UNDERSTANDING THE INEFFECTIVENESS OF CU AND ZN IN REDUCING UREA HYDROLYSIS IN GRAZED DAIRY PASTURE SOILS

Kamal P. Adhikari[1], Surinder Saggar[1,2], James A. Hanly[1] & Danilo F. Guinto[3]

[1] *Soil & Earth Sciences Group, Institute of Agriculture & Environment, Massey University, Palmerston North 4442, New Zealand*
[2] *Ecosystems & Global Change, Landcare Research, Palmerston North 4442, New Zealand*
[3] *Ballance Agri-Nutrients Limited, Hewletts Road, Mount Maunganui, New Zealand*
(Email: adhikari.kamal5@gmail.com)

Abstract

The commonly used urease inhibitor, N-(n-butyl) thiophosphoric triamide (nBTPT), (i.e. Agrotain®), does reduce ammonia (NH_3) emissions when applied with urea fertiliser or cattle urine but it is short-lived (7-14 days). In some studies micronutrients such as copper (Cu) and zinc (Zn) have been shown to inhibit soil urease enzyme activity (UEA) and reduce NH_3 emissions over a longer duration than nBTPT. However, Adhikari et al. (2016), in a survey of Waikato soils, did not observe any significant inverse relationships between inherent soil Cu and Zn levels and soil UEA, which was attributed to probable metal complexation by soil organic carbon (C). This current study was conducted to assess the Cu and Zn application effects on soil UEA and to investigate the influence of soil organic C on the effectiveness of Cu and Zn in reducing urea hydrolysis. Laboratory experiments were conducted using 4 dairy soils with contrasting soil C levels and soil supernatant from 2 dairy soils. Different amounts of Cu (5, 10, 20 mg kg^{-1} soil) and Cu + Zn (5 + 5 mg kg^{-1} soil) were added to soils and soil supernatant [Cu (5, 10, 20 mg kg^{-1} soil) and Zn (20 mg kg^{-1} soil)], and changes in soil UEA and urea hydrolysis were monitored, respectively. There was no significant reduction in soil UEA by Cu and Zn treatments added to soil. The highest Cu additions significantly reduced urea hydrolysis by 65.2 and 87.3%, after 32 days of treatment application and, by 49.9 and 54.8% after 16 days of treatment application in Rangitekei loamy sand and Egmont black loam soil supernatant, for the two N rates, 120 and 600 mg N kg^{-1} soil, respectively. However, Zn was also not effective with the soil supernatants. This result shows that while Cu does inhibit urea hydrolysis, its ineffectiveness in pasture soils could potentially be attributable to complexation with the high organic C present. Although most of the Zn added was bioavailable, the observed levels of bioavailable Zn had no effect on studied soil UEA.

Introduction

Intensification of dairy farming in New Zealand (NZ) over the last three decades has resulted in a substantial increase in use of urea nitrogen (N) fertiliser and, as a consequence, higher ammonia (NH_3) emissions. Following urea fertiliser application to pasture, the losses via NH_3 emissions have been estimated to account for an average of approximately 11% of N applied at rates of urea typically applied by NZ dairy farmers (i.e. 25-45 kg N ha^{-1} $application^{-1}$) (Sherlock et al. 2008). However, these losses can be up to 33.2% (Black et al. 1987) depending on soil and climatic conditions as well as rate of urea fertilisation applied. Similarly, urine patches cover approximately 20–30% of the grazed pasture area annually and contain the equivalent rate of N ranging from 200-2000 kg N ha^{-1} (Selbie et al. 2015).

Following urine deposition to pasture, the losses via NH_3 emissions have been estimated to account for an average of 15.9% of N deposited (Sherlock et al. 2008) but can be up to 25.7% (Laubach et al. 2012) depending on soil and climatic conditions.

