Effects of multiple but low pesticide loads on aquatic fungal communities colonizing leaf litter

Anne Talk, Susanne Kublik, Marie Uksa, Marion Engel, Rüdiger Berghahn, Gerhard Welzl, Michael Schloter, Silvia Mohr

1. Research Unit Environmental Genomics, Helmholtz Zentrum München, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany.
2. Umweltbundesamt, Schichauweg 58, 12307, Berlin, Germany
3. Umweltbundesamt, Dernburgstr. 7, 14057, Berlin, Germany

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Pesticides
Regulatory acceptable concentration
Fungal community structure
Leaf litter degradation

ABSTRACT

In the first tier risk assessment (RA) of pesticides, risk for aquatic communities is estimated by using results from standard laboratory tests with algae, daphnids and fish for single pesticides such as herbicides, fungicides, and insecticides. However, fungi as key organisms for nutrient cycling in ecosystems as well as multiple pesticide applications are not considered in the RA. In this study, the effects of multiple low pesticide pulses using regulatory acceptable concentrations (RACs) on the dynamics of non-target aquatic fungi were investigated in a study using pond mesocosm. For that, fungi colonizing black alder (Alnus glutinosa) leaves were exposed to multiple, low pulses of 11 different pesticides over a period of 60 days using a real farmer’s pesticide application protocol for apple cropping. Four pond mesocosms served as treatments and 4 as controls. The composition of fungal communities colonizing the litter material was analyzed using a molecular fingerprinting approach based on the terminal Restriction Fragment Length Polymorphism (t-RFLP) of the fungal Internal Transcribed Spacer (ITS) region of the ribonucleic acid (RNA) gene(s). Our data indicated a clear fluctuation of fungal communities based on the degree of leaf litter degradation. However significant effects of the applied spraying sequence were not observed. Consequently also degradation rates of the litter material were not affected by the treatments. Our results indicate that the nutrient rich environment of the leaf litter material gave fungal communities the possibility to express genes that induce tolerance against the applied pesticides. Thus our data may not be transferred to other fresh water habitats with lower nutrient availability.

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Introduction

The common EU risk assessment (RA) for pesticides only refers to effects of single substances despite the fact that in most cases a combination of different pesticides is repeatedly used in agriculture to protect crops from pests. For example, in fruit cultivation a considerable number of pesticides with different modes of action is repeatedly applied in short time intervals in the so called ‘spraying sequences’ over the entire growing season (Süß et al., 2006; Bayer, 2014). Multiple pesticide loads may reach fresh water system via various pathways, including spray drift and runoff (Huber et al., 2000;
Schulz, 2004). Here the compounds can impact biota on all trophic levels and thus negatively interfere with ecosystem services of aquatic environments.

Fungi are of utmost importance for aquatic food chains, since they contribute to a large extent to the breakdown of submerged leaf litter (Hieber and Gessner, 2002; Gessner et al., 2010). Thus, a loss of fungal abundance and diversity in aquatic ecosystems may influence the entire aquatic ecosystem as a result of reduced energy and nutrient flow (Bundschuh et al., 2011; Zubrod et al., 2011). Thus it is striking that fungi are not yet included in the RA of pesticides (EFSA, 2013), despite the fact that fungi might be highly impacted by xenobiotics. For example, Dijksterhuis et al. (2011) found that aquatic fungi were most sensitive to ergosterol inhibiting fungicides, which makes sense since ergosterol is obligatory for the biosynthesis of fungal membranes (Mille-Lindblom et al., 2004). More recent studies by Zubrod et al. (2015) have proven the sensitivity of aquatic hyphomycetes to pesticides.

For some fungicides, the HCS concentration (hazardous concentration to ≤5% of all tested species) derived from Species Sensitivity Distribution Curves (SSD, Posthuma et al., 2002) has been proven to be sufficiently protective also for fungal communities (Malby et al., 2009) and thereby regulatory acceptable. However these SSD are based on LC50 values or no-observed-effect concentrations (NOECs) for a small set of invertebrates, primary producers, and fish in single species standard tests with defined toxicants, whereas fungi are not taken into account so far. Thus taking these values as a basis for assessing the toxicity of compounds for fungal communities might be strongly biased.

