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Influence of different temperatures on metal tolerance measurements and growth response in bacterial communities from unpolluted and polluted soils

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Abstract The effects of temperature on the growth rate and metal toxicity in soil bacterial communities extracted from unpolluted and polluted soils were investigated using the thymidine and leucine incorporation techniques. An agricultural soil, which was contaminated in the laboratory with Cu, Cd, Zn, Ni or Pb, and an uncontaminated forest soil were used. Measurements were made at 0 °C and 20 °C. Leucine incorporation was found to be as sensitive to heavy metals as thymidine incorporation in the short-term trial used to indicate heavy metal tolerance. Similar IC_{50} values (the log of the metal concentration that reduced incorporation to 50%) were also obtained at 0 and 20 °C, independently of the technique used. Metal tolerance could thus be measured using both techniques at any temperature in the range 0–20 °C. In the long-term experiment different temperature–growth relationships were obtained on the basis of the rate of thymidine or leucine incorporation into bacterial assemblages from unpolluted and polluted soils, as judged from the minimum temperature values. This could not be attributed to the metal addition alone since different patterns were observed when different metals were added to the soil. Thus, the minimum temperature for thymidine incorporation was similar in Cu-polluted and unpolluted soil, while in soils polluted with Cd and Zn the minimum temperature increased by 2 °C, and Ni and Pb additions increased the minimum temperature by 4 °C compared to the unpolluted soil. This suggested that heavy metal pollution led to bacterial communities showing different temperature characteristics to those in the corresponding unpolluted soil. Similar observations were deduced from the minimum temperatures required for leucine incorporation. Three groups of bacterial communities were distinguished according to the growth response to temperature in polluted soils, one group in Cu-polluted soil, a second group in soil polluted with Zn and Cd, and a third group in soils polluted with Ni and Pb.

Key words Soil bacteria · [^{3}H]-Thymidine · [^{14}C]-Leucine · Temperature · Metal tolerance · Heavy metals · Soil

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Introduction

It is well known that physicochemical characteristics of the environment can modify the toxicity of heavy metals towards microorganisms by affecting metal availability and/or by changing the physiological state of the microorganisms. The influence of edaphic factors, such as pH, organic matter content, and the proportion and type of clay, on the susceptibility of soil microorganisms to heavy metals has been studied extensively (Babich and Stotzky 1980, and references therein). In contrast, temperature has not been taken into account in most toxicity studies at a soil microbial community level.

However, pure culture studies have indicated that temperature can affect the toxicity of metals to microorganisms. Babich and Stotzky (1978) found that the toxicity of Zn to *Aspergillus niger* was the same at 25 and 37 °C, but in a medium containing NaCl, Zn was more toxic at 25 °C than at 37 °C. In a later study, Babich and Stotzky (1982) observed that the resistance of *Aspergillus flavus* to Ni was increased by increasing the temperature from 23 to 33 °C. Similar observations showing that greater toxicity occurred at lower temperatures were also made by Giangiordano and Klein (1994) in a study on the effect of Ag on *Hyphomicrobium* sp. In contrast, Hsu et al. (1992) reported that raising the incubation temperature from 25 to 37 °C increased the susceptibility of *Saccharomyces cerevisiae* to Cd and Hg. Other studies have also shown increases in toxicity at higher temperatures, for example the toxicity of Ni to psycrophilic marine species of

Pseudomonas (Babich and Stotzky 1983), of Hg to *Scenedesmus acutis* (Huisman et al. 1980), and of Cu to *Paramecium tetraurelia* (Szeto and Nyberg 1979).

The effects of heavy metal pollution on the number, biomass, and activity of soil microorganisms have been reported both from laboratory and field experiments (Duxbury 1985; Doelman and Haanstra 1986; Bååth 1989; Doelman and Haanstra 1989). Under field conditions, any influence of temperature will obviously be included in the measurements. However, the relative importance of temperature in assessing heavy metal toxicity cannot usually be determined from field investigations since temperature data are often not available or since the simultaneous influence of factors others than temperature makes interpretation difficult. Laboratory experiments, in contrast, are often carried out at temperatures in the upper range of ambient temperatures found in most soils, in order to reduce the incubation time. It is then assumed that the incubation temperature has no significant effect on the toxicity.