Ammonia emissions represent economic losses of N fertiliser and have negative impacts on the environment. Annual losses of NH_3 from urea fertiliser used on NZ farms are estimated to have a value of about $30 million (Ballance Agri-Nutrients 2015). Atmospheric N deposition in adjacent native ecosystems of some intensive dairy farming areas of NZ (Christchurch, Hastings, Invercargill, Rotorua and Whangarei) are above the critical limit of 5 kg N ha^{-1} yr^{-1} (Ministry for the Environment 2000). The deposition of NH_3 occurs within 4-5 km from source of emissions (Krupa 2003) and can cause eutrophication of aquatic bodies and acidification of soil (Saggar et al. 2004). Ammonia is the major contributor for the generation of secondary aerosols in the atmosphere (Aneja et al. 2001; Menz and Seip 2004), which are detrimental to human and animal health and also minimise visibility (Aneja et al. 2001). Ammonia also acts as an indirect source of nitrous oxide (N_2O) (Martikainen 1985). Mitigation measures that reduce these losses would improve N use efficiency on farms and reduce the economic losses and environmental impacts.

The urease inhibitor N-(n-butyl) thiophosphoric triamide (nBTPT), sold under the trade name Agrotain[®], has recently been identified as one of the more promising and effective approaches for reducing NH_3 emissions by inhibiting soil UEA when applied with urea fertiliser or cattle urine (Saggar et al. 2013). However, the inhibitory effect of nBTPT on soil UEA and NH_3 emissions is effective for 7-14 days (Saggar et al. 2009) during which NH_3 emissions from urea are inhibited. Therefore, to reduce the NH_3 emitted from the deposition of urine following each grazing event, regular applications of nBTPT are required. Micronutrients such as copper (Cu) and zinc (Zn) have been shown to inhibit soil UEA and reduce NH_3 emissions over a longer duration (4-12 weeks) compared to nBTPT. This inhibitory effect has been associated with level of Cu and Zn applied. For example, 4 weeks at 11 mg Cu kg^{-1} soil (Junejo et al. 2013), 4 weeks at 16 mg Cu or 33 mg Zn kg^{-1} soil (Khariri et al. 2016), 8 weeks at 50 mg Cu or Zn kg^{-1} soil (Wyszkowska et al. 2006), and 12 weeks at 200 mg Cu kg^{-1} soil (Hemida et al. 1997). These metal ions react with the sulfhydryl groups (cysteine, essential for proper functioning of urease enzyme) on the active site of the urease enzyme in a similar way to the formation of metal sulfides. Thus, insoluble sulfides forming metals cause inactivation or inhibition of soil UEA (Shaw and Raval 1961; Tabatabai 1977; Takishima et al. 1988). But, there is minimal NZ & international information on Cu and Zn application effects on soil UEA and NH_3 emissions. In our recent study (Adhikari et al. 2016) using 24 dairy farm soils of the Waikato region, with contrasting inherent Cu and Zn levels and soil organic carbon (C), there was no significant inverse relationships between inherent bioavailable Cu and Zn levels and soil UEA. The lack of such a relationship could possibly be attributed to complexation of these metals with soil organic C and reduced bioavailability. Previous studies also reported the high complexation of Cu and Zn with soil organic matter (Hodgson et al. 1966; Sauvé et al. 1997). However, there are few studies conducted to assess the role of soil organic C on the effectiveness of Cu and Zn to inhibit urea hydrolysis. Therefore, the objectives of this study were to i) assess the effect of adding different amounts of Cu and Zn to pasture soils on UEA and ii) investigate the role of soil organic C on influencing the effectiveness of added Cu and Zn on reducing urea hydrolysis.

