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Effect of Heavy Metals on the Denitrification Process in Soil[1]

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ABSTRACT

The effect of Cd, Cu, Pb, and Zn on denitrification of three *Pseudomonas*-species in a liquid culture medium (Giltay) and in autoclaved soil, as well as on the denitrifying activity of native soil, was investigated. In Giltay medium, Cd in concentrations starting at 50 μg/ml strongly inhibited growth and denitrification of an unidentified *Pseudomonas* sp., *P. aeruginosa*, and *P. denitrificans*, while Zn and Pb did not influence the denitrifying activity even up to 500 μg/ml. Cu at 50 μg/ml inhibited denitrification of *P. aeruginosa* and the unidentified *Pseudomonas* sp.; however, it had no influence on *P. denitrificans*. When the three bacteria were inoculated into autoclaved soil their reaction to Cd and Cu was similar to that in Giltay medium, but the accumulation of nitrite was considerably higher. Zn, which showed no effect in the liquid medium at 500 μg/ml, inhibited the denitrifying activity of all bacteria in the soil at this concentration.

Denitrification in native (nonautoclaved) soil was also inhibited by the addition of increasing amounts of heavy metals. This was indicated by an accumulation of nitrite and nitrous oxide which appeared to a lesser degree and for a shorter time period in nontreated samples.

Additional Index Words: **heavy metal toxicity, nitrite accumulation, nitrous oxide formation, inhibition of denitrification.**

Heavy metals are inherent components of soils but today's concern is related to their accumulation from man-made applications. Although the average level of heavy metals can be quite low, their probable inhibitive or toxic impact on the metabolic activity of living systems or, in particular, to the natural microflora and microfauna in the soil can have a deleterious effect on the quality of the soil and, consequently, on nutrient cycling and soil fertility. There is only limited information on the specific physiological effects of heavy metals on microbes (7, 9, 10). Consequently, their precise impact under various environmental conditions is not yet clear.

It was the aim of this study to investigate the influence of heavy metals on the microbial growth under anaerobic conditions which are often predominant [illegible] the biota of the soil environment. To our knowled[illegible] such investigation has been reported. Since many [illegible] microorganisms are capable of adapting to the denitrification process for respiratory purposes, this physiological activity was essentially examined in order to determine the heavy metal effect on microbes in the absence of oxygen. Since denitrification causes the reduction of nitrate to nitrite and nitrogenous gases, this investigation includes the possible impacts of heavy metals on the nitrogen cycle.

Extrapolation of results from pure microbial cultures to the natural environment has often been questionable, but the complexity of a natural ecosystem such as soil does not often allow us to assign the formation of a certain product to a specific physiological process. Therefore, various systems were used to investigate the effect of cadmium (Cd), copper (Cu), lead (Pb), and Zinc (Zn) on denitrification. The experiments were performed with pure bacterial cultures in a growth medium and in autoclaved soil under anaerobic conditions. This approach, as outlined in a previous publication, [illegible] should clarify whether the change from a chemically defined liquid to the solid and complex structure of soil causes an essential change in the denitrifying pattern of the bacteria—in this case, to the additions of heavy metals. Since an inoculated, autoclaved soil is still quite different from a natural soil, the experiments were also done by incubation of native soil from which extrapolation to field conditions appear more feasible.

MATERIALS AND METHODS

The denitrifying bacteria used in the experiments originated from the following sources. The unidentified *Pseudomonas* sp. was isolated from Hublersburg silt loam (2), *P. aeruginosa* was obtained from the culture collection of the Department of Microbiology at the Pennsylvania State University, and *P. denitrificans* from Dr. C. C. Delwiche, University of California, Davis. The bacteria were transferred from nutrient agar to liquid Giltay medium and grown for 2 days at [illegible] before they were used for inoculation of liquid media or autoclaved soil. Giltay medium consisted of 1.0 g KNO_3 (140 μg NO_3^--N/ml), 1.0 g l-asparagine, 8.5 g Na-citrate, 1.0 g KH_2PO_4, 1.0 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2 \cdot 6H_2O$, and 0.05 g $FeCl_3 \cdot 6H_2O$ in 1 liter distilled water. The pH was adjusted to 7.2 with NaOH. Each bottle containing the

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liquid medium (50 ml) including the heavy metals was inoculated with one loop of bacterial culture. Growth of the bacteria was measured by determining optical density at 550 nm with a Bausch and Lomb Spectronic 20 spectrophotometer. For all experiments, 125-ml Pyrex bottles were used which were sealed with one-hole rubber stoppers equipped with cylindrical rubber septa for gas flushing and gas sampling. The heavy metals used were $Cd(NO_3)_2 \cdot 4\ H_2O$, $Zn(NO_3)_2 \cdot 6\ H_2O$, $Pb(NO_3)_2$, and $Cu(NO_3)_2 \cdot 3\ H_2O$.

