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Influences of Quinclorac on Culturable Microorganisms and Soil Respiration in Flooded Paddy Soil[1]

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Objective To investigate the potential effects of herbicide quinclorac (3,7-dichloro-8-quinoline-carboxylic) on the culturable microorganisms in flooded paddy soil. **Methods** Total soil aerobic bacteria, actinomycetes and fungi were counted by a 10-fold serial dilution plate technique. Numbers of anaerobic fermentative bacteria (AFB), denitrifying bacteria (DNB) and hydrogen-producing acetogenic bacteria (HPAB) were numerated by three-tube anaerobic most-probable-number (MPN) methods with anaerobic liquid enrichment media. The number of methanogenic bacteria (MB) and nitrogen-fixing bacteria (NFB) was determined by the rolling tube method in triplicate. Soil respiration was monitored by a 102G-type gas chromatography with a stainless steel column filled with GDX-104 and a thermal conductivity detector. **Results** Quinclorac concentration was an important factor affecting the populations of various culturable microorganisms. There were some significant differences in the aerobic heterotrophic bacteria. AFB and DNB between soils were supplemented with quinclorac and non-quinclorac at the early stage of incubation, but none of them was persistent. The number of fungi and DNB was increased in soil samples treated by lower than 1.33 $\mu g \cdot g^{-1}$ dried soil, while the CFU of fungi and HPAB was inhibited in soil samples treated by higher than 1.33 $\mu g \cdot g^{-1}$ dried soil. The population of actinomycete declined in negative proportion to the concentrations of quinclorac applied after 4 days. However, application of quinclorac greatly stimulated the growth of AFB and NFB. MB was more sensitive to quinclorac than the others, and the three soil samples with concentrations higher than 1 $\mu g \cdot g^{-1}$ dried soil declined significantly to less than 40% of that in the control, but the number of samples with lower concentrations of quinclorac was nearly equal to that in the control at the end of experiments. **Conclusion** Quinclorac is safe to the soil microorganisms when applied at normal concentrations (0.67 $\mu g \cdot g^{-1}$).

Key words: Quinclorac; Soil culturable microorganisms; Soil respiration; Flooded paddy soil

INTRODUCTION

Quinclorac (3,7-dichloro-8-quinoline-carboxylic) is a new class of highly selective auxin herbicides. It is used in rice to control dicot and monocot weeds, particularly barnyard grass. A high efficiency in weed mortality and an increase in rice grain yield following application of quinclorac in rice fields have been reported by Grossmann *et al.*[1]. According to David *et al.*[2,3], the behavior and action of quinclorac in paddy soil and semi-dried fields are affected by factors such as methods, times and rates of its application.

GL[4] reported on quinclorac absorption, translocation, metabolism, and toxicity in leafy spurge (Euphorbia esula). Quinclorac was toxic to *E. esula* when applied to leaves (LD_{50} 2

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kg/ha), soil (LD_{50} 1.7 kg), and both the leaves and soil (LD_{50} 1 kg). Irisarri *et al.*[5] observed that quinclorac addition suppressed oxygen evolution to about 31% at 0.5 mg·L^{-1} and 41% at 1 mg·L^{-1} in Anabaena culture. In Nostoc, both concentrations of quinclorac reduced photosynthetic oxygen evolution by 45%, and quinclorac did not slow down nitrogenase activity during incubation for 3 or 24 h, and up to 2 mg·L^{-1} of oxygen was evolved in Nostoc and Anabaena isolates. Toxicity studies on quinclorac summarized by the Registration Department[6] showed that there were no irritant effects on eyes or skin, no skin sensitivity, no inhalation risk, no adverse effects on reproductivity and teratogenic or oncogenic responses. Laboratory studies by Ooi GG *et al.*[7] indicated that quinclorac was mildly toxic (LC_{50} *of* $>10^{-6}$*mg·L*$^{-1}$*) to sepat siam (Trichogaster pectoralis) and keli (Clarias batrachus).* Chen *et al.*[8] found that quinclorac at its recommended concentrations had no toxic effect on S. furcifera. Ghini *et al.*[9] assessed the effects of quinclorac on soil microbial activity and biomass in rice paddy fields as well as laboratory microcosms. He found that differences detected in the variables were transient both in the field and in microcosm tests, and the effects of quinclorac on microbial community were minimal.

