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Use of Heterotrophic and Cyanobacterial Nitrogen Fixation To Study the Impact of Anthropogenic Substances on Soil Biological Processes

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Because of its rapidity and sensitivity, biological nitrogen fixation (BNF) has been studied widely in the laboratory and in the field since the invention of the acetylene reduction assay (ARA) (Hardy et al. 1973). The basis for the assay is the fact that nitrogenase, the enzyme complex in diazotrophic microorganisms that reduces nitrogen to ammonia, also reduces acetylene to ethylene (Schöllhorn and Burris 1967). The main objection is that it is an indirect measure of BNF, since no exact relationship exists between nitrogen fixation and acetylene reduction (Mayne 1984, Mårtensson and Ljunggren 1984). Nonetheless, ARA has been used in ecological studies on BNF, including studies in aquatic systems on bluegreen algæ, heterotrophic nitrogen fixing bacteria in soil, and legume symbiotic systems (Granhall and Lundgren 1971; Nohrstedt 1982; Wivstad et al. 1987). In particular, interesting field studies of ARA have been made on the effects of various pollutants on BNF activities by heterotrophic nitrogen fixing microorganisms and cyanobacteria (Lundkvist 1970; Skujins et al. 1986; Brookes et al. 1986; Mårtensson and Witter 1990).

Recently, increased environmental concern in society has resulted in a need for rapid and sensitive methods to study the impact of various anthropogenic substances, such as pesticides and heavy-metals, on soil biological systems. Some of these potentially hazardous substances may be applied intentionally to soil systems, such as agrochemicals, whilst others, for instance are air-borne and unintentionally deposited. The intended test systems for assessing effects on soil biological processes caused by environmentally hazardous chemicals have to include sensitive processes/organisms and will replace the conventional toxicity testing methods based on chemical analysis or in vitro tests of higher organisms.

An extension of these test systems will be to monitor the development of various soil systems in a longer perspective and on an international basis. This study reports the use of free-living heterotrophic diazotrophic soil microorganisms and soil surface colonizing cyanobacteria to determine impact of anthropogenic substances in soil.

MATERIALS AND METHODS

The soils used originated from a long-term field experiment at Ultuna, central Sweden 60°N, 17°E begun in 1956. The geological origin of the soil at the field

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experiment is a uniform post-glacial clay (clay content 35%, silt 44%, fine sand 21% and a pH of 6.6, organic content 1.5% in 1956). The design of the experiment is 4 blocks, with 2 m strips in between, plot size 2 x 2 m, separated by wooden frames sunk into the soil to a depth of 20 cm. Fourteen treatments are included, randomized within each block. The treatments include additions of different types of organic matter, with or without additional inorganic nitrogen fertilizer and three types of inorganic nitrogen fertilizers. Every second year the amount of organic materials added is equivalent to 4 metric tons ash-free organic matter ha^{-1}. The N-fertilizer plots receive 80 kg N $ha^{-1}yr^{-1}$, P and K are added to achieve equal applications to all fertilized plots. The crop rotation consists mainly of cereals and oilseed crops, with a minimum of pesticides applied, during the last decade the experiment has been treated once with 2 kg glyphosate ha^{-1} to suppress couch grass. As a result of these practises, great variabilities with respect to soil pH and C- and N-contents of the treatments have evolved since 1956, although the geological origin remains unaltered, Table 1. The differences obtained have been utilized in the following studies when investigating anthropogenic impact on BNF. The treatments represented in Table 1, including calcium cyanamide fertilization, calcium nitrate fertilization, farmyard manure amendment, peat addition, straw addition and the treatment without any fertilization, were sampled to a depth of 8-10 cm on two occasions in September and October 1991, all blocks being sampled. Each sample consisted of ca 0.5 kg wet weight of soil. The soil was sieved (2mm) in the field and immediately used for microbiological studies on effects of anthropogenic substances.

Chloride-salts of Cu, Ni and Zn in aqueous solutions were used. The agrochemicals studied are presented in Table 2 and were used as commercial formulations dissolved in water.