The soil used, native or autoclaved, was a Hagerstown silt loam with a pH of 6.75, which was air-dried and passed through a 2-mm sieve prior to the experiments. The indigenous NO_3^--N concentration was 65 μg/g soil; organic carbon, 1.8%; and the sand-, silt-, and clay-contents were 8.5, 63.4, and 28.1%, respectively.

For the native soil experiments 20 g of soil were introduced into each bottle and thoroughly mixed with 7 ml of distilled water which contained 135 μg nitrate/g soil (in order to achieve a total of 200 μg NO_3^--N/g soil) and the desired amount of heavy metals. For the autoclaved soil experiments the bottles containing 20 g of soil were autoclaved three times at 121°C for 45 min. at 24-hour intervals. Before the third autoclaving, 4 ml of distilled water containing 135 μg NO_3^--N/g soil and the desired amount of heavy metals were added to the soil samples. After the autoclaving process was complete, each flask was inoculated with 3 ml of bacterial cultures.

Three replicates were included for each treatment, and each experiment was repeated at least once; the reported results represent average values. Samples without heavy metals as well as noninoculated culture media and autoclaved soil served as controls.

For preparation of anaerobic conditions, a syringe needle was inserted in the septum and helium at 0.56 to 0.703 kg/cm² (8 to 10 psi) was flushed through the flasks for 1 min. After decreasing the pressure of helium to 0.14 kg/cm² (2 psi) the bottles were stoppered tightly allowing a slight positive pressure in order to avoid air contamination. Atmospheric contamination could easily be detected by gas chromatographic analysis.

Gas samples of 500 μl were withdrawn from each flask with a gas-tight syringe and analyzed in a gas-chromatograph (Varian Aerograph, model 1820) using two parallel columns (2 mm I.D.) at 50°C simultaneously. Porapak Q (600 cm, 50–80 mesh) separated CO_2 and N_2O and Molecular Sieve 5 A (450 cm, 45–60 mesh) N_2 and O_2. The filament current was 200 milliampere (mA) for the Porapak Q and 150 mA for the Molecular Sieve 5 A column. Helium was used as a carrier gas at a flow rate of 40 ml/min under 3.16 kg/cm² (45 psi). Dual thermal conductivity cells at 200°C served as detectors. The peak areas were integrated automatically (Varian Aerograph, model 477) and the gas quantities calculated from standard gas curves. Increased pressure in bottles rsulting from gas production was measured by a manometric device, and the total gas volume and amount of detected gases were calculated accordingly.

Nitrate-N, NO_2^--N, and pH were determined in the liquid media after removal of the cells and in the soil after extraction with distilled water at a ratio of 40 ml of 20 g air-dried soil for 30 min on a 'Wrist-Action' shaker. The soil was separated from the aqueous phase by centrifugation.

Nitrate-N disappearance was measured with a nitrate electrode (Orion Research, Inc., Cambridge, Mass.) after destroying interfering nitrite by addition of a 2% sulfamic acid solution. Nitrate-N production was determined colorimetrically by the α-naphthylamine-sulfanilic acid procedure (1).

RESULTS

Three *Pseudomonas* species were selected to investigate the effect of Cd, Cu, Pb, and Zn on the denitrification process of isolated bacteria. *P. aeruginosa* and *P. denitrificans* reduced nitrate to nitrogen gas, while the end-product for the nonidentified *Pseudomonas* sp. was N_2O (4).

Effect of Cd, Cu, Pb, and Zn on Denitrification in a Liquid Growth Medium

Different concentrations of the heavy metals were added to Giltay medium and, after several days of incubation under anaerobic conditions, NO_3^--N disappearance, NO_2^--N formation, evolution of gases, change of pH, and growth were measured. Growth and denitrification of the *Pseudomonas* sp. were strongly inhibited by Cu and Cd; Cu was already highly effective at 2 μg/ml while Cd showed clear inhibition at 5 μg/ml and complete suppression of growth and nitrate reduction was observed at 50 μg/ml (Table 1). Zinc and Pb had virtually no effect on denitrification of the *Pseudomonas* sp. at concentrations of up to 500 μg/ml, as indicated by the formation of N_2O.

