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RESEARCH

The role of an *Ascophyllum nodosum* extract in lowering the environmental impact and improving nitrogen use efficiency in pasture systems under a reduced nitrogen regime

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Abstract

Nitrogen is a macronutrient that is applied in substantial amounts as a chemical fertiliser to conventional agricultural systems. However, loss of nitrogen from agricultural systems to the environment, in the form of gases (N_2O) and leachate (NO_3), is detrimental to the environment. As such, improving the nitrogen use efficiency (NUE) of crops is an essential component of sustainable agriculture. Biostimulants are a category of products that improve NUE when applied to crops and/or soil. Here, we examined the effects of PSI-362, an *Ascophyllum nodosum* biostimulant mixed with and applied on calcium ammonium nitrate (CAN) granules, on grass yield, quality traits, and environmental impacts in lysimeter trials under natural weather conditions. By using PSI-362, it was possible to reduce nitrogen application by 25% without a significant loss in yield compared to a conventional 100% N system. We also observed an increase in NUE under a 75% N with PSI-362 co-application fertilisation regime, and a significant increase in crude protein content compared to the 100% N fertilisation controls. Furthermore, this reduced nitrogen application also resulted in reduced nitrogen loss through leachate (NO_3) and N_2O to the atmosphere. The total NUE for PSI-362 + fertiliser granules ranged from 58.7% to 78.6% depending on the biostimulant dose, which was significantly better than the fertiliser alone. Our results showed that this biostimulant, which can easily be incorporated into conventional agronomic practices, allows for a more sustainable approach to pasture-based systems.

Keywords Nitrogen use efficiency · *Ascophyllum nodosum* · Biostimulant · Greenhouse gas emissions · Grassland productivity · Sustainability

Introduction

The Green Revolution in agriculture in the middle of the last century enabled food production to keep pace with an increasing world population (Khush 2001). The resulting increase in food security was instrumental in preventing mass starvation. This Green Revolution was based on a number of principles including the use of improved, high-yielding crop varieties and more efficient agronomic practices, such as the application of chemical fertilisers. While its economic and social impacts have broadly been positive, its impact on the environment has been less so, resulting in declining water quality, soil degradation and increases in emissions of greenhouse gases (GHGs) (Pingali 2012). Many of these negative traits are associated with the excessive input of nitrogen into farming systems.

Agriculture is a major emitter of GHGs and accounts for up to a quarter (along with land use change) of all global anthropogenic emissions (Gołasa et al. 2021;

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Laborde et al. 2021). The major GHGs associated with agriculture include methane (CH_4), nitrous oxide (N_2O), and to a lesser extent carbon dioxide (CO_2). However, the levels of each of these gases and their warming potential will depend on the farming system (e.g., arable vs. dairy) and the type of gas, respectively. N_2O is the major GHG associated with the application of nitrogen in agriculture, and direct emissions of N_2O from agriculture across Europe make up almost 12% of total agricultural emissions (Boix-Fayos and de Vente 2023). Global N_2O emissions have been estimated to be 17 Tg N year^{-1}, with natural sources from soils and oceans accounting for 57% of total emissions over the last decade and the remaining 43% arising from anthropogenic sources (Tian et al. 2020). Agriculture is by far the largest anthropogenic source of N_2O, accounting for up to 90% of total anthropogenic emissions (De Klein and Eckard 2008). N_2O is considered a stock GHG due to its long residency time in the atmosphere (114 years) and a global warming potential that is 298 times higher than CO_2 (Signor and Cerri 2013). Its level within the atmosphere has risen from 270 ppb in pre-industrial times to 331 ppb in 2018 (Tian et al. 2020).

N_2O is emitted from the soil via microbial nitrification and denitrification processes which will be influenced by soil conditions such as soil temperature, moisture content and aeration (Signor and Cerri 2013). Butterbach-Bahl et al. (2013) detailed some of the processes, pathways and variables that affect N_2O emissions from soils, including soil temperature and moisture (the rate of water-filled pore space), and microbial diversity and activity. Strategies to reduce N_2O emissions from soils include fertiliser management (rate, source, and timing), soil management, (e.g., draining wetter soil to prevent N_2O emissions via denitrification, application of nitrification inhibitors (De Klein and Eckard 2008)), as well as using biological N fixation to provide N inputs (Rees et al. 2013). In addition, the increased application of nitrogen to agricultural land has led to excess N leaching to surface and ground waters, leading to increased eutrophication and incidences of algal blooms (Wurtsbaugh et al. 2019; Basu et al. 2022).

