Biodegradation of imidacloprid

Jennifer Allen Chaplin Anhalt
Iowa State University

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Biodegradation of imidacloprid

by

Jennifer Allen Chaplin Anhalt

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Microbiology

Major Professors: Thomas B. Moorman and Alan A. DiSpirito

Iowa State University

Ames, Iowa

2000
Graduate College
Iowa State University

This is to certify that the Master's thesis of

Jennifer Allen Chaplin Anhalt

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy
Dedicated to my father.
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CHAPTER 1. GENERAL INTRODUCTION

**History of imidacloprid**

Imidacloprid (1-[6-chloro-3-pyridyl)methyl]-N-nitro-2-imidazolidinimine) is a soil insecticide first introduced around 1984 by Nihon Bayer in Japan (Leicht, 1996). It is also abbreviated as NTN-33893 by Bayer Corporation. Imidacloprid was originally classified with the nitromethylene class of chemicals, but later became the first registered chemical of the chloronicotinyl insecticides because it contains a unique chloropyridyl group. Insecticides of the nitromethylene class were first presented in 1978 at the International Union of Pure and Applied Chemists (IUPAC) Conference in Zurich, Switzerland. The chloronicotinyl class was thought to be a novel type of insecticidal structures until epibatidine was discovered in 1992.

Epibatidine is a natural compound that is structurally related to imidacloprid. It was isolated from the skin of a poisonous Ecuadorian frog and contains the same unique chloropyridyl structure that imidacloprid does (Leicht, 1996) (Figure 1). Through molecular work on mode of action, it was discovered that not only epibatidine, but also nicotine, is a predecessor to imidacloprid (Figure 2).

Imidacloprid is an opaque crystal with a weak odor and is used to control sucking and biting insects. Examples of sucking insects include, ricehoppers, aphids, thrips and white flies. Biting insects include the rice water weevil and the Colorado potato beetle. In addition to these insects, imidacloprid is also effective against some soil insects (*Atomaria, Blaniulus, Agriotes* and *Pegomya* spp.), fleas and termites (Rouchaud et al., 1996). It is not effective against nematodes or spider mites. Imidacloprid is used to treat seeds, soil, and foliage in
Figure 1. Imidacloprid and the chloropyridyl structure that makes it effective.

Figure 2. Structures of epibatidine and nicotine.
crops such as cereals, cotton, sugar beets, potatoes, tomatoes, and rice (Ishii et al., 1994; Leicht, 1996; Scholz & Spiteller, 1992; Tomlin, 1994).

As mentioned previously, imidacloprid is very similar to nicotine in its structure, and the two chemicals interact at the same receptor site (Tomizawa & Yamamoto, 1993). The degree of binding of imidacloprid is much greater than that of nicotine, however. Imidacloprid has a safety factor 700 times greater for mammals than does nicotine. The safety factor is defined as a ratio of toxicity (LD$_{50}$) in rats to toxicity in aphids (LD$_{50}$), where 7000 times more imidacloprid is required to affect a rat compared to an aphid, but only 10 times more nicotine is required in the same case (Leicht, 1996).

The mode of action of imidacloprid is to irreversibly bind to the postsynaptic membrane and therefore not allow acetylcholine to bind (Leicht, 1996; Tomlin, 1994). Interestingly, imidacloprid’s molecular target (blocking acetylcholine binding to the postsynaptic membrane) is not specific to insects since this type of binding also occurs in mammals. Despite this fact imidacloprid provides excellent insecticidal control, while maintaining very low toxicity to mammals (Table 1) (Leicht, 1996). This phenomenon is explained by experimental profiles that have been completed on insects and vertebrates, indicating that physiological differences have been described in these two groups. In the case of imidacloprid the acetylcholine receptors in mammals are insensitive to the chemical, whereas in insects analogous receptors have a high sensitivity to imidacloprid (Bai et al., 1991; Leicht, 1996; Liu & Casida, 1993).

Imidacloprid has a relatively high water solubility of 0.51 g L$^{-1}$ (Tomlin, 1994; Bayer Corp.) (Table 1). This would seem to indicate a high potential to leach to the groundwater,
Table 1. Physicochemical properties of imidacloprid.

<table>
<thead>
<tr>
<th>Class</th>
<th>chloronicotinyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode of action</td>
<td>irreversibly binds to postsynaptic membrane blocking acetylcholine binding</td>
</tr>
<tr>
<td>Empirical formula</td>
<td>C₉H₁₀ClN₅O₂</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>255.7 g</td>
</tr>
<tr>
<td>Water solubility (at 20° C)</td>
<td>0.51 g L⁻¹</td>
</tr>
<tr>
<td>Kₐₒₑ¹</td>
<td>t₀² = 259</td>
</tr>
<tr>
<td></td>
<td>t₁₀₀² = 915</td>
</tr>
<tr>
<td>T ½³</td>
<td>48 d to &gt; 1 yr</td>
</tr>
<tr>
<td>pKₐ⁴</td>
<td>11.12 (+/-0.11)</td>
</tr>
<tr>
<td></td>
<td>1.56 (+/-0.12)</td>
</tr>
<tr>
<td>Toxicity for rats⁵ (LD₅₀ mg kg⁻¹)</td>
<td>450</td>
</tr>
<tr>
<td>Toxicity for aphids⁵ (LD₅₀ mg kg⁻¹)</td>
<td>0.062</td>
</tr>
</tbody>
</table>

¹ Oi, 1999
² t₀ = time 0 in days
³ t₁₀₀ = time 100 in days
⁴ T ½ = half-life
⁵ Chamberlain et al. 1996
⁶ Leicht, 1996
but this is not the case. Imidacloprid sorption to soil has $K_{oc}$ values reported to be around 259 upon initial addition of imidacloprid to soil and around 915 after 100 days of soil incubation (Oi, 1999). This indicates that imidacloprid goes from medium to low mobility in soil to a slightly mobile or immobile state with increased aging time in soil. $K_{oc}$ is defined as $K_{oc} = \frac{(K_d)}{(% \text{ organic carbon})} \times 100$ where $K_d$ is a ratio of the concentration of a compound in soil to the concentration of that compound in water.

Sorption is a very important factor in determining the leaching potential of imidacloprid. Imidacloprid tends to sorb more strongly to soils with higher concentrations of soil organic matter and tends to sorb more strongly over time (Oi, 1999; Cox, 1998 and Cox, 1997). A study by Hellpointer (1998) demonstrated imidacloprid’s low mobility under field conditions and suggested that when applied as recommended, imidacloprid will not contaminate the subsurface soil or groundwater, although more studies are needed to confirm this. Prior to this study, Rouchaud et al. (1996) demonstrated that imidacloprid does not leach into the subsoil.

**Pesticide degradation**

Pesticide dissipation often begins with the transport of the pesticide through leaching and volatilization. Biodegradation and chemical degradation may begin before this or in conjunction with it. Biodegradation is defined as the partial or total transformation or detoxification of a contaminant by a microorganism and/or plant (Skipper, 1998). As a microorganism consumes an environmental contaminant it may use it for a carbon or nitrogen source or as some other nutrient. When this occurs portions of the chemical are
incorporated into the microbial biomass while other parts of the chemical are mineralized to CO$_2$ or inorganic ions. Not all chemicals are able to be biologically degraded, however, and some chemicals that are degraded by microorganisms are only broken down through cometabolism. Cometabolism is the transformation of a substrate/chemical by a microorganism without that microorganism deriving any energy, carbon, or nutrients from the substrate (Skipper, 1998). Some compounds which contain pyridines, which includes imidacloprid, are able to be degraded by several microorganisms.

Several researchers have found microorganisms with the ability to degrade pyridine compounds in both aerobic and anaerobic conditions. *Alicaligenes* sp. (Ronen & Bollag, 1992), *Blastobacter* sp. (Ogawa et al., 1996) and *Streptomyces* sp. (Shelton et al., 1996) are all capable of pyridine degradation. An *Alicaligenes* sp., using pyridine as its sole carbon source, is able to mineralize 67% of $^{14}$C-pyridine in contaminated sediment after 44 hours while native sediment microorganisms are only able to mineralize 10% in seven days (Ronen & Bollag, 1992). A *Streptomyces* sp. has the ability to degrade 12 herbicides, including imazapyr, but uses 1% dextrin as its carbon source instead of the herbicides (Shelton et al., 1996). Kaiser & Bollag (1992) found that in surface and subsurface soils that had been exposed to pyridine compounds for decades, microorganisms were able to completely degrade these compounds within two weeks under aerobic conditions and within four weeks under anaerobic conditions. Engvild & Jensen (1969) isolated a bacteria capable of degrading pyrazon, a pyridine-based herbicide. This organism was able to use pyrazon, a chlorinated pyridine attached to a benzene ring, as its sole carbon source.