The rate of soil bacterial growth can be assessed by techniques based on the incorporation of thymidine or leucine into bacteria extracted from soil by homogenization-centrifugation (Bååth 1992b, 1994). The thymidine method has also been developed into a simple and fast method for estimating the metal tolerance of soil bacterial communities (Bååth 1992a; Díaz-Raviña et al. 1994a), using a short-term toxicity test consisting of a 2-h incubation of the extracted bacteria with labelled thymidine in the presence of different amounts of heavy metals. The influence of different abiotic factors such as pH or temperature on metal toxicity can be easily determined using this technique. However, one criticism of the thymidine incorporation technique is that not all bacteria are able to take up and incorporate added thymidine (Robarts and Zohary 1993). This problem is likely to be less critical with the leucine incorporation technique, since leucine incorporation is a more common property among bacteria. Leucine incorporation may therefore be a good alternative to the thymidine incorporation technique to study metal tolerance in the soil bacterial community.

In the present work, the thymidine incorporation technique was used to determine the effect of two contrasting temperatures (0° and 20 °C) on the short-term toxicity of heavy metals and thus on estimates of the tolerance of bacterial communities from unpolluted soils as well as from soil polluted with different metals. The results were also compared with those obtained using the leucine incorporation technique to assess the suitability of this method. A third objective was to determine how the growth rates of a metal-tolerant bacterial community in a heavy metal-polluted soil responded to different temperatures compared to bacteria in an unpolluted soil. This objective was achieved by measuring thymidine and leucine incorporation at different temperatues, and calculating the apparent minimum temperature required for incorporation of both these substances using Ratkowsky's square-root model (Ratkowsky et al. 1982). This model has been shown to adequately describe below optimum growth-temperature relationships for soil bacterial communities (Díaz-Raviña et al. 1994b).

Materials and methods

Soils and treatments

We studied two contrasting soils, an agricultural sandy loam with 4.4% organic matter and a pH of 7.8, and a forest soil with 12.2% organic matter and a pH of 5.8. The soils were initially sieved (<2 mm). Triplicate 100-g samples of the agricultural soil were then contaminated in October 1992 with one of five different metals by adding $CdSO_4$, $CuSO_4$, $ZnSO_4$, $Ni(NO_3)_2$ or $Pb(NO_3)_2$ solutions, at concentrations of 16 mmol kg^{-1} dry weight for Cd and 32 mmol kg^{-1} dry weight for the other metals. The metal concentrations were selected for their ability to cause similar reductions in the soil ATP content (Frostegård et al. 1993). Three untreated soil samples were used as controls. Both uncontaminated and metal-contaminated samples were stored at room temperature (approximately 22 °C) for up to 8 months to make sure that the bacterial community had altered in response to the metal addition. Distilled water was frequently added during storage to keep the soils at a constant moisture content. Measurements were taken several times during storage. Since only uncontaminated forest soil was studied, the measurements were taken in samples stored at 4 °C for no longer than 2 months.

Extraction of bacteria

Bacteria were extracted from the soil by a homogenization-centrifugation method (Bååth 1992b), resulting in a bacterial solution with approximately 10–20% of the total bacteria counted in the soil.

Bacterial growth rate measurements

Bacterial growth rates were simultaneously determined by two methods, incorporation of thymidine and leucine into cold trichloroacetic acid-insoluble material, following the procedure described by Bååth (1994). Two milliliters of the bacterial solution obtained were incubated with 100 n*M* [^{3}H]-thymidine (Amersham, 925 GBq mmol^{-1}) and 395 n*M* L-(U-^{14}C) leucine (Amersham, 11.9 GBq mmol^{-1}) at the two different temperatures tested, 0 and 20 °C. These temperatures were selected because they represent the approximate range of temperatures that may be encountered by these microorganisms in their natural environment. Three replicates for each temperature and one zero-time control were used. The incubation time was selected in order to give a similar incorporation of bacterial solution at both temperatures. Thus, the incorporation was stopped after 2 or 24 h for samples incubated at 20 and 0 °C, respectively, by adding 1 ml of 5% formalin. Filtration, washing of the filters, and scintillation counting was carried out as described by Bååth (1994). Since thymidine and leucine incorporation by these bacteria had proved to be linear between 0 and 20 °C using square-root transformed data (Díaz-Raviña et al. 1994b), the minimum temperature for bacterial communities extracted from soil was calculated from the corresponding regressions. The minimum incorporation temperature was used as the criterion for comparing the growth-temperature relationships of bacteria extracted from unpolluted and polluted soils.