Materials and Methods

Soil urease inhibition by Cu and Zn

Four dairy-grazed pasture soils (0-10 cm soil depth; two Manawatu soils [Recent (Rangitekei loamy sand, RLS) and Pallic (Tokomaru silt loam,TSL)], a Waikato soil [Typic Orthic Allophanic, Horotiu] and a Taranaki soil [Allophanic, Egmont black loam, EBL] with contrasting soil organic C (Table 1) were used to assess the effect of adding different amounts Cu and Zn to soils on UEA. Soil pH (1:2.5 soil: water ratio), and soil total C and total N (high temperature combustion) were measured as described by Blakemore et al. (1987). Pot maximum water holding capacity was determined using pressure plate apparatus (Loveday 1974). Exchangeable basic cations and cation exchange capacity (CEC) were measured following semi-micro leaching technique (Blakemore et al. 1987). Treatments included were 5, 10, and 20 mg Cu kg^{-1} soil, and 5 mg Cu + 5 mg Zn kg^{-1} soil. Copper sulphate ($CuSO_4.5H_2O$) and zinc sulphate ($ZnSO_4.7H_2O$) were used as a Cu and Zn source, respectively. Four replicates were included for each treatment.

Following treatment application, soil UEA was determined as the amount of urea hydrolysed with some modifications (incubated at 20°C instead of 37°C) of methods described by (Mulvaney and Bremner 1979; Zantua and Bremner 1975). Calcium nitrate ($Ca(NO_3)_2$) extractable Cu and Zn was determined on the soil (RLS soil) with the lowest organic C content after treatment application. Thirty mL of 0.05 M $Ca(NO_3)_2$ was added to 5 g moist soil treated with Cu and Zn, shaken on an end-over-end shaker for 2 hr and then centrifuged at 15000 rpm for 10 min. The soil supernatant solution was filtered using Whatman No. 42 filter papers and metal contents of the extract were measured (Cu at 327.395 nm and Zn at 481.053 nm) using microwave plasma atomic emission spectrometer (MP-AES).

Table 1 Selected physical and chemical properties of dairy-grazed pasture soils studied

Soil properties	Rangitekei loamy sand	Tokomaru silt loam	Horotiu	Egmont black loam
pH water	5.54	5.45	5.37	5.39
Pot maximum water holding capacity (%)	17.5	29.1	38.6	53.3
Total C (%)	1.81	3.02	4.60	9.67
Total N (%)	0.20	0.32	0.46	0.96
Exchangeable bases:				
Exchangeable K (meq 100 g^{-1})	0.32	0.21	1.47	0.58
Exchangeable Ca (meq 100 g^{-1})	5.80	7.11	7.72	12.25
Exchangeable Mg (meq 100 g^{-1})	0.79	1.34	1.54	1.88
Exchangeable Na (meq 100 g^{-1})	0.10	0.17	0.19	0.32
CEC (meq 100g^{-1})	15.1	20.4	29.1	42.9

Soil supernatant study

A study was conducted to determine whether the effectiveness of Cu and Zn at inhibiting soil UEA by using soil supernatant from 2 dairy farm soils (RLS and EBL soils). This was conducted to test whether reducing the potential for Cu to complex with soil organic C would increase Cu bioavailability and improve its effectiveness at inhibiting urea hydrolysis. Soil supernatant was extracted by adding 0.01 M potassium sulphate (K_2SO_4) (2:1, 0.01 M K_2SO_4:soil) to the moist soil with 80% of pot maximum water holding capacity and shaken (end-over-end shaker) for half an hour and allowed for settle down (2 hrs and 4 hrs for RLS

