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Fate of a novel strobilurin fungicide pyraoxystrobin in flooded soil

Tilong Yang,† Chao Xu,† Xunyue Liu,† Xia Chen,† Jianbo Zhang,† Xingcheng Ding*†

† Institute of Nuclear Agricultural Sciences, Key Laboratory of Nuclear Agricultural Sciences of Ministry of Agriculture, Zhejiang University, Hangzhou 310029, China

* Corresponding Author: Institute of Nuclear Agricultural Sciences, Zhejiang University, Kaixuan Road No. 268, Hangzhou 310029, Zhejiang Province, China; Phone: +86-571-86971201; Fax: +86-571-86971421;
E-mail: dingxch@zju.edu.cn
Environmental Impact Statement

Pyraoxystrobin is a novel strobilurin fungicide with a substituted pyrazole in the side chain, which has high efficiency and broad spectrum fungicidal activities against many crop diseases. The impact of new pesticide on the environment must be assessed before used in agricultural applications. In this study, the extractable residues, bound residues and mineralization, as well as the dissipation rates of pyraoxystrobin were investigated in three flooded soils using $^{14}$C tracing technique in order to understand its potential risks to environment.
Abstract

Pyraoxystrobin, ((E)-2-(2-((3-(4-chlorophenyl)-1-methyl-1H-pyrazole-5-yloxy)methyl)) phenyl)-3-methoxyacrylate) is a novel strobilurin fungicide with excellent and broad spectrum antifungal efficiency. Environmental behaviors of the new fungicide must be assessed to understand its potential risks to environment. In this study, the extractable residues, bound residues and mineralization, as well as the dissipation rates of pyraoxystrobin were investigated in three flooded soils using $^{14}$C tracing technique. Results showed that pyraoxystrobin didn’t undergo appreciable dissipation during the 100-d incubation in some tested soils, with the amount of 70.01%, 28.58% and 83.85% parent compound remaining in the solonchak, cambisol and acrisol soil at the end of the experiment, respectively. Almost no $^{14}$C-pyraoxystrobin was mineralized to $^{14}$CO$_2$ (< 0.5%) over the experimental period. Organic matter had a dominating influence on the bound residues formation and the fractions of bound residues increased as the soil organic matter content increased. Less than 9% of the radioactivity was found in the aqueous phase, while the majority of extractable residues (> 65.39%) were recovered in the organic extracts. This study aims to give a deep insight into the environmental behavior of pyraoxystrobin and may be beneficial to the risk assessment for other analogous fungicides.

Keywords: pyraoxystrobin, flooded soil, dissipation, persistence.
Introduction

Strobilurin fungicides are an important class of agricultural chemicals derived from a group of natural fungicidal derivatives of β-methoxyacrylic acid, which have been commonly used against diseases in many important economical crop diseases. In addition, pyrazole derivatives are widely used as fungicides, insecticides, and herbicides. Recently, a series of new strobilurin derivatives with a substituted pyrazole in the side chain are synthesized and found to exhibit excellent fungicidal activities as well as insecticidal and acaricidal activities. Despite soil represent a primary sink for environmental contaminants and a major source of nutrients for plants in terrestrial environment, information concerning fate of strobilurins in soils is limited.

Pyraoxystrobin (Code number: SYP-3343, (E)-2-(2-((3-(4-chlorophenyl)-1-methyl-1H-pyrazole-5-yloxy) methyl))phenyl)-3-methoxyacrylate) is a novel strobilurin fungicide containing a substituted pyrazole, which was developed by the Shenyang Research Institute of Chemical Industry, Shenyang, China. It shows high efficiency and broad spectrum fungicidal activities against Pseudoperonospora cubensis, Blumeria graminis, Erysiphe cichoracearum, Plasmopara viticola, and Phyricularia grisea. And the effect against Plasmopara viticola, and Phyricularia grisea was even better than azoxystrobin, a commercially available strobilurin fungicide. It has obtained authorized patents both in China and in other countries and has obtained temporary registration in China in 2009. In pesticide risk assessment, bound residues (BR) formation and...
mineralization are usually considered in current regulatory procedures. Furthermore, nonextractable residues for new pesticides are required in Europe. According to the EU directive no authorization shall be granted if the form BR in amounts exceeding 70% of the initial dose after 100 days with a mineralization rate of less than 5% in 100 days. However, little is known of the fate processes such as formation of BR and mineralization for pyraoxystrobin. Pyraoxystrobin will potential used for control of rice diseases and may be subjected to flooded conditions following heavy rainfall on poorly drained surface soils. Unfortunately, concern is the absence of literature on fate of pyraoxystrobin in flooded soil.

