

## Effect of the nitrification inhibitors dicyandiamide, nitrapyrin and thiourea on *Nitrosomonas europaea*

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### Abstract

Effects of the inhibitors dicyandiamide (DCD), nitrapyrin (N-Serve, NS), and thiourea (TU) on growth and metabolism of *Nitrosomonas europaea* were tested under laboratory conditions. Growth of a pure culture was completely suppressed by 10 ppm NS and 0.5 ppm TU; inhibition by 300 ppm DCD was 83%. Ammonia oxidation and respiration of *Nitrosomonas* cell suspensions were reduced by 93% (10 ppm NS), 95% (0.5 ppm TU), and 73% (300 ppm DCD).

Hydroxylamine oxidoreductase was not affected by high concentrations of inhibitors (200 ppm DCD, 100 ppm NS, 100 ppm TU). Cytochrome oxidase activity was increased by 10% with 200 ppm DCD, not affected by 100 ppm TU, and inhibited by 52% with 100 ppm NS.

The modes of action thus differed among the tested inhibitors. The merely bacteriostatic effect of dicyandiamide was demonstrated by the reversibility of growth inhibition.

### Introduction

Nitrogen in mineral and organic fertilizers is only partly utilized since losses by  $\text{NH}_4^+$  fixation and, following nitrification, by denitrification and nitrate leaching occur during the non-growing season [1, 6]. One possibility to reduce losses especially by denitrification and nitrate leaching is the inhibition of the first step of nitrification by so-called nitrification inhibitors [3, 7].

The following investigations were conducted to examine the influence of the inhibitor dicyandiamide in comparison with nitrapyrin (N-Serve, NS), and thiourea (TU) (Table 1).

### Material and methods

#### Pure culture

*Nitrosomonas europaea* was obtained from the American Type Culture Collection (Strain No.

19.718) and cultivated in the following nutrient solution (ATCC-medium 221, modified).

$(\text{NH}_4)_2\text{SO}_4$	3.0 g	$\text{CaCl}_2 \times 2 \text{H}_2\text{O}$	0.004 g
$\text{K}_2\text{HPO}_4$	0.5 g	Fe (Chelate)	0.1 mg
$\text{MgSO}_4 \times 7 \text{H}_2\text{O}$	0.05 g		

dissolved in 1 l distilled  $\text{H}_2\text{O}$   
 pH 8.2 (adjusted after autoclaving with 5%  $\text{Na}_2\text{CO}_3$  solution)

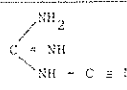
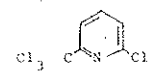
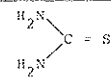
#### Growth rate in pure culture

After inoculation 300 ml Erlenmeyer flasks with 80 ml nutrient solution were shaken for 12 days on a rotary shaker (ca. 90 rpm) in a dark chamber (26°C). Main growth parameter was production of nitrite.

#### Cell suspensions

After approximately 14 days of accumulation in

Table 1. Nitrification inhibitor their formular, chemical and commercial product names

active agent	formula	chem. name	commercial product
dicyandiamide		1-cyanoguanidine	SEW - DIBIN
nitrapyrin		2-chloro-6-(trichloromethyl)-pyridine	N-SERVE
thiourea		thiocarbamide	TU

pure culture (with several pH corrections) the bacteria were centrifuged, washed twice with 0.1 M potassium phosphate buffer (pH 7.6), and resuspended in 20 ml of the same buffer. Protein was then determined according to Lowry [10]. The suspension was kept at 4°C and used within one day.

#### Ammonium oxidation of cell suspensions

amount of suspension: equivalent to 100 µg protein  
 substrate: 100 µM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>  
 inhibitor: 100, 200, 300 ppm DCD  
                   1, 5, 10 ppm NS  
                   0.1, 0.2, 1 ppm TU  
 0.1 M Tris/HCl-buffer, pH 8.0  
 total volume: 5 ml  
 6 replicates, 23°C

#### Respiration in cell suspensions

amount of suspension: equivalent to 150 µg protein  
 substrate: 50 µM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>  
 inhibitor: 100, 200, 300 ppm DCD  
                   1, 10 ppm NS  
                   0.5 ppm TU  
 0.1 M Tris/HCl-buffer, pH 8.0  
 total volume: 2.5 ml  
 6 replicates, 28°C  
 (Warburg apparatus, model V 166, Braun, Melsungen)