Measurements were also taken using different volumes of bacterial solution (0.25, 1, or 2 ml) to ensure that the level of incorporation measured did not affect the calculated minimum incorporation temperature. However, no differences were found in the bacterial growth rate or in the minimum temperatures calculated using different volumes of bacterial solution (data not shown).

Short-term toxicity measurements

The bacterial solution was poured, in 1.8-ml quantities, into plastic vials and 0.2 ml of distilled water (control) or of a metal solution was added. Heavy metal solutions with a range of concentrations (10^{-8} *M* to 2×10^{-3} *M*, final concentration) giving no inhibition to total inhibition of thymidine incorporation were prepared using the metal salts mentioned above. The bacterial suspensions with the added metals were then incubated at 0 or 20 °C with the labelled substrates. One replicate of each metal concentration at each temperature was used. Thymidine and leucine incorporation into total macromolecules was measured following the procedure as described above. Data were expressed as a percentage of the incorporation with no metal added (distilled water control). The logarithm of the concentration (*M*) resulting in 50% inhibition (IC_{50}) of thymidine or leucine incorporation was then calculated. The percentage of inhibition was plotted against the log metal concentration and the IC_{50} value was calculated from the slope of the decreasing linear part.

Statistical analysis

Data were analyzed by analysis of variance and when significant *F*-values ($P<0.05$) were obtained, Tukey's minimum significant difference test was used to compare individual means.

Table 1 Effect of temperature on short-term metal toxicity [50% inhibitory (IC_{50}) values expressed as log metal concentration] of soil bacteria extracted from unpolluted and polluted soils using thymidine (*TdR*) and leucine (*Leu*) incorporation techniques (*Agric.* agricultural soil). Cu and Pb-polluted soils were amended with 32 mmol kg^{-1} soil

Soil	Treatment	Date	Metal tolerance	Method	Temperature	
					0 °C	20 °C
Forest	Unpolluted	6/12/92	Cu	TdR	−6.16	−6.16
				Leu	−6.16	−6.12
Agric.	Unpolluted	17/12/93	Cu	TdR	−6.44	−6.36
				Leu	−6.46	−6.46
		27/2/93	Cu	TdR	−6.28	−6.40
				Leu	−6.42	−6.46
			Zn	TdR	−5.02	−4.92
				Leu	−4.92	−4.84
			Pb	TdR	−4.46	−4.50
				Leu	−4.58	−4.54
	Cu polluted	17/12/93	Cu	TdR	−5.60	−5.32
				Leu	−5.64	−5.34
		27/2/93	Cu	TdR	−5.24	−5.26
				Leu	−5.42	−5.22
	Pb pollutred	27/2/93	Pb	TdR	−4.46	−4.42
				Leu	−4.68	−4.56

Results and discussion

There was little variation in the IC_{50} values for the unpolluted soils measured after different storage times using either thymidine or leucine incorporation techniques, indicating that the measurements were reproducible (Table 1). IC_{50} values calculated by means of the leucine incorporation method were similar to those obtained with the thymidine incorporation technique (mean values of all measurements −5.49 and −5.44, respectively), whether the measurements were taken in unpolluted or polluted soils. Both leucine and thymidine incorporation were therefore suitable for detecting the toxicity of metals to soil bacteria and therefore have equal potential for measuring changes in the metal tolerance of the bacterial community. This is consistent with results reported by Riemann and Lindgaard-Jørgensen (1990) and Tubbing (1993) in studies on the effect of diverse toxic substances on bacterial assemblages from water, although differences in the exact level of the IC_{50} values were sometimes found for the two methods by these authors.

