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Aquatic degradation of Cry1Ab protein and decomposition dynamics of transgenic corn leaves under controlled conditions

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ABSTRACT

The increasing cultivation of genetically modified corn plants (*Zea mays*) during the last decades is suggested as a potential risk to the environment. One of these genetically modified variety expressed the insecticidal Cry1Ab protein originating from *Bacillus thuringiensis* (Bt), resulting in resistance against *Ostrinia nubilalis, the European corn borer*. Transgenic litter material is extensively studied regarding the decomposition in soils. However, only a few field studies analyzed the fate of the Cry1Ab protein and the impact of green and senescent leaf litter from corn on the decomposition rate and related ecosystem functions in aquatic environments. Consequently, a microbial litter decomposition experiment was conducted under controlled semi-natural conditions in batch culture using two maize varieties: one variety with Cry1Ab and another one with the appertaining Iso-line as control treatment. The results showed no significant differences between the treatment with Cry1Ab and the Iso-line regarding loss of total mass in dry weight of 43% for Iso-line and 45% for Bt-corn litter, lignin content increased to 137.5% (Iso-line) and 115.7% (Bt-corn), and phenol loss decreased by 53.6% (Iso-line), 62.2% (Bt-corn) during three weeks of the experiment. At the end of the experiment Cry1Ab protein was still detected with 6% of the initial concentration. A slightly but significant lower cellulose content was found for the Cry1Ab treatment compared to the Iso-line litter at the end of the experiment. The significant higher total protein (25%) and nitrogen (25%) content in Bt corn, most likely due to the additionally expression of the transgenic protein, may increase the microbial cellulose degradation and decrease microbial lignin degradation. In conclusion a relevant year by year input of protein and therefore nitrogen rich Bt corn litter into aquatic environments may affect the balanced nutrient turnover in aquatic ecosystems.

1. Introduction

Corn (*Zea mays* L.) is one of the most important plants for food and energy production in the world (Meise, 2003). About 158 million hectare of corn were cultivated worldwide in 2009 (James, 2009). An important pest, resulting in decreased yield, is the larvae of the European corn borer (*Ostrinia nubilalis*). As a consequence, a genetically modified corn has been cultivated to reduce the effects from the infestation of this pest. An important variety of transgenic corn often planted in agricultural systems includes the insecticidal Cry1Ab protein from *Bacillus thuringiensis* (Bt), resulting in a resistance increase against *O. nubilalis* (NASS, 2007). The cultivation area of these genetically modified corn plants increased during the last years to 9.2 million ha in 2009 (James, 2009). Litter of these transgenic plants is not completely removed by the farmers and it will subsequently be decomposed in soil. The litter decay of Bt-corn was extensively investigated for terrestrial ecosystems (Saxena and Stotzky, 2001; Flores et al., 2005; Tarkalson et al., 2008; Daudu et al., 2009; Zurbrügg et al., 2010). But only recently it was shown that pollen, leaves, and cobs of corn may enter the aquatic ecosystems, and will subsequently be transported and decomposed within streams, with a relevant share being accumulated downstream and still showing relevant concentration of Cry1Ab after six months of their entry (Rosi-Marshall et al., 2007; Griffiths et al., 2009; Tank et al., 2010).

The input of leaf and other plant litter is the most important energy input into the krenal and rhitral of streams as allochthonous ecosystems. During the primary decomposition made by microorganisms, both the organisms and their produced exudates

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form a heterotrophic biofilm (Kominkova et al., 2000). Although a lot is known about litter processing during aquatic decomposition, little is known about the effect of Cry1Ab-protein (Bt-corn) on the microbial decomposition of litter in aquatic ecosystems. Unfortunately, the results of studies that examined the effect of Bt-corn on litter decomposition are highly diverse and even contradictory (Rosi-Marshall et al., 2007; Griffiths et al., 2009; Swan et al., 2009). Several studies found a lower decomposition rate for plant parts of Bt-corn (Saxena and Stotzky, 2001; Flores et al., 2005; Swan et al., 2009), whereas other studies found that they were decomposing faster compared to its isoline or conventional non-Bt corn (Escher et al., 2000; Rosi-Marshall et al., 2007; Zwahlen et al., 2007; Griffiths et al., 2009). These studies were carried out in the field with probably quite varying experimental conditions due to factors like weather conditions, abundance of aquatic decomposers (zoobenthos, microorganisms), and pollutants. Hence, the data of the fate of Cry1Ab protein and impact on green/senescent litter decomposition of corn as well as related ecosystem function were partially contradictory.

