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

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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.

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;

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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 HC5 concentration (hazardous concentration to $\leq$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 (Maltby et al., 2009) and thereby regulatory acceptable. However these SSD are based on LC_{50} 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.) Gaertn.) 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 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 m^3 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