#### Enzyme activities

##### Hydroxylamine oxidoreductase:

*Nitrosomonas europaea* is capable of reducing animal cytochrome c. Hydroxylamine, the first metabolite of ammonium oxidation, serves as a substrate (electron donor). The electrons are transported by the enzyme hydroxylamine oxidoreductase.

experimental conditions:

1 cm cell, total volume 3.0 ml  
 0.15 ml Cyt c (2%, Sigma Type II a)  
 0.5 mM hydroxylamine  
 amount of cell suspension: equivalent to 150 µg protein  
 inhibitor: 200 ppm DCD, 100 ppm NS, 100 ppm TU  
 0.1 M Tris/HCl-buffer; pH 8.0  
 5 replicates, 25°C

The increase of extinction at 550 nm, which corresponds to the increase of reduced cytochrome c was measured for 5 min in a double beam spectrophotometer with temperature controlled cuvette holders. The reference cell contained buffer.

##### Cytochrome oxidase:

*Nitrosomonas europaea* can make use of reduced cytochrome c as an electron donor; the electrons are then transferred to oxygen by a cell-borne cytochrome oxidase.

experimental conditions:

1 cm cell, total volume 3.0 ml  
 0.15 ml Cyt c (2%, Sigma type II a)

reduced with  $10^{-5}$  M sodium ascorbate  
amount of cell suspension: equivalent to 150  $\mu$ g  
protein

inhibitor: 200 ppm DCD, 100 ppm NS,  
100 ppm TU, 0.1 mM KCN (equiva-  
lent to 217 ppm)

0.1 M Tris/HCl-buffer; pH 8.0

5 replicates, 25°C

Enzyme activity was measured as the decrease of  
extinction at 550 nm for 5 min. The reference  
cell contained oxidized cytochrome c.

#### Bacteriostatic effect of DCD

After inoculation of pure cultures (300 ml Erlen-  
meyer flasks with 80 ml nutrient solution), sam-  
ples were separated between untreated (control)  
and DCD-treated ones (3 replicates each). DCD  
treatment (100 ppm) was employed at the begin-  
ning of logarithmic growth and resulted in a  
retarded nitrite formation.

When the control samples reached the station-  
ary phase, untreated as well as DCD-treated  
bacteria were transferred to a fresh, DCD-free  
medium (50 ml Erlenmeyer flasks with 20 ml nu-  
trient solution). In this second experiment (again  
with 3 replicates) it was tested whether the DCD  
treated bacteria are able to recover from the  
inhibitor treatment by producing the same  
amounts of nitrite as the untreated bacteria in  
the control. Nitrite production was measured  
after five days of incubation.

## Results

### Growth in pure culture

Figures 1, 2 and 3 illustrate the growth of *Nitro-  
somonas europaea* (control) in pure culture. The  
initial (lag) phase was very long and the main  
growth phase did not start before 5 or 6 days.  
The stationary phase was reached when the con-  
centration of nitrite in nutrient solution amount-  
ed to approximately 35 mg N/l. This halt in  
growth was not caused by the high nitrite values,  
but by the acidification of the nutrient medium;  
pH values decreased from 8.0 to 5.8. Bacterial  
numbers as determined in a Neubauer counting  
cell were very low. They increased in control

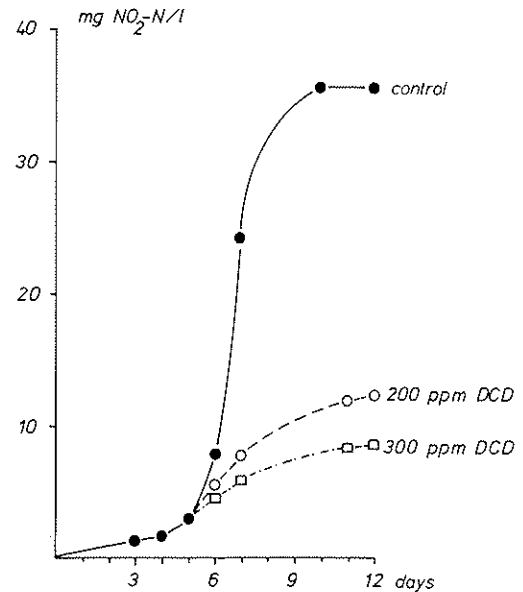


Fig. 1. Effect of dicyandiamide on growth of *Nitrosomonas europaea* in pure culture.

