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Evaluating the ecotoxicity of nitrification inhibitors using terrestrial and aquatic test organisms

Julia Elli Kösler, Olga C. Calvo*, Jürgen Franzaring and Andreas Fangmeier

Abstract

Background: The increasing demand for food and animal fodder worldwide has led to an intensified agriculture with an increasing use of nitrogen fertilizers. More recently, nitrate leaching and gaseous nitrogen emissions have become the focus of environmental discussions and climate politics. One approach to reduce such negative impacts is the use of nitrification inhibitors (NIs) that have shown to effectively reduce nitrogen losses to the groundwater and the air. However, ecotoxic effects of NIs have been studied to a limited extent only. Therefore, two commercial NIs (Piadin and Vizura) and an active ingredient of another NI, dicyandiamide (DCD), were assayed using various ecotoxicological biotests and test species: the *Lemna* Growth Inhibition Test (*Lemna gibba*), the Seed Germination/Root Elongation Toxicity Test (*Agrostemma githago*, *Fagopyrum esculentum*, *Glycine max*, *Hordeum vulgare*, *Lunaria annua*, *Zea mays*), the Seedling Emergence and Seedling Growth Test (*A. githago, F. esculentum, Z. mays*) and the marine Luminescent Bacteria Test (*Aliivibrio fischeri*). The fresh water *L. gibba* and the bacterium *A. fischeri* were exposed to different test concentrations in liquid growth media, whereas the terrestrial plants were exposed to the test substances diluted/dissolved in deionized water and added to the solid growth medium.

Results: Dicyandiamide did not show ecotoxic effects in any test conducted. Piadin and Vizura showed ecotoxic effects throughout all experiments. Frond number and frond area of *L. gibba* were inhibited with increasing concentrations of both substances with Piadin leading to an earlier inhibition and therefore lower EC_{50} values. In the Seed Germination Test, Vizura generally inhibited seed germination and root development more effectively than Piadin. Regarding both substances, the endpoint root length was much more sensitive than the endpoint germination. In the Seedling Emergence Test, *Z. mays* was the least sensitive and the rare weed species *A. githago* the most sensitive species with regard to the tested endpoints and both substances. *A. fischeri* was strongly inhibited by Vizura, whereas Piadin had barely effects on the bacteria.

Conclusion: All findings indicate ecotoxic effects of Piadin and Vizura, especially on the aquatic species *L. gibba* and on the root development of several terrestrial plant species. However, the origins of the ecotoxic properties remain unclear as both substances contain a mixture of—to some extent unknown—chemical compounds.

Keywords: *Lemna gibba*, *Aliivibrio fischeri*, Nitrification inhibitors, Ecotoxicity

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Background

Modern agriculture faces the challenge of guaranteeing food supply for a growing world population. Nitrogen (N) plays the most important role in plant nutrition by affecting both yield and plant quality like no other nutrient [41]. Therefore, to gain highest possible yields, N fertilization is essential. However, plants cannot take up all available N, resulting in a fertilizer efficiency of only 50% or even less [13, 39]. Furthermore, nitrate (NO_3^-), which is built from ammonium (NH_4^+) via nitrification, is easily leached with consequential threats for human health and the environment [11, 34]. Additional negative impacts such as nitric and nitrous oxide emissions occur due to (de)nitrification processes in the soil [33]. NH_4^+, in contrast to NO_3^-, is bound to the negatively loaded surface of clay minerals due to its positive charge [5]. It is, hence, desirable to maintain a certain level of ammonium in the soil and to reduce nitrate formation. One approach is the use of nitrification inhibitors (NIs), which are chemical substances that delay the nitrification process and, therefore, the nitrate formation in the soil by suppressing the responsible *Nitrosomonas* bacteria. NIs have been reported to efficiently reduce NO_3^- leaching and N_20 emissions [8, 27, 40] and to increase nitrogen use efficiency (NUE) and yield of crops [1]. On the other hand, some studies indicate that even though N losses are reduced, ammonia (NH_3) emissions are significantly higher and thus level out the reported positive effects [17, 18]. Nonetheless, the advantages of NIs seem to prevail and the worldwide development of new nitrification inhibitors has been encouraged over the last decades. Many substances have been identified as possible nitrification inhibitors during that time, mainly in the USA, Japan and Europe [35]. The specific search for nitrification inhibiting substances started as early as in the 1950s, leading to the introduction of nitrapyrin [2-chloro-6(trichlormethyl)pyridine] to the US American market in 1962 under the trade name N-Serve (Dow Chemical Company, USA, see Goring [10]. Dicyandiamide (DCD) on the contrary gained importance in the European market during that time [41]. The rather new NI 3,4-dimethylpyrazolephosphate (DMPP) was developed by BASF (trade mark ENTEC) in 1999 [14] and had since then been promoted as advantageous and practicable with regard to its low application rate, high efficiency and low (eco)toxicity [41]. Other pyrazoles and triazoles like 3-methylpyrazole (3-MP) and 1H-1,2,4-triazole have also been reported as nitrification inhibiting substances [26]. In Germany, the application and registration of nitrification inhibiting chemicals are regulated by the Fertilizer Ordinance, where currently eight substances are listed as NIs including nitrapyrin, DCD, DMPP and the mixture of 1H-1,2,4-triazole and 3-methylpyrazole [7]. The commercial products Piadin (SKW Piesteritz) and Vizura (BASF) were introduced in the early 2000s and 2016, respectively, dominating the German market of NIs today. Piadin contains a mixture of 1H-1,2,4-triazole and 3-MP, whereas Vizura contains a mixture of DMPP and 1H-1,2,4-triazole as active ingredients.

Most studies about nitrification inhibitors focus on the efficacy regarding nitrate leaching and nitrous oxide (N_2O) reduction. Only few studies consider the associated ecotoxic effects of commercially used products and active compounds, and most of them are not very recent [4, 21]. Therefore, not only recently developed or registered products lack information about their ecotoxicity. With regard to the discovered occurrence of some nitrification inhibitors in German surface waters [31], the ecotoxicity of NIs is questioned and urgently requires further research efforts. Moreover, as application of NIs is promoted as economically and ecologically sustainable [15], contamination and appearance in the aquatic and terrestrial environment might be underestimated.