Quinclorac, usually extraneous to soil component pools, are also expected to affect the behavior of natural ecosystem. As a normal agricultural procedure, direct application of these synthetic agrochemicals on soil can affect microbial activity and also cause an overall toxic effect on the environment[10]. Therefore, in this process the residual matter becomes a pollutant and may act as a potential environmental hazard, with a great tendency to accumulate in soil, to disturb the natural ecological equilibrium[10-12]. However it is rarely known about effects of quinclorac on soil microorganisms after it is applied in paddy soil.

The main objective of the present study was to investigate the effects of quinclorac application on microbial populations and microbial matter transformation in paddy rice soil.

MATERIAL AND METHODS

Soil and Soil Treatment

The herbicide powder containing 50% active quinclorac was obtained from Linyi Pesticide Manufactory, Jiangsu Province, China.

A Fluvio marine yellow loamy soil was collected from the upper 2-15 cm layer of a rice experimental field at Zhejiang University, Huajiachi Campus, Hangzhou, China, where no quinclorac had been previously used. The soil was air-dried at room temperature and sieved (2 mm) to remove plant debris, soil micro fauna and stones. After sieved, the soil samples were homogenized in a rotary cylinder and stored at 4℃ before use. The soil properties are described in Table 1.

TABLE 1

Main Physicochemical Properties of the Soil Sample Tested[a]

Soil	Organic Matter (g·kg^{-1})	Total Nitrogen (g·kg^{-1})	C/N	Total Potassium (g·kg^{-1})	Total Phosphonium (g·kg^{-1})	pH
Huangsong Paddy Soil	16.70(±0.12)	1.43(±0.01)	13.20(+0.22)	20.60(±0.34)	1.520(±0.025)	7.20(+0.10)

Note. [a]: Values were the means and standard deviations in the parenthesis.

The experiments were conducted using quinclorac concentrations of 0, 0.33, 0.67, 1, 1.33, and 2 μg·g^{-1} soil dry wt. For each treatment one microcosm, consisting 1 500 g soil,

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was established in a plastic pot 15 cm in diameter and 13 cm deep. Then 1 000 mL sterile distilled water was added to submerge the soil. The flooded soil was incubated in the dark at 28℃±1℃. Soil samples were taken from each pot at certain intervals after incubation for microbial numeration and microbial activity assays. A certain amount of distilled water was added to keep the soil flooded[10].

Enumeration of Aerobic Bacteria, Actinomycetes and Fungi

Total soil aerobic bacteria, actinomycetes and fungi were counted by a 10-fold serial dilution plate technique. The number of colony forming units (CFUs) of aerobic bacteria was determined by spreading 100 μL of diluted samples on Brewis peptone medium[13] ($g \cdot L^{-1}$): beef extract 5.0, peptone 10.0, NaCl 5.0, distilled water 1 000 mL, agar 18.0, pH 7.2-7.4. Medium for actinomycetes[13] ($g \cdot L^{-1}$) was: soluble starch 10.0, $(NH_4)_2SO_4$ 2.0, K_2HPO_4 1.0, $MgSO_4 \cdot 7H_2O$ 1.0, NaCl 7.0, $CaCO_3$ 3.0, distilled water 1 000 mL, agar 18.0 and pH 7.2-7.4. The number of CFUs of fungi was estimated on Martin's agar medium with 1.25 $g \cdot L^{-1}$ streptomycin and 0.033 $g \cdot L^{-1}$ Rose Bengal. Three replicates of the inoculated plates were incubated at 28℃ for 3 d for bacteria, 5 d for fungi and 7 d for actinomycetes after which colonies were counted[13].