samples from  $1.5 \times 10^6$  to  $7.5 \times 10^6$ /ml during  
the main growth phase. By applying a nitrifica-  
tion inhibitor at the beginning of the lag-phase,  
the growth curve more or less flattens depend-  
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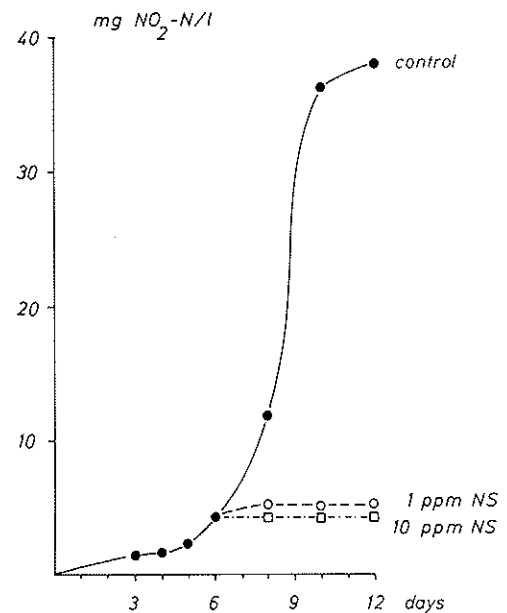


Fig. 2. Effect of N-serve on growth of *Nitrosomonas europaea* in pure culture.

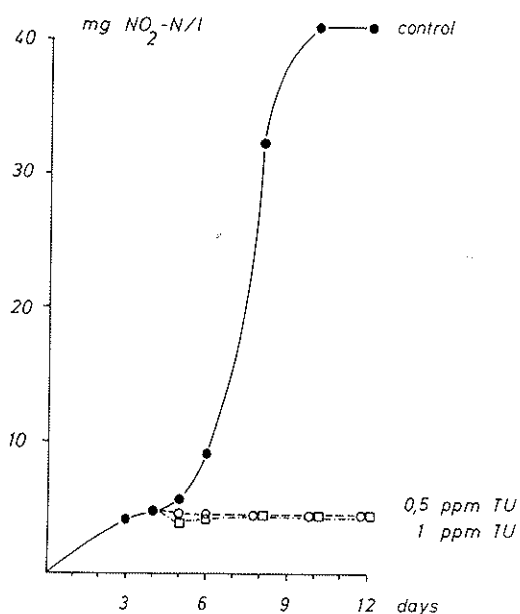


Fig. 3. Effect of thiourea on growth of *Nitrosomonas europaea* in pure culture.

DCD, NS and thiourea (Figs. 1, 2, 3) were very effective inhibitors. However, the application rates necessary for an inhibiting effect varied. While concentrations of 10 ppm N-serve or even of 1 ppm thiourea were sufficient, growth inhibition by 200 ppm DCD was only 71%; it could be increased to 83% with 300 ppm.

#### Ammonium oxidation in cell suspensions

The decrease in activities of inhibitor-treated cell suspensions is given in Table 2. With 100 ppm DCD, inhibition was only 56%. Increased concentrations of DCD (200 and 300 ppm) were even more effective in inhibiting nitrite production. Inhibition by N-serve and thiourea increased with increasing concentrations of the inhibitor. Thiourea was very effective in inhibiting nitrite production, and 1 ppm TU completely inhibited nitrite formation.

#### Respiration of cell suspensions

A clear inhibition of respiration was observed with inhibitors (Table 3). An increase in the amount of DCD increased the inhibition of respiration significantly. N-serve and thiourea again

Table 2. Production of NO<sub>2</sub><sup>-</sup>-N by *Nitrosomonas europaea* cell suspensions within 2 hours

Treatment	mg/l	%
Control	10.97	100
100 ppm DCD	4.85	44
200 ppm DCD	3.68	34
300 ppm DCD	3.25	30
L.S.D. <sub>1%</sub>	0.42	
Control	11.32	100
1 ppm NS	2.42	21
5 ppm NS	0.58	5
10 ppm TU	0.20	2
L.S.D. <sub>1%</sub>	0.58	
Control	11.37	100
0.1 ppm TU	0.53	5
0.2 ppm TU	0.35	3
1 ppm TU	0	0
L.S.D. <sub>1%</sub>	0.27	

Table 3. Oxygen consumption by *Nitrosomonas europaea* cell suspensions within 2 hours

Treatment	μl O <sub>2</sub>	%
Control	276	100
100 ppm DCD	117	42
200 ppm DCD	97	35
300 ppm DCD	75	27
L.S.D. <sub>1%</sub>	15	
Control	273	100
1 ppm NS	32	12
10 ppm NS	18	7
0.5 ppm TU	14	5
L.S.D. <sub>1%</sub>	20	

were highly effective even at low doses. As little as 1 ppm NS inhibited respiration by 88%, and 0.5 ppm TU almost completely stopped respiration.