The main objective of this study was, thus, to test the commercially used NIs Vizura and Piadin and the active compound dicyandiamide (DCD) involving different endpoints of biotests with aquatic and terrestrial plants and a bacterium. To evaluate their ecotoxicity and to determine EC_{50} values, four standardized biotests were conducted with Piadin, Vizura and DCD as test substances. The two liquid commercial products were diluted in deionized water when added to solid growth media (Seed Germination and Seedling Growth Test) or in the liquid growth medium (*Lemna* and Bacteria Test). The crystalline DCD was dissolved in deionized water and then added to all growth media, similarly.

Materials and methods

Test substances

Substances tested were the two liquid commercial products Piadin (SKW Piesteritz, Germany) and Vizura (BASF, Germany) and the active compound dicyandiamide (DCD) that is contained in some commercial products such as ALZON 46 (SKW Piesteritz).

Piadin contains 2.7–2.8% 1H-1,2,4-triazole ($C_2H_3N_3$) and 1.4–1.5% 3-methylpyrazole ($C_4H_6N_2$). The biggest part (38%) consists of ammonium nitrate and the remaining ingredients are not further specified. For this study, Piadin was kindly provided by SKW Piesteritz.

Vizura contains 15% of the two active compounds DMPP and 1H-1,2,4-triazole (1:1) and 50% phosphoric acid. The remaining compounds are also not declared. A sample of Vizura was kindly provided by BASF.

DCD is a colorless, crystalline chemical that is used not only as a NI, but also in the production of pharmaceuticals and other agrochemicals. For this study, a sample

of 99.5% purity was kindly provided by the AlzChem AG (Germany). Physical and chemical properties of all test substances can be obtained from the respective safety data sheets [2, 3, 32]. To prepare adequate test solutions, DCD was dissolved in deionized water and later on further diluted.

Lemna sp. Growth Inhibition Test

The *Lemna* sp. Growth Inhibition Test according to OECD guideline 221 [23] was performed to determine the toxicity of NIs on water plants, which are among the first higher organisms to encounter contaminants released into water bodies.

Plant material

Fronds of *Lemna gibba* L., commonly known as gibbous duckweed, were taken from a stock culture, grown from plants originally obtained from Eurofins, Germany. Plants were kept in a climate chamber (Fitotron, UK) in beakers with 400 mL of 20xAAP medium, a full growth medium described in the OECD guideline, under conditions following the OECD guideline 221 [23].

Test concentrations

After performance of range finding tests for all substances, the following concentrations of the respective substance in the growth medium were chosen in the main experiment in the individual substances, respectively: In the experiment conducted with Piadin, *L. gibba* was exposed to 0 (control), 0.001, 0.01, 0.1, 1 and 10 mL L^{-1} for a period of 7 days, respectively.

In the tests conducted with Vizura, *L. gibba* was exposed to 0 (control), 0.01, 0.1, 1, 10 and 100 mL L^{-1} again for a period of 7 days, respectively. Due to a lack of response of *L. gibba* to any of the DCD concentrations in the range finding test (0, 2, 10, 20, 40 and 100 mg L^{-1}), no further experiments were conducted.

Test procedure

To evaluate potential toxic effects of the test substances, a 7-day *Lemna* Growth Inhibition Test was performed. A semi-static renewal test was conducted by changing the test solutions after 3–4 days during the test. Experimental conditions and the procedures were similar in each of the test runs. The nutrient solution used was 20× AAP as recommended in the OECD guideline [23]. The temperature in the climate chamber was kept at 24 °C. Light was supplied continuously at an intensity of 100 ± 15 µmol^{-2} s^{-1} using fluorescence lamps (Philips, Netherlands).

The tests were carried out in 250-mL glass beakers (VWR, Germany) with an outer diameter of 6.5 cm and filled with 150 mL of a sample with pH adjusted to 7.5. Beakers were randomized to obtain a randomized block design. Each test was repeated four times and test endpoints were frond number and frond area, which were determined at day 7 of the experiment. Total frond area was determined using the Image J software (Wayne Rasband, National Institute of Mental Health, USA). Frond number was counted visually by means of the pictures taken for the area determination. Additionally, observations regarding frond color, chlorosis and necrosis were recorded at day 7.

The average specific growth rate and percent inhibition of growth rate were determined for both endpoints (frond number and frond area) according to the OECD guideline 221.

Seed Germination/Root Elongation Toxicity Test

The Seed Germination/Root Elongation Toxicity Test described by the United States Environmental Protection Agency [37] and the Seedling Emergence and Seedling Growth Test according to OECD guideline 208 [24] were chosen to determine the toxicity of NIs on early and later growth stages of terrestrial plants.

Plant material

Species were chosen after recommendations of the guideline and according to differences in taxonomy and usage: agriculturally relevant crops were represented by maize (*Zea mays* L., Poaceae), barley (*Hordeum vulgare* L., Poaceae) and soybean (*Glycine max* L., Fabaceae). Common buckwheat (*Fagopyrum esculentum* Moench, Polygonaceae) was chosen as a neglected and underutilized crop. Common corn-cockle (*Agrostemma githago* L., Caryophyllaceae) and annual honesty (*Lunaria annua* L., Brassicaceae) were employed as two representatives for wild species. Seeds of the latter were obtained from Appels Wilde Samen, Germany.

Test concentrations

In all three tests, plants were exposed to following concentrations of test substances diluted/dissolved in deionized water: 0 (control), 2, 10, 20, 40 and 100 mL L^{-1} (and accordingly mg L^{-1} for DCD).

Test procedure

To screen adverse effects of the chosen substances, a 7-day seed germination/root elongation toxicity test was performed [37]. Test conditions and procedures were similar in each of the experimental runs. Seeds of all species were kept in constant darkness and at a temperature of 24 °C in a climate chamber (Fitotron, UK) for a period of 7 days.

The tests were carried out in plastic Petri dishes with a diameter of 9 cm that were filled with 40-g quartz sand as

a substrate. Depending on their size, six to ten seeds were evenly distributed on one Petri dish and 10 mL of the solutions with the test concentrations were added prior to closing and sealing the samples with Parafilm tape. As it is not demanded in the guideline, the test sample's pH was not adjusted in this experiment. Each test had three replicates and Petri dishes were randomly distributed within one repetition to maintain a randomized bock design. The endpoints seed germination rate and root length (length of the longest root per seed) were determined at day 7 visually and using a ruler.