In general, microorganisms are able to degrade pyridines via an initial ring hydroxylation step. Pyridine compounds may go through various intermediate steps, but then
are ultimately degraded to a common chemical, maleamate, by opening of the ring. They are further degraded to fumarate and/or pyruvate. Pyridine-2,5-diol, for example, is transformed to formate and maleamate, then to NH₃ and maleate, and finally, in a reversible step, to fumarate (Cain et al., 1974). Imidazolidin structures undergo a similar transformation in which they generally degrade to succinate then fumarate, maleate, and finally pyruvate.

Bioavailability

Imidacloprid has a half-life ranging from 48 days in vegetated soil to greater than 180 to 190 days in nonvegetated soil (Oi, 1999; Scholz & Spiteller, 1992). Imidacloprid is extremely photolytic and will reach its half-life in a matter of hours upon exposure to sunlight. Its degradation is also more rapid in reduced environments than in aerobic environments. For example, when imidacloprid is placed in anaerobic, moisture-saturated, dark conditions, its half-life is approximately 27 days (M Oi, unpubl). The extremely long half-life of imidacloprid, which can be greater than one year in some cases, raises questions about the environmental and safety impact it may have.

Control of degradation seems to be through rate-limiting sorption-desorption factors. Among these factors are intraparticle-diffusion, intrasorbent diffusion, and chemisorption. These factors control the distribution of the pesticide in the soil matrix and thereby control the amount of pesticide available at any given time to dissipation processes (Beck et al., 1995).
Pesticide sorption in soil may occur in a biphasic pattern. There are two distinct processes that contaminants undergo: 1) the occurrence of a rapid rate of contaminant sorption to soil (readily bioavailable) and 2) a subsequent slow rate of contaminant diffusion into soil aggregates (not readily bioavailable) (Radosevich et al., 1997; Shelton & Doherty, 1997; White et al., 1997). The length of time a contaminant has been exposed to its environment is called aging. This is directly related to contaminant degradation. Laboratory experiments have demonstrated that degradation rates decrease the longer a pesticide is aged in soil (Hatzinger & Alexander, 1995; Oi, 1999; Radosevich et al., 1997). This is because the longer the residence time of a pesticide, the less biological activity it possesses, consequently making the compound even more resistant to degradation (Novak et al., 1995).

Laboratory studies also show that subsurface soils do not degrade pesticides as quickly as surface soils. Pesticides such as alachlor, metribuzin, fluometuron, and 2,4-D all degrade more slowly in subsurface than surface soils (Moorman & Harper, 1989; Mueller et al., 1992; Pothuluri et al., 1990; Welch, 1995). In general degradation is slower and pesticide half-lives are longer. This is due to factors such as a limiting amount of nutrients, soil temperature, soil water content, and soil type. Microbial biomass is also much reduced with depth, leading to reduced microbial populations and activity.

Biodegradation of a compound results in the transformation and breakdown of organic compounds which may in turn leave more recalcitrant metabolites than the parent. These metabolites may or may not be toxic. Bioavailability of a substance indicates the degree to which a microorganism, plant, invertebrate, or vertebrate is able to take up and utilize the compound and/or its metabolites (Alexander, 1995). Bioavailability is very hard to quantify and is dependent on the model organism. The use of microorganisms to indicate...
chemical bioavailability may allow us to predict the effect of the compound of interest on higher organisms (e.g. plants, mammals).

In general, bioavailability seems to limit degradation, but little is known about the bioavailability of imidacloprid. Cox et al. (1997, 1998) have done experiments looking at the available, sorbed, and strongly sorbed fractions of imidacloprid in soil. In these experiments soil aliquots were removed at various sampling times and then sequentially extracted with CaCl₂ (available), then acetonitrile and water (9:1) (sorbed), and finally with HCl (strongly sorbed). In addition, Cox et al. have indicated that imidacloprid sorption is concentration dependent and that sorption to soil increases with increasing organic carbon content.

A strongly sorbed compound, such as imidacloprid, is less available than a weakly sorbed compound and therefore will likely be less of a threat to the environment (Beck et al., 1995). The degree of sorption is often indicated by 

\[ K_{oc} \]

values. Imidacloprid has an initial \( K_{oc} \) value of 259 (Oi, 1999). Sorption of imidacloprid and its metabolites tends to increase with increasing organic matter. Examples of sorption values of other common insecticides are shown in Table 2. In general, though not always, \( K_{oc} \) values tend to indicate greater sorption with higher \( K_{oc} \) values.

The sorption of imazapyr, a pyridine compound similar in structure to imidacloprid, has been investigated and shows that sorption to soil is greatly increased with pH's of less than 5 and with increasing organic matter. Imazapyr also has a very high water solubility (11.3 g L⁻¹) and a half-life ranging from around 240 d to greater than two years in unvegetated soil, both properties shared by imidacloprid (Pusino et al., 1997). Despite these similarities, imazapyr has a \( K_{oc} \) of 8.81 and demonstrates considerable leachability in soil, unlike imidacloprid (Vizantinopoulos & Lolos, 1994).
Table 2. Sorption values for imidacloprid and other insecticides

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>$K_{oc}$</th>
<th>solubility</th>
<th>mobility$^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidacloprid</td>
<td>$t_0 = 259$</td>
<td>0.51 g L$^{-1}$</td>
<td>medium</td>
</tr>
<tr>
<td></td>
<td>$t_{100} = 915$</td>
<td></td>
<td>slight</td>
</tr>
<tr>
<td>Carbofuran*</td>
<td>141</td>
<td>0.32 g L$^{-1}$</td>
<td>high</td>
</tr>
<tr>
<td>Chlorpyrifos*</td>
<td>8900</td>
<td>0.00073 g L$^{-1}$</td>
<td>immobile</td>
</tr>
<tr>
<td>Isofenphos*</td>
<td>575</td>
<td>0.0238 g L$^{-1}$</td>
<td>low</td>
</tr>
<tr>
<td>Malathion*</td>
<td>407</td>
<td>0.145 g L$^{-1}$</td>
<td>medium</td>
</tr>
</tbody>
</table>

$^\ast$Montgomery, 1993
$^\dagger$McCall et al, 1980

The longer a compound remains in the soil the more likely it is to be less bioavailable (Kelsey et al., 1997). Over time bioavailability of a pesticide may change. This can occur as the compound partitions further into the inner soil surfaces, making tighter bonds, becoming irreversibly bound, or through enzyme-induced binding to humic substances (Alexander, 1995; Bollag, 1991; Novak et al., 1995). Additionally, bound pesticides may also be very slowly released from the soil over time (Bollag, 1991).

Problems with assessing bioavailability arise because there is no standard method to determine bioavailability of a pesticide. A traditional laboratory technique, used to assess the total chemical present, uses the strongest solvents possible to completely extract the target compound. This, however, is not a realistic indicator of the pesticide present in the environment since solvents will not be washing out the target compound in nature (Kelsey et al., 1997).
Conclusions

Imidacloprid is a very persistent insecticide in nonvegetated soil. Its half-life can be greater than one year in length, and the longer this compound ages, the less bioavailable it is to soil microorganisms. Despite its high water solubility, imidacloprid does not seem to leach into the groundwater. Instead it is very stable in the environment. In addition to this, its mode of action specifically targets insects and does not pose a hazard to mammals, making it very safe to use as an insecticide. Despite its apparent safety, imidacloprid does persist in the soil for long periods of time, making itself available for possible microbial use.

Objectives

The objective of this research was to determine the bioavailability of imidacloprid in surface and subsurface soils. The examination of different soil depths is important in assessing the biodegradation and bioavailability of imidacloprid because of the varying environmental factors encountered with soil depth. In an attempt to accomplish my objective, this thesis reflects the beginning stages of the research. The objective has been partially met by 1.) the discovery and isolation of a single microbial species with the ability to degrade imidacloprid and 2.) running a soil incubation study in order to relate long-term persistence of imidacloprid to water-extractable (available), solvent-extractable (sorbed), and acid-extractable (strongly sorbed) concentrations in soil using the microorganism as an indicator of bioavailability in those experimental soils.
Thesis Organization

This thesis is composed of a general introduction, two journal papers, a general conclusion and references for each section. The papers are formatted according to the directions of the journal Biodegradation. The general introduction gives an in-depth overview of imidacloprid. The first paper focuses on the discovery and isolation of a microbial isolate that is able to degrade imidacloprid, with isolation by soil enrichments and subsequent purification. The second paper discusses the degradation of imidacloprid in soil. In this study two soils at two depths each were treated with either 0.1 mg kg\(^{-1}\) or 1 mg kg\(^{-1}\) imidacloprid and monitored for loss of imidacloprid. The general conclusion attempts to tie in all aspects of imidacloprid discussed in the introduction and two papers.