#### Activity of hydroxylamine oxidoreductase

For enzymatic investigations, concentrations of N-serve and thiourea were increased to 100 ppm because no effect of 100 ppm was observed in preliminary experiments.

The enzyme activity of treated cells and untreated control samples was nearly identical (Fig. 4). Only the increase of extinction with thiourea was significantly higher than with dicyandiamide or N-serve.

reduced with  $10^{-5}$  M sodium ascorbate  
amount of cell suspension: equivalent to 150  $\mu$ g  
protein

inhibitor: 200 ppm DCD, 100 ppm NS,  
100 ppm TU, 0.1 mM KCN (equiva-  
lent to 217 ppm)

0.1 M Tris/HCl-buffer; pH 8.0

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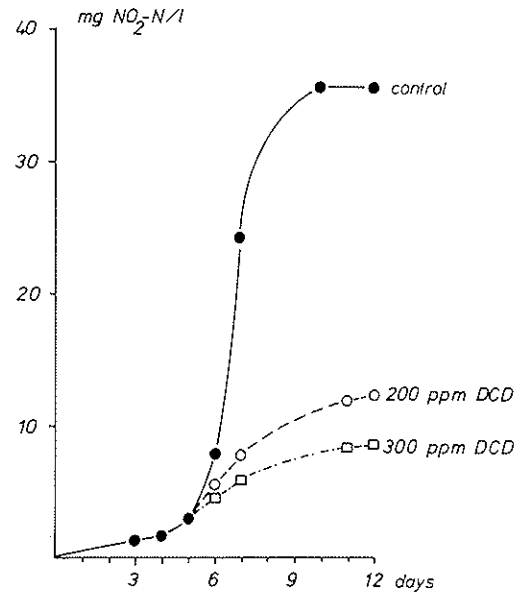


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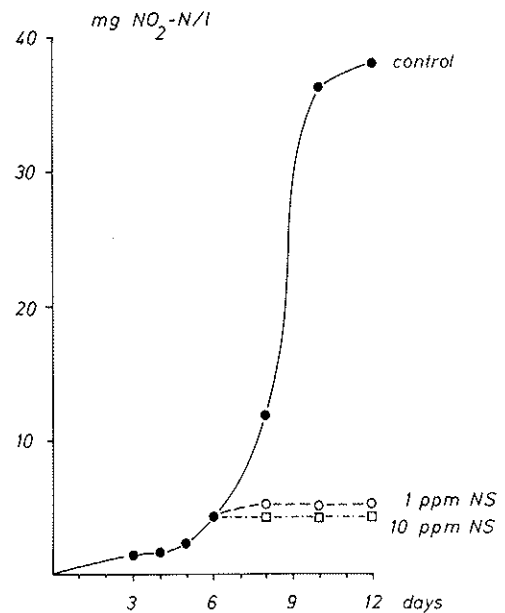


Fig. 2. Effect of N-serve on growth of *Nitrosomonas europaea* in pure culture.