Seedling Emergence and Seedling Growth Test

Plant material

To test the effects of NIs on early plant growth stages, another test was performed using established seedlings. Species tested were chosen based on the results from previous seed germination/root elongation toxicity test. To maintain plant group diversity, the following species covering wild, mono- and dicotyledonous species were chosen: *Z. mays*, *F. esculentum*, *A. githago* and *L. annua* were chosen for the greenhouse experiment. Unfortunately, *L. annua* was damaged by insects and therefore, results became invalid. Because of the absence of adverse effects of DCD in the seed test, only Piadin and Vizura were tested in the greenhouse experiment.

Test concentrations

After a range finding test with concentrations of 0, 0.1, 1, 10 and 100 mL L^{-1} for both substances, concentrations for each species and substance were defined: Piadin concentrations were 0, 5, 25, 50 and 100 mL L^{-1} for maize and buckwheat and 0, 0.1, 1, 5 and 25 mL L^{-1} for corncockle and annual honesty. Vizura concentrations were 0, 5, 25, 50 and 200 mL L^{-1} for maize, 0, 5, 25, 50 and 100 mL L^{-1} for buckwheat and 0, 0.1, 1, 5 and 25 mL L^{-1} for the two wild species.

Test procedure

To screen adverse effects of the chosen substances on establishing plants, a greenhouse experiment based on the OECD guideline 208 [24] was set up on September 4th 2018. Pots with a volume of 344 cm^{-3}, a height of 6.8 cm and a diameter of 9 cm were filled with 225 g of soil containing 50% quartz sand and 50% LD80 substrate (Fruhstorfer) consisting of peat, humus, volcanic clay, bark and slow release fertilizer with a pH value of 5.9. The guideline does not demand a pH adjustment. The test had five replicates. Five seeds of each species were sown in each pot. The five repetitions were arranged in five blocks in the greenhouse and randomized within each block. All pots were watered until saturation and 1 day later, 100 mL of the solutions with the test concentrations were added per pot and concentration to allow an even distribution throughout the substrate. Control pots received 100-mL pure water. Pots were watered every 2nd day and germination was documented. When all five seeds of the control had germinated, plants were thinned to one seedling per pot. The thinning out deviated from the OECD guideline, where all seeds planted remain in the pot throughout the experiment and the survival of the plants is an additional endpoint. However, in this experiment, we wanted to exclude intraspecific competition within the pots and only focus on the development of one seedling. Therefore, we neglected the plant survival. However, absolute results with/without this endpoint might differ, but due to the use of the % inhibition, the relative numbers do not influence the results. Harvests took place on September 24th, 26th, and 28th 2018 for *Z. mays*, *F. esculentum*, and *A. githago*, respectively, and the measured endpoints were plant height, fresh and dry weight.

Luminescent Bacteria Test

The Luminescent Bacteria Test according to the ISO guideline 11348-2 [9] was chosen because *Aliivibrio fischeri* is often used as first and reliable indicator for ecotoxicity because of its fast response [25] and the strong correlation with toxic effects on other aquatic organisms [16].

Bacterial strain

The strain of luminescent bacteria was *A. fischeri* NRRL-B-11177 (Biofix, Macherey–Nagel, Germany).

Test concentrations

After a range finding test with concentrations of 0, 25, 50, 100 and 200 mL L^{-1} (mg L^{-1} for DCD), individual concentrations for Piadin and Vizura were set. Due to a lack of reaction to any DCD concentration, no further experiments with higher concentrations were carried out.

In the test conducted with Piadin, concentrations of the range finding test were considered as appropriate and were maintained in the main experiment. In the test performed with Vizura, concentrations needed to be set lower and were, therefore, adapted to 0, 0.05, 0.5, 25 and 100 mL L^{-1}.

Test procedure

The luminescent bacteria test was performed following the ISO guideline 11348-2 [9]. Each test had two technical replicates, but was repeated over time three times independently. Liquid-dried *A. fischeri* bacteria were reactivated by adding a reactivation solution (Biofix, Germany). 0.5 mL of reactivated bacterial solution was mixed with 0.5 mL of test samples in glass cuvettes with a diameter of 12 mm prior to being incubated in

a thermoblock (Dr. B. Lange, Germany). Test solutions for this experiment were prepared by diluting the test substances in 2% NaCl solutions and adjusted the pH to 7. The % inhibition of luminescence was determined with a luminometer (Lumistox 300, Dr. B. Lange, Germany) at 15 °C after 30 min of incubation.

Statistical analysis

Statistical analysis was conducted using the free software R (https://www.r-project.org/). All analyses were based on the package 'drc' [29]. Graphs, curves and the estimates of EC_{50} values were also obtained from this package.

Results

Lemna Growth Inhibition Test

In the experiment conducted with DCD, no trend in inhibiting frond number or frond area was observed (Table 1). Furthermore, no visible effects like changes in color or plant fitness could be detected. However, no further tests with higher concentrations were performed. Therefore, no EC_{50} values for the test substance DCD could be derived. *L. gibba* exposed to both, Piadin and Vizura, showed a stronger inhibition of frond number and frond area with increasing concentrations of the substances. Piadin, however, inhibited frond number and frond area development stronger than Vizura, resulting in EC_{50} values of 0.42 and 0.40 mL L^{-1} for both endpoints, respectively. EC_{50} values for Vizura were 2.73 mL L^{-1} and 2.86 mL L^{-1}, respectively (Table 2). Due

Table 1 Inhibition of *Lemna gibba* (frond number and frond area) with DCD in the growth medium

Concentration of DCD (mg L^{-1})	0	2	10	20	40	100
Inhibition of frond number (%)	0	−0.60 ± 2.2	−1.11 ± 1.6	−2.21 ± 2.8	−3.31 ± 3.5	−0.84 ± 2.6
Inhibition of frond area (%)	0	0.07 ± 2.0	−0.49 ± 1.4	−2.34 ± 3.3	−3.98 ± 2.5	−1.12 ± 1.4

The values are mean ± standard error (SE) ($n = 4$)

Table 2 EC_{50} values for four biotests with the nitrification inhibitors Piadin and Vizura

Biotest	Species	Endpoint	Piadin EC_{50} (mL L^{-1})	Vizura EC_{50} (mL L^{-1})
Lemna	*Lemna gibba*	Frond number	0.4	2.7
		Frond area	0.4	2.9
Seed germination	*Agrostemma githago*	Germination	30.8	20.9
		Root length	2.5	1.3
	Fagopyrum esculentum	Germination	34.4	45.3
		Root length	5.7	2.3
	Glycine max	Germination	–	54.2
		Root length	–	3.7
	Hordeum vulgare	Germination	45.3	12.4
		Root length	3.9	1.4
	Lunaria annua	Germination	5.9	3.5
		Root length	1.7	1.8
	Zea mays	Germination	53.2	–
		Root length	12.3	9.4
Seedling growth	*Agrostemma githago*	Plant height	5.7	4.2
		Dry weight	3.1	2.2
	Fagopyrum esculentum	Plant height	6.2	8.7
		Dry weight	3.8	3.5
	Zea mays	Plant height	12.6	21.5
		Dry weight	18.1	22.7
Bacteria test	*Aliivibrio fischeri*	Luminescence	111	3

"–" EC_{50} values could not be derived due to invalid results (*G. max*) or absence of inhibition (*Z. mays*)

to a high correlation of both endpoints (R^2 of 0.99 and 0.98 in the Vizura and Piadin experiment, respectively), only the regression curve for inhibition of frond number (Fig. 1) is shown.