Although some bacterial species are unable to assimilate labelled thymidine (Robarts and Zohary 1993), similar dose-response curves and similar IC_{50} values, were obtained in the short-term toxicity test using either thymidine or leucine (Fig. 1, Table 1). This indicates that either most bacteria incorporating leucine also incorporated thymidine or that the bacteria able to incorporate thymidine had the same heavy metal tolerance as the bacteria incorporating leucine. This finding supports the use of the [^{3}H]-thymidine assay in measuring the metal tolerance of bacteria extracted from soil by homogenization-centrifugation. Furthermore, it is likely that other radiotracer methods that are often applied to estimate metabolic activity in different natural environments can

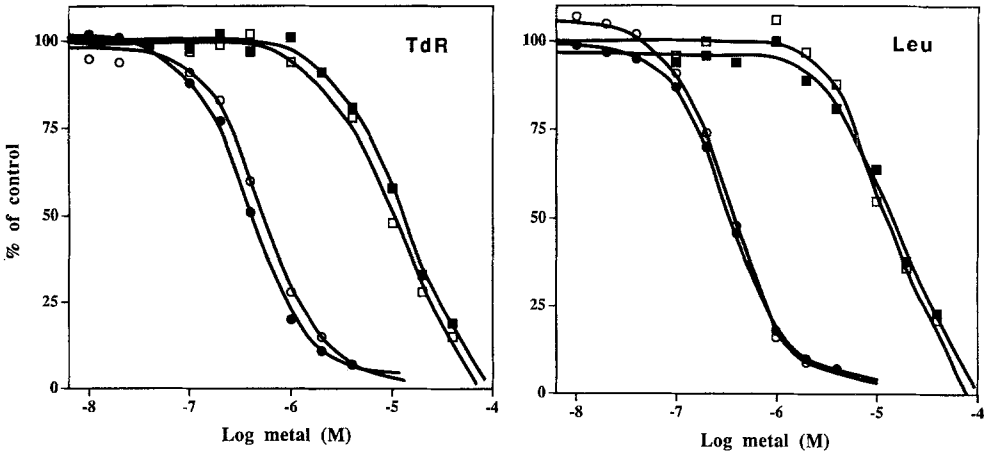

Fig. 1 Dose-response curves describing the short-term effect of added Cu and Zn on thymidine (*TdR*) and leucine (*Leu*) incorporation into macromolecules of bacteria extracted from unpolluted agricultural soil. Values are expressed as a percentage of the control (no metal added). (−○− Cu, −□− Zn; *open symbols* denote measurements made at 0 °C, *closed symbols* denote measurements made at 20 °C)

also be used to study the metal tolerance of soil bacteria using similar techniques to those used in the present study.

We found no temperature effects on the toxicity of the heavy metals to soil bacteria, irrespective of the technique used. The mean overall IC_{50} values (Table 1) were −5.50 at 0 °C and −5.43 at 20 °C. This is exemplified by the dose-response curves for leucine and thymidine incorporation at 0 and 20 °C (Fig. 1). In contrast, a temperature effect on metal toxicity has been shown in pure culture studies and this has been attributed to changes induced in the growth rate of the microorganisms and in membrane fluidity (see Introduction for references). The difference between studies may be attributed to the range of temperature used in the experiments. In the studies cited, temperatures both below and above the optimum for growth were usually used, while only temperatues below the optimum were used in the present study. Temperatures above the optimum might interact with metal toxicity in a different way from temperatures below the optimum. In addition, the data reported in previous studies represent the response by individual species of microorganisms, which may differ in their response from the overall response of the whole community. The present results therefore show that tolerance measurements can be made using either the thymidine or the leucine incorporation technique over an incubation temperature range of 0–20 °C, even though the higher temperature represents the upper temperature limit for bacteria from Swedish soils under field conditions. Since the incubation time can be reduced by using the higher temperature, the use of 20 °C is recommended.

As expected, differences were observed in the tolerance of the bacterial communities to the different metals studied. Since no differences were found between IC_{50} values calculated using different temperatures and incorporation methods, the different measurements were pooled to give an overall mean value. The results indicated that for bacteria extracted from the unpolluted agricultural soil, Cu was the most toxic of the three test metals (IC_{50} = −6.41, overall mean), followed by Zn and Pb (IC_{50} = −4.92 and −4.52, respectively). Different IC_{50} values were obtained for Cu in Cu-polluted soils (IC_{50} = −5.38) compared with unpolluted soils, indicating an increase of about 1 logarithmic unit in the tolerance of bacterial communities to this metal. In contrast, IC_{50} values for Pb obtained for bacteria extracted from Pb-polluted soils (IC_{50} = −4.53) indicated no increase in tolerance as a consequence of the Pb pollution. Similar effects on metal toxicity and on the extent of tolerance as a consequence of heavy metal pollution were obtained in a previous study (Díaz-Raviña et al. 1994a).