Nitrogen fixation by free-living biological nitrogen fixing microorganisms was determined. Fresh soil samples corresponding to 50 g dry weight of soil were placed in 100-mL polystyrene beakers fitted with a perforated lid. The heavy-metals and agrochemicals studied were added by gentle and uniform wetting of the soil surface with an aqueous solution containing the studied substance and supplied with glucose to give an addition of 2.5 mg glucose g dry weight of $soil^{-1}$. The water contents were set equal, and ranged between 28-35%, additions of sterile tap water being made if necessary. The soil was then gently and carefully mixed with a spatula, sealed with the perforated lid and incubated in darkness 25°C for 36 h. The soil samples were then placed in glass jars (300-mL) fitted with gas sampling

Table 1. Soil pH, total C and N content 35 years after the start of the long-term field experiment at Ultuna, mean values ± S.E. of four randomized replicates.

Treatment	pH	%C	%N
Calcium cyanamide ($CaCN_2$)	7.41±0.06	1.35±0.09	0.17±0.01
Calcium nitrate $Ca(NO_3)_2$	6.75±0.08	1.34±0.06	0.16±0.01
Farm Yard Manure	6.56±0.08	1.92±0.04	0.21±0.01
Peat	5.60±0.18	2.43±0.09	0.18±0.01
Straw	6.37±0.10	1.49±0.09	0.17±0.01
Unfertilized	6.16±0.08	1.16±0.08	0.14±0.01

Total heavy metal content of soils (μg g^{-1}): Cd not detectable, Cu 28±1, Ni 23±3, Pb 19±1, Zn 69±2.

ports. 10% of the apparent gas volume was replaced with acetylene and the soils were incubated in dark, 25°C, for 24-48 hours. ARA determined as ethylene formation was detected at intervals. Finally, the presence of endogenous ethylene formation was detected through the addition of 0.5% carbon monoxide to each sample, and incubating a further 6 h period (Nohrstedt 1983), followed by ethylene detections. The possible difference in ethylene production between the two treatments was considered to originate from spontaneous ethylene production from, for example, various fungi.

Cyanobacterial nitrogen fixation was studied by taking fresh soil samples from the long-term field experiment consisting of 20 g dry weight of soil and placing these in plastic Petri dishes (60 mm diameter). A cover-glass was placed on the soil surface. Three out of four blocks from the long-term field experiment were used for soil sampling. Heavy-metals and agrochemicals in aqueous solutions were added to the soil by pipetting and gently and carefully mixing the soil with a spatula. The Petri dishes were kept in permanent light and watered when necessary with 1:50 Stanier's medium (Stanier et al. 1971). The development of ARA was followed by repeated short-term incubations at intervals of 2 weeks during a period of 2.5 months. ARA was determined by placing each Petri dish in a glass jar (300-mL) supplied with a gas sampling port. The glass jars were sealed and 10% of the atmosphere was replaced with acetylene. Incubations were made for 1-2 h at room temperature, in light, before sampling for ethylene. The cover-glasses were examined microscopically for presence of bluegreen algæ. The ARA values obtained when maximum ARA was recorded in the control, after 7 weeks of incubation, were used when analyzing the experiments. The cover-glasses were examined microscopically for presence of bluegreen algæ.

Table 2. Agrochemicals used in the experiments.

Name	Active ingredient	Mode of action	Recommended dose (kg ha^{-1}) corresponding to a concentration in topsoil of ($\mu g\ g^{-1}$)
Fungicides			
Benomyl	Methyl 1-(butylcarbamoyl)-2-benzimidazolyl carbamate	Inactivates fungal hyphæ	0.5-4
Mancozeb	[Ethylene bis(dithio-carbamato)]-manganese plus zinc ion	Inhibits function of fungal microtublules	1-10
Herbicides			
Chlorsulphuron	2-chloro-N-[[84-methoxy-6-methyl-1,3,5-triazin-(2yl)amino]carbonyl]benzene-sulfonamide	Inhibits synthesis of acetolactate synthase	$(1\text{-}10)\cdot 10^{-3}$
2,4-D	(2,4-dichlorophenoxy)acetic acid	Auxin-derivate	0.5-3.5
Glyphosate	N-(phosphonomethyl)-glycine	Inhibits synthesis of aromatic aminoacids	1-4

RESULTS AND DISCUSSION

The highest potential nitrogen fixation measured as ARA by the free-living BNF was obtained in the soil fertilized with calcium cyanamide with a pH of 7.4 and the lowest ARA was found in the peat-amended soil with a pH of 5.6. A correlation between ARA and soil pH was evident, r^2=0.836, Fig. 1 A. ARA was independent of soil C and N contents. The highest cyanobacterial nitrogen fixation was obtained in soil fertilized with calcium cyanamide and the lowest activity was obtained in unfertilized soil, Fig. 1 B. ARA and soil pH were again correlated (r^2=0.745). No relationship between ARA and soil C or soil N was found.