Visible effects of increasing concentrations for both substances were a loss of green color (chlorosis) for the two highest concentrations and a separation of fronds for the second highest concentrations.

Seed Germination/Root Elongation Toxicity Test

In the experiment conducted with DCD, no trend in decreasing root length or germination rate of any species tested was observed (data not shown). Therefore, also in this experiment, no EC_{50} values could be determined.

For Piadin and Vizura, inhibiting effects on seed germination and root length occurred and EC_{50} values were determined, differing between species. EC_{50} values for Piadin ranged from 30.8 mL L^{-1} (*A. githago*) to 53.2 mL L^{-1} (*Z. mays*) for germination and from 1.7 mL L^{-1} (*L. annua*) to 12.3 mL L^{-1} (*Z. mays*) for root length (Table 2).

Soybean (*G. max*) did not meet the validity criteria in the Piadin experiment; therefore, an EC_{50} value is missing (Table 2). EC_{50} values for Vizura ranged between 3.5 mL L^{-1} (*L. annua*) and >100 mL L^{-1} (*Z. mays*) for germination and from 1.3 mL L^{-1} (*A. githago*) to 9.4 mL L^{-1} (*Z. mays*) for root length (Table 2).

We observed a decrease in germination rate and root length in all species with increasing concentrations of Piadin and Vizura. The curves differed from species to species, but all followed more or less a sigmoid shape (Additional file 1: Figures S1, S2).

Seedling Emergence and Seedling Growth Test

This biotest was conducted in a greenhouse with the four species *A. githago*, *F. esculentum*, *L. annua* and *Z. mays*. Due to damage of *L. annua* plants, results are presented for the other three species only. Endpoints were plant height, plant fresh and dry weight at harvest time. As dry and fresh weight correlated highly, only the endpoint dry weight was further analyzed. As *Z. mays* germinated earlier, plants were harvested 20 days after sowing (DAS). The other species needed more time to germinate and harvest, therefore, took place 22 DAS and 24 DAS for *F. esculentum* and *A. githago,* respectively.

Visible effects were leaf tip burns of all species for all concentrations higher than the control, except at the lowest concentrations (0.1 mL L^{-1}) for *A. githago*. With increasing concentrations of both substances, plant height and dry weight decreased in all species (Additional file 1: Figures S3, S4). However, plants differed in their response and substance sensitivity, leading to different EC_{50} values across endpoints and substances (Table 2). Again, *Z. mays* was the least sensitive species with highest EC_{50} values regarding both endpoints and both substances.

Luminescent Bacteria Test

The Luminescent bacteria test was conducted with all three substances. However, also in this experiment, an EC_{50} estimation for DCD was not possible due missing effects to the concentrations applied. For Piadin and Vizura, an increasing inhibition with increasing concentrations was observed. Whereas the trend for increasing concentrations of Piadin showed a linear form, the inhibition caused by Vizura showed a saturation curve with a high slope in the first part of the curve (Fig. 2). The EC_{50} values obtained for Piadin and Vizura differ highly from each other (111 mL L^{-1} for Piadin and 3 mL L^{-1} for Vizura, Table 2).

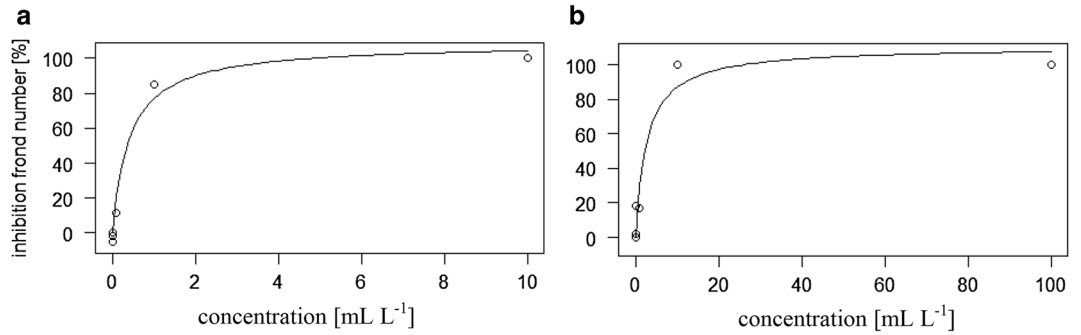

Fig. 1 Inhibition of *Lemna gibba* (frond number) after exposure to Piadin (**a**) and Vizura (**b**) for 7 days. The regression curves were modelled using a Michaelis–Menten function with two parameters ($n = 4$)

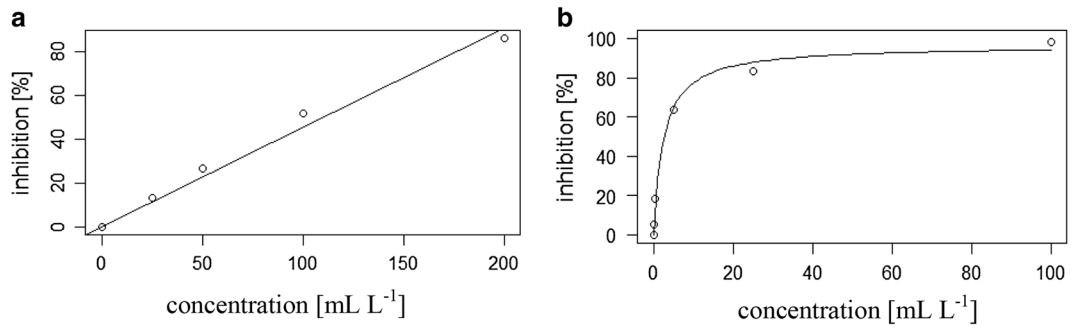

Fig. 2 Inhibition of *Aliivibrio fischeri* (luminescence) after exposure to Piadin (**a**) and Vizura (**b**) for 30 min ($n = 3$). The regression curves were modelled using a linear model (left) and a Michaelis–Menten function with two parameters (right)