However, developing standard tests for fungal communities is still a challenge due to the fact that the kingdom of fungi is diverse and most organisms are hard to cultivate using classical isolation based approaches. In the last decades tools in microbial ecology based on the direct analysis of extracted nucleic acids from environmental samples have been developed, which made a cultivation independent description of fungal communities in environmental samples possible (Marsh, 1999). Consequently, a large number of studies on the effects of biotic and abiotic stressors on terrestrial as well as aquatic fungal communities is available (Nikolcheva et al., 2003; Nikolcheva and Bärlocher, 2004; Solé et al., 2008a; Moreirinha et al., 2011), including studies on the effect of single pesticides (Sigler and Turco, 2002; Girvan et al., 2004).

However, as still effects of repeated multiple low pesticide concentrations on fungal communities are missing, in this study we addressed the question, how aquatic fungal communities colonizing leaf litter material are affected by a typical spraying scenario used in apple cultivation, where a high number of fungicides is applied (Süß et al., 2006; Bayer, 2014). Therefore we used a pond mesocosm system where half of the ponds where treated with the same spraying sequence used for apple cultivation in Germany and the other half was treated as control, where no pesticides were applied. We took alder (Alnus glutinosa L.) leaf litter in litterbags and measured the degradation rates at different time points after application of the pesticides to the ponds. In addition we used molecular fingerprinting approaches based on directly extracted deoxyribonucleic acid (DNA) from the litter material at different time points during the degradation process of the litter material to characterize the diversity of the fungal communities. We postulated that the applied fungicides affect mainly fungal diversity pattern, whereas degradation rates of the litter material stay mostly unaffected mainly at early stages of litter transformation due to the high functional redundancy of different microbes present on the litter material (Aneja et al., 2006).

1. Material and methods

1.1. Experimental outline

A real farmers’ application protocol for apple crop plantation from 2010 in the Bodensee region — one of the biggest regions for apple plantation in Germany, with a yield of 269.000 tons in 2012 (LEL, 2012) — was chosen including the spring scenario (April to June) where multiple fungicide applications are performed. Type of substances, order of application and repetition used in this study followed the original protocol from the field (Table 1).

Litter bags with A. glutinosa leaves were exposed to the water in eight indoor pond mesocosms and were periodically sampled (Table 1). The mesocosms are part of the artificial size stream and pond system of the German Federal Environment Agency (UBA) in Berlin (www.umweltbundesamt.de/fsa). Four ponds were treated with multiple pesticides using the original spraying protocol, 4 ponds served as controls. Stagnant water conditions were chosen since they allow ecotoxicologically relevant substances to act for longer time periods. This is considered as ‘worst case’ scenario in tier RA of pesticides and therefore preferred to lotic conditions.

1.2. Preparation of pond mesocosms

Eight open indoor mesocosm ponds (length 6.90 m × width 3.25 m × height 2.5 m) with a water volume of 12 m³ had been set up equally 1 year before the start of the experiment by introducing sand, sediment as well as macrophytes, benthos, periphyton and plankton from the field station of the UBA or from unpolluted freshwater sources in South Brandenburg (Germany; Mohr et al., 2012) in order to establish an aquatic pond community. The ponds were filled with a mixture of ground and deionized water and were spiced with nutrients prior to the start of the experiment to establish mesotrophic water conditions. Bulkhead openings in the side walls of each pond allowed for the water exchange between the ponds during the 1 year establishing phase in order to maintain the synchronous development of the plankton communities between the ponds (Mohr et al., 2008). Bulkheads were closed 4 weeks prior to first pesticide application. For each pond, ventilators blowing over the water surface during night ensured mixing of the water body. Ponds were illuminated by 2× 2000 W and 2× 400 W mercury-vapor lamps corresponding to a mean light intensity of 13,000 lx at the water surface.

1.3. Physico-chemical and biological parameters of the pond water

Water temperature, oxygen concentration, and conductivity were measured online throughout the experiment at 50 cm
Table 1 – Concentrations of the applied pesticides are given as recovery rates (%) 1 hr after application of the pesticides to the pond water starting in April.

