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ORIGINAL ARTICLE

Role of *Gordonia* sp JAAS1 in biodegradation of chlorpyrifos and its hydrolysing metabolite 3,5,6-trichloro-2-pyridinol

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Significance and Impact of the Study: Biodegradation of chlorpyrifos results in the formation of an antimicrobial compound 3,5,6-trichloro-2-pyridinol (TCP). It is more mobile compared with the parent molecule due to its higher water solubility thus, causing widespread contamination and has antimicrobial property. Therefore, biodegradation of TCP, the major metabolite of chlorpyrifos, is crucial as if left to accumulate, it will kill all the beneficial microbes in the soil. In this study, *Streptomyces* belonging to genus *Gordonia* sp JAAS1 strain capable of degrading not only chlorpyrifos but also TCP was observed. This is a first report pertaining to biodegradation of chlorpyrifos and its metabolite TCP from *Gordonia* genus. The ability to degrade chlorpyrifos and its metabolite TCP makes this strain a useful candidate for the remediation of contaminated sites.

Keywords
biodegradation, chlorpyrifos, diethylthiophosphoric acid, *Gordonia* sp JAAS1, TCP.

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Abstract

Paddy field soil with prior exposure to chlorpyrifos was chosen for the biodegradation of the pesticide by employing bacteria with special emphasis given to actinomycetes. Actinomycetes are organisms predominantly known for their bioactive compounds, but there is dearth of work pertaining to their role in bioremediation. So this work was carried out to screen for actinomycetes and assess their potential in degradation of the pesticide. Actinobacterial strains were isolated from paddy field soil, with capabilities to degrade chlorpyrifos and its major metabolite 3,5,6-trichloro-2-pyridinol (TCP). Two strains were successfully isolated among which one strain was efficient and was able to tolerate high concentrations of chlorpyrifos. This strain was selected for further investigation; it was identified as *Gordonia* sp based on 16S rRNA analysis and designated as *Gordonia* sp JAAS1. The actinobacterial strain was able to degrade 110 mg l^{-1} of chlorpyrifos within 24 h incubation, and TCP was found to accumulate in the culture medium. However, after 72 h of incubation, TCP was degraded, and finally, diethylthiophosphoric acid (DETP) was obtained.

Introduction

Organophosphorus group of pesticides are the most widely used all around the world, and it accounts for more than 38% of the total world market (Singh and Walker 2006). A reasonable amount of pesticide accumulates in the soil or water after application. The total amount of applied pesticide reaching the target insects is <0·1% and the rest remains in the environment (Pimentel 1995). Several ecosystems have been reported to be contaminated across the world as a result of indiscriminate use of organophosphorus pesticides, causing poisoning of millions of people and over 200 000 deaths annually (Singh *et al.* 2009). Chlorpyrifos is one of the most widely used broad spectrum chlorinated organophosphorus insecticide. The half-life of chlorpyrifos in soil is between 10 and 120 days but can range from up to 1 year depending on the type of the soil, climate and other environmental factors (Singh *et al.* 2006).

Earlier studies have reported that chlorpyrifos was hydrolysed to 3,5,6-trichloro-2-pyridinol (TCP) under alkaline conditions (Racke *et al.* 1996), but later, the

microorganisms are involved in the hydrolysis of chlorpyrifos (Singh *et al.* 2003). The hazardous effects of such toxic chemicals require remediation of chlorpyrifos contaminated sites. There are numerous methods available for detoxification of chlorpyrifos including chemical treatment, photodecomposition, volatilization and incineration, but most of them are not applicable for complete removal of contamination at low concentration because they are inefficient, expensive and not environmental friendly. Biodegradation is one of the most viable options for the remediation of chlorpyrifos in soil and water (Chishti *et al.* 2013).

