Environmental Toxicology and Chemistry, Vol. 34, No. 2, pp. 297–302, 2015
Published online 1 November 2014 in Wiley Online Library
(printed in the USA)

ENVIRONMENTAL FATE OF EMAMECTIN BENZOATE AFTER TREE MICRO INJECTION OF HORSE CHESTNUT TREES

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(Submitted 1 July 2014; Returned for Revision 7 August 2014; Accepted 30 October 2014)

Abstract: Emamectin benzoate, an insecticide derived from the avermectin family of natural products, has a unique translocation behavior in trees when applied by tree micro injection (TMI), which can result in protection from insect pests (foliar and borers) for several years. Active ingredient imported into leaves was measured at the end of season in the fallen leaves of treated horse chestnut (Aesculus hippocastanum) trees. The dissipation of emamectin benzoate in these leaves seems to be biphasic and depends on the decomposition of the leaf. In compost piles, where decomposition of leaves was fastest, a cumulative emamectin benzoate degradation half-life time of 20 d was measured. In leaves immersed in water, where decomposition was much slower, the degradation half-life time was 94 d, and in leaves left on the ground in contact with soil, where decomposition was slowest, the degradation half-life time was 212 d. The biphasic decline and the correlation with leaf decomposition might be attributed to an extensive sorption of emamectin benzoate residues to leaf macromolecules. This may also explain why earthworms ingesting leaves from injected trees take up very little emamectin benzoate and excrete it with the feces. Furthermore, no emamectin benzoate was found in water containing decomposing leaves from injected trees. It is concluded, that emamectin benzoate present in abscised leaves from horse chestnut trees injected with the insecticide is not available to nontarget organisms present in soil or water bodies. Environ Toxicol Chem 2015;34:297–302.

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Keywords: Insecticide Tree micro injection Environmental fate Bioavailability Biodegradation Earthworms

INTRODUCTION

The injection of chemicals into tree trunks has a long history and dates back as far as 1158 CE [1,2]. Several unsuccessful attempts have been made over the centuries to make use of this technology. In the 20th century, tree injection research and application was revisited in the 1940s following the spread of Dutch elm disease in the USA [1]. The application method experienced a second boost in the 1990s following the spread of invasive tree pests and diseases across the world. Notable examples include: the emerald ash borer and the Asian longhorn beetle in the USA, pine wilt nematodes in Asia and Europe, the horse chestnut leaf miner in Europe, and the red palm weevil in the Middle East, North Africa, and Europe [3–6]. Numerous methods of injecting liquids or placing implants have been explored, but the breakthrough was achieved with new classes of plant protection chemicals [7]. One such chemical is emamectin benzoate, a gamma-aminobutyric acid agonist that activates chloride channels in the insects’ nervous system. Emamectin benzoate is derived from the avermectin family of natural products [8]. Conventionally applied as a foliar spray, it degrades rapidly on leaf and soil surfaces by ultra violet radiation [9]. Protected from sunlight and due to its physicochemical properties and translocation behavior in trees, emamectin benzoate protects trees for several years from target pest infestation after micro injection [10–12]. The application is very specific, minimizing environmental hazard and operator exposure during application as it is conducted within a closed system, sequestering the chemical inside the tree’s tissue. Nevertheless the possibility remains that emamectin benzoate may be released into the environment via abscised leaves. To investigate this uncertainty, the present study was carried out using abscised leaves collected in autumn from a treated horse chestnut (Aesculus hippocastanum) tree. Fallen leaves were monitored through degradation in various environments, such as compost piles, in water, or on the ground in contact with soil. The insecticide emamectin benzoate was analyzed at intervals in the decomposing organic matter and corresponding environment.

MATERIALS AND METHODS

An 82-yr-old horse chestnut tree, with a 42 cm diameter at breast height (DBH), in Wüllfingerstrasse, Winterthur, Switzerland, was treated on 16 May 2012 (end of blooming) by tree micro injection of Revive® (Syngenta Crop Protection AG, Switzerland), a 4% microemulsion formulation of emamectin benzoate. At the base of the tree, within 30 cm of the soil, injections were made into intact, healthy sapwood. Injection points were prepared by drilling holes with a brad point drill-bit (diameter, 10 mm), 2.5 cm–4.0 cm into the sapwood. Before drilling, drill-bits were cleaned and disinfected in 70% alcohol. Immediately following drilling, a biodegradable micro-injection plug (Figure 1A) was inserted into the borehole to form a seal at the site of the phloem. The plug functions as a barrier for restricting any backflow of the liquid from the borehole through the orifice and protects the tree from

