Research Article

Edita Juknevičienė, Honorata Danilčenko, Elvyra Jarienė, Jürgen Fritz*

The effect of horn-manure preparation on enzymes activity and nutrient contents in soil as well as great pumpkin yield

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Abstract: This investigation was inspired by an increasing global issue on how to improve soil quality while using alternative preparations instead of synthetic fertilizers. The main aim of a three-year study was to investigate the influence of horn-manure preparation on enzyme activity and nutrient content in soil and pumpkin yield. The results showed that significantly higher amounts of P (respectively 106 and 79 mg kg^{-1} CAL), K (149 and 106 mg kg^{-1} CAL), nitrogen (5.41 and 3.21 mg kg^{-1}), ammoniacal nitrogen (9.38 and 3.45 mg kg^{-1}) and mineral nitrogen (7.97 and 5.67 mg kg^{-1}) were measured in the plots where the horn-manure preparation was used. A higher activity of the soil enzymes (urease activity was 1.93 times higher and the saccharase activity was 1.05 times higher) were identified with horn-manure. The average soil CO_2 flux (F_c) value, when using horn-manure preparation (from 56 till 70 day), was significantly higher by 5.32% in the middle of the growing season. The yield of pumpkin was significantly increased by 18% with horn manure treatments. Significant positive correlations were identified between pumpkin yield and urease activity, and saccharase activity, as well as soil P and K.

Keywords: Chlorophyll index; Horn-manure preparation; Pumpkins; Soil enzymes

1 Introduction

The most important aim of agriculture is to maintain soil fertility in order to guarantee food security for the growth of the human population, globally (FAO 2009). An increasing number of studies show that organic farming leads to higher soil quality and more biological activity in soil in comparison with conventional farming (Zaller and Köpke 2004; Brock et al. 2013; Heinze et al. 2010).

Biodynamic agriculture is the oldest form of organic farming with a history of more than 90 years (Sedlmayr et al. 2014). In 2013, the number of Demeter-certified farms increased to 4,800 with a total area of 153,246 ha. (Lüthi 2014). A horn-manure preparation, which is a spray preparation for the soil, is made from cow manure in a special preparation process. In analyses by Giannattasio et al. (2013), the microbial population of horn manure preparations was: 2.38 x 10^8 aerobic colony-forming units in each gram (dry weight), 7.85 x 10^7 anaerobic colony forming units and 1.2 x 10^6 fungal colony units. These values vary, however, because most of the preparations are made individually on farms.

According to Turinek et al. (2009), the horn manure preparation enhances the biological activity of the soil and improves root growth, although it is used in very small quantities. The primary purpose of all biodynamic preparations is not to add nutrients, but to stimulate the processes of nutrient and energy metabolism and improve soil and crop quality (Demeter e. V. 2013). In experiments by Jariene et al. (2015), higher antioxidant contents were determined by the application of biodynamic preparations in potatoes.

Since the 1970s, universities have been investigating the effects of biodynamic preparations. In most of the published experiments the treatments were carried out with all biodynamic preparations. Studies of Reganold et al. (1993), Goldstein (1990), Garcia et al. (1989) have shown that the topsoil of the biodynamically treated plots generally was higher in organic matter, microbial activity, enzyme activity (dehydrogenase and urease), earthworm channels, total N, and pH in comparison with the topsoil of chemically fertilized plots. In a four-year plot experiment the biodynamic plots were significantly higher in

***Corresponding author: Jürgen Fritz,** University of Kassel, Department of Organic Farming and Cropping Systems, Germany, E-mail: j.fritz@uni-bonn.de
Edita Juknevičienė, Honorata Danilčenko, Elvyra Jarienė, Vytautas Magnus University Agriculture Academy, Lithuania

soil enzyme activity and microbial biomass in comparison with organic plots (Koepf 1993).

Soil activity, measured by soil enzymes and soil respiration, is closely related to physical and chemical soil properties, soil type and fertilisation (Monokrousos et al. 2006). Soil respiration largely depends on soil temperature, moisture, and seasonal changes. Rates of soil CO_2 flux vary with vegetation (Raich and Tufekciogul 2000). Mäder et al. (2002) describe the soil activity of a comparative trial of systems in Switzerland over many years. The activity of dehydrogenase, protease, phosphatase, saccharase was higher in the biodynamic variant in comparison with the organic variant. With the Shannon index, which describes the functional variety of the soil microbiology, the biodynamic variant with the lowest quotients of soil respiration per microbiological biomass also had the highest energy efficiency.