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CHAPTER 2. BIODEGRADATION OF IMIDACLOPRID BY A SOIL MICROORGANISM

A paper to be submitted to the journal Biodegradation

Jennifer C. Anhalt and Thomas B. Moorman

ABSTRACT

Imidacloprid (1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine), a chloronicotinyl insecticide used to control biting and sucking insects, is very persistent in the soil with a half-life often greater than 100 days. Our objectives were to discover, isolate, and characterize microorganisms capable of degrading imidacloprid in soil. Soil-free stable enrichments in N-limited media were obtained which degraded 19 mg L\(^{-1}\) (43%) and 11 mg L\(^{-1}\) (16%) of the applied imidacloprid and produced about 19 mg L\(^{-1}\) 6-chloronicotinic acid in three weeks. Enrichment media without microorganisms had no loss of imidacloprid. Strain PC-21, obtained from the enrichment cultures, degraded 37% to 58% of 25 mg L\(^{-1}\) imidacloprid in tryptic soy broth containing 1 g L\(^{-1}\) succinate and D-glucose at 27° C incubation over a period of three weeks. Trace amounts of NO\(_3^-\)/NO\(_2^-\) were produced and six metabolites were characterized by HPLC using \(^{14}\)C-methylene-imidacloprid and LC-MS. Two of the metabolites were identified as imidacloprid-guanidine and imidacloprid-urea by HPLC standards and LC-MS. 6-chloronicotinic acid was not produced. Less than 1% of the applied \(^{14}\)C was incorporated into the microbial biomass and no \(^{14}\)CO\(_2\) was detected. Strain PC-21 cometabolizes imidacloprid. PCR amplification of a 500 bp sequence of 16s rRNA identified strain PC-21 to be of the genus *Leifsonia*. 
INTRODUCTION

Imidacloprid is a commercially available insecticide known as Admire, Confidor, Gaucho and Premise and is manufactured by Bayer Corporation. It is used to control sucking and biting insects by irreversibly binding to the post synaptic membrane, therefore inhibiting the binding of acetylcholine. It is effective at doses as low as 0.075 kg ha\(^{-1}\) (Moriya et al., 1992).

Imidacloprid has a very long half-life, usually greater than one year in dark, aerobic soil conditions and is extremely photolytic, reaching its half-life in a matter of hours when exposed to sunlight (Scholz & Fritz, 1998). Vegetation increases the rate of degradation of imidacloprid, yielding a half-life of 48 days compared to a half-life of up to 190 days in nonvegetated soil (Scholz & Spiteller, 1992). Oi (1999) measured a similar half-life of up to 180 days in nonvegetated soils.

Cox et al. (1997) used the batch equilibrium technique to determine the sorption-desorption of imidacloprid and its metabolites in three soils at concentrations of 0.05 to 1.5 mg L\(^{-1}\). These laboratory studies determined that imidacloprid and its metabolites become irreversibly bound to soil (Cox, et al., 1997 & 1998). Sorption of imidacloprid and its metabolites imidaclorpid-guanidine, imidaclorpid-guanidine-olefin, and imidaclorpid-urea, was determined to be affected by soil properties and sorption tended to increase with increasing organic carbon content. \(K_{oc}\) values for imidacloprid are reported to be around 259 upon initial addition of imidaclorpid to soil and around 915 after 100 days of soil incubation (Oi, 1999). As pesticides age in soil bioavailability is less and would seem to limit
degradation (Kelsey, et al., 1997). The strong sorption of aged imidacloprid in soil likely makes biodegradation of imidacloprid very difficult.

Imidacloprid and other pyridine compounds can be very difficult to degrade in the environment. Synthetic pyridines, such as some pesticides and industrial wastes, may be a potential health threat to our surface and subsurface groundwater due to their high water solubilities (Sims & O’Loughlin, 1989). Pyridines can enter water through synthetic fuel and coal tar processing, pesticide application, and chemical manufacturing and are major constituents (though in very small amounts) in natural plant alkaloids, pyridoxyl derivatives, and coenzymes like NAD⁺ (Kaiser et al., 1996; Kaiser & Bollag, 1992; Ronen & Bollag, 1992; Sims & O’Loughlin, 1989).

In general, microorganisms initiate pyridine degradation via an initial ring-hydroxylation step. Pyridine compounds may go through various intermediate steps, but then are ultimately degraded to a common chemical, maleamate, by opening of the ring, and then to fumarate and/or pyruvate. An example of a pyridine degradation pathway is demonstrated with nicotinic acid. Nicotinic acid is hydroxylated to produce 6-hydroxynicotinic acid. It is then oxidatively decarboxylated to 2,5-dihydroxypyridine, then oxidatively cleaved to form maleamate and formamate. Maleamate is then transformed to maleate and NH₃ and finally, in a reversible step, fumarate is formed (Behrman & Stanier, 1957; Cain et al., 1974). Imidazolidin structures undergo a similar transformation in which they generally degrade to succinate then fumarate, maleate, and finally pyruvate (Ogawa et al., 1996).

*Alcaligenes* sp., (Ronen & Bollag, 1992), *Blastobacter* sp., (Ogawa et al., 1996), and *Streptomyces* sp. (Sheiton et al., 1996) are all capable of pyridine degradation. *Alcaligenes* sp., using pyridine as its sole carbon source, is able to mineralize 67% of ¹⁴C
pyridine after 44 hours in a contaminated sediment while native microorganisms from the contaminated sediment are only able to mineralize 10% in seven days. A *Streptomyces* sp. has the ability to degrade 12 herbicides, including imazapyr, but uses 1% dextrin as its carbon source instead of the herbicides. Kaiser & Bollag (1992) found that in surface and subsurface soils that had been exposed to pyridine compounds for decades, microorganisms were able to completely degrade these compounds within two weeks under aerobic conditions and within four weeks under anaerobic conditions. Engvild & Jensen (1969) isolated a bacteria capable of degrading the herbicide pyrazon, a chlorinated pyridine attached to a benzene ring. This organism was able to use pyrazon as its sole carbon source.

The overall objective of our research was to determine the bioavailability of imidacloprid in soil. The objectives of the research described in this report were to discover, isolate, and characterize a single microbial species with the ability to degrade imidacloprid.

**MATERIALS & METHODS**

*Chemicals*

Analytical standards of imidacloprid (1-[(6-chloro-3-pyridinyl)-methyl-N-nitro-2-imidazolidinimine) (96.9% pure), as well as three metabolites, imidacloprid-guanidine (1-[(6-chloro-3-pyridinyl)methyl]-4,5-dihydro-1H-imidazol-2-amine), imidacloprid-guanidine-olefin (1-[(6-chloro-3-pyridinyl)methyl]-1H-imidazol-2-amine), and imidacloprid-urea (1-[(6-chloro-3-pyridinyl)-methyl-2-imidazolidinone) were obtained from Bayer Corporation, Stilwell, KS (Figure 1). Analytical grade (99% pure) 6-chloronicotinic
acid was purchased from Aldrich Company. Two radiolabeled forms of imidacloprid were also obtained from Bayer Corporation: $^{14}$C-methylene-imidacloprid (4.64 MBq mg$^{-1}$, 99.9% pure) and $^{14}$C-4,5-imidazolidin-imidacloprid (4.59 MBq mg$^{-1}$, 98% pure).

**Soils**

Agricultural soils from Indiana (Drummer silty clay loam) and California (Exeter sandy loam) were sampled at surface and subsurface depths by Bayer Corporation at field sites near Oxford, IN and Fresno, CA. The Drummer soil has never had imidacloprid applied to it and has had no pesticide application of any kind in 1997, when it was collected. The Exeter soil also has never been exposed to imidacloprid and has no pesticide application history for five years prior to its collection in 1997. Drummer (IN) and Exeter (CA) surface soils, (0-15 cm) and subsurface soils, (15-76 cm and 46-61 cm, respectively) were collected from the field site with hand shovels, placed in water impermeable bags and shipped to the National Soil Tilth Laboratory, Ames, IA. Soils were stored in a 7° C incubator before the study.

Total soil carbon and soil nitrogen were determined using a Carlo-Erba NA 1500 NCS elemental analyzer (Haake Buchler Instruments, Paterson, NJ) and total soil microbial biomass was measured by performing fumigation-extractions on the soil and then analyzing the extracts using a Dohrmann DC-180 carbon analyzer (Rosemount Analytical Services, Santa Clara, CA) (Soulas et al., 1984).
Figure 1. Structure of imidacloprid and its metabolites, imidacloprid-guanidine-olefin, imidacloprid-guanidine, and imidacloprid-urea.
**Soil slurries & soil-free enrichments**

Four agricultural soils, Drummer, Exeter, Monona, and one from an Iowa (IA) agricultural chemical dealership were initially used to create soil slurries for isolation of an imidacloprid-degrading microorganism(s). Three versions of Kaufman and Kearney's (1965) minimal salts media (MSM) were prepared. Fifty mL C-limited MSM with 83 mg L\(^{-1}\) imidacloprid as the sole carbon source, 50 mL N-limited MSM with imidacloprid as the sole nitrogen source and sodium citrate and sucrose added as supplemental carbon sources, or 50 mL MSM broth which contained all components plus sodium citrate, sucrose, and imidacloprid were inoculated with 2 g of soil to enrich for imidacloprid degrading microorganisms. Cultures were maintained on the shaker at 100 rpm and 27\(^\circ\) C. Subcultures were made monthly in order to obtain soil-free enrichments. Aliquots of the cultures were periodically removed from the shaker flasks for HPLC analysis.