P. aeruginosa was also inhibited by Cd and Cu if incubated for 3 days under anaerobic conditions, but Pb and Zn did not influence growth and denitrification at concentrations of up to 400 μg/ml (Table 2). It was interesting to observe that Cd had an inhibitory influence at 10 μg/ml, which caused the accumulation of 25 μg/ml of nitrite, while Cu interfered with denitrification at higher concentrations. This interference resulted in the formation of nitrite which apparently could not be further reduced to N_2 at 50 μg/ml Cu.

Table 1—Effect of different concentrations of Cd, Cu, Pb, and Zn on denitrification by a *Pseudomonas* sp. in Giltay medium (anaerobic incubation for 4 days at 28°C).

	Conc.	Nitrogen as NO_3^-	Nitrogen as NO_2^-	Nitrogen as N_2O†	Nitrogen as N_2	Total detected†	CO_2	Growth (OD 550 nm)	pH
	μg/ml								
Noninoculated		145.6	0	0	0	145.6	0	0	7.2
Inoculated		2.9	0.1	103.4	0	106.4	8.2	0.16	8.4
Cd	5	26.5	0.1	84.1	0	110.7	11.8	0.16	8.3
	10	74.6	0.1	54.4	0	129.1	16.5	0.11	8.1
	25	117.6	0.1	20.5	0	138.2	14.3	0.06	7.6
	50	147.3	1.2	0.3	0	148.8	2.6	0.01	7.2
Cu	2	111.4	tr	21.2	0	132.6	10.6	0.06	7.7
	5	127.0	tr	10.9	0	137.9	10.8	0.03	7.5
	10	126.8	tr	7.2	0	134.0	9.8	0.02	7.4
	25	135.0	tr	3.4	0	138.4	7.9	0.02	7.3
Pb	50	8.1	1.5	100.5	0	110.1	7.3	0.16	8.4
	100	10.9	1.5	95.5	0	107.9	8.9	0.18	8.4
	250	13.2	1.2	93.2	0	107.6	10.9	0.25	8.3
Zn	50	2.8	0.8	105.9	0	109.5	9.5	0.16	8.4
	100	5.2	0.1	103.4	0	108.7	11.5	0.17	8.3
	250	7.8	0.1	97.8	0	105.7	27.0	0.17	8.0

† No corrections were made for the solubility of N_2O and consequently it was not possible to detect the total N at an increasing pH with the applied analytical methods.

Table 2—Effect of different concentrations of Cd, Cu, Pb, and Zn on denitrification of *Pseudomonas aeruginosa* in Giltay medium (anaerobic incubation for 3 days at 28°C).

	Conc.	Nitrogen as NO_3^-	Nitrogen as NO_2^-	Nitrogen as N_2O	Nitrogen as N_2	Total N detected	CO_2	Growth (OD 550 nm)	pH
		μg/ml							
Noninoculated		139.4	0	0	0	139.4	0.4	0	7.2
Inoculated		1.8	0	0	140.5	142.3	14.8	0.44	8.3
Cd	10	33.5	25.0	0	62.2	120.7	27.9	0.26	8.2
	25	61.7	14.1	0	43.9	119.7	31.0	0.18	8.0
	50	121.3	1.2	0	1.1	123.6	20.9	0.03	7.4
	100	135.3	0.1	0	0	135.4	0.5	0.005	7.3
Cu	5	2.2	0	0	131.5	133.7	18.7	0.30	8.2
	10	1.8	0	0	130.4	132.2	20.6	0.24	8.2
	25	7.8	20.0	0	101.5	129.3	42.7	0.15	8.1
	50	117.3	20.9	0	0	138.2	15.6	0.04	7.2
Pb	50	2.0	0	0	139.8	141.8	20.3	0.54	8.3
	100	1.9	0	0	138.7	140.6	22.8	0.47	8.2
	200	2.2	tr	0	136.5	138.7	22.8	0.56	8.2
	400	2.1	tr	0	139.7	141.8	33.9	0.60	8.1
Zn	50	2.3	tr	0	141.0	143.3	24.1	0.41	8.3
	100	2.1	tr	0	142.4	144.5	27.2	0.40	8.2
	200	2.0	tr	0	138.4	140.1	41.6	0.42	8.1
	400	2.1	tr	0	182.7	184.8	69.5	0.46	7.9

P. denitrificans (Table 3) proved to be much more resistant to the inhibitory effect of the heavy metals at the investigated concentrations than the other two bacteria. Only Cd, at a concentration of 100 μg/ml, affected denitrification and caused the accumulation of nitrite, but growth was already reduced at lower concentrations. This appears to indicate that the suppression of denitrification is not correlated only to growth.