This paucity of information prompted us to assess the effect of plant litter of genetically modified corn plants with Cry1Ab-protein on leaf litter decomposition rates in aquatic environments under controlled temperature and dissolved oxygen conditions, tested using laboratory batch experiments.

2. Material and methods

2.1. Plant material

Leaves of the transgenic corn with Cry1Ab-protein (Bt), (PAN 6Q-321B) and of its isoline (Iso), (PAN 6Q-121) were used in the experiments. The green and senescent leaves were harvested from the same plants at the same time after the fourth month. The harvested leaves were immediately shock frozen and stored at −20 °C until start of experiments to prevent the decomposition of the Cry1Ab-proteins.

2.2. Experimental setup

Leaf disks of green and senescent plant material in natural proportion of 70:30 (green/senescent) (Rossini et al., 2011) with a diameter of 2 cm, excluding the major vein, were used. Both green and senescent leaves were used because they occur at same time of harvest under field conditions, and their destination would be the streams. These leaf disks were put into 10 L polyethylene vessels which were filled with 5 L of stream water from the Federal Environment Agency of Germany in Berlin-Marienfelde (52°39′72.71″N, 13°36′64.05″E) (Table A1 lists physical and chemical parameters of the stream water), for more information see Böttger et al. (2012). The experiment was conducted in a climate chamber at 18 °C. Aeration was performed by air diffusers and an aeration pump at a rate of 0.5 L min^{-1} to stabilize dissolved oxygen concentration in the water. A 12 h/12 h light–dark-cycle was set with 3.1–3.4 lx for the light period to simulated natural light conditions. Four replicates of Bt-corn litter and Iso-line litter were used, which were sampled repeatedly due to limitations of leaf material availability. The duration of the experiment was 21 days.

2.3. Sampling, sample preparation and analysis

The sampling grid of the experiment was closer at the start to document the first and fast processes of the corn degradation like leaching of phenols and proteins. The samples were taken according to Table A2, immediately frozen and subsequently freeze dried. Afterwards the leaf disks were partitioned and analyzed. All samples except those for protein analysis were dried at 50 °C to a constant weight. Prior to measure the carbon and nitrogen contents, the samples were grounded and analyzed using a Elementar Vario EL III (Hanau, Germany) elemental analyzer according to DIN-ISO-10694 (1995).

The lignin and cellulose contents were measured using the method from Van Soest (1963) according to Gessner (2005) and phenols were analyzed according to Bärlocher and Graca (2005). In short, Lignin and cellulose measurements were done using 250 mg of ground litter. Sulfuric acid 0.5 M plus 20 g L^{-1} CTAB (hexadecyltrimethyl-ammonium bromide) were added. Then samples were incubated with occasional stirring in a boiling water bath (96 °C for one hour). After filtering and washing (using hot distilled water and acetone) the samples were dried at 105 °C. The samples were cooled to room temperature using a desiccator and weighted again. The cellulose content in the remaining fraction was hydrolyzed for 3 h by overlaying with 72% H_2SO_4 and mixing in it. In the next step the samples were washed using hot water free from acids and dried at 105 °C to constant weight. After cooling (see above) and weighing, the samples (in crucibles) were placed in a muffle furnace at 550 °C for five hours to remove all organic material (lignin). Phenol measurements were also done using 250 mg of ground litter. The samples were extracted using 12.5 mL of 70% acetone at 4 °C for one hour. After this the samples were centrifuged at 14,000 revolutions per minute for 15 min. 500 μL of the extract was diluted by a factor of two to 1 mL using pure water. To this 1 mL solution 5 mL of another solution (2% Na_2CO_3 dissolved in 0.1 mol NaOH) were added. After five minutes 0.5 mL Folin–Ciocalteu-reagent was added and mixed with the sample. The phenol content was measured after twenty minutes at 760 nm using a photometer (Specord 200, Carl-Zeiss, Jena, Germany).