In this study, $^{14}$C-pyraoxystrobin was used as a tracer to investigate the kinetics of extractable residues (ER), formation of bound residues (BR) and mineralization of pyraoxystrobin in flooded soils.

Materials and Methods

Chemicals. $^{14}$C labeled pyraoxystrobin (Table 1; radiochemical and chemical purity are both over 98% and the specific activity is $5.042 \pm 0.076$ mCi/mmol) was synthesized according to the method of Liu et al. Two kinds of scintillation cocktail were used in this study. Scintillation cocktail I was prepared by dissolving 0.5 g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene (POPOP, Arcos Organics, Geel, Belgium) and 5.0 g of 2,5-diphenyloxazole (PPO, Arcos Organics, Geel, Belgium) in 350 mL of 2-methoxyethanol and 650 mL of dimethylbenzene. Scintillation cocktail II was prepared by dissolving 0.5 g POPOP and 5.0 g PPO in a mixture of 600 mL
dimethylbenzene, 225 mL 2-ethoxyethanol and 175 mL ethanolamine. Scintillation cocktail II was used for trapping and measuring $^{14}$CO$_2$ and scintillation cocktail I was used for measuring the radioactivity of the extracts. Dimethylbenzene, 2-methoxyethanol, ethanolamine, calcium chloride, acetonitrile, methanol, dichloromethane were all of analytical grade or chromatographic grade.

**Soils.** Three different representative agricultural soils, including a solonchak soil (soil A), a cambisol soil (soil B) and an acrisol soil (soil C), were used in this experiment. The soil samples were taken from the surface layer (0-15 cm) of agricultural fields in Zhejiang Province, which located in Eastern China and has a subtropical summer rain climate. All soils were not previously exposed to pyraoxystrobin. The bulk soil samples were air dried, mixed and passed through a 2-mm sieve before use. Selected physicochemical properties of the soils are given in Table 2.

**Soil incubation experiments and measurement of released $^{14}$CO$_2$.** The incubation experiments were performed similar to OECD Guideline 307. The setup employed in this study is shown in Figure S1 of the Supplementary Information. The tested soils were pre-incubated in a dark cultivation cabinet at 25 ± 1°C for 30 days after adjusting the soil moisture content at pF 5.5 to allow the microorganisms to acclimatize. Then an appropriate volume of prepared pyraoxystrobin methanol solution (8.2 × 10$^5$ Bq) was dripped into each soil (400 g, dry weight). The homogenized soil samples were left in a fume hood to completely remove methanol. After re-adjusting the soil moisture content at pF 3.8, a 10 g (dry weight) aliquot of
each treated soil was transferred into a 40-mL glass vial and water-logged (about 2 cm water layer). The uncovered vials were placed in a vacuum desiccator and incubated at 25 ± 1°C in the dark in a cultivation cabinet. Three replicates were used for each soil. The flow-through soil test system was flushed with a slow and continuous nitrogen stream (99.99% pure) for 30 min, at 0, 1, 5, 10, 20, 30, 45, 60, 75, and 100 d after pyraoxystrobin application. The traps containing 30 mL solutions connected with each incubation flask in the upstream section were used for scrubbing the trace amounts of CO₂ from the inlet gas (with 0.5 M NaOH, two traps) and for compensating the water loss (with water). And in the downstream section, ethylene glycol and H₂SO₄ (50%) were used to trap organic volatile compounds and alkaline volatile chemicals respectively, followed by two traps containing 30mL of 0.2 M NaOH to absorb ¹⁴CO₂ from the mineralization of the labeled pyraoxystrobin. The trap solutions were exchanged at each sampling date and radioactivity in the 0.2 M NaOH traps, ethylene glycol and H₂SO₄ were measured on an ultra-low level liquid scintillation counter (Quatalus-1220, Perkin Elmer, Turku, Finland) after mixing with 15 mL liquid scintillation cocktail I and removing chemiluminescence in dark for 24 hours.