Actinomycetes have great potential for the biodegradation of certain organic compounds, such as pesticides (Castillo *et al.* 2006). Recent research has shown that actinobacteria of genus *Streptomyces* have the ability to degrade pesticides such as chlorpyrifos (Briceno *et al.* 2012), cypermethrin (Lin *et al.* 2011) and methoxychlor (Fuentes *et al.* 2010). However, very little information is available about the degradation of chlorpyrifos by actinobacteria. The present study reports on the isolation and molecular characterization of chlorpyrifos degrading actinomycetes strain isolated from the paddy field soil.

Results and discussion

Isolation, screening and characterization of chlorpyrifos degrading actinobacterium

In the present study, two different types of actinobacterial isolates were obtained from agricultural soil after enrichment with chlorpyrifos at 60 mg l^{-1}. The isolated strains were screened for the highest tolerance of chlorpyrifos by MIC in which increasing concentrations of chlorpyrifos that ranged from 60 to 110 mg l^{-1} were used. The results revealed that one of the two isolates exhibited growth in the minimal salt medium (MSM) supplemented with 110 mg l^{-1} of chlorpyrifos. This isolate was selected for the further studies.

The isolate was identified based on 16S rRNA sequence analysis. The phylogenetic analysis (Fig. 1) based on 16S rRNA analysis indicated that the isolate had maximum similarity with *Gordonia* sp and was designated as *Gordonia* sp JAAS1. Many researchers have reported the degradation of chlorpyrifos using microbes such as *Alcaligenes* sp. JAS1 (Silambarasan and Abraham 2013a), *Cupriavidus* sp. DT-1 (Lu *et al.* 2013), *Bacillus pumilus* C2A1 (Anwar *et al.* 2009), *Paracoccus* sp. strain TRP (Xu *et al.* 2008), *Sphingomonas* sp. DSP2 (Li *et al.* 2007), *Aspergillus terreus* JAS1 (Silambarasan and Abraham 2013b), *Cladosporium cladosporioides* Hu-01 (Chen *et al.* 2012), *Verticillium* sp. DSP (Feng *et al.* 1997), *Streptomyces* sp. strains AC5 and AC7 (Briceno *et al.* 2012), etc. To our knowledge, this is the first report on *Gordonia* sp JAAS1 on facilitating degradation of chlorpyrifos.

Growth of *Gordonia* sp JAAS1

Growth in presence of chlorpyrifos as the only carbon source was seen in the MSM (Fig. 2). *Gordonia* sp JAAS1 grew in the MSM supplemented with 110 mg l^{-1} of chlorpyrifos during 120 h of incubation, which indicated that it utilized the pesticide as a carbon source, and ISP-2 medium was also used to monitor the actinobacterial growth (Fig. 2). Growth was measured as an increase in absorbance at 600 nm.

Degradation of chlorpyrifos by *Gordonia* sp JAAS1

The degradation of chlorpyrifos (110 mg l^{-1}) in the mineral medium was achieved by *Gordonia* sp JAAS1 (Fig. 3). After 24 h of incubation, chlorpyrifos was detected in the sample by High-performance liquid chromatography (HPLC). After 48 h, TCP was detected in the sample but after 72 h of incubation, TCP was also completely degraded (Fig. 4). TCP is a major metabolite in the chlorpyrifos degradation and has antimicrobial property which inhibits the microbial growth (Feng *et al.* 1997). Recently, Briceno *et al.* (2012) reported that the *Streptomyces* sp. strains AC5 and AC7 were able to degrade about 90% of chlorpyrifos in the liquid medium at the concentrations of 25 and 50 mg l^{-1} within 24 h of incubation, which was confirmed with the appearance of TCP. Such short time for the complete degradation of chlorpyrifos was observed in other investigations as well. For example, Silambarasan and Abraham (2013a) reported that 300 mg l^{-1} of chlorpyrifos was degraded within 12 h of incubation by *Alcaligenes* sp JAS1. *Paracoccus* sp. strain TRP showed the complete mineralization of 50 mg l^{-1} of chlorpyrifos within 4 days in addition to the accumulation of TCP (Xu *et al.* 2008). Xu *et al.* (2007) reported that 100 mg l^{-1} of chlorpyrifos was completely degraded by *Serratia* sp and *Trichosporon* sp within 24 h of incubation. However, in our studies, *Gordonia* sp JAAS1 completely degraded 110 mg l^{-1} of chlorpyrifos and its metabolite TCP within 72 h of incubation.