Freeze dried plant material (freeze dried, to avoid degradation of Cry1Ab) was used for determining Cry1Ab protein concentration. An ELISA-test (enzyme-linked immunosorbent assays) was used to quantify the Bt-toxin concentration in the leaves (Institute of Integrative Biology, ETH Zürich, Switzerland). As described in Zwahlen et al. (2003), extraction buffer [10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, 3 mM NaN_3, 2% (v/v) polyvinylpyrrolidone (M_r= 25,000), 0.05% (v/v) Tween 20, pH 7.4] was used to extract the Bt protein from the leaf disk. Microtitre immunoassay plates Immunolon 4 (Dynatech Laboratories Inc.) were used to measure the optical density at 405 nm with a MRX microplate reader. To measure the total protein, dried ground leaves were treated according to Nguyen and Jehle (2009) with a HEPES-Buffer. Afterwards the Compat-Able™ Protein Assay Preparation Set and the BCA Protein Assay Kit from Pierce was used according to Smith et al. (1985).

2.4. Statistical analysis

Due to temporal pseudo-replication statistical analysis of the data of normal distribution was done using repeated measure ANOVA and Mann–Whitney *U*-test with SPSS version 16.

3. Results and discussion

3.1. Experimental performance

No differences between the Bt corn and Iso-line litter were found for mean temperature of 17.6 ± 0.6 °C, pH of 8.3 ± 0.1, and oxygen saturation of 100 ± 6.0% during the experiment. The conductivity increased during the 21 days from 619 to 717 μS cm^{-1} not differing between the Bt corn and Iso-line litter. This