The formation of CO_2 could not always be related to growth. It has been previously shown that, under certain conditions, if the denitrification process was reduced or suppressed, a corresponding increase in CO_2 formation took place (3). There is no clear explanation for this observation.

Effect of Cd, Cu, Pb, and Zn on Denitrifiers in Autoclaved Soil

When autoclaved soil was inoculated with the three *Pseudomonas* species and incubated under anaerobic conditions, an inhibition of denitrification was observed at most levels of all the heavy metals tested. In control soil, to which no heavy metals had been added, practically all nitrate was volatilized to N_2O (unidentified *Pseudomonas* sp.) or N_2 (*P. aeruginosa* and *P. denitrificans*) during 4 days of incubation. The soil supplied with Cd, Cu, Pb, and Zn starting at 10 μg/g soil showed an inhibition of denitrification, as indicated by the remaining amount of NO_3^--N or the accumulation of NO_2^--N and N_2O (Fig. 1).

Copper was the most effective inhibitor of denitrification for the unidentified *Pseudomonas* sp.; Cd and Pb caused less inhibition, and the inhibitive influence of Pb was very limited even at a concentration of up to 1,000 μg/g soil (data not presented). Although a clearly inhibitive effect of denitrification could be determined with this bacterium, it was never possible to detect the accumulation of nitrite.

The relative inhibitive effect of the various heavy metals on denitrification of *P. aeruginosa* inoculated into the soil was similar to that of the unidentified

Table 3—Effect of different concentrations of Cd, Cu, Pb, and Zn on denitrification of *Pseudomonas denitrificans* in Giltay medium (anaerobic incubation for 3 days at 28°C).

	Conc.	Nitrogen as NO_3^-	Nitrogen as NO_2^-	Nitrogen as N_2O	Nitrogen as N_2	Total N detected	CO_2	Growth (OD 550 nm)	pH
		μg/ml							
Noninoculated		142.0	0	0	0	142.0	0	0	7.2
Inoculated		2.1	0	0	139.1	141.2	18.6	0.43	8.3
Cd	10	2.8	0	0	136.5	139.3	23.3	0.32	8.1
	25	2.8	0	0	139.4	142.2	22.4	0.19	8.1
	50	3.2	0	0	133.9	136.1	21.2	0.12	8.2
	100	7.5	20	0	103.4	130.9	42.8	0.12	8.1
Cu	5	2.1	0	0	131.3	133.4	18.3	0.28	8.4
	10	2.1	0	0	132.3	134.4	20.4	0.23	8.3
	25	2.1	0	0	139.1	141.2	24.3	0.23	8.2
	50	2.7	0	0	139.5	142.2	28.6	0.22	8.2
Pb	50	2.1	0	0	138.4	140.5	20.3	0.42	8.3
	100	2.1	0	0	138.0	140.1	23.4	0.45	8.2
	250	2.1	0	0	131.1	133.2	30.0	0.51	8.3
	500	2.1	0	0	132.6	134.7	43.0	0.58	8.1
Zn	50	2.1	0	0	134.0	136.1	24.6	0.32	8.3
	100	2.1	0	0	132.6	134.7	28.8	0.33	8.2
	250	2.1	0	0	138.9	141.0	53.0	0.34	8.0
	500	2.1	0	0	132.1	139.2	92.5	0.48	8.0