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Published online 1 November 2014 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/etc.2795

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secondary infections. Ten mL of undiluted Revive was injected (at 2 bar pressure) through the plug into the borehole using a tree micro injection gun (Figure 1). Eight injection points were placed around the tree at 12 cm–18 cm apart. From 4 October to 14 November 2012 the tree was wrapped in a plastic scaffold screen (Figure 2) to catch the naturally abscising leaves. The screen was emptied on 25 October and 14 November 2012, collecting all of the fallen leaves. Control leaves were collected from untreated horse chestnut trees. Over a 1-yr period, collected leaves were subjected to various decomposition pathways. Emamectin benzoate content was analyzed periodically.

**Compost**

Collected leaves were incubated from 14 November 2012 in compost piles. Four batches of compost were prepared, 3 with leaves from the emamectin benzoate treated-horse chestnut trees and 1 with leaves from the control nontreated (Table 1). Leaves were shredded with a handheld vacuum leaf shredder. The batches were piled on a lawn in silos consisting of a stainless steel wire frame (1 m high, 60 cm diameter), lined with a perforated black plastic film (200 holes/m², 1 cm diameter). The lawn soil below the compost was covered with a nylon screen. The pile was covered by a piece of nontransparent, nonwoven, PP fleece (Tencate Toptex® Compost Cover). Periodically the piles were turned and water, horn meal, and rock flour were added. The temperature in the compost piles and the temperature of the air (20 cm above ground) was measured. The quality and maturity of the compost was assessed using the cress test, a very sensitive phytotoxicity bioassay that can be used to estimate the maturity of compost degradation [13,14]. The cress test involves seeds of garden cress (*Lepidium sativum*), which were densely sown on the compost in a PP-container (10 cm × 12 cm × 5 cm) and germinated and grown for 6 d in a greenhouse. Compost quality was assessed by comparing the germination rate, growth, and color of the garden cress seedlings.

Earthworms (*Lumbricus terrestris*), naturally colonizing the compost piles, were collected and kept on various food sources (organic matter from the compost piles, either with leaves from treated or control trees) at room temperature in the dark.

**Leaves in water body**

Five batches of 70 g of leaves were placed in plastic baskets (diameter, 35 cm; height, 40 cm) and watered with 1.8 L of tap water. The baskets were held outdoors and covered loosely with a lid protecting them from precipitation and keeping the leaves in the dark, but allowing respiration. The water level was kept constant at 1.8 L, readjusted when decreased by transpiration or by sampling for analysis.

**Leaves on the ground**

Five batches of 70 g of leaves were kept on the ground of a lawn. A nylon net (mesh size, 1 cm) was placed between the leaves and soil, and a plastic cylinder (diameter, 35 cm; height, 40 cm) was placed around the leaves. In this arrangement, shaded leaves were in contact with the soil and exposed to precipitation.

**Quantitative analysis of emamectin benzoate in samples**

**Sample preparation.** Single samples were analyzed from pooled organic matter. They were homogenized in a blender for 2 min with acetonitrile and water (95:5) (leaves and compost 25 g in 100 mL; earthworm and earthworm feces 5 g in 20 mL). Thirty mL or 10 mL of the sample along with the drying agent (Agilent Part No. 5982–5755/50 mL) were shaken in a QuEChERS Extract Tube (Agilent Technologies). After centrifugation (2 min at 2500 g) 10 mL of supernatant was transferred to a QuEChERS Dispersive SPE Tube (Agilent Part No. 5982–5058/15 mL), and shaken. After centrifugation (2 min at 2500 g) the supernatant was transferred into autosampler vials using a 0.2 μL PTFE syringe filter. Water samples were diluted in acetonitrile (4 ×) and processed as the organic matter samples.

**Blank preparation.** Untreated horse chestnut leaves, prepared as the samples described above in Sample Preparation, were used for Blank and Standard solutions.

**Recovery.** The active ingredient dissolved in acetonitrile was injected into the petiole of a horse chestnut leaf. When prepared as a sample, recovery of emamectin benzoate was >90%.

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**Figure 1.** Revive® stem injection of a horse chestnut tree through a biodegradable plug (A) using the tree micro injection gun.