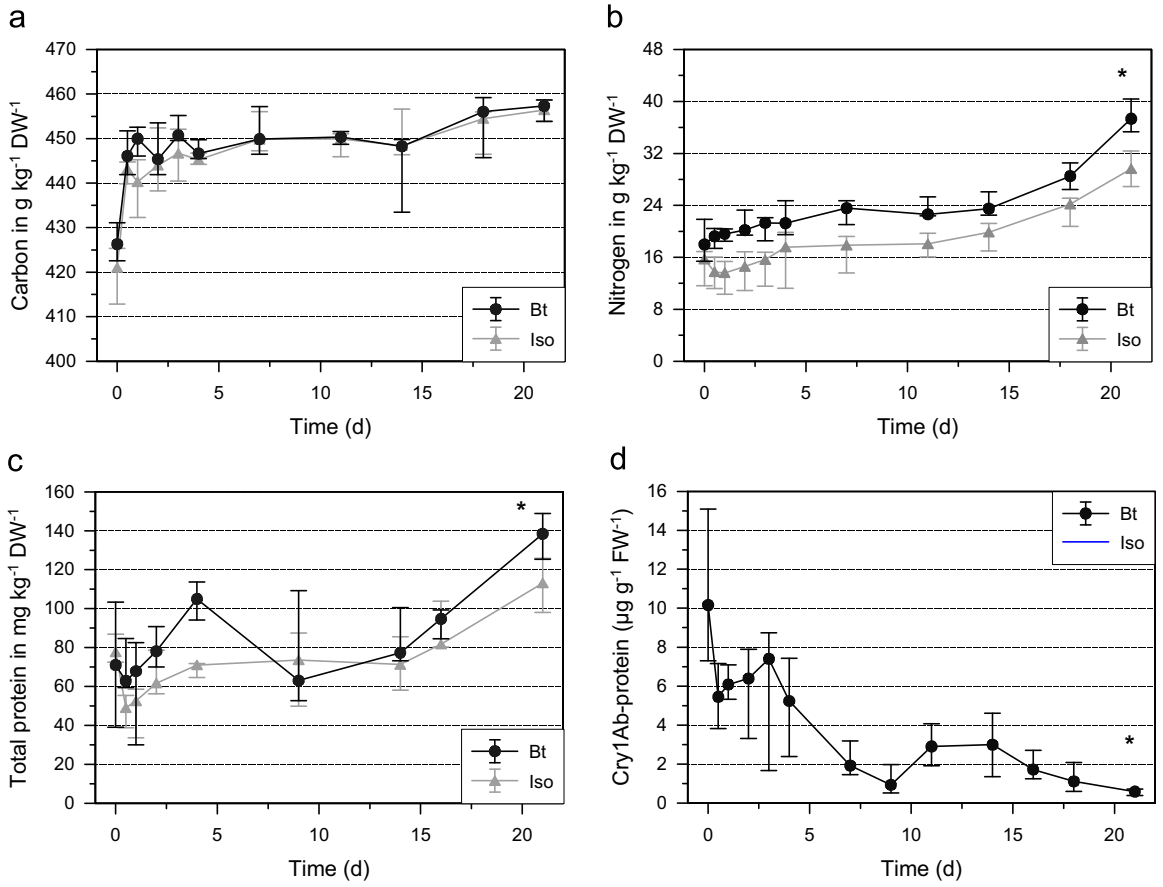

Fig. 1. Concentrations of carbon (a), nitrogen (b), total protein (c) and Cry1Ab-protein (d) in relation to dry weight of litter (DW) in water during the experiments in Bt- and Iso-treatment. The values are medians of four replicates and include minimum and maximum. Significant differences (*, ANOVA) were found for nitrogen, total protein, and Cry1Ab-protein (*, one factor ANOVA, with time as factor) during the experiment between Bt- and Iso-treatment. Cry1Ab-protein content for the Iso-line litter was always zero.

environment is comparable with field conditions in streams with proven Bt corn input (Griffiths et al., 2009; Swan et al., 2009).

3.2. Carbon, nitrogen and protein content during litter degradation

During the primary decomposition of litter, which is made by microorganisms colonizing plant tissues, both produced exudates and microbes form a heterotrophic biofilm (Kominkova et al., 2000). The carbon and nitrogen contents increased for both litter types, which may be explained by an increasing biomass of microorganisms colonizing decomposing plant litter (Geisseler et al., 2009). The carbon content in leaf litter was not different between Bt corn and Iso-line litter during decomposition (Fig. 1a), which is in accordance with studies in terrestrial ecosystems (Jung and Sheaffer, 2004; Mungai et al., 2005; Lehman et al., 2008; Poerschmann et al., 2009). However, the nitrogen content in the Bt-corn litter was significant higher than in the Iso-line litter during the experiment (Fig. 1b; $p < 0.001$, $F = 23.559$, ANOVA). At the end of the experiment a mean nitrogen content of 38 g kg^{-1} DW^{-1} in the Bt-corn litter and 30 g kg^{-1} DW^{-1} in Iso-line litter were found. Bruns and Abel (2003) identified a positive correlation between Bt δ-endotoxin content and total nitrogen due to specific Cry1Ab expression. Furthermore, they indicated a correlation between the nitrogen availability during the growth period and the Bt protein concentration in each plant. The significant increase of total nitrogen content in litter with biofilm is independent of the Cry1Ab concentration (see below) and was found in both litter types (Fig. 1b). Nitrogen is well known to accumulate during litter decomposition because of it is essentiality for the growth of microbes, as shown previously (Gessner, 2000). Therefore the nitrogen/carbon ratio narrowed in a range from 19.5–27.4 to 11.4–12.8 in Bt-corn and from 24.8–36.1 to 14.1–17 in the Iso-line as a result of increasing respiration of organic carbon and accumulation of nitrogen given by the decomposition.

The variation of protein content in both litter types may be due to a variation in protein expression in the plants, depending on e.g. leaf age and senescent (Abel and Adamczyk, 2004), but also by the biomass of the microbial decomposer community containing high amounts of proteins (Kjelleberg et al., 1987). The total protein content in residual litter biomass increased during litter degradation in both litter types (Fig. 1c). Dynamic processes of degradation of nitrogen containing compounds, especially readily degradable compounds like amino acids/peptides and the syntheses of proteins by microbes, may be the reason for the variance of the total protein content. The expression of the Cry1Ab protein is most likely not the direct reason for the significantly higher total protein content ($p < 0.05$, $F = 5.351$, ANOVA) in the Bt-corn litter (Fig. 1c) considering that Cry1Ab protein was in the range of microgram whereas total protein was in the range of milligram. However, the Cry1Ab protein my cause a shift in the microbes of the biofilm in the Bt-corn degradation and therefore in the syntheses of proteins my microbes. The observed increases of the Cry1Ab content during the first 3 days must be assigned to individual variability (Fig. 1d). Afterwards the Bt-protein content decreased significantly, but a small amount of Cry1Ab protein still remains after three weeks.