In an initial experiment, the effects of different additions of Cu, Ni and Zn to the experimental soils on ARA of free-living BNF were studied. Additions of Cu were equalized to give 50, 75 and 125 ppm Cu in the soils considering an initial content of 28 ppm. Additions of Ni corresponded to 75, 125 and 175 ppm Ni with respect to an inital concentration of 23 ppm. Additions of Zn corresponded to soil concentrations of 250, 450 and 850 ppm Zn with respect to an initial concentration of 69 ppm Zn. At a soil content of 75 ppm Cu , 75 ppm Ni and 450 ppm Zn, significant decreases in ARA by heterotrophic BNF occurred in all soils (P<0.010). Adverse effects of Cu and Ni were independent of soil pH, and C and N content of the soils when comparing minimum inhibitory concentration of the heavy-metals in all soils with soil characteristics (Figure 2 A). Negative effects on ARA by Ni were elevated with decreasing soil pH (r^2=0.601), but independent of soil content of C and N. Since the observed effects of Cu and Zn were independent of important soil chemical parameters such as pH, C and N, it is concluded that these are useful as internal standards when studying effects of free-living diazotrophic soil bacteria and cyanobacteria in various soils by anthropogenic substances.

In a subsequent experiment, other anthropogenic substances, i. e.,agrochemicals including fungicides and herbicides, and their effects on free-living BNF, were studied. The effects on ARA by free-living BNF in the presence of the agrochemicals studied differed. Of the fungicides studied, benomyl and mancozeb, mancozeb proved to be more hazardous to the soil heterotrophic BNF, additions of 400 ppm benomyl, which is equal to 100 times recommendations,

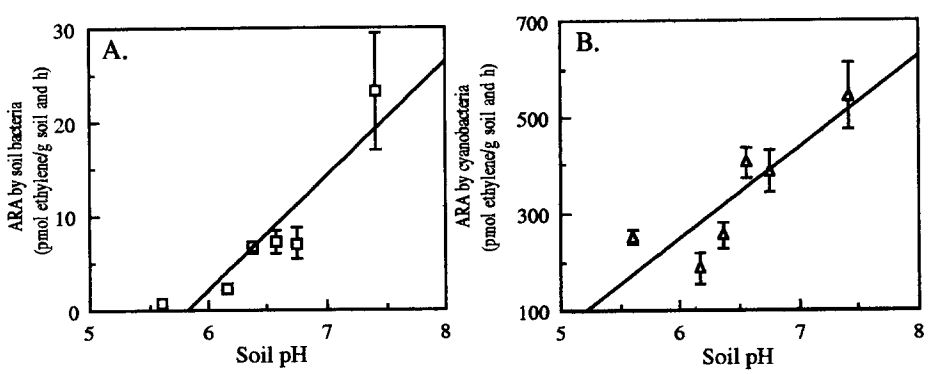

Figure 1. The relationship between soil pH and nitrogen fixation activity (ARA) by heterotrophic free-living soil bacteria (A) and cyanobacteria (B).

assuming 1 μg active substance·g^{-1} dry weight of soil corresponds to an addition of 1 kg active substance·ha^{-1} (Fletcher 1956), significantly reduced heterotrophic BNF in all soils ($P<0.010$). The other fungicide, mancozeb, significantly negatively affected the heterotrophic BNF at levels equal to 40 times recommendations corresponding to a soil concentration of 400 ppm mancozeb ($P<0.010$). The negative effects on ARA by the fungicide benomyl were independent of soil pH, C and N content. However, the effects of the other fungicide, mancozeb, were elevated at decreasing soil pH ($r^2=0.537$), but independent of C and N content (Figure 2 B).