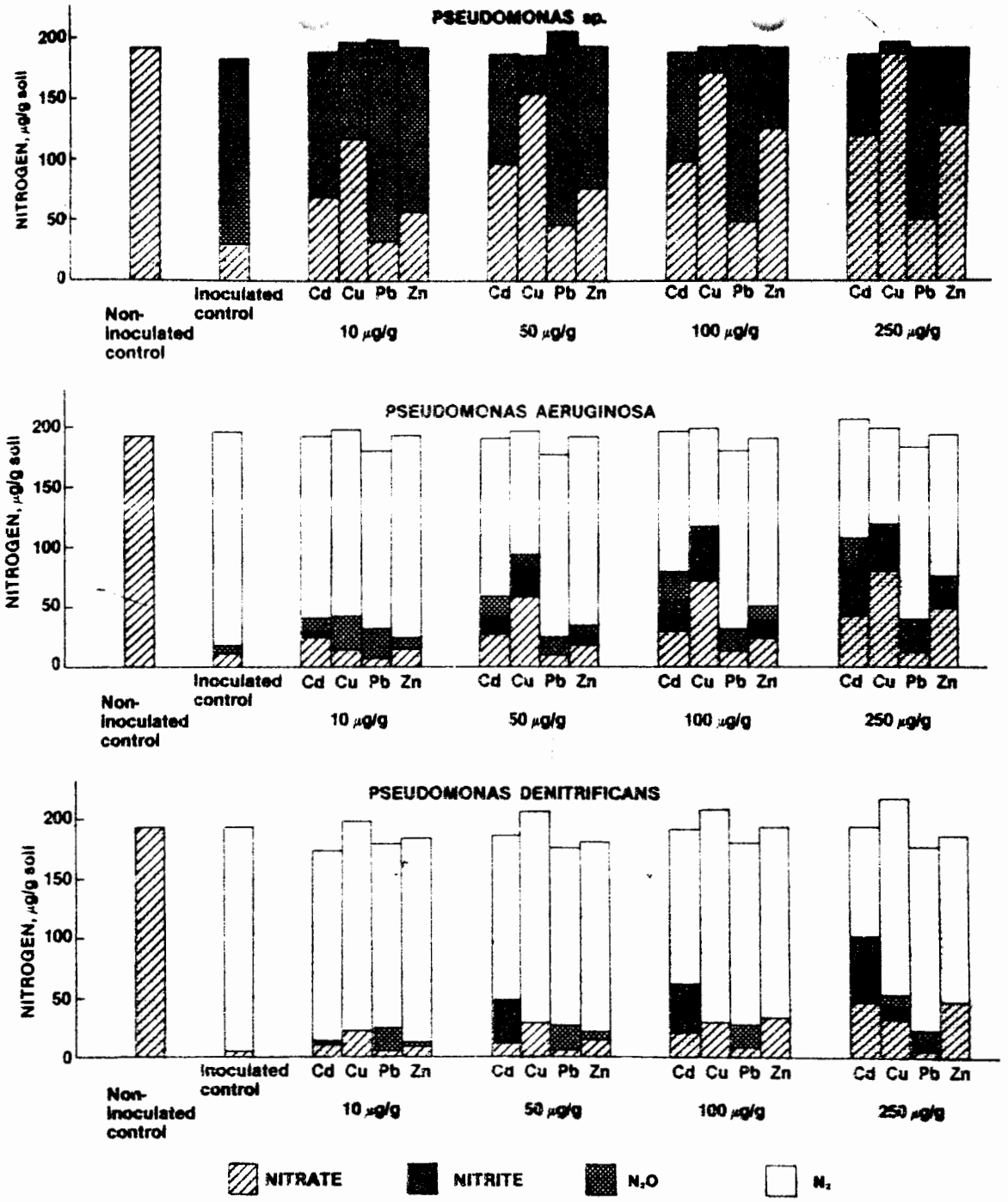

Fig. 1—Effect of various concentrations of Cd, Cu, Pb, and Zn on the denitrifying activity of an unidentified *Pseudomonas* sp., *P. aeruginosa*, and *P. denitrificans* in autoclaved soil. (Anaerobic incubation for 4 days at 28°C).

Pseudomonas species (Fig. 1). It could be determined that increasing concentrations of Cu, Cd, and Zn caused higher accumulation of nitrite. The addition of 100 µg Cu per g soil caused the formation of 41 µg/g NO_2^--N and 250 µg Cd per g soil resulted in the accumulation of 52 µg/g NO_2^--N (Fig. 2). The inhibitory influence of Pb was minor up to 500 µg/g soil, but at a concentration of 1,000 µg/g soil (data not presented), it caused the accumulation of about 17 µg NO_2^- per g soil. All investigated heavy metals caused the formation of N_2O by *P. aeruginosa*. However, this observation could not be made with Cu at concentrations above 100 µg per g soil.

