

# Synergistic Effects of Adding A Substrate and an Organic Reductant for Stimulating the Bioremediation of 4-Chloronitrobenzene-Contaminated Soil

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## Research article

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# Abstract

**Background:** Soil contaminated with 4-chloronitrobenzene (4NCB) is resistant to microbial degradation due to the electron-withdrawing properties of the nitro and chloro groups in 4NCB. Currently, sufficient information is not available on how to use biostimulation strategies to enhance the bioremediation of 4NCB-contaminated soil.

**Results:** In the present study, a novel strategy was developed by utilizing the synergistic effects of adding an organic reductant (ascorbic acid, VC) and an organic nitrogen source (peptone) to stimulate the biodegradation of 4NCB-contaminated soil. Using this strategy, the bioremediation of 1 g-4NCB/ kg-1 soil could be completed within 8 days in soil batch reactors. Furthermore, the study discovered two 4NCB cometabolic intermediates in the soil reactors added with peptone and VC, and for the first time, 4NCB was transformed to 4-chlorofromanilide.

**Conclusion:** The proposed strategy is promising because it is highly efficient, easy to control and involves a non-toxic, environmentally friendly substrate/reductant.). Finally, this approach warrants future studies to extend its applications to soils contaminated with other nitroaromatic compounds.

## Background

A typical chlorinated nitroaromatic intermediate compound, 4-chloronitrobenzene (4NCB), has been released into the environment for many years due to its extensive use in the chemical synthesis of drugs, herbicides, dyes, and other industrial chemicals [1]. In addition, the worldwide area of soil contaminated with 4NCB has substantially increased because of inappropriate use and accidental leakage. Soil contamination by 4NCB is resistant to microbial degradation due to the electron-withdrawing properties of its nitro and chloro groups [2]. However, the important role that the microbial community plays in the degradation of organic xenobiotics in the environment is now clearly recognized [3]. Therefore, exploring highly efficient bioremediation methods for accelerating the biodegradation rates of contaminated soils, which typically contain a complex microbial community structure, is important for soil remediation.

The aim of bioremediation is to accelerate the biodegradation rate via two approaches (bioaugmentation and biostimulation) [4]. Bioaugmentation is a promising approach for cleaning soil contamination [5]; however, the challenge with this approach is to introduce a specific strain or a consortium of microorganisms with low levels of survival in complex soil ecosystems [6]. There have been a number of studies to isolated and characterized microorganisms with the capacity to degrade 4NCB [7–11]. However, only two studies have successfully performed bioaugmentation using the inoculated strain at the laboratory scale [10, 11].

There are two opposing viewpoints in the comparison of biostimulation and bioaugmentation as strategies for remediation of soil contaminated by organic compounds and determining which strategy is more appropriate to enhance the organic biodegradation rate [12, 13]. However, to the best of our knowledge, no studies have been reported describing biostimulation strategies to address 4NCB-

contaminated soil. Biostimulation, which is an important bioremediation technology, has received extensive recognition because of its following advantages [14]. 1) The technology is simple, inexpensive and "green" and can be easily controlled by adding the active compounds; moreover, biostimulation does not require much time to isolate a specific strain or a consortium of microorganisms with the capability to biodegrade 4NCB [15]. 2) Biostimulation has been used to stimulate the activity and biomass of indigenous microorganisms to enhance the degradation rate. Thus, the survival of indigenous microorganisms used for biostimulation in the target soils is not problematic [16, 17]. However, the use of biostimulation faces a key problem of how to effectively enhance the ability of soil microorganisms to biodegrade.

In the present work, a simple and "green" method was utilized for effectively enhancing 4NCB removal in soil. A novel approach was previously established to utilize the synergistic effect of an organic reductant and a co-substrate that could effectively enhance the nitroaromatic compounds (4NCB and nitrobenzene) biodegradation rate of the strain as a pure culture [18, 19]. In the experimental stages, a system was established using a mineral salt medium (MSM) with soil microorganisms, as soil compounds could interfere with 4NCB removal by soil microbial biodegradation if an additional compound was used. To compare the two systems (soil and MSM), the results of the experiments identified the soil compounds that affected the 4NCB removal rate. Moreover, an additional reductant could affect the concentration of co-metabolite and the 4NCB biodegradation co-metabolic pathway; therefore, identifying the 4NCB biodegradation pathway involved in the approach is important. Certain studies have identified the pathway under aerobic conditions, but none have identified the 4NCB removal co-metabolic pathway. The objectives of the present study were 1) to evaluate whether this approach effectively enhanced the 4NCB biodegradation rate of the microbial communities in soil, 2) to evaluate whether 4NCB contamination in soil could be effectively removed using the approach, and 3) to evaluate the co-metabolic pathway of 4NCB removal from soil using the approach.

## Results

### Effect of the Substrate on 4NCB Reduction.

The microbial community plays an important role in the biodegradation of organic xenobiotics in soil [20]. A novel approach was previously established that could effectively enhance [the biodegradation rate of nitroaromatic compounds using pure strains](#) [10, 11]; however, complex microbial populations and complex soil compounds exist in the soil. Here, the intent was to evaluate effect on 4NCB degradation using an additional compound in two systems (MSM, soil).

The effect of the additional substrate on the 4NCB removal and the *OD* values of soil microorganisms were observed in the MSM after 3 days. At a concentration of 100 mg/L 4NCB, minimal indigenous microorganisms existed that could utilize 4NCB as carbon and nitrogen sources to grow, as shown in Fig. 1A ( $0.160 \pm 0.022$ , the *OD*<sub>600</sub> value for the indigenous microorganisms of the control group), and higher levels of 4NCB would be difficult to remove by indigenous microorganisms (the 4NCB degradation for the