**HPLC & LC-MS analysis**

Periodic analysis of imidacloprid levels was accomplished with a Waters HPLC (Division of Millipore, Milford, MA), using a Reverse Phase C-18 (RP18) Symmetry Shield column (Waters-Millipore) (3.9 x 150 mm), 30 min gradient of acetonitrile and acidified (pH 3) ultrapure water [(0 min) 20%/80% ACN:H\(_2\)O; (7 min) 22%/78% ACN:H\(_2\)O; (14 min) 30%/70% ACN:H\(_2\)O; (21 min) 40%/60% ACN:H\(_2\)O; (23-30 min) 20%/80% ACN:H\(_2\)O)], an injection volume of 25 µl, a flow rate of 0.6 mL min\(^{-1}\), and UV detection at wavelengths of 220, 247, and 270 nm. Media samples were filtered (0.2 µm) and diluted by half with methanol prior to analysis.
Analytical grade imidacloprid and imidacloprid-guanidine, imidacloprid-guanidine-olefin, and imidacloprid-urea metabolites, were used as standards, as well as technical grade 6-chloronicotinic acid. The retention times for imidacloprid and its metabolites are as follows: imidacloprid-guanidine and imidacloprid-guanidine-olefin co-elute at 1.65 min; imidacloprid-urea, 6.4 min; imidacloprid, 11.1 min; and 6-chloronicotinic acid, 18.2 min.

Radiochromatography was performed with the HPLC system previously described coupled with a Radiomatic detector (Packard Instrument Company, Downers Grove, IL) equipped with a flow cell of either 2.5 mL or 0.5 mL. Ultima-Flo M scintillation cocktail was used at a rate of 1.8 mL min^{-1} and a standard curve was generated using differing amounts of ^{14}C-methylene-imidacloprid as standards. Retention times are increased by 0.3 to 0.4 min compared to UV detection times.

Strain PC-21 acetonitrile extracts were analyzed on a liquid chromatograph-mass spectrometer (LC-MS) (Waters Micromass ZMD coupled to a Waters Alliance 2690 HPLC, Milford, MA) under the following conditions: Zorbax C-8 column (2.1 mm x 15 cm), 10 µl injection, flow rate 0.2 mL min^{-1}, and a gradient of acetonitrile and formic acid/water [(0 min) 85%/15% ACN:1% formic acid/H_{2}O; (3 min) 70%/30% ACN:1% formic acid/H_{2}O; (6 min) 60%/40% ACN:1% formic acid/H_{2}O; (12 min) 50%/50% ACN:1% formic acid/H_{2}O; (15 min) 20%/80% ACN:1% formic acid/H_{2}O; (20 min) 85%/15% ACN:1% formic acid/H_{2}O].

The type of LC-MS system used is an electrospray LC-MS. This method does not apply heat to the capillary column. Here the chemical is separated into ions in solution prior to reaching the mass spectrometer (MS). Ions then enter into the MS in a fine spray created
by nebulizing gas and a high voltage. In the electrospray technique one mass unit is added to each molecule. This must be taken into account when evaluating the ion spectrum output.

**Isolation of microorganisms**

Two mixed enrichment cultures that showed losses of imidacloprid during incubation, NTN-13 and NTN-16, were spread-plated using dilutions of $10^{-1}$ to $10^{-6}$ on N-limited agar plates containing 36 mg kg$^{-1}$ imidacloprid and either streptomycin or cyclohexamide. After a one-week incubation, the plates were screened for colonies that visually appeared different from one another. In total, 29 colonies (PC-1 through PC-29) were transferred onto new N-limited agar plates. After about three weeks PC strains 1-26 (bacterial isolates) were put into 25 mL of tryptic soy broth (TSB) and PC strains 27-29 (fungal isolates) were put into 25 mL Capex Dox broth containing 25 mg L$^{-1}$ imidacloprid. After three days growth, each of the 29 isolates was centrifuged in HDPE tubes for 10 min at 6700 x g. The supernatent was poured off, and the isolates were resuspended in sterile phosphate buffer for a total volume of 10 mL. A 2-mL sample of each isolate was inoculated into N-limited MSM, C-limited MSM, and TSB (described previously) all containing 30 mg L$^{-1}$ of imidacloprid in 25 mL total. Non-inoculated controls were also made by inoculating 2 mL of phosphate buffer into each of the media-filled flasks. All samples were wrapped in aluminum foil and placed on a shaker operated at 27$^\circ$C and 100 rpm. The three noninoculated controls were analyzed for imidacloprid concentration. After seven days of incubation, the 29 cultures were extracted by adding 25 mL of methanol to the flasks. The samples were then sonicated for six min
each at 50% duty cycle and centrifuged at 6700 x g. Four mL aliquots were filtered through a 0.2 µm filter and analyzed on the HPLC.

Using ¹⁴C-methylene-imidacloprid to verify whether or not imidacloprid degradation was truly occurring, a second experiment was performed using the only three PC strains that showed possible imidacloprid degradation. In this experiment the three strains (PC-17, PC-21, and PC-27) were inoculated into 25 mL TSB broth with 25 mg L⁻¹ imidacloprid and incubated on the shaker at 27° C and 100 rpm and were covered with aluminum foil. After three weeks time the cell growth was terminated by adding 25 mL of methanol to the cultures. Samples were sonicated, centrifuged, and the pelleted cells were removed and air dried. The ¹⁴C content in the dried pellets (biomass) was determined by using a Harvey Biological Oxidizer. Imidacloprid in the sonicated culture supernatants was extracted by liquid-liquid partitioning done three times per sample with a total of 200 mL methylene chloride. The partitioned organic fraction of the samples was evaporated to dryness on a rotary evaporator, and then redissolved in 4 mL of acetonitrile. The acetonitrile samples were analyzed by HPLC.

These preliminary experiments resulted in the loss of imidacloprid and production of a metabolite, imidacloprid-guanidine by strain PC-21, indicating that it was capable of degrading imidacloprid. A more detailed experiment was performed to investigate imidacloprid metabolite production by strain PC-21. In this experiment 2 mL of strain PC-21 was inoculated into 50 mL of either 10% TSB or full strength TSB. Cells were grown in TSB containing 25 mg L⁻¹ imidacloprid for three to five days. Cells were then harvested by centrifuging at 6700 x g to wash off the media. This step was repeated two more times, each
time pouring off the media and then resuspending the cells in phosphate buffer for a final concentration of $2.5 \times 10^7$ cells mL$^{-1}$.

Concentrated strain PC-21 (in phosphate buffer) was added to six replicates (three labeled with $^{14}\text{C}$) for each of three time points. Sample flasks were covered with aluminum foil and placed on a shaker operating at 100 rpm and 27° C. Four-mL aliquots of the media were removed at day 0, 3, 7, 14, and 21 and filtered through 0.2 µm filters for HPLC analysis. Growth was terminated in the culture flasks at day 7, 14 or 21 by adding 50 mL of methanol to the flasks. Cultures were also sampled for NO$_3^-$/NO$_2^-$ (Lachat Instruments, Zellwegger Analytics, Inc, Milwaukee, WI). The $^{14}\text{C}$-methylene-imidacloprid samples were then sonicated, centrifuged, and the microbial pellet was removed. Liquid-liquid partitioning was performed as described above, and the methylene chloride extracts were evaporated to dryness and redissolved in 4 mL of acetonitrile. Cell-free acetonitrile extracts were analyzed by HPLC with radioactivity quantification for metabolite determination. Radioactivity in the aqueous fraction following methylene chloride partitioning was measured by liquid scintillation spectroscopy (LSC) to determine radioactivity. $^{14}\text{CO}_2$ production was measured in cultures spiked with $^{14}\text{C}$-4,5-imidazolidin-imidacloprid by trapping the $\text{CO}_2$ in a base trap of 10 mL of 0.5 M NaOH. A mass balance was calculated.

Strain PC-21 was viewed under the scanning electron microscope (SEM). The cells were placed on a copper grid covered with Fomvar film for support and then stained with a negative stain of 1% phosphotungstic acid (pH 6). Excess media and stain were wicked away with a filter paper. Grids were viewed at 20,000X to 25,000X magnification.
RESULTS & DISCUSSION

Drummer and Exeter soils were sent to Midwest Labs, Inc for soil moisture analysis. Additional analysis was performed in the lab to determine the physicochemical characteristics of the soil. Soil organic carbon ranged from 0.11% in the Exeter subsurface soil to 4.49% in the Drummer surface soil. Soil nitrogen ranged from 0.02% in the Exeter subsurface soil to 0.36% in the Drummer surface soil. Total soil microbial biomass ranged from 10.2 µg C g\(^{-1}\) in Exeter subsurface soil to 704.7 µg C g\(^{-1}\) in Drummer surface soil.

Two soil-free enrichments, NTN-13 and NTN-16, were obtained from the Monona soil which degraded imidacloprid after several months of exposure to the insecticide. In these enrichment cultures, imidacloprid concentrations decreased by about 43% and 16%, respectively, from initial concentrations of 44 and 70 mg L\(^{-1}\) in a period of three weeks. These two cultures also produced about 19 mg L\(^{-1}\) 6-chloronicotinic acid. Further subcultures were able to degrade imidacloprid slightly faster with approximately 44% remaining after eight days. Cell-free controls showed no imidacloprid loss.