3.3. Cry1Ab concentration in leaf litter of corn in the course of submerse microbial degradation

The initial Cry1Ab-protein concentration of 10.2 (7.3–15.1) $\mu g\, g^{-1}\, DW^{-1}$ is comparable to data of other experiments (Then and Lorch, 2008), (Fig. 1d). During the experiment the Cry1Ab concentration decreased significantly by about 94% to 0.6 (0.4–0.7) $\mu g\, g^{-1}\, DW^{-1}$ ($p < 0.01$, ANOVA). This decrease of proteins is based largely on microbial degradation (Palm et al., 1996; Koskella and Stotzky, 1997). Variability due to individual plant growth conditions are common (Rossini et al., 2011) and may reflect near field conditions. Griffiths et al. (2009) investigated the fate of the Cry1Ab-protein in a stream field experiment. Their initial concentration of 5 $\mu g\, g^{-1}\, DW^{-1}$ was about halved in the first hour and remained at this level from 3 to 7 days, in waters of headwater streams with different nutrient concentrations (Griffiths et al., 2009). An equal result was found in our experiment, where 50% of the initial Cry1Ab concentration lasted until day 4 (Fig. 1d). However, in a field experiment a higher Cry1Ab-protein concentration remained longer (20% after 70 days), (Griffiths et al., 2009), whereas in our study after three weeks only 6% of the initial concentration was found. The differences in Cry1Ab degradation may be caused by different water conditions and a different heterotrophic community of microorganisms. In our study the constant oxygen saturation and the high temperature regime of 17.6 °C may result in higher decomposition rates through enhanced heterotrophic fungal and bacterial activity. Furthermore, nutrient availability for the microbes in our experiment was probably accelerated due to the absence of leaching as it might occur in running water systems. Other experiments, investigating the degradation of Cry1Ab-protein in soil, found a decrease from 21% to 60% within 20 days depending on the cultivar (Wandeler et al., 2002). Escher et al. (2000) showed a decline of Cry1Ab of about 25% within 8 weeks in soil. We confirm here a faster degradation of the Cry1Ab-protein in an aquatic environment compared to terrestrial ecosystems as reported by Douville et al. (2005). The variation of Bt-concentrations detected in the intervening period at day 11, 14, and 16 may be based on inhomogeneity of the leaf material. Generally, younger leaves contain more protein and chlorophyll per biomass than senescent leaves. In addition, the Cry1Ab concentration is correlated to chlorophyll concentration, age of the different leaves, and the longitudinal and diagonal dimension in leaf tissue (Abel and Adamczyk Jr., 2004; Székács et al., 2010a, 2010b). The different leaf ages (green, yellow and brown leave disks) used in our experiment represent natural field conditions and may cause the small inhomogenity of Cry1Ab content in the used leaf material.

3.4. Decay of structural and phenolic leaf carbon compounds in Bt-corn litter and Iso-line litter

The genetic modification had no significance influence on the leave degradation (DW based assay) and decay of related leaf structural compounds compared with Iso-line litter, with the exception of a small influence on cellulose content at day 18 (Figs. 1 and 2; $p < 0.05$, Mann–Whitney U-test). Our results showed a mass loss in dry weight of 43% for Iso-line litter and 45% for Bt-corn litter within 3 weeks (Fig. 2d). The total protein content significantly increased during the decomposition. These results contrast findings of Griffiths et al. (2009) with a significant