Discussion

DCD

DCD did not show ecotoxic effects in any of the tests conducted. In the *Lemna* Growth Inhibition Test, no phytotoxic effects on the endpoints frond number or frond area occurred. The test was chosen to determine the toxicity of DCD on higher water plants because after regular application and possible spills, DCD might be discharged into surface waters. The concentrations applied in our experiment (up to 100 mg L^{-1}) are higher than those reported for water bodies. Scheurer et al. [31] detected DCD concentrations from several µg L^{-1} up to 7.2 mg L^{-1}. However, concentrations in water bodies might increase in the future due to intensification of DCD usage or even due to mismanagement or (accidental) exceedance of application rates. The results obtained in present study indicate that DCD is not harmful to *Lemna* even under extreme concentrations. To verify our findings, further comparable experiments with other water plants and different endpoints (e.g., longevity, floral reproduction) need to be performed. Furthermore, a longer exposure time of plants to the substance could lead to phytotoxic effects and should be studied.

In the Seed Germination/Root Elongation Toxicity Test with terrestrial plants, neither restriction in root growth nor a reduced germination rate was observed. These findings correspond with those of Bremner and Krogmeier [4] who studied the seed germination response of several species (including maize and barley) to various concentrations of different nitrification inhibitors. They found that DCD did not affect germination of any species tested and concluded that this NI is, therefore, safe to use at early growth stages. However, several studies with DCD applied to growth media for a longer period of time, like greenhouse and growth chamber experiments, indicate that phytotoxic effects such as leaf chlorosis, reduced biomass and necrotic patches might occur. Maftoun and Sheibany [20] describe such effects for soybean (at 40 ppm DCD in the soil) grown in a greenhouse in alluvial calcareous silty clay loam, and Reeves and Touchton [28] studied corn, cotton and grain sorghum in a pot experiment with Norfolk sandy loam. Furthermore, Macadam et al. [19] described a yield reduction of around 16% for white clover at the recommended DCD application rate (25 kg ha^{-1}) in a laboratory experiment. However, since all these findings origin from very different experimental set-ups than in present study, no direct comparison of results is possible. Thus, it remains unclear if DCD would have shown phytotoxic effects if applied for a longer period of time like, for example, in a greenhouse trial. As results of laboratory tests can differ from those of field experiments, it is important to keep in mind that DCD availability in the soil might decrease, for example, due to leaching or absorption under field conditions. A study of Mason [22] resulted in decreased yield and dry matter of wheat under DCD application in some of the field experiments. On other sites dealt within the same study, vegetative yield was increased. Therefore, due to inconclusive results and a lack of current studies, it is rather difficult to state whether or not DCD can be considered as phytotoxic in the field.

To determine the toxicity on a non-target bacterium, the Luminescent Bacteria Test with the marine species *A. fischeri* was conducted with DCD. This also did not show effects even at concentrations of 100 mg L^{-1}. Guo et al. [12] found that after a long-term application of DCD, non-target microbes in the soil were unaffected. Therefore, the authors concluded that DCD only acts specifically on the ammonia oxidation. Because of the lack of *A. fischeri*'s reaction to DCD, this hypothesis can be supported. Tindaon et al. [36] applied DCD concentrations of 10 µg g^{-1} dry soil (recommended rate) to different soil samples under laboratory conditions and up to 1000 times higher concentrations in different treatments and measured the activity of dehydrogenase and dimethyl sulfoxide reductase activity as an indicator for non-target

microbial activity. The authors not only claimed that higher DCD rates might affect non-target microbial activity in the soil but they also state that DCD can be considered as safe to use at recommended application rates.

Piadin

In contrast, Piadin showed various ecotoxic effects throughout all experiments conducted.

In the *Lemna* Growth Inhibition Test, Piadin inhibited growth of *Lemna* plants already at low concentrations, with EC_{50} values of 0.42 mL L^{-1} for frond number and 0.40 mL L^{-1} for frond area (Table 2). The similarity of these endpoints is not surprising due to the high correlation between frond number and frond area. Visual toxicity symptoms consisted of chlorosis in all fronds at the two highest concentrations (1 and 10 mL L^{-1}) and disintegrated fronds at the second highest concentration of Piadin. As similar studies with *L. gibba* or related aquatic plants are missing, it is impossible to compare our results with other data. It is, therefore, crucial to repeat such experiments and to include other endpoints such as fresh and dry weight and chlorophyll content as they might differ in sensitivity.

In the Seed Germination/Root Elongation Toxicity Test, restrictions of germination and root development across all species were observed. All species tested had in common a much higher sensitivity with regard to the endpoint root length compared to the seed germination. It has been reported that dormant seeds show less vulnerability under unfavorable conditions, but as soon as germination starts, the first developing plant organs are very sensitive towards environmental stress in general [38]. This holds true also for Piadin application as a stress factor in young plant seedlings.

Generally, EC_{50} values for seed germination ranged between 31 and 53 mL L^{-1}. Only the wild species *L. annua* showed a much lower value of around 6 mL L^{-1}. As the values for *L. annua* and *A. githago* differ, it cannot be concluded that wild species in general would be more sensitive to Piadin. To make a clearer statement, tests with more wild species are needed. Germination of *L. annua* might be much more sensitive due to its small seed size. In general, larger seeds tend to be more stress-resistant still enabling the resulting seedlings more likely to establish [6]. The seed size-related sensitivity is supported by the fact that amongst the other species, the species with the largest seeds, *Z. mays*, was by far the least sensitive with regard to both endpoints.

To determine effects of Piadin at later plant growth stages, a greenhouse experiment with *A. githago, F. esculentum* and *Z. mays* was conducted. Results showed a similar pattern as for the Seed Germination Test. Maize was the least sensitive species regarding both endpoints (height and dry weight) and the wild species reacted faster with a reduction of growth. The species tested did not show a clear trend in endpoint sensitivity. *A. githago* and *F. esculentum* showed higher sensitivity with regard to the endpoint dry weight, whereas the effects on *Z. mays* were the other way round. Unfortunately, no other studies tested the phytotoxicity of Piadin or its active ingredients before, so that the results obtained from this study cannot be compared to other references. More greenhouse experiments also integrating other species than tested in the present study should be carried out in the future to understand how NIs affect plant performance later growth stages.