Enumeration of Anaerobic Bacteria

The number of anaerobic fermentative bacteria (AFB), denitrifying bacteria (DNB) and hydrogen-producing acetogenic bacteria (HPAB) was numerated by three-tube anaerobic most-probable-number (MPN) methods with anaerobic liquid enrichment media. The number of methanogenic bacteria (MB) and nitrogen-fixing bacteria (NFB) were determined by the rolling tube method in triplicate. Medium preparation was carried out using the Hungate anaerobic technique. Compositions of the medium were as follows: (1)AFB ($g \cdot L^{-1}$): glucose 10.0, peptone 5.0, beef extract 3.0, NaCl 3.0, cystein 0.5, resazurin 0.002, pH 7.2-7.4. The growth of bacteria appeared in tubes was checked after incubation at 28℃ for 7 d and used as the index of MPN; (2) HPAB ($g \cdot L^{-1}$): CH_3CH_2COOH 2.22, $CH_3(CH_2)_2COOH$ 2.64, sodium lactate 3.36, sodium succinate 8.1, CH_3CH_2OH 1.38, yeast extract 2.0, $MgCl_2$ 0.1, NH_4Cl 1.0, K_2HPO_4 0.4, cystein 0.5, resazurin 0.002, *trace element solution 10 mL, **soil extract solution 300 mL, pH 7.0-7.3. Hydrogen formation was monitored in the headspace of the tube by 102 G type gas chromatography with a thermo-conductor after incubation at 28℃ for 15 d. (3) DNB ($g \cdot L^{-1}$): sodium citrate 5, KNO_3 2.0, K_2HPO_4 0.4, $MgSO_4$ $7H_2O$ 0.2, distilled water 1 000 mL, pH 7.2-7.5. After incubation at 28℃ for 5 d, bubbles in the reversed Duhum tube were checked as described by Min[10,11]. (4) MB ($g \cdot L^{-1}$): HCOONa 5, CH_3COONa 5, CH_3OH 5 mL, H_2/CO_2 (80/20 v/v, full in the tube); **soil extract solution 300 mL, NH_4Cl 1.0, $MgCl_2$ 1.0, K_2HPO_4 4, KH_2PO_4 4, cystein 0.5, resazurin 0.002, yeast 1, agar 18, *trace element solution 10 mL; pH 6.8. The number of CFUs of MB was counted after incubation at 28℃ for 30 d. (5) NFB ($g \cdot L^{-1}$): KH_2PO_4 0.2, K_2HPO_4 0.8, $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 0.1, $FeCl_3$ 0.05, $Na_2MoO_4 \cdot 2H_2O$ 0.05, $CaSO_4 \cdot 2H_2O$ 0.1, mannite 10.0, agar 20, distilled water 1 000 mL. The number of CFUs of NFB was counted after incubation at 28℃ for 5 d.

*Composition of the trace element solution used was as follows ($g \cdot L^{-1}$): HCl (25%w/w) 10 mL; $FeCl_2 \cdot 4H_2O$ 1.5; $CoCl_2 \cdot 6H_2O$ 0.19; $MnCl_2 \cdot 4H_2O$ 0.1; $ZnCl_2$ 0.07; H_3BO_3 0.006; $Na_2MoO_4 \cdot 2H_2O$ 0.036; $NiCl_2 \cdot 6H_2O$ 0.024; $CuCl_2 \cdot 2H_2O$ 0.002.

**Preparation of the soil extract solution was as follows: several kilograms of fresh

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paddy soil were put in a barrel and submerged with tap water, stirred evenly, kept statically for 24 h and filtered. The filtrate was autoclaved at 121℃ for 30 min and stored at 4℃ before application[11,12].

Measurement of Respiration

7.5 g of each soil sample treated with different concentrations of quinclorac and 1 mL glucose solution (0.1 mol·L^{-1}) were placed into a 50 mL-serum bottle. The bottle was then sealed with isobutyl rubber stopper and aluminum cover, and incubated at 28℃ ± 1℃ for 24 h. CO_2 formed in the headspace of the serum bottle was monitored by a 102G-type gas chromatography with a stainless steel column filled with GDX-104 and a thermal conductivity detector[10,12].

Statistic Analysis

Values of microbial enumeration and soil respiration activity were expressed as the means (3 replicates) and compared statistically by *t*-test at 5% with SPSS 10.0 software.

RESULTS

Effect of Quinclorac Treatments on Soil Aerobic Heterotrophic Bacteria, Actinomycetes, and Fungi

The effects of various concentrations of quinclorac on the soil aerobic heterotrophic bacteria, actinomycetes, and fungi with the increasing incubation time are shown in Fig.1. The number of aerobic heterotrophic bacteria in soil samples treated with lower than 1.33μg · g^{-1} soil of quinclorac increased, being proportionate to the concentration of quinclorac during the initial period. The bacteria number in soil samples treated with 1.33 μg·g^{-1} soil and 2 μg·g^{-1} soil of quinclorac initially decreased, but on the 11th day rose to the highest recorded levels of 5.017×10^6 cfu/g dried soil and 3.976×10^6 cfu/g dried soil, respectively. Then the number of bacteria in all the samples declined to nearly the same level as that in the control on the 24th day, whereas there were no apparent differences ($P<0.05$) in the populations of aerobic heterotrophic bacteria.

In soil samples treated with 0.33 μg·g^{-1} soil and 0.67 μg·g^{-1} soil, the fungi were stimulated to such an extent that the population increased up to 4-fold on the 33rd day, which were somehow significantly different ($P<0.05$) to paddy soils amended with non-quinclorac at the early incubation stage. Significant differences ($P<0.01$) were also found in the soil fungi number after 33 days incubation between the samples incorporated with 2 μg·g^{-1} soil quinclorac and non-quinclorac. Meanwhile, the population of fungi in the soil sample treated with 1 μg·g^{-1} soil decreased in the first week. The number of fungi in the soil sample with a high-level of quinclorac, however, was suppressed from the beginning to the end.