and EBL soils, respectively). The 0.01 M K_2SO_4 was used for extraction because it has same ionic strength as the soil solution, and does not interfere with the soluble C measurement. Glucose (^{12}-C labelled) was added to the supernatant at the rate of 2 mg C g^{-1} soil and pre-incubated for 5 days at 20°C to build up microbial activity and thereby urease enzyme activity, prior to treatment application. Soluble C content in the supernatants were measured, using a TOC Analyser (Analytik Jena, Multi N/C 3100/1) before treatment application, and had values of 1580.68 mg C kg^{-1} soil for the RLS and 1551.22 mg C kg^{-1} for the EBL soil supernatants. Two urea N application rates, 120 mg N kg^{-1} soil and 600 mg N kg^{-1} soil, were selected and urea hydrolysis on each soil supernatant was measured as the amount of ammonium (NH_4^+-N) present. The treatments used were urea, 5 mg Cu kg^{-1} soil + urea (5-CuU), 10 mg Cu kg^{-1} soil + urea (10-CuU), 20 mg Cu kg^{-1} soil + urea (20-CuU), and 20 mg Zn kg^{-1} soil + urea (20-ZnU). Copper sulphate and $ZnSO_4.7H_2O$ were used as a Cu and Zn source, respectively. Four replicates were included in each treatment. Soil supernatants were analysed for $NH4^+$-N, nitrate (NO_3^--N), $Ca(NO_3)_2$ extractable Cu and $Ca(NO_3)_2$ extractableZn on day 1, day 2, day 4, day 8, day 16 and day 32 following treatment application. The temperature was maintained at 20°C throughout the experimental period. All the analyses were also measured on soil supernatants without addition of glucose (N =120 mg kg^{-1} soil) and supernatants without urea or, Cu and Zn.

Statistical analysis
The data were analysed using Analysis of Variance (ANOVA) and Kruskal–Wallis test (for data set not meeting normality requirement even after transformations) to detect any significant difference and different means in the treatments were compared using Duncan Multiple Range Test (DMRT) and Bonferroni (Dunn) t tests, respectively. All the analyses were conducted using Statistical Analysis System software (SAS 9.4).

Results

Soil urease inhibition by Cu and Zn
There was no significant reduction in soil UEA from any of the Cu and Zn treatments (Table 2). Soil UEA (control) values (16.81, 22.2, 51.63 and 61.54 mg kg^{-1} soil h^{-1}) increased with corresponding increase in soil organic C values (1.81, 3.02, 4.60 and 9.67 %) for RLS, TSL, Horotiu and EBL soils, respectively. To explore the reasons for the ineffectiveness of these metals at reducing soil UEA, $Ca(NO_3)_2$ extractable Cu and Zn in soil with the lowest organic C content (RLS soil) were measured after treating soil with these metals. The results of $Ca(NO_3)_2$ extractable Cu and Zn are shown in Table 3. Most of the added Cu, > 95% was not $Ca(NO_3)_2$ extractable but Zn remained highly $Ca(NO_3)_2$ extractable, 75.2% of Zn added.

Table 2 Effect of Cu and Zn application on soil urease enzyme activity of dairy-grazed pasture soils

Cu (mg kg^{-1} soil)	Soil urease enzyme activity (mg kg^{-1} soil h^{-1}) at 20^0C			
	Rangitekei loamy sand	Tokomaru silt loam	Horotiu	Egmont black loam
0	16.81	22.2	51.63	61.54
5	16.39	22.23	51.6	62.15
10	17.14	21.67	47.88	63.31
20	17.83	23.1	48.24	61.45
5+5 (Cu+Zn)	16.91	21.31	49.22	63.34

Table 3 Ca(NO$_3$)$_2$ extractable Cu and Zn after addition of these metal on the Rangitekei loamy sand soil

Cu (mg kg^{-1} soil)	Ca(NO$_3$)$_2$ extractable metals (mg kg^{-1} soil)	
	Cu	Zn
0	-	0.49
5	0.11	0.75
10	0.21	0.67
20	0.79	0.72
5+5 (Cu+Zn)	0.28	4.25