There is very little knowledge, presented in publications, of the influence of horn-manure preparation on the yield of different plants. A four-year experiment, in the past, has demonstrated unambiguous results (Spiess 1978). A significantly higher wheat yield was determined after spraying a horn-manure preparation four times on the soil. The carrot yield of a three-year cultivation did not differ significantly in the variant with horn-manure preparation compared with the variant without it. The yield of sugar beet and sugar beet leaf was significantly increased by horn-manure preparation treatments (Spiess 1978). Raupp and König (1996) reported that biodynamic preparations caused opposite yield effects depending upon yield levels. The preparations tended to increase the yields, which were generally low. When the yields reached a medium level, this positive effect was smaller. At higher yield levels preparations tended to lower yields. In carrot studies over two years, variants with horn-manure preparation led to significantly lower yields (Fleck et al. 2005). This effect of the horn-manure preparation at a high carrot yield level was interpreted as a balancing effect.

The application of the two biodynamic spray preparations significantly increased the seed yield of cumin (*Cuminum cyminum* L.) in both fertilizer variants (Sharma et al. 2012). The use of horn-manure preparation alone led to a significant increase in yield (+ 24%) in only one of the two fertiliser variants. In the case of Bacchus et al. (2010), the yield differences in three fertilization variants were not significant in the application of the biodynamic preparations to lettuce (*Lactuca sativa* L.). The germination properties of the newly formed seeds of bush beans were higher in three experimental years with the biodynamic horn silica preparation than in the control (Fritz and Köpke 2005). At a very low yield level, the yield of two soybean varieties in Vietnam was significantly increased, by more than 30% each, with the application of biodynamic spray preparations (Tung and Fernandes 2007). Both variants were not fertilized. Also, with nonfertilized variants at very low yield levels, the application of biodynamic spray preparations to two rice varieties led to a significant yield increase of 10% and 15%, respectively, in the Philippines (Valez and Fernandes 2008).

Cucurbita maxima is one of the most economically important species cultivated worldwide for human consumption. The Lithuanian climate is suitable for growing great pumpkins (Danilčenko et al. 2014). These pumpkins contain large amounts of fibre, free sugars, vitamins such as B_1, B_2, and C, as well as active ingredients, including carotenoids and phenols, all of which provide the fruit with various health-promoting functions (Nara et al. 2009). Pumpkins produce one of the highest yields in comparison with other vegetables and they are appreciated for their simple production technology. For effective photosynthesis in pumpkin plants a specific ratio and content of chlorophylls is necessary. Leaf chlorophyll content is described as a good indicator of photosynthesis activity, mutations, stress and nutritional state (Wu et al. 2008). The amount of chlorophyll per unit leaf area is related to the overall condition of the plant.

If we broaden the view then a basic statement of organic farming is that a healthy soil leads to better, healthy plant growth and this leads to a healthy diet. This leads to the question in biodynamic agriculture: Can biodynamic preparations make a positive contribution to this basic statement of organic farming? This leads to the hypothesis in the present paper: Horn-manure preparation treatment increases the soil activity and plant growth of great pumpkin. (Further studies on the effects of biodynamic preparations on the food quality of great pumpkin will be presented in a follow up article.)

2 Materials and methods

The investigations were carried out over the period of 2012–2014 in a Kaunas district organic farm. In the experimental field, three great pumpkin cultivars 'Justynka', 'Karowita' and 'Amazonka' were cultivated. These cultivars of *Cucurbita maxima* species have a bushy growth habit. Pumpkins were sown in plastic cups in May (2–3 seeds were put into one hole of 2–4 cm depth) and put in the greenhouse. The plants were placed in the field at the end of May. The field replications were arranged as a block system. The total area of one plot was 12 m^2, the width of the edge strip was 0.5 m, the area of the core plot was 6 m^2. There were 6 plants in each core plot.

