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# Impact of cycloheximide addition on adenylates in soil

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**Abstract**

Cycloheximide inhibits specifically the ribosomal protein synthesis of eukaryotic cells, i.e. the metabolism of soil fungi. We measured cycloheximide effects on adenylates in 20 different soils (0–10 cm depth) from arable, grass and forest land with a large variety of soil properties. The aims were (1) to assess the interactions between cycloheximide effects and soil properties and (2) to prove the relationship between cycloheximide effects on ATP and the ergosterol-to-microbial biomass C ratio, which is an indicator for the fungal proportion of the total microbial biomass. The adenylates ATP, ADP and AMP were measured 6 h after adding either 10 mg cycloheximide per gram soil in combination with 24 mg talcum per gram soil or 24 mg talcum per gram soil solely. The medians of the relative increases in AMP and ADP were 45 and 25% and the medians of the relative decreases in ATP and adenylates were −36 and −12%. These changes in adenylate composition lead to a cycloheximide-induced relative decrease in the adenylate energy charge level of 15%. The relative decrease in ATP content after cycloheximide addition was significantly correlated with the ATP-to-microbial biomass C ratio, but not with the ergosterol-to-microbial biomass C ratio. The absolute increase in ADP and the absolute decrease in ATP were affected by the clay content according to principal component analysis. The reduction of the ATP-to-microbial biomass C ratio indicates that this ratio had the potential of being an important ecotoxicological indicator of direct toxic effects of organic pollutants on soil microorganisms.


*Keywords:* ATP; ADP; AMP; Microbial biomass; Selective inhibition; Fungi; Ergosterol

## 1. Introduction

Cycloheximide inhibits specifically the peptidyl transferase activity of the 60 S subunit of eukaryotic 80 S-type ribosomes (Stryer, 1988). For this reason, cycloheximide has been used as a protein synthesis inhibitor for a large range of eukaryotic organisms in thousands of medical (Mukhopadhyay et al., 2002), zoological (Gossett et al., 1996), botanical (Chen et al., 2004) and mycological (Wellmann et al., 1996; Ali-Shtayeh et al., 1998) experiments, often with the intension of elucidating cell-internal physiological processes. In soil microbial ecology, cycloheximide was used by the selective-inhibition technique to inhibit fungal respiration after glucose addition (Anderson and Domsch, 1973).

Since its introduction, the selective inhibition technique has been used in a large variety of soils and experiments to estimate the relative proportion of fungi and bacteria in the total microbial biomass (Anderson and Domsch, 1975; Sakamoto and Oba, 1994; Blagodatskaya and Anderson, 1998; Nakamoto and Wakahara, 2004). The majority of the data indicate fungal dominance in most microbial communities, often contrasting the results of direct microscopic approaches (Stahl et al., 1995; Klein et al., 1998). The strong divergence between different methodological approaches and a variety of inherent methodological problems has led to an ongoing discussion, especially with respect to the completeness, the specificity and the interference of soil properties in the inhibitory effect of cycloheximide (Badalucco et al., 1994; Velvis, 1997; Bailey et al., 2003).

Cycloheximide was not only used to inhibit glucose-induced respiration, but also arginine ammonification (Lin and Brookes, 1999), $N_2O$ and $NO_3^-$ formation (Castaldi and Smith, 1998), denitrifcation and codenitrification (Laughlin and Stevens, 2002), and utilization pattern of aromatic compounds (Marwati et al., 2003). Although it is well known that cycloheximide affects ribosomal protein formation and thus a membrane-dependent ATP-generating

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system, nothing is known about specific cycloheximide effects on ATP in soil, but also on the total adenylate pool, including ATP, ADP and AMP. However, Autry and Fitzgerald (1993) used ATP after cycloheximide addition only as a microbial biomass indicator for analysing the prokaryotic contribution to organosulfur formation in forest soil, without investigating the specific effects of cycloheximide on the ATP-to-microbial biomass C ratio. In the present study, we measured cycloheximide effects on adenylates in different soils with a large variety of soil properties with the aim of (1) assessing the interactions between cycloheximide and soil properties and (2) proving the relationship between cycloheximide effects on ATP and the ergosterol-to-microbial biomass C ratio as an indicator for the fungal proportion of the total microbial biomass (Djajakirana et al., 1996; Bååth and Anderson, 2003).