Samples of the NTN-13 and NTN-16 enrichments were plated on N-limited agar containing imidacloprid. Twenty-nine pure colonies were selected from these enrichments to be tested for imidacloprid degradation by HPLC. Only strain PC-21 was found to have the ability to degrade imidacloprid.

In several experiments, strain PC-21 degraded an average of 35% of the insecticide, while no degradation was observed in the controls. Strain PC-21 grew slightly in TSB from \(1.86 \times 10^8\) cfu mL\(^{-1}\) to \(2.42 \times 10^8\) cfu mL\(^{-1}\) the first four days, then decreased to \(2.75 \times 10^4\)
cfu mL\(^{-1}\) by day 21. PC-21 was not able to degrade imidacloprid in 10% TSB, but was able to degrade 37% to 58% of imidacloprid in the full strength TSB (Figure 2).

Strain PC-21 produced seven metabolites, including imidacloprid, in TSB determined by HPLC and LC-MS. Metabolites not identified as parent were determined to be breakdown products of imidacloprid by \(^{14}\)C-HPLC analysis (Table 1). The metabolite at 2.2 min was determined to be imidacloprid-guanidine by matching the retention time of this metabolite to that of the imidacloprid-guanidine standard on the HPLC. Additionally, the

![Figure 2](image_url)

Figure 2. Biodegradation of imidacloprid by strain PC-21 in 10% TSB versus full strength TSB over 21 days.
Table 1. Production of imidacloprid and metabolites by strain PC-21 grown in tryptic soy broth, determined by LC-MS or by HPLC radiochromatography.

<table>
<thead>
<tr>
<th>ID</th>
<th>Rep.</th>
<th>Day</th>
<th>LC-MS Fragment (mol.wt)</th>
<th>LC-MS RT*</th>
<th>HPLC RT</th>
<th>metabolite ID</th>
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<td>7</td>
<td>211.26</td>
<td>2.3</td>
<td>2.2</td>
<td>Imidacloprid-guanidine</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>212.26</td>
<td>5.89</td>
<td>2.9</td>
<td>Imidacloprid-urea</td>
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<td>4.7</td>
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<td>5.8</td>
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<td>6.81</td>
<td>11.3</td>
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<td>2.3</td>
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<td>211.25</td>
<td>2.31</td>
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<td>256.24</td>
<td>6.83</td>
<td>11.4</td>
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</tr>
</tbody>
</table>

*Z C-8 = Zorbax C-8 column
*SS RP18 = Symmetry Shield reverse phase C-18 column
*RT = retention time in minutes
retention time and mass spectra of this metabolite indicated molecular weights comparable with the imidacloprid-guanidine standard on the LC-MS (Figure 3). As discussed earlier the LC-MS allows samples to be fragmented according to their molecular weight, giving a determinative mass spectra pattern that corresponds only to that compound.

Three of the seven metabolites did not match retention times our standards. For the HPLC system used in this study, their retention times are 2.9 min, 4.9 min, and 5.8 min. Additionally, imidacloprid-urea and an unidentified compound, Unknown-D, were characterized as metabolites on the LC-MS, but the retention times of these compounds could not be matched with the metabolite retention times obtained using $^{14}$C HPLC (Table 1). It is possible that the radiochromatography methods are not sufficiently sensitive to detect these compounds. Although 6-chloronicotinic acid was seen in the mixed culture enrichments, it cannot be identified as any of these metabolites and does not appear to be produced by strain PC-21. No degradation and consequently no metabolites were produced in the non-inoculated controls.

Table 2 shows a mass balance of imidacloprid and the six metabolites produced by strain PC-21. Metabolite Unknown-A is present at day 7 (19%) and then decreases to below the detection limit of the HPLC by day 21. This is in contrast to metabolite Unknown-C which is also present at day 7, but increases to more than double its day 7 concentration (15%) by day 21 (32%). This may indicate that as strain PC-21 produces metabolite Unknown-A, this metabolite is consumed to form metabolite Unknown-C. As mentioned previously, imidacloprid-guanidine is produced along with the concomitant production of $\text{NO}_3^-$/$\text{NO}_2^-$. Due to the instrumentation used it is not possible to determine if it is $\text{NO}_3^-$ or
Figure 3. Representative LC-MS spectra of imidacloprid-guanidine from a strain PC-21 sample (retention time = 2.317; electron spray).
NO$_2^-$ that is being produced. However, it seems more likely that NO$_2^-$ is produced since this would not require an oxidation step. No NO$_3^-/NO_2^-$ was produced in the non-inoculated controls. This indicates that strain PC-21 may be cleaving off the NO$_2$ group on the end of the imidazolidin ring of imidacloprid to produce this metabolite.

Table 2. Mass balance of $^{14}$C after 7, 14, and 21 d of incubation in strain PC-21 as a percentage of applied $^{14}$C-methylene-imidacloprid.

<table>
<thead>
<tr>
<th>Day</th>
<th>14</th>
<th>21</th>
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</thead>
<tbody>
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<td>Imidacloprid</td>
<td>35.6</td>
<td>44.3</td>
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<td>Metabolites</td>
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<td>Imidacloprid-guanidine</td>
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<tr>
<td>Imidacloprid-urea</td>
<td>BD*</td>
<td>BD</td>
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<td>Unknown-B</td>
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<td>Unknown-C</td>
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<td>23.6</td>
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<tr>
<td>Unknown-D</td>
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<tr>
<td>Microbial biomass</td>
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<td>0.4</td>
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<tr>
<td>CO$_2$</td>
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<td>0</td>
</tr>
<tr>
<td>Total $^{14}$C</td>
<td>100.6</td>
<td>100.4</td>
</tr>
</tbody>
</table>

*BD= below detection limit of 0.4 mg L$^{-1}$

$^{14}$C-4,5-imidazolidin-imidacloprid was applied to these samples
The microbial biomass, sonicated and removed from cultures by centrifugation prior to partitioning, was combusted in a biological oxidizer. Less than 1% of the total $^{14}$C-imidacloprid was determined to be in the microbial biomass after 7, 14, and 21 days of incubation (Table 2). Strain PC-21 incubated with $^{14}$C-4,5-imidazolidin-imidacloprid under similar conditions produced no $^{14}$CO$_2$ (Table 2).

Strain PC-21, which was isolated from a soil enrichment culture from soil removed from Treynor, IA (Monona), was determined to be able to degrade imidacloprid in TSB. Colonies are typically yellow or white, circular, convex, opaque and gram positive. Light microscopy showed that this strain was motile and rod shaped. Further identification of strain PC-21 was obtained by MIDI Labs (Newark, DE) using polymerase chain reaction (PCR) to amplify a 500 bp sequence of 16s rRNA. The amplified rRNA sequence was matched to known bacterial sequences and this analysis suggested this bacterium is most closely related to *Clavibacter michiganense* by a 94% match. This was not a good enough match to determine the genus so the sequence was then matched against two other data banks indicating that it could be either *Clavibacter xyli* or *Leifsonia poae*.

The match to *Leifsonia poae* was the closest at 98% similarity. *Clavibacter xyli* has been reclassified as *Leifsonia xyli*, indicating that strain PC-21 is likely in the genus *Leifsonia*, although the species is uncertain. From taxonomic information (Evtushenko et al., 2000) it was further determined that PC-21 is likely from the genus *Leifsonia*. This is consistent with it being a gram positive, obligate aerobe, rod shaped, motile organism. Observation under a scanning electron microscope (SEM) proved that PC-21 is indeed motile with a single, long, polar flagella (Figure 4). PC-21 was also tested for catalase activity and was found to be catalase positive. In general *Leifsonia* are non-sporeforming,
rod shaped, obligate aerobes that test catalase positive, and are usually motile. Extensive details of their genetic and molecular biology traits can be found in Evtushenko et al. (2000).

*Leifsonia* forms a coherent phylogenetic cluster attached to the branch of *Agromyces* spp. The genus *Agromyces* is classified as branched, slender, filamentous cells, that are gram positive, nonmotile, catalase negative, and oxidase negative. *Agromyces* spp. are considered to be fragmenting actinomycetes with diaminobutyric acid (DAB) in their cell walls (Zgurskaya et al., 1992). *Leifsonia* also contains DAB. *Agromyces* are also closely related phylogenetically to *Clavibacter* spp., one of which has recently been reclassified as a *Leifsonia* sp.
Strain PC-21 degrades imidacloprid cometabolically. Cometabolism is the transformation of a compound (imidacloprid) to intermediates without the organism (PC-21) deriving any energy, carbon, or nutrients from the compound. Evidence to support the cometabolic activities of strain PC-21 include the inability to produce $^{14}$CO$_2$, the production of stable metabolites, the inability to maintain and increase cell populations, and the inability to use imidacloprid as its sole carbon source and perhaps its sole nitrogen source. It is still unclear, however, whether or not strain PC-21 is able to use imidacloprid as its sole nitrogen source. Trace amounts of NO$_3^-$/NO$_2^-$ are produced as strain PC-21 degrades imidacloprid, presumably cleaving the NO$_2$ group off of imidacloprid. The NO$_3^-$/NO$_2^-$ is then consumed over time by strain PC-21 which is why NO$_3^-$/NO$_2^-$ levels are so low.