control=16.9%). Thus, an increase in the biomass of the indigenous microorganisms could be a key factor affecting 4NCB biodegradation. Therefore, testing the effect of a normal substrate on the biomass of the indigenous microorganisms to enhance 4NCB removal was required in the next stage of the experiment. First, with additional carbon (sucrose, starch), The biomass of indigenous microorganisms ( $OD_{600}$  values= $0.301\pm0.083$  and  $0.456\pm0.122$ , respectively) and the degradation of 4NCB residue (19.2% and 17.5%, respectively) indicated minimal increases compared with the data for the control (Fig. 1A). The results indicated that the indigenous microorganisms could not sufficiently utilize the additional carbon to enhance the strain's growth and therefore did not produce an increase in the 4NCB biodegradation rate. Second, with inorganic nitrogen sources (ammonium nitrate or urea) ( $OD_{600}$  values= $0.274\pm0.022$  and  $0.3446\pm0.0607$ , respectively), an increase in the 4NCB biodegradation rate was not apparent compared with the control data; however, the 4NCB data (11.7% and 14.0%, respectively) indicated that the biodegradation rates had been slightly suppressed. Finally, the effect of adding inorganic nitrogen sources on the 4NCB biodegradation rates was opposite to that of indigenous strains from soil. Supplementation with organic nitrogen (peptone) markedly improved the  $OD_{600}$  value (1.54), and the 4NCB degradation was 62.9% after 3 days. The results indicated that the substantial enhancement of indigenous microbial growth was induced organically and resulted in increased 4NCB biodegradation.

For all the biostimulated substrates in soil, the highest level of 4NCB degradation (78.3%) was found to be a result of supplementation with peptone. No substantial difference was observed in 4NCB degradation (51.9% and 46.2%, respectively) with a carbon source (sucrose or starch), and the degradation (48.8% and 43.1%, respectively) was limited with an inorganic source (urea or ammonium nitrate). Considering these results, peptone was determined to be the optimal primary substrate for our subsequent experiments.

### **Effect of the Reductant on 4NCB Reduction.**

4NCB is resistant to microbial degradation due to the simultaneous existence of chlorine and nitro groups, which may induce a partial reduction of 4NCB in the initial steps. Thus, a reductant could enhance 4NCB biodegradation by pure strains [11]. Therefore, we tested the effect of an additional reductant on the 4NCB removal and the  $OD$  values of the soil microorganisms. Fig. 1A shows that there were no statistically significant differences between the  $OD_{600}$  values for the additional reductant and those for the control group. This finding refutes the assumption that a reductant cannot be utilized as a substrate to support microbial growth. When reductants (D-glucose, mannitol, ascorbic acid (VC), and  $FeSO_4$  ( $Fe^{2+}$ )) were added to the MSM, the concentrations of the 4NCB residue were 75.6 mg/L, 70.9 mg/L, 75.8mg/L, and 83.7 mg/L, respectively. These results indicated that the additional reductant could catalyze the 4NCB biodegradation rate of soil microorganisms. However, in soil, the additional reductant had a negligible effect on 4NCB reduction due to the complex compounds in the soil (Fig. 1B). The 4NCB biodegradation rate ranged from 47.1% to 54.3% with reductant compared with 50.4% for the control.

However, the utilization of biostimulation with a substrate or a reductant to enhance 4NCB removal from soil warrants further evaluation.

### **Synergistic Effect of the Reductant and Peptone on 4NCB Reduction.**

With the synergistic effect of the optimal mixture, the 4NCB and nitrobenzene biodegradation rates for target strains from various environments were effectively enhanced [18]. Thus, it was important to evaluate whether the approach could be utilized to effectively enhance 4NCB biodegradation rates using soil microorganisms. When supplementing MSM with peptone and reductants (control, D-glucose, ascorbic acid, mannitol and  $\text{Fe}^{2+}$ ), the 4NCB residue concentrations from an initial concentration of 100 mg /L 4NCB were  $83.1 \pm 3.9$  mg/L,  $36.5 \pm 1.2$  mg/L,  $1.1 \pm 0.9$  mg/L,  $11.9 \pm 2.5$  mg/L and  $18.4 \pm 3.5$  mg/L, respectively, after 3 days. In addition, the  $OD_{600}$  data were  $0.160 \pm 0.017$ ,  $1.29 \pm 0.105$ ,  $1.404 \pm 0.015$ ,  $1.48 \pm 0.5244$  and  $1.49 \pm 0.102$ , respectively. When soil was amended with peptone and reductants (control, peptone, peptone + D-glucose, ascorbic acid + peptone, mannitol + peptone and  $\text{Fe}^{2+}$  + peptone), the 4NCB residue concentrations from an initial concentration of 100 mg  $\text{kg}^{-1}$  4NCB were  $49.6 \pm 2.8$  mg/kg,  $21.7 \pm 3.2$  mg/kg,  $22.5 \pm 4.5$  mg/kg,  $3.3 \pm 1.6$  mg/kg,  $12.7 \pm 2.4$  mg/kg and  $10.2 \pm 2.2$  mg/kg, respectively. These results show that the optimal mixture (VC + peptone) could be effectively utilized to enhance 4NCB removal in soil with a low initial content of 4NCB. The variations of residual 4NCB concentrations in soil were examined when the initial 4NCB concentration was 1 g/kg 4NCB (Fig. 4). After 4-8 days, the level of 4NCB treatment using the control was negligible. The treatment maintained a level of 72.9% ( $729.2 \pm 71.2$  mg/kg) of the initial concentration. As verification of the approach, the 4NCB content in soil had decreased from 1 g  $\text{kg}^{-1}$  to  $43.8 \pm 14.6$  mg  $\text{kg}^{-1}$  after 6 days (Fig. 4), and additional 4NCB was completely degraded after 8 days. Thus, the approach may be a potent technique to treat soil contaminated with 4NCB.

To identify the co-metabolic pathway in soil that contributed to effectively utilizing the approach, GC/MS analysis of 1 g/kg 4NCB biodegraded products in soil was performed using the organic extracts of the isolate. The retention time and m/z spectra of the identified co-metabolites are shown in Fig. 5. Two major co-metabolites were found: 4-chloroaniline and 4-chloroformanilide. No previous reports have described the 4-chloroformanilide metabolite.