The population of actinomycete was significantly inhibited within 5 days after application, and was then activated to varying extents, depending on the concentration of quinclorac. The actinomycete number declined in negative proportion to the concentrations of quinclorac applied after 4 days. The number of the sample treated with 2 μg·g^{-1} soil quinclorac decreased to 29.8% of that in the control. But 12 days later, there was a little increase in some samples with quinclorac. The second inhibition was found at the end of incubation time. The half life of quinclorac was 8-11 d in paddy soil[14], indicating that the metabolite of quinclorac might have effects on actinomycete in soil.

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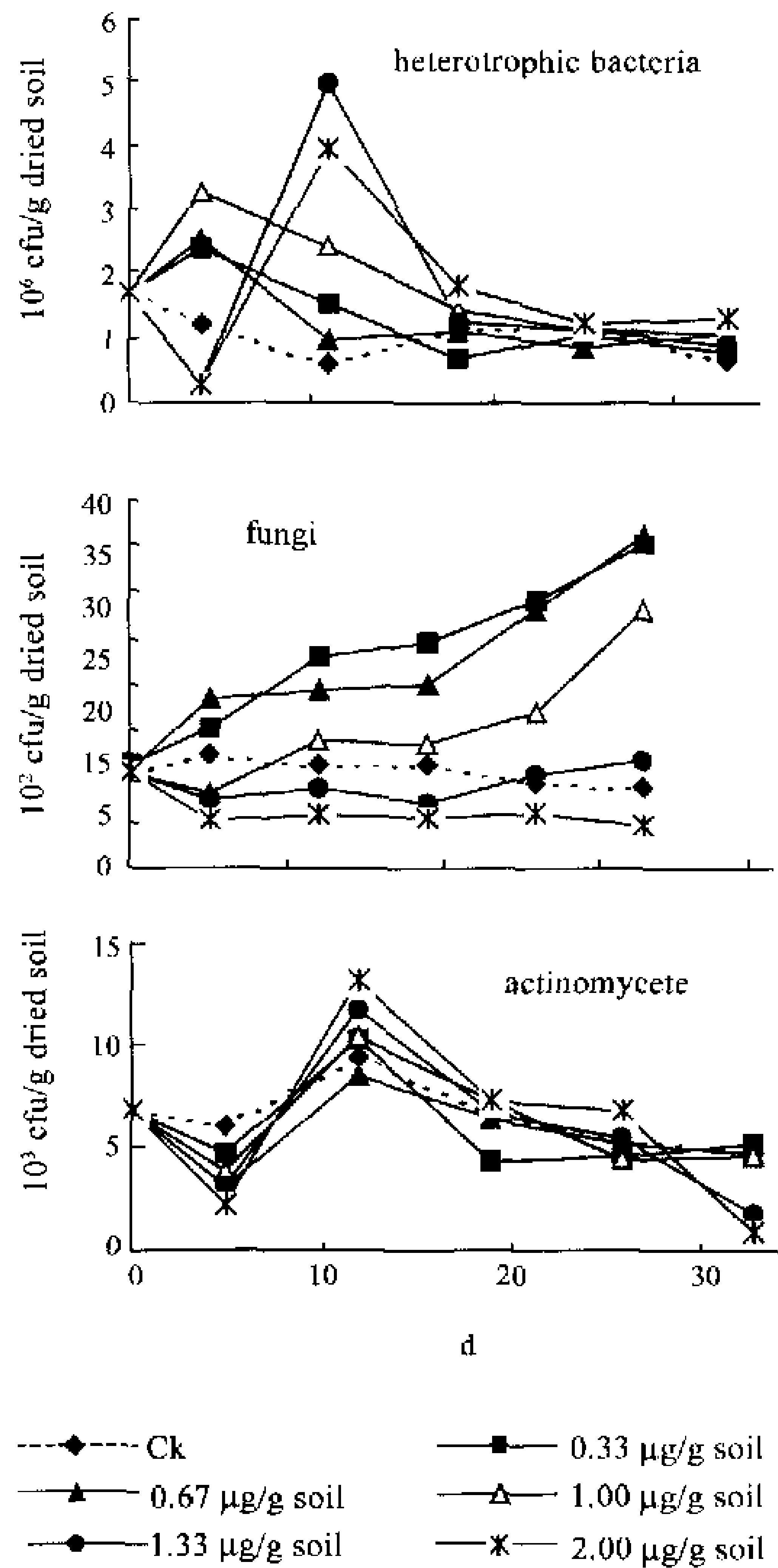

FIG. 1. Effect of quinclorac on the number of aerobic heterotrophic bacteria, fungi and actinomycetes.