The experiment was carried out in four replications. Pumpkins were harvested in the first decade of September. Pumpkin total yield ($t \cdot ha^{-1}$), marketable yield ($t \cdot ha^{-1}$) and average weight of marketable fruit (kg), with and without horn-manure preparation treatment, were measured. Healthy, undamaged and mature fruits were considered to be of marketable quality. The average weight of marketable fruit (kg) was obtained by dividing the total yield by the number of fruits.

The horn manure preparation for the study comes from a Demeter farm in Germany that specialises in the production of biodynamic preparations (CvW KG, Internationale Biodynamische Präparatezentrale, Künzelsau). Manure from several cows was collected and placed in cow horns, which were then buried in the soil during the winter and unearthed in spring. Horn-manure preparation is the 'humus mixture' that resulted from this fermentation. The soil was sprayed with 1% concentration solution (200 l solution/ha, 200 g horn-manure preparation for 1 ha) two weeks before planting pumpkin shoots. The solution was stirred 1 h before spraying. The soil was sprayed using fine sprays, in the afternoon. The control variant was sprayed with water. The horn-manure preparation used in the experiment was weakly acidic (pH_{KCl} 6.96), very high in phosphorus (1960 mg kg^{-1} total amount in dry matter), potassium (259 mg kg^{-1} total amount in dry matter), nitrogen (2.10% total amount in dry matter) and also high in enzyme activity (urease activity 1.56 mg NH_3 g^{-1} soil 24 h^{-1}; saccharase activity 32.7 mg glucose g^{-1} soil 48 h^{-1}). The soil of the experimental location (*Hapli-Epihypogleyic Luvisol*) was weakly acidic (6.81 pH), of high humus content (2.4%), limnoglace clay loam on the boulder clay, carbonate, deeply gleyic luvisol. The soil had average available nitrogen (0.29 %) and was high in available phosphorus (173 mg kg^{-1}) and available potassium (209 mg kg^{-1}).

Three replicated soil samples were randomly taken over the whole plot – 7 days, 14 days, 65 days and 130 days after spraying horn-manure preparation. Samples taken at 65 days after the horn manure treatment were not analysed for enzymes. The soil properties, related to the sample depth of 20 cm, are based on three replicates, which were composite samples (in each plot soil was taken in ten places; four plots = one composite sample per variety; three varieties = three composite samples per treatment). The following soil properties were identified in the air dried soil: pH_{KCl} measured using the potentiometric method; available P (mg kg^{-1}) and available K (mg kg^{-1}) concentration using the CAL method; nitrogen (sum nitrate nitrogen plus nitrous nitrogen) (mg kg^{-1}) and ammoniacal nitrogen concentration (mg kg^{-1}) using the flow analysis (FIA) spectrometric method followed by inductively coupled plasma mass spectrometer (ICP-MS, Thermo Finnigan MAT, Bremen, Germany); mineral nitrogen concentration (mg kg^{-1}) was calculated as a nitrogen (nitrate nitrogen plus nitrous nitrogen – NO_3 + NO_2) and ammoniacal nitrogen amount. The activity of soil urease and soil saccharase was determined spectrometrically (Schinner et al. 1991).

The CO_2 flux value and the soil temperature were measured at a depth of 5 cm. The measurements were taken with the portable photosynthesis system (LI-6400 XT). A mean value was calculated from 16 measurements in each plot. The measurements were performed during the pumpkins vegetative growth period every two weeks. Correlations between some of the above mentioned indicators were identified.

Chlorophyll index in pumpkin leaves (third leaves from the top of the plant) was established with a handheld chlorophyll meter CCM-200 (Opti-Sciences, Tyngsboro, Massachusetts, USA). The CCM-200 has a 0.71 cm^2 measurement area, and calculates a chlorophyll content index (CCI) based on absorbance measurements at 660 and 940 nm. The measurements were taken six times every two weeks. The average (n = 30) chlorophyll index of the three investigated cultivars was calculated, because the tendency was similar.

The statistical evaluation of the experimental data was done with Systat 10 (Systat 10, Statistics I, SPSS Inc., Chicago, IL, USA). The means were compared using the least significant difference test. For the evaluation of the data, the years were assumed to be "random". Interactions between the treatment of horn manure and the years have not developed.