## **Discussion**

In this study, indigenous microorganisms were a key factor in biostimulation affecting the degradation rate of organic compounds in soil. Due to the interference caused by complex compounds in soil, it is reasonable that these compounds would affect the removal rate of 4NCB as well as the biomass of indigenous microorganisms. Reducing the interference from these complex compounds in soil would help optimize 4NCB removal, but reducing the interference in the soil system to a level that could eliminate the effect of soil compounds is almost impossible. The soil bacteria were counted using a miniaturized most likely number (MPN) method [21, 22] and the soil microbial communities were determined by nucleotide sequence analyses and 16S rRNA-based denatured gradient gel electrophoresis

[23, 24]. However, the two methods could not be utilized to characterize the effect of the additional compound on the microbial biomass and 4NCB biodegradation. As a result, we evaluated several additional compounds to determine their effects on the biomass of the indigenous soil microorganisms and the 4NCB biodegradation rate in the MSM. In our experiment, the distribution of microbial communities of the same population was associated with the soil and MSM because the microbial communities were obtained from the same soil in two systems. The MSM results could be less influenced by soil compounds and directly reflect the effect of the additional compound on organic contaminant removal by the soil microbial communities. In the study, two strategies of biostimulation were utilized to enhance 4NCB removal. Firstly, we utilized co-metabolism to enhance the 4NCB removal rate. Generally, a key factor of successful co-metabolism is needed to enhance the biomass of microorganisms. In the experiment, an additional carbon source could not be used to enhance the 4NCB contaminant removal in soil because the organic carbon as a sole source was not sufficient to support the growth of indigenous microbes in the soil (Fig. 3). Conversely, a notable increase in both the biomass and biodegradation rate was observed because of supplementation with organic nitrogen. The reason appears to be that nitrogen from 4NCB was utilized by the strains to generate energy to drive metabolic reactions rather than to produce new biomass, while carbon from 4NCB was utilized by the strains to produce new biomass (18). A series of studies indicated that the addition of inorganic or organic nitrogen-rich nutrients (biostimulation) is an effective approach to enhancing the bioremediation process (25). However, in our experiment, the additional inorganic nitrogen or organic nitrogen produced opposite results regarding their effect on the biodegradation of nitroaromatic compounds. The reason may be that an inorganic nitrogen source has limited bioavailability to soil microorganisms. The findings suggested that only an organic nitrogen source could enhance the 4NCB biodegradation rate by increasing the biomass of indigenous microorganisms in soil.

The other strategy is to utilize additional reductant to enhance 4NCB degradation. Interesting findings on this strategy were as follows: 1) In MSM, the reductant (electron donors) as the sole additional agent could not support microbial growth but could enhance the 4NCB removal rate from soil by the microbial communities (Fig. 2A). These results are consistent with past studies reporting that the additional reductant could catalyze the nitroaromatic compounds biodegradation rate [18, 19, 26, 27]. 2) In soil, no positive effect on the 4NCB removal rate was observed when the reductant was added. This finding raises an interesting question as to why the addition of the same reductant in two systems has different effects. The reasons may be as follows: 1) The additional reductant could enhance 4NCB bioreduction while the indigenous microorganisms and the additional reductant encounter each other. In MSM, the additional reductant and the indigenous microorganisms continue to move, creating ongoing contact between the reductant and the microorganisms, which causes the 4NCB bioreduction rate to be enhanced; however, in soil, both the limited biomass of indigenous microorganisms and the additional agent could be adsorbed in a limited space [28], minimizing the contact between the microorganisms and additional reductant. 2) The other reason may be a chemical reaction of the additional reductant with complex soil compounds, the interaction between the additional reductant and complex soil compounds, etc.

An additional reductant (alkaline ascorbic acid) has been suggesting to exhibit the potential to reductively degrade similar compounds such as nitrobenzene in soil [29], but it is unclear why the additional ascorbic acid would have opposite results regarding its effect on similar compounds (nitrobenzene, 4NCB). In the present study, 80% of nitrobenzene was degraded in a soil slurry with a high water to soil ratio (> 85%, w/w); the soil slurry under these conditions was in an aqueous phase. Moreover, maintaining a high alkaline pH is essential for the ascorbic acid effect on 4NCB removal. Therefore, with a normal water to soil ratio (< 20%), the additional alkaline ascorbic acid has no obvious impact on the 4NCB removal in neutral soil in the experiment [29]. These results further demonstrate that effectively removing 4NCB in untreated soil solely using an additional reductant is impossible.

Finally, the use of two cooperative strategies can effectively remove 4NCB. The results of our experiments indicated a similar tendency consistent with previous MSM studies suggesting that a treatment using a combination of a reductant and a substrate is more effective than a treatment using either component individually [18, 19]. Thus, the liquid system could be utilized to reduce the interference of the soil compounds and to evaluate the effect of the additional compound on the biomass of indigenous microorganisms and the 4NCB degradation rate.

However, testing the synergistic effect is the key step in the experimental process. Three types of mixtures (reductant VC + peptone, mannitol + peptone, and  $\text{Fe}^{2+}$  + peptone) appeared to have a positive synergistic effect on 4NCB removal in soil, but only one mixture (D-glucose + peptone) had no positive synergistic effect on 4NCB removal. In soil, an existing organic reductant is present [30], which calls into question the need for an additional reductant. However, the additional optimal reductant was useful in effectively removing 4NCB from soil for the following reasons: 1) Using some additional reductant does not impact the synergistic effect on 4NCB removal in soil. For example, no noticeable difference was observed in the 4NCB removal rate in soil for the experimental data using additional glucose and peptone compared to the data using only additional peptone. Thus, organic reductant and peptone in soil may not always produce a synergistic effect on 4NCB removal from soil. 2) The process of adding peptone to soil to enhance the 4NCB removal rate still needs improvement. Luan *et al.* demonstrated that the enhancement of nitrobenzene bioreduction is dependent on the availability of excess electron donors [31]. Therefore, due to the excess number of electron donors using the additional reductant, the optimal combination of reductant and peptone will have an improved synergistic effect on the 4NCB removal from soil. 3) Based on our previous studies, the effects of the same mixture (reductant and substrate) on the ability of different strains to remove nitrobenzene or 4NCB may be different [18, 19]. The microbial communities under different soil conditions were different regarding the distribution of cells, causing the same mixture to have a different synergistic effect. Thus, identifying the optimal mixture to effectively enhance the reduction of 4NCB from soil was important. A series of studies on biostimulation through iron reduction have been published [9, 32]. In our studies, two mixtures (VC and peptone and mannitol and peptone) produced better results for the synergistic effect of 4NCB removal in soil than  $\text{Fe}^{2+}$  with peptone. These results are consistent with our previous studies and demonstrate that mixtures using an organic reductant allow more choices and are less toxic [18, 19]. With the optimal mixture (VC and peptone),