Soil supernatant study

The highest Cu treatment, 20-CuU, significantly reduced urea hydrolysis by 65.2% and 87.3% for the two N rates, 120 and 600 mg N kg^{-1} soil, respectively, after 32 days of treatment application to the RLS soil supernatant (Figs. 1 and 2). In comparison, the 20-CuU treatment significantly reduced urea hydrolysis by 49.9% and 54.8% for the two N rates, 120 and 600 mg N kg^{-1} soil, respectively, up to 16 days following treatment application to the EBL soil supernatant (Figs. 3 and 4). Overall, zinc had little influence on reducing urea hydrolysis.
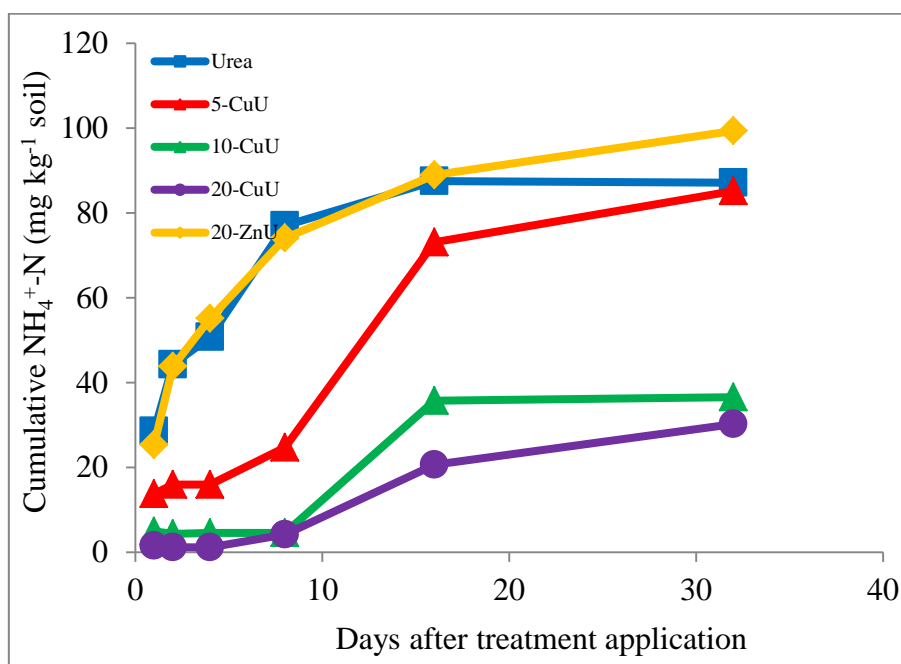

Fig. 1 Effect of Cu and Zn on mean cumulative NH$_4^+$-N concentration on Rangitekei loamy sand soil supernatant throughout the incubation period as affected by urea (N = 120 mg kg^{-1} soil) application