The degradation of imidacloprid by strain PC-21 is the first report of imidacloprid degradation by an isolated microorganism. This bacterium's transformation may give us an indication of how degradation occurs in soil. Laboratory studies in soil support the findings in culture that no CO$_2$ is produced affirming one aspect of cometabolism. Imidacloprid half-lives in vegetated soils may be shorter because of the rhizosphere effect which is large numbers of microorganisms congregating around the roots of plants for nutrition and shelter. Degradation in soil may begin with the cleavage of the NO$_2$ group, making NO$_3^-$/NO$_2^-$ available to microorganisms. Degradation of imidacloprid may then proceed by breaking apart the imidazolidin ring at its two adjacent carbons or perhaps the methylene group is broken leaving 6-chloronicotinic acid and the imidazolidin ring as separate compounds.
Acknowledgments

Funding for this research was provided by Bayer Corporation, Stilwell, KS. I thank Beth Douglass for her laboratory assistance. I also thank Tracey Pepper of the Bessey Microscope Facility for carrying out the SEM work.

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CHAPTER 3. BIODEGRADATION OF IMIDACLOPRID IN SOIL

A paper to be submitted to the journal Biodegradation

Jennifer C. Anhalt, William C. Koskinen, and Thomas B. Moorman

ABSTRACT

Imidacloprid \([1-\{(6\text{-chloro}-3\text{-pyridinyl})\text{methyl}\}N\text{-nitro}-2\text{-imidazolidinimine}]\), a chloronicotinyl insecticide used to control biting and sucking insects, is very persistent in the environment with half-lives often greater than one year. Research objectives were to determine the persistence of imidacloprid in Drummer and Exeter surface and subsurface soils. Soils were treated in triplicate with either 0.1 mg kg\(^{-1}\) \(^{14}\)C-methylene-imidacloprid or 1.0 mg kg\(^{-1}\) non-radiolabeled imidacloprid. Cumulative \(^{14}\)CO\(_2\) was less than 1.5% of applied \(^{14}\)C in all soils over 400 d. Soils treated with \(^{14}\)C-benzoic acid mineralized greater than 40% of this compound after 21 d. Drummer surface soil was able to mineralize greater than 60% of \(^{14}\)C-2,4-D after 40 d, while the other soils mineralized less than 5%. Sequential extractions were carried out to determine the bioavailability of imidacloprid. Water-, acetonitrile-, and acid-extractable fractions were collected. Less than 10% of the imidacloprid was degraded in the Drummer subsurface soil compared to about 40% in the Drummer surface soil and about 50% in both Exeter soils. Between 70% and 99% of imidacloprid in Exeter surface soil was present in the water-extractable fraction, compared to only 12% in the Drummer surface soil. The acetonitrile-extractable fraction increases with time in the Drummer surface soil much more than in the Exeter surface soil. Subsurface soils produced similar results, with 25% of the imidacloprid remaining after 400 d in a water-
extractable form in the Drummer subsurface soil. These data suggest that imidacloprid is bioavailable to microorganisms and not a limiting factor for biodegradation.

INTRODUCTION

Developed in 1984, imidacloprid is a commercially available insecticide known as Admire, Confidor, Gaucho and Premise. It is a chloronicotinyl insecticide that acts against sucking insects, soil insects, termites, and some species of chewing and biting insects. Imidacloprid acts as an agonist of acetylcholine by binding to acetylcholine receptors on the postsynaptic membrane. It is effective at doses as low as 0.075 kg ha$^{-1}$ (Moriya et al., 1992).

Relatively little is known about imidacloprid degradation in the environment. Despite its fairly high water solubility of 0.51 g L$^{-1}$, imidacloprid has a very long half-life in soil, usually greater than one year in dark, aerobic conditions. It is also extremely photolytic, reaching its half-life in a matter of hours when exposed to sunlight (Scholz & Fritz, 1998), and its degradation is more rapid in anaerobic environments, with a half-life of approximately 27 days (Oi, pers. comm.). Half-lives of 48 d have been reported when vegetation is present (Scholz & Spiteller, 1992). This is compared to a half-life of 180 or 190 d in nonvegetated soil (Oi, 1999; Scholz & Spiteller, 1992).

Bioavailability of a compound indicates the degree to which a microorganism, plant, invertebrate, or vertebrate is able to take up and metabolize the insecticide or chemical (Alexander, 1995). Bioavailability is difficult to quantify and is dependent on the model
organism. The use of microorganisms to indicate chemical bioavailability may allow us to predict the effect of the compound of interest on higher organisms (e.g. plants, mammals). The longer a compound remains in the soil the more likely it is to be less bioavailable (Kelsey et al., 1997).

There currently is no standard method to determine bioavailability of a pesticide. Over time bioavailability of a pesticide may change. This can occur as the compound partitions farther into the inner soil surfaces, making tighter bonds, or through enzyme-induced binding to humic substances (Alexander, 1995; Bollag, 1991). Additionally, bound pesticides may also be very slowly released from the soil over time (Bollag, 1991). Conventional solvent extractions used to quantify pesticides in soil are not a realistic indicator of bioavailability; this only tells us the total chemical present, not what is available for microbial use.

Persistence in soil can result from decreasing bioavailability over time or from low activity of the microbial community towards the pesticide. Extreme persistence may result from a combination of these factors. We use the sequential extraction methodology developed by Cox et al. (1998a) to ascertain the bioavailability of imidacloprid in soil. Cox et al. (1998a) investigated the available, sorbed, and strongly sorbed fractions of imidacloprid in soil and found that sorption tends to increase with increasing organic matter and that imidacloprid and its metabolites become increasingly bound to the soil. Extraction of soil with aqueous CaCl₂ removes imidacloprid present in the soil solution and imidacloprid adsorbed to soil on exchangeable sites. Acetonitrile extraction (following CaCl₂) removes additional adsorbed imidacloprid from sites that retain imidacloprid by stronger physical or chemical mechanisms.
Kelsey et al. (1997) related changes in microbial metabolism of atrazine aged in soils to extractability with a methanol-water mixture, which suggests that some solvent-extractable imidacloprid may also be bioavailable. Locke et al. (1992) showed that methanol extraction removed alachlor from soil after desorption with a 0.01 M CaCl$_2$ solution and that the methanol-extractable fraction increased over time. Cox et al. (1998a) found that further extraction of soil with acid following aqueous and solvent extracts removed still more of the compound. The acid-extractable fraction may represent insecticide adsorbed in the protonated form to soil by cation-exchange mechanisms (Cox et al., 1998b; Novak et al., 1995).

Sorption of imidacloprid and its metabolites is affected by soil properties and tends to increase with increasing organic carbon content (Cox et al., 1998a). Sorption $K_{oc}$ values of imidacloprid in soil are around 259 upon initial addition of imidacloprid to soil and around 915 after 100 d of soil incubation (Oi, 1999). These values indicate that imidacloprid goes from medium to low mobility in soil to a slightly mobile or an immobile state with increased aging time in soil. $K_{oc}$ is defined as $K_{oc} = [(K_d)/(\% \text{ organic carbon})]*100$ where $K_d$ is a ratio of the concentration of a compound in soil to the concentration of that compound in water. Imidacloprid and its metabolites become increasingly bound to the soil (Cox, et al., 1997). This makes biodegradation by microorganisms more difficult.

The objective of this study was to determine the persistence and bioavailability of imidacloprid in surface and subsurface soils. In order to partially accomplish this a soil incubation study was set up to relate long-term persistence of imidacloprid to water-extractable, solvent-extractable, and acid-extractable concentrations in soil. A microorganism will be applied to the soils later as an indicator of bioavailability.
MATERIALS & METHODS

Chemicals

Analytical standards of imidacloprid (1-[(6-chloro-3-pyridinyl)-methyl-N-nitro-2-imidazolidinimine) (96.9 % pure), as well as three metabolites, imidacloprid-guanidine (1-[(6-chloro-3-pyridinyl)methyl]-4,5-dihydro-1H-imidazol-2-amine), imidacloprid-guanidine-olefin (1-[(6-chloro-3-pyridinyl)methyl]-1H-imidazol-2-amine), and imidacloprid-urea (1-[(6-chloro-3-pyridinyl)-methyl-2-imidazolidinone) were obtained from Bayer Corporation Stilwell, KS. Analytical grade 6-chlornicotinic acid (99% pure) was obtained from Aldrich Company. Two radiolabeled forms of imidacloprid were also obtained from Bayer Corporation: $^{14}\text{C}$-methylene-imidacloprid (4.64 MBq mg$^{-1}$, 99.9 % pure) and $^{14}\text{C}$-4,5-imidazolidin-imidacloprid (4.59 MBq mg$^{-1}$, 98 % pure).

