref. X
Trichloracetic acid (TCA)	Bagnara & Finch, 1972
	Eiland, ref. I
Trichloracetic acid-phosphate-paraquat (TCA-PO_4-paraquat)	Jenkinson & Oades, 1979

The cold extractants with a pH in the neutral range do not always effectively stop the enzyme activity, which may result in hydrolysis of ATP during the extraction procedure. For extraction of ATP from soil, Paul & Johnson (1977) used cold bicarbonate to flood the soil colloidal system with an ion that interferes with the adsorption of ATP to soil particles and chloroform to lyse the cells. Jenkinson et al. (1979) compared this method with a trichloracetic acid-phosphate-paraquat extraction (Jenkinson & Oades, 1979) and found that the latter method extracted the highest amount of ATP, although the highest recovery of ATP was obtained by the $NaHCO_3$-$CHCl_3$ method.

Conklin & Macgregor (1972) compared a n-butanol-octanol reagent with formic acid (Klofat et al., 1969), hot Tris (Hamilton & Holm-Hansen, 1967) and n-bromosuccinimide-EDTA-arsenate (MacLeod et al., 1969). They found that n-butanol-octanol was most effective in extraction of ATP from soil.

A nucleotide releasing reagent (NRB®) has been used (Van de Werf & Verstraete, 1979; ref. III; Verstraete et al., 1983). The extraction of ATP from soil with the NRB reagent was performed by Eiland in a few studies (ref. III), because of its simplicity and because it had been suggested that the reagent only extracted ATP from microbial cells (Lumac Company). The method was based on a "direct" extraction performed under nearly neutral conditions at 0°C (page 167 , APPENDIX I). However, only a minor part of the ATP content present in the soil was extracted (ref. VIII). It was also found that clay and soil water content strongly influenced the extracted amount of ATP with this method (Table 5).

Most authors which have compared different ATP extractants found that boiling Tris-EDTA buffer had a poor efficiency as compared to acid extractants (Lee et al., 1971a; Conklin & Macgregor, 1972; Ausmus, 1973; Karl & LaRock, 1975; ref. I). The exception was the study by Kaczmarek et al. (1976); they preferred boiling Tris.

Although boiling of the soil extracts should have the advantage of stopping the enzyme activity, a loss of ATP in soil during the extraction procedure with Tris buffer (pH 7.5) has been observed (ref. I). When extracting ATP from a material with a high heat capacity, the temperature of the buffer extractant drops to that of the added material (e.g. soil) with a consequent loss in extracted ATP (Holm-Hansen & Booth, 1966). Karl & LaRock (1975) found that use of boiling Tris to extract ATP from beach sands and sediments yielded extremely variable results. They suggested that the variability may be due to effects of thermal gradients established in a fluid-solid mixture. When solids are submersed in liquids, a transition or boundary layer of fluid is established around the solid that does not have the thermal characteristics of the bulk fluid. When the fluid is heated, a sharp thermal gradient is established across the boundary layer. Bacteria adhering to the solid fall within this thermal gradient across the boundary layer, and thus the temperatures to which they are exposed may not be high enough to efficiently extract ATP and prevent its hydrolysis.

Most comparisons of different extractants have shown that cold acidic reagents with a pH below 2 is to be preferred to extract ATP from soil (Lee et al., 1971a; Ausmus, 1973; ref. I; Jenkinson & Oades, 1979; ref. X). These authors found that the best extractant of those tested was either sulphuric acid or trichloracetic acid (TCA). A pH below 2 was important to ensure that the phosphate groups of the ATP molecules were undissociated. The acid kills the cells, releases intact ATP and inactivates the ATP hydrolysing enzymes. During the extraction the most reactive P-retaining components such as the amorphous Fe-and Al-sesquioxides and $CaCO_3$ tend to be dissolved thus releasing more of the ATP than under neutral conditions (Lee et al., 1971a).

TCA, sulphuric acid, DMSO-acid and perchloric acid extracted

ATP efficiently from soils (ref. I). However perchloric acid proved unsuitable; this was either caused by the strong inhibitory effect on the luminescence reaction by the neutralized extract (ref. I) or by considerable loss of ATP by co-precipitation with perchloric acid (Wiener et al., 1974). Sulphuric acid and TCA were found to be most suitable for soil ATP extractions (ref. I). To avoid a co-precipitation of ATP with e.g. Fe, Al, or Ca in the acid soil extracts on adjustment to pH 7.5, a dilution is necessary (ref. II). The ions can also be removed by cation exchange before pH-adjustment. The method found to be most efficient among different extractant methods tested , was based on sulphuric acid extraction, followed by filtration, cation exchange clean-up on Na^+ resin, and pH adjustment with ethanolamine (ref. I). However, it was difficult to obtain quantitative values in all soil types by the mentioned sulphuric acid ATP method (ref. VIII). This was probably caused by a combined effect of an extractant to soil ratio (ml/g wet weight soil) of 1.2:1.0 and use of sulphuric acid without phosphate and NRB®. The used ratio was originally chosen to obtain enough sensitivity for measuring ATP with the equipment earlier used. Using more sensitive equipment for measuring ATP it was found that more ATP could be extracted by increasing the extractant to soil ratio and filtration and cation-exchange could be avoided (ref. X). The suggested ATP method is discussed in a separate section (page 20; see also APPENDIX I, page 160).

To avoid adsorption onto the soil colloids of released ATP from the microorganisms, compounds can be included in the extractant which are known to be adsorbed on the soil colloids, resulting in a desorption of ATP. Both ATP and phosphate molecules can be bound on positively charged sites in soil and thus compete for the adsorption on the soil colloids.

Jenkinson & Oades (1979) used an addition of both phosphate and paraquat dichloride to a trichloracetic acid (TCA) extractant. In a red brown loam soil under permanent pasture, they found that

addition of phosphate increased the quantity of soil ATP extracted from 0.16 to 0.64 µg ATP/g d.wt. soil, and the amount extracted was further increased to 1.10 µg ATP/g d.wt. by addition of paraquat. The method is very effective for extracting ATP from soil microbial populations but both TCA and paraquat are toxic reagents. Furthermore, the procedure for recrystallizing the technical grade paraquat dichloride is time consuming, and the pure chemical (methyl viologen, Sigma) is very expensive.

Verstraete et al. (1983) included 0.1 M pyridine and 0.1 M decane,1,10-bistrimethylammonium iodide in case of very heavy textured soils (>25 % clay) with potential ATP sorption problems. This compound was chosen instead of paraquat dichloride. Eiland (ref. X) did not find an increased ATP content by including paraquat dichloride in the sulphuric acid-phosphate-NRB procedure for extraction from a soil containing 11.9 % clay. NRB® acts in a similar manner to paraquat dichloride and pyridine together with decane,1,10-bistrimethylammonium iodide, because it can be bound to clay and other soil colloids.

A method for extraction and measurement of ATP from soil and from cultures of microorganisms

Materials and methods (see APPENDIX I)

Discussion of the methodology

The experience from the earlier used methods (refs I & III) and the introduction of more sensitive ATP photomultiplyers have resulted in a development of a new improved procedure for extraction of ATP from soil. It has great advantages, compared with most other techniques available for ATP extraction from soil samples. The method is simple, rapid and gives reproducible results. After correction for recovery of added ATP (internal standards), the results probably representing the total amount of ATP in soil. Use of internal standards corrects for: 1) Degradation and adsorption of ATP during the extraction procedure, and 2) inhibition of the luminescence reaction by ions

and other components in the soil extracts and the extractant solution used. In addition no toxic reagents were used (ref. X). Some characteristics of the soils used to examine the proposed ATP method are given in Table 2.

To compete with ATP molecules for adsorption on the soil colloids, phosphate was added to the sulphuric acid extractant and a quaternary ammonium compound (NRB®) was also used in the extraction procedure for the same purpose.

In preliminary experiments, paraquat was added to different extractants used for ATP extraction from a silt loam soil (Table 3). Addition of paraquat to a sulphuric acid-phosphate extractant did not increase ATP extraction, when the NRB reagent was used. Paraquat dichloride slightly depressed the light emission in all extractants. Use of NRB reagent in the suggested procedure increased the light emission and caused a decaying light signal after 30-40 s for all the extractants. A very slow decay of the light signal was observed for all extracts without use of NRB® in the procedure. Because of the fast decline of the light emission with use of NRB®, the suggested method including NRB® cannot be used in liquid scintillation spectrometers in which ATP is measured over periods exeeding 1 min after mixing of the soil extract with the enzyme. As far as is known the mechanism which caused the decline has not been examined. It could be due to a catalytic effect of the NRB reagent.

NRB® enhanced the sensitivity of the measurement by giving higher counts and also increased the extraction of ATP from soil in the suggested method due to the adsorption of NRB® to the soil colloids (Table 3), and thus competing with the ATP molecules.

Table 2. Some characteristics of the soils used (ref. X)

Soil (Location)		pH ($CaCl_2$)	Moisture content* (%)	Clay <0.002 mm (%)	Silt 0.002-0.02 mm (%)	Fine sand 0.02-0.2 mm (%)	Coarse sand 0.2-2.0 mm (%)	CEC (m.equiv./ 100 g d.wt. soil)	Organic C (%)	Total N (%)	P_i (µg P/ g d.wt. soil)	K_i (µg K/ g d.wt. soil)
Coarse sandy soil	I	5.2	10.0	3.9	4.1	12.2	76.8	9.3	1.7	0.11	180	189
(Jyndevad, Denmark)	II	ND	10.5						1.7	0.10	183	80
Fine sandy soil	I	5.4	19.7	3.7	6.2	75.8	12.0	8.4	1.6	0.13	363	355
(Tylstrup, Denmark)	II	ND	32.1						1.5	0.11	318	186
Sandy loam soil	I	6.8	22.7	10.1	22.8	57.7	6.8	14.4	1.8	0.17	216	178
(Roskilde, Denmark)	II	ND	15.5						1.7	0.17	225	94
Loam soil	I	6.9	22.2	16.3	21.1	36.7	24.0	13.4	1.6	0.15	243	221
(Rønhave, Denmark)	II	ND	13.6						1.4	0.15	267	224
Silty loam soil	I	6.9	26.7	11.9	13.2	72.5	0.4	13.1	1.9	0.19	216	147
(Højer, Denmark)	II	ND	33.8						2.1	0.18	237	248
Humus soil (Sweden)	III	3.2	193.7	ND	ND	ND	ND	ND	40.00	0.55	1.0×10^3	6.2×10^3

*Determined prior to analysis.

Results are means of duplicate samples. ND = No determination.

I. A group of soils from ploughed plots, sampled in September 1981, and stored at 5°C for 90 days, and then at 25°C for 5 days.

II. A group of soils from rotavated plots, sampled in May 1981, and stored at 5°C for 210 days.

III. The humus soil from a forest was sampled in September 1981, and stored at 5°C for 90 days, and then at 25°C for 5 days.

I, II. Arable soils sampled from the 0-5 cm layer, and carry continuous barley, with a mustard catch crop. Only inorganic fertilizers have been applied. Soil texture was quoted from Hansen (1976).

Table 3. Comparison of ATP extraction efficiency from soil by different extractants (ref. X)

	Silt loam soil (Højer)		
Extractant	Extracted ATP with NRB[1] light units/30 s	Extracted ATP without NRB[1] light units/30 s	Extracted ATP with NRB/without NRB ratio
H_2SO_4-PO_4	55542	25539	2.17
ATP standard in H_2SO_4-PO_4	47562	26214	1.81
H_2SO_4-PO_4-paraquat	46014	21954	2.10
ATP standard in H_2SO_4-PO_4-paraquat	41490	22428	1.85
TCA[2]-PO_4	43092	21990	1.96
ATP standard in TCA[2]-PO_4	46470	25548	1.82
TCA[2]-PO_4-paraquat	40956	20973	1.95
ATP standard in TCA[2]-PO_4-paraquat	40398	21762	1.86
ATP standard in Tris-EDTA buffer	55256	31604	1.75

Results are means of triplicate samples.

All ATP measurements in the different extract were made by the proposed procedure. Control ATP standards were made in the extractant solutions and in Tris-EDTA buffer, respectively. Standards were added to the extractants without soil.

1) NRB® = A quaternary ammonium detergent (Lumac)

2) TCA = trichloracetic acid.

The effects of shaking and ultrasonification on extraction of ATP were examined on a sandy loam soil (Table 4). Extraction of ATP increased with duration of shaking, giving 1.39 µg/g d.wt. soil after 15 min shaking (corrected for recovery of added ATP by internal standards). The shaking time in this experiment was not continued until a constant ATP content was obtained. This has been done in an experiment using the same soil type and the same experimental conditions. Similar results were found after 15 and 20 min shaking time, whereas 25 and 30 min shaking time resulted in 10 % decrease in the ATP content. Shaking increased the temperature of the soil suspension from $0^{o}C$ to $3^{o}C$ irrespective of the time of shaking. Ultrasonification (MSE 150 W ultrasonic disintegrator with a 8 mm probe tip) for 1 min with full power gave after correction for recovery 1.33 µg ATP/g d.wt. soil, with 86 % recovery of added ATP, and an increase in temperature from $0^{o}C$ to $16^{o}C$. When the soil was ultrasonified for 3 min, the added ATP was recovered with 73 %, whereas the quantity of ATP extracted was nearly the same as when ultrasonified for 1 min or shaken for 15 min. Ultrasonification for 3 min indicated a loss of the added ATP standard solution, which resulted in misleadingly high values for the extracted soil ATP. This is probably caused by hydrolysis of added ATP when a prolonged time of ultrasonification has been used. Shaking for 15 min was found to be most suitable for the soils examined, provided soil dispersion occurs. If this is not the case, ultrasonification for 1 or 2 min can be used to disperse the soil aggregates.

The effect of extractant to soil ratio on extraction of ATP was examined on the sandy loam soil (Roskilde). An extractant to soil ratio of 10:1 removed the same amount of ATP from the soil as found by use of a ratio of 25:1 (1.4 µg/g d.wt. soil). However it was difficult to obtain reproducible results with the latter ratio. A ratio of 5:1 only removed 0.9 µg/g d.wt.

Table 4. Effect of shaking and ultrasonification on the extraction of ATP from sandy loam soil from Roskilde (ref. X)

Treatment	Duration of treatment (min.)	Temperature in soil suspension after treatment (^{o}C)	Added ATP recovered (%)	ATP content of soil* (µg/g d.wt. soil)
Shaking	1	3	99	0.64
Shaking	2	3	99	0.68
Shaking	3	3	89	0.97
Shaking	5	3	93	1.11
Shaking	10	3	95	0.90
Shaking	15	3	90	1.39
Ultrasonification	0.5	10	100	0.66
Ultrasonification	1	16	86	1.33
Ultrasonification	2	23	93	1.30
Ultrasonification	3	28	73	1.65

*Corrected for recovery of added ATP by the internal standard procedure.

Results are means of triplicate samples.

The effect on the ATP content of filtering soil suspensions was examined (Table 5). The filtration procedure of the acid extracts resulted in 57-60 % decrease in different soil types. It is likely that a smaller decrease would have been observed if NRB® or Rodalon® had been added before the filtration. Without a filtration procedure, the soil suspensions contained from 1.4 to 1.6 % soil particles of the suspension used for measuring ATP in different soils (Table 5).

An experiment was also performed to see whether the ATP molecules were retained in the filter or adsorbed to sand/bentonite (Table 6). Filtration of ATP disodium salt solutions did not decrease the relative ATP light units, indicating that ATP is not retained in the filter. On the contrary, filtering of a sand/bentonite suspension decreased the relative ATP light units with 88 %, indicating that ATP mainly is adsorbed to the sand/bentonite mixture.

Table 5. The effect of filtration on the content of ATP

Localization and soil type (0-20 cm depth)	Measuring ATP in soil suspension µg ATP/g d.wt. soil	Measuring ATP in soil extract after filtration[1)] ug ATP/g d.wt. soil	Decrease in ATP content after filtering %	Soil particles in the suspension used for ATP (w/v) %
Jyndevad, coarse sand	0.91	0.39	57	1.6
Korntved, coarse sand	0.86	0.35	60	1.5
Ballum, sandy loam	2.75	1.17	58	1.4
Højer, silty clay	2.46	1.02	59	1.4

Means of duplicate samples.

ATP method (ref. X).

1) Whatman no. 5 filterpaper was used: The acid soil suspensions were filtered before the neutralization.

The soils were sampled in May 1985, adjusted to 60 % of w.h.c. and preincubated for 5 day at 25°C.

Table 6. The effect of filtration on the content of ATP added to a mixture of sand/bentonite

ATP disodium solutions extracted	Without filtration rel. light counts	After filtration[1)] rel. light counts
ATP (0.5 ml of 8 µM) added to 10 ml extractant agent and passed the ATP extraction procedure	871	909
ATP (0.5 ml of 8 µM) added to 9.5 ml extractant agent and 1 g portion of sterilized sand/bentonite (ratio 4/1)	562	67

Means of duplicate samples.

ATP method (ref. X).

1) Whatman no. 5 filterpaper was used. The acid solutions were filtered before the neutralization.

No correction with standards.

Measurement of ATP in soil suspensions

It was not necessary to filter neither the sulphuric acid-phosphate soil extract nor any of the other suspensions tested (Table 3). After shaking of the soil suspensions for 15 min each of the tubes were shaken for another 5 s to obtain a homogeneous solution. A 50 µl aliquot was taken immediately from the upper part of the suspension (1-2 cm layer) and added to a heavily buffered solution containing Tris and EDTA, to neutralize and dilute the soil suspension. It was possible to take representative samples between 40 and 100 µl of the acid suspension from the soils examined. These amounts could be neutralized in 1.5 ml buffer. Then 50 µl of the latter mixture was transferred into 50 µl of NRB® and ATP measured after a 10 s exposure period. Results from the same acid soil suspension gave for a coarse sandy soil (Jyndevad) and a sandy loam soil (Ballum) coefficients of variation of 1.7 % and 2.4 %, respectively.

If low amounts of ATP were determined, less dilution of the soil suspension need to be applied in the steps after the acid extraction procedure. Portions of the acid suspensions (e.g. 0.5 ml) were added to a Tris-EDTA buffer (e.g. 2 ml), and pH was adjusted to 7.5 with 1.0 M NaOH. The sensitivity can also be improved by increasing the concentration of the luciferin-luciferase reagent.

Measurements of ATP content in cultures of microorganisms

Extraction of ATP from microbial cultures is performed as the method for ATP extraction from soil (ref. X). The sensitivity of the method can be improved by adding a decreased amount (4 ml) of the sulphuric acid-phosphate extractant to 1 ml of the culture. For standardization 3.5 ml of the extractant plus 0.5 ml of an ATP standard solution are added to 1 ml of the culture. This mixture is treated as the proper culture.

Measuring bioluminescence of ATP

The luminescence from extracted soil ATP emitted after mixing of sample and enzyme is most conveniently measured with a sensitive photomultiplier especially adopted for bioluminescence measurements (ref. X). Alternatively, a liquid scintillation spectrometer can be used (Jenkinson & Oades, 1979). Use of the liquid scintillation spectrometer is more time consuming than use of a photomultiplier adopted for bioluminescence measurements, as it is not possible to measure the light emission immediately after mixing of the ATP extract with the enzyme solution. Values can either be determined from the peak height of the light emission (Rasmussen & Nielsen, 1968), or from the integration of light over a pre-set time (Δt), starting the time t after mixing of the enzyme with the ATP solution. The following values of Δt and t have been used in soils:

1) Eiland (ref. X), Δt = 10 s or 30 s and t = 1 s,
2) Greaves et al. (1973), Δt = 5 s and t = 60 s,
3) Jenkinson & Oades (1979), Δt = 6 s and t = 3600 s,
4) Lee et al. (197la), Δt = 5 s and t = 10 s,
5) Paul & Johnson (1977), Δt = 60 s and t = 15 s,
6) St. John (1970), Δt = 30 s and t = 1 s.

Such procedures can be used only when the light emission of ATP from the various samples show the same time dependence. The light emission of ATP from a soil, from a culture of bacteria, and from a disodium ATP solution does not always show the same time dependence, when crude luciferin-luciferase is used. Therefore, Eiland & Nielsen (ref. II) suggested a measurement of the total light emission by an iterative fit of experimental data or graphically after plotting the data on a semilogarithmic plot.

However, for determination of ATP in soil samples, the integration system is the most convenient to use (ref. X). This requires the use of purified luciferin-luciferase.

Crude or purified enzymes

The procedures for light measurements can only be used, when the light emission from samples and ATP standards show the same time dependence (ref. II). This is not always the case, when measurements are performed within the first 4 min after injection of crude enzymes. A prolonged delay in measuring the light emission gives the ATP producing and consuming enzymes in the crude preparation an opportunity to operate. Crude firefly enzyme contains adenylate kinase and nucleoside diphosphate kinase both of which may catalyse the formation of ATP from other nucleotides present in the extract (Rasmussen & Nielsen, 1968). Holm-Hansen & Booth (1966) suggested that ATP determinations made with crude enzyme can give results between 5-35 % too high.

Due to these errors caused by impurities in the crude enzyme, use of purified enzymes must be strongly recommended for ATP determinations in soil. The reactions of various samples and ATP standards show the same type of kinetics in this enzyme in which the light intensity remains relatively constant during the measuring period. Use of NRB® caused a faster decline of emitted light, but the same reaction kinetics of different samples and ATP standards were still observed. Purified enzymes ensure that ATP is specifically measured and differential measurements can be made with the most convenient measuring time, provided that the maximum light intensity (the plateau) is reached.

Effects of ions and temperature on the luminescence reaction

Several ions reduce the light emission. Aledort et al. (1966) found an inhibition by cations decreasing in the following order of $Ca^{++} > K^{+} > Na^{+} > Rb^{+} > Li^{+} > chloline^{+}$. Anion inhibition occurred in the order of $I^{-} > H_2PO_4^{-} > Br^{-} > ClO_3^{-} > Cl^{-} > F^{-} > HCO_3^{-} > COOCH_3 \cdot H_2O^{-}$. Denburg & McElroy (1970) found that the following anions inhibited the reaction: $SCN^{-} > I^{-} \backsim NO_3^{-} > Br^{-} > Cl^{-}$.

The buffer effect on ATP analysis has been examined by Webster et al. (1980). They found a higher light emission in Tricine, Tris and glycylglycine than in phosphate and Hepes buffers.

Interfering ions have been diluted out (Van Dyke et al., 1969; Patterson et al., 1970; ref. X), or removed by cation exchange resin (Ausmus,1973; ref. I) or by Sephadex G-10 column (Van Dyke et al., 1969). To avoid adsorption of extracted ATP to inorganic and organic particles in soil, compounds are included in the extractants, which are known to be adsorbed to those particles (Jenkinson & Oades, 1979; ref. X, Verstraete et al.,1983).McElroy & Strehler (1949) found that a pH of 7.5 and a temperature of approximately 25°C are optimal for measuring luminescence. The reaction was inhibited by pH values below 5.5 (Seliger & McElroy, 1960) and by temperatures more than 35°C (McElroy & Strehler, 1949).

ATP content in soil

For determination of ATP in soil, either living microbial cells of known ATP content or the pure ATP chemical can serve as a standard. Some authors (e.g. Lee et al., 1971a; Conklin & MacGregor, 1972; Christian et al., 1975; Paul & Johnson, 1977) have added microbial cells to the soil, while others have added the pure ATP chemical (e.g. Jenkinson & Oades, 1979; refs I,III & X; Verstraete et al., 1983). The pure chemical is to be preferred as it is easier to make a standard with the chemical, than to maintain a culture of microbial cells with a constant content of ATP.

Determination of ATP content in soils by use of various ATP extraction procedures have shown a wide range. Jenkinson & Oades (1979) reported values of from 0.64 to 7.00 µg ATP/g d.wt. soil using the trichloracetic acid-phosphate-paraquat method, Eiland (Table 7) found 0.37 to 7.52 µg ATP/g d.wt. by the sulphuric acid-phosphate-NRB method, Paul & Johnson (1977) 0.11 to 4.73 µg ATP/g wet soil, using cold bicarbonatechloroform, (ref. III) 0.08 to 0.40 µg ATP/g d.wt. with Tris-NRB extractant, Verstraeten et al. (1983) 0.02 to 0.7 µg ATP/g d.wt., and Knight & Skujins (1981) 0.002-0.028 µg ATP/g dry soil by cold sulphuric acid; the soils examined by Knight & Skujins (1981) were arid soils, where lower values should be expected than for the other soils. However it seems that these results are exceptionally low. The different extraction methods, utilizing crude or purified enzymes as well as different procedures for measuring light emission might partly explain the varying results. The methods giving the highest amounts of ATP were the sulphuric acid-phosphate-NRB method (ref. X), and the trichloracetic acid-phosphate-paraquat method (Jenkinson & Oades, 1979).

Table 7. Biomass C, recovery of ATP, ATP content and biomass C/ATP values in some Danish and Swedish soils (ref. X)

Soil (Location)		Biomass C[1] (µg C/g d.wt. soil)	Proposed ATP method: Added ATP recovered (%)	Proposed ATP method: ATP content of soil* (µg/g d.wt. soil)	Proposed ATP method: Biomass C/ATP ratio	NRB®ATP method: Added ATP recovered (%)	NRB®ATP method: ATP content of soil* (µg/g d.wt. soil)	NRB®ATP method: Biomass C/ATP[1] ratio
Coarse sandy soil	I	89	93	0.37	240	28	0.09	1047
(Jyndevad, Denmark)	II	76	92	0.35	217	ND	ND	ND
Fine sandy soil	I	163	68	1.31	124	40	0.16	1019
(Tylstrup, Denmark)	II	62	91	0.13	477	ND	ND	ND
Sandy loam soil	I	169	94	1.04	163	42	0.13	1300
(Roskilde, Denmark)	II	96	82	0.56	171	ND	ND	ND
Loam soil	I	193	55	1.35	143	29	0.05	3860
(Rønhave, Denmark)	II	258	48	0.60	430	ND	ND	ND
Silt loam soil	I	202	88	1.44	140	61	0.05	4040
(Højer, Denmark)	II	445	72	1.12	397	ND	ND	ND
Humus soil (Forest soil Sweden)	I	ND	65	7.52	ND	30	0.72	ND

*Corrected for recovery of added ATP by the internal ATP standard procedure.

1) Biomass carbon estimated by the $CHCl_3$ fumigation method.

I. A group of soils sampled in September 1981, adjusted to 60 % of the w.h.c. and stored for 90 days at 5°C, and then incubated for 5 days at 25°C.

II. A group of soils sampled in May 1981, adjusted to 60 % of the w.h.c. and stored for 210 days at 5°C.

Results are means of triplicate samples. ND = no determination.

C. THE RATIO BETWEEN THE NUCLEOTIDES ATP, ADP AND AMP IN SOIL

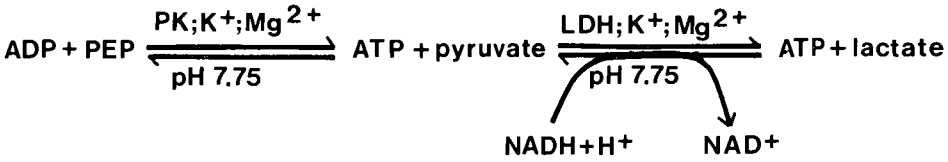
Introduction

The ATP content of soil determined by the luciferin-luciferase method is believed to be a useful measure of soil microbial biomass or activity in various ecosystems. Measurement of the adenylate energy charge, $AEC = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$ (Atkinson & Walton, 1967; Atkinson, 1968; 1969, Chapman et al., 1971), may give additional information about the metabolic state of the organisms. The reactions involved in the processes are simplified in Fig. 3.

The AEC value provides a measure of the total amount of metabolic energy momentarily stored in the adenine nucleotide pool. The theoretical range of AEC values is from 0 (all AMP) to 1 (all ATP) although these extreme values may not be found. In actively growing cells, reported AEC values range from about 0.80 to 0.95 but in stressed and senescent organisms, the values can be as low as 0.5-0.7. Dead or dying cells have AEC less than 0.5 (Karl, 1980; Karl & Holm-Hansen, 1977; Wiebe & Bancroft, 1975). For endospores of bacteria AEC values as low as 0.08 have been reported (Setlow & Kornberg, 1970).

I Measurements of ATP (reactions, see Fig.2, page 12).

II Measurements of ADP plus ATP (reaction for converting ADP to ATP):



III. Measurements of AMP plus ADP plus ATP (reactions for converting AMP to ATP):

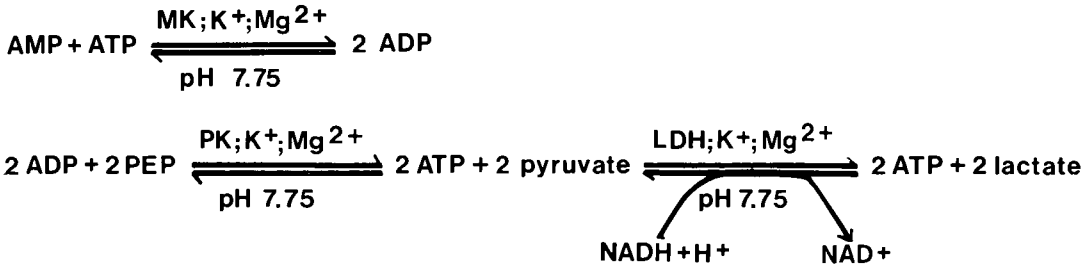


Fig. 3. Scematic presentation of the reactions for converting ADP and AMP to ATP.

Pyruvate was reduced by NADH and LDH to promote the speed of the reactions.

The mixtures including samples and standards were incubated for 30 min at 30°C and the ATP concentrations measured.

Mixtures for the reactions (see APPENDIX II)

PEP = phosphoenol pyruvate

NADH = β-nicotinamide-adenine dinucleotide

PK = pyruvate kinase

LDH = lactate dehydrogenase

MK = myokinase

The validity of the AEC concept as a mechanism for the control of cellular metabolism has been attacked on theoretical grounds (Fromm, 1977). Knowles (1977) argued that the unitless of the AEC limits the usefulness of this parameter. It has also been argued (Lowry et al., 1971) that the AEC is an insensitive metabolic indicator. Despite of these objections, most data indicate that the relative molar concentrations of the adenine nucleotides in actively metabolizing cells are maintained within the limits predicted by the AEC hypothesis (e.g. Chapman & Atkinson, 1977; Chapman et al., 1971).

The aim of this study was to develop a method to measure AEC of the soil microbial biomass and examine the size of the AEC in soils by enzymatic conversions of ADP and AMP to molar concentrations of ATP, followed by quantitative analysis of ATP by the luciferin-luciferase system.

Materials and methods (see APPENDIX II)

Discussion of the methodology

The adenine nucleotides were basicly extracted from soil with sulphuric acid-phosphate, as used in the suggested ATP method. Soil samples and standards (ATP, ATP/ADP and ATP/AMP) with sterilized soil were added to the extraction agent, shaken for 15 min and heated at 100^{o}C for 2 min. Then all mixtures were neutralized and aliquots were incubated together with the different enzymes at 30^{o}C for 30 min. Without a heat deactivation step, part of the ATP in the soil extract was

hydrolized during the incubation in the reaction mixtures, presumably due to a re-activation of enzymes in the neutralized extracts during the incubation at 30°C for 30 min. Blind values of sterilized soil treated as the proper soil samples were used to correct for ATP present in the reagents. To ensure that the sterilized soil did not contain measurable amounts of the nucleotides, blind values were also made from the sulphuric acid-phosphate extractant alone treated as the samples. The background light emission from the extractant without sterilized soil was in most experiments higher or similar to that of the extractant with sterilized soil, indicating an inhibition of the light emission from compounds in some soil types.

Myokinase is used in the procedure to convert AMP to ADP. A very high endogenous background light emission was found in preliminary experiments when a high concentration of myokinase (5 µg MK/50 µl) was used. This was probably caused by ATP bound to MK. It was also necessary to reduce the concentration of $(NH_4)_2SO_4$, to ensure a quantitative conversion of AMP to ATP because $(NH_4)_2SO_4$ inhibites the MK enzyme reaction. The reduction was achieved by centrifugation of the MK solution. The supernatant was removed and the pellet containing MK was diluted. A final concentration of 0.4 µg MK/50 µl was chosen, which ensured a quantitative conversion of AMP to ATP.