In the Luminescent Bacteria Test, a mean EC_{50} value of around 111 mL L^{-1} was observed. This is a rather high value indicating that Piadin has hardly any toxic effect on the non-target bacterium *A. fischeri*. Studies testing the effect of other nitrification inhibitors such as DCD and DMPP concluded that these NIs at the recommended application rates do not affect non-target soil bacteria and act specifically on ammonia oxidation [12, 36]. As *A. fischeri* was barely affected, this can be assumed for Piadin as well. However, other non-target species (bacteria and soil fauna) living in the soil right where application takes place should be studied to assure usage safety. Results obtained for *A. fischeri* seem to be of importance because of the strong correlation with toxic effects on other aquatic organisms and the general high sensitivity towards toxicants [16, 25].

All in all, it remains unclear which individual components in Piadin caused the observed phytotoxic effects and it might be useful to test different ingredients and mixtures separately. Piadin contains a compound with aromatic structure (1H-1,2,4-triazole), which can cause phytotoxic effects. However, not all ingredients are known as the company does not provide the corresponding information.

Furthermore, results showed species-specific trends across the four biotests. EC_{50} values differed not only between biotests and plant species, but also between different endpoints. Among all biotests conducted with Piadin, *L. gibba* was shown to be the most sensitive plant species regarding both endpoints. This contrasts to the stated low hazard to waters [32].

Vizura

Vizura also showed different ecotoxic effects across the experiments conducted.

In the *Lemna* Growth Inhibition Test, Vizura reduced growth of *Lemna* plants at higher concentrations than Piadin. EC_{50} values were 2.7 mL L^{-1} for frond number and 2.9 mL L^{-1} for frond area. Again, there existed a

high correlation between frond number and frond area. Visual toxicity symptoms were the same as for Piadin, namely chlorosis at all fronds of the two highest concentrations (10 and 100 mL L^{-1}) and disintegrated fronds at the second highest concentration of Vizura. It is, however, questionable whether Vizura would still have shown less phytotoxic effects than Piadin if the pH had not been neutralized. Comparable data for Vizura and *Lemna* are missing, but BASF [3] gives some information about the effects of the active ingredients DMPP and 1H-1,2,4-triazole (1:1) on the cyanobacterium *Anabaena flos-aquae* (EC_{50} 25 mg L^{-1}) and the microalgae *Pseudokirchneriella subcapitata* (EC_{50} > 79 mg L^{-1}). To make direct comparisons possible, further studies on the effect of the active compounds of Vizura on *Lemna* or the effect of Vizura on other water plants would be needed.

In the Seed Germination Test conducted with Vizura, a decrease in root length and germination with increasing concentrations of the substance was found for all species except for *Z. mays*, which still germinated under the highest concentrations. This is surprising because all other EC_{50} values were lower for Vizura than for Piadin. All species showed higher sensitivity towards Vizura regarding the root length, supporting the statement from above that germination in general might be less sensitive in stress situations. For the other species except in maize, EC_{50} values for the germination ranged between 3.5 and 54 mL L^{-1}. The lowest values were observed in *L. annua*, which has been shown to be the most sensitive species. The other wild species *A. githago* showed the second lowest EC_{50} value for germination followed by the crops. However, the rather big difference between the two wild species does not allow general conclusions about the vulnerability of wild species towards Vizura in general. Rodrigues et al. [30] studied the seed germination of lettuce, watercress and clover under different DMPP concentrations and observed negative effects only for lettuce at the highest concentration (100 mg L^{-1}). EC_{50} values for the endpoint root length were generally very low (around 1.5–3.5 mL L^{-1}) except for *Z. mays*. This indicates that Vizura affects root development and germination generally stronger than Piadin does. An underlying reason might be the extremely low pH of Vizura that was not neutralized in the Seed Germination Test. Since only few taxa were studied, no clear trend with regard to a specific plant group like monocotyledons vs. dicotyledons regarding their sensitivity could be discovered. However, the data suggest a clear species specificity. Therefore, to gain a better insight of plants' reactions towards Piadin and Vizura application, more experiments with different plant species are required in greenhouses and in the field.

In the greenhouse experiment conducted with Vizura the same species like in the Piadin trial were tested. Among the three species, no clear trend was observed. *A. githago* and *F. esculentum* were more sensitive regarding the endpoint dry weight, whereas *Z. mays* reacted more sensitive regarding the endpoint plant height. Furthermore, the species differed in ecotoxic effects regarding both substances tested: *A. githago* showed lower EC_{50} values for both endpoints tested with Vizura, *Z. mays* reacted earlier when tested with Piadin. Despite a more complex and time-consuming experimental setup, results of the effect of NIs in a later developmental stage of plants are environmentally relevant and should be repeated to clarify different plant reactions. Rodrigues et al. [30] studied the phytotoxicity of DMPP in a hydroponic system with red clover and did only observe negative effects after applying very high concentrations (100 mg L^{-1}). Zerulla et al. [41] showed non-toxic effects of DMPP at concentrations eightfold above than the recommended application rate. Therefore, the reason for phytotoxic symptoms in the present study remains unclear and the question if formulations in the product were involved remains unanswered.

The Luminescent Bacteria Test conducted with Vizura resulted in a very low EC_{50} value of around 3 mL L^{-1}. As mentioned above, different studies showed non-toxic effects of DMPP on non-target soil bacteria [12, 36]. Furthermore, Rodrigues et al. [30] determined an EC_{50} of 16.6 mg DMPP L^{-1} on *A. fischeri* that was considered as harmful but negligible due to the low mobility of DMPP in soil. As pH was neutralized for this experiment, it cannot be the reason for the low EC_{50} and it can therefore be assumed that phytotoxic properties of Vizura observed in all biotests originate from other (unknown) ingredients. At this point it should be stated that also Vizura contains aromatic compounds (1H-triazole) which could be the reason for phytotoxic effects.

Conclusions

It can be stated that none of the conducted biotests showed ecotoxic effects of DCD but all tests conducted with Piadin and Vizura allowed the determination of different ecotoxic effects across the species and endpoints tested. Among all biotests, frond number and frond area of *Lemna* were the most sensitive endpoints for Piadin and root length of some species (*A. githago, H. vulgare*) was the most sensitive endpoint for Vizura.