P. denitrificans (Fig. 1) was, as in the liquid growth medium, more resistant to the inhibitive influence of the heavy metals than the other two bacteria. However, it is remarkable that Cd was the most effective inhibitor for *P. denitrificans* and the accumulation of a considerable amount of NO_2^- occurred.

Simultaneously with the gas chromatographic determination of N_2O and N_2, the evolution of CO_2 was measured. Generally, it could be observed that an in-

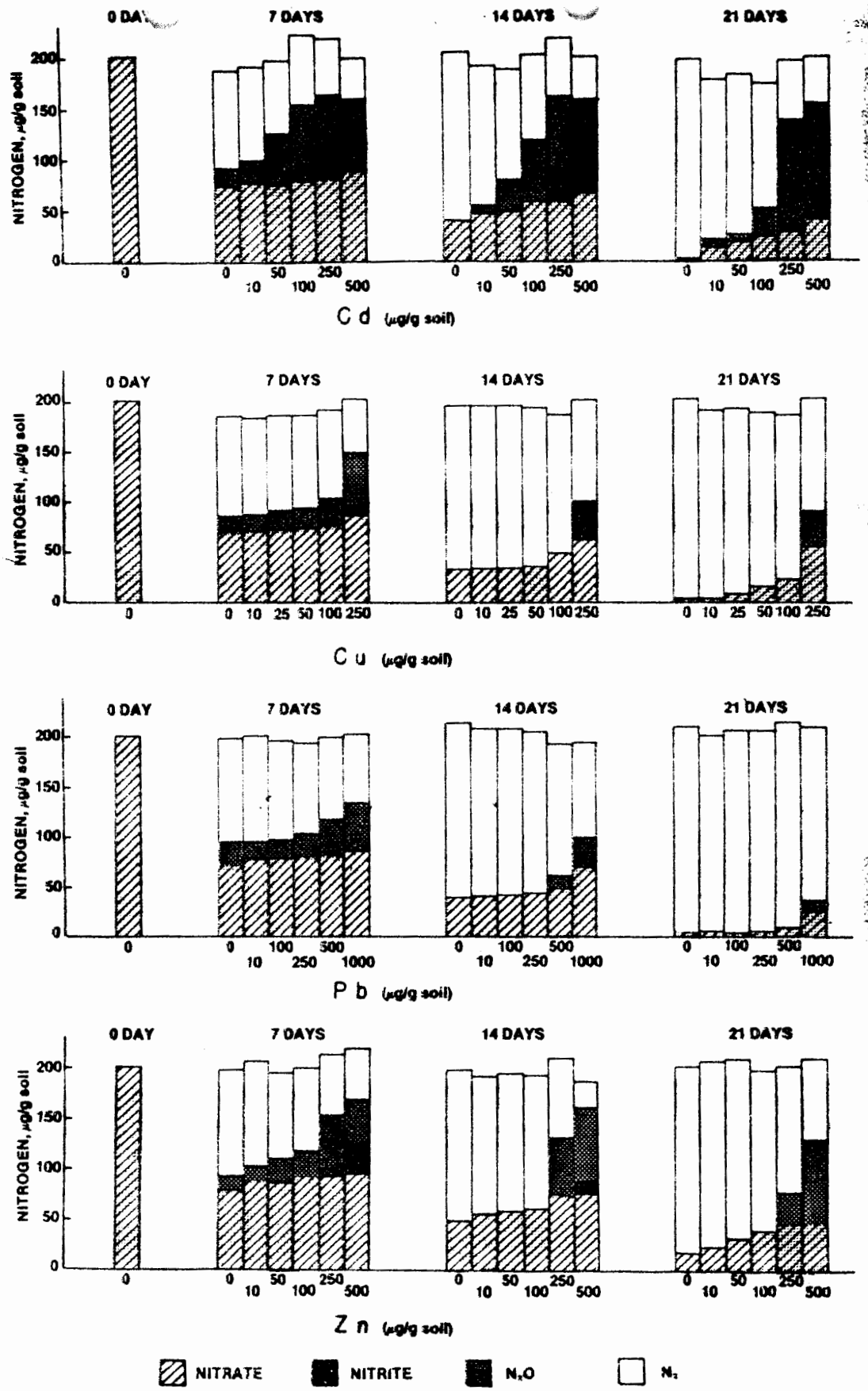

Fig. 2—Effect of various concentrations of Cd, Cu, Pb, and Zn on denitrification in native soil. (Anaerobic incubation at 28°C).

hibitive effect of the heavy metals on denitrification caused a decrease of CO_2 formation.