To promote the speed of the reactions for conversion of AMP and ADP to ATP, both lactatedehydrogenase (LDH) and B-nicotinamide-adenine dinucleotide (NADH) were included in the mixture for reaction II (determination of ADP plus ATP) and for reaction III (determination of AMP plus ADP plus ATP). ATP was included in standards used for reaction mixtures II and III. A comparison between mixed standards of ATP/ADP (ratio 4/1) and ADP standards gave similar results. Addition of ATP to reaction mixture III promotes the conversion from AMP to ADP. Karl & Holm-Hansen

(1977) suggested the use of addition of ATP to mixtures for conversion of AMP to ATP from soil samples. Use of LDH and NADH for determination of AEC values in seeds was proposed by Lunn & Madsen (1981). The efficiency of the conversion of ADP and AMP to ATP was always 95-100 %. This is in agreement with many papers, where conversion efficiency was between 90 and 100 % (Karl & Holm-Hansen, 1978; Kimmich et al., 1975; Lundin & Thore, 1975).

Karl & Holm-Hansen (1978) proposed the use of a heat deactivation step for low-level adenine nucleotide analyses after incubation of the reaction tubes, prior to the ATP assays. They found that in absence of heat deactivation before addition of crude firefly luciferin-luciferase, both the linearity of the standard curve and the lower limit of ATP detection were greatly affected. This was caused by immediate production (0-3 s) of ATP in the presence of phosphoenol pyruvate (PEP) and pyruvate kinase, presumably from ADP contained within the crude luciferin-luciferase enzyme.

In the suggested method, where purified luciferin-luciferase enzyme was used, it was not necessary to use the heat deactivation step after incubation of the reaction tubes.

The sensitivity of the method can be improved by increasing the concentration of the luciferin-luciferase reagent by use of a smaller volume of buffer (e.g. 5 ml instead of 8 ml). It is very important to use a very sensitive photomultiplier. The Lumacounter applied (M 2080) had a detection limit of less than 0.2×10^{-15} moles ATP, while a new model (Biocounter M 2010) was not sensitive enough (lower limit 0.4×10^{-15} moles ATP).

Recovery of added ATP, ADP and AMP gave 80.9 %, 84.8 % and 88.9 %, respectively, for the fresh sandy loam soil (Table 7). The same trends with percentage of recovery increasing in the following order, ATP < ADP < AMP, were also found in the different preincubated soils although the percentage of recovery was not the same. Similar trends between the three nucleotides were reported by Brookes et al., (1983).

Adenine nucleotides and adenylate energy charge in selected soils

Some characteristics of the soils used for the adenine nucleotide experiments are given in Table 8. A sandy loam soil was studied in great details comparing different soil treatments (Table 9). The "fresh" soil analysed 3 h after sampling had an AEC of 0.83 and ATP, ADP and AMP were 65.6 %, 33.9 % and 0.5 % of A_T (ATP plus ADP plus AMP), respectively. When the soil was air-dried for 10 days at 25°C, A_T decreased from 2.18 to 0.56 nmol ATP/g d.wt. soil. AEC decreased in the same period from 0.83 to 0.46 and the relative proportions of the three nucleotides were 17.9 % (ATP), 55.4 % (ADP) and 26.7 % (AMP) of A_T. Air-drying of the soil is known to kill part of the soil microorganisms (Powlson & Jenkinson, 1976). This agrees with the low AEC value where microorganisms begin to die. After the air-dried soil was remoistened to 60 % of w.h.c. and incubated for 24 h at 25°C, A_T increased from 0.56 to 1.80 nmol /g d.wt. soil and AEC rose from 0.46 to 0.73, indicating a formation of new biomass on the expense of dead microorganisms and substrate liberated from the soil by the drying and rewetting procedures. A_T and AEC were after the 24 h incubation period only slightly lower than those obtained for the "fresh" soil. ATP, ADP and AMP were 55.0 %, 35.6 % and 9.4 % of A_T, respectively. The relative proportions of the adenine nucleotides were fairly similar to those found for the "fresh" soil. The increase in content of nmol ATP/g d.wt. soil was not counterbalanced by a decrease in ADP and AMP. Therefore, ATP may be produced from degradation of substrate by de novo synthesis. Brookes et al. (1983) obtained similar AEC values in a park grass soil, although not exactly the same experimental conditions were used. However, after rewetting of air-dried soil and incubation for 2.5 h they found that ATP was only produced by conversion of ADP and AMP to ATP.

Table 8. Some characteristics of the soils used

Soil (Location)	Soil treatments	pH ($CaCl_2$)	Moisture (At sampling time) %	Organic C %	Total N %	C/N ratio	P_i µg P/g d.wt. soil	K_i µg K/g d.wt. soil
Coarse sandy soil [1] (Jyndevad, Denmark)	Ploughing and NPK fertilizers	6.4	11.6	1.9	0.11	17.7	258	105
Sandy loam soil (Askov, Denmark)	Ploughing and slurry manure	6.7	19.2	2.1	0.17	12.3	174	94
Sandy loam soil [2] (Askov, Denmark)	Ploughing and NPK fertilizers	6.6	19.3	2.3	0.18	12.5	216	108
Sandy loam soil [1] (Roskilde, Denmark)	Ploughing and NPK fertilizers	6.4	18.8	1.9	0.15	12.7	300	129
Silty loam soil [1] (Højer, Denmark)	Ploughing and NPK fertilizers	6.5	18.6	1.8	0.19	9.7	221	203
Humus soil [3] (Sweden)	No treatment	3.6	210.0	40.6	0.60	67.7	0.6×10^3	6.0×10^3

Means of duplicate samples.

1) The arable soils were sampled in June, 1983, from the 0-5 cm layer, and carry continuous barley with a mustard catch crop. The field experiment was initiated in 1973.

2) The arable soils were sampled by August, 1983, from the 0-20 cm layer. A four years rotation of sugarbeets (1982), Italian ryegrass (1983) and barley (1984) were applied. The field experiment was initiated in 1973.

3) The humus soil from a forest was sampled in June, 1983.

The examined plots are not the same as those mentioned in Table 2.

See Table 2 and APPENDIX II page 168, for the soil textures.

Table 9. Adenine nucleotides and adenylate energy charge values of a sandy loam soil

Sandy loam soil (Roskilde)	Soil adenine nucleotide content (nmol/g d.wt. soil)			Total soil adenine nucleotide content (A_T)	Soil adenine nucleotide content as % of A_T			AEC values
	ATP	ADP	AMP	(nmol/g d.wt. soil)	ATP	ADP	AMP	
"Fresh"soil[1] containing 60 % of w.h.c.[2]	1.43	0.74	0.01	2.18	65.6	33.9	0.5	0.83
Air-dried soil after incubation for 10 days at 25°C	0.10	0.31	0.15	0.56	17.9	55.4	26.7	0.46
Air-dried soil after incubation for 10 days at 25°C, remoistened to 60 % of w.h.c.[2] and incubated for 24 h at 25°C	0.99	0.64	0.17	1.80	55.0	35.6	9.4	0.73

Means of duplicate samples.

1) The soil was analysed 3 h after sampling.

2) w.h.c. = water holding capacity.

Recovery of added ATP, ADP, AMP for the "fresh" soil were 80.9 %, 84.8 % and 88.9 %, respectively.

The effects on A_T and AEC of glucose added to a sandy loam soil was examined (Table10). After sampling, the soil was moistened to 60 % of the w.h.c. and stored for 90 days at 5°C. The soil was then incubated for 5 days at 25°C, where glucose (1.0 % of soil d.wt.) was added followed by incubation at 25°C for further 3 days. A control without glucose was incubated similarly. The contents of ATP and ADP increased due to the incubation with glucose, whereas the content of AMP remained fairly constant. The A_T increased with 29.6 % due to the soil incubation with glucose. The AEC value increased from 0.65 in the soil without glucose to 0.70 in the soil with glucose added. The small increase in the soil added glucose is consistent with the assumption that the soil microorganisms to a large extent are old dormant or resting cells with an AEC of about 0.7, and only a minor part is in active growth with an AEC of about 0.8. The AEC value, which is an average value for the total biomass will then depend on the proportion of young cells to old and resting cells.

Thus, Leps & Ensign (1979a) found that addition of glucose to spherical cells of a pure culture of the bacterium Arthrobacter crystallopoietes increased both the ATP content per cell and their rate of endogenous metabolism. The ATP content fluctuated and then remained at a level higher than maintained during starvation while endogenous metabolism quickly declined. Leps & Ensign (1979b) also examined the AEC value of A. crystallopoietes during growth and starvation. AEC of rod-shaped cells rose during the first 4 h of growth, then remained constant during subsequent growth and decreased in the stationary growth phase. During starvation both spherical and rod-shaped cells excreated AMP but not ATP or ADP. AEC was 0.73 after 168 h of starvation. Both cell forms remained more than 90 % viable. Addition of glucose to starving cells resulted in an increase both in the ATP content and in the AEC value.

Table10. Adenine nucleotides and adenylate energy values of a sandy loam soil without and with addition of glucose

Askov sandy loam soil NPK fertilized	Soil adenine nucleotide content (nmol/g d.wt. soil)			Total soil adenine nucleotide content (A_T)	Soil adenine nucleotide content as % of A_T			AEC values
	ATP	ADP	AMP	(nmol/g d.wt. soil)	ATP	ADP	AMP	
Without addition of glucose	1.10	1.28	0.29	2.67	41.2	47.9	10.9	0.65
With addition of glucose	1.68	1.49	0.29	3.46	48.9	37.9	9.4	0.70

Means of duplicate samples.
The soil was sampled in June 1983, adjusted to 60 % of the water holding capacity, stored for 90 days at 5°C and incubated for 5 days at 25°C. Glucose (1 % of soil d.wt.) was added to one soil portion and soil without and with glucose was further incubated for 3 days at 25°C.

The minor increase of AEC in the soil with glucose added compared to the AEC of the soil without glucose are consistent with these observations, when it is assumed that only a certain part of the microorganisms in soil are young cells capable of much growth. The results from the soils also suggest that high amounts of extracellular AMP probably not are present in soil.

The contents of ATP, ADP and AMP and AEC values in different soils are shown in Table11. The ATP content increased when the soils were incubated at 25°C for 24 h, as compared to incubation at 5°C. This agrees with other results (Chapter E; ref. X). The percentages of ATP, ADP and AMP of A_T showed that a raise in temperature from 5°C to 25°C resulted in an increase in the percentage of ATP and a decrease in the percentage of either AMP or ADP. The AEC values were increased in the soils, when the temperature was raised. AEC in the soils incubated at 5°C ranged from 0.58 to 0.74 and suggests a biomass consisting mainly of dormant organisms. At 25°C, AEC ranged from 0.78 to 0.83 in different soils and suggest an increased metabolism for some of the organisms. The total soil adenine nucleotide content (A_T) was nearly the same in soils incubated at 5°C and 25°C, indicating a conversion of ADP or AMP to ATP and that ATP not was produced by de novo synthesis. Therefore, the biomass was not increased under these experimental conditions.

These results explain other experiments, where increase in ATP content in soil was observed within 2-3 h, when the soil temperature was raised (Chapter E, Fig. 12). It was not likely that the soil biomass increased so much during such a short period.

Chapman et al. (1971) found that AEC in exponentially growing cells of Escherichia coli was near 0.8. When growth stopped due to exhaustion of the carbon source in the medium, the AEC decreased to 0.6-0.7. After addition of glucose, the AEC rose rapidly to the level of the growing cells. Chapman & Atkinson (1977) discussed the possibility that AEC values for normal metabolizing cells are near 0.9 and the values published in a

Table 11. Adenine nucleotides and adenylate energy charge values in some Danish and Swedish soils

Soils (Location)	Temperature of preincubated soils °C	Soil adenine nucleotide content (nmol/g d.wt. soil) ATP	ADP	AMP	Total soil adenine nucleotide content (A_T) (nmol/g d.wt. soil)	Soil adenine nucleotide content as % of A_T ATP	ADP	AMP	AEC values
Coarse sandy soil	5	0.56	0.35	0.36	1.27	44.1	27.6	28.3	0.58
(Jyndevad, Denmark)	25	0.72	0.53	N.D.	1.25	57.6	42.4	N.D.	0.79
Sandy loam	5	2.25	0.53	1.46	4.24	53.1	12.5	34.4	0.59
(Askov, Denmark)	25	2.85	1.40	0.30	4.55	62.6	30.8	6.6	0.78
Silt loam soil	5	2.49	1.82	0.30	4.61	53.9	39.5	6.6	0.74
(Højer, Denmark)	25	3.45	1.09	0.30	4.84	66.0	20.9	6.2	0.83
Humus soil	5	13.27	8.04	4.82	26.13	50.8	30.8	18.4	0.66
(Sweden)	25	17.90	9.74	N.D.	27.64	64.8	35.2	N.D.	0.82

Means of triplicate samples.

N.D. = Not detectable.

See Table 6 for characteristics of the soils.

The soils were adjusted to 60 % of the water holding capacity, stored for 90 days at 5°C and then incubated for further 24 h at 5°C and 25°C, respectively.

range of papers e.g. the results of Chapman et al. (1971) are somewhat low because of an unrecognized loss of ATP during handling by use of poor extraction methods. Niven et al. (1977) observed that a culture of Beneckea natriegens maintained its AEC at a relatively constant level for several hours after substrate depletion. Ball & Atkinson (1975) reported that Saccharomyces cerevisiae cells grown on different carbon sources and under aerobic or anaerobic conditions maintained AEC above 0.8. When this eukaryotic organism was grown aerobically on ethanol or glucose and allowed to pass into the stationary phase, with utilization of accumulated ethanol, it maintained a normal value (0.8-0.9) of the AEC during prolonged starvation. Wiebe & Bancroft (1975) determined AEC in the upper layers of salt marsh sediments, and found values below 0.6 for the average community; these low values may partly be due to the boiling $NaHCO_3$ extraction method used (Bancroft et al., 1976).

Multicellular animals appear to maintain high AEC values in the range between 0.75 and 0.9 until they are moribund (for a review see Chapman et al., 1971). Karl et al. (1978) found in two species of copepods a higher ATP content of the cellular carbon (C/ATP ratios ≈ 30-40) than results reported in unicellular microorganisms; the corresponding AEC values in the copepods ranged from 0.89-0.93. The pool of animals in a soil may thus contribute to a somewhat higher community AEC value than expected for bacteria and fungi in soil. Better extraction methods using cold acid extractants, preserve a greater percentage of adenine nucleotides as ATP, than methods based on boiling with neutral extractants (Karl & Holm-Hansen, 1977; Karl et al., 1978). This is probably because acidic extractants are more effective in denaturing ATP-ases.

Brookes et al. (1983) found that the soil biomass, although mainly a resting population, has an ATP content and an AEC of the same size of that of actively growing microorganisms in vitro and they suggested that in soil metabolically active and

inactive populations have similar ATP contents and AEC values. This seems to be consistent with the results obtained for some of the soils incubated at 25°C before extraction (Tables 9 and 11) However, the somewhat lower AEC values obtained for the other soils (Tables 10 and 11) indicate that AEC values might be lower than that of actively growing microorganisms although the ATP content in old cells may be comparable to that of active growing microorganisms. This indicate rather complex mechanisms related to the physical soil conditions.

Martens (1983) found that AEC values in five agricultural soils were 0.3-0.4. This is values, where the microbial populations should die. He did not mention the soil temperature but the very low AEC value could be caused by a less effective extraction method (slightly alkaline $CHCl_3$-$NaHCO_3$ extractant) or by methodological problems in determination of all three adenine nucleotides.

A factor of importance for the size of the AEC value in a soil is the soil animals because they maintain a high AEC until there are moribound. AEC in endospores of bacteria has been found to be as low as 0.08 (Setlow & Kornberg, 1970). But spores might not have a significant effect on the AEC of the total biomass as only a minor part of the soil organisms survive as spores.

When the different factors mentioned are taken into account, the content of adenine nucleotides and the AEC values reported in this work seem to agree well with the general concept of the soil biomass, where most of the soil microorganisms are starving or dormant populations living most of the time in a substrate-depleted environment.

D. EXTRACTION OF ATP FROM BACTERIA AND FUNGI GROWN IN THE PRESENCE OF BENTONITE

Introduction

Clay minerals affect microbial growth but the influence depends very much upon the type of the clay mineral and the amount in the system (Lynch & Cotnoir, 1956; Estermann & McLaren, 1959), as well as on the microorganisms present (Estermann et al., 1959). Extensive studies have been made on the effects of clay on microbial biomass and activity (e.g. Nováková & Ettler,1974; Marshman & Marshall 1981a; 1981b) but there is still a lack of understanding of the mechanisms involved.

Extraction of ATP from soil has sometimes been fraught with low ATP recoveries which are either due to a poor extraction efficiency or due to adsorption phenomena (see Chapter B). Conklin & MacGregor (1972) and Anderson & Davies (1973) found that clay minerals affected soil ATP in such a way that complete desorption of ATP could not be achieved. However, they were not able to establish a relationship between clay content and ATP recovery from soil. Clay minerals adsorb phosphate anions by exchange of OH-groups, probably those which are linked to aluminum ions at the crystal edges (Muljadi et al., 1966). Similarly, triphosphate anions are adsorbed to clay (Lyons, 1964). Graf & Lagaly (1980) found that adenosine-5-phosphates are

adsorbed to clay minerals at very low nucleotide concentrations and that part of the ATP not recovered were dephosphorylated to ADP (45 % of ATP added to a suspension of calcium montmorillonite was thus recovered as ADP).

The aim of this study was to examine the effects of bentonite on the extraction of ATP from bacteria and fungi as well as the effects on a ATP sodium salt. Bacteria and fungi were grown in a mineral medium with and without 0.5 % and 1.0 % of the clay mineral bentonite prior to ATP extraction and determination. The extraction of ATP from dried cells and pure ATP in the presence and absence of bentonite was also examined.

Materials and methods (see APPENDIX III)

ATP content of a bacterium and a fungus

Addition of 0.5 % and 1.0 % bentonite to a culture of Pseudomonas fluorescens grown in a liquid medium, resulted in maximum O_2 uptake after 2 days of incubation (11.8 and 10.0 µg O_2/h/ml, respectively), and after 14 days of incubation the respiration rate decreased to 2-3 µg O_2/h/ml in all the cultures (Fig.4). The cultures with bentonite maintained a higher respiration during the first 2 days than the culture without bentonite but from the third to the fourteenth day only small differences were observed between the cultures. The culture grown in absence of bentonite had the maximum O_2 uptake after 3 days of incubation (8.5 µg O_2/h/ml) and then decreased.

The number of bacteria and the ATP content in the cultures added bentonite reached the maximum after 3 days (30-40 x 10^8 bacteria/ml and 1.4-1.7 µg ATP/ml)(Figs 5 and 6), and then decreased to 8-12 x 10^8 bacteria/ml and 0.3-0.4 µg ATP/ml after 6 days. From the sixth to the eleventh day, bacterial number and ATP content slightly increased or remained constant, and then decreased again from the eleventh to the fourteenth day to 6-8 x 10^8 bacteria/ml and 0.2-0.4 µg ATP/ml. After incubation for 41 and 49 days, the number of bacteria was 4-5 x 10^8 bacteria/ml and the ATP content was 0.2-0.3 µg ATP/ml. The cultures with bentonite maintained a higher bacterial number after third, fourtione and fourtinine days of incubation than the culture without bentonite. Bacterial numbers in the culture without bentonite increased during the first 2 days of incubation to 13 x 10^8 bacteria/ml, remained fairly constant up to the sixth day and then slowly decreased to the lowest level after 41 and 49 days (2 x 10^8 bacteria/ml).

The results show that the presence of a clay mineral temporarily stimulate the respiration, and preserve a higher

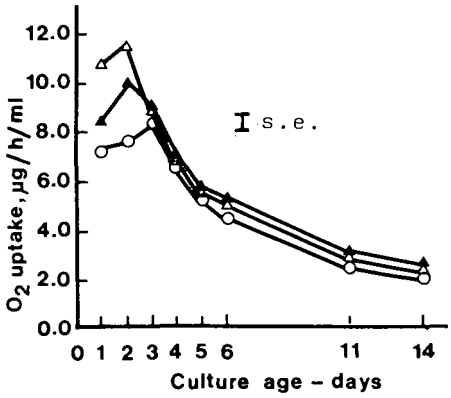


Fig. 4. Oxygen uptake in cultures of Ps. fluorescens grown in liquid medium in absence and presence of bentonite. (O) 0 %, (Δ) 0.5 % and (▲) 1.0 % bentonite. s.e. = standard error.

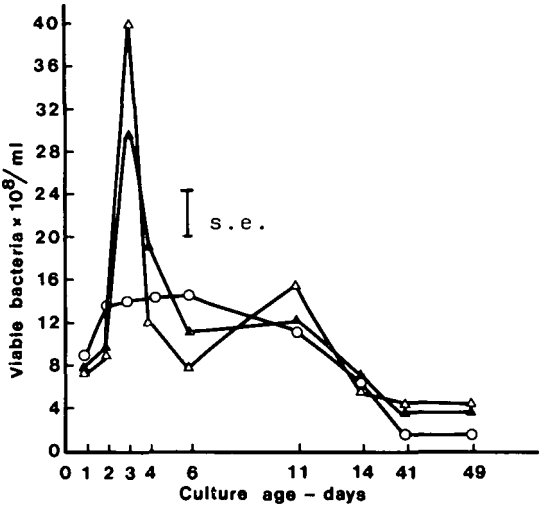


Fig. 5. Numbers of bacteria determined by plate counts in cultures of Ps. fluorescens grown in liquid medium in absence and presence of bentonite. (O) 0 %, (Δ) 0.5 % and (▲) 1.0 % bentonite. s.e. = standard error.

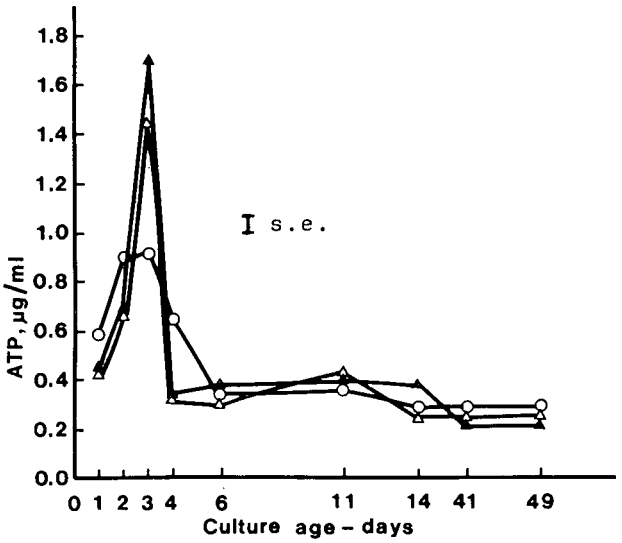


Fig. 6. ATP contents in cultures of Ps. fluorescens grown in liquid medium in absence and presence of bentonite. (O) 0 %, (Δ) 0.5 % and (▲) 1.0 % bentonite.
s.e. = standard error.

number of bacteria during growth in a liquid medium. The respiration rate of the bacteria was, however, far from being constant. The greatest variations were observed in the cultures added bentonite.

Nováková & Ettler (1974) also found that addition of bentonite increased the respiration rate in the early phases of incubation. The number of microorganisms did not increase due to the clay addition at the beginning of the experiments. However, they tended to rise later on during the incubation period.

The ATP content was in the culture without bentonite during the 0-14 days of incubation significantly positively correlated to the O_2 uptake ($r = 0.92$; $p < 0.01$). This was not found for the cultures with bentonite, where the O_2 uptake reached the maximum values earlier than the ATP content. However, a close relationship between ATP content and number of bacteria (0-49 days of incubation) was found in the cultures with bentonite (average results from the cultures with 0.5 % and 1.0 % bentonite:$r = 0.88$; $p < 0.01$), as opposed to the culture without bentonite, where ATP content and number of bacteria was not significantly correlated.

Presence of clay had a preserving effect on the number of bacteria; the number of cells were during the 41th and 49th day higher in the cultures with bentonite than in the culture without bentonite. This was not the case for the ATP content during the 41th and 49th day, where similar ATP contents were found in presence and absence of bentonite. It may be because of an ATP content in non-viable cells.

The ATP content per cell in cultures with bentonite was in the range of $0.2\text{-}0.8 \times 10^{-9}$ µg ATP/cell with the highest content during the exponential growth (Fig. 7). The culture without bentonite showed the same trends, the exception were after 41 and 49 days of incubation, where some decreases occurred.

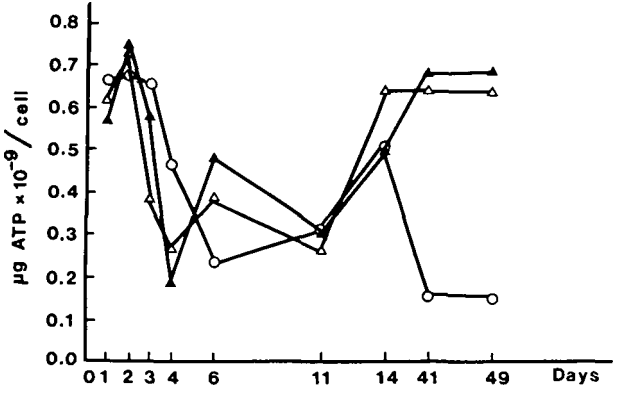


Fig. 7. ATP content per cell in cultures of Ps. fluorescens grown in liquid medium in absence and presence of bentonite.
(O) 0 %, (Δ) 0.5 % and
(▲) 1.0 % bentonite.

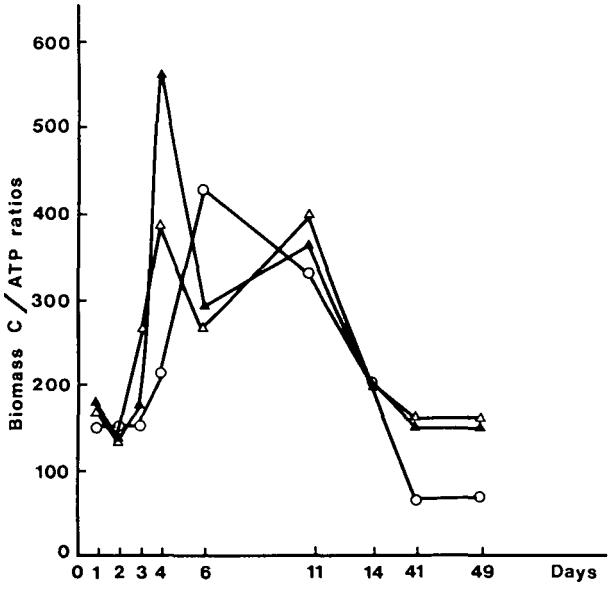


Fig. 8. The ratio between biomass C and ATP in cultures of Ps. fluorescens grown in liquid medium in absence and presence of bentonite.
(O) 0 %, (Δ) 0.5 % and
(▲) 1.0 % bentonite

D'Eustachio et al. (1968) found a relatively constant level of 0.5 x 10^{-9} µg ATP/cell throughout all phases of growth for thirteen species of bacteria. However, Lee et al. (1971b) using Aerobacter aerogenes in a sediment-free system observed a high ATP content of 4.0 x 10^{-9} µg/cell during the late lag-early log phase after 5 h in a nutrient broth medium, followed by a rapid decline to less than 1.0 x 10^{-9} well before the late log phase was reached, and a further decline to 0.3 to 0.5 x 10^{-9} µg/cell in the early stationary phase. Although in the early growth stages, the ATP content of bacteria cells can reach levels higher than at the later and less active growth stages, this initial high level was very short, and the ATP content dropped rapidly and equilibrated within a relatively narrow range.

The ratio between biomass C and ATP varied in the presence of bentonite between 153 and 585 (Fig. 8); without bentonite, biomass C/ATP ratios of the same magnitude were found with some exceptional low values after incubation for 41 and 49 days. The ratios in the cultures added bentonite were in the same order of magnitude as those found in soil (124-240), when biomass C was determined by the $CHCl_3$ fumigation method and ATP with the luciferin-luciferase method (ref. X).

The O_2 uptake and the ATP content of a fungus Cladosporium sp. were measured during the incubation for 2 weeks (Figs 9 and 10). Bentonite in the cultures stimulated the respiration and the ATP content during the first 2-3 days, but thereafter had no influence. However, the culture added 0.5 % bentonite contained more ATP, than the control and the culture with 1.0 % bentonite. The highest values were reached after 2 to 3 days of incubation and they then decreased to a constant level after 10 days. Although minor differences were observed between the cultures in absence and presence of bentonite, the results show a fairly good correlation between the respiration rate and the ATP content (for all results, $r = 0.91$; $p < 0.001$).

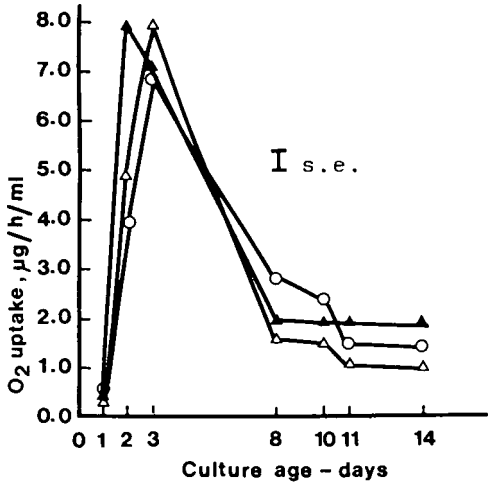


Fig. 9. Oxygen uptake in cultures of Cladosporium sp. grown in liquid medium in absence and presence of bentonite. (O) 0 %, (△) 0.5 % and (▲) 1.0 % bentonite. s.e. = standard error.

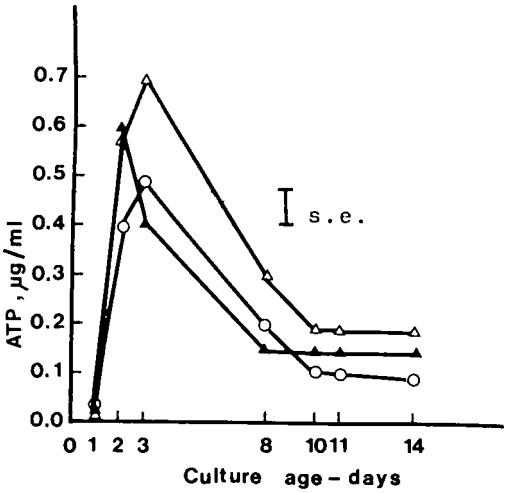


Fig. 10. ATP contents in cultures of Cladosporium sp. grown in liquid medium in absence and presence of bentonite. (O) 0 %, (△) 0.5 % and (▲) 1.0 bentonite. s.e. = standard error.

ATP content and number of cells in bacterial cultures air-dried in presence and absence of bentonite

Bacterial cells (Ps. fluorescens) air-dried in the presence of bentonite for 7 days had an ATP content/cell very similar to that of a non-dried culture (Table 12). Bentonite had a preserving effect on the viability of the cells in the dried state. The high ATP content and low number of bacteria found in the culture dried for 1 and 2 weeks could be caused by existence of "free" ATP molecules or existence of cells not able to multiply but still containing ATP. This is possibly the explanation of the unrealistically high ATP content/cell (Table 12).

Recovery of ATP (disodium salt solutions) added to samples of sand with and without bentonite and air-dried were examined (Fig. 11). Immediately after addition of ATP to samples of sand, ATP was fully recovered, while less ATP was recovered in the samples mixed with bentonite (Fig. 11). It is possibly because parts of the ATP was hydrolized to ADP in presence of bentonite or that the extraction efficiency was reduced. Graf & Lagaly (1980)

Table 12. The influence of drying cultures of Ps. fluorescens with and without presence of bentonite on the ATP content/cell

Bentonite %	Treatments of the cultures	Number of cells Bacteria/ ml medium	µg ATP/ ml medium	µg ATP/cell
0.00	The batch culture	14×10^8	0.24	0.2×10^{-9}
0.50	was incubated for	8×10^8	0.30	0.4×10^{-9}
1.00	6 days	11×10^8	0.32	0.3×10^{-9}
0.00	After incubation for	3×10^7	0.13	4.0×10^{-9}
0.50	6 days, the cultures were	4×10^8	0.12	0.3×10^{-9}
1.00	dried for 7 days at 25°C 1)	3×10^8	0.10	0.3×10^{-9}
0.00	After incubation for	4×10^3	0.11	27.5×10^{-6}
0.50	6 days, the cultures were	6×10^6	0.13	21.7×10^{-9}
1.00	dried for 14 days at 25°C 1)	5×10^6	0.08	16.0×10^{-9}

ATP is extracted with sulphuric acid-phosphate and NRB® (ref. X).

1) The cultures were centrifuged and the supernatant removed before drying.