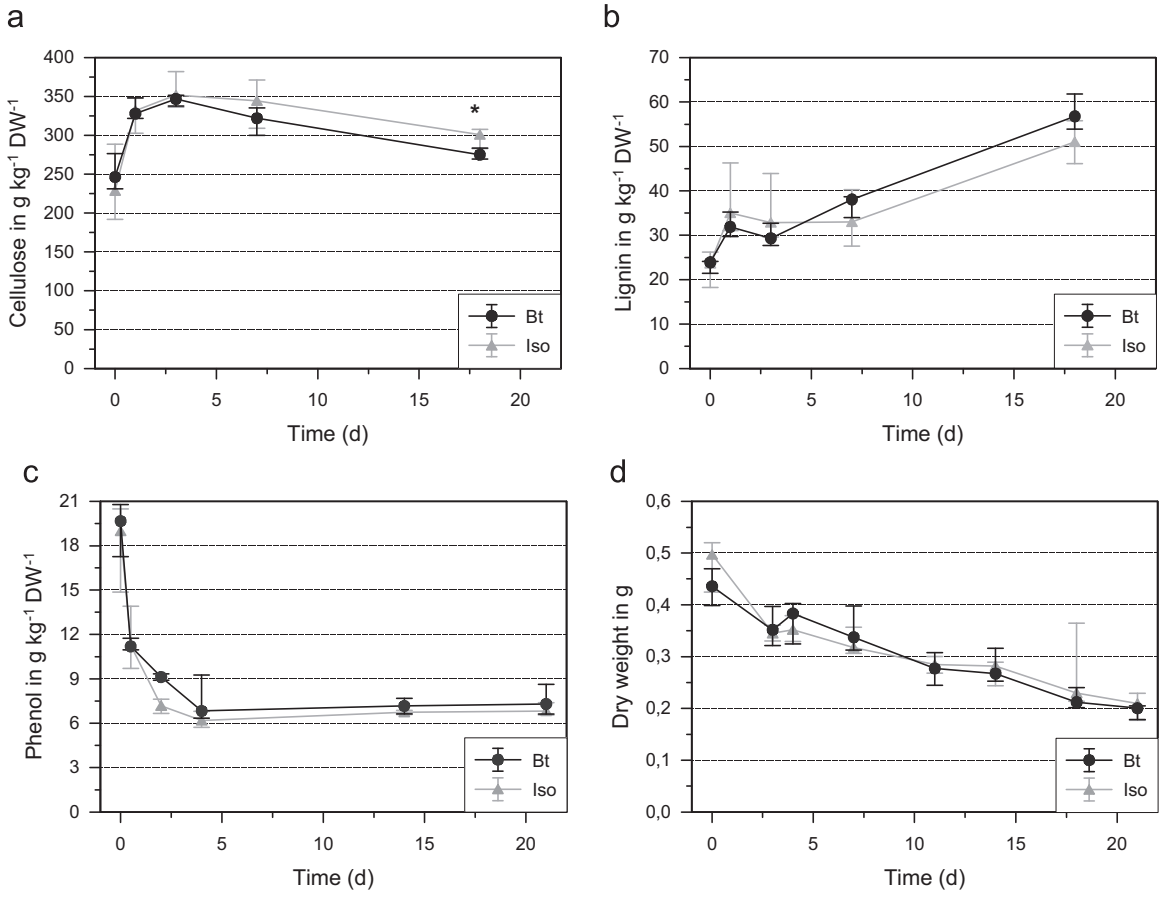

Fig. 2. Cellulose (a), lignin (b), and phenol (c) contents in relation to dry weight litter (DW) (d) in water during the experiments in Bt- and Iso-corn leaves. The values are medians with minimum and maximum ($n=4$). Significant differences ($p < 0.05$, Mann–Whitney U-test, * found at day 18).