1 g/kg 4NCB in soil was almost completely removed after 8 days. Niu *et al.* performed bioaugmentation of a soil contaminated with 4-chloronitrobenzene using a *Pseudomonas putida* ZWL73 strain, which produced similar effects on the 4NCB removal rate [33], but the success of this approach was limited because the inoculated microbes could not survive in the target soils. However, soil with indigenous microorganisms with the ability to biodegrade 4NCB may exist in various environments [18]; thus, biostimulation using their approach proved to be an effective strategy for treating soil contaminated with 4NCB as well as soil contaminated with other nitroaromatic compounds.

A series of studies on 4NCB metabolic pathways under aerobic conditions have reported the following findings: 1) 4NCB can be transformed into 4-chloroaniline, N-acetyl-4-chloroaniline, and 4-chloronitrosobenzene without any further degradation using the *Pseudomonas* sp. CBS3 strain [34]. 2) A reductive pathway was identified in which yeast (*Rhodospiridium* sp.) transforms 4NCB to produce 4-chloroacetanilide and 4-chloro-2-hydroxyacetanilide [11]. 3) The *Sphingomonas* sp. strain NCB3 can transform 4NCB into 4-chloroaniline [7]. However, no information on the 4NCB co-metabolic pathway has been reported. GC/MS analysis of co-metabolites were utilized to identify two compounds (4-chloroaniline and 4-chloroformanilide). 4-Chloroaniline has been reported to function as a normal metabolite under aerobic conditions. For example, two co-metabolic pathways exist for 4-chloroaniline: 1) 4NCB can be transformed into 4-chloroaniline by the microbial strains. The first pathway is the transformation of 4-chloroaniline into 2-amino-5-chlorophenol, after which this species can be further transformed into 5-chloropicolinic acid [33]. 2) Certain strains directly transform 4-chloroaniline via ring cleavage pathways [35]. In the experiment, 2-amino-5-chlorophenol was not detected by GC/MS in the soil; therefore, 4NCB may have been directly transformed into 4-chloroaniline via ring cleavage in this experiment. Here, we utilized the approach of transforming 4NCB into 4-chloroformanilide in soil to demonstrate the presence of a novel co-metabolic pathway for 4NCB due to the function of a novel metabolite. In addition, the metabolites indicated that the initial phase of 4NCB biodegradation is based on reductive pathways in soil; therefore, the additional reductant may be a catalyst for 4NCB biodegradation.

## Conclusion

In the previously studies, we established a novel approach that could efficiently enhance the rate of the strains' nitroaromatic compounds biodegradation by the synergistic effect of combining an organic reductant with a substrate. In the manuscript, utilizing the approach, 1 g 4-chloronitrobenzene kg<sup>-1</sup> soil could be effectively removed from soil after 8 days. Furthermore, in soil, our results disclosed that there were two 4-chloronitrobenzene co-metabolic pathways by utilizing the approach, and one of the ways is a novel metabolic pathway that 4-chloronitrobenzene was transformed to 4-chloroformanilide. In addition, the approach may provide a potential method for treating soil contaminated with 4-chloronitrobenzene as well as other nitroaromatic compounds.

## Methods



## Soil

The selected soil was classified as a light clay loam soil collected from the vegetable garden of Chongqing, and did not contain 4NCB, even in trace amounts. The sample was taken from the top layer (0–15 cm) and stored in the dark at 4°C until use. Its main properties were as follows: real alkalinity [in water], pH 7.8; water content, 18%; total organic carbon, 1.3%; total nitrogen 0.089%; soil texture: sand, 38.6%; silt, 25.2%; and clay, 40%.

## Experimental process

The experiment was designed to be divided into two experimental stages. The first stage was intended to analyze the effect of exogenous compounds (substrate, reducing agent or substrate + reducing agent) on 4NCB biodegradation by indigenous microorganisms from soil. Batch experiments for the first stage were conducted using 250 mL bottles filled with 50 mL of reaction solution and placed in a rotary shaker at 28°C and 180 rpm for 3 days (Table 1). This part of the testing was conducted in four sets, as shown in Table 1. All of the 50 mL reaction solutions (MSM) contained 0.1 g of soil and 100 mg/L 4NCB. Some of the solutions also contained additional reagents (Table 1).

In the second stage of experiments, we evaluated the effect on 4NCB biodegradation rates in the soil resulting from the addition of exogenous compounds (organic reducing agent, substrate). The biostimulation experiments in soil were conducted in 250 mL bottles at 28°C, and 50 g of contaminated soil was used without sterilization to maintain the water content (4NCB and the soil were mixed to complete homogenization, and 4NCB/soil=100 mg/1kg or 1g/1kg, w/w). Each of the 50 g of contaminated soil components contained additional reagents (Table 1), and this part of the testing was conducted in five sets, as shown in Table 1. The 4NCB residue contents in soil were extracted as previously described [11].

The densities of the indigenous microorganisms ( $OD_{600}$ ) and the 4NCB concentrations were measured after 72 hours in all sets except Set 5 in the second stage of the experiment (samples were withdrawn periodically for the analysis the 4NCB contents). In addition, the samples of Set 5 in the second stage of the experiment were utilized after 8 days to identify the intermediates of 4NCB. Each experiment was replicated four times (n=4).

## Analytical procedures

The concentrations of 4NCB were measured using high-performance liquid chromatography (HPLC) (Agilent 1260, Wilmington, DE, USA) on an instrument equipped with an Agilent Extend-C18 column (150 mm×4.6 mm). The analyses were performed with a flow of methanol/water (v/v, 7:3) at a rate of 1.0 mL min<sup>-1</sup>. The bacterial cell density was measured as OD<sub>600</sub>.