After 72 h of incubation, chlorpyrifos and its metabolite TCP degraded were subjected to the Fourier transforms infrared (FTIR) analysis is depicted in the Fig. 5. The bands at 1265 and 1085 cm^{-1} support the hydrolytic cleavage of chlorpyrifos. This hydrolytic cleavage was reported previously at 1047 cm^{-1} in chlorpyrifos and its metabolite TCP degradation in the mineral medium by *Alcaligenes* sp. JAS1 (Silambarasan and Abraham 2013a). Bhalerao and Puranik (2009) reported the intense bands at 1242 and 1047 cm^{-1} represent the hydrolytic cleavage

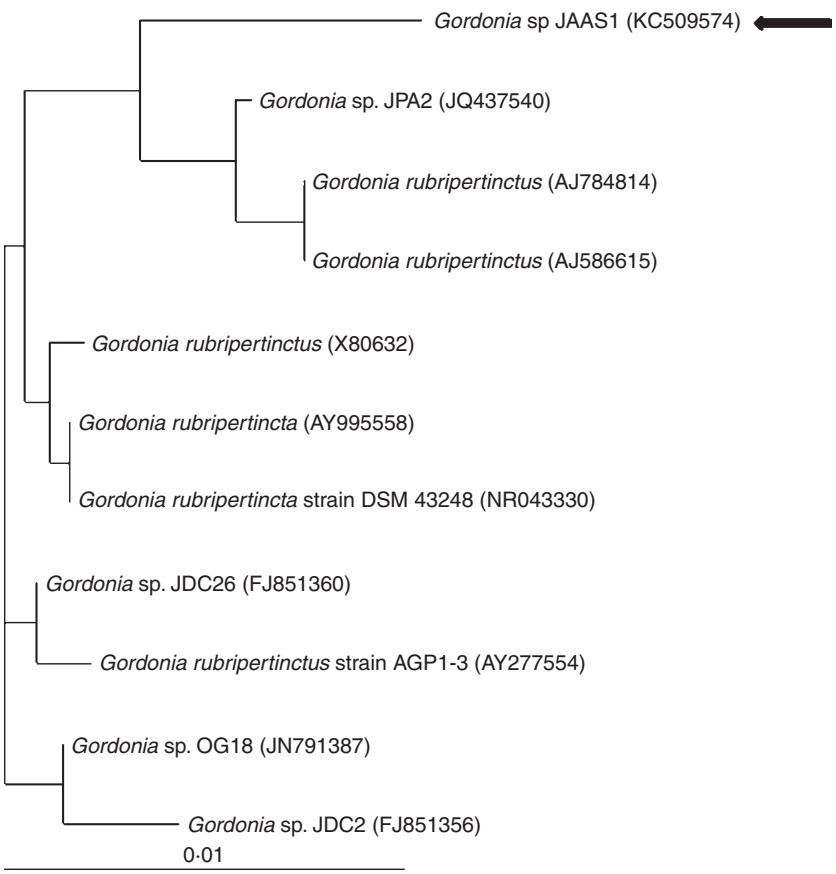

Figure 1 Phylogenetic relationship of *Gordonia* sp JAAS1 based on 16S rRNA gene nucleotide sequences.