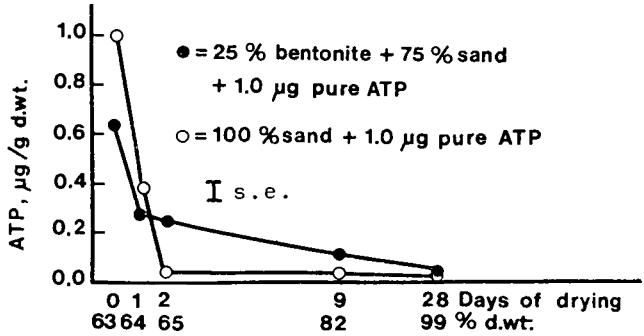


Fig.11. Recovery of ATP from samples of sand in the presence and absence of bentonite after drying of the mixtures. s.e. = standard error.

observed this for a range of clay suspensions. After two days at 25°C, nearly all ATP had disappeared from the sand, whereas ATP in the sand-clay mixture decreased much slower reaching zero after 28 days. These results indicate that extracellular ATP can be adsorbed onto bentonite when it is dried and that ATP was hydrolized very slowly in the adsorbed state.

Although ATP is a component of living cells and presumed to be absent from dead cells (Hamilton & Holm-Hansen, 1967), the results from the experiments suggest that extracellular ATP can accumulate in dry clay soils protected by adsorption to the clay minerals.

E. ATP CONTENT OF SOILS AS INFLUENCED BY ENVIRONMENTAL FACTORS

Introduction

When soil samples were analysed for ATP immediately after removal from the field, confusing results were often obtained which might be related to soil temperature or moisture content. Therefore, measurements were performed under laboratory conditions, to examine some possible factors which may influence the results of ATP determinations in soil, e.g. soil temperature, soil moisture content, clay content (see Chapter D), aerobic-anaerobic conditions and freezing of soil samples.

Materials and methods (see APPENDIX IV)

Effect of soil temperature on ATP

Determinations of the ATP content over a 24 h period showed that the content could vary much within a few hours (Fig. 12). ATP content as determined by ref. I, air temperature measured 2 cm above the surface, soil temperature and soil moisture were determined in April, 1979, every 2 h during a 24 h period in three depths (0-3 cm, 3-10 cm and 10-20 cm) of a fallow garden soil. The highest ATP contents were found in the top layer and the lowest ones in the 10-20 cm depth, with ATP contents of the 3-10 cm depth in between. ATP contents in the soil increased about four-fold from 7 am to 17 pm, and then decreased again to the initial values at 21 pm. Air and soil temperature (0-3 cm depth) reached maximum values 4 h before the ATP content in this depth, whereas the maximum soil temperatures were reached in the deeper layers at the same time as the max ATP contents. Soil moisture varied between 7.5 % and 10.5 % of d.wt. soil and pH ($CaCl_2$) of the soils averaged 6.5.

If the soil biomass mainly consists of dormant cells, it is not possible to get a four-fold increase in the size of the biomass during 10 h, as indicated by the ATP concentrations. A reasonable explanation could be that the soil temperature affect the ATP content in the microorganisms, as also indicated in experiments where contents of adenine nucleotides and AEC values were determined in soil (see Chapter C).

The influence of a soil preincubation for 5 days at 25°C of a sandy loam soil and a coarse sandy soil under four different management treatments is shown in Table 13. The ATP content in

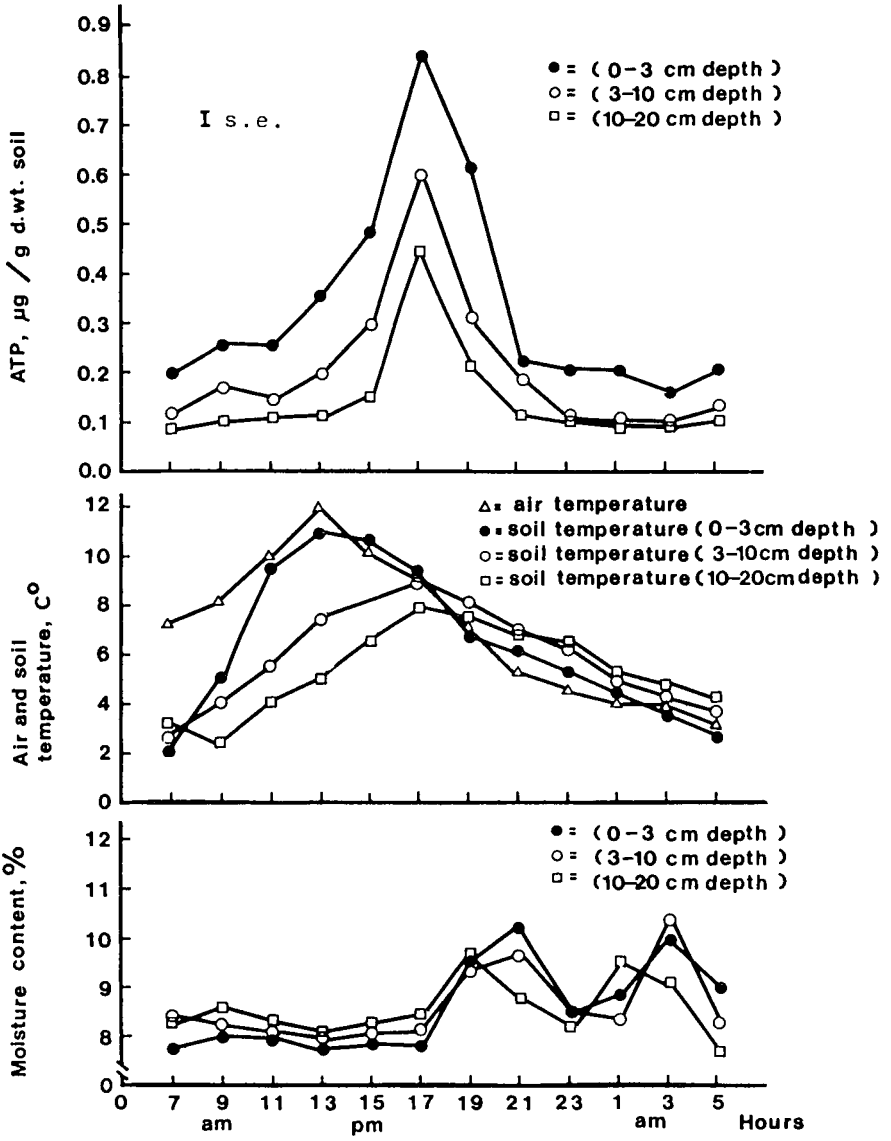


Fig. 12. ATP content, air and soil temperature and moisture content in a garden soil measured over a 24 h period.

s.e. = standard error (see page 188).

the soils were measured at the ambient field temperature of 5°C and then again after a preincubation of the soils for 5 days at 25°C. The moisture contents at the sampling time were not adjusted in these experiments. The preincubation resulted in an increased ATP content in most of the soil samples and the ratios between the ATP contents before and after changing of the soil temperature varied for the different soil samples (0.6-6.2). The results from the sandy loam soil could suggest that the ATP content may be used as an activity measure, when determined immediately after sampling at the ambient field temperature and the ATP content may be applied as a measure of the size of the biomass after preincubation of the soil for 5 days at 25°C with a moisture content equivalent to 60 % of w.h.c..

To verify the observations mentioned above, sandy loam soil and sandy soil were stored with 60 % of w.h.c. for 60 days at 5°C and then incubated for 5 days at 5°C and 25°C, respectively (Fig. 13). ATP content and biomass ($CHCl_3$ fumigation) were measured daily during 5 days in the soils incubated at 5°C and 25°C. The ATP content in the sandy loam soil increased 1.5 times from 0.67 (0 day, soil temperature 5°C) to 1.00 µg ATP/g d.wt. soil after 3 days of incubation at 25°C and then remained fairly constant to the fifth day. The ATP content in the soil incubated at 5°C, only increased from 0.67 (0 day) to 0.75 µg ATP/g d.wt. soil after 3 days, and then remained at that level to the fifth day. In the sandy soil ATP content increased from 0.45 (0 day, soil temperature 5°C) to about 0.54 µg ATP/g d.wt. soil after 1 day of incubation at 25°C, and then both soil samples at 5°C and 25°C slightly decreased up to the fifth day. The biomass in the sandy loam and the sandy soils contained around 186 µg and 90 µg biomass C/g d.wt. soil, respectively. The size of the biomasses as determined by $CHCl_3$ fumigation were independent of the soil temperature and also of the time of analysing during the 5 days.

Table 13. The influence of preincubation of soil samples on the measurement of ATP contents

Treatments		Moisture content (At sampling time) %	ATP content: Soil samples measured at the ambient field temperature of 5°C µg ATP/g d.wt. soil	ATP content: Soil samples stored at 25°C for 5 days µg ATP/g d.wt. soil	ATP content: Soil storage at 25°C for 5 days / Soil measured directly ratio
Sandy loam soil Roskilde					
Ploughing		19.4	0.28	0.89	3.2
Ploughing including a catch crop		19.5	0.14	0.85	6.2
Rotavation		18.5	0.47	1.32	2.8
Rotavation including a catch crop		19.4	0.64	1.28	2.0
Coarse sandy soil Jyndevad					
Ploughing	(1)	8.1	0.19	0.12	0.6
	(2)	9.6	0.34	0.40	1.2
Ploughing including a catch crop	(1)	9.0	0.22	0.29	1.3
	(2)	9.3	0.32	0.40	1.3
Rotavation	(1)	10.5	0.23	0.46	2.0
	(2)	10.1	0.54	0.72	1.3
Rotavation including a catch crop	(1)	8.1	0.33	0.73	2.2
	(2)	10.3	0.67	0.56	0.8
Coefficient of variation within soil sampling (%)			9.6	5.8	

Means of 4 replicated samples.

The soils were sampled in April, 1982, from the 0-20 cm depth.

(1) = Non irrigated soil; (2) = Irrigated soil.

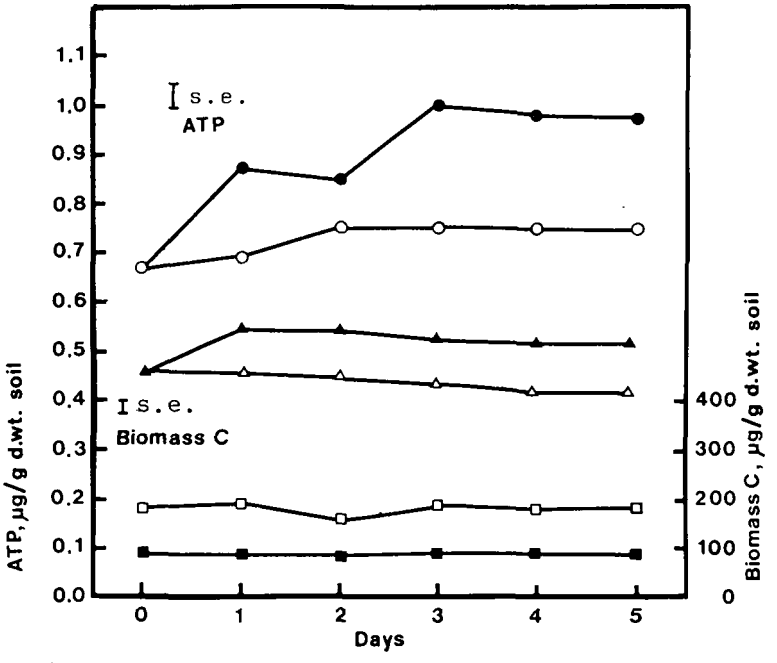


Fig. 13. The effect of soil temperature on ATP content and biomass C ($CHCl_3$ fumigation) in a Roskilde sandy loam and a Jyndevad coarse sand stored with 60 % of w.h.c. for 60 days at 5°C, and then incubated for 5 days at 5°C and 25°C, respectively. ATP content and biomass C were measured every day during the 5 days period. (The soil samples used for biomass C were fumigated with $CHCl_3$ every day according to the procedure).

Roskilde soil: ○ ATP (5°C); ● ATP (25°C) and
□ Biomass C (5°C and 25°C)
Jyndevad soil: △ ATP (5°C); ▲ ATP (25°C) and
■ Biomass C (5°C and 25°C)

s.e. = standard error.

The results indicated that the soil temperature influence the ATP content and that a relatively constant ratio between ATP content and biomass C exists in a soil, when the soil samples have been stored at 25°C for 3-5 days before ATP is determined.

The effects of soil temperature on the ATP contents in Roskilde sandy loam incubated for 68 days at 5°C, 10°C and 15°C with different moisture contents (10 %, 16 %, 22 % and 28 %) and also after a further incubation of the soil portions for 5 days at 25°C were examined (Fig. 14). The ATP contents were increased after preincubation of the samples for 5 days at 25°C, when the soils had been stored at 5°C, 10°C and 15°C, respectively. The soil moisture only influenced the ATP content to a minor extent. These results indicate that the ATP content of the biomass in soil differ depending on growth temperature, and that the soil temperature appears important for calculation of conversion factors between ATP and biomass. Therefore, the ATP content measured at different temperatures without an adjustment of the temperature, cannot be used as an expression of the size of the soil biomass. These results agree with the AEC experiments, where an increased temperature from 5°C to 25°C stimulated the ATP content whereas the AEC value only increased to a minor extent. This indicated that only a small part of the biomass was in active growth, and the changes in ATP concentration was caused by changes in contents of ADP or AMP.

The CO_2 production from arable soils incubated at different temperatures (Fig. 15) was used for each soil type for adjustment of the CO_2 production from the soil incubation temperature to the ambient soil temperature, in order to compare the microbial activity determined by CO_2 production with the ATP content measured at the ambient field soil temperature. The Q_{10} varied between 1.5 and 2.1 (5-25°C) dependent of the temperature interval and the soil types. The CO_2 production in a sandy loam soil (Fig. 15) had a Q_{10} (5-15°C) of

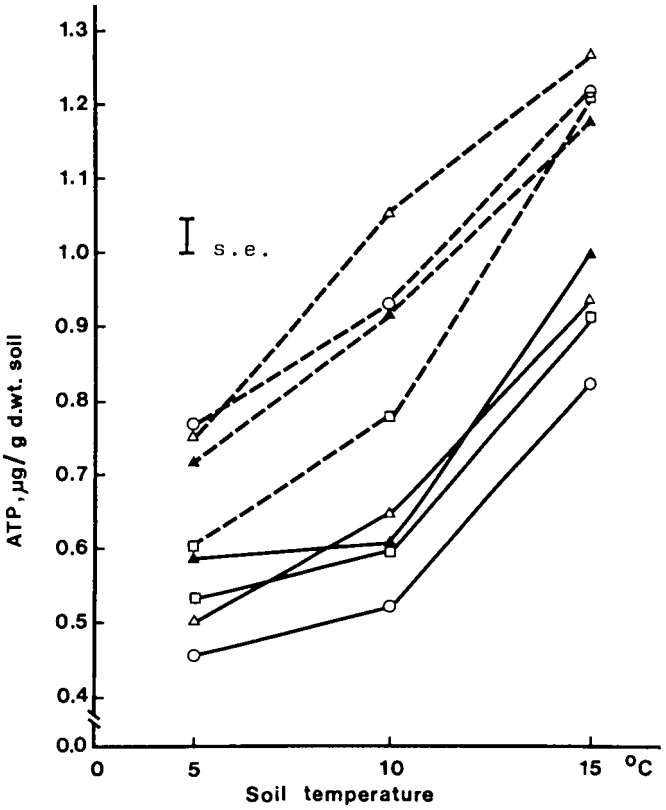


Fig. 14. The effects of soil temperature and soil moisture on the ATP contents in a Roskilde sandy loam soil incubated for 68 days at 5°C, 10°C and 15°C with different moisture contents and also after a further incubation of the soil portions for 5 days at 25°C.

——— Without a soil incubation for 5 days at 25°C

– – – – With a soil incubation for 5 days at 25°C.

(O) 10 %, (Δ) 16 %, (▲) 22 % and (□) 28 % moisture contents.

s.e. = standard error.

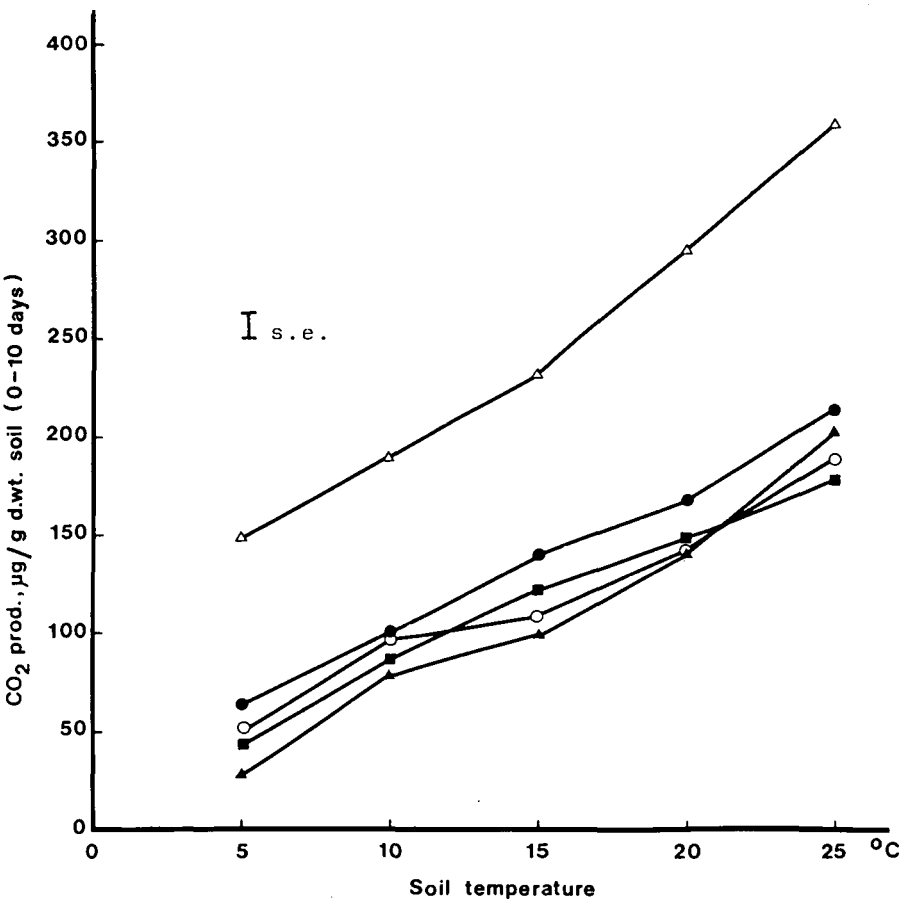


Fig. 15. The effect of the soil temperature on the CO_2 production from different soils.
(■) Coarse sand (Jyndevad), (▲) Fine sand (Tylstrup)
(O) Sandy loam (Roskilde), (●) Loam (Rønhave)
(△) Silty loam (Højer).
s.e. = standard error.

about 2.1 and the corresponding ATP content (Fig. 14) a Q_{10} of about 1.6. The results indicated a fairly good relationship between ATP content and CO_2 production as influenced by different soil temperatures, although the ATP content will be dependent both on the activity and the biomass of the soil microorganisms. This is consistent with results from the AEC work, where an increased soil temperature increased ATP content in the cells and this was not always correlated to an increased biomass.

The temperature effect observed on the ATP content in soil was further examined in a pure culture of the bacterium Pseudomonas fluorescens grown in a Nutrient Broth medium and at different temperatures (Table 14). The same growth stages from cultures grown at different temperatures were used at the measuring times (determined by turbidity measurements). The ATP content/cell was fairly constant in the different growth phases at the same growth temperatures. When cells begin to die, there was a tendency towards a lower ATP content/cell at 15°C and 25°C, as compared to the same temperature in earlier growth phases. This could either be due to counts of non-viable cells or that the ATP content decreased in old cells. The ATP content/cell in exponential and stationary growth phases was highest at 25°C and lowest at 5°C, with ATP content/cell from growth at 10°C, 15°C and 20°C in between. These results support the examinations in soil, where the temperature showed a marked effect on the ATP content in cells. Tate & Jenkinson (1982b) examined a park grass soil from England and a Placaquod soil under native grasses and shrubs from New Zealand and found similarly that temperature had a marked effect on the ATP content of the biomass in soil. They found that incubation at 10°C resulted in a 16 % increase in biomass C and a 19 % increase in ATP, when compared to the freshly sampled soil. However at 25° there was a 73 % increase in ATP and only 23 % increase for the biomass.

Table 14. The influence of different incubation temperatures on the ATP content/cell of the bacterium Pseudomonas fluorescens

Growth temperature °C	Exponential growth phase				Stationary growth phase				Dead phase			
	Incubation time h	AO-stained bacteria number x 10^8/ml	ATP content µg ATP/ml	µg ATP x 10^{-9}/cell	Incubation time h	AO-stained bacteria number x 10^8/ml	ATP content µg ATP/ml	µg ATP x 10^{-9}/cell	Incubation time h	AO-stained bacteria number x 10^8/ml	ATP content µg ATP/ml	µg ATP x 10^{-9}/ cell
5	16-37	4.6	0.47	1.0	67-94	8.9	0.8	0.9	160-165	5.4	0.4	0.8
10	2-5	3.3	0.69	2.1	50-53	3.6	0.5	1.3	75-80	2.5	0.5	2.0
15	2-5	7.2	0.97	1.3	19-22	8.9	1.2	1.3	42-45	10.0	0.4	0.4
20	2-5	7.8	1.43	1.8	19-22	8.0	1.3	1.6	42-45	N.D.	N.D.	N.D.
25	2-5	6.4	1.66	2.6	19-22	4.7	1.4	3.0	42-45	1.4	0.2	1.4

N.D. = No determination.

AO-stained bacteria = Acridine orange stained bacteria.

The cultures were incubated at different temperatures and were measured at the same optical density in each growth phase. Results are averages of three samplings taken during the mentioned incubation time. Triplicated analyses were performed from each sample.

Optical density was used to follow the growth.

Effect of the soil moisture content on ATP

The ATP content and the CO_2 production increased with increasing moisture both in a sandy loam and a sandy soil (Fig. 16). In the sandy loam soil, the two parameters reasonably well were correlated between 8 % and 26 % moisture. The soil with 1 % moisture contained 0.55 µg ATP/g d.wt. soil and the CO_2 production was close to zero. The high ATP content and low CO_2 production may be due to a large biomass with a low microbial activity or because "free" ATP is present and adsorbed to the clay. In the coarse sandy soil, ATP content and CO_2 production both increased when the moisture content was increased from 0 % to 16 %. ATP remained at 26 % moisture at the same level as found at 16 % moisture, whereas the CO_2 production increased from 16 % to 26 % moisture. The ATP content and the CO_2 production at different moisture contents were not well correlated in the coarse sandy soil.

The results indicate that ATP content cannot be used as an activity measure in dry soils containing clay and in sandy soils with different moisture contents.

In a sandy loam (Roskilde) preincubated at different moisture contents and temperatures for 68 days, the ATP content as determined by the NRB extraction method described in ref. III , was negatively correlated to respiration rate and dehydrogenase activity (Bååth, E., Eiland, F., Lundgren, B. & Söderström, B., unpublished). A very high ATP content was found in the driest treatment (10 % moisture). Knight & Skujins (1981) reported similar results in two arid soils (silt loam and sandy loam), where the ATP content decreased with increasing moisture content. This was evident only in the water potential which corresponded to about 10 % moisture. The used extraction method (ref. III),

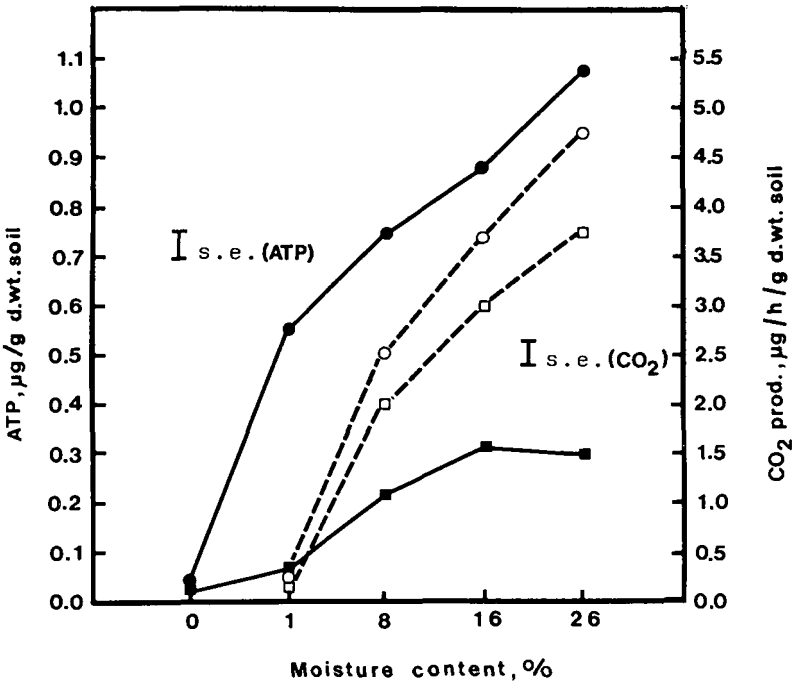


Fig. 16. ATP content and CO_2 production in sandy loam (Roskilde) and coarse sandy soil (Jyndevad) measured under different moisture regimes after the soils were incubated with different moisture contents for 5 days at 25°C.

ATP content and CO_2 production in sandy loam
ATP content and CO_2 production in coarse sand

s.e. = standard error.

however, gave very low values of ATP in soil, probably because of a low extraction efficiency. The low extraction efficiency for the latter method might be due to the clay content of the arable soils, since the extraction efficiency with this method decreased with increasing clay content (ref. X). The more effective ATP extraction method (ref. X), which was used on the sandy loam, gave an increased ATP content with increasing moisture content (Fig. 16). Vogt et al. (1980) reported negative correlations between ATP contents and soil respiration in summer and autumn in some forests in Washington, U.S.A. They did not explain these results, but it is likely that adsorption phenomena, use of a less effective extraction method (chloroform and Tris buffer) or that the soil respiration was not recalculated to the soil temperature at the sampling time, could explain these results.

Effect of aerobic-anaerobic conditions in soil on ATP

ATP content in arable soils was only slightly influenced by anaerobic conditions within a short incubation period; a sandy loam and a coarse sandy soil were incubated under aerobic and anaerobic conditions for 2 days at 25°C (Table 15). On average of the two soils the CO_2 production was decreased 14 % and the ATP content 5 % after anaerobic incubation as compared to the corresponding aerobic incubation.

Reports in the literature state generally that the ATP content is particularly dependent on good aeration in strictly aerobic organisms such as Bacillus subtilis, where Klofat et al. (1969) observed a 15-fold decrease of ATP on incubation in a N_2-atmosphere. In Aerobacter aerogenes a 2-fold decrease (Strange et al., 1963) and in Escherichia coli a 1.5-fold decrease (Cole et al., 1967) in the ATP content has been observed during the change from aerobic to anaerobic conditions. The small decrease in ATP content found in the soil experiments is probably due to the great number of inactive microorganisms existing in a soil and with only a small number of microorganisms actively growing.

Table 15. Effects of anaerobic and aerobic conditions on ATP content and CO_2 production in soil

Locality (Soil type)	ATP content*		CO_2 production*	
	Aerobic incubation µg ATP/ g d.wt. soil	Anaerobic incubation µg ATP/ g d.wt. soil	Aerobic incubation µg CO_2/h/ g d.wt. soil	Anaerobic incubation µg CO_2/h/ g d.wt. soil
Jyndevad (Coarse sand)	1.36±0.12	1.30±0.11	1.0±0.06	0.9±0.03
Roskilde (Sandy loam)	1.27±0.01	1.20±0.03	1.1±0.10	0.9±0.06

Means of triplicate samples ± standard deviation.
*ATP content and CO_2 production measured after 2 days of incubation at 25°C.

Effect of freezing of soil samples on ATP

Soils with a varying content of clay and sand were kept at -20°C for 1,7 and 14 days after a preincubation of "fresh" soil for 5 days at 25°C with 60 % of w.h.c. (Fig. 17). ATP was extracted without allowing the soils to thaw. Freezing had greater effect in the clay and humus soils than in the sandy and sandy loam soils. The sandy and the sandy loam soils (Jyndevad, Tylstrup and Roskide) did not change significantly during the 14 days period, whereas the ATP content in Rønhave loam decreased after 1 day of storage at -20°C, remained at that level up to 7 days, and had decreased again after 14 days. The Højer silt loam soil remained relatively constant during the first 7 days, unaffected by the freezing, and then decreased in a similar manner to the Rønhave soil from 7 to 14 days of incubation. The greatest fluctuations in ATP content were found in the humus soil, where the ATP content decreased strongly from 1 to 14 days of incubation.

It appears possible to store the examined sandy soils and the sandy loam soil for two weeks without significant changes in ATP. On the contrary, the ATP content in humus soil and one loam soil changed very much, and it is not possible to store these soil samples at -20°C without appreciable changes in ATP content. It is known that cold shock to microbial cells in addition to the injury or death of organisms, is accompanied by the release of low molecular weight intracellular solutes, e.g. the nucleotides (Strange & Dark, 1962). It is possible that parts of the liberated ATP from the cells in soils stored at -20°C are denaturated by ATP-ases.

Sparrow & Doxtader (1973) stored soil extracts at -20°C after the soil suspensions were extracted with butanol-octanol. After 2 days of storage the ATP content increased slightly and then

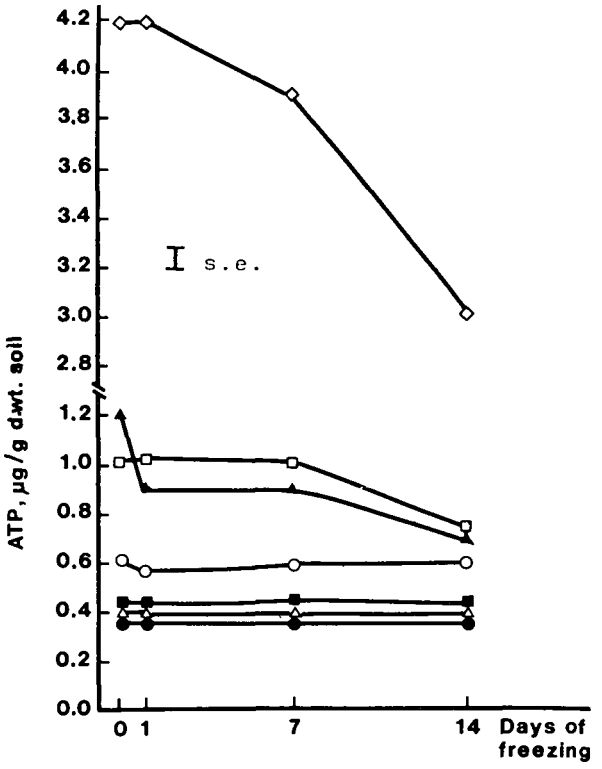


Fig. 17. Effect of freezing soil samples at -20°C after a preincubation of the "fresh" soils for 5 days at 25°C (time zero is the "fresh" soil).

▲ Rønhave loam soil.
○ Roskilde sandy loam soil.
● Jyndevad irrigated coarse sandy soil.
△ Jyndevad non irrigated coarse sandy soil.
□ Højer silty loam soil.
■ Tylstrup fine sandy soil.
◇ Forest humus soil.
s.e. = standard error.

decreased again up to 116 days. Ahmed et al. (1982) found that storage of freeze-dried soils at -15°C for 100 days led to substantial losses of ATP. Ross et al. (1980a) found in four soils from tussock grasslands that storage at -20°C was most suitable for retaining ATP content and biomass up to 28 days of storage.

The results indicate that different soil types react in a different manner on the soil freezing but it should be possible to freeze some types of soils without appreciable changes in ATP. It is advisable as a general rule to determine the ATP content in "fresh" soil without previous freezing.

F. ATP CONTENT AS A MEASURE OF MICROBIAL ACTIVITY AND SIZE OF BIOMASS IN SOIL

Introduction

The use of ATP as a measure of soil microbial biomass depends, in part, on the assumption that ATP is present as a relatively constant component of the biomass of diverse microorganisms and that it is neither associated with dead cells nor adsorbed onto soil components. Although measurements of the ATP content differs theoretically from the activity measurements, it has also been used to estimate the activity of the microorganisms in soil (Ausmus, 1973; Hersman & Temple, 1979). ATP is a biochemical compound which can be determined momentarily, whereas the respiration rate is a process which must be measured over a period of time.

To evaluate the relationships between ATP content and microbial biomass and activity in soil, correlations between ATP content and results of other methods, reflecting microbial biomass and activity in a range of arable soils were examined. Furthermore, conditions for use of the ATP method as an activity or biomass measure are discussed, and different methods available are mentioned. Results from field experiments are included, to illustrate the possibility of using the ATP content as a measure of microbial abundance in soil.