Effect of Quinclorac Treatments on Anaerobic Soil Bacteria

The amount of AFB, HPAB, DNB, MB and DNB in the paddy soil incorporated with different concentrations of quinclorac was comparatively recorded (Figs. 2 and 3). It showed that application of quinclorac greatly stimulated the growth of hydrolytic-fermentative bacteria, and that the higher the concentration of quinclorac, the greater the stimulation to hydrolytic-fermentative bacteria. The highest level of hydrolytic-fermentative bacteria was observed on the 11th day after application of quinclorac at a rate of 1 $\mu g \cdot g^{-1}$ soil. The extent of activation of bacteria ranged from 3.8×10^6 cfu/g dried soil to 9.2×10^6 cfu/g dried soil in soils. The results demonstrated that although there were some significant differences

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(P<0.05) in the number of hydrolytic-fermentative bacteria between soils supplemented with quinclorac and non-quinclorac at the early stage of incubation, none of them was persistent.

Application of quinclorac might reduce the number of denitrifying bacteria on the 5th day. When applied at a concentration lower than 1.33 $\mu g \cdot g^{-1}$ soil, stimulation appeared after 11 days incubation. While the amount of denitrifying bacteria decreased 3 weeks after application of quinclorac, the number was still lower than that in the control. Nevertheless, the population of denitrifying bacteria in soil treated with 2 $\mu g \cdot g^{-1}$ soil quinclorac was significantly different (P<0.05) from that in the control. Afterwards, no significant differences (P<0.05) were observed in other soils.

Effect of quinclorac on hydrogen-producing acetogenic bacteria seemed to have a same trend as that on denitrifying bacteria. Fig. 3 illustrates that the number of hydrogen- producing acetogenic bacteria in soil treated with 0.33 $\mu g \cdot g^{-1}$ soil and 0.67 $\mu g \cdot g^{-1}$ soil could reach up to 4.1-fold and 2.7-fold as that of the control by the 11th day respectively, and they were still higher than that of the control after 33 days incubation. It indicated that a small quantity of quinclorac acted as a stimulus to hydrogen-producing acetogenic bacteria after a period of adaptation.

It was observed that the amount of nitrogen-fixing bacteria differed markedly in their response to different concentrations of quinclorac. In the same way, lower concentrations of quinclorac enhanced the population of nitrogen-fixing bacteria. Higher concentration of quinclorac applied within this range resulted in a more significant stimulation of the nitrogen -fixing population. A large quantity of quinclorac decreased the nitrogen-fixing bacteria number in earlier period, followed by stimulation, then by a secondary inhibition after 26 days incubation. Observations of the secondary stimulation on the 33rd day indicated that the metabolites of quinclorac might easily be used by nitrogen-fixing bacteria and enhanced the nitrogen-fixing population.