**Analysis of ¹⁴C-extractable residues.** At 0, 1, 5, 10, 20, 30, 45, 60, 75, and 100 d after treatment, three replicates from each soil were sampled. The soil-water slurry from each sample was transferred into a 100-mL polypropylene centrifuge tube and centrifuged at 4000 rpm for 10 min using an Eppendorf 5840R centrifuge (Eppendorf, Hamburg, Germany), after which the supernatant was decanted and ¹⁴C activity in the
water phase was determined on a liquid scintillation counter (LSC, Wallac WinSpectral-1414, PerkinElmer). The soil phase was consecutively extracted by sequential extraction solvents (50 mL) with 0.01 M CaCl$_2$ solution, acetonitrile/water (9:1, v/v), methanol and dichloromethane. Each extracts constituted of shaking for 24 h, followed by centrifugation at 4000 rpm for 10 min. The dichloromethane phase was left in a fume hood until completely volatilized and re-dissolved in 25 mL methanol. A 1.0 mL aliquot of the extract from each step was removed and mixed with 10 mL scintillation cocktail I to measure $^{14}$C-radioactivity on LSC. The ER was defined as the sum of radioactivity from all extracts and the water phase.

**HPLC (High Performance Liquid Chromatography)-LSC analysis.** To prepare samples for identification and quantification of parent pyraoxystrobin, the surface water and the 0.01 M CaCl$_2$ extract were mixed, passed through a 0.45-μm filter (Millipore, Ireland) and extracted with an equivalent volume of dichloromethane until no further radioactivity was detected in the aqueous phase. The dichloromethane phase was then combined with other organic extracts. The combined extracts were further concentrated to near dryness on a vacuumed rotary evaporator (Eyela SB-1000; Eyela, Tokyo, Japan) at 37°C and then recovered in about 10 mL methanol and then condensed to 1.0 mL under a gentle stream of nitrogen at 45°C for further analysis. The final extracts were centrifuged at 14,000 rpm for 10 min. The supernatant was passed through a 0.22-μm filter and transferred to another clean centrifuge tube (1 mL). A 20-μl aliquot was injected into Waters 2695 multisolvent delivery unit coupled with a Waters Faction Collector III (Waters, Milford, MA, USA). The details of the
HPLC analysis were stated in Text S1 and Table S1 of Supplementary Information. A pyraoxystrobin standard sample was also injected for confirmation of the retention time of the parent pyraoxystrobin. The post-column eluents were collected at 1.0 min intervals into glass scintillation vials. The collected fractions were measured for radioactivity on LSC after addition of 10mL scintillation cocktail I. The measured $^{14}$C-activity was used for the quantification of parent compound.

**Analysis of $^{14}$C-bound residues.** After the sequential extraction for extractable residues, the soils were left in a fume hood to allow the volatilization of the residual organic solvents. Aliquots of 1.0 g homogenized air-dried soils were combusted on a biological oxidizer (OX-600, R.J. Harvey Instrument, Hillsdale, NJ) at a combustion temperature of 850°C and a catalysis temperature of 650°C for 4 min. The evolved $^{14}$CO$_2$ was trapped in 15 mL scintillation cocktail II and then measured for $^{14}$C radioactivity by LSC. The recovery was determined to be 95.0 ± 2.6% (n=3) by combusting the known radioactivity of $^{14}$C-pyraoxystrobin.

**Statistical analysis.** All measurements were in three replicates and the arithmetic means and standard errors of means (mean ± SEM) were calculated from the repeated measurements. SAS statistical software (SAS Institute, Cary, NC) was used for analysis of the significant difference between treatments tested and a one-way analysis of variance (ANOVA) was applied. The dissipation of pyraoxystrobin was fitted to the first-order equation:

$$y = a \cdot e^{-kt}$$

where $a$ represents the percent of parent molecule pyraoxystrobin at 0 d, $y$ represents
the percent of parent molecule pyraoxystrobin at time $x$, $x$ is the time in days, and $k$ is dissipation rate constant.