Materials and methods (see APPENDIX V)

Correlations between ATP content and microbial activity and biomass in field soils

The influence of addition of manure

Microbial activity and biomass as well as physical-chemical parameters were measured in arable soils, where the effects of addition to the soil of farmyard manure, slurry and inorganic fertilizers were examined (refs IV, V & VI).

In these studies, ATP content was measured at the ambient soil temperature 24 h after sampling, while O_2 uptake and CO_2 production were measured after soil incubation at 25°C for 1 and 3 days, respectively. Dehydrogenase activity was determined after incubation at 30°C for 45 min, as described in the procedure.

Correlations between ATP content and results of the other methods before and after adjustment for time of soil sampling and different experiments are seen in Table 16. Use of all data from all manure experiments without any adjustments resulted in significantly positive correlations between ATP content and biomass determined by $CHCl_3$ fumigation, and between ATP and NH_4-N, while significantly negative correlations were observed between ATP and bacteria determined by plate counts, O_2 uptake, CO_2 production, dehydrogenase activity, NO_3-N and soil pH, respectively.

Table 16. Correlation coefficients between ATP content and other characteristics in soil from four field experiments with addition of manures

Methods	Correlation coefficients	
	All individual data from four experiments without any adjustments	With data after adjusting for effects of time and different experiments (Over manures)
ATP-Biomass ($CHCl_3$ fumigation)	0.19**	0.31**
ATP-Bacteria (platecounts)	-0.16***	-0.25** 1)
ATP-O_2 uptake	-0.42****	0.19**
ATP-CO_2 production	-0.26****	0.41**
ATP-Dehydrogenase activity	-0.52****	0.23**
ATP-Total C	0.09	0.19**
ATP-Total N	0.06	0.26**
ATP-NO_3-N	-0.36***	0.23**
ATP-NH_4-N	0.17***	0.16*
ATP-H_2O	0.07	0.43**
ATP-pH	-0.13*	0.01

*, **, ***, **** = $P < 0.1$, 0.05, 0.01 and 0.001, respectively.

ATP content was determined by the H_2SO_4-cation exchange method (ref. 1).

1) The negative correlation between the adjusted values of ATP and bacteria is caused by just 4 single values out of 144 values. If these 4 values are excluded, the correlation coefficient becomes 0.026.

After the necessary statistical corrections (the effects of time of soil samplings and different experiments were eliminated by additive adjustment; see page 184, APPENDIX V). The addition of manures then showed significantly positive correlations between ATP and biomass determined by fumigation, O_2 uptake, CO_2 production, dehydrogenase activity, total organic C, total-N, NO_3-N, NH_4-N and moisture content, respectively, but ATP content and viable bacteria were still negatively correlated. The results indicate that great variations in the relationship between the ATP content and the size of the microbial biomass and activity may occur, dependent on the time of sampling and different experiments (Table 16). Furthermore, the results indicate that there is a relationship between the ATP content, microbial activity and biomass, in soil containing a high amount of easily decomposible organic material (Table 16). However, due to the adjustments (different sampling times and experiments), the results have to be interpretated with caution.

The seasonal variations in the relationships between ATP content and other methods may in part be caused by differences in the soil temperature, because the ATP content was measured with the ambient soil temperature and O_2 uptake, CO_2 production and dehydrogenase activity were measured after a pre-set incubation at different temperatures, as described in the procedures for the latter methods. However, the correlations also indicate that additional factors may well have affected the results (e.g. effects of soil moisture and clay content).

The influence of reduced tillage and use of a catch crop

The correlation between ATP content and other microbial parameters were also examined in two soil types subjected to different cultivations (Table 17). The effect of ploughing and rotavation and use of a catch crop (Sinapis alba L.) on the microbial biomass and activity were examined in fields regularly sown with barley (ref. IX). The ATP content was in a sandy loam soil (0-5 cm depth) significantly positively correlated to CO_2 production, dehydrogenase activity, cellulase activity, phosphatase activity, number of bacteria determined by plate counts, biomass determined by $CHCl_3$ fumigation and FDA-active fungi (Table 17). However, in the 5-20 cm depth, positive correlations were found only between ATP content and phosphatase activity, number of bacteria and the biomass determined by $CHCl_3$ fumigation. The ATP content was neither correlated to physical-chemical parameters in Roskilde sandy loam nor to the microbial and physical-chemical parameters in the Jyndevad sandy loam soil (depths, 0-5 cm and 5-20 cm); this may be ascribed to the small variations observed between soil treatments and over time (ref. IX).

Table 17. Correlation coefficients between ATP content and other characteristics in soil samples from a field experiment with four different soil treatments performed at Roskilde sandy loam over the period 1979-1980

Location (depth)	ATP-Biomass ($CHCl_3$ fumigation)	ATP-CO_2 prod.[1)]	ATP-Bacteria (platecount)	ATP-Dehydrogenase activity	ATP-Cellulase activity	ATP-Phosphatase activity	ATP-FDA active fungi
Roskilde (0-5 cm)	0.54***	0.70****	0.67****	0.68****	0.72****	0.67****	0.45****
Roskilde (5-20 cm)	0.39**	0.12	0.22**	0.16	-0.09	0.48****	0.08

, *, **** = $P < 0.05$, 0.01 and 0.001, respectively.

1) The CO_2 production was recalculated to the ambient soil temperature (see Fig. 15).

ATP content was determined with the H_2SO_4-cation exchange method (ref. I).

Physical-chemical characteristics of the soils are shown in Table 18. Biomass determined by $CHCl_3$ fumigation, ATP content and CO_2 production (Table 17) were determined in the 0-5 cm depth over the period 1981-1982 in the above mentioned experiments located on 5 different soil types. The soil samples were stored at the ambient soil temperature for 24 h after sampling. Then the soil samples were adjusted to 60 % of the w.h.c. and microbial and physical-chemical parameters determined. ATP content was also measured after a preincubation for 5 days at 25°C. When the results from the different times of samplings were used (Table 19), ATP content measured 24 h after sampling was significantly positively correlated to the CO_2 production ($r = 0.70$; $p < 0.01$) (Fig. 18), whereas these measurements were not significantly correlated, when ATP was measured after a preincubation period of the soils ($r = 0.39$)(Fig. 18). The ATP contents determined without a preincubation period of the soils ("directly") and also after a preincubation of the soil were significantly positively correlated to the biomass determined by the $CHCl_3$ fumigation method (Figs 19 and 20). However a preincubation of the soils before measuring ATP resulted in a closer relationship between ATP content and biomass ($r = 0.97$; $p < 0.001$)(Fig. 20), than between ATP content measured without a preincubation period "directly" and the biomass ($r = 0.81$; $p < 0.001$)(Fig. 19).

Table 18. Some physical-chemical characteristics of the soils used

Soil (Location)	Date of sampling	Mean weekly air temperature at the sampling time °C	Moisture content at the sampling time %	Organic C %	Total N %	C/N ratio	NO_3-N µg/g d.wt. soil	NH_4-N µg/g d.wt. soil	P_i µg P/g d.wt. soil	K_i µg K/g d.wt. soil
Coarse sandy soil	19/5-1981	14.7	10.7	1.9	0.14	13.7	58	53	265	102
Jyndevad	21/9-1981	13.2	12.5	1.7	0.10	17.9	13	6	215	124
Averages of irrigated and non irrigated soils	24/5-1982	14.1	11.2	N.D.	N.D.	N.D.	45	18	268	N.D.
Means		14.0	11.5	1.8	0.12	15.8			249	113
Fine sandy soil	11/5-1981	15.6	7.2	1.5	0.12	12.8	141	85	361	282
Tylstrup	19/9-1981	14.3	19.7	1.6	0.12	13.0	8	6	332	275
	10/5-1982	10.7	19.3	N.D.	N.D.	N.D.	113	53	381	N.D.
Means		13.5	15.4	1.6	0.12	12.9			358	279
Sandy loam soil	4/5-1981	10.7	18.0	1.7	0.15	11.4	137	74	269	161
Roskilde	7/9-1981	14.7	13.0	1.7	0.16	10.8	48	6	251	186
	17/5-1982	11.6	10.0	N.D.	N.D.	N.D.	43	10	299	N.D.
Means		12.3	13.7	1.7	0.16	11.1			273	174
Loam soil	19/5-1981	14.5	14.2	1.4	0.14	10.2	85	90	300	274
Rønhave	21/9-1981	14.0	24.8	1.6	0.15	10.5	10	10	267	274
	24/5-1982	14.0	19.0	N.D.	N.D.	N.D.	17	19	301	N.D.
Means		14.2	19.3	1.5	0.15	10.4			289	274
Silt loam soil	19/5-1981	14.3	31.2	1.8	0.18	10.7	21	43	233	188
Højer	21/9-1981	13.6	29.8	1.9	0.18	10.1	14	10	214	166
	25/5-1982	13.6	13.9	N.D.	N.D.	N.D.	190	85	252	N.D.
Means		13.8	25.0	1.9	0.18	10.4			233	177

Results are means of duplicate samples.

N.D. = No determination.

Table 19. Biomass C, CO_2 production, ATP content and biomass C/ATP ratios in soils

Soil (Location)	Date of sampling	Biomass C* µg C/g d.wt. soil	CO_2 prod.* ug CO_2/g d.wt. soil (0-10 days)	ATP (1)* measured in soil without a preincubation µg ATP/g d.wt. soil	ATP (2)* measured in soil after a preincubation µg ATP/g d.wt. soil	Biomass C/ATP (2) ratios
Coarse sandy soil	19/5-1981	104	179	0.31	0.44	236
Jyndevad	21/9-1981	119	656	0.43	0.44	270
Averages of irrigated and non irrigated soils	24/5-1982	82	161	0.48	0.48	171
Means		102	332	0.41	0.45	226
Fine sandy soil	11/5-1981	117	199	0.32	0.49	239
Tylstrup	19/9-1981	83	561	0.81	0.81	102
	10/5-1982	120	270	0.45	0.62	194
Means		107	343	0.53	0.64	178
Sandy loam soil	4/5-1981	174	243	0.39	0.93	187
Roskilde	7/9-1981	164	601	0.75	0.75	219
	17/5-1982	182	238	0.49	1.00	182
Means		173	361	0.54	0.89	196
Loam soil	19/5-1981	295	231	0.80	1.84	160
Rønhave	21/9-1981	153	739	1.14	1.14	134
	24/5-1982	142	286	1.11	1.11	128
Means		197	419	1.02	1.36	141
Silt loam soil	19/5-1981	521	617	2.32	3.60	145
Højer	21/9-1981	229	979	1.73	1.41	162
	25/5	165	505	1.05	1.05	157
Means		305	700	1.70	2.02	155

*) Means of 8 analyses from the 0-5 cm depth (four soil cultivations, see ref. IX). All the soils were adjusted to 60 % of the water holding capasity 24 h after sampling and ATP content (1) measured. Furthermore, CO_2 production (unfumigated and fumigated soil) was started. After a soil preincubation for 5 days at 25°C, ATP content (2) was measured again. Biomass was determined by $CHCl_3$ fumigation and ATP content by the H_2SO_4-PO_4-NRB extraction method (ref. X).

The relationship between ATP content and microbial activity and biomass in soil

When ATP content was measured in soil after storage of the soil for 24 h at the ambient soil temperature and moisture content, and adjusted to 60 % of w.h.c. before measurement, correlation coefficients from the field experiments (Tables 16 and 17) indicated that there was an over-all positive correlation between the ATP content and the microbial activity (recalculation of CO_2 production to the ambient soil temperature). Results of the field studies were fairly consistent with those obtained in laboratory studies using pure cultures of a bacterium (Pseudomonas fluorescens) and a fungus (Cladosporium sp.) grown in liquid substrates. ATP concentrations followed the changes in O_2 uptake in both organisms if available C was present. In a pure culture a good correlation between respiration rate and biomass is to be expected especially during the first period of growth. When different concentrations of bentonite were included in the substrates the relationship between ATP content and O_2 uptake still holds for the fungus but not for the bacterium.

The results suggest that the ATP content is a reasonably measure of microbial activity when determined shortly after sampling at the ambient soil temperature and soil moisture content. However, ATP content cannot be used as an activity measure in dry clay soils (Chapter D), as it was found that these soils can have a high ATP content not related to the activity and the size of biomass present. It is probably caused by adsorption of ATP to clay and other soil colloids.

The biomass determined by $CHCl_3$ fumigation in different soil types was hightly correlated to the ATP content determined after the soil moisture had been adjusted to 60 % of the w.h.c. and the soils preincubated for 5 days at 25°C (Fig. 20). An over-all

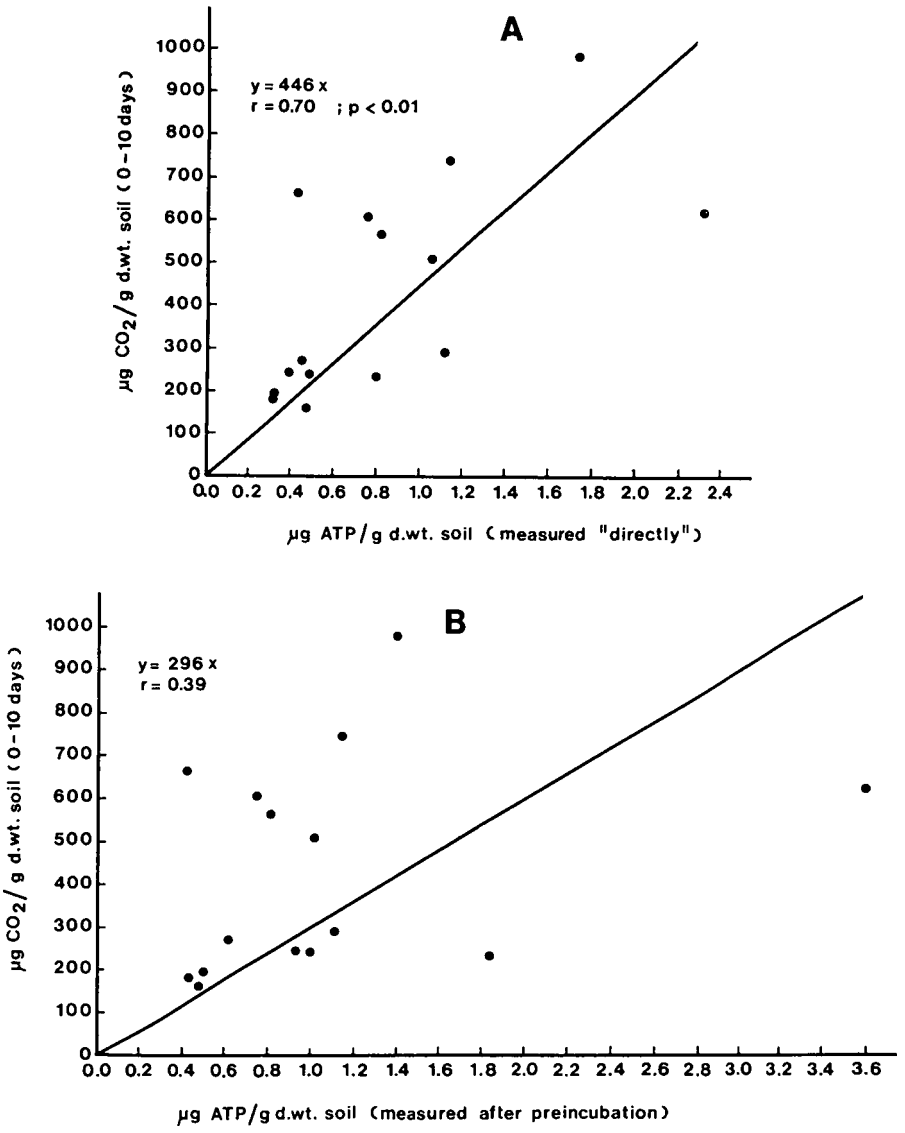


Fig. 18. Scattergram and linear regression through the origin for ATP content and CO_2 production (0-10 days) from 5 soils sampled at different times of the year (Table

19). The soils were adjusted to 60 % of w.h.c. and measuring of the CO_2 production started. ATP content was measured at the beginning ("directly") and again after a preincubation period of the soils for 5 days at 25°C.
The correlation coefficient for A was significantly different from 0 (0.01 level) but this was not the case for B.

average biomass C/ATP ratio of 154 was found, when the results from the different sampling times were used (Table 19).

Although the biomass/ATP ratios are not exactly the same in all soils, a close linear relationship between ATP and biomass was found for the 5 Danish arable soils sampled at different times of the year. The regression equation through the origin (Fig. 20) for the soils was; $y = 154x$, where x is the ATP content of the soil and y is the biomass C content, both in µg/g d.wt. soil (unfumigated soil measured in 0-10 days; k-factor 0.45). However, the biomass C/ATP ratios in the sandy and sandy loam soils were higher than in the loamy and silt loam soils; therefore, the regression equation through the origin and the correlation coefficient were determined for two groups of soils, group I: coarse sand, fine sand and sandy loam soils, group II: loam and silt loam soils. The soils in group I showed a close relationship between the two parameters ($r = 0.69$; $p < 0.05$), $y = 186x$. The soils in group II was even more closely correlated ($r = 0.99$; $p < 0.001$), $y = 148x$.

The higher ATP content per unit biomass C in soil group II could possibly be caused by the presence of "free" ATP molecules associated with the clay. A calculation of the specific activity of the soil biomass using the results for CO_2 production and biomass C (CO_2 production/biomass C; Table 19) indicated that the higher ATP content seemed not to be related to a more active microbial population. The specific activity was lower in soils from group II than in soils from group I.

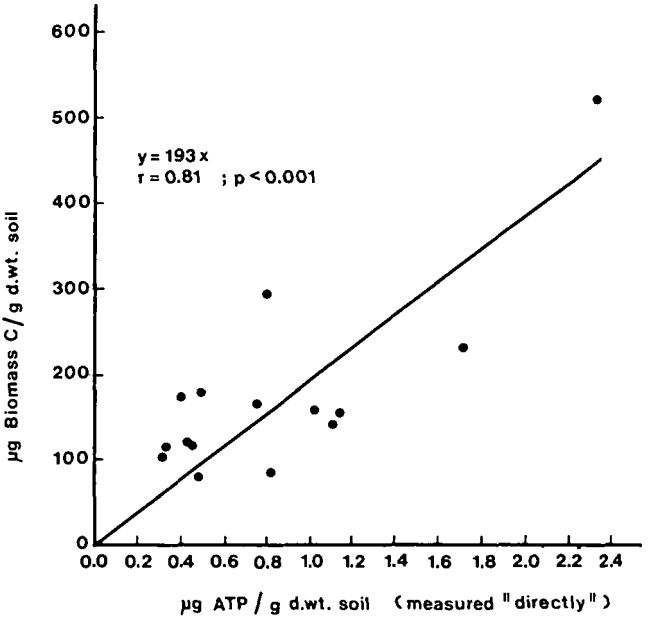


Fig. 19. Scattergram and linear regression through the origin for ATP content and biomass C ($CHCl_3$ fumigation method) determined in 5 soils sampled at different times af the year (see Table 19). The soils were adjusted to 60 % of w.h.c. and ATP and biomass C measured "directly" without a preincubation period. Biomass C was measured in 0-10 days from unfumigated soil, k = 0.45. The correlation coefficient is significantly different from 0 (0.001 %-level).

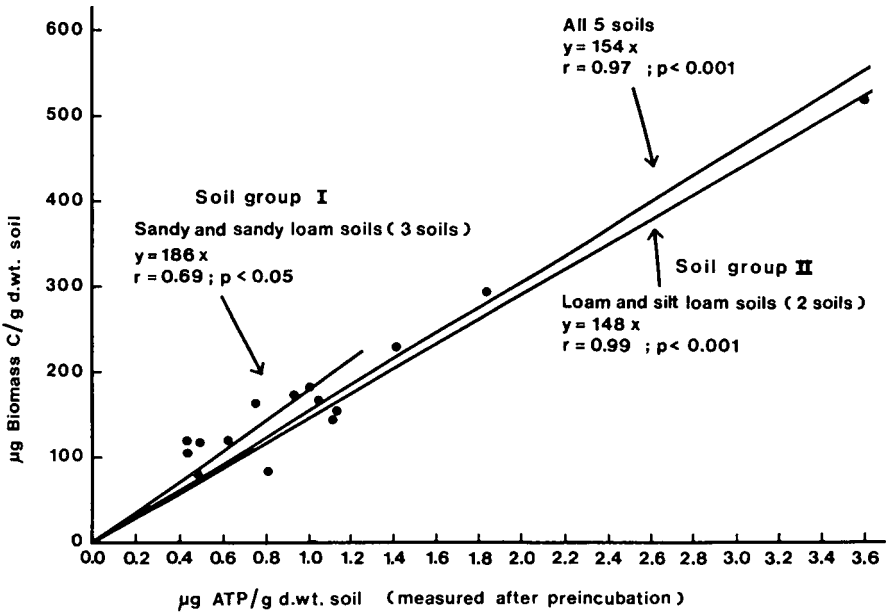


Fig. 20. Scattergram and linear regression through the origin for ATP content and biomass C ($CHCl_3$ fumigation method) resulted from 5 soils sampled at different times of the year (Table 19). The soils were adjusted to 60 % of w.h.c. and biomass C measured(unfumigated soil in 0-10 days; k = 0.45). ATP content was determined after preincubation of the soils for 5 days at 25°C. The correlation coefficient for all the soils was significantly different from 0 (0.001 % level). This was also found for the loam and silt loam soils (0.001 % level), whereas the correlation coefficient in the sandy and sandy loam soils was significantly different from 0 at 0.05 % level.

These parameters have to be interpreted with caution. If the biomass is determined with a CO_2 production from unfumigated soil from the 10th-20th day after sampling, instead of from the 0th-10th day, the biomass becomes higher. In Roskilde sandy loam soil the biomass increased thus with 30 %, when CO_2 production from unfumigated soil 10th-20th day was used instead of the CO_2 production from 0th-10th day. This resulted in higher conversion factors between biomass C and ATP content. The arbitrary k-factor can also be a source of error. However, the very close relationship regularly observed between biomass C and ATP content seems to indicate that the two parameters are related to the same fraction of living organisms in soil.

Biomass C, CO_2 production and ATP content were determined in Belgian soils with different soil textures (Eiland et al., 1983). The correlations between the parameters were similar to these from Danish soils, and suggest that the proposed ATP method is a useful tool for determination of the biomass in soils.

Oades & Jenkinson (1979) found, with two exceptions, that the ATP content of the soil biomass was relatively constant in different soils stored at 20°C for 5-130 days. If all the soils were considered, biomass to ATP ratios in the range of 77-191 were found. Jenkinson et al. (1979) examined 6 English and 11 Australian soils, and found a close linear relationship between ATP and biomass with an average ATP/C ratio of 138 after soil storage under aerobic conditions for 6 days at 25°C in large closed drums containing soda-lime. They had excluded two English woodland-soils and two Australian forest soils, which all contained more ATP per g biomass than in the other soils. On the contrary, Ross et al. (1980b) found less consistent relationships between ATP and biomass with ATP to C ratios of from 163 to 425 in grassland soils stored overnight at 4°C. Sparling (1981a) obtained ratios of between 201 to 858 in Scottish soils stored at 5°C. Eiland (ref. X) found ratios between 124 and 240 in five

Danish soils stored for 90 days at 5°C and then for 5 days at 25°C, while the same soil types stored for 210 days at 5°C varied between 171 and 477.

The ATP analyses (ref. X) were performed by means of purified luciferin-luciferase enzyme, whereas results from all other papers were achieved by use of crude enzyme. Tate & Jenkinson (1982a) concluded that the ATP results obtained by the procedure of Jenkinson & Oades (1979), using crude enzyme should be corrected by a factor of 1.24, giving a biomass C to ATP ratio of 171 instead of 138, as found by Jenkinson et al. (1979).

The ratios of 186 and 148 found between the biomass C determined by $CHCl_3$ fumigation and ATP content after preincubation of soil adjusted to 60 % of the w.h.c. and incubated for 5 days at 25°C after sampling agreed reasonably well with that of Tate & Jenkinson (1982a). As observed in the experiments, the factor for conversion of ATP results into biomasses, as determined by $CHCl_3$ fumigation may not always be the same.

If the pretreatments are changed, or if the soil has recently recieved large addition of substrate or contains a large amount of roots and root exudates, the ratio between biomass (fumigation method) and ATP content may vary. In addition, dry clay soils seem to maintain a pool of "free" ATP, adsorbed to the clay. However, the ATP content seems to be one of the most reliable measure of soil microbial biomass, when ATP content is measured after the soils have been adjusted to standard conditions and as far as the precautions earlier mentioned are taken into account. Furthermore, it was found that ATP content measured shortly after sampling generally is a meaningsful measure of the activity of microorganisms under different environmental conditions.

If the aim of a study is to examine a possible effect of different soil treatments (e.g. manuring and different soil tillage), the soil samples should be subjected to standard

conditions before the analyses are carried out. The soil samples should be adjusted to 60 % of w.h.c. as fast as possible after sampling. It was found most convenient to determine the CO_2 production from the adjusted soils over a 24 h period and measure the concentrations in a gaschromatograph. Determination of microbial activity with these adjustments has the avantage that the results not are influenced by the actual soil temperature and moisture content found at the sampling time at different times of the year. The activity will then be dependent on other factors, e.g. the presence of easily decomposible organic matter. The microbial biomass was assessed by determination of ATP content after the soils had been preincubated for 5 days at 25°C with 60 % of w.h.c..

When studies of effects of climatic conditions on the soil microbial activity and biomass are wanted, e.g. determination of the energy flow in a system, it is suggested that CO_2 production is measured over a 24 h period at the ambient soil temperature and moisture content, as fast as possible after sampling. This measure is of cause influenced by the disturbance of the soil when sampled but give a relative measure of the soil microbial activity during the year as influenced by soil temperature and moisture content. Instead of measuring CO_2 production, the ATP content can be determined shortly after sampling without any soil treatments. This measure will not be influenced as much by the sampling procedure as the CO_2 production but it cannot be used in very dry soils containing clay and in extreme sandy soils. The ATP measure is dependent on the biomass as well, and therefore, it is not possible to measure very low activities. A good correlation between ATP content and microbial activity can only be found within certain limitations for the soils. It is probably the explanation for the missing correlations between these parameters in the Jyndevad coarse sandy soil. The microbial

biomass can be determined by the ATP content after a preincubation for 5 days at 25°C with 60 % of w.h.c..

When the biomass is determined by ATP content in soil types, where the biomass has not previously been analysed by the $CHCl_2$ fumigation method, the conversion factor between biomass C and ATP content should be examined. One factor may not always will be reliable, as observed in the Danish soils.

The ATP content in relation to the amounts of carbon and minerals in soil microorganisms

When ATP measurements are utilized to assess the microbial biomass, amounts of carbon and different plant nutrient element such as nitrogen, phosphorus, sulphur, potassium and calcium immobilized in soil can be calculated by use of the ratios of ATP to these elements in microbial cell material. Luria (1960) found that the ratios in an Escherichia coli cell were: N:C = 0.28; P:C = 0.06; S:C = 0.02; K:C = 0.02 and Ca:C = 0.01. Lee et al. (1971b) obtained ATP:C:N:P:S ratios of 1:250:42:8.6:2.6 by averaging data cited by other workers (Spector, 1956; Strickland et al., 1969). These ratios are equivalent to the following ones: N:C = 0.17; P:C = 0.03 and S:C = 0.01. The latter authors assumed that the ratios are the same for cells grown in liquid culture and in soil. Anderson & Domsch (1980) examined the average biomass C to mineral content ratios of pure cultures of 24 species of soil microorganisms and found the following factors: N:C =0.15; P:C = 0.116; K:C = 0.098 and Ca:C = 0.014. These factors were used by Eiland (ref. VII) in estimation of the nutrient elements immobilized in the microbial biomass (0-20 cm depth) in arable soils from different field management systems. The N:C ratios obtained by Lee et al. (1971b) and by Anderson & Domsch (1980), were of the same size but different from the Escerichia coli data. The values for the phosphorus content of the biomass carbon were of different size in the above mentioned examinations. Anderson & Domsch (1980) found a much higher content of P than observed in the other studies mentioned.

The contents of biomass C and biomass N in the microbial biomass (depth, 0-20 cm) were estimated in different Danish arable soils to 200-800 kg C/ha and 30-120 kg N/ha (refs VII & IX). The biomass C was determined by the $CHCl_3$ fumigation method (non fumigated soil in the 0th-10th day; k factor 0.45) and biomass N calculated by a N:C factor of 0.15. Examinations performed in other arable soils either by the fumigation method or by the ATP method fell inside the mentioned range. Total organic C ranged from 36 t/ha to 64 t/ha in various soils. Carbon immobilized in the microbial biomass contained 0.4-1.6 % of the total organic carbon content. These results were similar to those from other Danish soils examined by Sørensen (1983b), where carbon in biomass ranged from 0.5 to 1.4 % of the total C in native soil organic matter.

Some examples to illustrate the application of the ATP method

Determination of microbial biomass by the ATP method is illustrated with soils from field experiments, which were subjected to different soil treatments (effects of slurry (SLU) or farmyard manure (FYM) added to soil and ploughed versus direct drilled soil). The microbial biomass was also determined in soil profiles using the ATP method.

The effects of amendment with SLU or FYM to coarse sandy soil and sandy loam soil were examined. Characteristics of the soils are shown in Table 20 and microbial biomass (ATP content) and activity as determined by the CO_2 production are shown in Fig. 21. The contents of total organic C, total N, phosphorus and potassium were generally higher in FYM than in SLU treatments, when the soils were amended with high amounts of manures (100 t/ha/yr, 200 t/ha/2 yrs and 400 t/ha/4 yrs),(Table 20).

The ATP content and the CO_2 production were in the coarse sandy soil higher in FYM amended soils than in SLU and NPK fertilized soils (Fig. 21). In the sandy loam soil the ATP content was of approximately the same size in SLU and FYM amended soils but a lower content was found in NPK fertilized soil; the CO_2 production was higher in FYM than in SLU amended and NPK fertilized soils. The soil biomass (ATP content) in the sandy loam soil was about two times that of the sandy soil (average of all treatments). The microbial activity as determined by the CO_2 production followed the same trend in between the two soil types for SLU and NPK amended soils. In FYM amended sandy soil there was found an activity in the order of that in the corresponding sandy loam soil.

More than one soil sampling is necessary to assess effects of soil management practices. These results and results from previously published papers (refs IV, VI & VII) indicate that

Table 20. Some characteristics of the soils

Soils and treatments	pH ($CaCl_2$)	Organic C (%)	Total N (%)	C/N ratio	P_i µg/g d.wt. soil	K_i µg/g d.wt. soil
Lundgård coarse sandy soil						
NPK fertilizers 80 kg N/ha/yr	6.0	1.3	0.10	13.8	276	50
25 t SLU/ha/yr	6.5	1.3	0.11	12.4	282	54
50 t SLU/ha/yr	6.3	1.5	0.12	12.8	264	84
100 t SLU/ha/yr	6.2	1.8	0.14	13.3	318	104
200 t SLU/ha/2 yrs	6.0	1.6	0.12	13.4	279	114
400 t SLU/ha/4 yrs	6.1	1.7	0.13	13.4	330	71
25 t FYM/ha/yr	6.0	1.5	0.11	13.2	333	86
50 t FYM/ha/yr	6.2	1.7	0.12	13.4	387	86
100 t FYM/ha/yr	6.2	1.8	0.16	11.5	468	115
200 t FYM/ha/2 yrs	6.0	2.1	0.17	12.4	573	218
400 t FYM/ha/4 yrs	6.2	2.1	0.17	12.4	570	121
Askov sandy loam soil						
NPK fertilizers 80 kg N/ha/yr	6.2	2.2	0.17	13.3	225	63
25 t SLU/ha/yr	6.3	2.6	0.19	13.6	207	97
50 t SLU/ha/yr	6.5	2.5	0.19	12.8	219	135
100 t SLU/ha/yr	6.8	2.6	0.19	13.4	276	173
200 t SLU/ha/2 yrs	6.4	2.7	0.21	13.0	249	153
400 t SLU/ha/4 yrs	6.4	2.4	0.20	12.2	249	129
25 t FYM/ha/yr	6.1	2.7	0.20	13.5	267	74
50 t FYM/ha/yr	6.6	2.7	0.21	12.9	294	89
100 t FYM/ha/yr	6.3	3.5	0.26	13.6	495	279
200 t FYM/ha/2 yrs	6.2	3.0	0.23	12.9	519	210
400 t FYM/ha/4 yrs	6.5	2.7	0.22	12.4	441	101

Results are means of duplicate samples.

The soils were sampled 8/3-1984 (depth, 0-20 cm).