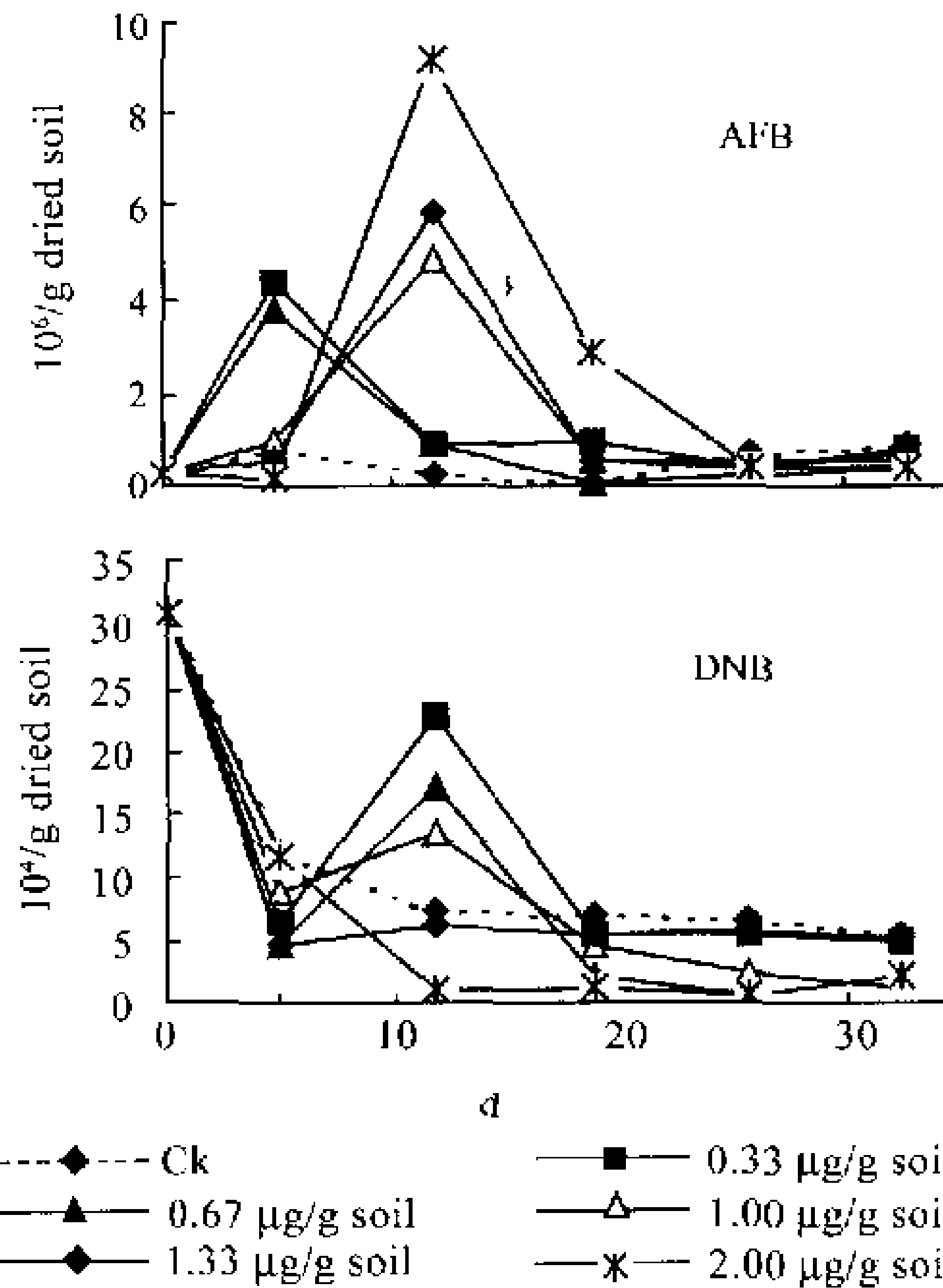

FIG. 2. Effect of quinclorac on the number of AFB and DNB in paddy soil.