Also the three herbicides studied, chlorsulphuron, 2,4-D and glyphosate, affected the free-living BNF. The negative responses of BNF differed between substances. Comparing them, 2,4-D was most harmful, significant reduction of ARA occurring at 6 times recommended application rates, chlorsulphuron was intermediate, with effects occurring at 20 times recommended concentrations, and glyphosate less harmful with effects occuring at 100 times recommendations ($P<0.010$). Negative effects on ARA by chlorsulphuron were correlated with soil pH ($r^2=0.657$), which is unexpected since the degradation of chlorsulphuron will increase at low pH through hydrolysis (Mårtensson and Nilsson 1989). The adverse effects of 2.4-D and glyphosate were independent of soil pH and C and N content of soil (Figure 2B).

The effects of additions of anthropogenic substances to soil on nitrogen fixing soil surface colonizing blue-green algal populations were studied by adding different amounts of the heavy metals Cu, Ni and Zn to the experimental soils. Additions of Cu were equalized to give 50, 75 and 125 ppm Cu in the soils considering an initial content of 28 ppm. Additions of Ni corresponded to 75, 125 and 175 ppm Ni with respect to an inital concentration of 23 ppm. Additions of Zn corresponded to soil concentrations of 250, 450 and 850 ppm Zn with respect to an initial concentration of 69 ppm Zn. At 125 ppm Cu and at 450 ppm Zn respectively, cyanobacterial nitrogen fixation was significantly reduced and found to be independent of pH, C- and N- contents of the soils. Nickel was inhibitory at 125 ppm Ni ($P<0.010$). The adverse effects of Ni on cyanobacterial ARA were

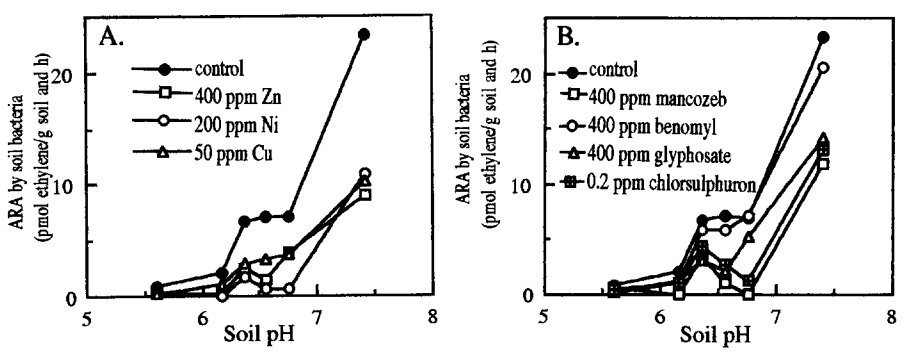

Figure 2. The relationship between soil pH and the effects of additions of Cu, Ni, and Zn (A) and the fungicides benomyl and mancozeb and the herbicides chlorsulphuron and glyphosate (B) on the nitrogen fixing activity (ARA) by free-living heterotrophic soil bacteria.

dependent of soil pH (r^2=0.681) but independent of soil C and N (Figure 3 A). This observation is similar to that found for the heterotrophs above, which makes the additions of either Cu or Zn as an internal standard recommendable when studying effects of various anthropogenic substances on cyanobacteria in different soils. No differences in variability of bluegreen algal species were observed microscopically between treatments.

Additions of agrochemicals including fungicides (benomyl, mancozeb) and herbicides (chlorsulphuron, 2,4-D, glyphosate) and their effects on cyanobacterial ARA were studied. The effects on ARA by cyanobacteria with regard to their disposition towards the fungicides and the herbicides differed. Minimum inhibitory concentrations of cyanobacterial ARA were obtained at 0.2 ppm chlorsulphuron, 200 ppm 2,4-D, 800 ppm glyphosate, 800 ppm benomyl and 400 ppm mancozeb, which corresponds to 20 times (chlorsulphuron), 60 times (2.4-D), 200 times (glyphosate), 200 times (benomyl) and 40 times (mancozeb) ($P<0.010$). The adverse effects on cyanobacterial ARA by herbicides were unaffected by soil pH and soil C and N. Considering the fungicides, similar to the heterotrophic BNF, the negative effects by the fungicide mancozeb were elevated at decreasing soil pH (r^2=0.590) (Figure 3 B). None of the negative effects on cyanobacterial ARA by the anthropogenic substances studied was dependent on soil organic matter or soil nitrogen status. No differences in variability of bluegreen algal populations were observed microscopically between treatments.