Bulk density in 0-20 cm depth: Lundgård soil 1.50 g/cm^3
and Askov soil 1.55 g/cm^3

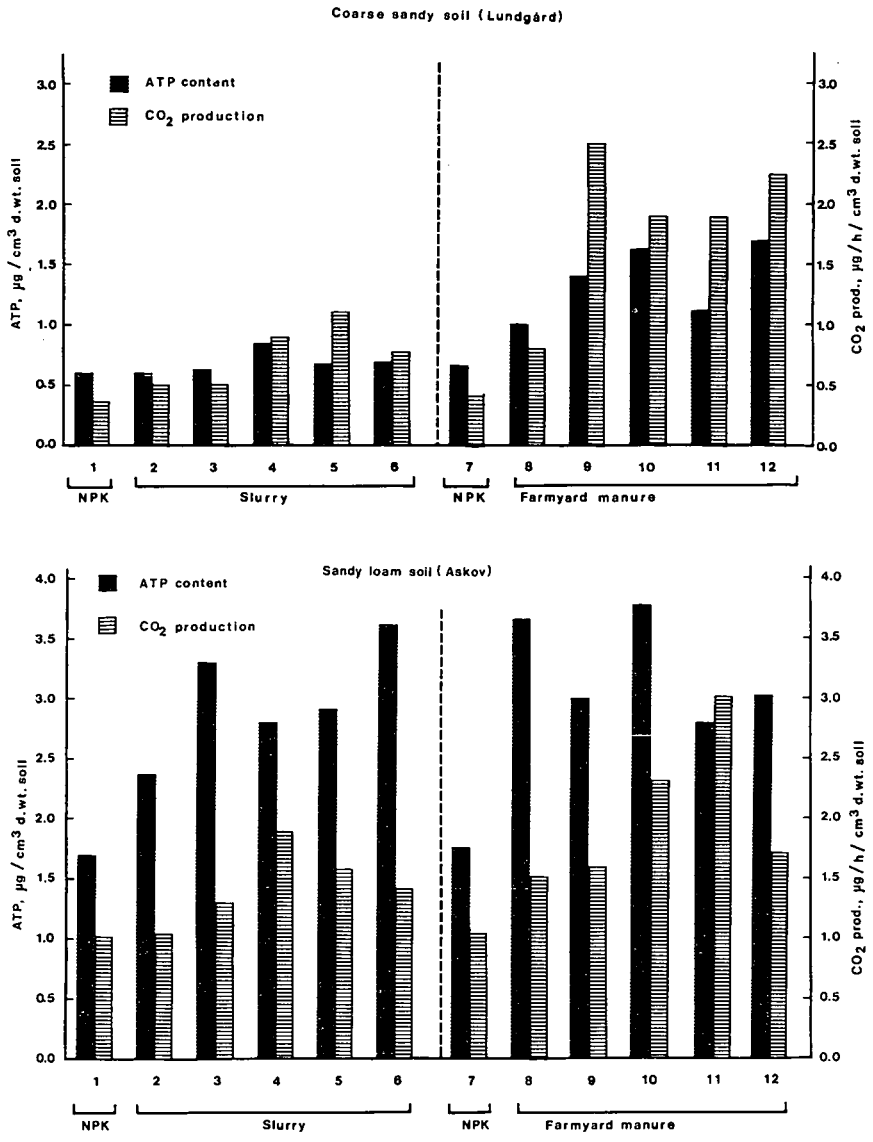


Fig. 21. ATP content and CO_2 production in soil from field experiments amended with slurry or farmyard manure. The soils were sampled 8/3-1984 (depth, 0-20 cm) from coarse sandy soil (Lundgård) and sandy loam soil (Askov).

The results are means of 4 replicated analyses (2 samples were analysed from each of 2 replicated plots). Materials and methods (see APPENDIX V).
1, 7 = (Control) 80 kg N/ha/yr in NPK fertilizers
2, 3, 4 = 25, 50 and 100 t/ha/yr in slurry, respectively
5, 6 = 200 t/ha/2 yrs and 400 t/ha/4 yrs in slurry, respectively
8, 9, 10 = 25, 50 and 100 t/ha/yr in farmyard manure, respectively
11, 12 = 200 t/ha/2 yrs and 400 t/ha/4 yrs in farmyard manure, respectively
200 t slurry or farmyard manure/ha/2 yrs applied last time 12/11-1982 (100 t) and 8/8-1983 (100 t).
400 t slurry or farmyard manure/ha/4 yrs applied last time 3/12-1980.

high doses of SLU and FYM resulted in an increased number of microorganisms; the effect is not permanent but restricted to a period of some month after addition.

The effects of ploughing and direct drilling were also examined. Characteristics of the soils are shown in Table 21 and ATP content and CO_2 production are shown in Figs 22 and 23 from two soil samplings. There were in some of the soils both in the 0-2 cm and 2-10 cm depth a slightly higher content of total organic C and total nitrogen in direct drilled soil than in ploughed soil (Table 21). This is probably due to smashed plant residues in the surface.The biomass as determined by the ATP content was for all soils and samplings (Figs 22 and 23) higher or about the same in the 0-2 cm depth as compared to the 2-10 cm depth. Direct drilled soil had in the 0-2 cm depth a

Table 21. Some characteristics of the soils

Soils, plant growth, treatments and depth		pH ($CaCl_2$)	Organic C (%)	Total N (%)	C/N ratio	P_i µg/g d.wt. soil	K_i µg/g d.wt. soil	Bulk density g/cm^3 soil
Korntved coarse sand								
(Barley)	Depth							
Ploughed	0- 2 cm	5.4	1.0	0.09	10.3	300	63	1.44
"	2-10 cm	6.0	0.9	0.09	10.7	300	53	1.44
Direct drilled	0- 2 cm	6.7	1.2	0.10	11.6	492	115	1.47
" "	2-10 cm	5.4	1.0	0.09	11.8	336	88	1.47
(Rye)	Depth							
Ploughed	0- 2 cm	5.9	1.1	0.11	10.5	402	175	1.44
"	2-10 cm	6.0	1.0	0.09	10.4	342	115	1.44
Direct drilled	0- 2 cm	6.6	1.3	0.11	12.1	540	148	1.47
" "	2-10 cm	6.0	1.1	0.10	11.8	360	115	1.47
Ballum sandy loam								
(Barley)	Depth							
Ploughed	0- 2 cm	5.9	1.2	0.13	9.6	588	198	1.51
"	2-10 cm	5.7	1.3	0.12	10.3	570	118	1.51
Direct drilled	0- 2 cm	5.5	1.4	0.14	9.7	570	296	1.51
" "	2-10 cm	5.8	1.4	0.13	10.5	588	159	1.51
(Wheat)	Depth							
Ploughed	0- 2 cm	5.6	1.4	N.D.	N.D.	615	270	1.51
"	2-10 cm	5.8	1.3	N.D.	N.D.	546	145	1.51
Direct drilled	0- 2 cm	6.0	1.5	N.D.	N.D.	588	300	1.51
" "	2-10 cm	6.0	1.3	N.D.	N.D.	546	189	1.51
Højer silty loam								
(Barley)	Depth							
Ploughed	0- 2 cm	6.9	1.9	0.20	9.5	330	259	1.35
"	2-10 cm	7.1	1.7	0.19	8.7	330	173	1.35
Direct drilled	0- 2 cm	6.9	2.0	0.21	9.3	354	303	1.48
" "	2-10 cm	7.0	1.8	0.20	9.1	330	200	1.48
(Wheat)	Depth							
Ploughed	0- 2 cm	7.2	1.7	0.18	9.3	270	230	1.35
"	2-10 cm	7.3	1.6	0.18	8.9	264	110	1.35
Direct drilled	0- 2 cm	7.2	1.8	0.19	9.3	312	275	1.48
" "	2-10 cm	7.2	1.6	0.18	9.0	270	121	1.48

Results are means of duplicate samples.
N.D. = Not determined. The soils were sampled 25/7-1983.

larger biomass than ploughed soil or sometimes very similar biomasses. There was an effect of the crop in Højer (soil sampling 22/5-1984, Fig. 23), where plots with spring barley contained a higher biomass than plots with winter wheat both in the 0-2 cm and 2-10 cm depth.

Only small differences were found between the management practices in the 2-10 cm depth for all the soils. The microbial activity determined by the CO_2 production in the 0-2 cm depth followed essentially the trends found for the biomass determined by ATP content in all soils. In the 2-10 cm depth only small differences were observed between soils and treatments.

Powlson & Jenkinson (1981) suggested that microbial biomass should give a measurable response to changes in soil management practices long before differences in total carbon or nitrogen became measurable. Although more soil samplings are necessary, the results from the ATP and the CO_2 measurements seem to indicate that a change from ploughed to direct drilled soil resulted in a slow accumulation of organic matter in the 0-2 cm depth. However in the 2-10 cm depth little differences were observed between the two management practices as determined by ATP content and CO_2 production, and the conclusion by Powlson & Jenkinson (1981) that direct drilling had little overall effect on the amount of organic matter in the soil agreed resonably well with these results.

In Danish experiments the yield of grain has often been found to be lower in direct drilled soil than in ploughed soil (K.J. Rasmussen, unpublished). The explanations could be as follows: 1) The high microbial activity and biomass occasionally found in the top 2 cm layer of direct drilled soils may leed to anaerobic soil conditions, 2) the large soil biomass in this layer immobilize inorganic nitrogen fertilizer when supplied in the spring, or 3) there is a lower mineralization in the deeper layer, difficulties for the plant roots etc.

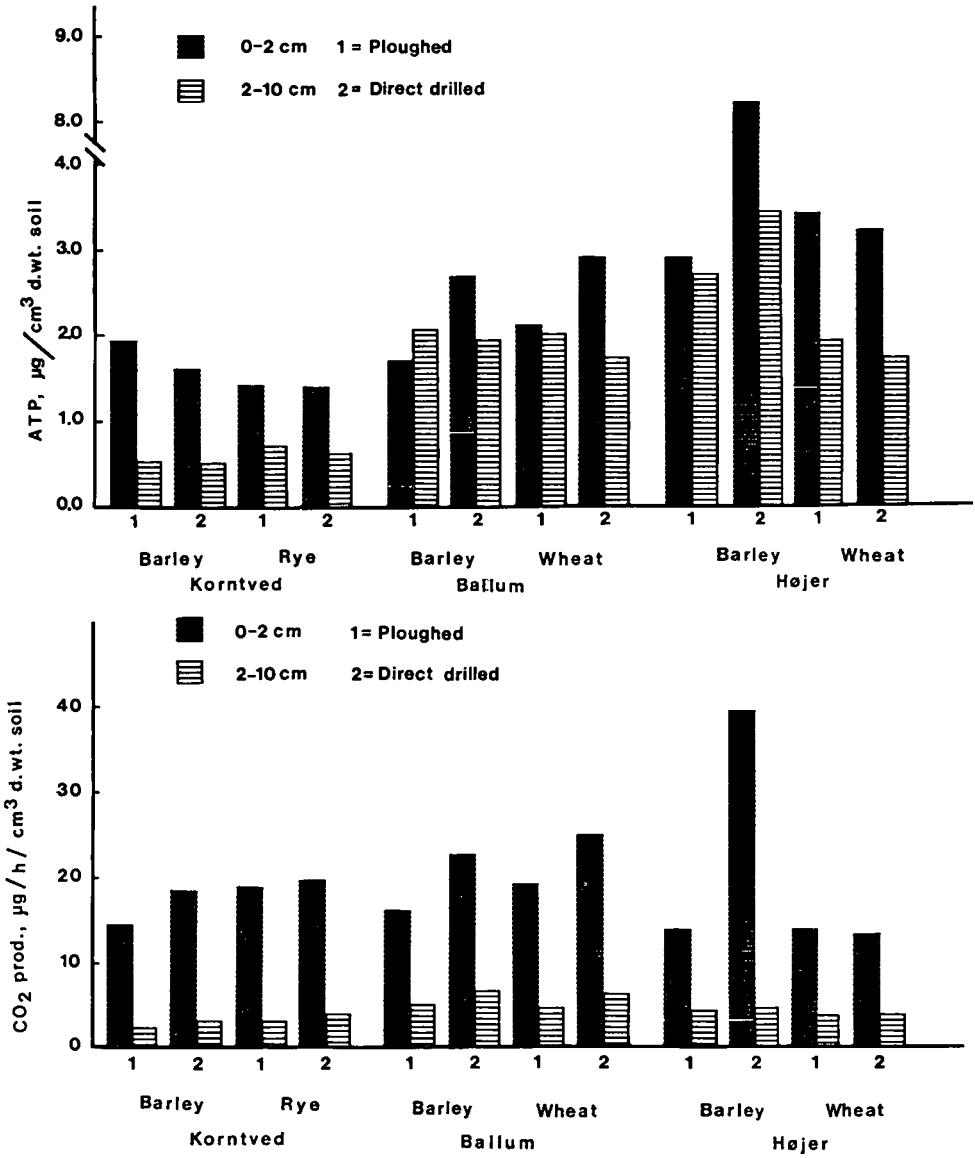


Fig. 22. ATP content and CO_2 production in field experiments, where ploughed and direct drilled treatments were compared in 3 soil types cropped with spring barley, rye or winter wheat. The soils were sampled 25/7-1983 (depth, 0-2 cm and 2-10 cm). The results are means of 4 replicated analyses (2 samples were analysed from each of 2 replicated plots). Materials and methods (see APPENDIX V).

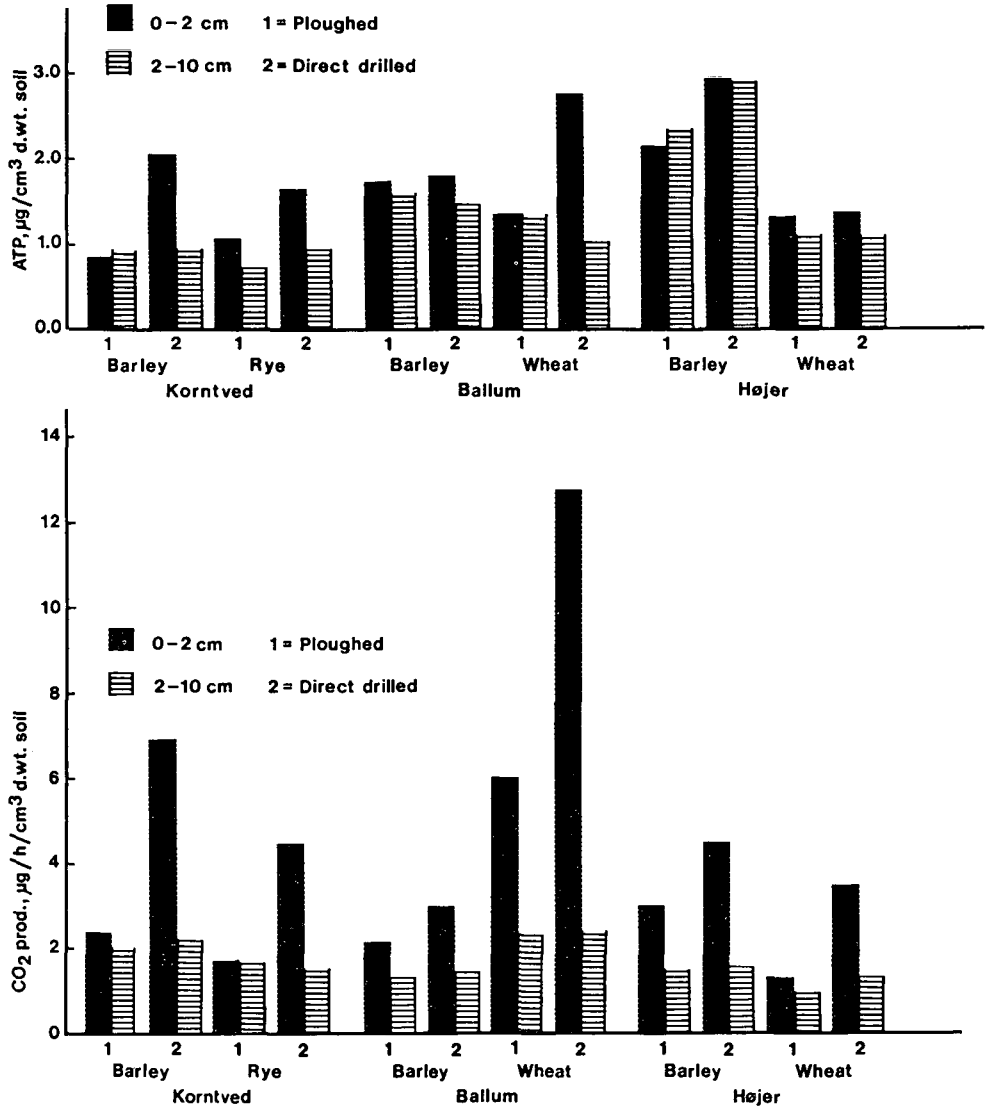


Fig. 23. ATP content and CO_2 production in the field experiment mentioned in Fig. 22.
These samples were taken 22/5-1984 from the same plots as in Fig. 22 and treated similarly. Materials and methods (see APPENDIX V).

These experiments show that changes in land practices predominantly are mirrored in the microbial biomass and activity in the 0-2 cm soil layer.

The microbial abundance was examined in two soil profiles in Gudum and in Borsholm. The soil type in Gudum was in the different depths as follows: 0.0-2.0 m loam soil; 2.0-9.0 m calcareous loam; 9.0-9.5 m fine sand; 9.5 -13.0 m clay with lime and 13.0-38.0 m white chalk. The soil profile in Borsholm was a loam soil in the 0.0-1.5 m depth and loamy sand in the 1.5-6.0 m depth.

The vertical distribution of ATP content, number of bacteria and FDA-active fungi was determined in the two soil profiles (Fig. 24). The ATP content was measurable down to 24.5 m in the Gudum profile, indicating that the method is very sensitive. Bacteria as determined by plate counts could be observed to about the same depth; below 14 m, only very small colonies appeared on the plates. Acridine orange stained bacteria (AO-bacteria) were measurable downwards to 13 m depth and FDA-stained fungi to 5 m.

Numbers of AO-bacteria in Borsholm profile was lower than the number found in Gudum. In contrast, ATP content did not differ very much between the two profiles.

The results from the agricultural management systems and the profiles in this session are only thought to be examples, to show how the ATP method can be used for different biological investigations. The mentioned examinations are all parts of studies going on and will later be reported in details.

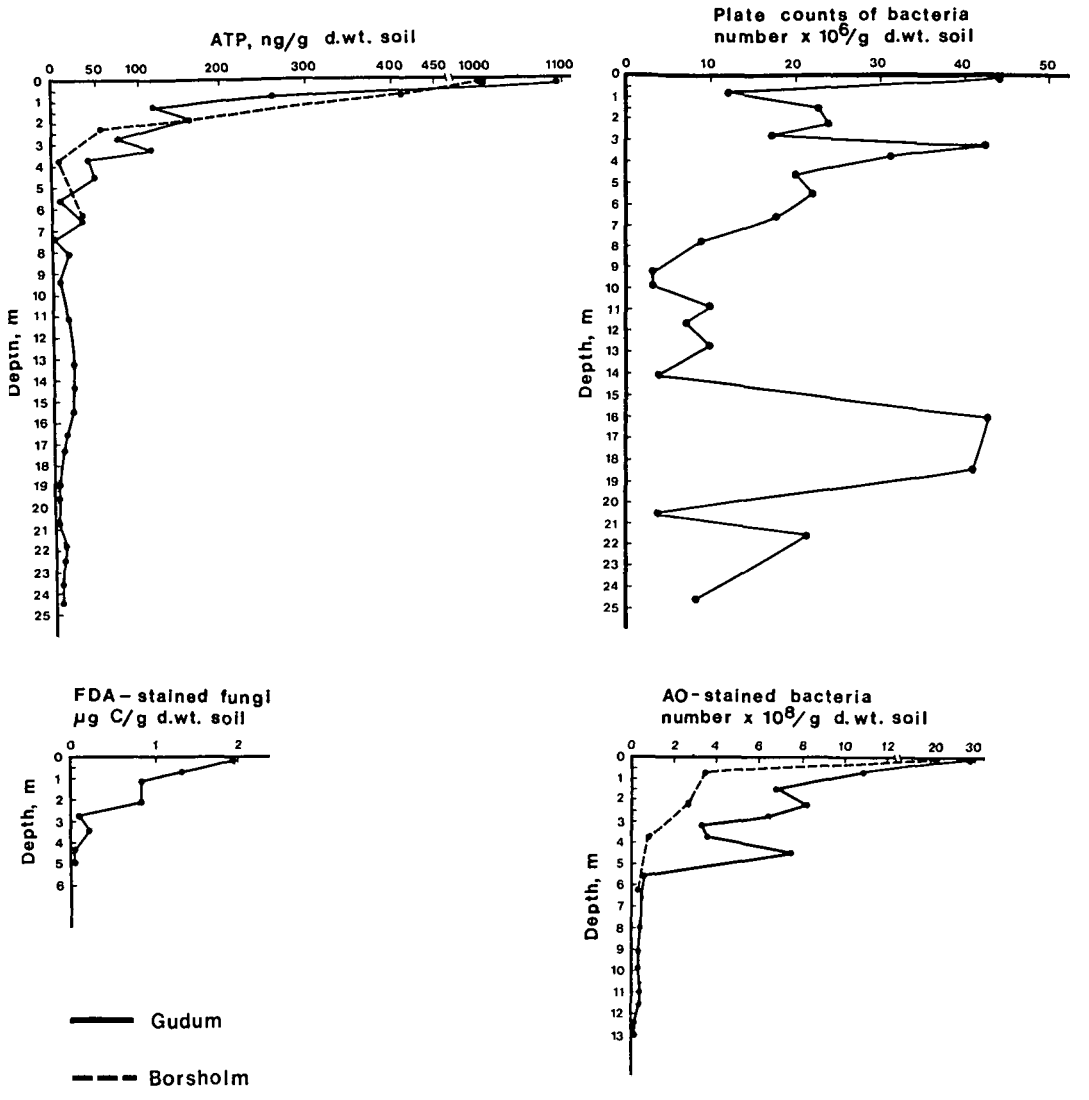


Fig. 24. Vertical distribution of ATP content, plate counts of bacteria, FDA-stained fungi and number of AO-bacteria in a soil profile (Gudum) down to 25.5 m. ATP content and AO-bacteria were also analysed in a profile down to 6.5 m (Borsholm). Materials and methods (see APPENDIX V).

Methods for determination of microbial activity and biomass in soil

The ATP content has in this work been related to results of other methods, which express microbial activity or biomass in soil. Therefore, the aim with this section is to give a short review over the main groups of organisms in soil and different methods used for determination of microbial activity and biomass in soil.

Many different organisms are living in soil, including microorganisms and animals. The microorganisms, belonging to innumerable genera and species, can be divided into the following major groups: Bacteria including actinomycetes, fungi, algae and protozoa. The soil fauna other than protozoa is mainly represented by nematodes, enchytraeids, earthworms and arthropods. Bacteria and fungi predominate in numbers and in the variety of activities over the other groups of microorganisms. Different examinations suggest that fungi make up a dominant part of the biomass. It has been found (ref. IX) that the fungi/bacteria ratio in four Danish soils averaged 80/20, as determined by the method suggested by Anderson & Domsch (1975). They found in three German arable soils that the fungi/bacteria ratios were 70/30, 65/35 and 80/20. The activity of the soil biomass seems to be severely limited by nutrient availability and many soil organisms apparently have very low respiration rates or spend most of their lifetime in dormant or resting phases (Gray & Williams, 1971; Gray, 1976).

The size of the microbial biomass do not necessarily give a measure of the activity of the microbial populations. Several methods exist for determination of microbial activity and biomass in soil (Fig. 25). A detailed description of methods for

Fig. 25. Some methods for determination of microbial activity and biomass in soil.

Microbial activity	Microbial biomass
Respiration (O_2 uptake and CO_2 production)	Direct measurements (light- or fluorescence microscopy)
Respiration using isotopically labelled substrates (^{14}C)	Chloroform fumigation technique
Microcalorimetry	Dilution plate count technique
Adenylate energy charge (AEC) (see Chapter C)	Substrate-induced respiration
ATP content (see Chapter F)	ATP content (see Chapter F)
Enzyme activities (e.g. amylase, cellulase, dehydrogenase, phosphatase and urease)	DNA content
	Muramic acid content

measuring microbial biomass and activity with all their associated problems, is beyond the scope of this work. Therefore, only a very brief outline of different methods are given. A review of methods for determination of the microbial biomass is worked out by Jenkinson & Ladd (1981). The over-all microbial activity has usually been estimated from the rate of CO_2 production or by the rate of O_2 uptake of soil samples in the laboratory (e.g. Petersen, 1926; Antoun & Jensen, 1979; refs V & IX). CO_2 production has also been measured in the field without removing of the soil (e.g. Redmann, 1978; DeJong et al., 1979). Microbial activity can also be determined by isotopically labelled substrates (e.g. Sørensen, 1983a) and by microcalorimetry, where the heat output of the soil biomass is measured (Mortensen et al., 1973; Ljungholm et al., 1979; Sparling, 1981a, 1981b). Measuring of adenylate energy charge (Brookes et al.,1983; Martens, 1983; Eiland, see Chapter C), ATP content (see Chapter F), or enzyme activities (refs III, IV, V, VI & IX; Nannipieri et al., 1978; Domsch et al., 1979; Sparling et al., 1981; Ross et al., 1982), have also been used as activity measures, although these methods do not give a direct measure of the microbial activity.

Estimates of the biovolume of soil bacteria and fungi have been made by direct microscopic methods. Fungal biomass has been determined by staining with phenol aniline blue and measured by light microscopy (Jones & Mollison, 1948; Jenkinson et al., 1976) or phase-contrast microscopy (Frankland, 1974). Use of an epifluorescence microscope and fluorescent dyes, e.g. acridine orange (Strugger, 1948; Trolldenier, 1973), fluorescein isothiocyanate (Babiuk & Paul, 1970), and water-soluble aniline blue (Paul & Johnson, 1977) makes it easier to separate microorganisms from the soil particles. There are often difficulties in distinguishing between living and dead cells by use of these methods and a number of assumptions have to be made

before counts can be converted into weights or volumes. Attempts have been made to determine the active fungal biomass by staining with fluorescein diacetate (FDA) (Söderström, 1977), as well as a determination of the FDA-active bacterial biomass (Lundgren, 1981).

The chloroform fumigation method (Jenkinson & Powlson, 1976) is an empirical method based on a constant mineralization of killed microorganisms and not nescessarily indicating the true biomass under all circumstances. Various incubation periods have been selected for determination of the CO_2 production from fumigated and non fumigated samples incubated at 25°C (Jenkinson & Powlson, 1976; Ross et al., 1980b). Use of a k-factor of 0.45 (Jenkinson & Powlson, 1980; Jenkinson & Ladd, 1981) for converting values of CO_2-C flush to biomass C contents has also been discussed and changed. Originally, Jenkinson (1976) suggested the use of a k-factor of 0.50. The k-factor and the above mentioned background CO_2 value from non fumigated samples are both critical values in the method. In addition, it was found that this method did not work in acid woodland soils (Jenkinson, 1976). However, the method has proved useful for determination of microbial biomass in arable soils (e.g. ref. X).

The dilution plate count technique only accounts for a small part of the total content of microorganisms in soil as determined by direct microscopy counting methods. This discrepancy is based on the fact that the nutrient medium used for the plate count method exert a selective effect, and consequently only a small part of the total microflora will multiply in the media. On the contrary, microscopic methods (e.g. acridine orange stained bacteria) include both active and inactive organisms as well as dead ones.

Determination of the biomass can also be obtained by the method of Anderson & Domsch (1978) from the respiration rate after addition of increasing concentrations of glucose. The results from this method have been found to be closely correlated to the

biomass, as measured by the $CHCl_3$ fumigation method (Anderson & Domsch,1978).

ATP content used as a measure of biomass or activity in soil is discussed earlier in this section (Chapter F). The ATP content from soil originates from all the soil microorganisms and probably also from small soil animals. When soil samples are preincubated as suggested for biomass measurements, it is assumed that small plant roots in the soil are dead and only to a minor extent influence the ATP content.

Determination of nucleic acid content (DNA and RNA) in soil has been tried as measure of the biomass (e.g. Anderson, 1979) as well as muramic acid from prokaryotic organisms (Millar & Casida, 1970). But none of these methods have shown good prospects for use as measures of soil microbial biomass (for a review see Jenkinson & Ladd, 1981).

Each method for measuring the activity and the size of soil biomass have naturally its limitations. Plate counts of bacteria, and FDA-active fungi account only for a very small proportion of the size of the biomass determined by microscopic counts using acridine orange, by the $CHCl_3$ fumigation method, determination of ATP content, or by the substrate-induced respiration method. The bacterial proportion derived from plate counts represented only 5 % of the value obtained from the $CHCl_3$ fumigation biomass in Roskilde sandy loam soil, 0-20 cm depth (ref. IX). FDA-active fungi in the same experiment accounted for only 0.8 % of the biomass determined by fumigation (ref. IX). (Because of a calculation error, results of FDA-active fungi in refs VII and IX should be divided with 10). It seems that the different methods are expressions of different parts of the soil biota. Jenkinson and Ladd (1981) suggested that four methods (direct counts, ATP content, the fumigation method and the substrate-induced respiration method) are available for measuring soil biomass, although much work has to be done to elucidate the conditions under which a method may or may not be used.

G. CONCLUSIONS AND PERSPECTIVES

An improved method has been developed for quantitative determination of soil ATP and ATP from cultures of microorganisms by the luciferin-luciferase enzyme system. The method is simple, rapid and gives reproducible results probably representing the total amount of ATP in soil after correcting for recovery of ATP by use of internal standards. In addition, no toxic reagents are included in the procedure. ATP is extracted from the soil by vigorous shaking with a sulphuric acid-phosphate solution. It was found that most ATP could be removed from a sandy loam soil with an extractant to soil ratio of 10:1; therefore, this ratio was always used for nucleotide extractions from soil. Shaking was found to be most suitable for the soils examined, provided soil dispersion occurs. The advantage of shaking soil suspensions instead of use of ultrasonification is that many soil samples can be analysed simultaneously. In soils, where soil dispersion do not occur, it is possible to use ultrasonification for 1 or 2 min instead of shaking. Phosphate and nucleotide-releasing reagent (NRB®) are included in the procedure to compete with ATP molecules for adsorption onto the soil colloids. The sulphuric acid-phosphate soil suspension is neither filtered nor centrifuged after the extraction of ATP, as normally used in other ATP methods for quantitative ATP determinations (e.g. Paul & Johnson, 1977; ref. I; Jenkinson & Oades, 1979). After shaking the soil suspension, an aliquot is taken immediately from the upper part of the suspension and added to a heavily buffered solution containing Tris and EDTA, to neutralize and dilute the soil suspension. Then an aliquot of the latter mixture is transferred into the NRB® reagent and the ATP content measured. The "direct" extraction procedure, where the soil particles are maintained in the mixture without any filtering or centrifuge step, ensures that ATP molecules adsorbed to the soil components are not removed thus resulting in a more complete extraction of

ATP. If low amounts of ATP in soil are determined, less dilution of the soil suspension need to be applied in the steps after the acid extraction procedure. Portions of acid suspensions (e.g. 0.5 ml) are added to a Tris-EDTA buffer (e.g. 2 ml), and pH is adjusted with NaOH.

The method developed for extraction of ATP from soil can also be used to extract ATP from microbial cultures. If small amounts of ATP is determined, less sulphuric acid-phosphate (4-8 ml) can be added to the culture (1 ml) to increase the amount of ATP for the measurement.

A method has also been developed for determination of adenylate energy charge (AEC) in soil defined as $AEC = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$, (all ATP, AEC = 1; all AMP, AEC = 0). The adenine nucleotides are basicly extracted with sulphuric acid-phosphate, as used in the suggested ATP method. For setting up standard curves, ATP standards and mixtures of ATP/ADP and ATP/AMP standards are added to portions of acid suspensions with sterilized soil. These mixtures are treated as the proper soil samples. Blind values of sterilized soil treated as the proper soil samples are used to correct for ATP in the reagents. To ensure that the sterilized soil not contains measurable amounts of nucleotides, blind values are also made with the extraction agent alone treated as the samples. The background light emission from the extractant without soil was in most experiments higher or similar to the light emission from the extractant with sterilized soil, indicating an inhibition of the light emission from compounds in some of the soil types. After shaking, all mixtures are heated (100°C for 2 min) and cooled. This is necessary to avoid hydrolysis of ATP in the soil extracts during the incubation in the reaction mixtures, presumably due to a re-activation of enzymes in the neutralized extracts. All mixtures are then adjusted to pH 7.5 with Tris-EDTA buffer and NaOH. ADP in the neutralized extracts is converted to ATP by incubation in Heepes buffer with KCl, phosphoenolpyruvate kinase-lactate dehydrogenase

and β-nicotinamide-adenine dinucleotide. AMP is converted to ATP by use of the mentioned reagents plus myokinase. To promote the speed of the reactions for conversion of AMP and ADP to ATP both lactate dehydrogenase and β-nicotinamide-adenine dinucleotide are included. To reduce an inhibition from $(NH_4)_2SO_4$ in a solution of myokinase, it is centrifuged, the supernatant removed, and the pellet containing myokinase diluted. After incubation of the mixtures, the ATP content is measured by the luciferin-luciferase method. The amounts of ATP enzymatically produced from ADP and AMP are calculated as differences between the measured values and molar concentrations of the adenine nucleotides (ATP, ADP and AMP) are used to calculate adenylate energy charge.