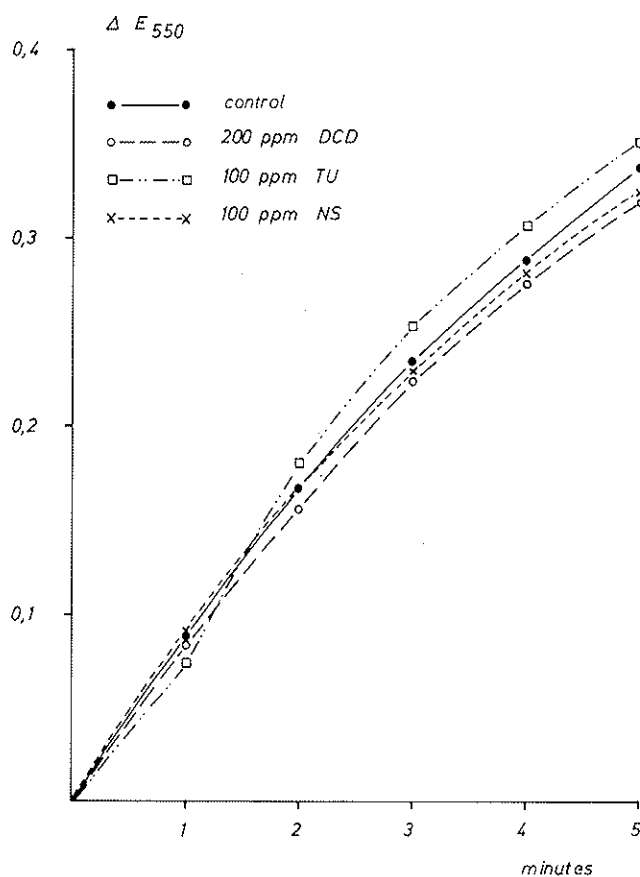


Fig. 4. Effects of inhibitors on reduction of cytochrome c in *Nitrosomonas europaea* cell suspensions.

#### Activity of cytochrome oxidase

In addition to nitrification inhibitors, 0.1 mM potassium cyanide was included in order to compare a known specific cytochrome oxidase inhibitor with the nitrification inhibitors. Fig. 5 clearly indicates, that N-serve and potassium cyanide, but not DCD and thiourea, reduce the enzyme activity.

N-serve and potassium cyanide resulted in a significant inhibition of 52 or 64%, respectively. Thiourea did not give significant effects. With dicyandiamide, the activity of the cytochrome oxidase was increased.

#### Bacteriostatic effect of DCD

Dicyandiamide evidently had a bacteriostatic effect when used at concentrations of 100 ppm (Table 4).

In the first experiment, addition of 100 ppm DCD resulted in lower nitrite production (by 61%). In the second experiment, DCD-treated bacteria taken from the first experiment into a DCD-free medium showed a strong nitrite production which was only 11% lower than in the control.

The growth inhibition by N-serve and thiourea in the first experiment was so severe that no active inoculum could be obtained for the second trial. A test similar to that described for dicyandiamide was therefore not possible.

#### Discussion

The investigated inhibitors differed in many respects, particularly with regard to effective doses and mode of action. Growth of *Nitrosomonas europaea* in pure culture as well as metabolic

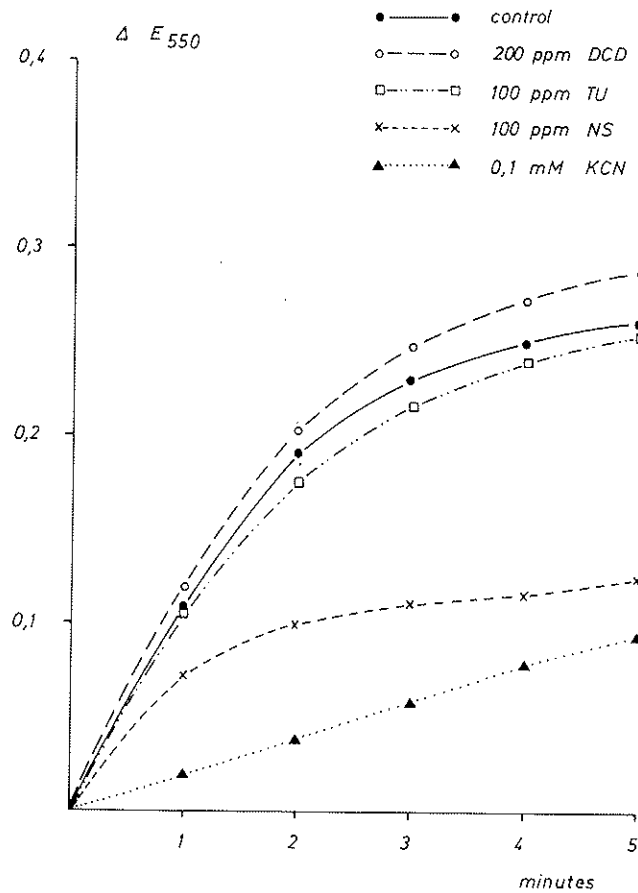


Fig. 5. Effects of inhibitors on oxidation of cytochrome c in *Nitrosomonas europaea* cell suspensions.

activity of cell suspensions could be retarded only by relatively high DCD-concentrations. However, even then ammonium oxidation and respiration were not completely inhibited and growth was not abruptly stopped after application. Concentrations of 200 to 300 ppm DCD correspond to customary application rates of 25 to 35 kg/ha in the field calculated as follows: Assuming an incorporation zone of normally 6–8 cm of soil, 30 kg DCD/ha is approximately equivalent to a concentration of about 30 mg DCD/kg soil. Since 1 kg soil usually contains around 200 ml of available soil water, the DCD concentration amounts to 150 ppm in the soil solution (i.e. 30 mg DCD/kg soil = 150 mg DCD/l soil solution). This concentration is higher with lower soil moisture or when using granules.