differences in decomposition between Bt-corn litter- and Iso-line. A significant difference was found at the end of the experiment for cellulose content between the Bt-corn litter and the Iso-line litter, whereas for lignin and phenol contents no significant differences were found during the experiment (Fig. 2a–c; $p=0.08$ for lignin and $p=0.25$ for phenol, Mann–Whitney *U*-test). The cellulose content in the Bt-corn litter increased from 246 g kg^{-1} DW^{-1} to about 346 g kg^{-1} DW^{-1} (day 3) and decreased to about 276 g kg^{-1} DW^{-1} (day 18) for the remaining time of the experiment (Fig. 2a). Equally the cellulose content of the Iso-line increased from 229 g kg^{-1} DW^{-1} (start of experiment) to about 355 g kg^{-1} DW^{-1} in the first three days, followed by a decrease to about 298 g kg^{-1} DW^{-1} after 18 days. The cellulose content of the Bt-corn litter after 18 days was significantly lower with 275 g kg^{-1} DW^{-1} compared to the Iso-line litter with 300 g kg^{-1} DW^{-1} ($p<0.05$, $p<0.05$, Mann–Whitney *U*-test). Comparing the results to data of other experiments, no clear conclusion can be made. Daudu et al. (2009) found higher cellulose content in genetically modified corn, whereas Zurbrügg et al. (2010) reported no differences between Bt-corn litter and Iso-line litter. Both litter types had an initial lignin content of 24 g kg^{-1} DW^{-1}, which increased to 137% in Bt-corn litter and to 116% in Iso-line litter during the experiment with no significance differences ($p<0.05$, Mann–Whitney *U*-test; Fig. 2b). These findings are confirmed by several studies (Jung and Sheaffer, 2004; Lehman et al., 2008; Zurbrügg et al., 2010). A higher lignin content in genetic modified corn reported by Saxena and Stotzky (2001), and Flores et al. (2005) may be due to the use of other corn tissues. In addition the natural variability of the tissue should be considered as a factor altering the content (Icoz and Stotzky, 2008). The higher nitrogen content in the Bt-corn litter compared with the Iso-line litter may also be responsible for the small differences in the cellulose and lignin content in the course of decomposition as described previously. Furthermore, the higher nitrogen content in our experiment may negatively affect the lignin degradation and increase the cellulose degradation via microorganisms (Carreiro et al., 2000).

There were no significant differences in the phenol concentration and degradation between the Bt-corn litter and Iso-line litter (Fig. 2c). The phenol content decreased from start to the end of experiment as described previously by Abelho (2001).

4. Conclusions

The input of genetically modified plant material in aquatic ecosystems may change the energy and element cycles as a consequence of the interaction with genetically modified proteins and the altered amount of available proteins as discussed above. Predominantly, intensive agriculture with genetically modified plants may result in higher organic nitrogen and total N input, which in turn may result in changed conditions for decomposition in aquatic ecosystems. The additional mass input may also result in higher biomass accumulation in the aquatic food web (secondary producer community), which in turn may change the habitat/microhabitat conditions like the oxygen concentration. Such potential variation on dissolved oxygen concentration probably influence the organisms involved in the degradation process (Abdel-Raheem, 1997; Abelho, 2001). Cry1Ab-protein had no effect on litter mass loss and both lignin and phenol contents of corn leaves under the tested experimental conditions. But it affected the nitrogen and total protein contents of leaf litter during decomposition process and therefore may affect the carbon turnover and nutrient spiraling in freshwater ecosystems in the long-term. Furthermore, a slightly decreased cellulose content of leaves during aquatic decomposition was found. Based on our finding the input, effect, and path of transgenic corn in the aquatic ecosystem, especially the fate of Cry1Ab and the impact on litter decomposition should be studied in more detail in future experiments to gain more knowledge for risk assessment.

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Appendix

See appendix Tables A1 and A2.

Table A1
Physical and chemical parameters of stream water.

Parameter	Unit	Value
pH	-/-	8.2
NH_4–N	mg/L	0.0
NO_2–N	mg/L	0.0
NO_3–N	mg/L	0.7
PO_4–P	mg/L	0.0
Alkalinity	mmol/L	2.1
Cl^-	mg/L	41.9
SO_4^{2-}	mg/L	94.0
Na^+	mg/L	41.1
K^+	mg/L	2.4
Ca^{2+}	mg/L	58.7
Mg^{2+}	mg/L	7.3

Table A2
Sampling grid for the different analysis. Two to three leaf disks for Cry1Ab were taken separately and freeze dried. 50 Leaf disks for the other analyzes were taken at once and partitioned after freeze drying.

Time (day)	0	0.5	1	2	3	4	7	9	11	14	16	18	21
Cry1Ab-protein	x	x	x	x	x	x	x	x	x	x	x	x	x
Total protein	x	x	x	x		x		x		x	x		x
Lignin	x		x		x		x					x	
Cellulose	x		x		x		x					x	
Phenol	x	x		x	x					x			x
Nitrogen	x	x	x	x	x	x	x		x	x		x	x
Carbon	x	x	x	x	x	x	x		x	x		x	x
Dry weight	x				x	x	x		x	x		x	x

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