Results and Discussion

Mineralization of pyraoxystrobin.

Throughout the incubation period, good mass balance was consistently obtained by the sum of the radioactivity of recovered $^{14}$CO$_2$, BR and ER (95.6 ± 0.9% to 104.8 ± 0.7%, Figure 1). During the whole incubation period, no radioactivity was detected in the ethylene glycol and H$_2$SO$_4$ solution, indicating that little pyraoxystrobin or its metabolites were volatilized or no alkaline volatile molecules was formed. The mineralization, defined as the fraction of recovered $^{14}$CO$_2$ in initially applied amount, gradually increased with incubation time in all tested soils. However, the cumulative $^{14}$CO$_2$ did not exceed 0.5% of the spiked radioactivity in all tested soils throughout the incubation under flooded conditions (Figure 2), indicating the mineralization of the pyraoxystrobin pyrazole ring was negligible and pyraoxystrobin might be persistent in soil or exist in the form of metabolites. The low amount of mineralization could be also due to the bound residues formation of pyraoxystrobin or its metabolites.

Furthermore, the mode of action of strobilurins is that these compounds inhibit the mitochondrial respiration of fungi. $^{13}$Pyraoxystrobin, as a novel fungicide with high efficiency and broad spectrum fungicidal activities, was likely able to block respiration of certain microorganisms and thus the mineralization of pyraoxystrobin is limited.
**Bound residues formation.**

Bound residues (BR) are measured by the total radioactivity obtained by the combustion of the extracted soil samples. In the present study, the formation of BR in all three soils was slow at the beginning of incubation and increased with the incubation time after 5 days of the incubation (Figure 3). For example, the fractions of BR were 1.76 ± 0.13%, 1.55 ± 0.05% and 1.07 ± 0.04% of the initial applied \(^{14}\)C activity at 5 d in soil A, B and C, respectively. No significant increase was found in the first 5 days of the incubation period (\(p > 0.05\)). After 5 d, BR was gradually formed with time and reached 14.3 ± 0.6%, 31.5 ± 0.3%, and 3.0 ± 0.1% of the initially spiked activity at the end of incubation in soil A, B, and C, respectively. The organic matter content in soil is considered to be one of the most important factors for the BR formation because it contains reactive functional groups and internal nanopore structures.\(^{14, 15}\) The formation of BR in soil B, which contains a higher content of soil organic matter (3.23%), is much higher than that in soil A (2.41%) and soil C (2.06%) (\(p < 0.05\)). It suggested that organic matter had a dominating influence on the BR formation of pyraoxystrobin or its metabolites. In addition, the BR formation is also influenced by soil pH. For example, the fraction of BR was the highest in neutral soil (soil B, pH 6.95), followed by alkaline soil (soil A, pH 8.20) and acid soil (soil C, pH 5.36). However, the order varied for different pesticides such as metsulfuron-methyl, \(^{16}\) bromoxynil, \(^{17}\) and ZJ0273, \(^{18}\) which suggests the chemical structure is also an important factor on the environmental fate of a pesticide.
The formation of BR may cause a loss of fungicide activity and bioavailability and may be considered as a detoxification process.\textsuperscript{19} However, a partial release of BR may induce relevant ecotoxicological effect and environmental pollution.\textsuperscript{20} At the end of the 100-d incubation, BR in all soils was much lower than 70\%, which meet the non-accumulative criteria in the directive by the European Commission.\textsuperscript{11}