Adenine nucleotides and adenylate energy charge values were studied in different soils. A moist Roskilde sandy loam soil analysed 3 h after sampling had an AEC value of 0.83 and the content of ATP, ADP and AMP was 65.6 %, 33.9 % and 0.5 % of the total amount of A_T (ATP plus ADP plus AMP), respectively. After air-drying for 10 days at 25°C, A_T decreased from 2.18 to 0.56 nmol/g d.wt. soil. AEC decreased in the same period from 0.83 to 0.46. Remoistening of the air-dried soil and incubation for 24 h at 25°C resulted in an increase in A_T from 0.56 to 1.80 nmol/g d.wt. soil and AEC rose from 0.46 to 0.73, indicating a formation of new biomass on the expense of dead microorganisms and substrate liberated from the soil by the drying and rewetting procedures.

Addition of glucose to a Askov sandy loam soil and incubation for 3 days at 25°C resulted in an increase in the AEC value from 0.65 in the soil without glucose to 0.70 in the soil with glucose added. The small increase is consistent with the assumption that the soil microorganisms to a large extent are old dormant and resting cells with an AEC of about 0.7, and only a minor part is in active growth with an AEC value of about 0.8. The AEC value, which is an average value for the total biomass will then depend on the proportion of young cells to old and resting cells.

The content of ATP, ADP and AMP as well as the AEC values were determined in different soils adjusted to 60 % of w.h.c., stored for 90 days at 5°C and then incubated for further 24 h at 5°C and 25°C, respectively. The ATP content increased when the soils were incubated at 25°C for 24 h, as compared to incubation at 5°C. The proportions of ATP, ADP and AMP of A_T showed that a raise in temperature from 5°C to 25°C resulted in an increase in the percentage of ATP and a decrease in the percentage of either AMP or ADP. The AEC values were increased in the soils, when the temperature was raised. AEC in the soils incubated at 5°C ranged from 0.58 to 0.74 and suggests a biomass consisting mainly of dormant organisms. At 25°C, AEC values ranged from 0.78 to 0.83 in the different soils and suggest an increased metabolism and growth for some of the organisms. The total soil adenine nucleotide content (A_T) was nearly the same in soils incubated at 5°C and 25°C, indicating a conversion of ADP or AMP to ATP. This indicates that the biomass is fairly similar in a soil stored for a short period at the two temperatures, as also observed by a determination of the soil biomass ($CHCl_3$ fumigation method) in a Roskilde sandy loam and a sandy sandy loam soil incubated for 5 days at 5°C and 25°C, respectively.

It is likely that the somewhat high AEC value found in some of the soils could be caused by a large population of soil animals. They are known to maintain a high AEC value until they are moribund. Although the ATP content changed in the soils dependent on the soil conditions, the results show that the soil biomass maintains a fairly high ATP content and sometimes a very high AEC value can be observed.

The results indicate that temperature, soil moisture and energy supply are factors which are important for the amount of adenine nucleotides and for the size of AEC in soils. When the different factors mentioned are taken into account, the content of adenine nucleotides and the AEC values reported seem to agree well with the general concept of the soil biomass, where the main part of the soil microorganisms are starving or dormant cells living most of the time in a substrate depleted environment.

The effect of clay minerals on the ATP content from cultures of bacteria and fungi grown in presence of bentonite was examined. Addition of bentonite to a culture of the bacterium Pseudomonas fluorescens stimulated both respiration, ATP content and number of bacteria as determined by plate counts. The ATP content was fairly closely related to the respiration in the culture in absence of bentonite, whereas a closer relationship between ATP content and number of bacteria was found in the presence of bentonite. The ATP content per cell in cultures added bentonite varied with a factor of about 3 during the growth period.

Drying of the bacterial cultures in absence and presence of bentonite indicated that bentonite had a preserving effect on the viability of the cells. ATP content remained at the same level after one and two weeks of drying of the cultures with bentonite, although the number of bacteria decreased. This apparent increase in the proportion between ATP content and bacterial numbers could be caused by the presence of "free" ATP or existence of non viable cells in the dried cultures adsorbed to the bentonite.

Recovery of ATP from samples of sand plus bentonite after drying indicated that ATP can be adsorbed onto bentonite and that it can be hydrolized to ADP very slowly in the absorbed state.

A fairly good over-all relationship between increase in the ATP content and the respiration rate was observed during growth of a fungus (Cladosporium sp.) in presence of bentonite.

Although ATP is a component of living cells and presumed to be absent from dead cells, the results from the experiments with pure cultures of bacteria in presence of bentonite suggest that extracellular ATP can accumulate in dry clay soils protected by adsorption to the clay minerals.

The ATP content in soil was influenced by the soil temperature. An increased soil temperature from about 5°C to 25°C resulted in higher concentrations of ATP in different field

soils as well as in cultures of Pseudomonas fluorescens. The effect of the soil temperature on ATP content and CO_2 production was examined in a Roskilde sandy loam incubated for 68 days at 5^oC, 10^oC and 15^oC with different moisture contents. A Q_{10} ($5-15^oC$) of about 1.6 was found for the ATP contents, and the corresponding CO_2 production gave a Q_{10} of 2.1. This indicated a fairly good relationship between these parameters, although the ATP content will be dependent both on the activity and the number of the soil microorganisms. The results also show that ATP content cannot be used as a measure of the soil biomass without a preincubation of the soil with adjusted moisture content and temperature.

The effect of soil moisture content was examined in a sandy loam soil. A relatively high ATP content was found at 1 % moisture either because "free" ATP is present and adsorbed to the clay or the size of the soil biomass is not much affected by the drying procedure.

Anaerobic conditions in a sandy loam soil and a coarse sandy soil only slightly decreased the ATP content and the CO_2 production. This is probably due to the great number of inactive microorganisms existing in a soil, with only a small number of microorganisms actively growing.

Freezing had no or little influence on the ATP content in sandy and sandy loam soils but in one humus and one loam soil the content decreased very much. ATP should preferably be determined on "fresh" soil without freezing.

The results show that the ATP content is a reasonable measure of microbial activity, when determined immediately after sampling at the ambient soil temperature and soil moisture content. However, ATP content cannot be used as an activity measure in dry clay soils and in sandy soils. It was found that dried soils containing clay can maintain ATP not related to the microbial activity, probably because of adsorption of ATP molecules to clay and other soil colloids or because of a large biomass with a low

microbial activity. ATP content and CO_2 production were not well correlated in sandy soils with different moisture contents. More fundamental work is required on the existence of "free" ATP molecules. Can "free" ATP be present in clayey soils, which have not been dried? In addition, the small variations observed over time in sandy soils from field experiments did not correlate with other parameters of soil microbial activity and biomass.

The ATP content in five soils adjusted to 60 % of w.h.c. and preincubated for 5 days at 25°C before measuring ATP was significantly correlated to the soil biomass C ($CHCl_3$ fumigation). The fumigation was started immediately after the adjustment of the water content and biomass C was measured with unfumigated and fumigated soil in 0-10 days with a k-factor of 0.45. The correlation coefficient was significantly different from 0 (0.001 % level).

In support of the general conclusions the following findings should be emphasized:

1) ATP can be extracted from soil and from microbial cultures by the developed sulphuric acid-phosphate-NRB extraction method.

2) Adenylate energy charge , $AEC = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$ can be determined in soil by the developed method. AEC is an expression of the metabolic status of the soil biomass. ATP, ADP and AMP are basicly extracted with sulphuric acid-phosphate and ADP and AMP are converted to ATP by incubation of the soil extract with different enzymes.

3) AEC values in different moist soils which had been preincubated ranged from 0.58 to 0.83. Temperature, soil moisture and energy supply are factors which are important for the amounts of adenine nucleotides (A_T) and for the size of AEC in soils. A_T was nearly the same in soil incubated at 5°C and 25°C, indicating a conversion of ADP or AMP to ATP. Remoistening of an air-dried

soil and incubation for 24 h at 25°C resulted in an increase in A_T and AEC rose from 0.46 to 0.73, indicating a formation of new biomass on the expense of dead microorganisms and substrate liberated from the soil. Soil incubation with glucose only resulted in a small increase in the AEC value (from 0.65 to 0.70). These results are consistent with the assumption that the soil microorganisms to a large extent are old dormant and resting cells with an AEC of about 0.7, and only a minor part is in active growth with an AEC value of about 0.8. The AEC value, which is an average value for the total biomass will then depend on the proportion of young cells to old and resting cells.

4) Presence of the clay mineral bentonite in a dried bacterial culture preserved some "free" ATP molecules either released from dead cells or because of the presence of non-viable cells still containing ATP. Recovery of ATP added to samples of sand with and without bentonite and air-dried indicated that ATP remained adsorbed onto the clay when it was dry. These results suggest that extracellular ATP can accumulate in clay soils by adsorption to the clay, when the soils are dried.

5) Environmental factors influence the ATP content in the following ways: Short term changes in the ATP content (e.g. by an increased soil temperature) can be caused by conversion of ADP or AMP to ATP. This did not result in a change in the size of the soil biomass. The ATP content/cell in a bacteria culture was fairly constant in different growth phases when the same growth temperature was used. The growth temperature influenced the ATP content/cell in the early phases of growth, resulting in the highest ATP content/cell at 25°C and the lowest one at 5°C. Growth at temperatures at 10°C, 15°C and 20°C yielded ATP content/cell in between. Dry soils containing clay can preserve ATP. It may be due to a large biomass or because "free" ATP is present and adsorbed to the clay. Anaerobic incubation of sandy

and sandy loam soils showed that the ATP content is only slightly influenced by the content of oxygen in soil. This is probably due to the great number of inactive microorganisms existing in a soil.

6) ATP is a reasonable measure of microbial activity, when determined immediately after sampling at the ambient soil temperature and soil moisture content. However, ATP content cannot be used as an activity measure in dry soils containing clay and in extreme sandy soils at different moisture levels.

7) ATP content is a measure of the soil biomass as determined by the chloroform fumigation method, when soil is preincubated with 60 % of the water holding capacity for 5 days at 25^{o}C, so that a proper balance between ATP, ADP and AMP is reached. The average C/ATP ratio was 186 for the sandy and sandy loam soils and 148 for loam and silt loam soils.

The present study has demonstrated the utility of the ATP method as a very useful tool for estimating microbial abundance in a range of soils subjected to different agricultural practices. It was shown that differences in biomass and activity as determined by the ATP method in different soil types and management systems (e.g. manuring, fertilization, tillage and irrigation) can be detected. Powlson & Jenkinson (1981) suggested that the biomass may indicate changes in soil organic matter before they become measurable in the total organic carbon and total nitrogen contents. The soil biomass and its activity are important measures for following decomposition of organic matter. The biomass contribute to plant nutrition far more than its size might suggest. The biomass acts as a catalyst for the decomposition process and a labile source-sink for the nutrients (e.g. N,P, K and S). The ATP method can be used to assess immobilization of nutrient elements in the soil organisms, such

as nitrogen, phosphorus, potassium, calcium and sulphur. The discrepancies between the amounts of elements per cell found in different investigations indicate that a more extensive study of the relationship between ATP content and other cellular constituents, especially phosphorus and potassium, for diverse soil microorganisms under various environmental conditions is needed. ATP content may also be used in determination of the energy flow in the systems. ATP can be quantitatively related to soil respiration rates as measured in the laboratory. Detailed studies should be done to examine root contribution to the ATP pool in soil as well as the amount of ATP derived from different compartments (e.g. protozoa, enchytraeids and nematodes). It is likely that the size of biomass and the microbial activity are parameters related to the amount of mineralized nitrogen, which moves through the biomass. The ATP method may be a useful measure in determination of the nitrogen turn-over at different times of the year in arable systems. The ATP method has shown promise in determination of microbial life in subsoils. A better knowledge of microbial biomass and activity in deeper soil layers is important in the work of protection the groundwater from pollution of e.g. nitrate originating from the cultivated soil layer. The method should also be useful to detect harmful effects on the microorganisms originated from heavy metal pollution of soils or influence of other soil contaminations. In the future, the ATP content in soil may serve as a biological index of soil fertility, which can be determined routinely. When the ATP measure is combined with other methods and mathematical modeling is used, it may be possible to improve the understanding of soil processes and ecosystem functioning. The advantage of the ATP method, as compared with other methods, is that the analyses can be carried out with great speed and low cost in operator time. The disadvantage of the method is the rather high cost of reagents. Use of the adenylate energy charge method might yield additional informations on the metabolic status of the soil biomass.

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SUMMARY

The adenosine triphosphate (ATP) content in cells has been found to be a useful tool in many fields of the microbiology, e.g. for determination of the size of the biomass in the aquatic environment. This thesis describes a number of investigations related to determination of ATP and AEC in soil. When these parameters were used on soil, a number of special problems related to methodology and interpretation of the results in a meaningsful way have been found. The aim of this work was to contribute to an elucidation of some of these problems e.g. the influence of different environmental factors for the use of the ATP content as a measure of microbial biomass and microbial activity in soil. Parts of the results have been published separately in the ten papers listed on pages 6 and 7. References are made to these papers by Roman numerals, I-X.

Methodology

A simple method has been developed for quantitative determination of soil ATP by the luciferin-luciferase enzyme system. The ATP content is extracted from the soil by vigorous

shaking for 15 min with a sulphuric acid-phosphate solution. An aliquot of the soil suspension is neutralized with a Tris-EDTA solution and mixed with a special ATP releasing reagent (NRB®). ATP is measured in the soil suspension after a 10 s exposure to the NRB reagent, followed by addition of luciferin-luciferase and integration over 10 s in a Lumacounter M 2080. The method can also be used for determination of ATP content in cell material from cultures. The ATP content in a range of soils varied between 0.37 and 7.52 µg ATP/g d.wt. soil.

A method has also been developed for determination of "adenylate energy charge" (AEC) in soil. AEC is defined as $\frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$. If all adenine nucleotides (A_T) is on the ATP form, AEC = 1; if all is on AMP form, AEC = 0. AEC is an expression of the metabolic status of the soil populations. The adenine nucleotides in the soil samples are basicly extracted with sulphuric acid-phosphate as used in the suggested ATP method. The extracts containing the soil particles are boiled for 2 min, cooled, and adjusted to pH 7.5 with Tris buffer and NaOH. ADP in the neutralized soil extracts is converted to ATP by incubation in Heepes buffer with KCl, phosphoenol pyruvate kinase-lactate dehydrogenase and β-nicotinamide-adenine dinucleotide (NADH). AMP is converted to ATP by use of the reagents for determination of ADP and myokinase (MK).

For setting up standard curves, ATP standards and mixtures of ATP/ADP and ATP/AMP standards are added to suspensions of sterilized soil. These mixtures are treated in the same way as the proper soil samples. After incubation of the tubes at 30°C for 30 min, the ATP content is measured by the luciferin-luciferase method. The concentrations of ADP and AMP are found by differences, and the AEC values are calculated.

Content of ATP, ADP and AMP in soil as influenced by temperature, moisture and energy supply

Adenine nucleotides and adenylate energy charge values (AEC) were studied in a sandy loam soil. The "fresh" soil analysed 3 h after sampling had an AEC value of 0.83 and the content of ATP, ADP and AMP was 65.6 %, 33.9 % and 0.5 % of A_T (ATP plus ADP plus AMP), respectively. After air-drying of the soil for 10 days at 25°C, A_T decreased from 2.18 to 0.56 nmol/g d.wt. soil. AEC decreased in the same period from 0.83 to 0.46 and the relative proportions of the three adenine nucleotides in the air-dried soil were 17.9 % (ATP), 55.4 % (ADP) and 26.7 % (AMP) of A_T. The air-dried soil was then remoistened to 60 % of the water holding capacity (w.h.c.) and incubated for 24 h at 25°C; this resulted in an increase in A_T from 0.56 to 1.80 nmol/g d.wt. soil and AEC rose from 0.46 to 0.73, indicating a formation of new biomass on the expense of dead microorganisms and substrate liberated from the soil by the drying and rewetting procedures. A_T and AEC were after the 24 h incubation period only slightly lower than those results obtained for the "fresh" soil. The relative proportions between the adenine nucleotides were fairly similar to those found for the "fresh" soil. The increase in the content of nmol ATP/g d.wt. soil was not counterbalanced by a decrease in ADP and AMP. Therefore, ATP may be produced from degradation of substrate by de novo synthesis.

The contents of ATP and ADP increased in a soil due to the incubation with glucose for 3 days at 25°C, whereas the amount of AMP remained fairly constant. The small increase in the AEC value from 0.65 in the soil without glucose to 0.70 in the soil with glucose added is consistent with the assumption that the soil microorganisms to a large extent are old dormant and resting cells with an AEC of about 0.7, and only a minor part is in active growth with an AEC value of about 0.8. The AEC value, which is an average value for the total biomass will then depend on the proportion of young cells to old and resting cells.

The contents of ATP, ADP and AMP and AEC values were determined in different preincubated soils. The ATP content increased when the soils were incubated at 25°C for 24 h, as compared to incubation at 5°C. The proportions of ATP, ADP and AMP of A_T showed that a raise in temperature from 5°C to 25°C resulted in an increase in the percentage of ATP and a decrease in the percentage of either AMP or ADP. The AEC values were increased in the soils, when the temperature was raised. AEC in the soils incubated at 5°C ranged from 0.58 to 0.74 and suggests a biomass consisting mainly of dormant organisms. At 25°C, AEC values ranged from 0.78 to 0.83 in the different soils and suggest an increased metabolism and growth for some of the organisms. The total soil adenine nucleotide content (A_T) was nearly the same in soils incubated at 5°C and 25°C, indicating a conversion of ADP or AMP to ATP. The soil biomass will probably be similar in a soil stored for a short period at the two temperatures. This was also found in a sandy loam and a sandy soil, when the biomass was determined by chloroform fumigation.

The high AEC value of 0.83 found in the "fresh" sandy loam soil is comparable to AEC values of actively growing microorganisms in vitro and it could suggest that in soil both dormant and metabolically active cells maintain a high AEC value. However, in the different moist soils examined, AEC values varied from 0.58 to 0.83, indicating that AEC values probably not are similar in active and inactive cells, but will depend on the proportion of young cells to old and resting cells. It is likely that the somewhat high AEC value found in some of the soils could be caused by a large population of soil animals. They are known to maintain a high AEC value until they are moribund. Although the ATP content changed in the soils dependent on the soil conditions, it has been found (ref. X), that the soil biomass maintains a fairly high ATP content.

The results indicate that temperature, soil moisture and energy supply are factors which are important for the amounts of adenine

nucleotides and for the size of AEC in soils. When the different factors mentioned are taken into account, the content of adenine nucleotides and the AEC values reported here seem to agree well with the general concept of the soil biomass, where the main part of the soil microorganisms are starving or dormant cells living most of the time in a substrate-depleted environment.

The influence of the clay mineral bentonite on biomass, activity and the ATP content of bacteria and fungi

Presence of bentonite in a culture of the bacterium Pseudomonas fluorescens stimulated the respiration during the initial growth phase. The ATP content of the cells and the number of bacteria as determined by plate counts were larger in the presence of bentonite after 3 days of incubation than without bentonite. The ATP content in the cultures added bentonite was in the range of 0.2-0.8 x 10^{-9}µg ATP/cell with the highest ATP content during the exponential growth. The ATP content was fairly closely related to the respiration in the culture in absence of bentonite, whereas a closer relationship between ATP content and counts of bacteria was found in the presence of bentonite. The biomass C/ATP varied in the bacterial cultures with bentonite between 153 and 585. In Danish soils which were adjusted to 60 % of w.h.c. and preincubated for 5 days at 25°C before measurement, the ratio varied from 124 to 240.

The number of bacteria in a dried culture material decreased less if bentonite was added before the drying than if not. The content of ATP/cell increased in the cultures dried for 2 weeks, possibly because the clay preserved some "free" ATP molecules released from dead cells or existence of cells not able to multiply but still containing ATP.

A fungus, Cladosporium sp., was grown for two weeks in absence and in presence of bentonite. Both the O_2 uptake and the ATP

content during the first 2-3 days of growth were stimulated. Although minor differences were observed between the cultures in absence and presence of bentonite, the results indicate a fairly good correlation between the respiration rate and the ATP content.

Recovery of ATP added to samples of sand with and without bentonite and air-dried indicated that ATP remained adsorbed onto the clay when it was dry, but a slow hydrolysis seems to take place. The results suggest that extracellular ATP can accumulate in clay soils by adsorption to the clay minerals, when the soils were dried.

The influence of environmental factors on the ATP content

Temperature, oxygen, moisture content of the soil and freezing of soil samples influenced the ATP concentration. The soil temperature at the sampling time appears of importance for calculation of conversion factors between ATP and biomass. However, a relatively constant ratio between ATP content and biomass C as determined by the fumigation method can be obtained if the soil samples were preincubated with 60 % of w.h.c. for 5 days at 25°C before determination of ATP. In this way a proper balance between ATP, ADP and AMP is reached. Determination of CO_2 production gave Q_{10} values between 1.5 and 2.1 (5-25°C) in the arable soils. The CO_2 production in a sandy loam soil had a Q_{10} (5-15°C) of about 2.1 and the corresponding ATP content a Q_{10} of about 1.6. This suggests that a fairly good correlation may exist between CO_2 production and ATP content.

Different temperatures influenced the ATP content in cells of the bacterium Pseudomonas fluorescens. The ATP content/cell in exponential and stationary growth phases was highest at 25°C and lowest at 5°C, with values from growth at

$10^{o}C$, $15^{o}C$ and $20^{o}C$ in between. The ATP content/cell was fairly constant in the different growth phases at the same growth temperature. When cells begin to die, there was a tendency towards a lower ATP content/cell at $15^{o}C$ and $25^{o}C$, as compared to the corresponding temperatures in the earlier growth phases. This could be due to counts of non-viable cells or because the ATP content was reduced in old cells.

The ATP content and the CO_2 production increased with increasing moisture both in a sandy loam and a sandy soil. In the sandy loam soil, the two parameters reasonably well were correlated between 8 % and 26 % moisture. At 1 % moisture, a relatively high ATP content and a very low CO_2 production were found in the sandy loam soil. It may be due to a large biomass with a low microbial activity or because "free" ATP is present and adsorbed to the clay. The two parameters as affected by different moisture contents were not well correlated in a sandy soil. Therefore, the ATP content cannot be used as a measure of microbial activity in dry soils containing clay and in sandy soils with different moisture contents. Anaerobic incubation of a sandy and a sandy loam soil for 2 days decreased the ATP content with 5 % and the CO_2 production with 14 % , as compared with the corresponding aerobic incubation (average of the 2 soils). These results show that the ATP content is only slightly influenced by the anaerobic incubation. This is probably due to the great number of inactive microorganisms existing in a soil with only a small number of microorganisms actively growing.

Freezing of the soil samples at $-20^{o}C$ for up to 2 weeks before the analyses of ATP, was possible for the sandy and the sandy loam soils without significant changes in ATP. However, the ATP

content in a humus soil and a loam soil decreased much. It is not possible to store these soils at -20°C without appreciable changes in ATP content. In general, the ATP content should preferably be determined without freezing of the soils.

The nucleotide contents as measures of soil microbial biomass and activity

The ATP content in arable soils added organic manures showed significantly positively correlations to O_2 uptake, CO_2 production, dehydrogenase activity, biomass determined by $CHCl_3$ fumigation, total organic C, total N, NO_3-N, NH_4-N and soil moisture. This indicate a relationship between the ATP content, microbial activity and biomass if soil contains easily decomposible organic material.

The effects on microbial biomass and activity of reduced tillage and use of a catch crop were examined in a sandy loam and a sandy soil. Only inorganic fertilizers have been applied. The ATP content was in the upper 5 cm layer of the sandy loam soil significantly positively correlated to CO_2 production, dehydrogenase,- cellulase- and phosphatase activity, bacteria determined by plate counts, biomass ($CHCl_3$ fumigation) and FDA-active fungi. In the 5-20 cm depth, the ATP content was not correlated to CO_2 production, dehydrogenase activity, cellulase activity and FDA-active fungi but only to the biomass ($CHCl_3$ fumigation), bacteria on plates and phosphatase activity. The ATP content was not correlated to physical-chemical parameters in the sandy loam soil and in the sandy soil in both depths. In addition, no correlations between microbial parameters were found in the sandy soil. The missing correlations may be ascribed to

the small variations observed between the soil treatments and over time in this soil type.

There was a significant correlation between ATP content and CO_2 production ($r = 0.70$; $p < 0.01$) for 5 different arable soils. The soil samples had been stored for 24 h at the ambient soil temperature and then adjusted to 60 % of w.h.c. immediately before the measurements were carried out. When ATP was measured after preincubation of the soils for 5 days at 25°C, there was not a significant correlation between ATP content and CO_2 production ($r = 0.39$). ATP measured without and with a preincubation of the soils was significantly correlated to biomass C determined by chloroform fumigation. The preincubation of the soils before measurements of ATP resulted in a more close relationship ($r = 0.97$; $p < 0.001$), as opposed to ATP measured without a soil preincubation ($r = 0.81$; $p < 0.001$).

The regression equation through the origin and the correlation coefficient were determined for two groups of the 5 different soils; I) coarse sand, fine sand and sandy loam soils and II) loam and silt loam soils. Soil group I had a regression equation of $y = 186x$ with a close relationship between ATP content and biomass C ($r = 0.69$; $p < 0.05$). Soil group II had a regression equation of $y = 148x$, and ATP content and biomass C was even more closely correlated ($r = 0.99$; $p < 0.001$).

The ATP content is a measure of the size of the soil biomass if the soil samples have been adjusted to standard conditions (60 % of w.h.c.) and preincucated for 5 days at 25°C. Changes in the size of the soil biomass, resulting from management practices and environmental variations can be detected by measurements of the ATP concentrations. Differences in the size of the biomass in different soil types is also reflected in the ATP concentrations. There was also a fairly good relationship between ATP content and various measures of the microbial activity, if ATP is measured immediately in "fresh" soil at the ambient soil temperature and moisture content. The positive correlation between ATP content

and microbial activity was not very good in dry soils containing clay; in extreme sandy soil the correlation is also limited presumably because there is not a good relationship between ATP content and a low microbial activity.

The adenylate energy charge (AEC) is an expression of the metabolic state of the soil biomass. The AEC determinations are fairly consistent with the view that the bulk of the biomass consist of dormant organisms, which are unable to grow and the organisms capable of growth account only for a minor proportion of the total soil biomass. The AEC value is believed to be a useful tool in research of the over-all activity state of the soil biomass.

DANSK RESUMÉ (DANISH SUMMARY)

BESTEMMELSE AF ADENOSIN TRIFOSFAT (ATP) OG ADENYLAT ENERGILADNING (AEC) I JORD OG ANVENDELSE AF ADENIN NUKLEOTIDER SOM MÅL FOR MIKROORGANISMERNES BIOMASSE OG AKTIVITET I JORDEN

Indholdet af adenosin trifosfat (ATP) i celler kan anvendes indenfor mange mikrobiologiske områder, f.eks. til at bestemme biomassens størrelse i akvatiske økosystemer. I denne afhandling er beskrevet en række undersøgelser, der omhandler bestemmelse af ATP og AEC i jord. Når disse parametre blev anvendt på jordprøver, er det fundet at der er specielle problemer forbundet med metodikken og tolkningen af resultaterne på en meningsfuld måde. Formålet med dette arbejde har været at bidrage til en klarlæggelse af nogle af disse problemer, f.eks. forskellige miljøfaktorers indflydelse på brugen af ATP indholdet som et mål for mikrobiel biomasse og mikrobiel aktivitet i jord. En del af de opnåede resultater er tidligere publiceret i 10 artikler, der er angivet på side 6 og 7 med romertal I til X.

Metoder

En simpel metode er blevet udviklet til kvantitativ bestemmelse af jordens ATP indhold ved brug af luciferin-luciferase enzymsystemet. ATP indholdet blev ekstraheret fra jorden ved

kraftig rystning i 15 minutter med en svovlsyre-fosfat opløsning. En mængde af jordsuspensionen blev neutraliseret med en Tris-EDTA opløsning og derefter blandet med et specielt ATP frigørende reagens (NRB®). Efter en kontaktperiode på 10 sekunder mellem jordsuspensionen og NRB, blev der tilført luciferin-luciferase enzym og ATP indholdet blev målt ved integration over en 10 sekunders periode i en Lumacounter M 2080. Denne metode kan også bruges til at bestemme ATP indhold i celler fra kulturer. ATP indholdet i jorder der er opbevaret i 90 dage ved 5°C og derefter inkuberet i 5 dage ved 25°C lå imellem 0,37 og 7,52 µg ATP/g tørvægt jord.

En metode er også blevet udviklet for bestemmelse af adenylat energiladning (AEC) i jord.
AEC er defineret som $\frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$.
Hvis alle adenin nukleotider (A_T) er på ATP form er AEC = 1; hvis alle er på AMP form er AEC = 0. AEC er et udtryk for stofskiftet i jordens organismer. Adenin nukleotiderne i jordprøverne ekstraheres med svovlsyre-fosfat, på samme måde som foreslået for ATP metoden. Ekstrakterne der indeholder jordpartiklerne koges i 2 minutter, afkøles, og pH justeres til 7,5 med Tris buffer og NaOH. ADP indholdet i neutraliserede ekstrakter omdannes til ATP ved inkubering i Heepes buffer med KCl, fosfoenol pyruvat kinase-laktat dehydrogenase og β-nikotinamid-adenin dinukleotid (NADH). AMP omdannes til ATP ved brug af reagenserne til bestemmelse af ADP, hvortil der er tilsat myokinase (MK).

For at kunne lave standardkurver er ATP standarder og blandinger af ATP/ADP og ATP/AMP standarder tilsat suspensioner af steriliseret jord. Disse blandinger behandles på samme måde som jordprøverne, der undersøges.

Efter inkubering af blandingerne ved 30°C i 30 minutter, måles ATP indholdet med luciferin-luciferase metoden. Koncentrationerne af ADP og AMP bestemmes ved subtraktion og AEC værdierne kalkuleres.

Indhold af ATP, ADP og AMP i jord ved påvirkning af temperatur, fugtighed og energitilførsel

Adenin nukleotider og adenylat energiladning (AEC) blev undersøgt i en lerblandet sandjord. Den "friske" jord der blev analyseret 3 timer efter prøveudtagning havde en AEC værdi på 0,83 og indholdet af ATP, ADP og AMP var henholdsvis 65,6 %, 33,9 % og 0,5 % af det totale indhold af adenin nukleotider (A_T). Efter at jorden var lufttørret i 10 dage ved 25°C faldt A_T fra 2,18 til 0,56 nmol/g tør jord. AEC faldt i den samme periode fra 0,83 til 0,46 og det relative indhold af de tre nukleotider i den lufttørrede jord var 17,9 % (ATP), 55,4 % (ADP) og 26,7 % (AMP) af A_T. Den lufttørrede jord blev derefter genopfugtet til 60 % af vandholdende evne og inkuberet i ydeligere 24 timer ved 25°C. Dette resulterede i en stigning i A_T fra 0,56 til 1,80 nmol/g d.wt. soil og AEC steg fra 0,46 til 0,73, hvilket indikerer en dannelse af ny biomasse på bekostning af døde mikroorganismer og substrat frigjort fra jorden ved tørring og genopfugtningsprocesserne. A_T of AEC var efter de 24 timers inkubering kun lidt mindre end de resultater, der blev opnået for den "friske" jord. Det relative forhold imellem adenin nukleotiderne svarede ret godt til det, der blev fundet for den "friske" jord. Stigningen i indholdet af nmol ATP/g d.wt. jord modsvarede ikke et fald i ADP og AMP. Derfor må ATP blive produceret fra nedbrydning af substrat ved de novo syntese.

Indholdet af ATP og ADP steg i en jord ved inkubering med glukose i 3 dage ved 25°C, hvorimod AMP indholdet forblev ret konstant. Den lille øgning i AEC værdien fra 0,65 i jorden uden glukose til 0,70 i jorden med glukose er konsistent med den antagelse at jordens mikroorganismer i stor udstrækning er gamle og hvilende celler med en AEC på omkring 0,7, og kun en mindre del er i aktiv vækst med en AEC værdi på omkring 0,8. AEC værdien, som er en gennemsnitsværdi for hele biomassen, vil således afhænge af andelen af unge celler til gamle og hvilende celler.