Globally, for every 100 units of nitrogen used in agriculture, only 17 are ultimately consumed by humans as dairy, crop or meat products (Braun 2007). Therefore, improving nitrogen use efficiency (NUE) will enhance food security as well as mitigating the environmental damage (including climate change) associated with synthetic nitrogen fertilisers. Current strategies for improving NUE include modifying the soil chemistry (e.g., remedying the pH to correct for acidity), using controlled release fertiliser (or NH_4/NO_3 inhibitors), adopting an appropriate soil management strategy (correct fertiliser type, rate, method and timing of application), using nitrogen efficient genotypes and planting more nitrogen fixing crops (Fageria and Baligar 2005).

In recent years, the "seeds" of a new Green Revolution have started to germinate; these include the New Green Deal in the USA and the European Green Deal (along with its associated strategies such as Farm to Fork, Biodiversity Strategy and the Soil Strategy). The driving force for these initiatives is the urgent need to reduce GHG emissions to prevent further a rise of global temperature. At the heart of this new Green Revolution is the need for sustainable agriculture and sustainable food production. For example, key targets in the European Green Deal (and its associated strategies) include a target of 25% of agricultural land to be under organic farming, a 50% reduction in pesticide usage, and a 20% reduction in the use of fertilisers (European Commission 2020). However there are key challenges associated with the transition to more sustainable farming; chief amongst these is the need to maintain yields to support a growing world population that is expected to peak at *ca.* 9.7 billion by 2064 (Vollset et al. 2020). Bio-based products like biostimulants have been shown to improve crop performance, including NUE, and they can form part of the strategy to support the transition to more sustainable farming practices (Tolisano and Del Buono 2023). Plant biostimulants can be divided into various classes depending on origin, and include humic acids, chitosan, protein hydrolysates, seaweed extracts, inorganic compounds, and beneficial fungi and bacteria (Du Jardin 2015). The EU Regulation 2019/1009, defines a biostimulant as: "A plant biostimulant shall be an EU fertilising product the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: (i) nutrient use efficiency, (ii) tolerance to abiotic stress, (iii) quality traits, and (iv) availability of confined nutrients in the soil or rhizosphere" (Regulation 2019).

There is a long history of seaweeds or seaweed-based products being used in agriculture (Craigie 2011; Tiwari and Troy 2015). Liquid seaweed extracts account for 33% of the total worldwide biostimulant market (El Boukhari et al. 2020). While there are over 9,000 species of macroalgae, they are broadly classified into three main types based on their pigmentations: brown, red, and green algae. Brown seaweeds are the second most abundant group, but are the most widely used in agriculture, of which *Ascophyllum nodosum* is the most researched (Khan et al. 2009) with a number of research papers detailing its effect (and possible mode of action, including enhanced metabolism and dysregulation of various stress responsive genes and genes associated with nutrient uptake) on plant quality, growth, yield, nutrient acquisition and mitigation of abiotic stress (Shukla et al. 2019; De Saeger et al. 2020; Pereira et al. 2020). Many of these positive effects of *A. nodosum* extracts (ANEs) have been attributed to their bioactive compounds such as laminarin, fucoidan and alginate. Conversely the composition of these polysaccharides will depend on harvesting dates as well as extraction techniques (Rioux et al. 2009; Sharma et al. 2014). It has also been shown that the type of ANE can affect its performance traits in the field, including how it affects NUE (Łangowski et al. 2022). Much of the research of ANEs on plant growth and yield has been confined to high value crops such as tomato, citrus, and cereals due to their traditional mode of application (foliar spray and fertigation) with fewer studies on grass. Additionally, we are not aware of any study involving biostimulants and their potential role in mitigating nitrogen losses to the environment through enhanced use efficiency in grass. In this study, the effects of the algal biostimulant, PSI-362 on NUE in a commercial grass seed mixture under reduced N fertilisation, including its impact on N_2O emissions and nitrate leachate were investigated. Our results showed that PSI-362 has a positive impact on NUE under reduced N fertilisation without significant yield penalties, and consequently results in reduced N loss through leachate (NO_3) and N_2O to the atmosphere, thereby decreasing the environmental footprint of the pasture system.