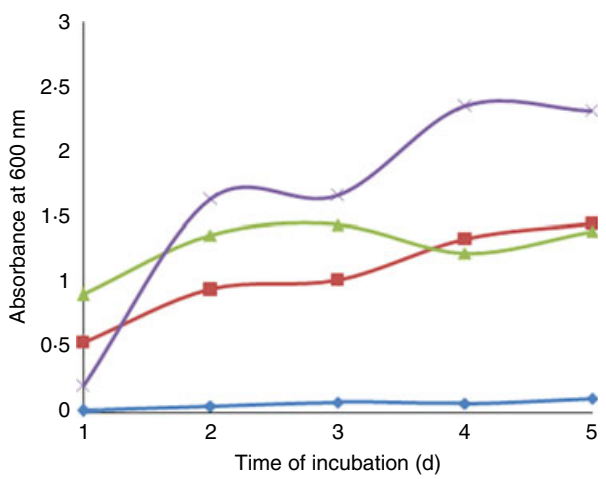

Figure 2 Growth of *Gordonia* sp JAAS1 in the presence and absence of chlorpyrifos. (–◆–) MSM without chlorpyrifos; (–■–) MSM with chlorpyrifos; (–▲–) ISP-2 without chlorpyrifos; (–×–) ISP-2 with chlorpyrifos.

of organophosphorus insecticide such as monocrotophos by *Aspergillus oryzae* ARIFCC 1054.

Further, the metabolic product formed during the degradation of chlorpyrifos and its metabolite TCP by *Gordonia* sp JAAS1 was analysed by GC–MS (Fig. 6). After incubation for 72 h, the final metabolite of chlorpyrifos was identified with mass ion at m/z of 172 [$(M\text{-}3H)^-$, where M = 169] which corresponds to diethylthiophosphoric acid (DETP). Chen *et al.* (2012) reported that chlorpyrifos was first metabolized by hydrolysis to produce TCP and DETP. Subsequently, the hydrolysis product TCP was further transformed by ring cleavage, resulting in its complete detoxification. However, in our study, the DETP was obtained from complete degradation of chlorpyrifos and its metabolite TCP.

Gordonia sp JAAS1 was isolated from agricultural soil that revealed rapid degradation of chlorpyrifos and its metabolite TCP from the mineral medium, which was confirmed with the presence of DETP. These results suggested that *Gordonia* sp JAAS1 would be helpful in the practical application of bioremediation of chlorpyrifos contaminated environment.

Materials and methods

Sampling

The soil sample was collected from a paddy field that was located in the city of Vellore in Tamil Nadu, India. This particular field of choice was exposed to continuous