The identification of the degradation intermediates of 4NCB was conducted using gas chromatograph–mass spectrometry (GC/MS 7890B, Agilent, USA). The GC/MS was configured with an Agilent HP-5 MS

(30 m×0.25 mm i.d.×0.25 μm) column. Helium (purity 99.999%) was employed as the carrier gas with a constant column flow of 1.0 mL/min. The initial temperature was held at 50°C for 5 min, after which the temperature was increased to 150°C and held for 20 min and then increased again to 270°C and held for 20 min.

## Abbreviations

4NCB

4-chloronitrobenzene; VC:ascorbic acid

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

The data sets used and analysed during the current study are available from the corresponding author upon reasonable request.

### Competing interests

The authors declare that they have no competing interests.

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### Authors' contributions

T.L. and P. Z. were involved in the experiments, manuscript writing, and data analysis. TC. Z. contributed to the study design and manuscript correction. All authors read and approved the final manuscript.

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## Tables

**Table 1. Experimental sets and conditions**

Test set	Substrate	Reductant	4-NCB	Experimental purpose
The first stage (MSM): each batch reactor contained: 1) 0.1 g soil; 2) 4NCB of 100 mg L <sup>-1</sup> ; and 3) 49 mL mineral salt medium (MSM) with or without adding substrate and/or reductant:				
Set 1	☐	☐	+	Testing the capacity of the indigenous microorganisms for biodegradation of 4NCB; results were used as a benchmark (the control tests)
Set 2	+	☐	+	Testing the biostimulation of the indigenous microorganisms for 4NCB biodegradation by adding an additional substrate (e.g., 1% of sucrose or starch or ammonium nitrate or urea or peptone)
Set 3	☐	+	+	Testing the biostimulation of the indigenous microorganisms for 4NCB biodegradation by adding one of the reductant [e.g., 0.02% of Fe <sup>2+</sup> or D-glucose or VC (=ascorbic acid) or mannitol]
Set 4	+	+	+	Testing the biostimulation of the indigenous microorganisms for 4NCB biodegradation under the influence of adding one of the 5 reductants and one substrate (peptone)
The second stage (soil): each batch reactor contained 50 g soils mixed with 100 mg-4NCB Kg <sup>-1</sup> soil (W/W) with a moisture content of 20% for sets 1-4, and 1 g-4NCB Kg <sup>-1</sup> soil (w/w) for set 5 with or without adding substrate and/or reductant with the concentration the same as the corresponding sets in the first stage:				
Set 1			+	Testing the capacity of the soil for 4NCB biodegradation; results were used as a benchmark
Set 2	+		+	Testing the biostimulation of the soil for 4NCB biodegradation by adding each of the five substrates
Set 3		+	+	Testing the biostimulation of the soil for 4NCB biodegradation by adding each of the five reductants
Set 4	+	+	+	Testing the biostimulation of the soil for 4NCB biodegradation by adding one of the 5 reductants and one substrate (peptone)
Set 5	+	+	+	Testing the biostimulation of the soil for biodegradation of 4NCB with a high concentration [1 g-4NCB Kg <sup>-1</sup> soil (w/w)] by adding VC and peptone and finding the associated metabolite of 4NCB

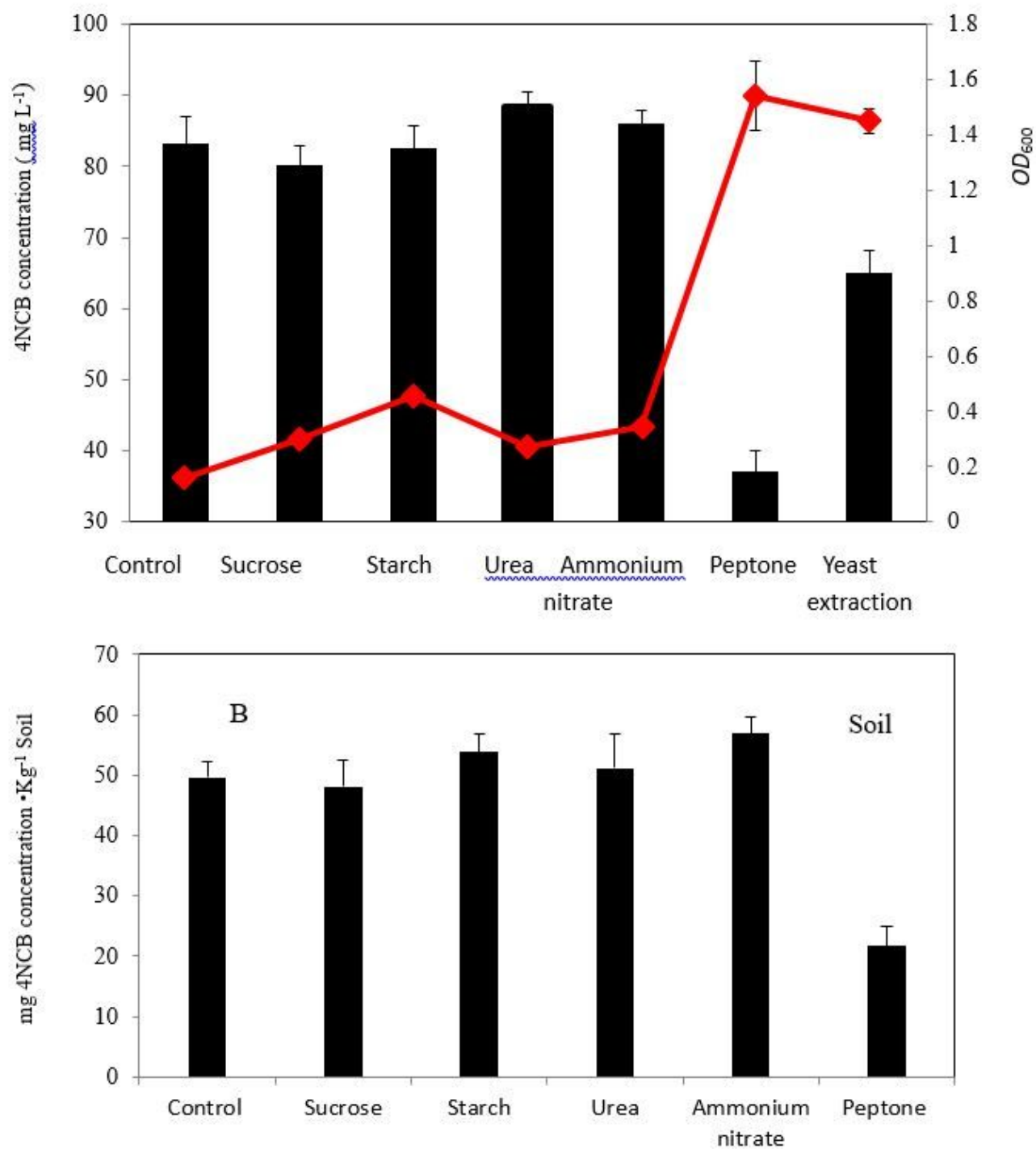
Note:

- In the first stage: All of the 50 mL reaction solutions contained: 1) 0.1 g soil; 2) 4NCB (final concentration = 100 mg L<sup>-1</sup>; and 3) 49 mL mineral salt medium (MSM). + represents the additional substance (substrate or reductant); ☐ represents without the additional substance. In Set 2, substrate represents 1% sucrose or 1% peptone or 1% starch or 1% ammonium nitrate or 1% urea (W/W); in Set

3, reductant represents 0.02%  $\text{Fe}^{2+}$ , or 0.02% D-glucose, or 0.02% VC (= L-lactose) or 0.02% mannitol (W/W); In set 4, substrate represents 1% peptone (w/w), and reductant represents 0.02% of each of the 4 reductants [i.e.,  $\text{Fe}^{2+}$ , D-glucose, VC, mannitol]. The 4-NCB concentration in the batch reactor was  $100 \text{ mg L}^{-1}$  for sets 1-4.

- In the second stage: All of the 50 g soils (4NCB and the soil were mixed up to complete homogenization) contained the following: 1) the same concentration of additional substance as sets 1-4 of the first stage; 2) set 5: substrate represents 1% peptone (w/w), and reductant represents 0.02% VC (w/w). The 4NCB concentration in the soil was  $100 \text{ mg-4NCB Kg}^{-1}$  soil (W/W) for sets 1-4, and  $1 \text{ g-4NCB Kg}^{-1}$  soil (w/w) for set 5; the moisture of the all reactors in the second stage was 20%.