3 Results and discussion

The three-year study shows that quantities of phosphorus and potassium were significantly higher during the whole vegetative growth period with the horn-manure preparation (HMP), which was compared with the water treatment (Table 1), except for potassium after 14 days. The amounts of these substances after 7 days were highest and then decreased until the end of the vegetation. Nitrogen (nitrates + nitrites), ammonia nitrogen and total mineral nitrogen was significantly higher at all dates with the horn manure treatment, with the exception of ammonia nitrogen after 130 days. The level of pH was significantly lower

Table 1: Soil data during great pumpkin vegetative period

Indicator	Terms after spray							
	7 days		14 days		65 days		130 days	
	water	HMP	water	HMP	water	HMP	water	HMP
P (mg·kg^{-1})	153	174**	152	159**	83	110**	79	106**
K (mg·kg^{-1})	238	251**	237	225**	125	178**	106	149**
Nitrogen (mg·kg^{-1}) (nitrate + nitrite)	25.95	24,85**	26,02	29.27**	3.90	5.99**	3.21	5.41**
Ammonia nitrogen (mg·kg^{-1})	4.55	4.96**	4.60	5.39**	3.45	9.38**	2.46	2.56
Mineral nitrogen (mg·kg^{-1})	30.50	29.81*	30.72	34.66**	7.35	15.37**	5.67	7.97**
pH	6.81	6.68**	6.78	6.64*	6.90	6.82	6.66	6.52**

Note: spray treatments: water – sprayed with water, HMP – sprayed with horn manure. Differences between the means of treatments marked by one asterisk are significant, p ≤ 0.05; two asterisks p ≤ 0.01

Table 2: The influence of spraying with horn-manure preparation on the activity of soil enzymes

Enzyme	Terms after spray					
	7 days		14 days		130 days	
	water	HMP	water	HMP	water	HMP
Urease activity (mg NH_3 g^{-1} soil 24 h^{-1})	0.45	0.62**	0.46	0.52*	0.28	0.54**
Saccharase activity (mg glucose g^{-1} soil 48 h^{-1})	33.79	35.64**	33.82	35.64**	33.22	35.00**

Note: spray treatments: water – sprayed with water, HMP – sprayed with horn manure. Differences between the means of treatments marked by one asterisk are significant, p ≤ 0.05; two asterisks p ≤ 0.01

where the biodynamic preparation was used. Differences in treatment with the horn manure treatment were therefore already apparent after 7 days.

Soil enzyme activity is one of the main indicators of biological activity and soil fertility. Enzyme activity is closely related to other important indicators of biological activity: respiration intensity, nitrification ability, total amount of microorganisms and even more associated with soil humus content, amounts of mobile P and K, soil acidity and crop yield (Karlen et al. 2001; Peregrina et al. 2014). Our findings show that the horn-manure preparation (P 500) significantly increased soil enzymes activity. The activity of urease and saccharase, after 7 days, was significantly higher in the variant sprayed with the fermented manure preparation (with preparation up to 37.78% and up to 5.33% accordingly) (Table 2). At the end of the pumpkins vegetation, urease activity was 1.93 times higher and the saccharase activity was 1.05 times higher, compared with the water sprayed plots.

Significant changes, 7 days after spraying horn-manure, were analysed. It could be based on some studies that soil enzymes may respond to changes in soil management more quickly than other soil variables and therefore might be useful as early indicators of biological changes (Trasar-Cepeda et al. 2000). Jin et al. (2009) report a key role of urease in nitrogen (N) cycling in soils, which explains the higher difference during the same period compared with saccharase.

Soil CO_2 production is the sum of the respiration from free-living microbes and plant roots and it is strongly dependent on the temperature, soil moisture, soil organic content, and growth activity of plants (Xu et al. 2006). The soil CO_2 flux (F_c) ranged from 2.10 to 5.90 μmol m^{-2} s^{-1} during pumpkin vegetation (Figure 1). The average F_c value, with the horn-manure preparation (P 500), in the middle of the growing season (from 56 till 70 day) was significantly higher, by 5.32%. The soil CO_2 flux shows a strong diurnal pattern and closely follows the soil temperature variations; this is because microbial respiration increases exponentially with temperature (Raich et al. 2002). The horn-manure preparation had a significant effect on the soil temperature (T_{soil}) from the 56th till the 70th vegetation day – the average T_{soil} value was higher by 0.56°C.

Chlorophyll index is an important indicator of photosynthesis as it is directly proportional to chlorophyll content in leaves of higher plants (Ciganda et al. 2009).