<table>
<thead>
<tr>
<th>Days after 1st formulation</th>
<th>Formulation product</th>
<th>Active substance</th>
<th>Type of pesticide and class of active substance</th>
<th>Log ( P_{100%} )</th>
<th>RAC (mg/L) of the product</th>
<th>Recovery (%) as mean of ponds 1, 3, 5, 7</th>
<th>Standard deviation (%)</th>
<th>Litter bag sampling time</th>
<th>Derivation of RAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>-8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>Funguran</td>
<td>Copper</td>
<td>F (copper)</td>
<td>0.44</td>
<td>6.00</td>
<td>83.81</td>
<td>32.59</td>
<td>Exposure of litter bags 0</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Delan WG</td>
<td>Dithianon</td>
<td>F (chalcone)</td>
<td>3.20</td>
<td>1.11</td>
<td>&lt;LOQ**</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>Syllit</td>
<td>D Citadel</td>
<td>F (guanidine)</td>
<td>1.25</td>
<td>1.40</td>
<td>32.42</td>
<td>6.57</td>
<td>-</td>
<td>Algae (EC50, RAC (active substance) converted to RAC (product))</td>
</tr>
<tr>
<td>20</td>
<td>Syllit</td>
<td>D Citadel</td>
<td>F (guanidine)</td>
<td>1.25</td>
<td>1.40</td>
<td>32.42</td>
<td>6.57</td>
<td>-</td>
<td>Algae (EC50, RAC (active substance) converted to RAC (product))</td>
</tr>
<tr>
<td>20</td>
<td>Calypso</td>
<td>Thioclyprid</td>
<td>I (neonicotinoid)</td>
<td>1.25</td>
<td>0.65</td>
<td>71.28</td>
<td>3.17</td>
<td>-</td>
<td>Microcosm, Chloroform (NOEAFB hatching rate, RAC on basis of test with product)</td>
</tr>
<tr>
<td>25</td>
<td>Malvin WG</td>
<td>Captan</td>
<td>F (pyralimide)</td>
<td>2.50</td>
<td>6.20</td>
<td>2.02</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>Flint</td>
<td>Trifluroxystrobin</td>
<td>F (oxime acetate)</td>
<td>4.50</td>
<td>0.30</td>
<td>44.06</td>
<td>5.17</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>31</td>
<td>Consistent plus</td>
<td>Captan</td>
<td>F (pyralimide)</td>
<td>2.50</td>
<td>8.30</td>
<td>2.01</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>31</td>
<td>Flint</td>
<td>Trifluroxystrobin</td>
<td>F (oxime acetate)</td>
<td>4.50</td>
<td>8.30</td>
<td>35.39</td>
<td>1.95</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>31</td>
<td>Runner</td>
<td>Methoxyfenozide</td>
<td>I (diacylhydrozine)</td>
<td>3.72</td>
<td>18.00</td>
<td>1.16</td>
<td>0.00</td>
<td>-</td>
<td>Algae (EC50, RAC (active substance) converted to RAC (product))</td>
</tr>
<tr>
<td>34</td>
<td>Syllit</td>
<td>D Citadel</td>
<td>F (guanidine)</td>
<td>1.25</td>
<td>1.40</td>
<td>8.62</td>
<td>0.29</td>
<td>-</td>
<td>Fish (O. mykiss, LC50, RAC on basis of test with product)</td>
</tr>
<tr>
<td>41</td>
<td>Delan WG</td>
<td>Dithianon</td>
<td>F (chalcone)</td>
<td>3.20</td>
<td>1.11</td>
<td>&lt;LOQ**</td>
<td>0.00</td>
<td>-</td>
<td>Fish (O. mykiss, NOEC, RAC (active compound) converted to RAC (product))</td>
</tr>
<tr>
<td>49</td>
<td>Delan WG</td>
<td>Dithianon</td>
<td>F (chalcone)</td>
<td>3.20</td>
<td>1.11</td>
<td>&lt;LOQ**</td>
<td>0.00</td>
<td>-</td>
<td>Fish (O. mykiss, NOEC, RAC (active compound) converted to RAC (product))</td>
</tr>
<tr>
<td>54</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>56</td>
<td>Delan WG</td>
<td>Dithianon</td>
<td>F (chalcone)</td>
<td>3.20</td>
<td>1.11</td>
<td>&lt;LOQ**</td>
<td>0.00</td>
<td>-</td>
<td>Fish (O. mykiss, NOEC, RAC (active compound) converted to RAC (product))</td>
</tr>
<tr>
<td>56</td>
<td>Topas</td>
<td>Penconazole</td>
<td>F (triazole)</td>
<td>3.72</td>
<td>32.00</td>
<td>98.82</td>
<td>2.66</td>
<td>-</td>
<td>Daphnia magna (NOEC, RAC (active substance) converted to RAC (product))</td>
</tr>
<tr>
<td>56</td>
<td>Insegar</td>
<td>Fenoxycarb</td>
<td>I (carbamate)</td>
<td>4.07</td>
<td>0.88</td>
<td>77.82</td>
<td>6.53</td>
<td>-</td>
<td>Microcosm, D. magna (NOEC, RAC (active substance) converted to RAC (product))</td>
</tr>
<tr>
<td>59</td>
<td>Glyfas</td>
<td>Glyphosate</td>
<td>H (phosphonomat)</td>
<td>-0.80</td>
<td>28.40</td>
<td>77.85</td>
<td>1.17</td>
<td>-</td>
<td>Lemma (EC50, RAC (active substance) converted to RAC (product))</td>
</tr>
<tr>
<td>59</td>
<td>U 4 M Fluid</td>
<td>MCPA</td>
<td>H (arsenolanic acid)</td>
<td>-0.80</td>
<td>28.40</td>
<td>77.85</td>
<td>1.17</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*The target concentrations were derived from RAC (regulatory acceptable concentration) for each single substance in freshwaters (based on referred test and most sensitive species; number in parentheses indicates the applied assessment factor, **LOQ: limit of quantitation, LOQ for dithianon = 6.40% recovery). F: fungicide, I: insecticide, h: herbicide, log_{oct/wat} = octanol/water partition coefficient.
water depth using the following instruments (WTW, Germany): SensoLyt 700 IQ for pH, FDO 700 IQ for oxygen concentration and TetraCon 700 IQ for conductivity. Water temperature was measured by all three instruments. The water temperature increased for all ponds from 11°C in April to around 20°C in mid-June (Table S1). The conductivity of the pond water did not change over time or as a result of the pesticide application and varied around 500 μS/cm over the complete experimental period (Table S1). The pH values of the ponds were slightly alkaline and were in the range of pH = 8 in all ponds at all sampling time points (Fig. 1a). Only at day 7 after the first application increased pH values (above 9) were measured in two of the ponds (ponds 1 and 2).