Effect of Cd, Cu, Pb, and Zn on Denitrification in Native Soil

Soil, which was not autoclaved and not inoculated with bacteria, was also supplied with various concentrations of heavy metals and incubated for 3 weeks. Every 7 days the denitrification activity of the soil was measured in individual samples (Fig. 2). Increasing concentrations of heavy metals caused inhibition of denitrification as indicated mainly by accumulation of N_2O or nitrite. The response varied with the various elements: Pb was least effective followed by Zn, Cd, and Cu. Nitrite accumulation was not observed in the nontreated and Pb-supplied soil samples, but Cu and Zn caused a temporary formation of nitrite, and soils treated with 500 μg Cd per g soil retained a considerable amount of nitrite even after 3 weeks. Simultaneously, it could be observed that while N_2O formation decreased in untreated soil during 3 weeks, an accumulation of this gas occurred with increasing concentrations of Cd, Cu, Zn, and Pb.

Again it was possible to observe (data not presented) that with an increasing inhibition of the denitrifying activity a decrease of CO_2 formation took place.

DISCUSSION

The denitrifying activity of various microorganisms in Giltay medium and autoclaved soil was compared with that of native soil after exposure to different concentrations of heavy metals. The selected bacteria showed different responses to the addition of heavy metals in the investigated environments. The unidentified *Pseudomonas* sp. which produced N_2O as the end product of denitrification was most sensitive to the influence of Cd, Cu, Pb, and Zn, while *P. aeruginosa* was less inhibited in its growth and denitrification by the tested heavy metals and *P. denitrificans* was affected in Giltay medium by only Cd.

In general, it could be determined that Cd was the metal with the strongest inhibiting influence on denitrification. It reduced the growth of bacteria, decreased the evolution of N_2O, N_2 and generally of CO_2, and caused the accumulation of large amounts of nitrite. Nitrite accumulation was mostly observed in the liquid medium and in soil inoculated with *P. aeruginosa* and *P. denitrificans*.

Zinc, which had no effect on denitrification in Giltay medium at concentrations of up to 500 μg/ml, inhibited nitrate reduction of all bacteria in the inoculated soil as well as in the native soil. Cu affected the tested denitrifying bacteria to a similar extent in Giltay medium and in the autoclaved soil. Inhibition of the denitrifying activity by Cu was most pronounced with unidentified *Pseudomonas* sp., while *P. aeruginosa* and, in particular, *P. denitrificans* were less affected.

It is remarkable that Cd and Cu caused inhibition of denitrification in Giltay medium at lower concentrations than were necessary for suppression of denitrification in soil, while Zn did not affect the bacteria in the liquid medium, but caused inhibition of denitrification in the soil.

Lead was the heavy metal in our study that had the least effect on denitrification. It caused virtually no inhibition of the nitrite-reducing enzyme system (e.g., compare the denitrification activity of *P. denitrificans* in autoclaved soil) but, in several cases, an accumulation of N_2O was observed in the presence of Pb. Foully (5) found under aerobic conditions that Pb at 100 ppm completely suppressed gram positive bacteria in vitro, but it did not inhibit bacteria in soil. He concluded that Pb is attracted by clay-humic complexes and, therefore, becomes unavailable for microorganisms.

All these observations confirm the conclusions from Sadler and Trudinger (9) that inhibition of microbial activity by heavy metals is specific, both for each organism and each metal.

The accumulation of nitrite during denitrification under adverse conditions has been observed in our previous investigations. Certain unfavorable environmental growth conditions (2), various pesticides (4), nitrification inhibitors (6), and heavy metals, as reported herein, can cause the accumulation of nitrite which is usually not found during the denitrification process. This observation might indicate that the nitrite reducing-enzyme system is more sensitive to inhibitory influences than the nitrate reductase system. It is worthwhile to emphasize this fact, since the temporary or permanent accumulation of nitrite can cause concern from an environmental point of view.

The reported results with pure cultures of denitrifiers in a liquid medium and autoclaved soil, as well as with native soil, clearly indicate that the investigated heavy metals reduce denitrification activity of microorganisms. It can be concluded that heavy metals interfere in the microbial activity under anaerobic conditions, which implies an additional potentially disturbing impact of these compounds in the environment.

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