Materials & methods

PSI-362 coated granular N fertiliser

PSI-362 (a water soluble biostimulant extracted from the brown seaweed, *Ascophyllum nodosum,* supplied by Brandon Bioscience, Ireland) was mixed with and applied on granular calcium ammonium nitrate (CAN) fertiliser (27% N + 4% S) and allowed to air dry, as previously described by Quille et al. (2022). It was applied at two different rates: 1.0x, and 1.5x (the 1.0x rate refers to the rate on the commercially available products; Supplementary Figure 1). Compositional analysis of PSI-362 was previously reported by Goñi et al. (2021) and contained 43.7 % (w/w) ash, 26 % (w/w) carbohydrates, 12.3 % (w/w) polyphenols, and 18 % (w/w) other organic compounds. The N:P:K macronutrient content of PSI-362 was 0.4:0.1:8.0 (Goñi et al. 2021). For the purposes of this study, the N content of PSI-362 was found to be 0.4% w/v (ranging from 0.8 g to 2.4 g ha^{-1} depending on application rate) while the carbon content of the applied biostimulant ranged from 40 g to 120 g ha^{-1} depending on application rate.

Modified lysimeter grass trial setup and treatments

The lysimeters were located on the south campus of Munster Technological University (coordinates: 52.271495, -9.690538). Four different soils (2 x loam and 2 x silt loam) were collected from four different dairy enterprise farms in Co. Kerry, Ireland in 2013 (site coordinates are given in Supplementary Table 1). Each treatment comprises 3 lysimeter replicates for each of the four soils. Soils were classified for sand, silt and clay content (Supplementary Table 1) using the dispersion (sodium hexametaphosphate method) particle size distribution test (Kroetsch and Wang 2008) which involves digesting the soil organic matter with hydrogen peroxide before using sodium hexametaphosphate to increase soil dispersion so the fine earth particles of sand, silt and clay can be determined by sieving and weighing. The modified lysimeters (Supplementary Figure 2) were filled by placing 30 kg of soil (taken from the A horizon (the first mineral soil horizon below the soil surface) to a max depth of 45 cm) into a 20-litre lysimeter (surface area of 0.003 m^2 per lysimeter). To ensure adequate drainage, a grommet was placed at the bottom of the lysimeter, over which a layer of coarse sand and pea gravel was added (about 6 to 9 cm) before the soils were added on top. The grommet and associated plumbing (Supplementary Figure 3) also allowed for leachate collection. All soils were kept under permanent grass growth until 2019 when reseeding commenced with a commercial grass seed mixture, Diamond Hi Digestibility (*Lolium perenne)* varieties: Astonenergy (29%), Meiduno (23%), Oakpark (21%), Astonking (21%), and white clover (*Trifolium repens*): Crusader (6%); supplied by Kellihers Feed and Agricultural Supplies, (Tralee, Co. Kerry, Ireland) applied at a rate of 34.6 kg ha^{-1} (or 14 kg acre^{-1}) in September 2019. Target Fertilisers (Enniscorthy, Ireland) supplied all granular fertilisers used in the study except for protected urea which was distributed by Grassland Agro (Cork, Ireland) and sourced from Kerry Agribusiness (Charleville, Co. Cork, Ireland).

Treatments for the lysimeter trials included two rates (1.0x and 1.5x) of PSI-362 mixed with calcium ammonium nitrate (CAN) and applied to the sward under the following N regimes: (1) 0% N (no fertiliser applied), (2) CAN (100% N), (3) CAN (75% N), (4) PU (75% N, protected urea with the urease inhibitor that contains the active ingredients of *N*-(*n*-butyl)thiophosphoric triamide and N-(n-propyl) thiophosphoric triamide), (5) CAN (75% N + 1.0x PSI-362), and (6) CAN (75% N + 1.5x PSI-362). N application rates were taken from Wall and Plunkett (2021) (Supplementary Table 2), where rates for the 100 % N positive control is equal to an annual application rate of 306 kg of N ha^{-1}. Treatments for each month were hand applied after weighing granules of each respective fertiliser per lysimeter. During the dry months (from April to August) an irrigation regime was established where each lysimeter received a maximum of 300 ml of water three times daily. The irrigation regime was modified where appropriate to ensure a consistent water filled pore space (WFPS) for all lysimeters of 70 to 80% using the following equation (Fichtner et al. 2019):

$$\mathrm{WFPS} = \frac{SWC}{1 - \frac{BD}{PD}} \times 100$$

where SWC =volumetric soil water content, BD =bulk density (g mL^{-1}) and PD =particle density (g mL^{-1}).