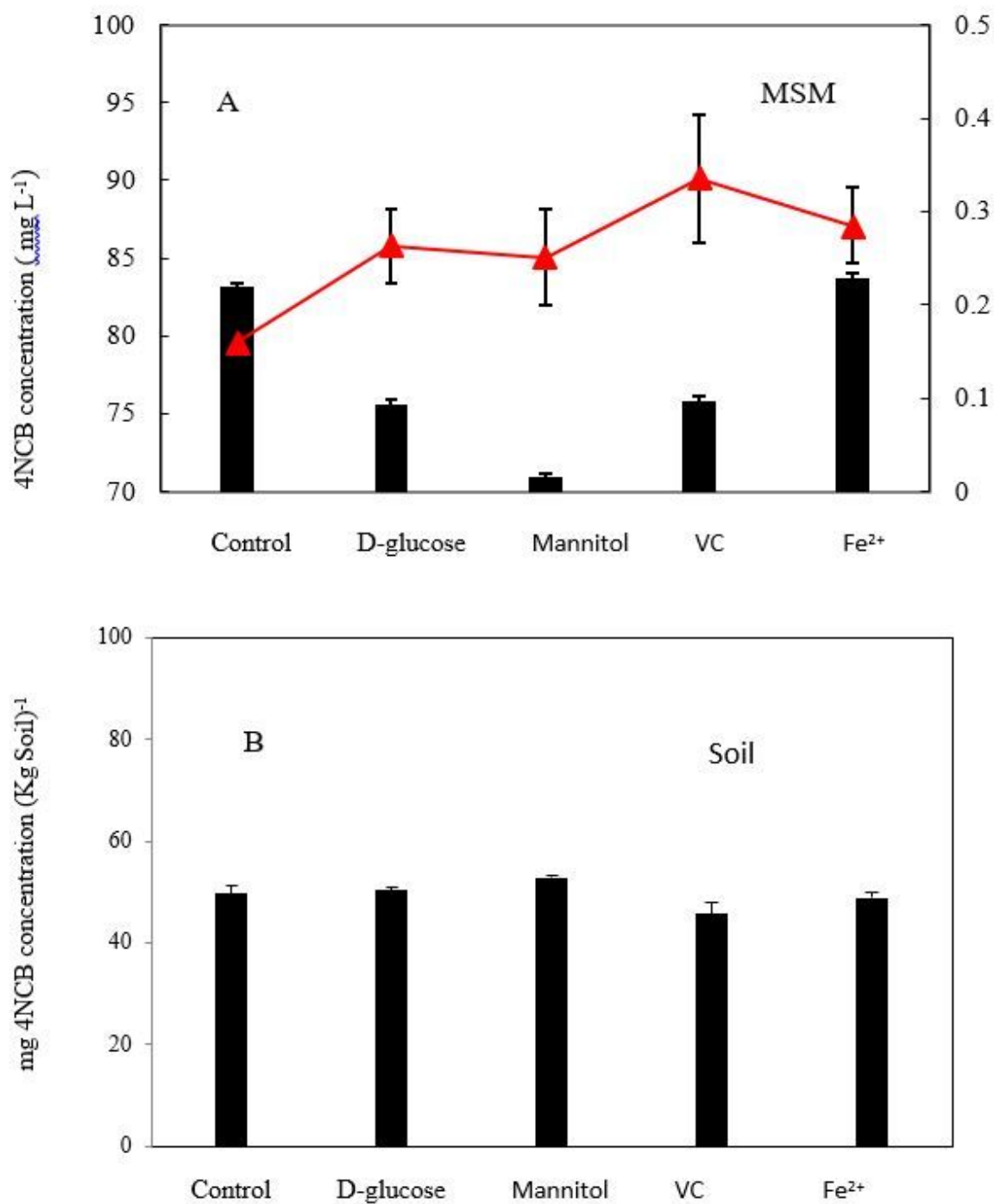
## Figures



**Figure 1**

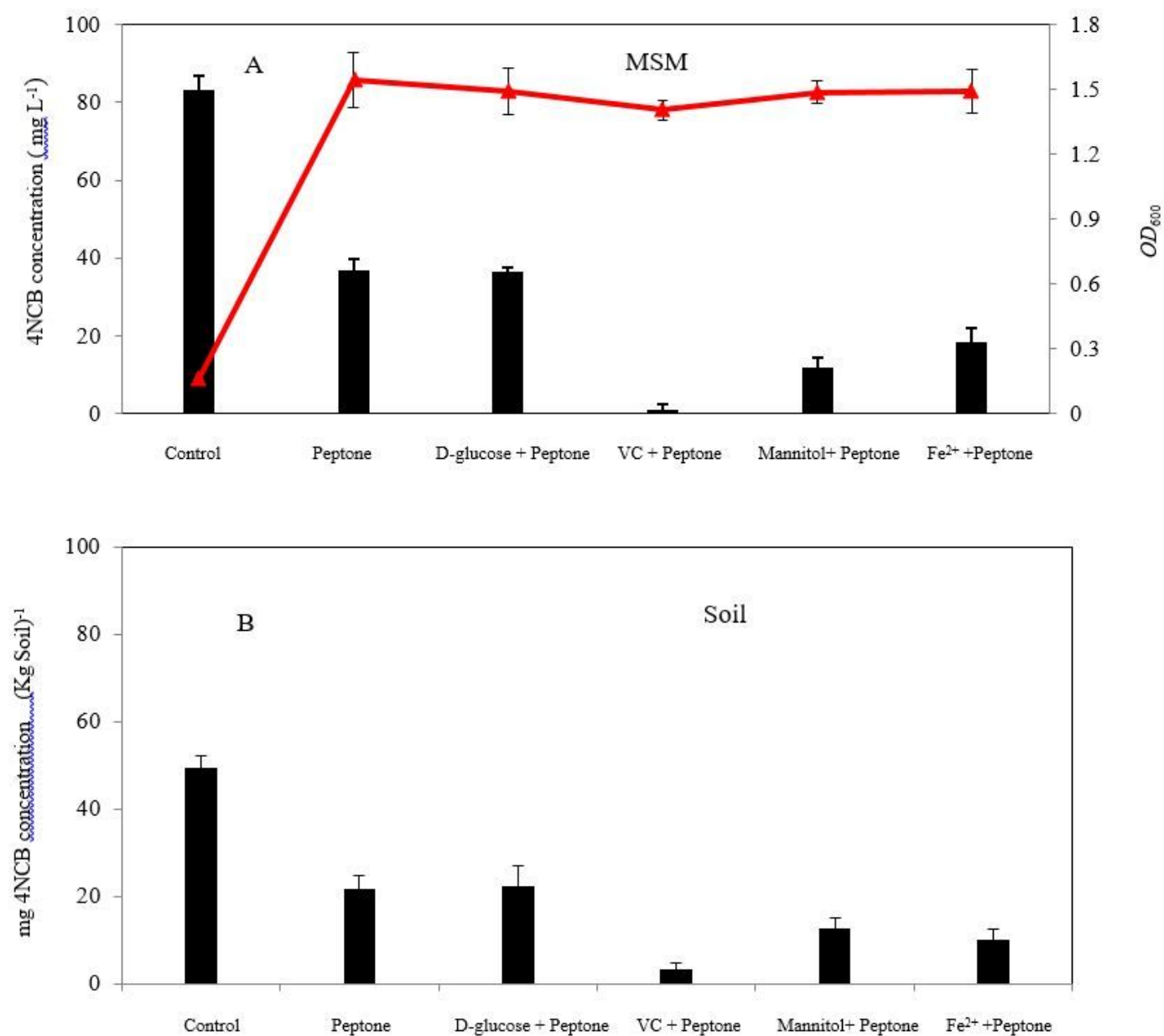
Effects of reductants (Control, D-glucose, Mannitol, VC, and Fe<sup>2+</sup>) on 4NCB degradation and microbial biomass in MSM after 3 days. (A) The amount of residue from 100 mg/L 4NCB and the OD<sub>600</sub> of the indigenous microorganisms. (B) The amount of residue from 100 mg 4NCB/1 kg soil.