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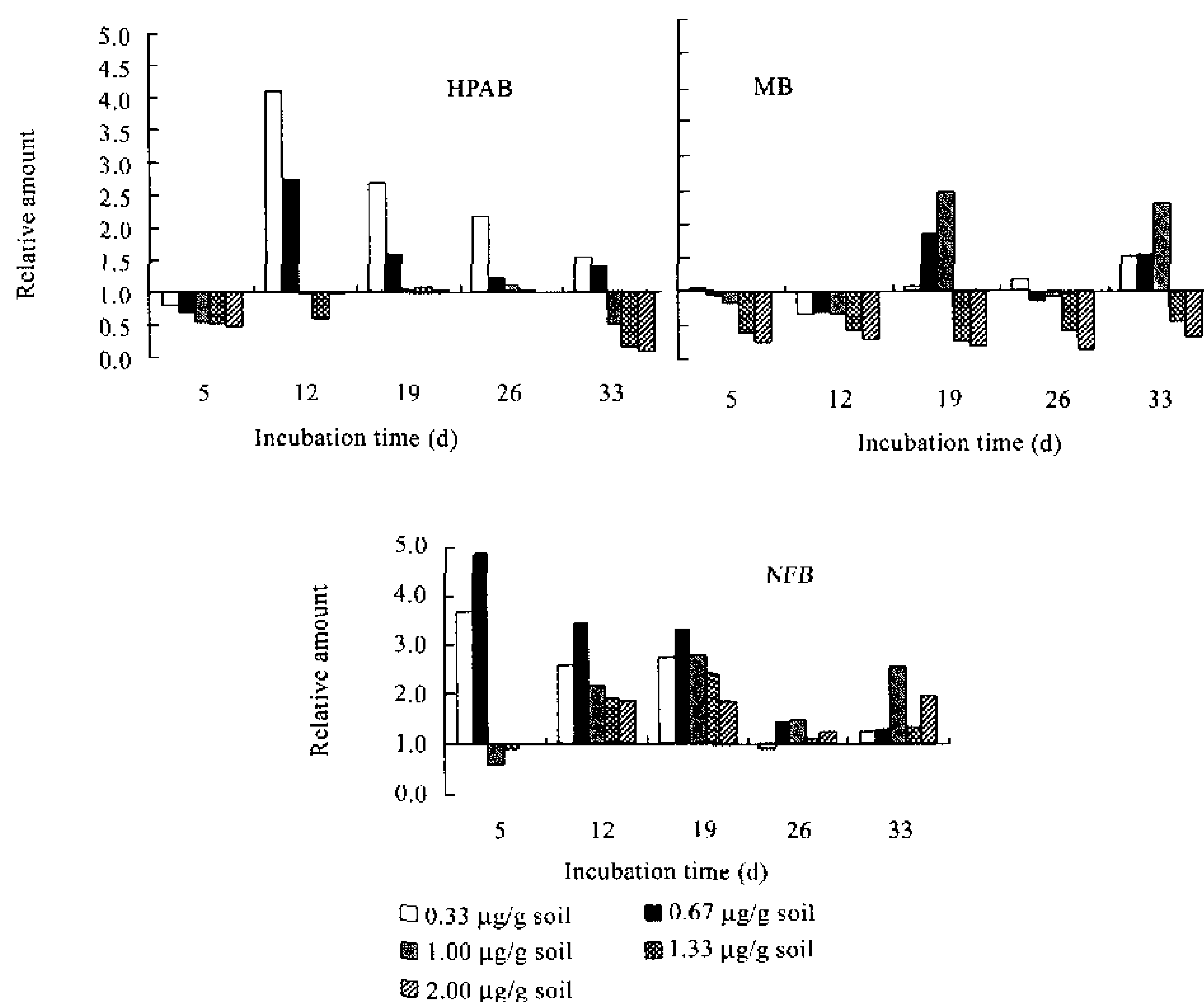

FIG. 3. Effect of quinclorac on the number of HPAB, MB and NFB in paddy soil.

Fig. 3 indicates that the temporary inhibition of quinclorac to methanogenic bacteria appeared immediately after its application. The amount of methanogenic bacteria decreased by 60.5% and 72.9% in soil samples treated with 1.33 $\mu g \cdot g^{-1}$ soil and 2 $\mu g \cdot g^{-1}$ soil after 5 days incubation. The population of the soil sample treated with 1.33 $\mu g \cdot g^{-1}$ soil recovered a little until 33 days later, but was still only 42.7% of that in the control. It was obvious that higher concentrations of quinclorac were toxic but a lower concentration of quinclorac could be a stimulus to methanogenic bacteria in paddy soil during a period of incubation.

Effect of Quinclorac Treatments on Soil Respiration

Respiration is one of the important indexes for pesticide environmental security evaluation. The results showed that quinclorac stimulated soil respiration when added to soil at normal field concentrations and inhibited respiration at higher concentrations (Fig. 4). Microbial respiration of soil treated with 2 $\mu g \cdot g^{-1}$ soil of quinclorac was inhibited and not recovered within the monitoring period. Respiration of the soil sample treated with 0.67 $\mu g \cdot g^{-1}$ soil of quinclorac reached its highest level on the 11th day, which was similar to the heterotrophic bacteria and denitrifying bacteria number.

DISCUSSION

It was observed that the bacteria differed markedly in their response to quinclorac. The

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concentration of quinclorac applied was an important factor affecting populations of various microorganisms, except for those characteristics of quinclorac itself. It might be considered that quinclorac and its metabolites or intermediate products had co-effects on bacteria in the soil. At the same time, the available nutrient in soil might reduce because of utilization by other bacteria.