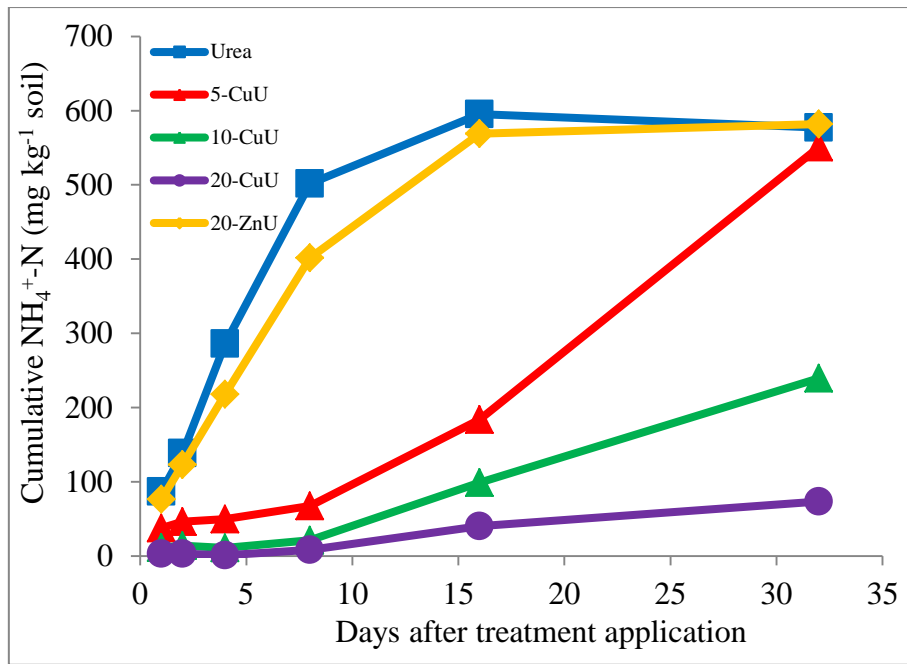**Fig. 2** Effect of Cu and Zn on mean cumulative NH_4^+-N concentration on a Rangitekei loamy sand soil supernatant throughout the incubation period as affected by urea (N = 600 mg kg^{-1} soil) application
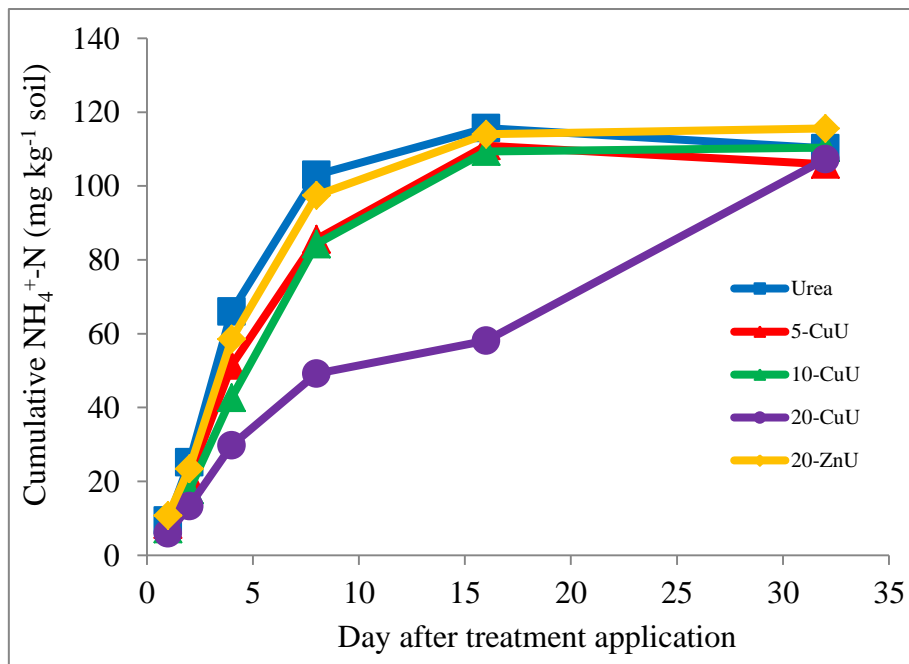