Comparing the sensitivity of heterotrophic BNF and cyanobacteria, heterotrophic nitrogen fixers triggered a response at lower concentrations of additions of heavy-metals than the bluegreen algæ. Negative effects by the heavy-metals on heterotrophic nitrogen fixation were determined at a soil content of 75 ppm Cu , 75 ppm Ni and 450 ppm Zn ($P<0.010$). The cyanobacteria, however, were reduced at 125 ppm Cu, 125 ppm Ni and 450 ppm Zn ($P<0.010$), which indicates that the cyanobacteria are less sensitive than the heterotrophs with respect to response at increasing concentrations of heavy metals. This has also been shown elsewhere when studying the impact of heavy-metal contaminated sewage sludge in arable soils (Mårtensson and Witter 1990). Considering the pesticides,

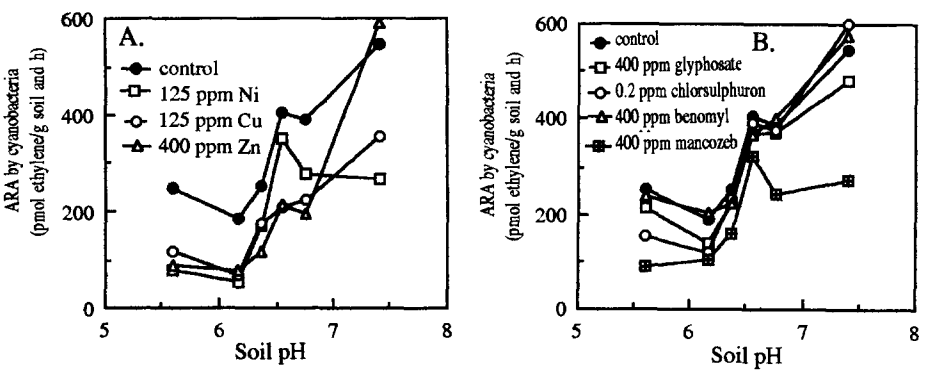

Figure 3. The relationship between soil pH and the effects of additions of Cu, Ni, and Zn (A) and the fungicides benomyl and mancozeb and the herbicides chlorsulphuron and glyphosate (B) on the nitrogen fixing activity (ARA) by cyanobacteria.

the heterotrophic nitrogen fixing processes were again more sensitive than the bluegreen algæ towards most substances except for mancozeb, which triggered a responses on BNF at 100 times recommendations on the heterotrophic BNF but on the cyanobacteria at 40 times recommendations. Reasons for the greater tolerance of the bluegreen algæ against agrochemicals may be that microbial degradation of the agrochemicals occurs before the establishment of the bluegreen algal populations, which takes at least 1 month under optimum growth conditions, i.e., permanent light and humid conditions which probably also will favour the microbial degradation. In general, pesticide concentrations must exceed recommended concentrations before negative effects occur. Heavy metals, however, must be observed since adverse effects occur at levels below or close to recommended critical values. However, if the aim is to evaluate hypothetically hazardous substances, both processes, i.e., heterotrophic BNF and cyanobacteria, are recommendable to use because of the different biochemical reactions in the two groups of organisms.

A suitable way to study impact of anthropogenic substances on heterotrophic nitrogen fixing soil bacteria and cyanobacteria is to use additions of Cu and Zn as internal standards, the effects being independent of pH, C and N contents of the soil. The heterotrophic nitrogen fixation was affected at lower concentrations of studied substances than the cyanobacteria. In general, pesticide concentrations must exceed recommended concentrations before negative effects occur. Heavy metals, however, must be observed since adverse effects occur at levels below or close to recommended critical values. Investigating effects of different agrochemicals and heavy-metals in various soils on soil heterotrophic nitrogen fixing bacteria and cyanobacteria soil C and N content are less regulating but pH will elevate adverse effects.

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