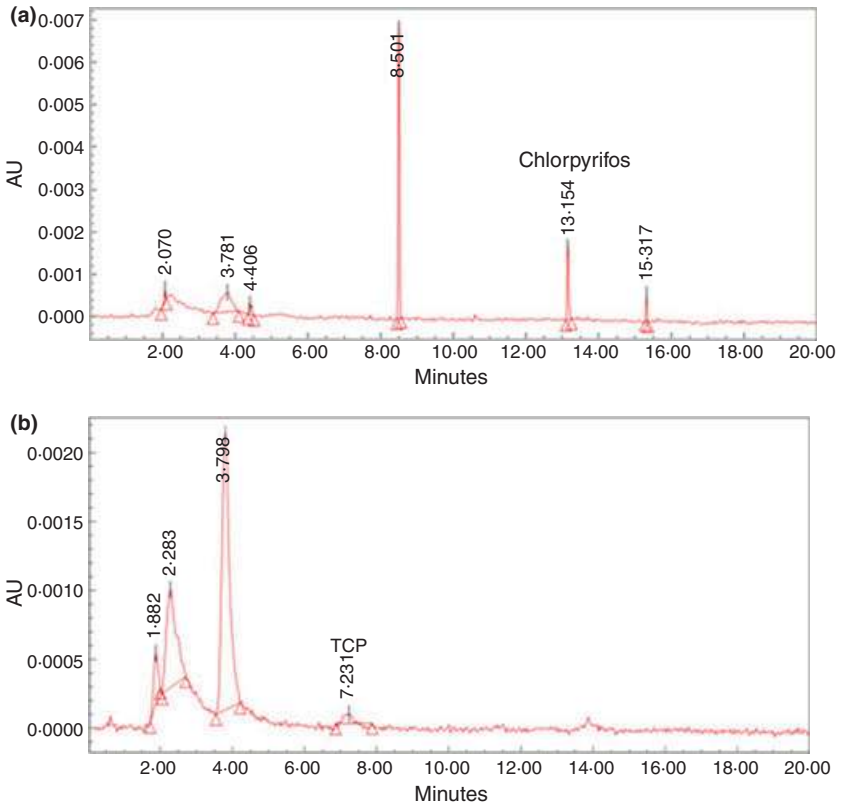

Figure 3 (a) High-performance liquid chromatography of chlorpyrifos detected at 24 h and (b) 3,5,6-trichloro-2-pyridinol detected at 48 h.

applications of chlorpyrifos for a considerable period of time, and the initial concentration of chlorpyrifos residue in the soil was 0·27 mg l^{-1}. Soil was air-dried and sieved through a 2-mm mesh and stored at 4°C until further use.

Chemicals

Certified standard of analytical-grade chlorpyrifos (99% chemical purity) and TCP (99% chemical purity) were purchased from Sigma Aldrich (St Louis, MO, USA). Technical-grade chlorpyrifos, a 20% emulsifiable concentrate, was used in this study which was obtained from Isagro (Asia) Agrochemical Pvt. Ltd., Mumbai. All other reagents used in this study were of high purity and analytical grade.

Enrichment and isolation of chlorpyrifos degrading actinobacterium

The enrichment and isolation of actinobacterial strain were carried out in ISP-2 medium (Malt extract 10 g; yeast extract 4 g; glucose 4 g; distilled water 1000 ml). 10 g of soil was added to 100 ml of ISP-2 medium supplemented with 60 mg l^{-1} of chlorpyrifos in the 250 ml Erlenmeyer flask on a rotary shaker at 120 rev min^{-1} and incubated at room temperature for 1 week. Serial dilutions were prepared using sterilized distilled water, and 0·1 ml of aliquots (10^{-5} dilution) was inoculated in petriplates that contained ISP-2 agar medium. The inoculated plates were incubated at 28°C for 6 days, and isolated colonies were further purified by streaking onto the ISP-2 agar.

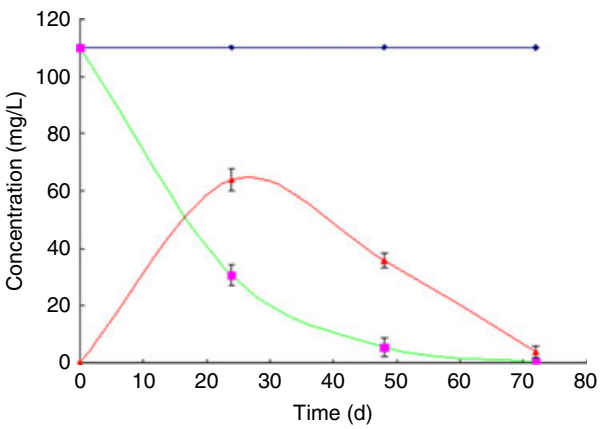

Figure 4 Degradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol (TCP) by *Gordonia* sp JAAS1 in minimal salt medium (MSM). The data are the mean ± standard deviation for triplicate treatments. (—◆—) Control; (—■—) chlorpyrifos; (—▲—) TCP.