SWC was determined by drying overnight at 80 degrees in a convection oven until a constant weight, BD was determined by dividing the dry mass by the volume of soil taken, and PD was assumed to be 2.65 g cm^{-1}.

Lysimeter soil nutrient analysis

Prior to experiments beginning in 2021, all soils were analysed for pH using the SMP buffer test, and P and K content via Morgan's extraction method using colorimetric and atomic spectroscopy, respectively (Shoemaker et al. 1961) by Southern Scientific Services (Farranfore, Co. Kerry, Ireland; Supplementary Table 1).

Weather data

Weather (rainfall and soil temperature) data (Supplementary Table 3) were collected weekly on site using a rain gauge and a soil thermometer (Amarell, VWR, Ireland). Soil temperature was taken to a depth of 10 cm.

Grass growth rate, yield measurement and nitrogen content

The experiment was setup to simulate a rotational pasture grazing system in terms of fertiliser application timing and rates, as well as date of harvesting. Grass was harvested from March to October in 2021 and 2022. At the beginning of both years, grass in all lysimeters were cut to a height of 4 to 6 cm from the soil level. Fertiliser was applied according to the rates listed in Supplementary Table 2. Application rates were based on an intensive livestock system stocked at rates of 2.47 livestock units ha^{-1} (Wall and Plunkett 2021). The grass growing period per rotation (harvest) varied from 18 to 30 days depending on seasonal growth rates. Grass in lysimeters was harvested at the 3-leaf growth stage (≈ 1200 to 1600 kg of dry matter (DM) ha^{-1}) to simulate maximum utilisation efficiency under a pasture grazing system at 4 to 6 cm in height (Curran et al. 2010). Grass was collected after each harvest/rotation in a plastic bag and weighed. Dry weight (DW) was calculated by taking representative samples after each harvest and drying overnight in a convection oven at 100°C. The DW was then used to calculate yield (kg DM ha^{-1}) by multiplying the area of the lysimeter to 10,000 m^2 (1 hectare). Samples were also taken for determination of crude protein (CP) after each harvest. CP was determined using an N analyser (Leco CNS 928 Analyzer; Leco, USA). Subsequent to harvesting, fertiliser was applied to all lysimeters within 48 h according to rates listed in Supplementary Table 2, once again to simulate typical agronomic activity, before the pasture cycle continued once more.

Nitrate leachate collection and analysis

Leachate was collected from the months of April until December in 2022 for two soils (1 x loam and 1 x silt loam as representative samples; Soil 1 and Soil 3 from Supplementary Table 1; 3 replicates for each treatment and soil type). Leachate was collected from each lysimeter via the fitted grommet at the bottom of the lysimeter and were held in a collection bottle attached via a tube and fitting (see Supplementary Figure 3). Samples were collected at least on a weekly basis, or more frequently during times of high rainfall, as required. Samples were either frozen or analysed within 24 h for nitrate via the salicylic acid-sulphuric acid method, which involves the nitration of salicylic acid under acidic conditions, before determining absorbance of the yellow chromophore at 410 nm (Zhao and Wang 2017). A standard curve was constructed using potassium nitrate ranging from 0.05 to 2 mM to determine the concentration of nitrate in the leachate samples.

Nitrous oxide sampling and analysis

Nitrous oxide (N_2O) was collected via the static chamber technique (as described by Zaman et al. 2021) for 2 soil types (1 x loam and 1 x silt loam; same soils used for leachate nitrate analysis. Specially fabricated chambers were constructed to fit over the lysimeters during gas collection and provide an airtight seal (Supplementary Figure 4). These chambers had a height of 45 cm with a diameter of 30 cm giving a total volume of 27.17 L. Gas sampling took place between 10.00 to 14.00 hrs for each collection. Gas samples were taken at 0, 15, 30 and 45 min (Rahman et al. 2021) after the closure of the chamber using a 20 mL syringe and Luer lock attached to both the syringe and chamber. Prior to sampling the tube and chamber was repeatedly flushed 10x with the syringe to ensure mixture of the gasses contained within. Samples were then injected into evacuated 12-mL glass vials (Exetainers, Labco, UK), stored under ambient conditions before N_2O analysis using gas chromatography (Scion Instruments; The Netherlands) equipped with a ^{63}Ni electron capture detector with high-purity helium as the carrier gas. N_2O flux was calculated according to the following equations taken from the Kellogg Biological Station (Kahmark et al. 2023):