**Figure 2.** All leaves from a treated horse chestnut tree were caught in a screen at leaf fall in autumn.
Reference stock and standard solutions preparation. A standard sample of emamectin benzoate of >98% purity (91.75% emamectin B1a benzoate and 5.02% emamectin B1b benzoate), supplied by Syngenta Crop Protection AG, was used to prepare reference stock solutions of 1 ppm, 5 ppm, 100 ppm, and 1000 ppm in acetonitrile. Standard solutions were prepared by dilution of reference stock solutions using blank solution (40 ppb, 160 ppb, 800 ppb, and 4000 ppb).

**Chromatography instrumentation and chromatographic conditions.** Emamectin benzoate was quantified by the analysis of the free base of Emamectin B1a in 5 mL of extract solution (2 aliquots per sample) using high-performance liquid chromatography tandem mass spectrometry (HTS-PAL Autosampler with Flux Pump Rheso 2200 and Finnigan LTQ). The column was a Kinetex C8 (Phenomenex Part No. 00D-4497-E0) and the temperature was maintained at 24 °C. The mobile phase was 0.1% aqueous trifluoroacetic acid – acetonitrile (gradient 50 + 50 to 0 + 100) at a flow rate of 1.2 mL min -1 for 9 min, followed by a cleaning part at a flow rate of 1.7 mL min -1 for 7 min. Scan range was 156.5 m/z to 159.5 m/z (158 + C6 1.5 m/z) as a daughter ion of 886.5 m/z. Retention time for emamectin benzoate B1a was 2.5 min (Figure 3). The limit of quantitation (LOQ) was 10 μg/kg.

| Leaves from | Emamectin benzoate treated HCN tree | Control HCN tree | None HCN broad leaf trees |
|-------------|------------------------------------|------------------|--------------------------|
| Fresh compost| 6.5 kg                             | 7.3 kg           | 28.0 kg                  |
| Wood chips  | 21.5 kg                            | 22.5 kg          | 21.5 kg                  |
| Rock flour  | 1.0 kg                             | 1.0 kg           | 0.5 kg                   |
| Horn meal   | 0.5 kg                             | 0.5 kg           | 0.5 kg                   |
| Inoculation broth | 4.0 L                           | 4.0 L            | 3.0 L                    |

Table 1. Four compost piles with horse chestnut leaves were prepared

Compost

The temperature in the compost piles rose shortly after building them (Figure 4). After 1 wk, the temperature at 10 A.M. was 3 °C to 9 °C higher in the compost piles than the temperature of the air 20 cm above ground. The difference in temperature remained within this range for over 3 mo, until the end of February. No differences in temperature between that of the compost piles and the air temperature 20 cm above ground were registered until the end of April. From April onward, the temperature in the compost piles ranged from 1 °C to 5 °C below the air temperature at 10 A.M. Temperature courses in the different piles were comparable. A slight difference was found in the control pile during the summer months, it was on average 1.4 °C warmer than the temperature measured in the other 3 compost piles.

In the 3rd compost pile, which contained fresh compost, a somewhat accelerated physical decomposition of leaves was observed at the beginning of the process. The differences disappeared over time and after 1 yr of feeding the compost piles, the physical decomposition stage was comparable in all 4 piles, including the control; leaves were fairly decomposed, and compost reached 90% to 95% maturity (Figure 5). The uniformity of appearance was confirmed by a bioassay using garden cress as a quality indicator (Figure 6). Cress germination, growth, and color did not vary when sown and grown in the compost of the different piles. No emamectin benzoate above the LOQ of 10 μg/kg could be detected in any of the cress plants.

Periodic analyzes of organic matter contained in the piles revealed a decreasing emamectin benzoate content in a biphasic pattern with a fast dissipation at the beginning and a slowing
down with time (Table 2). The cumulative degradation half-life time was calculated to be at 20 ± 5 d derived from the linear regression model with untransformed values.

**Earthworms**

In spring, earthworms were found colonizing the compost piles. No differences in earthworm density were detected between the treated and control piles. At the beginning of April, earthworm populations were established in all 4 compost piles. The worms living in compost piles with treated leaves were also ingesting leaves containing emamectin benzoate. Indeed, worms collected on 12 June 2013 from the 3 piles containing treated leaves registered 78 ± 7 µg/kg emamectin benzoate. Samples of compost from treated piles collected on the same day contained 347 ± 7.9 µg/kg emamectin benzoate. No emamectin benzoate was detected in worms or compost from the control pile.

When earthworms were transferred from a pile with treated horse chestnut tree leaves to the control pile, the emamectin benzoate content in the worms decreased rapidly (Table 3). Within 4 d, emamectin benzoate concentration dropped by 73% and within 14 d concentrations dropped by over 95%.