Fig. 3 Effect of Cu and Zn on mean cumulative NH_4^+-N concentration on the Egmont black loam soil supernatant throughout the incubation period as affected by urea (N = 120 mg kg^{-1} soil) application
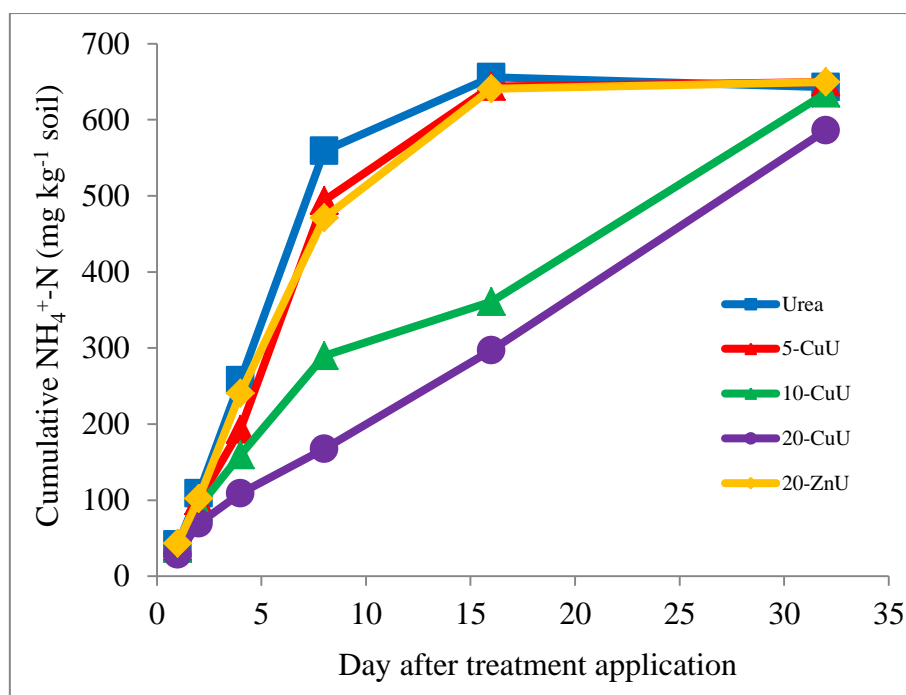
Fig. 4 Effect of Cu and Zn on mean cumulative NH_4^+- N concentration on the Egmont black loam soil supernatant throughout the incubation period as affected by urea (N = 600 mg kg^{-1} soil) application

Nitrate levels in the soil supernatants were also monitored to see the treatments effect on microbial growth. Higher NO_3^--N level in soil supernatants indicates reduced microbial growth and vice-versa, as microorganisms consume NO_3^--N for their growth and development. Activities of soil microorganisms are enhanced by the addition of organic substrates thereby accelerating the mineralisation of native soil organic matter (positive priming effect), mainly in combination with N supply. Nitrate levels were significantly higher in Cu treatments than the urea treatment for the N rate 120 mg N kg^{-1} soil, in both soil supernatants except in EBL soil supernatant for day 1 after treatments application (Tables 4 and 5). A similar trend was found for NO_3^--N levels at N rate 600 mg N kg^{-1} soil in both soil supernatants.

Table 4 Effect of Cu and Zn on mean NO_3^--N concentration on the Rangitekei loamy sand soil supernatant throughout the incubation period as affected by urea (N = 120 mg kg^{-1} soil) and C (glucose) application

	NO_3^--N (mg kg^{-1} soil)					
Treatments	Day 1	Day 2	Day 4	Day 8	Day 16	Day 32
Urea	9.99 [d]	1.49 [d]	1.12 [c]	-	-	-
5-CuU	22.37 [b]	14.66 [b]	7.31 [b]	-	-	-
10-CuU	25.59 [a]	18.78 [a]	12.16 [a]	-	-	-
20-CuU	25.95 [a]	19.82 [a]	11.83 [a]	-	-	-
20-ZnU	18.52 [c]	6.78 [c]	1.49 [c]	-	-	-

Means followed by different small letters in a column are significantly different by DMRT test

Table 5 Effect of Cu and Zn on mean NO_3^--N concentration on the Egmont black loam soil supernatant throughout the incubation period as affected by urea (N = 600 mg kg^{-1} soil) and C (glucose) application

Treatments	NO_3^--N (mg kg^{-1} soil)					
	*Day 1	Day 2	Day 4	Day 8	Day 16	Day 32
Urea	88.68 [a]	67.92 [b]	13.07 [d]	-	-	-
5-CuU	85.71 [a]	70.42 [ab]	17.92 [c]	-	-	-
10-CuU	86.11 [a]	74.99 [a]	43.48 [b]	-	-	-
20-CuU	86.92 [a]	76.07 [a]	58.00 [a]	3.36	-	-
20-ZnU	85.13 [a]	66.38 [b]	10.38 [d]	-	-	-