Nitrate, nitrite, ammonium, phosphate, and silicate were determined at fortnightly intervals. Concentration of macro- and micronutrients, alkalinity and turbidity were also monitored biweekly employing the corresponding DIN EN ISO standard techniques. Alkalinity was measured using the titrimetric method (TitrIC System, Deutsche Metrohm, Germany) according to DIN EN ISO 9963-1. PO₄-P, NH₄-N, NO₃-N and silicate was analyzed employing colorimetric continuous flow analysis CFA (San++, SKALAR, Netherlands) using the following methods: DIN EN ISO 13395 (1996), DIN EN ISO 15681-1 (2004), DIN EN ISO 16264 (2004), and DIN EN ISO 11732 (2005). Phosphate and ammonium concentrations were stable over the experimental period and were lower than 0.05 mg/l (PO₄-P), 0.1 mg/L (NH₄-N) (Fig. 1b and c). Only at day 68 that an increase in ammonium and phosphate values was measured in ponds 1 and 2. Based on the obtained data for nutrients, the trophic status of all ponds was classified as mesotrophic. This could be also confirmed by the measured oxygen concentrations, which were in the range of 8–10 mg/L over the experimental period (Fig. 1d). Slightly different oxygen concentrations were measured for ponds 1 and 2 at day −7 and 68, with values up to 13 mg/L at day −7 and less than 6 mg/L at day 68.

1.4. Leaf litter and preparation of the litter bags

A. glutinosa leaves were picked in autumn directly from a freshly chopped tree which originated from a private forest in
South Brandenburg (Germany) dominated by A. glutinosa leaves were air dried at 4°C for 5 weeks and frozen at −22°C until needed. For the preparation of the litter bags leaf material was heat-treated (80°C for 120 hr) in order to reduce plant derived DNA (Darzynkiewicz et al., 1975; Royere et al., 1988), which could interfere with the analysis of fungal communities mainly at early stages of litter degradation (Bellemain et al., 2010), shredded (maximum size 10 mm²), and sieved (0.63 mm²). Leaves were shredded to ensure homogenous leaf material for each of the 192 bags.