IC_{50} values for Cu in the unpolluted forest soil (IC_{50} = −6.16) were rather similar to those obtained in the agricultural soil (Table 1), which may be a coincidence. This is suggested by the very different IC_{50} values for Cu found in other unpolluted forest soils (Pennanen and Bååth, personal communication). Moreover, caution is required in comparing IC_{50} values obtained for bacterial communities extracted from contrasting soils, since the different pH values and different organic matter contents found in different soil types are likely to alter the effective concentration of the metal in the bacterial suspensions.

Table 2 shows the effect of temperature on the bacterial incorporation of thymidine and leucine and the related minimum temperatures calculated for the different experiments. Bacteria extracted from the two unpolluted soils showed a similar response to incubation temperature, with an increased growth bacterial rate, as measured by thymidine and leucine incorporation techniques, at 20 °C compared to 0 °C. Somewhat different results were obtained, however, depending on the technique used. The

Table 2 Growth rate at 0 and 20 °C and minimum incorporation temperature (T_{min}) for bacterial communities extracted from unpolluted and polluted soils measured by thymidine and leucine incorporation techniques (*Agric.* agricultural soil). Cd-polluted soil was amended with 16 mmol kg^{-1} soil, while for the other metals 32 mmol kg^{-1} was used. Data reported for 27/2/93 and 5/6/93 represent means of two to five measurements made with different volumes of bacterial solution in the range 0.25–2 ml

Soil	Treatment	Date	Thymidine ($\times 10^{-14}$ mol h^{-1} ml^{-1})			Leucine ($\times 10^{-13}$ mol h^{-1} ml^{-1})		
			0 °C	20 °C	T_{min}	0 °C	20 °C	T_{min}
Forest	Unpolluted	6/11/92	0.64	5.58	−10.3	1.80	21.58	−8.1
		12/11/92	0.85	8.78	−9.0	1.31	18.34	−7.3
		18/11/92	0.78	7.65	−10.0	1.93	28.27	−7.1
Agric.	Unpolluted	6/11/92	0.88	10.42	−8.2	2.07	38.18	−6.1
		17/12/92	1.46	16.58	−8.4	2.99	47.79	−6.7
		27/2/93	1.23	15.67	−7.8	1.29	26.26	−5.7
		5/6/93	2.27	22.97	−9.2	2.53	47.08	−6.2
	Cu polluted	28/2/93	0.31	3.93	−7.8	1.50	31.00	−5.6
		5/6/93	0.83	8.55	−8.8	2.23	49.50	−5.0
	Cd polluted	28/2/92	1.00	17.99	−6.2	2.07	29.94	−7.1
		5/6/93	2.27	37.92	−6.4	7.37	96.24	−7.6
	Zn polluted	28/2/93	1.62	30.44	−6.0	3.34	48.11	−7.1
		5/6/93	1.90	30.60	−6.6	4.79	62.37	−7.7
	Ni polluted	28/2/93	1.05	32.06	−4.4	1.28	61.13	−3.4
		5/6/93	1.98	59.74	−4.5	2.42	110.15	−3.5
	Pb polluted	28/2/93	3.24	114.20	−4.0	2.00	71.44	−4.0
		5/6/93	5.43	203.53	−3.9	2.38	168.30	−2.8

mean minimum temperatures calculated on the basis of the thymidine incorporation date were -9.8 ± 0.4 °C (mean ± SEM) and -8.4 ± 0.3 °C for the forest and agricultural soil, respectively. The corresponding values for leucine incorporation were significantly higher ($P<0.05$), by about 2.2 °C than those obtained using the thymidine incorporation technique (-7.5 ± 0.3 and -6.2 ± 0.2 °C for the forest and agricultural soil, respectively). This is in accord with results obtained for the same soils in a previous study, in which possible explanations for this difference between thymidine and leucine incorporation were discussed (Díaz-Raviña et al. 1994b).