Furthermore, it has to be considered that in

Table 4. Nitrite formation by *Nitrosomonas europaea* during (experiment I) and after (experiment II) DCD-treatment

	I		II	
	Measured after 6 days mg NO <sub>2</sub> <sup>-</sup> -N/l	%	Measured after 5 days mg NO <sub>2</sub> <sup>-</sup> -N/l	%
Control	36.4	100	36.7	100
DCD	14.1	39	32.8	89
L.S.D. <sub>1%</sub>	7.0		13.4	

our laboratory experiments conditions for growth of *Nitrosomonas* were optimal, and in pure cultures densities of 10<sup>5</sup>–10<sup>7</sup> bacteria/ml were obtained. In the soil, population densities are only between 10<sup>2</sup> and 10<sup>4</sup>/ml [2, 13].

Nuti [12] obtained complete growth inhibition with 10 ppm DCD-sulfate. N-serve was described as highly toxic for NH<sub>4</sub><sup>+</sup> oxidizing bacteria with effective concentrations ranging from 0.05 ppm to 20 ppm [6]. Other authors [4, 12] obtained

complete growth inhibition of *Nitrosomonas* in pure culture by 0.2 ppm NS. 1 ppm NS is regarded as sufficient for an effective inhibition in soils [5, 9].

By measuring ammonium oxidation and respiration, it could be clearly shown that oxidation of the substrate was reduced or completely stopped under the influence of inhibitors, thiourea being the most efficient one with only 1 ppm required for complete inhibition. As a consequence, the flow of electrons slowed down, thus, the gain of energy by respiratory phosphorylation was lower. This greatly impaired metabolism especially since *Nitrosomonas* as such has already a low energetic efficiency and depends on high metabolic turnover rates.

With respect to enzyme activities, thiourea at a high concentration of 100 ppm surprisingly exerted no influence on hydroxylamine oxidoreductase, consequently no complexation of Fe atoms of the hemo-groups or of cytochrome P-460 occurred.

It is also remarkable that dicyandiamide did not inhibit cytochrome oxidase. It may therefore be concluded that dicyandiamide impairs the uptake or utilization of substrate but not the flow of electrons in the respiratory cycle if  $\text{NH}_4^+$  is missing and electrons are supplied by another donor. This result contradicts other authors [12] who obtained an inhibition by approximately 50% with 100 ppm of DCD-sulfate. Dicyandiamide did inhibit the metabolic turnover of *Nitrosomonas europaea* (Fig. 1 and Table 2). However, when measuring cytochrome oxidase, only an electron donor and no real substrate (ammonium sulfate) was supplied.

The inhibition of cytochrome oxidase by N-serve was already demonstrated earlier [4]. These authors, however, obtained a 70% inhibition with 1.5 ppm NS. Still, it can be established that N-serve has a different mode of action than dicyandiamide and thiourea.

By proof of its bacteriostatic effect dicyandiamide fulfills one of the main requirements for a nitrification inhibitor. The sometimes reported classification of dicyandiamide as a "nitrifide" cannot be accepted on the background of our results. The bactericidal effect of N-serve and thiourea could not be demonstrated by us due to technical problems. Thiourea has been classified

earlier as very toxic for  $\text{NH}_4^+$ -oxidizing bacteria [8]. Lethal effects on *Nitrosomonas sp.* have been suspected [11] since cells treated with 1 ppm TU did not take up nitrite production even after 4 weeks in fresh medium. Presumably irreversible enzyme damages by chelation are the reason for the high toxicity on *Nitrosomonas sp.* [14]. These reports agree well with our own observations. A toxic (bactericidal) effect of N-serve and thiourea would explain why these two substances were so effective at low concentrations especially in nutrient solution. Without soil-borne influences (e.g. adsorption to organic matter, degradation) the toxicity can fully take effect. The different effect of dicyandiamide as compared to N-serve and thiourea was also evident from the form of the growth curve. With 300 ppm DCD, growth was not halted abruptly but the curve ended in a gentle bow (Fig. 1) while with 10 ppm NS and 0.5 ppm TU the curve was interrupted immediately after application, and continued horizontally until the end of the experiment (Fig. 2 and Fig. 3).

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