Means followed by different small letters in a column are significantly different by DMRT test
Days with * indicate means followed by different small letters in a column are significantly different by Bonferroni (Dunn) t Tests

Discussion

Soil urease inhibition by Cu and Zn
The rates of Cu and Zn used in this study, added to all soils [RLS, TSL, Horotiu and EBL] were not effective at inhibiting soil UEA. In another recent study, Guimarães et al. (2016) reported that Cu and Zn (applied at 2 and 10 mg kg^{-1} soil) were not effective at significantly inhibiting soil UEA in pasture soil. The ineffectiveness of the Cu was likely attributable to the high organic C content of the dairy-grazed pasture soils used, which promoted immobilisation of metal ions through adsorption and chelation (Arias et al. 2006; Vytopilova et al. 2015). In the present study, < 5% of Cu added was measured as $Ca(NO_3)_2$ extractable Cu in the RLS soil, supporting low bioavailability of the added Cu being a cause of its ineffectiveness. Even though the bioavailability of added Zn remained high (75.2% of Zn added was $Ca(NO_3)_2$ extractable), it was still ineffective at inhibiting UEA. Wyszkowska et al. (2006) also found that Zn did not show significant inhibitory effects on soil UEA, even when applied at a rate of 50 mg kg^{-1} soil.

Soil supernatant study
The use of soil supernatants with low organic C content reduced the complexation of Cu with soil organic C and enhanced Cu bioavailability. This resulted in the higher Cu treatments reducing the rate of UEA and urea hydrolysis for both the RLS and EBL soil supernatants. The effectiveness of Cu was sustained longer in the RLS soil supernatant, compared to the EBL soil supernatant. This is attributed to faster microbial growth in EBL soil supernatant 2 days after treatment application, could indicate greater Cu immobilisation rates because of higher availability of NO_3^--N in EBL soil supernatant than RLS soil supernatant. Copper inhibits soil UEA and reduces urea hydrolysis not only by deactivating extracellular urease activity but also by supressing microbial growth. This is supported by evidence of higher NO_3^--N levels on soil supernatant treated with Cu as a result of less consumption of NO_3^--N by reduced microbial growth.

Urease activity on soil supernatants with added C in this study is cumulative effect of extracellular urease and microbial urease (intracellular) which can be evidenced by lower urea hydrolysis on urea treatments without addition of C (48.59 and 36.26% less relative to urea treatment with glucose in RLS and EBL soil supernatants respectively, N = 120 mg kg^{-1} soil). Soil UEA could be differentiated into two parts; microbial urease directly linked with

microorganisms and adsorbed urease, apparently adsorbed on soil colloids (Paulson and Kurtz 1969). Intracellular and extracellular urease activity represented 54 and 46% of total urease activity, respectively (Klose and Tabatabai 1999).

Overall, the results from this study indicated that Cu was more effective than Zn at inhibiting soil UEA and urea hydrolysis. Similar results have been reported in previous studies (Hemida et al. 1997; Tabatabai 1977) stating that Cu was more effective than Zn at reducing soil UEA. This has been attributed to Cu having a higher affinity for amino groups that occur in both urea and urease (Daif and Van Beusichem 1981). Hughes et al. (1969) reported that the Cu ion forms a more stable metal sulphide with the sulfhydryl group of urease than the Zn ion, and, therefore is a stronger urease inhibitor.

Conclusions

Copper added to dairy-grazed pasture soils had low bioavailability and, consequently, was not effective at reducing UEA. When Cu bioavailability was increased by reducing soil organic C in the soil supernatant experiment, urea hydrolysis was inhibited. Although most of the Zn added was bioavailable, the observed levels of bioavailable metal had limited effect on soil UEA. Therefore, neither Cu nor Zn will be practical urease inhibitors for NZ dairy-grazed pasture soils.

Acknowledgements

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