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Use of *Azotobacter sp.* as an indicator to detect the toxicity of heavy metals in soils

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Abstract— A physiological strain of microorganism — *Azotobacter sp.* has been adopted as an indicator to detect the toxicity of heavy metals in soils. The concentration of heavy metals to which *Azotobacter sp.* was behaving initially to have the resistance to heavy metals is defined as the critical poisoning concentration. The method of physiological threshold adopted can have a quantitative determination with reproducible results. The determined critical poisoning concentration is basically consistent with the results of heavy metals and arsenic toxicities to the bacteria reported recently in literatures. Total 9 typical soils, including 6 zonal soils and 3 purple soils, in the whole country were determined for the toxicities of 5 heavy metals and arsenic to *Azotobacter sp.* that resulted in 48 critical poisoning concentrations.

Keywords: soil microorganisms; *azotobacter sp;* heavy metals; poisoning concentration.

INTRODUCTION

The concentration of heavy metals to which *Azotobacter sp.* is behaving initially to have resistance is identified as the critical poisoning concentration. This concept has never been found in the literatures being used to study the carrying capacity of soil environment. In 1961, Somers, E. (Somers, 1961) determined the fungicidal property of 24 metal cations to 2 fungi, and suggested ED_{50}, i.e., the concentration of heavy metals inhibiting 50% of microorganism, as the measure of the toxicity. In 1985, Haanstra, L. *et al.* (Haanstra, 1985) provided a number of evidences that showed the inhibition of nickel to soil respiration and suggested again ED_{50}, i.e., the concentration of heavy metals inhibiting 50% of maximum respiration strength, to measure the toxicity of heavy metals. However, Haanstra found that ED_{50} ignored the initial reduction in respiration while simply taking the inhibiting rate of 50% in account. For bridging

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this gap, the Netherlandish auther suggested the ecological dose range (EDR) being used to describe the toxicity of heavy metals suppressing 10–90% of maximum respiratory rate was used to measure the toxic dose of heavy metals studied. Nevertheless, either ED_{50} or EDR are quite different from the "critical" poisoning concentration to be described in this paper. This is because ED_{50} in use to measure the toxicity ignored the fact that the microorganism in the presence of heavy metals would generate the genetic mutation and resistance that might lead to an increased poisoning concentration, while EDR referred to a dose range that is also not exactly as we need. Eventually, they give us a clue that to measure toxicity will need a criteria.

In 1981, Sysina, A. N. *et al.* (Sysina, 1981) in the Moscow University and the Institute of Public Health carried out the experiments of 3 heavy metals in 2 soils, using *Azotobacter sp.* as an indicator to detect the toxicity of heavy metals. They found that the species of microorganism was a sensitive and representative indicator. This is consistent with our practice for several years. However, there were some shortcomings in their experiments. For example, few and optional concentration levels were designed for heavy metals of which copper had three levels of 5, 30 and 100 ppm only. In the next place, varied standards were used to measure the toxicity of different heavy metals. 5ppm of copper inhibiting 81% of microorganism, 10ppm of zinc inhibiting 50% of microorganism, and even 100 ppm of nickel without indicating an inhibitory rate all were considered being the poisoning concentration. It seems that this method is impossible to be adopted in our study on the carrying capacity of soil environment.

The results from their experiments are not in the sense of "critical" concentration while being the toxic one. For this reason, we have suggested the concept of "critical" poisoning concentration which can be higher precise and more reproducible in terms of microorganism having resistance. During the period of near two years with a great deal of experiments, it has proved preliminarily feasible. This is concluded from the analysis of the results from the numerous pot and indoor planting experiments in recent years, and from the reports abroad on the laws of resistance taking place of microorganism. For example, in 1965, Ashida, J. (Ashida, 1965) found that yeast could produce resistence at all concentrations. The course in which resistant microorganism is produced could have the first delaying phase and the first growing phase, and also have the second delaying phase and the second growing phase. For over 50 hours of culturing period, it entered the second growing phase. In this case, the microorganism produced consisted of a large proportion of resistant microorganism although it could be reproduced in a large amount on the media with toxic heavy metals. On the culture media containing copper, the mutants resistant to copper were reproduced in a certain proportion. In 1980, Chiburi Iki (Chiburi, 1980) found that the wax gemmabacillus growing in broth had the best growth under the conditions as control. As mercury chloride was added to the media, numbers of microbic strain were becoming inhibited. When $HgCl_2$ was at the concentration of 5×10^{-5} mol/L, the microbe stopped growing for 6 hours whereas it had the same counts of microbe as those in control by 24 hours. This is due to the appearance of the