Indholdet af ATP, ADP og AMP og AEC værdier blev målt i forskellige forinkuberede jorder. ATP indholdet steg når jorderne blev inkuberet ved 25°C i 24 timer, sammenlignet med inkubering ved 5°C. Andelen af ATP, ADP og AMP af A_T viste at en stigning i temperatur fra 5°C til 25°C resulterede i en øgning i procentdelen af ATP og et fald i procentdelen af enten AMP eller ADP. AEC værdierne steg i jorderne, når temperaturen var øget. AEC i jorderne inkuberet ved 5°C varierede fra 0,58 til 0,74 og dette peger på en biomasse, der hovedsagelig består af hvilende organismer. Ved 25°C varierede AEC værdierne fra 0,78 til 0,83 i forskellige jorder og det tyder på et øget stofskifte og vækst for nogle af organismerne. Indholdet af adenin nukleotiderne (A_T) var næsten det samme i jorder inkuberet ved 5° og 25°C, hvilket indikerer at der sker en omdannelse af ADP eller AMP til ATP. Jordens biomasse vil sandsynligvis være ens ved opbevaring i en kort periode ved de to temperaturer. Det var også fundet i en lerblandet sandjord og en sandjord, hvor biomassen var bestemt ved kloroformdampningsmetoden.

Den høje AEC værdi på 0,83, der blev fundet i den nyudtagne lerblandede sandjord er sammenlignelig med AEC værdier i aktivt voksende mikroorganismer in vitro og det kunne se ud til at både inaktive og stofskifteaktive celler opretholder en høj AEC værdi i jord. Imidlertid varierede AEC værdierne i de forskellige fugtige jorder mellem 0,58 og 0,83, hvilket indikerer at AEC værdier sandsynligvis ikke er ens i aktive og inaktive celler. Det er mere sandsynligt at den noget højere AEC værdi der blev fundet i nogle af jorderne kunne skyldes en stor population af dyr i jorden. De er kendt for at opretholde en høj AEC værdi indtil de dør. Skønt ATP indholdet ændredes i jorderne afhængig af jordbundsforholdene, er det blevet fundet (ref. X), at jordens biomasse opretholder et temmeligt højt ATP indhold.

Resultaterne viser at temperatur, fugtighed og energitilførsel er faktorer som er væsentlige for mængden af adenin nukleotider og for størrelsen af AEC i jorder. Når de forskellige faktorer

der er nævnt er taget i betragtning, synes indholdet af adenin nukleotider og AEC værdierne der er fundet i disse undersøgelser, at stemme godt med den herskende opfattelse af jordens biomasse, hvor størstedelen af jordbundsmikroorganismerne er udsultede og hvilende celler, der lever det meste af tiden i et næringsfattigt miljø.

Virkning af lermineralet bentonit på bakteriers og svampes biomasse, aktivitet og ATP indhold

Tilstedeværelsen af bentonit i en kultur af bakterien Pseudomonas fluorescens stimulerede respirationen i den indledende vækstfase. ATP indholdet i cellerne og bakterietallet bestemt ved pladespredninger var større, når bentonit var tilstede efter 3 dages inkubering end uden bentonit. ATP indholdet i kulturerne med bentonit var i størrelsesordenen 0,2-0,8 x 10^{-9} µg ATP/celle med det største ATP indhold i den exponentielle vækst. Koncentrationen af ATP var korreleret til respirationen i kulturen uden bentonit, hvorimod der i kulturerne med bentonit var en bedre sammenhæng mellem ATP indholdet og bakterietallet.

Biomasse C/ATP forholdet i bakteriekulturerne med bentonit varierede mellem 153 og 585. I danske jorder som var justeret til 60 % af vandholdende evne og forinkuberet i 5 dage ved 25°C før ATP måling, blev der fundet værdier mellem mellem 124 og 240.

Udtørring af bakteriekulturerne med bentonit mindskede både bakterietallet og ATP indholdet sammenlignet med en ikke-tørret kultur. Indholdet af ATP/celle steg i den kultur, der var tørret i 2 uger, muligvis fordi leret beskyttede "frie" ATP molekyler, der var frigjort fra døde celler eller fordi nogle celler var hvilende og havde mistet deres evne til at danne kolonier på et agar medium, skønt de stadig var levende.

Under 2 ugers vækst af svampen Cladosporium sp. var der en god sammenhæng mellem ATP indholdet og respirationen, både i kulturer uden og med bentonit. Bentonit i kulturen stimulerede både O_2 forbrug og ATP indhold under de første 2-3 dages vækst. Skønt mindre forskelle blev set mellem kulturerne uden og med bentonit, viste resultaterne en temmelig god korrelation mellem respirationen og ATP indholdet.

Genfinding af ATP tilsat prøver af sand med og uden bentonit og lufttørret, viste at ATP forblev adsorberet til leret, når det var tørt, men en langsom hydrolyse synes at foregå. Skønt ATP er en kemisk forbindelse, der har været antaget kun at findes i levende celler, peger resultaterne fra de forskellige forsøg på, at extracellulært ATP kan findes i udtørrede lerjorder, hvor det er beskyttet mod nedbrydning ved adsorbtion til lermineraler.

Miljøfaktorers indflydelse på ATP indholdet

Temperatur, iltforhold, fugtighedsindhold i jorderne og frysning af jordprøver indvirkede på ATP concentrationen. Justering af jordtemperaturen til en standardtemperatur er væsentlig for at kunne beregne omsætningsfaktorer mellem ATP og biomasse. Et ret konstant forhold kunne imidlertid findes mellem indholdet af ATP og biomasse C bestemt med kloroformdampningsmetoden, når jordprøverne blev forinkuberet med 60 % af vandholdende evne i 5 dage ved 25°C, før ATP indholdet blev bestemt. På denne måde blev der opnået en balance mellem ATP, ADP og AMP. Bestemmelse af CO_2 production gav en Q_{10} mellem 1,5 og 2,1 (5-15°C) i landbrugsjorderne. CO_2 produktionen i en lerblandet sandjord havde en Q_{10} (5-15°C) på omkring 2,1 og det tilsvarende ATP indhold gav en Q_{10} på omkring 1,6. Dette tyder på at der kan være en temmelig god sammenhæng mellem CO_2 produktion og ATP indhold.

Forskellige temperaturer påvirkede ATP indholdet i celler af

bakterien Pseudomonas fluorescens. ATP indholdet/celle i de exponentielle og stationære vækstfaser var højest ved 25°C og lavest ved 5°C. Inkubering ved 10°C, 15°C og 20°C gav ATP indhold/celle, der lå imellem de ovennævnte værdier. ATP indholdet/celle var temmeligt konstant i de forskellige vækstfaser indenfor hver af vækstemperaturerne. Når cellerne begyndte at dø var der en tendens til et lavere ATP indhold/celle ved 15°C og 25°C, sammenlignet med de tilsvarende temperaturer i de tidligere vækstfaser. Det kunne være på grund af ikke-levedygtige celler eller fordi ATP indholdet blev reduceret i aldrende celler.

ATP indholdet og CO_2 productionen steg med et øget vandindhold både i en lerblandet sandjord og en sandjord. I den lerblandede sandjord var der en rimelig god korrelation mellem de to parametre, når jorden indeholdt mellem 8 % og 26 % vand. Ved 1 % vandindhold blev der fundet et relativt højt ATP indhold og en meget lav CO_2 produktion. Det må skyldes, at der er en stor biomasse med en lav mikrobiel aktivitet eller også fordi "frit" ATP er tilstede og adsorberet til leret. I sandjorden var ATP indholdet og CO_2 produktionen ikke godt korreleret ved de forskellige vandindhold. Derfor kan ATP indholdet ikke blive anvendt som et mål for mikrobiel aktivitet i tørre jorder, der indeholder ler og i sandjorder med forskellige vandindhold.

Anaerob inkubering af en sandjord og en lerblandet sandjord i 2 dage fik ATP indholdet til at falde med 5 % og CO_2 produktionen med 14 % i forhold til den tilsvarende aerobe inkubering (gennemsnit af 2 jorder). Disse resultater viser at ATP indholdet kun ændres lidt af den anaerobe inkubering. Det skyldes sandsynligvis at der er et stort antal inaktive organismer i en jord, med kun et lille antal mikroorganismer, der er i aktiv vækst.

Det viste sig muligt at fryse de undersøgte sandjorder og en lerblandet sandjord ved -20°C og opbevare jordprøverne i op til 2 uger før analysering af ATP uden nævneværdige ændringer i ATP

indholdet. Modsat blev ATP indholdet i en humusjord og en lerjord reduceret meget. Derfor er det i almindelighed bedst at tilstræbe målinger af ATP uden en nedfrysning af prøverne.

Nukleotidindhold som mål for jordens biomasse og aktivitet

ATP indholdet i landbrugsjorder tilsat organiske gødninger viste signifikante positive korrelationer til O_2 forbrug, CO_2 produktion, dehydrogenase aktivitet, biomasse bestemt med kloroformdampning, total organisk C, NO_3-N, NH_4-N og jordfugtighed. Dette peger på en sammenhæng mellem ATP indholdet, mikrobiel aktivitet og biomasse, når jord indeholder let nedbrydeligt organisk stof.

Virkning på mikrobiel biomasse og aktivitet af reduceret jordbehandling og brug af en efterafgrøde blev undersøgt i en lerblandet sandjord og en sandjord. Kun uorganiske gødninger blev anvendt. ATP indholdet var i det øverste 5 cm lag af den lerblandede sandjord signifikant positivt korreleret til CO_2 produktion, dehydrogenase,- cellulase- og fosfataseaktivitet, bakterier bestemt med pladespredningsmetoden, biomasse (kloroformdampning) og FDA-aktive svampe. I 5-20 cm laget var ATP indholdet ikke korreleret til CO_2 produktion, dehydrogenase aktivitet, cellulase aktivitet og FDA-aktive svampe,men kun til biomasse (kloroformdampning), antal bakterier og fosfataseaktivitet. ATP indholdet var ikke korreleret til fysiske-kemiske egenskaber i den lerblandede sandjord og sandjorden (0-5 cm og 5-20 cm dybde). Desuden blev der ikke fundet nogen sammenhæng mellem mikrobiologiske parametre i sandjorden. De manglende korrelationer må tilskrives de små variationer mellem jordbehandlingerne og over tid i denne jordtype, fordi jordprøverne var for ens.

Der var en signifikant korrelation mellem ATP indhold og CO_2 produktion ($r = 0,70$; $p < 0,01$) for 5 forskellige landbrugsjorder. Jordprøverne var opbevaret i 24 h ved udtagningstemperaturen og så justeret til 60 % af vandholdende evne umiddelbart før målingerne blev udført. Når ATP blev målt efter en forinkubering af jorderne i 5 dage ved 25°C, var der ikke en signifikant korrelation mellem ATP indhold og CO_2 produktion ($r = 0,39$). ATP målt både uden og med en forinkubering af jorderne var signifikant korreleret til biomasse C bestemt med kloroformdampningsmetoden. Forinkubering af jorderne før ATP målingerne resulterede i en bedre sammenhæng ($r = 0,97$; $p < 0,001$), end når ATP blev målt uden en forinkubering ($r = 0,81$; $p < 0,001$).

Regressionen gennem 0-punktet og korrelationskoefficienten blev bestemt for to grupper jord; I) grovsand, finsand og lerblandede sandjorder og II) lerjord og siltagtig lerjord. Gruppe I havde en regressionsligning på $y = 186x$ med en tæt sammenhæng mellem ATP indhold og biomasse C ($r = 0,69$; $p < 0,05$). Gruppe II havde en regressionsligning på $y = 148x$, og gav en endnu bedre korrelation mellem ATP og biomasse C ($r = 0,99$; $p < 0,001$).

ATP indholdet er et mål på størrelsen af jordens biomasse, hvis jordprøverne er blevet justeret til standardbetingelser (60 % af markkapacitet) og forinkuberet i 5 dage ved 25°C. Ændringer i størrelsen af jordens biomasse, som et resultat af landbrugspraksis og miljøbestemte variationer, kan undersøges ved måling af ATP koncentrationerne. Forskellen i biomassens størrelse i forskellige jordtyper viser sig også i ATP koncentrationerne. Der var også en temmelig god sammenhæng mellem ATP indholdet og forskellige målinger af mikrobiel aktivitet, hvis ATP måles i nyudtagne jordprøver ved det temperatur-og fugtighedsindhold, der var i marken. Den positive korrelation mellem ATP indhold og mikrobiel aktivitet var ikke så god i jorder med et højt lerindhold, fordi "frit" ATP adsorberes til lerpartikler; i ekstreme sandjorder er korrelationen også begrænset, sandsynligvis fordi der ikke er nogen god sammenhæng

mellem ATP indhold og en lav mikrobiologisk aktivitet.

Adenylat energiladning (AEC) er et udtryk for biomassens aktivitetsniveau i jord. AEC bestemmelserne er ret konsistent med den herskende opfattelse at størstedelen af biomassen består af hvilende organismer, som er ude af stand til at vokse og de organismer, der er i stand til vækst udgør kun en mindre del af den totale biomasse i jorden. AEC værdien menes at være et nyttigt mål til undersøgelser af biomassens generelle aktivitetsniveau i jorden.

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APPENDIX I

THE FIREFLY BIOLUMINESCENCE ASSAY FOR MEASURING ATP CONTENT

(A method for extraction and measurement of ATP from soil and from cultures of microorganisms)

Materials and methods

Soils

The proposed ATP method was tested on different soil types (Table 7, page 34) Soils were sampled (depth, 0-5 cm) in September 1981 and stored moist at 5°C in closed polyethylene bags. After 90 days, the soils in the bags were incubated at 25°C for 5 days. In addition, soils (depth, 0-5 cm) were sampled in May 1981 and stored moist at 5°C for 210 days in closed polyethylene bags. After the respective incubation periods, the soils were sieved (<2 mm) and all analyses were carried out.

Chemical analyses

The following chemical analyses were made by Danish standard procedures (Ministry of Agriculture, Copenhagen,1972). Organic C by dry combustion (Ter Meulen), total N by Kjeldahl digestion, soil pH ($CaCl_2$), P_i (P soluble in 0.1 M H_2SO_4), K_i (exchangeable K in 0.5 M CH_3COONH_4) and soil moisture content (drying of soil samples at 105°C for 24 h).

Determination of ATP in soil

Reagents. - All reagents were of analytical grade and distilled water was used throughout.

Buffer A for neutralization of extractant A. - 37.7 g Trizma® (T-4128, Sigma) and 1.5 g Titriplex® III (EDTA, Merck) were dissolved in 1000 ml water, giving a solution 250 mM with respect to Tris and 4 mM to EDTA. The solution had a pH of 7.5 at 25°C.

Buffer B for ATP standards. - Buffer A diluted ten times with water and sterilized by autoclaving for 20 min at 121°C.

Extractant A. - 27.3 ml of a 96 % H_2SO_4 and 44.5 g $Na_2HPO_4 \cdot 2H_2O$ were dissolved in 1000 ml water, forming a solution of 0.5 M H_2SO_4 and 0.25 M Na_2HPO_4.

Extractant B. - A quaternary ammonium detergent NRB® (Lumac). Alternatively, a 10 % Rodalon® disinfectant (Ferrosan, Copenhagen) was dissolved in Tris-EDTA buffer B to give a 0.005 % solution. The disinfectant contains a quaternary ammonium compound (alkyldimethyl-benzylammonium chloride).

ATP standards.- Solutions were prepared by dissolving 10 µg crystalline disodium ATP (Lumac) in buffer B (25 mM Tris-0.4 mM EDTA; pH 7.5 at 25°C), giving an ATP range of from 0.2 to 10.0 µM. Standard ATP in these concentrations gave a reproducible linear response.

Soil blank suspensions for correcting of ATP present in the enzyme solution. - For each soil type, a sample (1 g) was added to 10 ml of extractant A. The suspension was shaken for 15 min and sterilized by autoclaving for 20 min at 121°C. Then the solution was cooled and treated as mentioned under "Measurement of ATP".

Enzyme.- The purified luciferin-luciferase enzyme Lumit (Lumac) was dissolved in 5 ml Heepes buffer, containing 25 mM (N-2-Hydroxyethylpiperazine-N-1-2-ethanesulphonic acid), 10 mM $MgSO_4$ and 0.02 % NaN_3, pH 7.75 (Lumac), and stored in the dark for 2 h at 20°C before use. For determination of ATP content in the 0-20 cm soil layer, the enzyme can be dissolved in 8 ml Heepes buffer instead of 5 ml buffer.

ATP was measured in a photometer (Lumacounter M 2080 or a Biocounter M 2010).

Extraction of ATP

The extraction procedure is as follows:

1) Soil samples (1 g) are added to plastic tubes (72 x 22 mm) containing 10 ml of extractant A and kept on ice in plastic boxes.

2) ATP standards -Soil samples (1 g) are added to tubes containing 9.5 ml of extractant A and 0.5 ml of ATP standard solutions, similarly. The assay was routinely standardized by using 0.5 ml of an ATP solution which contained about the same amount of ATP as present in the soil suspension (e.g. 4 µM or 8 µM ATP).

3) All soil suspensions are shaken for 15 min on a reciprocating shaker at 0°C with 164 movements/min (Gerhardt LS 20).

4) Recovery of added ATP- 0.5 ml of an ATP solution (e.g. 4 µM ATP) is added to tubes containing 10 ml of extractant A.

All suspensions are measured as described below.

Measurement of ATP

Soil blank suspensions, samples and ATP standards are all measured using the following procedure: Shake the suspension for 5 s which results in a homogeneous suspension, and pipette immediately 50 µl from the upper part of the suspension (100-200 mm layer) into 1.5 ml of buffer A, kept in an ice bath. After shaking for 3 s (1), pipette 50 µl of the latter mixture into a cuvette containing 50 µl NRB extractant, and shake the mixture gently for 5 s (2). Place the cuvette in the photometer measuring chamber for a further 5 s (3) and then inject 100 µl of the luciferin-luciferase enzyme. The shaking times used in experiments performed after publication of ref. X have been changed to the following ones, because there are more convinient to use: (1) = 5 s, (2) = 10 s and (3) = 10 s. This procedure did not change the amount of ATP measured. The ATP contents is measured over a 0-10 s integration period. If the maximum light intensity is not reached during the first 10 s after injection of the enzyme, the ATP content is measured over a 0-30 s integration period. Two samples are taken from each acid suspension and measured. Three replicate soil samples are analysed.

ATP procedure and calculation of results

For converting the relative values from the soil samples to ATP, the results were read from an internal standard curve, which was used to compensate for interfering factors.

The standardization method is based on measurement of samples without and with known amounts of ATP added. The added ATP concentrations should at least be in the same concentration or up to the double amount of the sample ATP. The assay was routinely standardized by one ATP concentration. The differences in readings between measurements of "sample ATP + standard ATP" - "sample ATP" give the calibration parameters.

Procedure for determination of ATP:

For standardization	(sample 1 ATP + standard ATP) 1 g soil + 9.5 ml extractant agent A + standard ATP (0.5 ml of e.g. 8 µM ATP) 1 g soil + 9.5 ml extractant agent A + standard ATP (0.5 ml of e.g. 8 µM ATP) (sample 1 ATP) 1 g soil + 10 ml of extractant agent A 1 g soil + 10 ml of extractant agent A
Soil blank	(soil blank suspension ATP) 1 g soil + 10 ml of extractant agent A (15 min shaking and sterilized by autoclaving for 20 min at 121°C. Alternatively, 1 g soil portions can be sterilized by autoclaving and the soil can be treated as the other soil samples Many soil types do not inhibite the background ATP. Therefore, a blank value can also be obtain from the extractant agent A treated as the soil samples.
Soil samples	(sample 2 ATP) 1 g soil + 10 ml of extractant agent A 1 g soil + 10 ml of extractant agent A
For recovery (not used for calculation of ATP content in samples)	0.5 ml standard ATP + 10 ml extractant agent A (diluted as the samples, no shaking)

Example with one ATP concentration (see the procedure for determination of ATP and Fig. 26).

	Rel. light intensity analyses		mean ±SD
sample 1 ATP + standard ATP =	3889	3839	3864 ± 35
sample 1 ATP =	1573	1637	1605 ± 45
standard ATP =			2259
sample 2 ATP =	1603	1629	1616 ± 18
soil blank suspension ATP =	106	112	109 ± 4
sample 2 ATP			1507

S.D. = standard deviation

To obtain a linear ATP standard curve, (sample 1 ATP + standard ATP) - (sample 1 ATP). When "sample 1 ATP" is subtracted from "sample 1 ATP + standard ATP", the relative light intensity for 2 ug ATP/g wet soil is obtained (Fig. 26, I). The ATP standard curve is then made by drawing a streight line between the two points [(2, 3864) and (0, 1605) in Fig. 26, I].

For calibration of the soil samples, (sample 2 ATP) - (soil blank suspension ATP).

When "soil blank suspension ATP" is substracted from "sample 2 ATP", the relative light intensity (1507 in Fig. 26, II) is used to read the ATP content of the soil sample from the standard curve (1.33 µg ATP/g wet weight and then corrected for moisture content in the soil. The results are given directly in µg ATP/g d.wt. soil corrected for recovery of added ATP (Fig. 26, II).

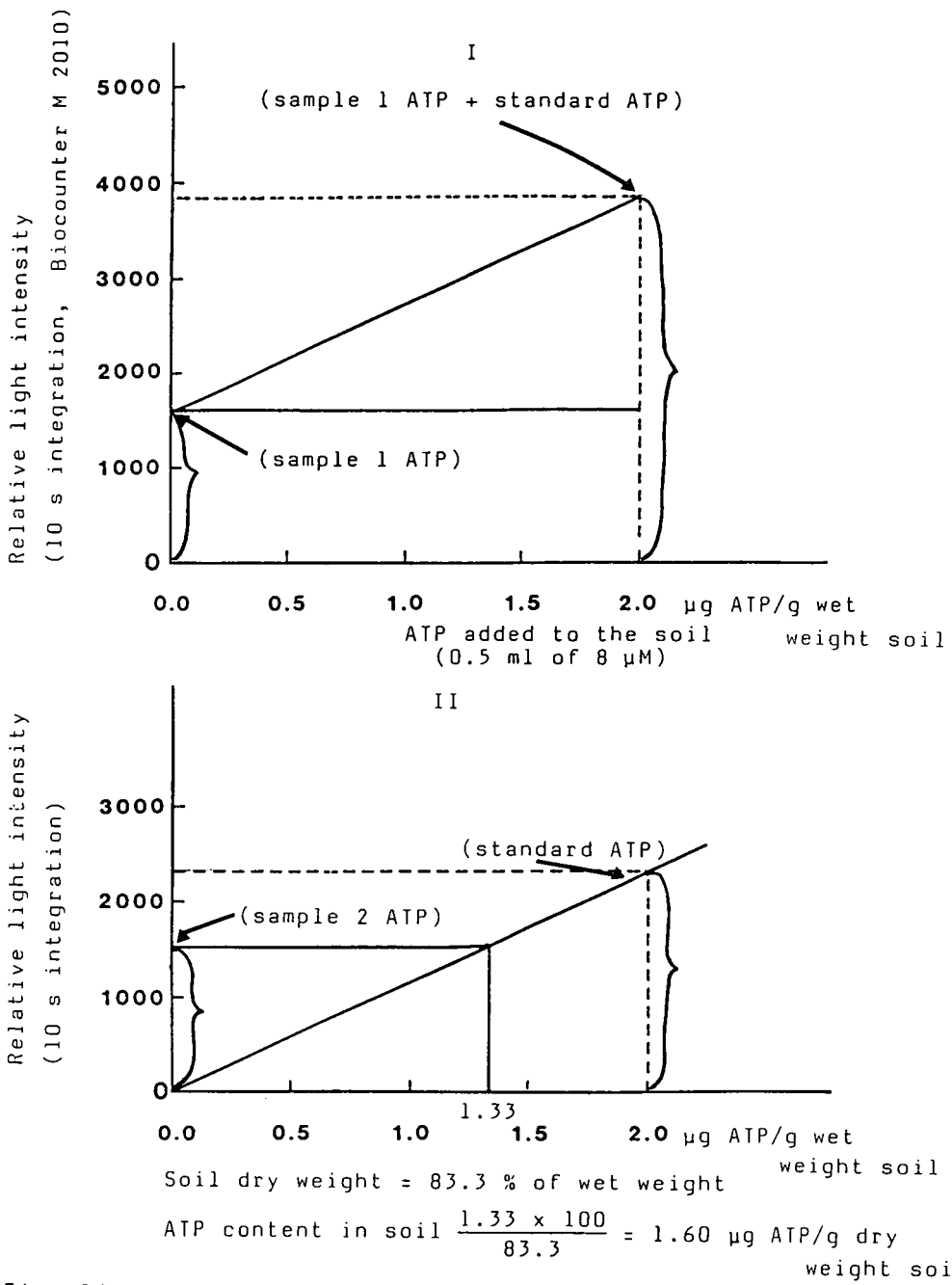

Fig. 26. Graphic illustration of the ATP standadization procedure.

For comparative purposes, percent recovery of added ATP was calculated by the following formula, where "sample ATP + standard ATP" refers to soil suspensions containing added ATP standard solutions. "Standard ATP measured in the extract" refers to ATP standard solutions added directly to the sulphuric acid-phosphate extractant, neutralized with the buffer and measured in the same way as for the samples. "Reagent blank" refers to ATP measured in the neutralized extractant A.
Percent recovery of ATP = (sample ATP + standard ATP)-(sample ATP)x100/(standard ATP measured in the extract)-(reagent blank)

Other ATP methods

The ATP contents in soil were determined by the NRB method as described by Nielsen & Eiland (ref. III), except that ATP standards were added to the soil suspensions and treated in the same way as the soil samples. The ATP content of a sandy loam soil was also determined by the TCA-phosphate-paraquat extraction method (Jenkinson & Oades, 1979).

Soil biomass

Biomass C was estimated by the chloroform fumigation technique (Jenkinson & Powlson,1976) with minor modifications. Before fumigation, the soils were stored as described under "soils". Fumigated and non fumigated soil samples, 60 g portions in tubes (100 x 30 mm), were placed in the upper part of a 2 l glass jar containing 50 ml 25 mM $Ba(OH)_2$. Incubation were done with adjustment of the water contents of the soil samples to 60 % of w.h.c. (Table 7). Biomass C was calculated from CO_2 evolved by fumigated and non fumigated soils incubated at 25°C for 0-10 days, using a k-factor of 0.45 (Jenkinson & Powlson, 1980).

APPENDIX II

THE RATIO BETWEEN THE NUCLEOTIDES ATP, ADP AND AMP IN SOIL

Materials and methods

Soils

The nucleotides ATP, ADP and AMP were determined on different soil types from the 0-5 cm depth (Table 8). A Roskilde sandy loam was sampled in June 1983. The soil temperature at the sampling time was 25°C and the soil was analysed 1) 3 h after sampling 2) after airdrying for 10 days at 25°C and 3) after the soil was remoistened to 60 % of w.h.c. and incubated for further 24 h at 25°C (Table 9).

An Askov sandy loam soil was sampled from the 0-20 cm depth in August 1983, stored with 60 % of w.h.c. for 90 days at 5°C, and then incubated at 25°C for 5 days. The soil was divided in two 500 g portions and 1.0 % glucose was added to one of the portions (on d.wt. basis). Then both of the soil portions were incubated for 3 days at 25°C and analysed (Table 10).

Different soil types were sampled from the 0-5 cm depth in June 1983, stored with 60 % of w.h.c. for 90 days at 5°C and then incubated for further 24 h at 5°C and 25°C, respectively, and analysed (Table 11). The incubation of the soils were performed under aerobic conditions in loosely closed polyethylene bags, and sterilized distilled water was added when necessary. Some characteristics of the soils used are given in Table 8 and the soil textures are shown in Table 2 except for Askov sandy loam. The texture in this soil was as follows: Clay $<$0.002 mm 10.6 %; silt 0.002-0.02 mm 11.8 %; fine sand 0.02-0.2 mm 37.0 % and coarse sand 0.2-2.0 mm 37.6 %. CEC was 12.1 m.equiv./100 g d.wt. soil.

Chemical analyses (see APPENDIX I)

Reagents for nucleotide determinations

All reagents were of analytical grade and distilled water was used throughout. Unless otherwise stated, biochemicals were obtained from Sigma.

Extraction agent. - 27.3 ml of 96 % H_2SO_4 and 44.5 g $Na_2HPO_4 \cdot 2H_2O$ was dissolved in 1000 ml water, to give final concentrations of 0.5 M H_2SO_4 and 0.25 M Na_2HPO_4.

Buffer A for neutralization of the extraction agent. - 37.7 g Trizma® (T-4128) and 1.5 g Titriplex® III (EDTA, Merck) were dissolved in 1000 ml water, giving a solution 250 mM with respect to Tris and 4 mM to EDTA. The solution had a pH of 7.5 at 25°C.

Buffer B for nucleotide standards. - Buffer A was diluted ten times with water, giving a solution 25 mM with respect to Tris and 0.4 mM to EDTA (pH 7.5). The solution was sterilized by autoclaving for 20 min at 121°C and cooled.

Standards. -Sodium salts of adenosine 5'-triphosphate, ATP (Lumac), adenosine 5'-diphosphate, ADP, and adenosine 5'-monophosphate, AMP, were dissolved in buffer B, giving standard solutions of 0.5 mM and frozen at -20°C. Prior to use, individual vials were thawed and further diluted with buffer B to obtain a range of 0.5-10.0 µM of ATP, ADP and AMP in terms of free acids.

Use of sterilized soil for correction of ATP present in the reagents. - For each soil type, samples (1 g portions) were sterilized by heating at 160°C for 48 h followed by autoclaving for 20 min at 121°C, and then the soil was cooled, and treated as mentioned under "Extraction of ATP,ADP and AMP".

Purified luciferin-luciferase enzyme (Lumit PM) was dissolved

in 8 ml Heepes buffer, containing 25 mM N-2-Hydroxyethylpiperazine-N-1-2-ethanesulphonic acid, 10 mM $MgSO_4$ and 3.1 mM NaN_3, pH 7.75 (Lumac), and stored in the dark for two h at 20°C before use.

Reaction mixture I for ATP determinations. - KCl (0.0186 g) was dissolved in 25 ml Heepes buffer to give a solution 10 mM with respect to KCl. The solution could be stored at 2-4°C for several weeks.

Reaction mixture II for determination of ADP plus ATP. - Phosphoenol pyruvate, PEP (2.6 mg) and β-nicotinamide-adenine dinucleotide, NADH (8.85 mg) were dissolved in 25 ml of reaction mixture I, forming concentrations of 0.5 mM, respectively. The stock solution could be kept for two weeks at 2-4°C. Before use, 100 µl of pyruvate kinase-lactate dehydrogenase (PK/LDH) from rabbit muscle (4 mg/ml) was added to 5 ml of the stock solution giving 4 µg PK/LDH/50 µl. The reaction mixture could be stored for 12 h at 20°C.

Reaction mixture III for determination of AMP plus ADP plus ATP. - 0.5 ml of the myokinase suspension, MK (rabbit muscle, 2 mg/ml 1,000 units/mg protein) was centrifuged at 3,000 g (0°C) for 15 min and the pellet was dissolved in Tris-EDTA buffer B forming a concentration of 1 mg/ml. The resulting reduction of the $(NH_4)_2SO_4$ concentration was necessary to decrease enzyme inhibition. After preparation, the enzymes were stored at 0°C. Before use, 20 µl of the centrifuged MK was added to 2.5 ml of reaction mixture II, giving 0.4 µg MK/50 µl. The solution could be stored for 3 h at 20°C.