To study the composition of fungal communities colonizing A. glutinosa leaves, six enclosure mounts carrying four litter bags each (plastic frame of 15 cm length × 6.7 cm diameter, covered with gauze of 0.2 mm mesh size) were placed in every pond 8 days before the first application of the pesticides. Enclosure mounts consisted of a stainless steel rod with specially constructed mounts to fix the bags and to keep them in the same place over the ground and without interference to other bags. Each litter bag was filled with 7 g of shredded A. glutinosa leaf litter material (dry weight). Loose filling of the litter bags allowed for full contact of the pond water with the leaves’ surface, so that water borne fungal spores as well as the pesticides could get in contact with the A. glutinosa leaves. The enclosure mounts were placed in the non-lighted area of the ponds to avoid algal growth on the surface of the bags. The enclosure mounts ensured that the litter bags were fixed in the same position of the ponds at a distance of around 20 cm above the sediment.

Six litter bags per pond were retrieved at each of four sampling dates from April to June 2013, and treated as technical replicates, namely: 7 days before the 1st pesticide application in order to have an initial state of the fungal community before the pesticide application (equivalent to 1 day after the exposure of the litter bags to water), 7 days, 54 days and 68 days after the first pesticide application (Table 1).

The obtained litter material was drained in a spin-drier (C240R, Electrolux, Germany) with 1450–1740 r/min for 15 sec, and the wet weight was determined. The 1 g of the drained litter material was directly stored at −22°C for molecular analysis. For dry weight determination the rest of the drained A. glutinosa leaf litter material was dried at 80°C for 24 hr.

For the calculation of the remaining litter dry mass the calculated dry weight per litter bag at the different sampling time points was divided by the amount of initial litter material per bag (7 g dry weight) and expressed in %.

1.5. Pesticide application and analysis

As it was intended to mimic a typical ‘spraying sequence’ scenario, which is frequently used in the respective apple crop region in Germany, the used low concentrations of the pesticides were selected according to the regulatory acceptable concentrations (RACs) of the individual substances. Therefore pesticide formulations, dissolved in 0.5 L water as stock solution, were diluted with 40 L tap water for each application and mixed in a stainless steel tank. Finally the solution was applied homogeneously 10 cm below the water surface to four of the eight pond mesocosms (ponds 1, 3, 5 and 7) using a spraying lance in order to realistically simulate the spray drift deposition of multiple pesticide applications to surface waters. Formulations were used, since they are actually applied on the field and the additives may influence the potential effect of the active ingredients on non-target organisms. The pesticides, their formulations and the corresponding RAC are listed in Table 1. In some cases, solvents were added to the stock solution. The 4 control ponds (ponds 2, 4, 6, and 8) were treated in the same way with tap water (and if used with solvents) but without pesticide formulations.

After pesticide application the pond water was mixed for 1 hr by means of compressed air from a nozzle. With this method, air bubbles mechanically mixed the water body to achieve a quite homogenous pesticide distribution. Thus water samples for chemical analysis were taken 1 hr after applying the pesticides to measure the bioavailable fraction of the applied pesticides using inductively coupled plasma mass spectrometry (ICP-MS) for copper oxychloride, liquid-liquid extraction (LLE), gas chromatography coupled with mass spectrometry (GC-MS) for captan and sulfur, and liquid chromatography–mass spectrometry (LC-MS/MS) for the other pesticides (Table 1). All pesticides were analyzed using standard test protocols for the appropriate pesticides. Analyses were done by Eurofins/SOFIA GmbH, Germany. Whereas for the fungicide penconazole almost 100% and for the fungicide copper oxychloride around 80% of the applied concentrations were measured, for most of the other pesticides lower values were detected, which ranged between 70% and less than 10% of the applied concentrations (Table 1). For some substances high loss rates were expected. For example dithianion and capton hydrolyzes fast with a half-life time of less than a day, respectively less than 1 hr at pH > 8 (PAN pesticide info, https://extension.usu.edu/files/publications/factsheet/AG_Pesticides_14.pdf) while other substances like trifloxilritin, methoxyfenoxizide and dithianion are known to absorb quickly to surfaces including the litter material itself due to high logPow (Dimitrov et al., 2014).

1.6. Nucleic acid extraction and fingerprinting of fungal Internal Transcribed Spacer (ITS) amplicons

DNA was extracted from 0.5 g A. glutinosa litter material (wet weight) using the Nucleo Spin® Soil Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer’s instructions except for the following modifications. Cell lysis was performed with the Precellys 24 homogenizer (Peqlab, Erlangen, Germany) at 4°C for molecular analysis. For dry weight determination the rest of the drained A. glutinosa leaf litter material was dried at 80°C for 24 hr.