Plant species used in the two terrestrial plant tests (Seed Germination and Seedling Emergence) differed highly in their response to Piadin and Vizura application and showed differing sensitivity with regard to substance and endpoints. The trend of wild and small seed-sized species being generally more vulnerable needs to be verified in further studies. However, the use of wild species could be problematic regarding the lack of

standardization of for example the seed material. Additionally, a broader range of monocotyledonous and dicotyledonous species should be tested to see if there is a plant group-related reaction trend. In any case, legumes, which depend on N fixing bacteria, should be looked at in greater detail in future studies.

Based on the results obtained in the present study, all four methodologies proved adverse effects of two out of three substances tested. However, results cannot be directly transferred to real field conditions. This requires checking in outdoor experiments. Investigations of the effects of individual compounds and mixtures of the substances tested are furthermore needed to identify the origin of the observed ecotoxicity. A possible reason might be the aromatic compounds which are used in the formulations of Piadin and Vizura. However, also other unknown ingredients of both products could be the cause of ecotoxic properties.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12302-019-0272-3.

Additional file 1: Figure S1. Inhibition of root length (left) and germination rate (right) of different plant species exposed to different concentrations of Piadin: a) *Agrostemma githago*, b) *Fagopyrum esculentum*, c) *Hordeum vulgare*, d) *Lunaria annua*, e) *Zea mays*. The regression curves were modelled using a log logistic function with four parameters (n=3). **Figure S2.** Inhibition of root length (left) and germination rate (right) of different plant species exposed to different concentrations of Vizura: a) *Agrostemma githago*, b) *Fagopyrum esculentum*, c) *Hordeum vulgare*, d) *Lunaria annua*, e) *Zea mays*. The regression curves were modelled using a log logistic function with four parameters (n=3). **Figure S3.** Inhibition of plant height (left) and dry weight (right) of different plant species exposed to different concentrations of Piadin: a) *Agrostemma githago*, b) *Fagopyrum esculentum*, c) *Zea mays*. The regression curves were modelled using a log logistic function with four parameters (n=3). **Figure S4.** Inhibition of plant height (left) and dry weight (right) of different plant species exposed to different concentrations of Vizura: a) Agrostemma githago, b) Fagopyrum esculentum, c) Zea mays. The regression curves were modelled using a log logistic function with four parameters (n=3).

Abbreviations

3-MP: 3-methylpyrazole; DAS: days after sowing; DCD: dicyandiamide; DMPP: 3,4-dimethylpyrazolephosphate; EC_{50}: half maximal effective concentration; NI: nitrification inhibitor; NUE: nitrogen use efficiency; US EPA: United States Environmental Protection Agency.

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Authors' contributions

JEK performed all experiments, analyzed the data and was a major contributor to writing the manuscript. OCC was major contributor to supervision, guided the laboratory experiments and contributed to writing the manuscript. JF guided the greenhouse experiment and contributed to writing the manuscript. AF contributed to supervision, administrative issues regarding the experiments and writing the manuscript. All authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

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References

1. Abalos D, Jeffery S, Sanz-Cobena A, Guardia G, Vallejo A (2014) Meta-analysis of the effect of urease and nitrification inhibitors on crop productivity and nitrogen use efficiency. Agr Ecosyst Environ 189:136–144. https://doi.org/10.1016/j.agee.2014.03.036
2. AlzChem (2019) Dicyandiamide. Edited by AlzChem. https://www.alzchem.com/de/dicyandiamide. Accessed 4 June 2019
3. BASF (2017): Sicherheitsdatenblatt Vizura. Edited by BASF Germany. https://www.agrar.basf.de/agroportal/de/media/migrated/de/productfiles/sdb/sdb-vizura.pdf. Accessed 4 June 2019
4. Bremner JM, Krogmeier MJ (1989) Effects of nitrification inhibitors on germination of various seeds in soil. Biol Fertil Soils 8:369–372
5. Cameron KC, Di HJ, Moir JL (2013) Nitrogen losses from the soil/plant system: a review. Ann Appl Biol 162(2):145–173. https://doi.org/10.1111/aab.12014
6. Dalling JW, Hubbell SP (2002) Seed size, growth rate and gap microsite conditions as determinants of recruitment success for pioneer species. J Ecol 90:557–568
7. Deutscher Bundestag (1/6/2017) Zulassung von Düngemitteln mit Nitrifikations- und Ureaseinhibitoren, pp. 1–38
8. Di HJ, Cameron KC (2012) How does the application of different nitrification inhibitors affect nitrous oxide emissions and nitrate leaching from cow urine in grazed pastures? Soil Use Manag 28(1):54–61. https://doi.org/10.1111/j.1475-2743.2011.00373.x
9. DIN EN ISO 11348-2 (1999) Water quality-determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (luminescent bacteria test). Part 2: Method using liquid-dried bacteria. Beuth Verlag GmbH, Berlin, Germany
10. Goring CAI (1962) Control of nitrification by 2-chloro-6-(trichloro-methyl) pyridine. Soil Sci 3:211–218
11. Grizzetti B (2011) Nitrogen as a threat to European water quality. Cambridge: Cambridge University Press. http://centaur.reading.ac.uk/20869/. Accessed 4 June 2019
12. Guo YJ, Di HJ, Cameron KHC, Li B, Podolyan A, Moir JL et al (2013) Effect of 7-year application of a nitrification inhibitor, dicyandiamide (DCD), on soil microbial biomass, protease and deaminase activities, and the abundance of bacteria and archaea in pasture soils. J Soils Sediments 13(4):753–759. https://doi.org/10.1007/s11368-012-0646-2
13. Hodge A, Robinson D, Fitter A (2000) Are microorganisms more effective than plants at competing for nitrogen? Trends Plant Sci 5(7):304–308
14. Hofmair W (2000) DMPP - ein neuer Nitrifikationsinhibitor (Wirkstoff-Wirksamkeit-Einsatzgebiet). Edited by Arbeitsgemeinschaft landwirtschaftlicher Versuchsanstalten. Gmunden
15. IPCC (2014) Climate Change 2014: Mitigation of Climate Change. Contribution of Working Group III to the Fifth Assessment. Report of the Intergovernmental Panel on Climate Change. Edenhofer, O., R. Pichs-Madruga, Y. Sokona, E. Farahani, S. Kadner, K. Seyboth, A. Adler, I. Baum, S. Brunner, P. Eickemeier, B. Kriemann, J. Savolainen, S. Schlömer, C. von Stechow, T. Zwickel and J.C. Minx (eds). Cambridge University Press, Cambridge
16. Kaiser KLE (1998) Correlations of *Vibrio fischeri* bacteria test data with bioassay data for other organisms. Environ Health Perspect 106:583–591
17. Kim D-G, Saggar S, Roudier P (2012) The effect of nitrification inhibitors on soil ammonia emissions in nitrogen managed soils: a meta-analysis.