To verify if the emamectin benzoate dissipation in the earthworms was due to metabolism or excretion, the earthworms were fasted for 5 d (Table 4), during which feces was collected and analyzed. After 5 d the earthworms’ feces accounted for 64 ± 11% of emamectin benzoate present at the beginning of the test.

**Leaves in water body**

The physical degradation of leaves aged in water in the dark under outdoor conditions, was similar between the control and the treated trees. The decomposition was very slow. Even after 1 yr some leaf pieces were still recognizable. The emamectin benzoate content in these leaves decreased in a biphasic pattern (Table 5) with a cumulative degradation half-life time of 94 d (Linear regression analyzes with untransformed values). No emamectin benzoate was detected (LOQ = 10 µg/kg) in water.

**Leaves on the ground**

Leaves placed in contact with soil and exposed to precipitation did not visually decompose much within a yr, as pieces of the leaf were still recognizable and nearly intact. During the first 30 d, the dissipation rate of emamectin benzoate in leaves on the ground was similar to the dissipation of emamectin benzoate in leaves kept in water (Table 6). Afterwards dissipation slowed down, resulting in a cumulative degradation half-life time of 212 d derived from the linear regression model with untransformed values.
Table 2. Emamectin benzoate content (µg active ingredient/kg fresh wt) in compost piles

| Date                  | DOC            | Pile 1a | Pile 2b | Pile 3c | Control piled |
|-----------------------|----------------|---------|---------|---------|--------------|
| 14 November 2012      | 0              | 2758    | 100%    | 1809    | 100%         |
| 12 December 2012      | 28             | 1006    | 36%     | 727     | 40%          |
| 06 March 2013         | 112            | 541     | 20%     | 303     | 17%          |
| 12 June 2013          | 210            | 415     | 15%     | 261     | 14%          |
| 10 September 2013     | 300            | 310     | 11%     | 180     | 10%          |
| 04 1 4 6             |                |         |         |         |              |

Without fresh compost.
Control pile with horse chestnut leaves of untreated trees.
Limit of quantitation (LOQ) 10 µg/kg.
Limit of quantitation (LOQ) 10 µg/kg
DOC = days of composting.

DISCUSSION

In the present study, the physical decomposition of fallen horse chestnut leaves was very slow and comparable in both the treated and control leaves. Even in compost piles, leaf decomposition to humus required more than 1 yr whether treated or control. Under some conditions, the decomposition of organic matter of plant origin, including leaves, takes a few weeks to a few months to reach humus stage [15,16]. The main determinants for the degradation process are the composition of the organic matter and external factors such as temperature, moisture, oxygen, and the biological activity of the surroundings. Some of these parameters (i.e., oxygen and water) were controlled in the present study. Microbial activity was stimulated using an inoculum made from freshly started organic household compost. Temperature was not controlled as we investigated, in outdoor conditions, the degradation of fallen horse chestnut leaves. The experiment was initiated after leaf fall in November 2012 at the beginning of winter. The winter of 2012 to 2013 was unusually long at the trial site with low temperatures until May 2013. The long winter could have delayed the degradation process in comparison with an average year with a shorter winter. The slow decomposition of leaves observed under these field conditions might be explained by plant secondary metabolites, for example, by the high content of tannic acids and lignin in horse chestnut leaves, which have been shown to delay decomposition [17]. The presence of emamectin benzoate had no impact on the decomposition of fallen leaves. Progress and quality of decomposition was comparable in all of the various batches and set ups, independent of emamectin benzoate content in leaves.

After micro injection of Revive into tree trunks, emamectin benzoate moves apically in the xylem into leaves [11,18]. Once in the leaves, emamectin benzoate may begin to metabolize before leaf abscission in the fall, as shown in ash trees treated in Michigan [11]. It has also been shown that emamectin benzoate in the leaves of vegetables, like lettuce and cabbage, and of sweet corn is metabolized into nontoxic components and incorporated into natural products [19]. The ratio between import and metabolic rate might determine if emamectin benzoate content is increasing, constant, or decreasing over time in leaves after tree micro injection. In horse chestnut trees, emamectin benzoate import to leaves seems to be higher than metabolism because it is accumulating. After an injection of 2 mL Revive per cm DBH in May 2012, emamectin benzoate content reached 8194 µg/kg until leaf fall of the same year.