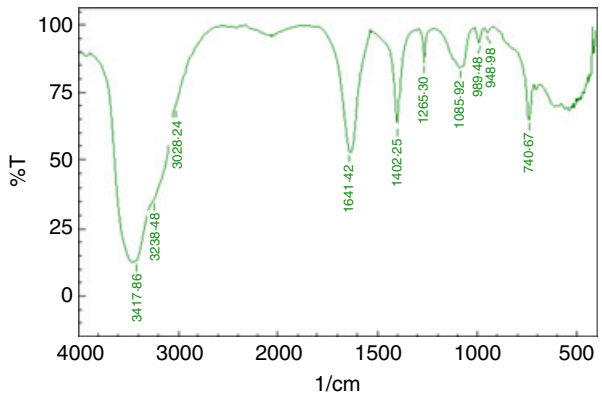

Figure 5 Fourier transforms infrared spectrum of biodegradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol in mineral medium.

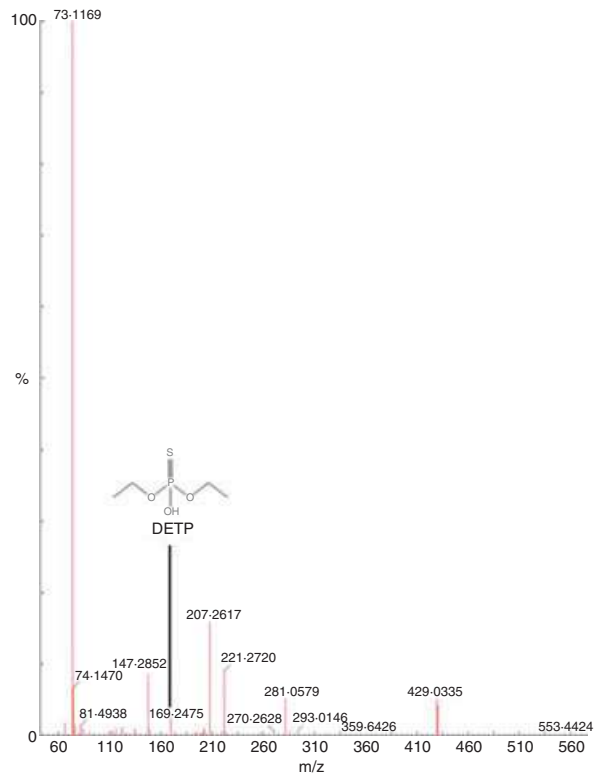

Figure 6 Mass Spectrometry detection of diethylthiophosphoric acid metabolite appeared during the degradation of chlorpyrifos and its metabolite 3,5,6-trichloro-2-pyridinol in the minimal salt medium by *Gordonia* sp JAAS1.

Minimum inhibitory concentration and growth of actinobacterium

The MSM containing (g l^{-1}) Na_2HPO_4, 5·8; KH_2PO_4, 3·0; NaCl, 0·5; NH_4Cl, 1; $MgSO_4$, 0·25 and with increasing concentrations of chlorpyrifos as the sole carbon source was used for the determination of minimum inhibitory concentration (MIC) for the isolated actinobacterial strain. For determination of growth, 1 ml of culture was inoculated into 100 ml of MSM and ISP-2 medium separately with and without chlorpyrifos. The culture was incubated at 28°C on a rotary shaker at 120 rev min^{-1} and at regular time intervals, the optical density at 600 nm was measured.

Characterization of chlorpyrifos degrading actinobacterial strain

The pure culture of the actinobacterial strain was grown in ISP-2 broth for 4 days, and chromosomal DNA was extracted. The genomic DNA was extracted using Chromus Genomic DNA Isolation kit (Chromus Biotech Pvt, Ltd., Bangalore, India). The isolated genomic DNA was loaded and run on 1% agarose gel. For amplification of 16s-rRNA gene, forward primer (400 ng) 5′AGAGTRT GATCMTYGCTWAC-3′ and reverse primer (400 ng) 5′-CGYTAMCTTWTTACGRCT-3′, 2·5 mmol l^{-1} each of dNTPs, 10X Taq polymerase assay buffer and Taq DNA polymerase enzyme were combined keeping the reaction volume to 100 μl. The amplification reaction was further followed by initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s leading to final extension at 72°C using $MgCl_2$ with 1·5 mmol l^{-1} final concentration. The amplified product was sequenced with the primer using ABI 3130 Genetic Analyzer (Chromous Biotech Pvt. Ltd.). The phylogenetic position of the *Gordonia* sp JAAS1 was assessed by performing a nucleotide sequence database search using the BLAST program from NCBI GenBank. The nucleotide sequencing result was submitted to the GenBank national Centre for Biotechnology Information (NCBI), and accession number obtained is KC509574.