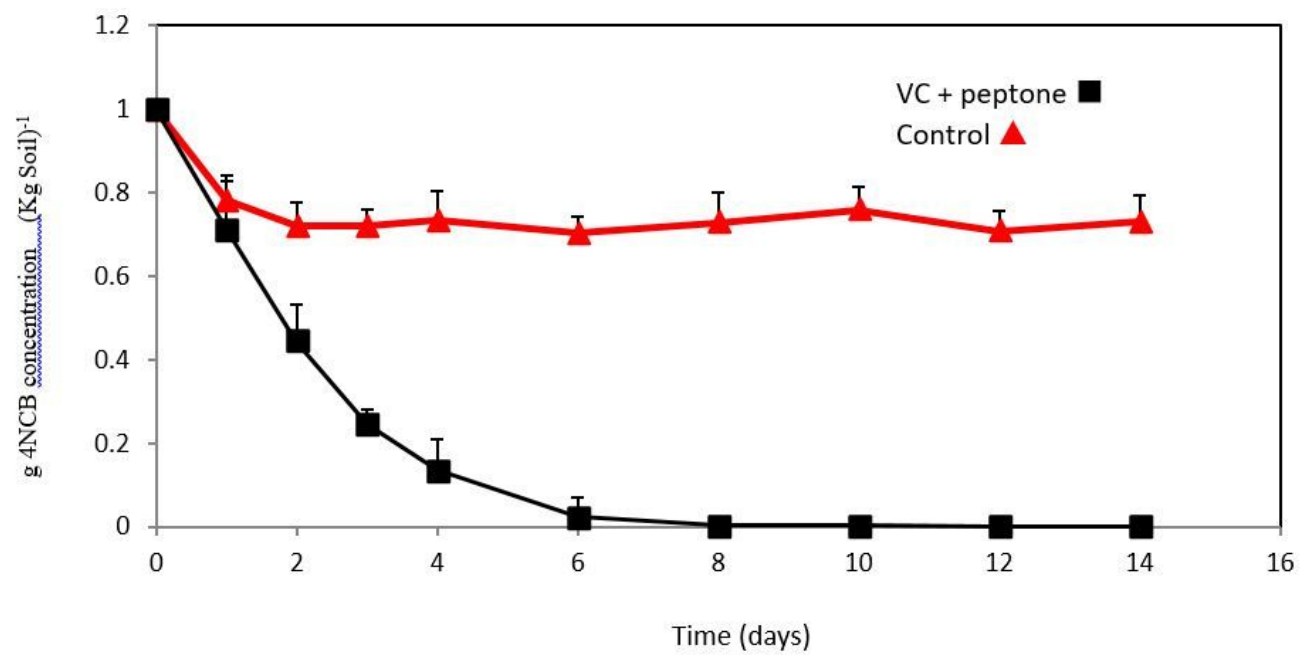
**Figure 2**

Effect of the substrate (Control, Sucrose, Starch, Urea, Ammonium nitrate, and Peptone) on 4NCB degradation in the two systems after 3 days. (A) The concentrations of 4NCB residue from an initial level of 100 mg /L and the OD600 of the indigenous microorganisms. (B) The residue content from 100 mg 4NCB/1 kg soil.



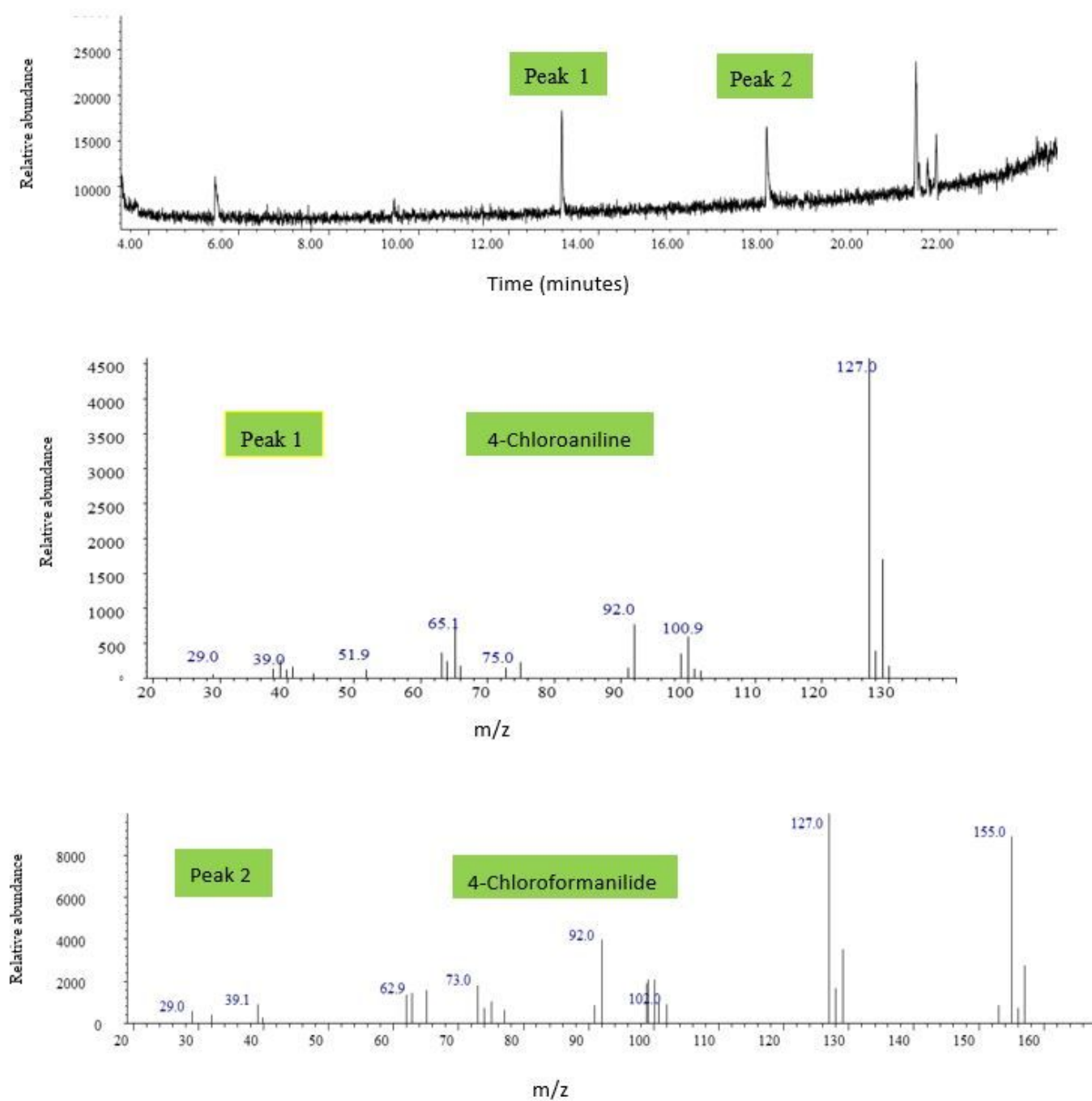
**Figure 3**

Synergistic effect of a reductant and peptone (Control, Peptone, D-glucose + Peptone, VC + Peptone, mannitol + Peptone and Fe + Peptone) on 4NCB degradation after 3 days. (A) The concentrations of 4NCB residue from an initial level of 100 mg /L and the OD<sub>600</sub> of the indigenous microorganisms. (B) The residue content from 100 mg 4NCB/1 kg soil.



**Figure 4**

Utilizing the developed approach to treat laboratory-scale 4NCB-contaminated soil.



**Figure 5**

GC/MS analysis of the metabolites of 4NCB.