Dissipation of emamectin benzoate in fallen leaves was relatively slow and seemed to follow a biphasic pattern. During the first 3 mo to 4 mo emamectin benzoate content decreased quickly, and subsequently slowed. This pattern is similar to the dissipation of emamectin benzoate in soils [9]. This biphasic dissipation profile in soils has been attributed to the rapid and extensive sorption of emamectin benzoate residues to soil macromolecules [20], and, subsequently, reduced bioavailability of the sorbed residue to soil microorganisms. Similar mechanisms may be occurring in leaves. Sorption to macromolecules in leaves might be responsible for reduced biological availability and dissipation of emamectin benzoate. Indeed, this view is supported

Table 3. Emamectin benzoate content (µg active ingredient/kg fresh wt) in earthworms

| Day     | Pile compost 3 | Pile compost control pile |
|---------|----------------|---------------------------|
| 0       | 124            | 124                       |
| 4       | 132            | 34                        |
| 14      | 168            | 6                         |

Earthworms from Pile 3 transferred to the laboratory on 11 July 2013 (Day 0) and kept on compost of pile 3 and control pile. Emamectin benzoate content of compost pile 3 on 11 July 2013 was 325 ppb.

Table 4. Recovery of emamectin benzoate in feces of earthworms

| Worm | Fresh weight mg | EMA ng | EMA % |
|------|-----------------|--------|-------|
| Days | Avg. SE | Avg. SE | Avg. SE | Avg. SE |
| 2    | 927 165 | 62 16 | 50 15 | 57 10 |
| 5    | 544 171 | 25 4 | 59 12 | 42 5 |

Earthworms were transferred from compost piles to the laboratory and fasted for 5 d. Three replicate samples of worms were analyzed. No emamectin benzoate was found in worms from the control pile.

EMA = emamectin benzoate Avg. = average; SE = standard error.
Table 5. Horse chestnut leaves, aged outdoor in water in the dark\textsuperscript{a}

| Date               | Time in days | Leaves in water |
|--------------------|--------------|-----------------|
| Date               | Time in days | Litter μg/kg    | Water μg/L | % Unfiltered | Filtered     |
| 14 November 2012   | 0            | 8194            | 100        | <LOQ         |              |
| 12 December 2012   | 28           | 3849            | 47         | <LOQ         |              |
| 10 April 2013      | 147          | 2340            | 29         | <LOQ         |              |
| 16 October 2013    | 336          | 1115            | 14         | <LOQ         |              |

\textsuperscript{a}Emamectin benzoate content in leaves and water.

\textsuperscript{b}Limit of quantitation (LOQ) 10 μg/kg.

Table 6. Emamectin benzoate content in leaves kept on the ground under outdoor conditions

| Date               | Time in days | Leaves μg/kg % |
|--------------------|--------------|----------------|
| 14 November 2012   | 0            | 8194 100       |
| 12 December 2012   | 28           | 4665 57        |
| 10 April 2013      | 147          | 2736 33        |
| 11 July 2013       | 239          | 2876 35        |

CN = horse chestnut.

by several observations 1) earthworms ingesting leaves take up little emamectin benzoate and excrete it in the feces; 2) no emamectin benzoate was found in water when leaves were kept and degraded in water; and 3) degradation of emamectin benzoate in leaves decomposing on the ground in contact with soil was comparable with emamectin benzoate degradation in the soil. In addition, it has been observed that emamectin benzoate dissipation parallels the decomposition process of the leaves. In compost piles, where decomposition of leaves was fastest, a cumulative emamectin benzoate degradation half-life time of 20 d was calculated. In leaves decomposing in water a degradation half-life time of 94 d was observed. In leaves decomposing on the ground in contact with soil the degradation half-life time was 212 d. This may indicate that emamectin benzoate is biologically available primarily during the breakdown of leaf macromolecules and degraded with them. This pattern indicates that bioavailability will be very low following leaf fall onto soil or into water, thus limiting exposure of terrestrial and aquatic organisms to emamectin following tree injection applications.

Acknowledgments—We would like to thank P. Hirsgier of the Municipal Gardens Department, Winterthur (CH), for the test trees he made available to us. We thank E. Farrelly, D. Cox and A. Cornish for reviewing this manuscript prior to submission for publication and providing editorial comments for improving the presentation of the present study.

Disclaimer—R. Burkhard, H. Binz, C. A. Roux and P. Wyss are employees of a Syngenta group company. M. Brunner and O. Ruesch have received consultancy fees from a Syngenta group company.

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