## 2. Materials and methods

### 2.1. Soil sampling and analysis

Soil samples from 20 areas surrounding Göttingen (Germany) were collected in March 2003. The soils belonged to three types of ecosystems: arable soils, forest soils, and grassland soils. They were taken with a spade to a depth of about 10 cm; the overlying organic layer was removed before the samples were taken. All field moist soil samples were sieved (<2 mm), homogenized and stored in polyethylene bags at 4 °C, until the analyses started. Clay was measured by Andreasen–Koehn analysis (Schlichting et al., 1995). Soil pH was measured in 25 ml of distilled water added to 10 g of soil. Subsamples of dried soil were finely ground in a ball mill. Total C and total N were determined using an Elementar Vario Max analyser. Soil organic C was measured as total C minus carbonate C, which was measured gas-volumetrically after the addition of 4 M HCl (Schlichting et al., 1995).

### 2.2. Soil microbial properties

Microbial biomass C was estimated by fumigation–extraction (Vance et al., 1987). Two portions equivalent to 25 g soil were taken from the soil sample, one portion was fumigated for 24 h at 25 °C with ethanol-free $CHCl_3$. Following fumigant removal, the soil was extracted with 100 ml 0.5 $K_2SO_4$ by 30 min horizontal shaking at 200 rev $min^{-1}$ and filtered. The non-fumigated portion was extracted similarly at the same time fumigation commenced. Organic C in the extracts was measured as $CO_2$ by infrared absorption after combustion at 850 °C using a Dimatoc 100 automatic analyser (Dimatec, Essen). Microbial biomass C was $E_C/k_{EC}$, where $E_C$ = (organic C extracted from fumigated soils) − (organic C extracted from non-fumigated soils) and $k_{EC} = 0.45$ (Wu et al., 1990).

Ergosterol was measured in 2 g soil; the sample was extracted with 100 ml ethanol for 30 min by oscillating shaking at 250 rev $min^{-1}$ (Djajakirana et al., 1996). Quantitative determination of ergosterol was performed by reversed-phased HPLC analysis using 100% methanol as the mobile phase and a resolution in detection of 282 nm.

Measurements of adenine nucleotides and calculations of the adenylate energy charge (AEC) were made according to the procedure of Bai et al. (1988) as described by Dyckmans and Raubuch (1997). A moist sample equivalent to 3 g oven-dry soil was extracted with a mixture of 4 ml dimethylsulfoxide (DMSO), 16 ml buffer (10 mm $Na_3PO_4$-buffer + 20 mM EDTA adjusted to pH 12 with KOH). Then, 500 µl of this suspension was mixed with 500 µl benzalkonium chloride solution (Martens, 2001) in a centrifuge tube and mixed for 5 s in an ultrasonic bath. After derivatisation with chloracetaldehyde, the adenine nucleotides were determined by HPLC. The chromatography was performed isocratically with 50 mm ammonium acetate buffer containing 1 mm EDTA and 0.4 mm TBAHS mixed with methanol (89.5/10.5, v/v) as the mobile phase. Fluorometric emission was measured at a wavelength of 410 nm with 280 nm as the excitation wavelength.

### 2.3. Cycloheximide addition

Two treatments were carried out at 50% water holding capacity: (1) 3 g soil + 24 mg talcum per gram soil and (2) 3 g soil + 10 mg cycloheximide per gram soil + 24 mg talcum per gram soil. Adenylates were determined 6 h (25 °C in the dark) after the amendments were carefully mixed into the soil. The concentration of cycloheximide and incubation time was chosen as optimum conditions according to Lin and Brookes (1999) tested in a previous study (Raubuch et al., 2004). The adenylate energy charge (AEC) was (ATP + 0.5ADP)/(ATP + ADP + AMP). The effects of cycloheximide on adenylates were calculated as the absolute difference (adenylate with cycloheximide addition − adenylate without cycloheximide addition) or as the relative difference in % [(adenylate with cycloheximide addition − adenylate without cycloheximide addition) × 100]/(adenylate without cycloheximide addition).

### 2.4. Statistical analysis

The results presented in the tables and figures are expressed on an oven-dry soil basis (about 24 h at 105 °C). They are arithmetic means of duplicate analyses (texture and soil chemical analyses) and triplicate analyses (soil biological properties). Data were log-transformed for statistical analysis if they did not fit to a normal distribution. All statistical calculations, such as linear regression analysis, principal component analysis (orthotran/varimax transformation), and analysis of variance were carried out using StatView 5.0 (SAS, Inc.). Cycloheximide effects on soil adenylates were assessed by a one-way ANOVA for