Extraction of ATP, ADP and AMP

The adenine nucleotides in soil samples were basicly extracted as described by Eiland (ref. X). Soil samples (1 g portions) were

added to tubes containing 10 ml of the extraction agent. For internal standardization, sterilized soil (1 g portions) was added to 9.5 ml of the extraction agent together with 0.5 ml of different concentrations of ATP (e.g. 0.2-4.0 µM), ATP/ADP (4/1 ratio, e.g. 0.2-4.0 µM for ATP plus ADP), ATP/AMP (4/1 ratio, e.g. 0.2-4.0 µM for ATP plus AMP), respectively. The efficiency of conversion of ADP and AMP to ATP was checked by adding ATP standards to reaction mixtures II and III after they were treated as the samples. The standards were in the same concentrations as the mixed standards (ATP/ADP and ATP/AMP). For determinations of the recovery of the adenine nucleotides, portions of 1 g sterilized soil were added to tubes containing 9.5 ml of the extraction agent and treated as the soil samples. Aliquots (0.5 ml) of standards were added immediately before the neutralization step as described below. Blind samples with sterilized soil (1 g sterilized soil and 10 ml of the extraction agent) were treated as the proper soil samples to correct for ATP present in the reagents and inhibition from compounds in the soils. To ensure that the adenine nucleotides were not present in the sterile soils, blind samples with the extraction agent without sterilized soil were also treated as the soil samples. All the solutions were shaken at 0°C for 15 min on a reciprocating shaker at 164 rev./min, immersed into a boiling water-bath and heated at 100°C for 2 min. After cooling in an icebath 0.5 ml of the different solutions were adjusted to pH 7.5 by addition of 2 ml of 250 mM Tris-4 mM EDTA buffer followed by addition of drops of 1.0 M NaOH.

Enzymatic conversion of ADP and AMP to ATP and measuring ATP

All the plastic tubes (47 x 12 mm) were kept at -20°C for at least 2 h before use. For determination of adenine nucleotides in soil samples, sets of four tubes were used for each of the

determinations, labelled I_A-I_O, II_A-II_O and III_A-III_O, respectively. The sets, I, II and III contained 50 µl of the reaction mixtures for determination of ATP, ATP plus ADP, and ATP plus ADP plus AMP, respectively. Determination of the adenine nucleotides from soil was performed by addition of 50 µl of the neutralized soil suspensions to reaction mixtures I_A-I_D, II_A-II_D and III_A-III_D, ATP standards in three different concentrations were added to tubes containing reaction mixtures I,II and III, respectively (I_E-I_G, II_E-II_G and III_E-III_G tubes), ATP/ADP standards, ratio 4/1 to tubes (II_H-II_J) and ATP/AMP standards, ratio 4/1 to tubes (III_H-III_J). All standards were added to sterilized soil and treated as the soil samples before addition to the reaction mixtures (see Chapter C, page 37). The sterilized soil suspension served as a blank (I_K, II_K and III_K). To ensure that the sterilized soil did not contain measurable amounts of the nucleotides, blind values were also made from the extractant alone, treated as the soil samples (I_L, II_L and III_L). For determination of recovery of ATP, ADP and AMP, 0.5 ml of the different standards (ATP, ATP/ADP and ATP/AMP) were also added to sterilized soil suspensions in the tubes immediately before the neutralization step and incubated as the other standards for enzymatic conversion of ADP and AMP to ATP. The concentrations were equivalent to the standards passing the procedure and diluted (I_M-I_O, II_M-II_O and III_M-III_O). The recovery is showing the loss of standards during the procedure. However, the recovery percent was not used in the calculations because the standards were also added to the sterilized soil and treated as the soil samples. To ensure that the conversion of ADP and AMP to ATP were quantitative, ATP standards with sterilized soil were also added to tubes containing reaction mixtures II_E-II_G and III_E-III_G.

All reaction tubes were then incubated at 30°C for 30 min. The tubes were regularly shaken during the incubation.

The ATP content was measured in a Lumacounter M 2080 using a 0-10 s integration period after injection of 100 µl of luciferin-luciferase.

Procedure for determination of adenine nucleotides in soil samples.

Sets of four tubes were used for each of the determinations, labelled I_A-I_0, II_A-II_0 and III_A-III_0, respectively. The reaction mixtures, I, II and III (see page 170) were added (50 µl pr. tube) for determination of ATP, ATP + ADP, and ATP + ADP + AMP, respectively.

	(ATP)	(ATP + ADP)	(ATP + ADP + AMP)
Two different soil samples each in duplicates. Addition of 50 µl of the neutralized soil suspension	I_A	II_A	III_A
	I_B	II_B	III_B
	I_C	II_C	III_C
	I_D	II_D	III_D
Addition of 50 µl of ATP standards in three different concentrations (E-G). ATP standards have been treated as the soil samples together with sterilized soil	I_E	II_E	III_E
	I_F	II_F	III_F
	I_G	II_G	III_G
Addition of 50 µl of standard of ATP/ADP (II) and ATP/AMP (III), ratio 4/1, in three different concentrations (H-J). Standards have been treated as the soil samples together with sterilized soil	-	II_H	III_H
	-	II_I	III_I
	-	II_J	III_J
Blind; addition of 50 µl of sterilized soil suspension treated as the soil samples	I_K	II_K	III_K
Blind; addition of 50 µl of extractant agent treated as the soil samples	I_L	II_L	III_L
Recovery; not used in the calculations. Addition of standards of ATP, ATP/ADP and ATP/AMP added to sterilized soil suspension immediately before neutralization and incubation as the other standards	I_M	II_M	III_M
	I_N	II_N	III_N
	I_0	II_0	III_0

Calculation of results

Rectilinear ATP standard curves for each of the reaction mixtures were obtained by plotting counts of the light emission from the standards on the ordinate versus ATP concentrations on the abcissa. For the calibration of the samples, the counts of the sterilized soil suspensions were subtracted from the respective standards and samples. From these curves, the ATP content in each of the three reaction mixtures (ATP; ATP plus ADP; ATP plus ADP plus AMP) were determined, and the amounts of ATP enzymatically produced from ADP and AMP were calculated as differences between these measured values.

The AEC values were calculated from the equivalent concentrations of the adenine nucleotides determined by differences, using the formula $AEC = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$ (Atkinson & Walton, 1967). AEC can also be determined from the equation of Ball & Atkinson (1975), $AEC = \frac{\frac{1}{2}[(ATP) + (ATP + ADP)]}{[(ATP + ADP + AMP)]}$. The latter one has been used in order to reduce the propagation of errors.

The recovery was calculated from the loss of nucleotides during the procedures, assuming that the nucleotides added to the incubation tubes with sterilized soil gave 100 % recovery.

$$\text{Percentage recovery of ATP} = \frac{I_{E-G} \times 100}{I_{M-O}}$$

$$\text{Percentage recovery of ADP} = \frac{II_{H-J} \times 100}{II_{M-O}}$$

$$\text{Percentage recovery of AMP} = \frac{III_{H-J} \times 100}{III_{M-O}}$$

See the procedure at page 173

APPENDIX III

EXTRACTION OF ATP FROM BACTERIA AND FUNGI GROWN IN THE PRESENCE OF BENTONITE

Materials and methods

Experiments

A chemoheterotrophic aerobic, gram negative bacterium, Pseudomonas fluorescens, and a fungus Cladosporium sp., originally isolated from soil were used (Figs 4,5,6,9 & 10). A preculture period of the bacterium for 24 h, and 48 h for the fungus was performed at 25°C by shaking of Erlenmayer flasks (300 ml capacity) containing 100 ml of a glucose-mineral salt medium inoculated directly with cells from agar slant stock cultures (Difco Nutrient Agar, 23 g/l). Portions of 5 ml of the precultures were inoculated into 250 ml of a mineral salt medium that contained, per liter of distilled water, 1 g NH_4NO_3, 1 g $K_2HPO_4 \cdot 3H_2O$, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 20 mg $CaCl_2 \cdot 2H_2O$, 10 mg $FeCl_2 \cdot H_2O$, 0.5 g yeast extract and 2 g glucose. $K_2HPO_4 \cdot 3H_2O$, KH_2PO_4 and glucose were prepared separately. Bentonite (0.5 % and 1.0 % (w/v)) was added to some of the cultures. The solutions were sterilized by autoclaving for 20 min at 121°C, and the volumes were readjusted before the inoculation. The cultures were placed in a Voith Sapromat B 12 (J.M. Voight GMBH, Heidenheim a.d. Brenz) at 25°C and were stirred continuously during the incubation period.

Oxygen consumption was monitored continuously in the Sapromat and the other measurements were performed regularly over a period of 2 weeks. Two portions of 10 ml of the culture were removed from each of two flasks after 6 days of incubation, centrifuged at 3,000 g for 5 min, and the precipitate dried at 25°C for 1 and 2 weeks, respectively.

Bacteria were enumerated by the standard dilution plate count method using agar (Difco Bacto-Agar, 15 g/l) added to the above mentioned mineral medium. Two subsamples (1 ml portions) of the bacteria culture were taken from each of two flasks and four plates were inoculated from each of the dilutions (10^7-to 10^8-fold). All plates were incubated for 6 days at 25°C and the bacteria were counted. For conversion of the number of bacteria to biomass C, it was assumed that the dry weight of one bacterial cell is 0.2×10^{-6} µg and the cell contains 50 % C of the dry weight (Fig. 8).

The ATP content of bacteria and fungi was determined by the sulphuric acid-phosphate-NRB method (ref. X). Three portions of one ml of the cultures were taken from each of two flasks. The fungi were disrupted by 1 min of sonification, instead of 15 min of shaking. The ATP content was measured in a Lumacounter M 2080 (Lumac).

The influence of drying cultures of Ps. fluorescens with and without presence of bentonite on ATP content and number of bacteria was examined (Table 12). Preparation of the cultures and determination of ATP and number of bacteria are described above. In these experiments 10^2-to 10^8-fold dilutions were used. Before drying, the cultures were centrifuged (3,000 g for 5 min) the supernatant removed and the precipitate dried at 25°C for 1 and 2 weeks, respectively.

The recovery of ATP from sand and bentonite during a drying period was examined (Fig. 11). Solutions of 1.0 µg ATP (disodium

ATP) were added to 1 g portions of sterile washed sand and also to mixtures of sterile washed sand (75 %) and bentonite (25 %) (Sigma B 3378). As controls served the same mixtures added the corresponding amounts of sterilized water without ATP. All mixtures were adjusted to 63 % of d.wt. and then dried by incubation at 25°C in tubes closed with cotton. ATP and moisture contents were measured at 0,1,2,9 and 28 days of drying. Three replicate flasks with samples were analysed for ATP content and moisture content, respectively. ATP content was determined by the proposed procedure (ref. X) and moisture content by drying of the mixtures for 24 h at 105°C.

APPENDIX IV

ATP CONTENT OF SOILS AS INFLUENCED BY ENVIRONMENTAL FACTORS

Materials and methods

Effect of soil temperature on ATP

The ATP content was measured every 2 h during 24 h in April 1979 in a fallow garden soil (Fig. 12). Ten soil cores (28 mm dia.) were taken in the 0-3 cm, 3-10 cm and 10-20 cm depth and mixed into one sample from each of the layers. Three samples were analysed from each layer . Air and soil temperatures were measured by an electrical thermometer (Fluke®) and soil moisture by drying of soil samples at 105°C for 24 h. All measurements were performed immediately after sampling.

Soils were sampled (depth, 0-20cm) in April 1982 from Roskilde sandy loam and Jyndevad coarse sand (non irrigated and irrigated plots). The soils and field treatments are described by Andersen et al. (ref. IX) and some characteristics are given in Table 2. Shortly after sampling the ATP content of the soil samples was determined at the ambient field temperature of 5°C, and the soils were then incubated in loosely closed polyethylene bags for 5 days at 25°C and the ATP content measured again (Table 13). The water content was not adjusted in these experiments.

Roskilde sandy loam soil and Jyndevad coarse sandy soil (rotavated treatment) were sampled in the field experiment as mentioned above. Portions of 500 g were adjusted to 60 % w.h.c. and stored at 5°C for 60 days in loosely closed polyethylene bags and distilled sterilized water added when necessary. Afterwards ATP content and biomass C by the fumigation method were determined. Soil samples were then stored for 5 days at 5°C and 25°C, respectively, and the above mentioned analyses were started daily on subsamples during the 5 day period (Fig. 13). The results are means from triplicated samples.

The sandy loam soil (ploughed treatment; depth, 0-20 cm) was also sampled in March, 1983. It was divided into four aliquots to which distilled water was added to obtain water contents of 10 %, 16 %, 22 % and 28 % of dry soil. These corresponds to approximately 30 %, 50 %, 70 % and 90 % w.h.c.. The soil was thoroughly mixed, left overnight and then each aliquot was divided into 12 subsamples, about 250 g d.wt. each. Each subsample was placed in a loosely closed polyethylene bag and incubated at 5°C, 10°C or 15°C for 68 days and water being added when necessary (four different soil moistures x 3 different temperatures with three replicated samples in each treatment). The ATP content was measured and all the soil samples were then incubated at 25°C for another 5 days and the ATP contents were measured again (Fig. 14).

The CO_2 production was measured from different soil types (Jyndevad, coarse sand; Tylstrup fine sand; Roskilde sandy loam; Rønhave loam and Højer silty loam) (Fig. 15). The soils were sampled in March 1983 from the field experiment mentioned in ref. IX (ploughed treatment; depth, 0-20 cm). The soils were adjusted to 60 % of w.h.c., mixed, and after preincubation for 5 days in closed polyethylene bags at 5°C, 10°C, 15°C, 20°C and 25°C, respectively, triplicate samples each of 60 g wet weight were incubated in flasks over a 10 day period and CO_2 production was measured by titration (Eiland et al., 1979).

The ATP content of bacterial cells grown at different temperatures was examined (Table 14). Cells of Pseudomonas fluorescens were obtained by 24 h shake-incubation at 25°C of Erlenmayer flasks (300 ml capacity) containing 100 ml of a Bacto Nutrient Broth medium (8 g/l) inoculated directly with cells from an agar slant stock culture (Difco Bacto-Agar, 23 g/l). The culture (50 ml aliquots) was used to inoculate 500 ml portions of the Bacto Nutrient Broth medium contained in 2 l capacity Erlenmayer flasks. The flasks were incubated with shaking at 5°C, 10°C, 15°C, 20°C and 25°C, respectively, until the dead phase was reached. The flasks were closed with cotton plugs during the incubation. All procedures were performed under sterile conditions. Aliquots of the cultures at the different temperatures were removed at the time of the growth phases and analysed immediately for ATP and acridine orange stained bacteria. Growth was followed by measuring the turbidity of the culture at 420 nm. ATP concentrations were determined by the sulphuric acid-phosphate-NRB method (ref. X) as described at page 29 for determination of ATP from microbial cultures. Three portions of 1 ml from each of 2 flasks were analysed. The ATP content was measured in a Lumacounter M 2080 (Lumac). Acridine orange stained bacteria were measured in an epifluorescent microscope as described at page 192. Three portions of 1 ml from each of the flasks were analysed. Ten to twenty bacteria from each of twenty microscopic fields were counted.

Effect of soil moisture content on ATP

The effect of soil moisture content on ATP was examined on Roskilde sandy loam (ploughed treatment) and on Jyndevad coarse sandy soil (non irrigated ploughed treatment),(Fig. 15). The soils were sampled in May 1982 with 60 % of the w.h.c., divided into 500 g soil portions and adjusted to 0 %, 1 %, 8 %, 16 % and 26 % of moisture either by drying or by addition of distilled water. The soils without any moisture were obtained by drying soil samples at 105°C for 24 h. After incubation in loosely closed polyethylene bags for 5 days at 25°C with the adjusted moisture contents, ATP contents and CO_2 production were measured, the latter by titration (Eiland et al., 1979) after incubation over a period of 3 days. Two analyses from each of two replicate soil portions were analysed.

Effect of aerobic-anaerobic conditions in soil on ATP

Sandy loam soil (Roskilde) and coarse sandy soil (Jyndevad) sampled from the ploughed treatment (depth, 0-20 cm) in May 1982 were used to examine the effect of aerobic-anaerobic conditions on the ATP content in soil (Table 15). After soil incubation in loosely closed polyethylene bags for 5 days at 25°C with 60 % w.h.c., soil portions (60 g) from each soil type were placed in 6 flasks (300 ml volume) and the air in 3 of the flasks was replaced with helium. All the flasks were then incubated at 25°C for 2 days and the CO_2 production was measured in a Hewlett Packerd 5730A-gaschromatograph (TC detector, 50°C; Poropak® Q column; 800 mm x 2 mm; flow 30 ml He/min). All the soil samples were then frozen at -20°C for 3 h and the ATP content determined by adding 1 g portions of the frozen soil into cold acid extractant (ref. X).

Effect of freezing soil samples on ATP

The effect of freezing soils on ATP contents was examined on soils sampled in May 1981. The samples were stored moist (60 % of w.h.c.) in loosely closed polyethylene bags at 60 % w.h.c. for 90 days at 5°C by addition of sterilized distilled water when necessary , and the samples were then preincubated for 5 days at 25°C (Fig. 17). The ATP contents of the soils were then measured, and soil portions were frozen and kept at -20°C for 1, 7 and 14 days, respectively, and measured. Two soil samples from each of three replicated soils were analysed. The frozen soil samples were not allowed to thaw before the extractant for ATP extraction was added.

APPENDIX V

ATP CONTENT AS A MEASURE OF MICROBIAL ACTIVITY AND SIZE OF BIOMASS IN SOIL

Materials and methods

Correlations between ATP content and microbial activity and biomass in field soils (experimental data)

The microbial biomass and activity were measured in arable soils from Askov Experimental Station, where the effects of addition of farmyard manure, slurry or inorganic fertilizers to the soil were examined. The experimental conditions and methods used are given in refs IV, V and VI.

The influence of reduced cultivation and use of a catch crop were examined in field experiments regularly sown with barley, and with application of inorganic fertilizers only. The experiment was located on different soil types. Details on the experimental methods are given in ref. IX.

Belgian soils of different textures, with clay contents ranging from 6.1 % to 34.0 % and different organic matter contents, varying from 1.3 % to 6.3 % were studied. Preincubation lasted for 14 days at approximately 14^{o}C and 70 % of w.h.c..

Method of adjustment of experimental data

Many chemical and biological parameters in soil must be expected to be influenced by the environment. For example the content of NO_3-N may change from time to time due to supply of fertilizer or uptake of nitrogen indepently of the content of ATP. This can be illustrated with the following constructed example. Assuming that a high content of NO_3-N may be found early in the spring due to a recent supply of fertilizer and at the same time there is a relatively low content of ATP. At the next sampling time the NO_3-N may be reduced because part of the nitrogen has been taken up by the plants and at the same time the ATP content has increased.

In this example a negative correlation coefficient could be expected (see Fig. 27) even if there is a possitive correlation coefficient when each time is considered separately.

Therefore, to calculate one correlation coefficient, which could tell about the dependence between ATP and NO_3-N at both times, it is necessary to adjust for changes in the level of ATP and NO_3-N from sampling time 1 to sampling time 2.

The means of the NO_3-N and ATP contents from Fig. 27 are:

	Sampling time		
	1	2	1 and 2
NO_3-N	8.09	3.09	5.59
ATP	3.75	4.75	4.25

Therefore the adjustment can be performed by adding 2.5 to NO_3-N and subtracting 0.5 from ATP at sample time two; and by subtracting 2.5 from NO_3-N and adding 0.5 to ATP at sample time one. This is done in Fig. 27. Then if we only regards the adjusted values (circled points) we have a picture of the dependence between ATP and NO_3-N when the adjustment for changes in level of contents from time to time has been performed.

For the actual data arising from 4 experiments each with 4 sampling times, 3 treatments and 3 replications, the adjustments and the calculation of the correlation coefficient can be formulated in the following way:

$$x_{ijkl} = X_{ijkl} - (\bar{X}_{ij..} - \bar{X}....) = X_{ijkl} - \bar{X}_{ij..} + \bar{X}....$$

$$y_{ijkl} = Y_{ijkl} - (\bar{Y}_{ij..} \div \bar{Y}....) = Y_{ijkl} - \bar{Y}_{ij..} + \bar{Y}....$$

where,

i = 1,2,3 or 4 is number of experiments

j = 1,2,3 or 4 is number of sampling times

k = 1,2 or 3 is treatment number

l = 1,2 or 3 is replication (plot) number

n = 4 4 3 3 = 144 is total number of observations in plots

X_{ijkl} and Y_{ijkl} is the unadjusted values in experiment i at sample time j in the l th plot with treatment k.

x_{ijkl} and y_{ijkl} is the adjusted values in experiment i at sample time j in the l th plot with treatment k.

$$\bar{X}_{ij..} = \frac{1}{3 \cdot 3} \sum_{k} \sum_{l} X_{ijkl}$$

$$\bar{X}.... = \frac{1}{4 \cdot 4 \cdot 3 \cdot 3} \sum_{i} \sum_{j} \sum_{k} \sum_{l} X_{ijkl}$$

$$\bar{Y}_{ij..} = \frac{1}{3 \cdot 3} \sum_{k} \sum_{l} Y_{ijkl}$$

$$\bar{Y}.... = \frac{1}{4 \cdot 4 \cdot 3 \cdot 3} \sum_{i} \sum_{j} \sum_{k} \sum_{l} Y_{ijkl}$$

The correlation coefficient can then be calculated using the usually formulars, thus we have

$$r = \frac{\sum\sum\sum\sum (x_{ijkl} - \bar{x}....)\cdot(y_{ijkl} - \bar{y}....)}{\sqrt{\sum\sum\sum\sum (x_{ijkl} - \bar{x}....)^2 \cdot \sum\sum\sum\sum (y_{ijkl} - \bar{y}....)^2}}$$

where $\sum\sum\sum\sum$ denotes summing over all combinations of i,j,k and l.

$$\bar{x}.... = \frac{1}{4\cdot4\cdot3\cdot3} \sum\sum\sum\sum x_{ijkl} = \bar{X}....$$

$$\bar{y}.... = \frac{1}{4\cdot4\cdot3\cdot3} \sum\sum\sum\sum y_{ijkl} = \bar{Y}....$$

Because of the adjustments r does not have the usually n-2 degrees of freedom but has n-(4 4)-1 = 127 degrees of freedom reflecting the fact that we have estimated 16 means instead of only the usually over all mean. For the r-values based on the unadjusted values we have n-1-1 = 142 when data are available for all experiments, sampling times, treatments and replications.

If we assume normality the hypothesis of a true zero correlation coefficient can be tested. This is done using the critical values of student t-distribution with the degrees of freedom for the r value in quistion in (e.g. Table IV in Hald, 1967). Here t is calculated as: $t = r\sqrt{\frac{f}{1-r^2}}$, where f is the degrees of freedom for r.

The statistical method used is described by Bonnier & Tedin (1940; In Swedish) in chapter 13, see especially page 155, where hen-races are equivalent to the conbinations of time of soil samplings and experiments. The method of adjustment is also described in English by Snedecor & Cochran (1967) in chapter 14, although it is only for continuous covariables in the latter book. The correlation coefficients in Table 16 can be looked at as a type of weighed means of the individual correlation coefficients for each combination of time of sampling and different experiments.

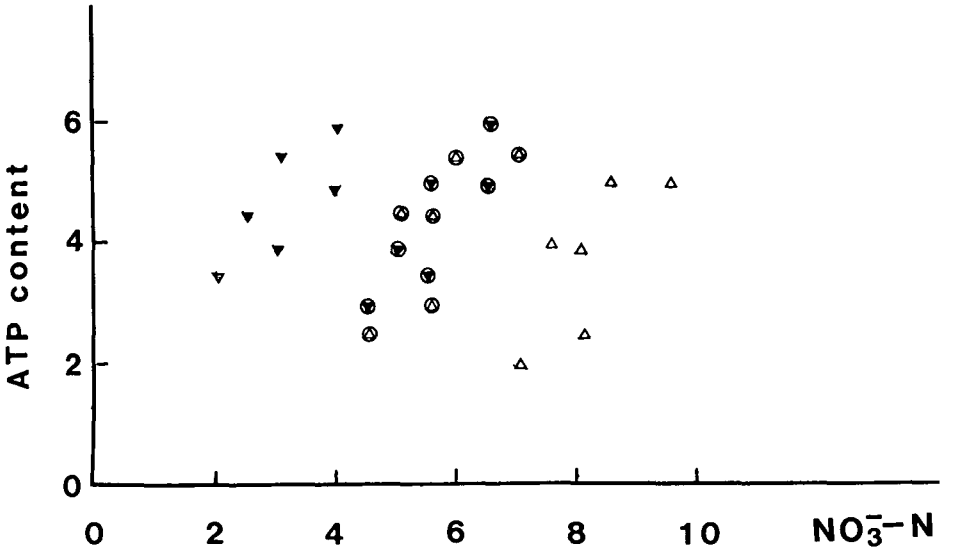

Fig. 27. Artifical data showing a negative correlation coefficient when all individual data from two sampling times are analysed without adjustment. A positive correlation coefficient is obtained after adjustment for sampling time.

△ = sampling time 1 unadjusted
▼ = sampling time 2 unadjusted
⊘ = sampling time 1 adjusted
⊛ = sampling time 2 adjusted

Calculation of standard error:

$$SAK_i = (X_{i1} - \bar{X}_{i\cdot})^2 + (X_{i2} - \bar{X}_{i\cdot})^2 \quad \text{(med 1 frihedsgrad)}$$

$$SAK_e = \sum_{i=1}^{n} SAK_i \quad \text{(med n frihedsgrader)}$$

$$s.e. = \sqrt{\frac{SAKe}{n} \cdot \frac{1}{2}}$$

where $\bar{X}_{i\cdot} = \frac{1}{2}(X_{i1} + X_{i2})$

n = number of treatments

X_{i1} and X_{i2} are the first and second recorded value from treatment i.

Comments to Fig. 12. From a statistical point of view, the sampling procedure has not been satisfactory. Ten soil cores were drawn from each depth and mixed into one sample. Three subsamples were taken from each depth and sampling time. When the soil in the cores are mixed to one sample, the field variation is removed. Then standard error becomes too small, when the variation between the tubes is not taken into account.

Some examples to illustrate the application of the ATP method

To assess the effects of different management practices (e.g. addition of slurry and farmyard manure to the soil and soil tillage methods), ATP content as determined in ref. X was used as a biomass measure (after preincubation of the soil for 5 days at 25°C with 60 % of w.h.c.) and the CO_2 production was used as an activity measure (Figs 21, 22 and 23).

The CO_2 production was started the day after sampling. The soil water was adjusted to 60 % of w.h.c. and soil portions (60 g) were incubated in 300 ml flasks over a 24 h period at 25°C. CO_2 production was measured in a Hewlett Packard 5730A gaschromatograph (TC detector, 50°C; Poropak® Q colomn, length 800 mm x dia. 2 mm; flow, 30 ml He/min). A measure of the activity is obtained which is not influenced by the soil temperature and moisture content at the sampling time. Soil samples were taken from each of 2 replicated plots. From each plot 8 cores (250 mm dia.) were taken at random and pooled to give one sample. Two subsamples were analysed per plot. The samples in the experiment with addition of organic manures were drawn from the 0-20 cm depth, as opposed to the samples in the experiment with ploughed and direct drilled treatments, which were taken in the 0-2 cm and 2-10 cm depth.

The field experiments at Askov and Lundgård Experimental Stations were initiated in 1972 to study the effects of heavy

applications of SLU and FYM on arable soil. The following amounts of SLU and FYM were used from the start of the experiment: 100 t/ha every year, 200 t/ha every two years or 400 t/ha every four years. These amounts are equivalent to 500 kg N/ha, 1000 kg N/ha and 2000 kg N/ha, respectively. Control plots, receiving 80 kg N/ha in NPK fertilizers were included in the experiments. The crop in 1984 recieved organic manures added in December 1983 at the two localities. Inorganic fertilizers were supplied in May 1984. The cropping system at Askov Experimental Station was a four years rotation of sugarbeets (1981), barley (1982), Italian ryegrass (1983) and barley (1984). At Lundgård, the four years rotation was barley (1981), sugarbeets (1982), barley (1983) and corn (1984). The soil temperature (10 cm depth) at the sampling time (8/3-1984) was 4°C for both localities. The experiment with direct drilled and ploughed treatments were initiated in 1981. The cropping system was continuous barley, wintherwheat or rye. The soil temperature at the sampling time (25/7-1983) was on an average of the three localities and 2 cm and 10 cm depths 21°C. The corresponding temperature for the soils sampled 22/5-1984 was 15°C.

Soil profiles

Vertical distribution of microorganisms was examined in two soil profiles. One soil profile was taken in arable land grown with continuous barley at Gudum, North Jutland. The samples were taken and stored in steel cylinders (480 mm length x 100 mm inner dia.) at 5°C for a few days and analysed. All samples were sealed in polyethylene bags in order to avoid air contamination and water loss. Soil used for the analyses was taken out from the inner section of the soil cores. All handlings were performed under sterile conditions.

Methods

ATP was extracted as described at page 29 to be able to measure small amounts. To improve the reproducibility in these samples, 100 µl of the neutralized soil suspension was added to 100 µl of NRB® instead of 50 µl soil supension added to 50 µl NRB®. The measurement was performed in a Lumacounter M 2080, which had a detection limit of less than 0.2×10^{-15} moles ATP.

Bacteria were enumerated by the standard dilution plate count method. The following plating medium was used: Quarter strength, Tryptone Soya Agar (TSA, Oxoid Ltd.), 10 g/l, with addition of extra 9.0 g/l Bacto Agar (Difco Lab.); Actidion (cycloheximide) 50 mg/l agar medium (0.25 g actidion was dissolved in 10 ml of a 95 % ethanol solution and 0.4 ml of this solution was added to 200 ml agar medium). Three subsamples of 1 g were taken from each examined depth and two dilutions (2×10^5 and 4×10^5-fold) were applied. The soil was diluted in sterile tap water containing 0.1 % peptone and 0.2 % calgone (sodiumhexametaphosphate). Further dilutions were performed in sterile tap water added 0.1 % peptone. Six plates were inoculated from each dilution. All plates were incubated for 6 days at 25°C.

FDA-stained fungi were determined mainly as described by Söderström (1977). Dependent of the soil depth 1-5 g soil portions were used and three soil samples were analysed from each depth. Each soil sample was suspended into 100 ml peptone-calgone water (peptone (0.1 %), calgone (0.2 %) and distilled water; sterilized) and homogenized at 20°C in a MSE ATO-mix run at half speed (approximately 6,000 rev/min) for 2 min. Söderström (1977) suggested the use of phosphate buffer; however, in these examinations, peptone-calgone water was applied in the first soil dilution and distilled water in the further dilutions. The same amount of hyphae were observed independent of the dilution liquid

used, and it was possible to use the same dilutions for counts of acridine orange stained bacteria. The soil suspension was transferred to a sterile flask and after soil particles were settled for 1 min, 5 ml of the suspension was pipetted into 50 ml of sterile distilled water with magnetic stirring (Stiromatic). Then 0.5-2.0 ml of the latter suspension was pipetted into 8 ml of sterile distilled water. The amount used depended on the number of hyphae in the preparation. In the final dilution 50 µl fluorescein diacetate (FDA) (Koch-Light Laboratories Ltd, Colnbrook, England) was added at a concentration of 10 µg/ml. Preparation of FDA was performed by dissolving of FDA in acetone (2 mg/ml) and storing at -20°C. The suspension was stained for 3 min and then filtered through a 0.8 µm black Millipore® filter kept in a Pyrex microanalysis filter holder. As a blind value surved sterilized distilled water treated as the soil suspension. The wet filter was placed on a glass slide with a drop of sterilized distilled water on the topside and covered with a coverglass. The preparation was immediately studied in an epifluorescent microscope with incident UV light (Reichert Fluovar with a HBO 50W Hg vapour burner, magnification 315x, objective 40x, NA = 0.90). A dichoic mirror was used. As primary filter surved a 3.5mmFITC490nm, and as secondary filter a 3mm(GG9 + OG515).

Both the length and the diameter of the hyphae were measured for determination of the specific biovolume. From each of the soil samples, 20 microscope fields were examined. For conversion to biomass C, the following factors were used: A density of 1.2 g/cm^3, dry weight of mycelium 15 % of wet weight and the carbon content was assumed to be 50 % of the dry weight.

Acridine orange stained bacteria were measured with an epifluorescent microscope as above (magnification 788x, with an oil immersion objective, 100x NA = 1.25). The soil samples and

the blind value (sterilized distilled water) were treated and diluted as mentioned for the FDA-stained fungi and the bacteria stained with acridine orange (AO, BDH Chemical Ltd) by adding 50 µl of a concentration of 67 µg AO/ml. After 5 min, the suspension was filtered through a dyed filter (Millipore® 0.22 µm) and the wet filter placed on a glass slide as for the FDA stained fungi and immediately studied in the microscope. The number of bacteria from twenty microscopic fields were counted.

The filter used has been dyed by dissolving 0.25 g Dyloncolour no. 8 (Ebony Black, Dylon International Ltd, London) and 0.25 g NaCl in 90-100°C of 100 ml sterile distilled water. This solution was filtered through a glass microfibre filter (Whatman, 55.0 mm dia. GF/A) and then through a 0.45 µm milliporefilter. The filters (0.22 µm) were stained for 5 min in the colour solution after the solution was heated to 60-70°C, and then rinsed in sterile distilled water and dried in an exicator on filter paper. The acridine orange solution (67 µg AO/ml) could be stored for at least 1 week at 5°C.

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