For the calculation of the remaining litter dry mass the calculated dry weight per litter bag at the different sampling time points was divided by the amount of initial litter material per bag (7 g dry weight) and expressed in %.
of 1:400 and denatured at 95°C for 5 min in a PeqStar96 Universal thermocycler (Peqlab, Erlangen, Germany) followed by fragment analysis running in an ABI 3730 capillary sequencer (Life Technologies, Carlsbad, USA). Size and relative abundances of terminal restriction fragments (t-RFs) were analyzed using the GeneMapper 4.0 Software (Life Technologies, Carlsbad, USA) and the T-REX Software (http://trex.biohpc.org/, 30.11.2014; Culman et al., 2009). All fragments of a size smaller than 56 bp were excluded in order to prevent the display of primer dimers.

1.7. Statistical analysis

All statistics for the analysis of fungal community structure are based on the statistical software R (http://www.R-project.org/) using the relative abundance of t-RFs normalized by the total peak height of the corresponding t-RF profiles. t-RFs with a relative abundance <1% and all fragments <56 bp were excluded from the analysis, since they may have been produced as artifacts by the sequencer (Bennett et al., 2008). Significant differences between the treatments \( (p < 0.05) \) were tested by PerMANOVA. A multifactorial comparison principle component analysis (PCA) based on Hellinger-transformed relative terminal Restriction Fragment Length Polymorphism (t-RFLP) data was performed to display the similarity of the fungal community compositions (t-RFLP profiles) with and without pesticide treatment.

2. Results

2.1. Litter degradation rates

Remaining litter dry mass in control and pesticide treated ponds did not differ significantly from each other \( (p > 0.30; \text{Fig. 2}) \). At the end of the exposure in the ponds (day 68) dry mass was around 70% of the initial litter material. As expected, the highest litter degradation rates were measured at the earlier exposure times up to day 54, whereas after day 54 litter degradation rates were significantly reduced.

2.2. Fungal community composition

Fungal communities colonizing A. glutinosa leaves showed a clear succession over the exposure time of the leaves in the pond water (Fig. 3). The underlying PCA explained 53% of the total variation in fungal t-RFLP profiles, for which ‘time’ is the dominating factor of principal components 1 and 2. Fungal communities at day \(-7\) clustered closely together in the PCA plots, whereas at later sampling time points the grouping was less tight, indicating also differences between replicates and high dynamics in fungi colonizing the litter material. These dynamics of fungal communities were comparable in control ponds and those from pesticide treated ponds.

A pronounced shift from the dominant t-RF ‘B213’ (48% relative abundance) to the dominant t-RF ‘B352’ (30% to 60% relative abundance) occurred from day \(-7\) to day 7, being mainly responsible for the observed community shifts between these two sampling time points (Fig. 4) both in litter samples obtained from control and treated ponds. Fungal communities colonizing the litter material at days 54 and 68 were more evenly distributed again independent from the treatment (highest relative abundance was for t-RF ‘B477’ which was in a range of 10% to 20%).

![Fig. 2 – Degradation of black alder leaf litter in the ponds \(-7\) days, 7 days, 54 days and 68 days after first pesticide application. Ponds 1, 3, 5, and 7 were treated with the described mixture of pesticides, ponds 2, 4, 6, and 8 acted as controls.](image)

![Fig. 3 – Multifactorial comparison of the fungal t-RFLP profiles using principle component analysis (PCA) for ponds treated with pesticides (p) and control ponds (c) \(-7\) days, 7 days, 54 days and 68 days after the first pesticide application. Percentage of total variance explained by principal components (PC) 1 and 2 is indicated in parentheses. Circles underline the clustering of the fungal t-RF fingerprints according to the time points; arrows indicate the dynamics of the fungal t-RF fingerprints over time. t-RFLP: terminal Restriction Fragment Length Polymorphism; t-RF: terminal restriction fragment.](image)
In addition to the clear shifts in the overall diversity pattern also strong shifts in richness (based on the total number of t-RFs per sample and sampling point expressed as the mean of all control and pesticide treated samples) were observed over time: from day $-7$ to day 7 a decrease in richness was observed, followed by an increase from day 7 to day 54 and stagnation from day 54 to day 68. These shifts were again independent from the application of the pesticides. Only for day 68 a tendency for higher fungal richness was observed for the litter material, which was obtained from the control ponds, however this difference was not significant due to the high standard deviation (Fig. 5).