$$a_m = \left(a_v \times M \times P\right)/(R \times T)$$

where a_m is the flux expressed in terms of ug N L^{-1} min^{-1}; a_v is the slope for T0 to T45 against the concentration change in N_2O; M is the molar mass of the gas (28 g of N_2O-N mol^{-1}); P is the pressure of the atmosphere (measured in atm); T is the temperature (K); and R is the gas constant (0.0821 L.atm/mol.K). Gases were sampled in 2022 around the application of fertiliser; 24 h pre fertiliser application and 24 and 48 h post fertiliser application. Samples were taken in 2022 between the months of April and September.

Nitrogen use efficiency and total emissions calculations

Nitrogen use efficiency (% NUE) was calculated by subtracting the determined N in grass from the unfertilised lysimeters (N_0) from the determined N in the various treated lysimeters (N_x), and dividing this figure by kg of N applied: [(grass yield at N_x - grass yield at N_0)/kg of N applied at N_x]*100 (Zemenchik and Albrecht 2002).

Statistical analysis

Statistical analysis was carried out using the XLSTAT statistical software (Lumivero, Paris, France) for Microsoft Excel. Where appropriate tests ranged from the t-test for two independent samples (when comparing the means of two independent groups) to Multiple (pairwise) comparisons using Tukey's Honest Significant Difference (HSD) test. Where appropriate, a false discovery rate correction (0.05) test was applied. A two-way ANOVA was used for correlations between treatments, soil type and grass measurements (yield, crude protein, NUE) and nitrous oxide and nitrate. In the text, the term 'significant' refers to $p \leq 0.05$. Unless stated otherwise, all data are expressed as average ± standard error of mean (SEM). Details of the individual sample size for each analysis and statistical test used are mentioned in the table and figure legends.

Results

Nitrogen response curve

The impact of increasing nitrogen application on grass yield in the lysimeters was assessed by generation of a nitrogen response curve. As anticipated, there was a clear correlation between nitrogen applied and total grass dry matter yield was observed during 2021 and 2022 for both soil types ($R^2 = 0.987$ for loam and $R^2 = 0.998$ for silt loam; Supplementary Figure 5). The increased grass yield response was linear up to 100% nitrogen, with no evidence of any plateau at the top end of the graph.

Grass dry matter yields

The average grass biomass (dry matter) yield from the lysimeter trials in 2021 and 2022 was found to be significantly impacted by fertiliser treatment and soil type, with no significant interaction between these two variables (Table 1). Supplementary Table 4 provides further information on the individual yearly grass yield for each soil type. For both soils, the biggest effect on yield was the level of nitrogen

Table 1 Comparisons between nitrogen fertiliser treatments and soil type on yield, crude protein, % NUE, nitrate leaching and emissions (N_2O and CO_2)