Soils

Agricultural soils from Indiana (Drummer silty clay loam) and California (Exeter sandy loam) were sampled at surface and subsurface depths by Bayer Corporation at field sites near Oxford, IN and Fresno, CA. The Drummer soil has never had imidacloprid applied to it and has had no pesticide application of any kind in 1997, when it was collected. The Exeter soil also has never been exposed to imidacloprid and has no pesticide application history five years prior to its collection in 1997. Soil from the following depths was collected: Drummer and Exeter surface soils, 0-15 cm; Drummer subsurface soil, 15-76 cm; and Exeter subsurface soil, 46-61 cm. Soils were removed from the field site with hand
Soil treatment

Soil aliquots of 30 g dry weight were treated in triplicate with either 0.1 mg kg \(^{-1}\) \(^{14}\)C-methylene-imidacloprid or 1.0 mg kg \(^{-1}\) non-radiolabeled imidacloprid. A treating solution for each of the two treatments was made by dissolving imidacloprid in 100 ml of HPLC grade acetonitrile to yield either 3 µg or 30 µg of chemical per jar of soil, depending on the treatment. Soils were placed into polypropylene beakers and treated with imidacloprid. Treated soils were mixed thoroughly with a spatula and then each jar was moistened with ultrapure water to bring it to -50 kPa water potential. Soils were then thoroughly mixed again in order to ensure uniform distribution of the water and imidacloprid.
Beakers of soil were then placed inside 946 ml mason jars and a vial containing 10 ml of 0.5 M NaOH was placed next to the beaker. The mason jars were sealed tightly and placed into boxes in the incubator. Soils were stored at 25°C in the dark for 1, 28, 84, 112, 308, or 400 days. Jars were opened to the air periodically to maintain aerobic conditions. \(^{14}\text{CO}_2\) trapped in NaOH vials was measured by adding 1 ml aliquots of NaOH to liquid scintillation cocktail and measuring radioactivity by liquid scintillation spectroscopy (LSC). Soil moistures were also maintained.

**Soil extraction**

At either 1, 28, 84, 112, 308, or 400 days soil samples were removed from the beakers and put into Whirlpack bags and stored in the freezer until they were extracted. Soils were extracted using an Automated Solvent Extractor (ASE) (Dionex Corp., Sunnyvale, CA). Conditions for the ASE were as follows: preheat 5 min, static 5 min, flush 60%, purge 60 s, 3 cycles, pressure 2050 psi, and temp. 75°C. Prior to sample extraction, imidacloprid recoveries from soil were determined using the ASE. Soil was treated with \(^{14}\text{C}-\text{methylene-imidacloprid}\) and allowed to equilibrate for 24 h. Treated soils were then put into ASE cells and extracted with acetonitrile. The acetonitrile was measured for radioactivity by LSC. Recoveries were greater than 86% for all soils.

Sequential extractions of 0.01 M CaCl\(_2\), acetonitrile, and a 10% 0.02 M phosphoric acid: 90% acetonitrile and ethyl acetate (80:20) mix were chosen to carry out the soil extractions with the ASE. The 30 g soil samples were divided in half and approximately 15 g were put into two stainless steel cells for extraction along with 2 g of hydromatrix.
Hydromatrix is comprised of inert diatomaceous earth and was used as a filler to keep the soil from packing into the cells. Each cell was extracted three times with each of the three solvents. A total of approximately 40 ml of extract for each of the three solvent systems was collected per vial.

The combined aqueous CaCl2 fractions were processed through C-18 solid phase extraction (SPE) cartridges using the method described by Baskaran et al. (1997). Slight alterations of the method were made by eluting the cartridges with 5 ml methanol instead of three and dissolving the concentrated samples in 83% ACN:17% H2O. The acetonitrile and acid fractions were concentrated under nitrogen gas to volumes between 2 and 4 ml.

Soil extracts were processed on the liquid chromatograph-mass spectrometer (LC-MS) (Waters Micromass ZMD coupled to a Waters Alliance 2690 HPLC, Milford, MA) under the following conditions: Zorbax C-8 column (2.1 mm x 15 cm), 10 µl injection, flow rate 0.2 ml min⁻¹, and a gradient of acetonitrile and formic acid/water [(0 min) 85%/15% ACN:1% formic acid/H₂O; (3 min) 70%/30% ACN:1% formic acid/H₂O; (6 min) 60%/40% ACN:1% formic acid/H₂O; (12 min) 50%/50% ACN:1% formic acid/H₂O; (15 min) 20%/80% ACN:1% formic acid/H₂O; (20 min) 85%/15% ACN:1% formic acid/H₂O].

RESULTS & DISCUSSION

Drummer and Exeter soils were sent to Midwest Labs, Inc for soil moisture analysis. Additional analysis was performed in the lab to determine the physicochemical characteristics of the soil. Soil organic carbon ranged from 0.11% in the Exeter subsurface
soil to 4.5% in the Drummer surface soil. Soil nitrogen ranged from 0.02% in the Exeter subsurface soil to 0.4% in the Drummer surface soil. Total soil microbial biomass was lowest at 10.2 µg C g⁻¹ in Exeter subsurface soil compared to the highest amount found in Drummer surface soil of 704.7 µg C g⁻¹ (Table 1).

At time points (1, 28, 84, 112, 308, and 400 d) the soils were extracted sequentially with an ASE. An extraction recovery of greater than 86% was determined for all soils. ASE recoveries are comparable to those determined by Ishii et al. (1994), who found that 89% of imidacloprid could be extracted with 80% ACN: 20% H₂O in an ultra-sonic bath.

Table 1. Soil physicochemical analysis of Drummer and Exeter soils.

<table>
<thead>
<tr>
<th></th>
<th>Exeter</th>
<th>Exeter</th>
<th>Drummer</th>
<th>Drummer</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0-15 cm</td>
<td>46-61 cm</td>
<td>0-15 cm</td>
<td>15-76 cm</td>
</tr>
<tr>
<td>Field capacity:</td>
<td>7.39</td>
<td>7.49</td>
<td>39.0</td>
<td>29.9</td>
</tr>
<tr>
<td>(-33 kPa) %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moisture Holding:</td>
<td>5.86</td>
<td>6.18</td>
<td>36.1</td>
<td>27.3</td>
</tr>
<tr>
<td>(-50 kPa) %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Texture:</td>
<td>Sandy loam</td>
<td></td>
<td>Silty clay loam</td>
<td></td>
</tr>
<tr>
<td>% Nitrogen:</td>
<td>0.03</td>
<td>0.02</td>
<td>0.36</td>
<td>0.08</td>
</tr>
<tr>
<td>% Organic carbon:</td>
<td>0.21</td>
<td>0.11</td>
<td>4.49</td>
<td>0.81</td>
</tr>
<tr>
<td>Total soil microbial biomass (µg C g⁻¹):</td>
<td>43.22</td>
<td>10.23</td>
<td>704.65</td>
<td>104.71</td>
</tr>
<tr>
<td>pH:</td>
<td>4.64</td>
<td>5.61</td>
<td>5.63</td>
<td>5.88</td>
</tr>
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</table>
Soil samples were sequentially extracted in order to determine the amount of imidacloprid in the available or water-extractable, sorbed or acetonitrile-extractable, and strongly sorbed or acid-extractable fractions. Imidacloprid extracted in the available fraction will be accessible for use by microorganisms as a carbon, nitrogen or energy source. We presume that any imidacloprid extracted in the sorbed and strongly sorbed fractions will not be available for microbial use. The available, sorbed, and strongly sorbed fractions will be influenced by soil properties with higher organic matter soils tending to have less imidacloprid available for microbial use (Cox et al., 1997 & 1998a).

The degradation of imidacloprid over 400 days is shown in Figure 1. In all soils there is a general trend for imidacloprid concentrations to decrease with time. The Drummer subsurface soil however, showed very little degradation (10%) while the Drummer surface soil degraded about 40% of imidacloprid. The Exeter surface and subsurface soils degraded up to 50% of imidacloprid.

Figures 2-5 show the imidacloprid extracted by the three solvent systems, expressed as a percentage of the total imidacloprid recovered. More imidacloprid was in a water-extractable form in the Exeter surface soil than the Drummer surface soil. This is likely due to the higher organic carbon content in the Drummer soil (Table 1), which would increase sorption of imidacloprid (Cox et al., 1998a). More than 70% of the imidacloprid remaining after 400 days was water-extractable in the Exeter surface soil (Figure 2), compared to 12% in the Drummer surface soil (Figure 3).