\textbf{Kinetics of extractable residues.}

Extractable residues (ER) are considered to be more available for organisms and susceptible to degradation.\textsuperscript{21} The fractions of ER of pyraoxystrobin in all tested soils are shown in Figure S2. The ER kept constant in soil C with no significant decrease over the experimental period ($p > 0.05$), while there were prominently decrease in both soil A and soil B ($p < 0.05$). For instance, the radioactive amount of ER of \textsuperscript{14}C-pyraoxystrobin were determined to be $98.4 \pm 2.0\%$ and $100.0 \pm 0.8\%$ on day 5 in soil A and soil B, respectively, while ER were found to be $82.0 \pm 2.1\%$ in soil A and $71.2 \pm 0.8\%$ in soil B on day 100. Radioactivity recovered in the water phase was $1.99 \pm 0.22\%$, $1.01 \pm 0.07\%$ and $1.18 \pm 0.18\%$ of the introduced \textsuperscript{14}C activity in soil A, B and C at 1 d after incubation (Figure 4). The fraction of \textsuperscript{14}C activity in water phase in soil B increased slightly with time, from $0.93\%$ on day 5 up to $2.78\%$ on day 100 (Figure 4b), while the fractions decreased over time in soil A (Figure 4a) and soil C (Figure 4c). The increase in soil B may due to the formation of more hydrophilic metabolites or the solubility enhancement\textsuperscript{22} of pyraoxystrobin by dissolved organic matter. The decrease in radioactivity of the aqueous extracts in soil A and soil C was
likely due to the conversion of the $^{14}$C activity into BR. The decrease could be also
due to the movement of radioactivity into the other organic fractions in this extraction
procedure. The $^{14}$C activity recovered in CaCl$_2$ was defined as the readily available
soil fraction by Mordaunt et al. The end of the incubation, only $3.46 \pm 0.20\%$, $3.02 \pm 0.20\%$ and $2.89 \pm 0.20\%$ of the introduced activity in CaCl$_2$ was detected in
soil A, B and C, respectively, indicating that pyraoxystrobin residues are poorly
water-soluble and not readily available. Pesticides in solution are more available to
microorganisms in soils. The low fractions (< $8.17\%$ in soil A, < $5.80\%$ in soil B,
and < $5.55\%$ in soil C, Figure 4) of pyraoxystrobin residues in aqueous phase (water
and CaCl$_2$), can partly predict a low bioavailability of pyraoxystrobin in flooded soil.
The majority of ER was presented in the organic extractable fraction
(acetonitrile/water, methanol and dichloromethane), especially in acetonitrile/water
(9/1) (Figure 4). The high lipophilic property of pyraoxystrobin (Table 1) could
account in part for the high extraction of the $^{14}$C activity in the organic fraction. The
radioactivity detected in the organic phase in soil C remained in the range of $92.78\%$
to $96.37\%$ of the applied activity over time, while it decreased from approximately
$90\%$ to $77.30\%$ in soil A and approximately $97\%$ to $65.39\%$ in soil B over the
incubation period. Because trace or no detectable radioactivity was detected in
mineralization and volatile pyraoxystrobin, the decline radioactivity of ER may be
caused by the conversion of pyraoxystrobin and its metabolites to BR.

Dissipation of pyraoxystrobin.
The combination of volatilization, mineralization and BR, as well as degradation contributed to pyraoxystrobin dissipation in soil. The parent compound content was calculated by the radioactivity measured on the LSC in the fraction of pyraoxystrobin collected by HPLC. As shown in Figure 5, the parent compound in soil A, B and C remained at 70.01 ± 4.65%, 28.58 ± 5.94% and 83.85 ± 4.99% of the introduced $^{14}$C activity at the end of the experimental period respectively. Dissipation of pyraoxystrobin was well fitted to the first-order decay model in the three soils. Pyraoxystrobin underwent limited dissipation in soil A and C during the experimental period under flooded conditions, with the estimated half-lives ($t_{1/2}$) exceeding the incubation of 100 days. The fungicide dissipation in soil B was more rapid compared to soil A and C, with a $t_{1/2}$ value of 56 days. The different dissipation rates in the three soils may be attributed to their physicochemical properties. Soil organic matter can directly result in the dissipation of pyraoxystrobin due to their dominating role on the process of BR. Furthermore, pyraoxystrobin degraded faster in the soils with more organic matter content than in the soils containing lower organic matter content (Figure S3). Consequently, the dissipation rate in soil B (organic matter 3.23%) was the fastest, followed by the soil A (2.41%) and soil C (2.06%). All the dissipation processes of pyraoxystrobin, such as mineralization ($< 0.17%$), degradation (Figure S3) and BR ($< 3.13%$), were negligible in soil C. It may result from that the activities of some specific microbial species or at least one or more enzymes may be inhibited in this acidic soil which contains the lowest organic matter content. Previous studies reported that the $t_{1/2}$ values of azoxystrobin, a strobilurin
fungicide, in soils were between 56 and 279 days, depending on the properties of the soils and different soil conditions. Pyraoxystrobin was stable in this study, with the estimated $t_{1/2}$ values at 214, 56 and 670 days in soil A, B, and C respectively under the flooded conditions. The long persistence of the parent compounds may have a potential toxic effect on soil microbes or a risk for accumulation into plant. The evaluation of the ecotoxicological relevancy of the residual parent compounds may need further investigation.