resistant microorganism. In 1971, Den Dooren de Jong, L. E. (Den Dooren de Jong, 1971) during the experiments of *Azotobacter sp.* inhibiting ring, found that *Azotobacter Vinelandii Lipmann* in the inhibiting ring in the present of Na_2SeO_3 could produce the second colony. He believed that the colony appeared in the inhibiting ring was due to a prolonged cultivating period during which *Azotobacter sp.* was affected by the chemical agent that led to a genetic mutation to produce the resistant cells. From the reports by many authors, it can be seen that the heavy metals as Hg, Cd, Cu, Cr, Pb and others could cause microorganisms to have resistance that might relate to the existence of resistant genes.

Therefore, the initial resistance of microorganism which was produced at low concentration of heavy metals shows that the microorganism has had a qualitative change in its genetic genes. The method of physiological threshold to be used has been provided on an objective basis to determine the critical poisoning concentration of heavy metals to microorganism (or enzyme activity) in soils.

APPROACHES

Since 1970s, due to the development of industry, the discharge of heavy metals has caused soils being increasingly polluted that has drawn all governments to concern. Heavy metals may damage microorganisms in soil and, in turn, affect the functions and fertility of the soil as well as the growth of crops and human health. In order to control such damages, it is necessary to extensively study the effects of heavy metals on soil microorganisms and crops so as to find out the concentrations at which they will have such effects, and the countermeasures that can be taken. How to develop the methods for measuring the microorganisms on a qualitative basis has been less reported abroad so far and is just at the exploration stage in our country. In recent years, a number of pot planting experiments and field surveys have been carries out from which it was found (Liao, 1987) that *Azotobacter sp.* and urease were well sensitive to heavy metals and also representative in soils. For this reason, *Azotobacter sp.* was selected as an indicator and the nitrogenase improving method was used to determined the harmful concentrations of heavy metals in Beijing meadow drab soil. The observational experiments show that such harmful concentrations have the almost same trend as the results from the measurements for pot planting soil by using serial dilution, can be quantitatively determined, and are characterized by being more accurate, simpler, faster and more economical than those by the serial dilution method. Our systematic and extensive studies can provide a reliable basis for our country to establish the permissible levels of heavy metals in soils. It is expected that we can keep our steps with the world in the development and improvement of the methods for measuring the microorganisms.

Major technical considerations

1. Try best to keep the consistent experiment conditions, such as soil extraction, inoculation, cultivated period, and temperature.

2. Set up the concentration levels of heavy metals in according to specific conditions. Firstly, designate a series of different concentration, generally at 12–14 levels. Some reasonable levels in narrowing interval must be timely added in the vicinity of the levels at which the resistance has been found.

3. Improve the precision of determination by using sophisticated instruments. For example, GC has been used to determine the activity of nitrogenase with the precision in the order of nanomolarity.

4. Fix the cultivated period of *Azotobacter sp.* in 72 hours during which the resistant *Azotobacter sp.* grows in a large amount.

5. Take the mineral media as control to determine the critical poisoning concentration of heavy metals to *Azotobacter sp.* because of such media without the interferences of organic molecules or heavy metals in soils. Then check the critical poisoning concentration in the presence of soil. This is simply because both of the critical poisoning concentrations are usually quite close to each other.

6. Identify whether *Azotobacter sp.* has produced the resistance or not by analysing the peaks if appeared after the inhibition of nitrogenase activity, or by observing the cut sections in an electron microscope.

7. Try best to find out the lowest concentration at which *Azotobacter sp.* to have the reasistance to heavy metals if the microbic species could have multiple resistance. The lowest concentration would be considered as the critical poisoning concentration of *Azotobacter sp.*.

APPLICATION OF THE METHOD

The method developed by the authors has been used to detect the critical poisoning concentrations of heavy metals in soils to *Azotobacter sp.* (enzyme activity) as an indicator, carrying out in total 9 typical soils consisting of 6 zonal soils and 3 purple soils in our country. The results from the literatures are listed in Table 1.