Nutr Cycl Agroecosyst 93(1):51–64. https://doi.org/10.1007/s10705-012-9498-9
18. Lam SK, Suter H, Mosier AR, Chen D (2017) Using nitrification inhibitors to mitigate agricultural N_2O emission: a double-edged sword? Glob Change Biol 23(2):485–489. https://doi.org/10.1111/gcb.13338
19. Macadam XMB, Prado A, Merino P, Estavillo JM, Pinto M, González-Murua C (2003) Dicyandiamide and 3,4-dimethyl pyrazole phosphate decrease N2O emissions from grassland but dicyandiamide produces deleterious effects in clover. J Plant Physiol 160(12):1517–1523. https://doi.org/10.1078/0176-1617-01006
20. Maftoun M, Sheibany B (1979) Comparative phytotoxicity of several nitrification inhibitors to soybean plants. J Agric Food Chem 27(6):1365–1368
21. Maftoun M, Yasrebi J, Darbeheshti M (1981) Comparative phytotoxicity of nitrapyrin and ATC to several leguminous species. Plant Soil 63:303–306
22. Mason MG (1987) Effects of dicyandiamide (a nitrification inhibitor) on leaching of nitrogen and growth of cereals. Aust J Agric 27(1):127–133
23. OECD (2006a) Test No. 208: Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test
24. OECD (2006b) Test No. 221: *Lemna* sp. Growth Inhibition Test
25. Parvez Shahid, Venkataraman Chandra, Mukherji Suparna (2006) A review on advantages of implementing luminescence inhibition test (*Vibrio fischeri*) for acute toxicity prediction of chemicals. Environ Int 32:265–268
26. Prasad R, Power JF (1995) Nitrification inhibitors for agriculture, health, and the environment. Adv Agron 54:233–281
27. Qiao C, Liu L, Hu S, Compton JE, Greaver TL, Li Q (2015) How inhibiting nitrification affects nitrogen cycle and reduces environmental impacts of anthropogenic nitrogen input. Glob Change Biol 21(3):1249–1257. https://doi.org/10.1111/gcb.12802
28. Reeves DW, Touchton JT (1986) Relative phytotoxicity of dicyandiamide and availability of its nitrogen to cotton, corn, and grain sorghum. Soil Sci Soc Am J 50:1353–1357
29. Ritz C, Streibig JC (2012) Dose response curves and other nonlinear curves in Weed Science and Ecotoxicology with the add-on package *drc* in R. www.bioassay.dk. Accessed 20 Mar 2019
30. Rodrigues JM, Lasa B, Aparicio-Tejo PM, González-Murua C, Marino D (2018) 3,4-Dimethylpyrazole phosphate and 2-(N-3,4-dimethyl-1H-pyrazol-1-yl) succinic acid isomeric mixture nitrification inhibitors: quantification in plant tissues and toxicity assays. Sci Total Environ 624:1180–1186. https://doi.org/10.1016/j.scitotenv.2017.12.241
31. Scheurer M, Brauch H-J, Schmidt CK, Sacher F (2016) Occurrence and fate of nitrification and urease inhibitors in the aquatic environment. Environ Sci Process Impacts 18(8):999–1010. https://doi.org/10.1039/c6em00014b
32. SKW Piesteritz (2015) Piadin Sicherheitsdatenblatt. Edited by SKW Piesteritz. http://www.raiffeisen.com/php/agrar_sdb/pdf/269/54ceae83417bbc125c6a57491034527c. Accessed 4 June 2019
33. Smith P, Martino D, Cai Z, Gwary D, Janzen H, Kumar P et al (2008) Greenhouse gas mitigation in agriculture. Philos Trans R Soc London Ser B Biol Sci 363(1492):789–813. https://doi.org/10.1098/rstb.2007.2184
34. Smith VH (2003) Eutrophication of freshwater and coastal marine ecosystems A global problem. Environ Sci Pollut Res 10(2):126–139
35. Subbarao GV, Ito O, Sahrawat KL, Berry WL, Nakahara K, Ishikawa T et al (2006) Scope and strategies for regulation of nitrification in agricultural systems—challenges and opportunities. Crit Rev Plant Sci 25(4):303–335. https://doi.org/10.1080/07352680600794232
36. Tindaon F, Benckiser G, Ottow JCG (2012) Evaluation of ecological doses of the nitrification inhibitors 3,4-dimethylpyrazole phosphate (DMPP) and 4-chloromethylpyrazole (ClMP) in comparison to dicyandiamide (DCD) in their effects on dehydrogenase and dimethyl sulfoxide reductase activity in soils. Biol Fertil Soils 48(6):643–650. https://doi.org/10.1007/s00374-011-0655-0
37. US Environmental Protection Agency (1996) Ecological effects test guidelines. OPPTS 850.4200. Seed germination/root elongation toxicity test. EPA 712–C–96–154
38. Wang W (1991) Literature review on higher plants for toxicity testing. Water Air Soil Pollut 59:381–400
39. Wiesler F (1998) Comparative assessment of the efficacy of various nitrogen fertilizers. J Crop Prod 1(2):81–114. https://doi.org/10.1300/J144v01n02_04
40. Zaman M, Blennerhassett JD (2010) Effects of the different rates of urease and nitrification inhibitors on gaseous emissions of ammonia and nitrous oxide, nitrate leaching and pasture production from urine patches in an intensive grazed pasture system. Agr Ecosyst Environ 136(3–4):236–246. https://doi.org/10.1016/j.agee.2009.07.010
41. Zerulla W, Barth T, Dressel J, Erhardt K, Horchler von Locquenghien K, Pasda G et al (2001) 3,4-Dimethylpyrazole phosphate (DMPP)—a new nitrification inhibitor for agriculture and horticulture. Biol Fertil Soils 34(2):79–84. https://doi.org/10.1007/s003740100380

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