Conclusion

This study provides the first evidence for the environmental behaviors of the novel strobilurin fungicide, pyraoxystrobin, in flooded soils. Pyraoxystrobin was relatively stable in flooded soils and the mineralization rate was less than 0.5 % in 100 days. The fraction of BR of pyraoxystrobin was less than 70% of the initially applied amount, which was the maximum residual level after 100-d incubation proposed by the European Commission. The majority of extractable residues were recovered in the organic extracts and less than 9% of the radioactivity was found in the aqueous phase. The high persistence of pyraoxystrobin can give us some implications that the fate of some strobilurins in flooded soils may also merit further investigation.

Acknowledgments

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Supplementary Information

Additional details on description of methods and results is available free of charge via the internet at http://www.rsc.org.
References:


Figure 1. Dynamic characterization of $^{14}\text{CO}_2$, extractable residues (ER) recovered in the aqueous phase, extractable residues (ER) recovered in the organic phase, and bound residues (BR) in (A) soil A, (B) soil B, and (C) soil C under flooded conditions during the 100-d incubation.
**Figure 2.** Cumulative mineralization rates of $^{14}$C-pyraoxystrobin under flooded incubations.
Figure 3. Formation of bound residue in flooded soils treated with $^{14}$C-pyraoxystrobin.
Figure 4. $^{14}$C-ER distribution characterization for the three soils: (a) Soil A; (b) Soil B; (c) Soil C.
Figure 5. Dissipation of parent molecule pyraoxystrobin in flooded soils over the incubation period.
Table 1. Physicochemical properties of pyraoxystrobin

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
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<tr>
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<tr>
<td>Chemical structure</td>
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<tr>
<td>Chemical formula</td>
<td>C$<em>{22}$H$</em>{21}$ClN$_2$O$_4$</td>
</tr>
<tr>
<td>Solubility</td>
<td>Insoluble in water</td>
</tr>
<tr>
<td>$K_{ow}$</td>
<td></td>
</tr>
<tr>
<td>pH=5</td>
<td>L$<em>g$K$</em>{ow}$=4.37</td>
</tr>
<tr>
<td>pH=9</td>
<td>L$<em>g$K$</em>{ow}$=5.36</td>
</tr>
<tr>
<td>Melting point (°C)</td>
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</tr>
</tbody>
</table>

* The $^{14}$C labeling position

* Octanol/water partition coefficient
Table 2. Basic physical and chemical properties of the soils used in the study

<table>
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<tr>
<th>No.</th>
<th>Soil type</th>
<th>pH (water)</th>
<th>OM&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>CEC&lt;sup&gt;b&lt;/sup&gt; (cmol kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>clay (%)</th>
<th>silt (%)</th>
<th>sand (%)</th>
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<td>Solonchak</td>
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<td>2.41</td>
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<td>Cambisol</td>
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<td>3.23</td>
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<tr>
<td>C</td>
<td>Acrisol</td>
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<td>2.06</td>
<td>8.93</td>
<td>41.25</td>
<td>18.62</td>
<td>40.13</td>
</tr>
</tbody>
</table>

<sup>a</sup> organic matter. <sup>b</sup> cation exchange capacity
Graphical and Textual Abstract

The mineralization and degradation of pyraoxystrobin was negligible and a large proportion of pyraoxystrobin can persist in flooded soil.