Degradation of chlorpyrifos in liquid medium

The actinobacterial strain grown in ISP-2 broth was used as inoculum for the degradation of chlorpyrifos in liquid medium. One millilitre of culture was inoculated into 250 ml Erlenmeyer flask containing 100 ml of MSM with addition of 110 mg l^{-1} chlorpyrifos and then incubated at 28°C, 120 rev min^{-1}. Samples were recovered from the culture flask at an interval of 24 h, and the degradation was determined by HPLC.

Chemical analysis

The sample aliquot of 10 ml was centrifuged at 7200 ***g*** for 10 min, and the supernatant obtained was extracted with equal volume of dichloromethane twice. Organic

layer of dichloromethane was evaporated at room temperature. The residues were dissolved in acetonitrile and analysed by HPLC. Sample injection volume was 20 μl, and the mobile phase was programmed at flow rate of 1 ml min^{-1}. Removal of chlorpyrifos and accumulation of TCP were detected at 230 nm wavelength by HPLC.

To evaluate the method of chlorpyrifos and TCP analysis, the validation studies were performed concerning the linear range of calibration curves, recoveries, limit of detection (LOD) and limit of quantification (LOQ). Each of chlorpyrifos and TCP in different known concentrations was spiked separately in 50 ml of the MSM medium (100, 200 and 300 mg l^{-1}). Extraction and analysis were performed in triplicate with the analytical procedure described previously. The average recoveries of chlorpyrifos from the MSM at levels of 100, 200 and 300 mg l^{-1} were measured to be 97·3 ± 4·6, 96·6 ± 4·7 and 98·6 ± 1·3%, respectively, and the calibration curve proved to be linear with these average recoveries. Average recoveries of TCP (100, 200 and 300 mg l^{-1}) from MSM were 100·2 ± 3·3, 98·6 ± 1·5 and 96·2 ± 5·1%, respectively. The LOD and LOQ of chlorpyrifos were 0·024 and 0·072 mg l^{-1}, respectively. The LOD and LOQ of TCP were 0·009 and 0·027 mg l^{-1}, respectively.

The instrument GC (Clarus 680 GC model, Perkin Elmer, Waltham, MA, USA) and the mass spectrometer (Clarus 600 with an Elite-5MS model (30·0 m, 0·25 mmID, 250 μm df) column) were used for analysis. The metabolites produced during chlorpyrifos degradation with reference to TCP degradation was analysed by GC–MS (Gas Chromatography–Mass Spectrometry). The sample from the DCM extraction of MSM was injected into the GC–MS. In this technique, the mass spectrometer was set to scan over range of one unit. The ion current resulting from this very small range of mass was detected and plotted. GC operating parameters were as follows: initial temperature 60°C for 2 min; ramp 10°C per min to 300°C; hold 4 min; total run time 30 min; sample injection volume 1 μl; split 10:1 and Helium (carrier gas). MS was operated in the solvent delay at 2 min; transfer temperature at 200°C; source temperature at 200°C and scanned by 50–600 Da.

The parent compound (chlorpyrifos) before and after degradations was recorded in the frequency range of 4000–500 cm^{-1} by a FTIR spectrophotometer (8400 Shimadzu, Tokyo, Japan).

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Conflict of Interest

No conflict of interest declared.

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