3. Discussion

In this study, the dynamic in fungal communities colonizing plant litter was analyzed in an artificial pond system over time in the presence and absence of a number of pesticides typically used in apple farming by using a molecular DNA based approach to obtain fingerprints of fungal communities. The dynamics of fungal communities colonizing A. glutinosa leaves in our study responded well with the temporal succession pattern and were not significantly influenced by the application of the pesticides. The observed 30 to 40 t-RFs indicate a complex fungal community composition throughout the course of the experiment. During the first 2 weeks (comparing results of days $-7$ and 7) a shift in the t-RFLP profiles and the fungal richness was observed, which was independent from the application of the pesticides. The replacement of the dominant t-RF ‘B213’ by the dominant t-RF ‘B352’ was mainly responsible for this shift, most likely presenting the replacement of terrestrial by aquatic fungal communities. This goes in line with a study by Nikolcheva et al. (2005) where aquatic fungi started to replace terrestrial fungi on day 5 after immersion of leaf litter to water. In our study A. glutinosa leaves had been taken directly from the tree without reaching the soil to simulate direct litter input from the tree to the surface water and were not sterilized before immersion in order to maintain the starting conditions as natural as possible. From literature it is known that terrestrial fungi already colonize the buds of the leaves by aerial dispersal of the spores (Warren, 1976).

In general the temporal dynamics of fungal communities occurred naturally due to changes in the chemical constitution of the leaves in the course of litter degradation. From forest ecosystems it is known that soil fungal community composition changes due to the chemical constitution of the leaves. During the first 6 months primary saprophytic sugar fungi colonize the leaves, followed by late colonizers, which occur after 6 up to 12 months (partly up to 18 months) (Osono, 2005). Late colonizers are cellulose decomposers and associated secondary saprophytic sugar fungi, followed by lignin decomposers in the last stage of litter decomposition (Hudson, 1968). In the study at hand the litter was exposed to the water for a total of 10 weeks, since litter degradation in aquatic ecosystems proceeds faster than in soils (Gessner et al., 1993; Sridhar et al., 2001). Thus not surprisingly fungal communities became stagnant after 8 weeks (days 54 and 68) of litter exposure to the pond water in our study. This plateau may most likely be formed by fungi which act as k-strategists.
and induce the degradation of the remaining recalcitrant litter materials. The presence of mostly k-strategists at that sampling time point is also confirmed by the even distribution of t-RFs in the respective profiles (high evenness).

Overall, our data indicated no differences between controls and the low dosed multiple pesticide load treatments. A significant effect of the multiple pesticide loads could neither be seen regarding litter breakdown rates nor on diversity and richness of fungal communities colonizing the litter material. This is in line with results from a former crop-based approach study by Arts et al. (2006) for selected aquatic endpoints (leaf litter decomposition, macroinvertebrates, phytoplankton, zooplankton and macrophytes) after exposure to multiple pesticide applications from a potato crop protecting program. Van Wijngaarden et al. (2004) also found no significant effects on aquatic communities in another study investigating realistic pesticide exposures from the flower bulb crop protecting program. They concluded that sensitive populations are sufficiently protected by the first-tier RA procedure for the individual compounds.

In contrast to these publications, Artigas et al. (2012) could show that the fungicide tebuconazole revealed effects on both the fungal community structure and litter breakdown at concentrations of \((33.1 \pm 12.4) \mu g/L\). A study by Zubrod et al. (2015) confirmed effects of tebuconazole on communities of aquatic hyphomycetes, showing effects already at a concentration of \(1 \mu g/L\). This indicates an extraordinary low effective fungicide concentration. Moreover, the authors could show that the mixture toxicity of the products of the active substances azoxystrobin, carbendazim, cyprodinil, quinoxyfen and tebuconazole resulted in a significantly reduced number of fungal species already at the lowest tested mixture concentration of \(6 \mu g/L\). In fact, several studies have already found out that aquatic fungi sensitively respond to anthropogenic pollutants in rivers (Pascoal et al., 2005; Solé et al., 2008b).

However, there are indications that potential pesticide effects in this study were hidden or not detectable due to several reasons:

1. Heterogeneity caused by nutritional differences between the ponds, which may have resulted from earlier macrophyte development along with faster growth in ponds 1 and 2, could have masked potential effects of the low multiple pesticide loads on the fungal communities. These differences between ponds 1 and 2 were very obvious mainly at days 54 and 68, which should reflect the late phases of litter breakdown, where fungal communities are of utmost importance. That these differences may influence fungal community composition has been confirmed by Bärlöcher (1992) who could show that fungal species colonizing leaves in rivers vary due to differing water conditions. Similarly, Suberkropp (1998) found significant differences in sporulation of fungi and leaf weight losses for e.g. nitrate nitrogen concentrations between 0.14 and 1.4 mg/L for two tested species of aquatic hyphomycetes at a wide range of phosphorus concentrations (0.1 and 10 mg/L). Gessner et al. (2007) also pictured the positive correlation of dissolved nutrients and fungal activity, especially litter decomposition, in nutrient-limited aquatic environments.