The growth of bacteria extracted from polluted soils also increased with temperature (Table 2). The minimum incorporation temperatures differed, however, and were significantly affected by both soil treatment and the technique used, as well as the interaction between soil treatment and method (treatment effect: $F=40.45$, $P<0.0001$; method effect: $F=15.88$, $P<0.005$; interaction: $F=11.94$, $P<0.005$). The lowest minimum temperature for thymidine incorporation was found for the Cu-polluted soil (-8.3 ± 0.5 °C, overall mean from Table 2 ± SEM), followed by Cd- and Zn-polluted soils with values of -6.3 ± 0.1 °C and -6.4 ± 0.3 °C, respectively. The Ni- (-4.4 ± 0.1 °C) and Pb-polluted soils (-3.9 ± 0.1 °C) had the highest values. Thus, for Cd- and Zn-polluted soils the minimum thymidine incorporation temperature was about 2 °C higher than in unpolluted soils ($P<0.05$), whereas for Ni and Pb an increase of 4 °C was found ($P<0.05$). The results obtained with the leucine incorporation technique were different. Zn- and Cd-polluted soils had the lowest values (-7.4 ± 0.3 °C), followed by Cu (-5.3 ± 0.3 °C), Ni (-3.4 ± 0.1 °C), and Pb (-3.4 ± 0.3 °C). Thus, a significant increase of 2.5 °C in the minimum incorporation temperature compared with unpolluted soils was observed for Ni and Pb pollution whereas a decrease of 1 °C was detected for Cd and Zn. No significant temperature differences were found between Cu-polluted and unpolluted soils, irrespective of the method used. Thus, according to the minimum temperatures observed for bacterial communities extracted from polluted soils measured by both methods, three different groups of bacterial communities were distinguished: one group formed in the Cu-polluted soil, a second group formed in the Zn- and Cd-polluted soils, and a third group in the Ni- and Pb-polluted soils. Similar results were deduced after grouping the bacterial communities on the basis of tolerance pattern measurements and phospholipid fatty acid pattern analysis (Frostegård et al. 1993; Díaz-Raviña et al. 1994a), although with these methods the effect of Pb contamination appeared more closely related to Zn and Cd pollution than to Ni pollution.

In the same soil, differences in growth rates were often observed in experiments performed on different days. This may have been an effect of the incubation conditions, such as changes in water content or nutrient levels. It has been observed that thymidine and leucine incorporation values can be affected by the soil moisture content (F. Duke and E. Bååth, personal communication). However, the similarity in minimum incorporation temperatures indicated that the growth-temperature relationships of soil bacteria did not change appreciably during storage. For example, incorporation values for the Cd-polluted soil varied by a factor of 2 to 3 between measurements made on different days. However, there were no differences in the minimum incorporation temperature calculated at different times (Table 2). Differences in the growth rate were also observed for bacterial communities extracted from soil contaminated with the different metals. For example, the growth rates observed in Pb-polluted soil were about eight times higher than those obtained in Cu-polluted soil. However, this did not affect the results since, as mentioned above, similar minimum incorporation temperatures were obtained using different volumes of the same bacterial solution.

Differences in the minimum incorporation temperature were observed between bacterial communities extracted from metal-contaminated and uncontaminated soils and between soil bacteria from soils contaminated with different metals. It is well known that the species composition of the microbial community can change appreciably as a consequence of heavy metal pollution (Frostegård et al. 1993) and that the temperature-growth dependence of bacteria varies between species. The results reported here might thus indicate that heavy metal pollution leads to metal-tolerant communities showing different temperature responses from those in unpolluted soils. Since a different temperature response was obtained depending on the metal added to the soil, the different metals affected the microbial communities in different ways. To our knowledge, no studies on temperature responses by microbial communities from metal-contaminated soils have been reported so far. However, evidence from pure culture studies appears to support our findings. In a recent study on the effect of manure on soil microbial populations, Huysman et al. (1994) observed significant differences in the upper temperature limit for growth between Cu-resistant and Cu-sensitive bacterial strains. Our data are also consistent with observations by Atlas et al. (1991), who examined the physiological tolerance of bacterial populations in disturbed aquatic and soil environments by characterizing randomly selected bacterial isolates. They found that microbial populations disturbed by chemical pollutants exhibited a different growth response to temperature that those from the undisturbed control. We therefore suggest that these distinct temperature characteristics can be used as a supplementary aid to study the effect of heavy metal pollution on soil bacterial communites.

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