It can be found that the critical poisoning concentrations as determined above are quite similar to the data reported in the literatures for the poisoning concentrations of heavy metals to bacteris in soils. In 1981, Ross, D. S. *et al.* (Ross, 1981) reported that 10–12 ppm of Cr (VI) inhibited most of the separated microbes on the soil extractive or synthetic media. The critical poisoning concentrations of Cr (VI) to *Azotobacter sp.* in 6 soils that we determined are in the range of 3–10 ppm and close to the data reported in the literatures. In 1981, Williams, S. E. *et al.* (Williams, 1981) separated 154 bacteria and actionomyces which were studied at 5 levels of cadmium concentration in soils. They found that 29 of 58 bacteria had their inhibitins at 5 ppm while having the resistance at more than 5 ppm, so that 5 ppm was suggested for a threshold tolerable concentration of cadmium. This is exactly the critical poisoning concentration as we defined. In our study by using 9 soils, the critical poisoning concentration was determined by the range of 1–10 ppm for cadmium that is close to the cited data. Hiroki Yamamoto *et*

al. (Hiroki, 1981) found that the counts of bacteria in soils had a decreasing trend at 1 ppm of Cu and were decreased in a large amount at 10 ppm. In our study for copper in 9 soils, the critical poisoning concentration was in the range of 1–6.25 ppm, closing to the cited data. Doelman, P. *et al.* (Doelman, 1979) suggested that the strain of bacteria separated from soils was considered to be a lead-sensitive one if could grow on the media with 4 ppm of Pb, and to be a lead-resistant one if could grow on the media with 30ppm of Pb. This implied that the soil bacteria could be resistant to the concentration of about 30 ppm of Pb. In our study for lead in 9 kinds of soil, the critical poisoning concentration to *Azotobacter sp.* was in the range of 6.3–75 ppm, closing to the cited data. Yang Jurong *et al.* (Yang, 1982) reported that the counts of soil bacteria began being reduced when the concentration of mercury in soils was up to 0.7ppm. In our study for mercury in 6 kinds of soil, the critical poisoning concentration was determined in the range of 0.3–0.7ppm of Hg to *Azotobacter sp.*. The counts of bacteria were also reported to start a reduction at 10 ppm of As in soils that is close to the critical poisoning concentration of to *Azotobacter sp.* in the range of 2.5–10ppm from our study in 9 kinds of soil.

Furthermore, the research workers in the Institute of Geography, Chinese Academy of Sciences, applied recently our points of view on the resistance to their reasonable explanation of the resistances which were produced by peroxidase and dehydrogenase activities. The critical poisoning concentrations that they determined are almost the same as we did.

Table 1 Critical poisoning concentrations of heavy metals in soils to *Azotobacter sp.* as an indicator, ppm

Soils	Cd^{2+}	Cr^{6+}	Hg^{2+}	Cu^{2+}	Pb^{2+}	As^{5+*}
Lateritic red soil	10.0	10.0	0.3	1.0	30.0	5.0
Yellowish brown forest soil	1.0	3.0	0.3	3.0	12.5	2.5
Meadow burozem	1.0	3.0	0.3	3.0	35.0	5.0
Chestnut	3.0	3.0	0.7	5.0	6.25	10.0
Blackearth	3.0	5.0	0.5	5.0	35.0	2.5
Meadow drab soil	5.0	5.0	0.5	6.25	25.0	5.0
Acid purple soil	1.0	/	/	6.25	12.5	2.5
Neutral purple soil	3.0	/	/	6.25	12.5	10.0
Alkali purple soil	5.0	/	/	5.0	75.0	2.5
Mineral media	3.0	5.0	0.3	3.0	12.5	5.0
Cited data	5.0	10–12	0.7	1–10.0	30.0	10.0

* Arsenic is classified as the heavy metal here due to higher metallicity and toxicity than phosphorus.

BENEFITS

Based on the critical concentrations determined for the soil microbes and soil enzymes as mentioned above, coupling with the critical concentrations of individual heavy metals for the crop effects in paddy and wheat pot planting and for the hygienic standards of agricultural

product quality, a comprehensive analysis and consideration can be carried out finally to determine the critical content of heavy metals in soils. In addition, a further analysis on the heavy metal input and output in a region and the calculation of the coefficiency of heavy metal residues can lead to a prediction of the environmental carrying capacity of heavy metals in the region for 50–100 years, and to the establishment of the quality standards of wastewater for irrigation in different regions and the permissible levels of heavy metals in sludged for application to cropfields. By comparison of the total heavy metals discharged from factories with the carrying capacity of soil environment, it will be easy to work out the amount of heavy metals needed to be cut down and to know what measures should be taken to control pollution. Thus, the implement of the carrying capacity of environment as one of the environmental standards may produce economic benefits. It was estimated that this will lead to a sum of RMB yuan 0.5–1 billion a year to be saved for our country by reducing the investment for pollution control, saving the research funding, and increasing the fertility of soils in the expanded irrigation area. At the same time, this will also benefit to human health.

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