Over time, the acetonitrile-extractable fraction increases in the Drummer surface soil to a much greater extent than the Exeter surface soil (Figures 2 & 3). The distribution of imidacloprid in the Drummer surface soil is consistent with previous studies (Cox et al., 1997
Figure 1. Degradation of imidacloprid in Drummer and Exeter surface and subsurface soils over 400 days.
Figure 2. Percentage of imidacloprid recovered in aqueous (AQS), acetonitrile (ACN), and acid (ACID) solvent fractions from Exeter surface soil based on total imidacloprid recoveries.
Figure 3. Percentage of imidacloprid recovered in aqueous (AQS), acetonitrile (ACN), and acid (ACID) solvent fractions from Drummer surface soil based on total imidacloprid recoveries.
Acid-extractable imidacloprid increases more rapidly in the Drummer surface soil than the Exeter surface soil, but both Drummer and Exeter surface soils have only 10% to 12% of imidacloprid in this fraction at day 400 (Figures 2 & 3).

Aging of pesticides tends to decrease their bioavailability to microorganisms and higher organisms. This bioavailability decrease occurs by the fairly rapid sorption of the pesticide to external soil surfaces and then a slower partitioning of the pesticide into the inner soil surfaces and organic matter. This is referred to as a biphasic model of sorption (Alexander, 1995; Kelsey et al., 1997; Radosevich et al., 1997; White et al., 1997). Our results are generally consistent with this model of pesticide behavior. The Drummer surface soil, with greater organic carbon and clay content, would be expected to adsorb more imidacloprid than the Exeter soil. The Drummer soil shows a pattern of declining bioavailability over time, whereas the large percentage of water-extractable imidacloprid in the Exeter soil suggests that bioavailability does not decline.

Generally, microorganisms in surface soils are able to degrade pesticides more readily than in subsurface soils due to their higher populations and greater organic matter content. Pesticides such as alachlor, metribuzin, fluometuron, and 2,4-D all degrade more slowly in subsurface than surface soils (Welch, 1995; Mueller et al., 1992; Pothuluri et al., 1990; Moorman & Harper, 1989). In general degradation is slower and pesticide half-lives are longer in subsurface soils due to lacking nutrients, soil temperature, soil water content, and soil type. In the Drummer subsurface soil degradation of imidacloprid is slower than in the Drummer surface soil, but no difference was seen between the Exeter surface and subsurface soils (Figure 1). The distribution of imidacloprid in the water-, acetonitrile-, and acid-fractions of the Exeter and Drummer subsurface soils was similar to that seen in the surface
soils (Figures 4 & 5). Only 10% of the imidacloprid was degraded in the Drummer subsurface soil, but 25% of the imidacloprid was still water-extractable after 400 days, suggesting that bioavailability was not limiting.

Microbial biomass and activity is often lower in subsurface soils than in surface soils. Microbial biomass in these two soil profiles follows this general trend (Table 1). Benzoic acid was mineralized very quickly by the Drummer surface soil with more than 25% mineralized within 24 h, compared to the Drummer subsurface soil which took more than 3 days to mineralize the same amount of chemical (Figure 6). In contrast, benzoic acid was mineralized in the low biomass Exeter soils after longer lag periods. The Drummer surface soil was able to mineralize 60% of $^{14}$C-2,4-D after 40 days, while the other three soils mineralized less than 5% of this chemical (Figure 7). In contrast to our 2,4-D results, Wilson & Cheng (1978) and Ou (1984) found that after a short lag period surface soils were able to mineralize up to 72% of $^{14}$C-2,4-D after 30 to 80 days. Welch et al. (1995) found that 30% to 45% of $^{14}$C-2,4-D was mineralized in surface soils after 168 days while subsurface soils mineralized less than 15% of $^{14}$C-2,4-D. Although imidacloprid mineralization is very low, both the Exeter and Drummer surface soils mineralized more than twice the amount of that mineralized in the subsurface soils (Figure 8). $^{14}$CO$_2$ evolved from $^{14}$C-methylene-imidacloprid treated soils was less than 1.5% over 400 days of incubation (Figure 8).

Imidacloprid appears to be degraded by cometabolism. A study with an imidacloprid-degrading microorganism, a *Leifsonia* sp., demonstrates that imidacloprid is cometabolized in soil-free liquid media culture (Anhalt, Chapter 2). Evidence to support the cometabolic activities of this organism include the inability to produce $^{14}$CO$_2$, the production of stable metabolites, the inability to maintain and increase cell populations, and the inability
Figure 4. Percentage of imidacloprid recovered in aqueous (AQS), acetonitrile (ACN), and acid (ACID) solvent fractions from Exeter subsurface soil based on total imidacloprid recovered.
Figure 5. Percentage of imidacloprid recovered in aqueous (AQS), acetonitrile (ACN), and acid (ACID) solvent fractions from Drummer subsurface soil based on total imidacloprid recoveries.
Figure 6. Cummulative mineralization of $^{14}$C-Benzolic Acid to $^{14}$CO$_2$ in Drummer and Exeter surface and subsurface soils.
Figure 7. Cumulative mineralization of $^{14}$C-2,4-D to $^{14}$CO$_2$ in Drummer and Exeter surface and subsurface soils.
Figure 8. Cumulative mineralization of $^{14}$C-methylene-imidacloprid to $^{14}$CO$_2$ in Drummer and Exeter surface and subsurface soils.
to use imidacloprid as its sole carbon source. In our four soils very little CO₂ was produced from imidacloprid and despite the large amount of microbial biomass in the Drummer surface soil, little degradation occurred. Cometabolism of imidacloprid therefore, seems likely as the primary means of degradation in the Drummer and Exeter soils. The absence of exposure to imidacloprid may have prevented microbial adaptation to breakdown imidacloprid.

Imidacloprid persistence in soil is likely controlled by biological factors, such as a lack of imidacloprid-degrading microorganisms in these soils, in combination with bioavailability. In view of the fact that three of the four soils had a high concentration of imidacloprid present in the water-extractable fraction at day 400, it seems reasonable to assume that these imidacloprid quantities are bioavailable. Despite these results, degradation was still limited in these soils. This indicates that the microorganisms necessary for imidacloprid degradation are either present in low populations or are limited in their activity.

Acknowledgments

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Pesti. Sci. 55:482-485
A microorganism was isolated from soil-free enrichments with the ability to degrade imidacloprid. Strain PC-21 was determined to be a *Leifsonia* sp., and is able to degrade 37% to 64% of imidacloprid in culture. No $^{14}$CO$_2$ is produced from $^{14}$C-imidacloprid, but six stable metabolites, including imidacloprid-guanidine and imidacloprid-urea are produced, as determined by HPLC and LC-MS. Less than 1% of the $^{14}$C applied as $^{14}$C-imidacloprid was found to be in the microbial biomass. Strain PC-21 also produces NO$_3^{-}$/NO$_2^{-}$, indicating that it is cleaving off the NO$_2$ group from the imidazolidin ring, which would be a necessary step in formation of the guanidine metabolite. Degradation in soil may begin with the cleavage of the NO$_2$ group, making NO$_3^{-}$/NO$_2^{-}$ available to microorganisms. Strain PC-21 will not grow in dilute media, but instead requires all nutrients provided to it. It is not able to use imidacloprid as its sole carbon source. Additionally, strain PC-21 is not able to maintain or increase its population when in the presence of imidacloprid. All of these factors indicate that strain PC-21 cometabolizes imidacloprid.

The degradation of imidacloprid by strain PC-21 is the first such report for this compound. This bacterium's transformation of imidacloprid provides an indication of how degradation occurs in soil. Laboratory studies in soil also show that no CO$_2$ is produced, affirming one aspect of cometabolism.

A soil incubation study demonstrated that the degradation of imidacloprid was limited to 10% in the Drummer subsurface soil compared to about 40% in the Drummer surface soil and about 50% in both Exeter soils. The Drummer surface soil has a greater organic carbon and clay content than the other soils. These soil characteristics are consistent
with greater imidacloprid adsorption. More imidacloprid was water-extractable in the Exeter surface soil than the Drummer surface soil. Over time, the acetonitrile-extractable fraction of imidacloprid increases in the Drummer surface soil to a much greater extent than the Exeter surface soil. Additionally, acid-extractable imidacloprid increases more rapidly in the Drummer surface soil than the Exeter surface soil, but both soils have only 10% to 12% of imidacloprid in this fraction at day 400. Imidacloprid in acetonitrile- and acid-extractable fractions is more sorbed to the soil than the imidacloprid in the water-extractable fraction, making these fractions less bioavailable to microorganisms.

The distribution of imidacloprid in the water-, acetonitrile-, and acid-extractable fractions of the Exeter and Drummer subsurface soils was similar to that seen in the surface soils. Only 10% of the imidacloprid was degraded in the Drummer subsurface soil, but 25% of the imidacloprid was still water-extractable after 400 days, suggesting that bioavailability was not limiting.

Soil microbial biomass was 43 µg C g\(^{-1}\) in the Exeter surface soil and 10 µg C g\(^{-1}\) in the subsurface soil, but the microbial biomass in the Drummer surface and subsurface soil was much larger at 705 and 105 µg C g\(^{-1}\), respectively. Less than 1.5% \(^{14}\)CO\(_2\) was produced in all soils over 400 d, but the Drummer and Exeter surface soils were able to mineralize twice as much imidacloprid as the subsurface soils. Despite the large water-extractable imidacloprid fractions observed in three of the four soils, imidacloprid degradation was limited in these soils by biological factors.
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