The average chlorophyll index value, with the fermented manure preparation after 70 and 84 days spraying, was significantly higher (in average 70 days after spraying with horn-manure preparation – 3.75 chlorophyll units more; 84 days after spraying – 1.80 chlorophyll units more compared with unsprayed plots) (Figure 2).

Justynka cv. produced more and bigger fruits than Karowita and Amazonka cv., which resulted in significantly higher total and marketable yield (Table 3). The yield of pumpkin was significantly increased by 18% on average of the three varieties with horn manure treatments. The horn-manure preparation had no influence on average weight of pumpkin fruit.

Strong significant correlations between pumpkin yield and available phosphorus ($r = 0.948$, $p < 0.01$), and between pumpkin yield and available potassium ($r = 0.910$, $p < 0.01$) were evident (Table 4). Positive strong significant relationships were established between urease activity and pumpkin yield ($r = 0.871$, $p < 0.01$), and between saccharase activity and pumpkin yield ($r = 0.954$, $p < 0.05$). Available phosphorus, available potassium and nitrogen were closely correlated. No significant correlation between pumpkin yield and soil CO_2 flux was determined.

Our results of the three year experiment show that the variant sprayed with the horn-manure preparation had significant higher values of P, K, mineral nitrogen, urease activity, saccharase activity, soil CO_2, soil temperature, chlorophyll index in great pumpkin leaves and higher pumpkin yield. Higher values of urease activity, saccharase activity, soil CO_2 and soil temperature with the horn-manure preparation shows that the horn-manure preparation stimulated biological soil activity. According to the experimental results, significant changes were identified within 7 days after spraying the horn-manure preparation. The horn-manure preparation probably works through the regulation of soil bacteria, which can be explained by bacteria identification and reaction to extremely low levels of signal molecules in their environment (Tejada et al. 2011). Pumpkin yields that we received in the project are normal yields for organic farms in Lithuania. The reason for the higher pumpkin yield with the horn-manure preparation application was presumably due to the increase of soil activity with the horn-manure preparation. Our test results thus confirm the working hypothesis that the horn-manure preparation treatment increases the enzyme activity of the soil and thus also increases the chlorophyll content and the yield of great pumpkin.

A basic question is how a substance can have an effect in such a small amount of application as horn-manure. According to our experimental results, the significant changes were identified within 7 days after spraying the horn-manure preparation. Possible explanations for an effect of horn manure could be plant hormones or bioactive substances or through bacterial regulatory effects.

One hypothesis is that biodynamic preparations work through hormonal effects. Radha and Rao (2014) analysed the composition of the microbial community in horn-manure preparations. They found that all strains of bacteria, which were analysed, produced indoleacetic acid. This result is supported by the study by Giannatasio et al. (2013). They found that horn manure showed strong auxin-like effects. Spaccini et al. (2012) found large amounts of ungraded lignin residues in horn-manure. It is known that they show IAA-like activity and can explain biostimulations against microbes and plants.

A second possibility of the mode of action of the biodynamic preparation is the stimulation of natural defence substances. While studying biodynamically (using all preparations) compared with organically cultivated vines, Botelho et al. (2016) showed increased activities of several enzymes, which typically correlate with biotic and abiotic stress and are associated with induced plant resistance. These results are in accordance with Soustre-Gacougnolle

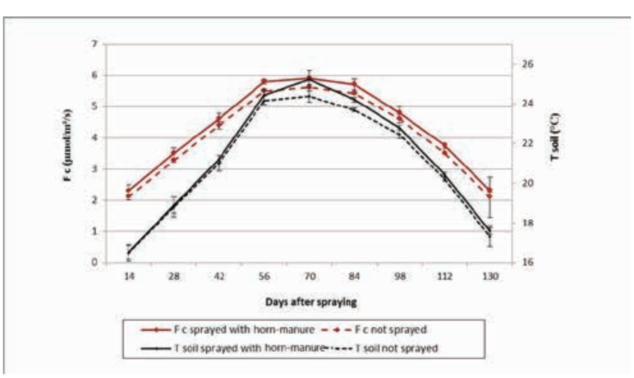

Figure 1: Soil CO_2 flux (F_c) and soil temperature (T_{soil}) as affected by spraying with horn-manure preparation