2. The chosen nominal pesticide concentrations were intentionally low by using RAC since each single pesticide should not significantly harm the aquatic community but multiple low concentrations of pesticides may do. The actually measured pesticide concentrations in the pond water, 1 hr after application revealed that the nominal concentrations had not been reached for many substances, especially fungicides, in this study. The formulations of the active substances dithianon and dithianon showed very low recovery rates in the pond water (8.4%–32.4% for dithianon and <6.40% for dithianon). These products contain additives, which ensure high surface adhesion to the target plant. This, however, caused major problems in the application of the substances to the pond water, since there was considerable sorption at inner surface of the application device. Even though this difficulty was realized in pre-tests, the problem could not be solved completely. The fact that dithianon quickly hydrolyzed when added to the water and the hydrophobicity of methoxyfenozide (\(\log_{\text{oct/wat}} \geq 3.7\)) (Health Canada, 2008) resulted in recovery rates of only 2% for both substances in the pond water. Many of the applied pesticides also have fairly low \(DT50_{\text{water}}\) values (\(DT50_{\text{water}} \leq 1\) day: dithianon, captan, dodine, trifloxystrobin; \(DT50_{\text{water}} \geq 2\) days: benconazole, fenoxycarb; \(DT50_{\text{water}} \geq 8\) days: glyphosate, thiachlodorid; pesticide property data base: http://sitem.herts.ac.uk/aera/ppdb/en/index.htm). This can be the result of sorption of the pesticides to organic particles and their fast degradation after the application. Anyhow, similar processes may also happen in nature when the compounds are used in agriculture and enter aquatic ecosystems by run-off.

3. Fungal fingerprinting based on amplified DNA from the ITS region reflects the fungal response at the community level. For that reason, very slight effects of the low pesticide loads on individual fungi would most likely not be detected by means of this approach. Also changes at higher taxonomic levels might be hard or not detectable due to the well-known primer and PCR bias. Indeed, for soil fungal populations it has been demonstrated that the fungicide chlorothalonil had no significant effect on the overall community structure of dominant fungal species, while several dominant species may respond sensitively to the applied fungicide (Sigler and Turco, 2002). Therefore, despite the fact that the resolution of t-RFLP based methods and sequencing based approaches is comparable if amplicons are analyzed (Gschwendtner et al., 2014), a further analysis using high-throughput sequencing to validate the results presented here would be advisable in order to identify the taxa of the regarded fungal communities. The power of this approach has been proven recently in a study by Dimitrov et al. (2014) where the effects of the fungicide tebuconazole to heterotrophic microbes in an aquatic microcosm have been studied.

4. Conclusions and implications for the RA and the environment

From our results it cannot be concluded, whether the consideration of multiple low concentration pulses of pesticides in the
RA should be recommended. The fungal community patterns did not differ between in affected and control ponds, nor did the litter degradation. However, due to several reasons in the study at hand the pesticide concentrations were very low, in some cases even lower than the RAC. In field, the situation differs from this: Süß et al. (2006) showed that 17 out of 26 pesticides, which were analyzed in a monitoring study in the ‘Altes Land’ region with intensive apple cultivation in northern Germany temporarily, exceeded the precautionary levels for the protection of surface waters. In fact, field studies investigating streams in the neighborhood to agricultural sites or vineyards showed that the actual concentrations of the variety of pesticides may affect aquatic fungi and lead to reduced leaf litter decomposition (Rasmussen et al., 2012). Both field studies regarding the multiple pesticide stressors are comparable to the study at hand since fungicides were detected as the most frequent pesticide group. Thus, how to include the multiple low concentration loads of pesticides and/or fungi as key organisms for litter breakdown in ecosystems in the RA requires further investigation. Developing a standard would be an important next step. The molecular fingerprinting method was feasible to detect fungal communities in this study and might therefore be a useful cost effective fungal endpoint for the RA of pesticides.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2015.11.028.

REFERENCES