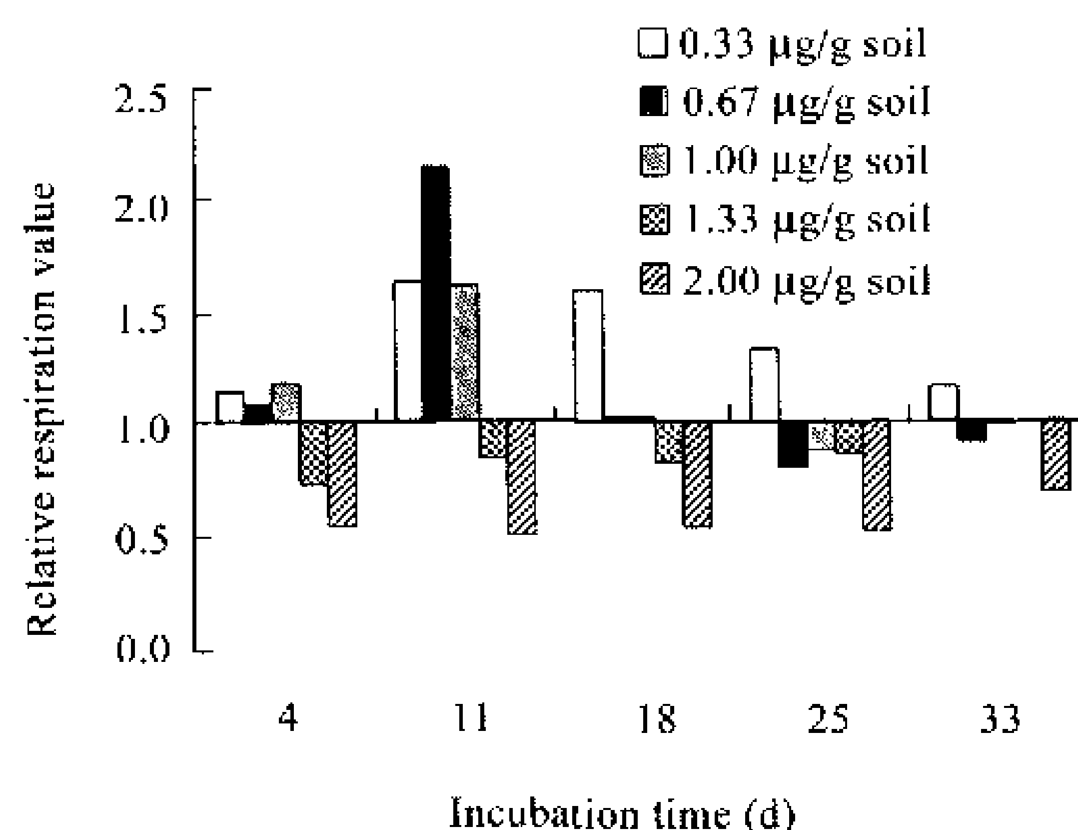

FIG. 4. Effect of quinclorac on microbial respiration in paddy soil.

The results demonstrated that despite some significant differences in the aerobic heterotrophic bacteria, AFB and DNB between soils supplemented with quinclorac and non-quinclorac at the early stage of incubation, none of them was persistent. The number of fungi and DNB was increased in soil samples treated by lower than 1.33 $\mu g \cdot g^{-1}$ dried soil, while the CFU of fungi and HPAB was inhibited in soil samples treated by dried soil higher than 1.33 $\mu g \cdot g^{-1}$. The population of actinomycete declined in negative proportion to the concentrations of quinclorac applied after 4 days, however, application of quinclorac greatly stimulated the growth of AFB and NFB. The MB numbers in the samples with lower concentrations of quinclorac were nearly equal to those in the control at the end of experiments. It can be concluded that quinclorac is relatively safe to soil microorganisms when applied at normal concentrations (0.67 $\mu g \cdot g^{-1}$ dried soil) and higher concentrations have some effects on different bacteria in soil.

Quinclorac, as a kind of herbicides, stimulates some bacteria growth rather than just causing inhibition. On the one hand, soil adsorption reduces the concentration of quinclorac contacted by microorganisms, while on the other hand, microorganisms could effectively degrade quinclorac in paddy soil, which reduces the toxicity of quinclorac. Moreover, the metabolites of quinclorac might be used as growth factors by some microbes. The bacteria number increases and the enzyme activities in the soil also increase. It also means that a certain amount of quinclorac can be degraded by this kind of bacteria.

Numerous researchers have pointed out that majority of bacteria in environmental samples cannot be isolated or cultured[15,16] by using traditional cultivation techniques. Amann *et al.*[16] reported that the culturability of bacteria from natural habitats ranged from 0.001% in seawater to 0.3% in soil. Most microorganisms that remain inaccessible are species for which the applied cultivation conditions are just not suitable or which have entered a non-culturable state. The results of culturable microorganisms were obtained under limited conditions and on limited media, which could not indicate the variation of unculturable microorganisms. Some culture-independent methods, such as terminal restriction

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fragment length polymorphism (T-RFLP), temperature or denaturing gradient gel electrophoresis (TGGE or DGGE), would be valuable to further assess the influence of quinclorac on the composition and structure of microbial community in the paddy soil.

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