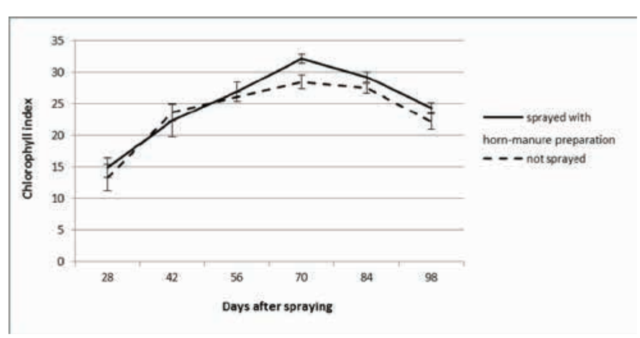

Figure 2: The effect of spraying with horn-manure preparation on chlorophyll index in great pumpkin leaves

Table 3: The effect of spraying with horn-manure preparation on great pumpkin yield

Cultivation variant	Total yield ($t \cdot ha^{-1}$)	Marketable yield ($t \cdot ha^{-1}$)	Average weight of marketable fruit (kg)
Justynka			
Control	46.00	40.25	2.30
Horn-manure	58.25	52.21	2.33
Karowita			
Control	34.05	31.95	2.13
Horn-manure	36.20	33.00	2.20
Amazonka			
Control	23.55	20.15	1.57
Horn-manure	27.63	24.20	1.70
LSD_{05}: A cultivar; B variant; interaction AxB			
A	1.29	2.83	0.16
B	1.05	2.31	ns
AxB	1.82	4.00	ns

Table 4: Correlation between pumpkin yield and soil properties

	P	K	N	Pumpkin yield	Urease activity	Saccharase activity	Soil CO_2
P	1.000	0.983**	0.947**	0.948**	ns	ns	ns
K	0.983**	1.000	0.920**	0.910**	ns	ns	ns
N	0.947**	0.920**	1.000	ns	ns	ns	ns
Pumpkin yield	0.948**	0.910**	ns	1.000	0.871**	0.954**	ns
Urease activity	ns	ns	ns	0.871**	1.000	ns	ns
Saccharase activity	ns	ns	ns	0.954**	ns	1.000	ns
Soil CO_2	ns	ns	ns	ns	ns	ns	1.000

** – $p < 0.01$, ns – not significant

et al. (2018). They found that biodynamic cultivation, using all preparations, leads to a high expression of silencing and immunity genes, and higher anti-oxidative and antifugal secondary metabolite levels, compared with conventional agriculture.

A third possibility is that horn-manure may act through bacterial regulatory effects, as bacteria recognize and react to extremely low signalling molecules in their environment that may contain the biodynamic preparations (Tejada et al. 2011; Reeve et al. 2010). According to Giannatasio et al. (2013), the concentration in which the horn manure preparation is used is sufficient to induce biological activities in soil bacteria or plants. The high content of carbohydrates and peptides, resulting from the microbially mediated slow maturation under oxygen-poor conditions during production of the preparation, may promote greater rhizospheric activity (Spaccini et al. 2012). It is possible that bacterial cultures and bioactive substances in horn-manure preparations, among others, are responsible for the increase in soil activity after treatments with the horn-manure preparation. These results are in accordance with the results of the long-term field trial by Mäder et al. (2002). The activity of dehydrogenase, protease, phosphatase, saccharase was higher in the biodynamic variant in comparison to the organic variant. As in many experiments, however, not only the horn-manure preparation but all biodynamic preparations were used (Birkhofer et al. 2008; Joergensen et al. 2010; Sradnick et al. 2013; Laghi et al. 2014; Fritz et al. 2017). More studies on the horn-manure preparation are necessary with different experimental approaches in field, pot and laboratory experiments to better understand the mode of action of the preparation.

4 Conclusion

Finally, we look again at the basic statement of organic farming that a healthy soil leads to better, healthy plant growth and this leads to a healthy diet and the question of whether biodynamic preparations can make a positive contribution to this basic statement of organic farming? The results of the present study showed that the horn-manure preparation increased the soil activity and plant growth of great pumpkin. Further studies about the effects of biodynamic preparations on the food quality of pumpkin will follow in another article.

Conflict of interest: Authors declare no conflict of interest.

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