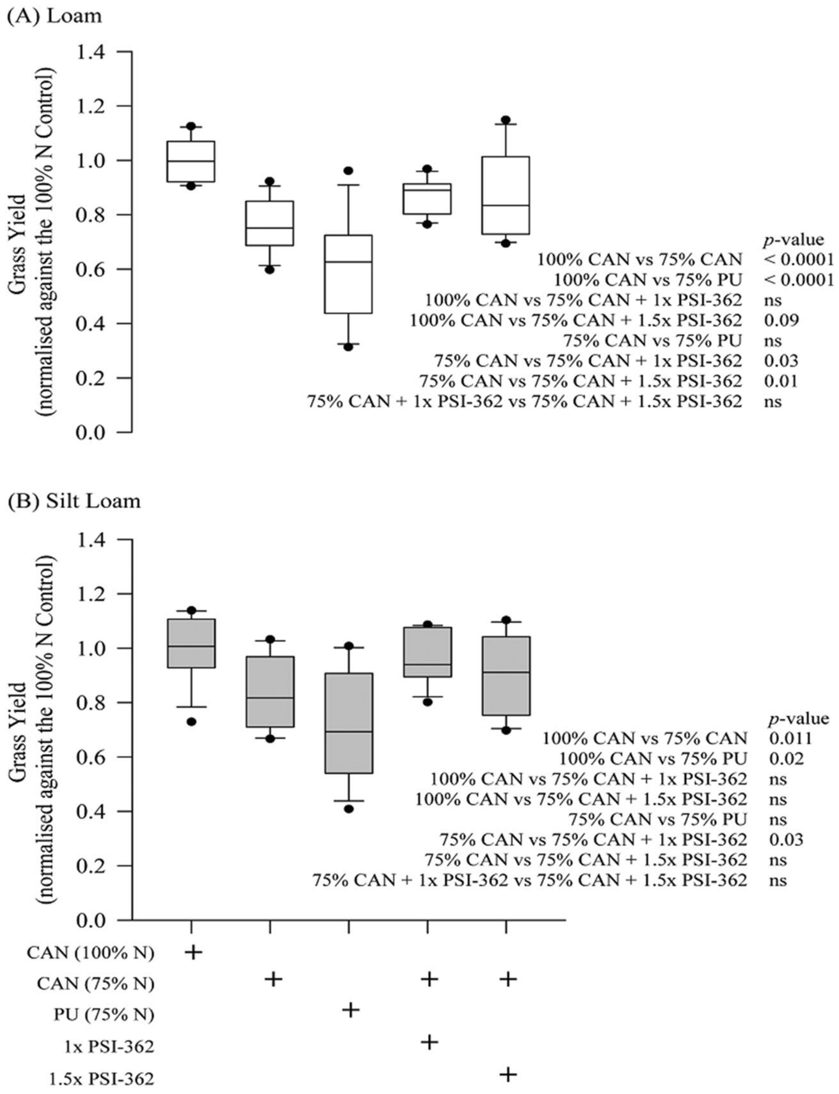
Factor	Dry Biomass Yield kg DM ha^{-1}	Crude Protein kg ha^{-1}	% NUE	N_2O g ha^{-1} day^{-1}	NO_3 kg ha^{-1}	CO_2 kg ha^{-1} day^{-1}
Treatment (T)	***	***	***	***	ns	ns
Soil Type (ST)	***	***	***	*	ns	ns
T x ST	ns	***	***	ns	ns	ns
Treatment (T)						
0% N	4395 ± 458^{d}	110 ± 6^{a}	N/A	29.3 ± 3.9^{b}	23.9 ± 2.4^{a}	1.34 ± 0.19^{a}
PU (75%N)	7974 ± 554^{c}	154 ± 9^{ab}	26.9 ± 2.7^{a}	47.9 ± 6.7^{b}	32.9 ± 1.7^{a}	1.54 ± 0.19^{a}
CAN (75% N)	9662 ± 387^{b}	167 ± 10^{b}	25.0 ± 2.5^{a}	41.7 ± 6.0^{b}	31.9 ± 2.5^{a}	1.50 ± 0.19^{a}
CAN (75% N) + 1.0x PSI-362	11133 ± 399^{a}	255 ± 19^{c}	58.7 ± 2.7^{b}	44.1 ± 4.9^{b}	37.5 ± 2.8^{a}	1.78 ± 0.22^{a}
CAN (75% N) + 1.5x PSI-362	10707± 409^{ab}	292 ± 16^{d}	70.6 ± 2.7^{c}	42.1 ± 3.7^{b}	35.7 ± 3.5^{a}	1.64 ± 0.21^{a}
CAN (100% N)	12116 ± 393^{a}	251 ± 10^{c}	46.3 ± 2.5^{d}	84.4 ± 14.8^{a}	41.8 ± 5.6^{a}	1.75 ± 0.22^{a}
Soil Type (ST)						
Loam	8176 ± 1137 ^{a}	170 ± 7 ^{a}	36 ± 2.5 ^{a}	41.8 ± 3.8 ^{a}	34.0 ± 2.1 ^{a}	1.26 ± 0.09 ^{a}
Silt Loam	10486 ±1155 ^{b}	263 ± 11 ^{b}	53 ± 3.3 ^{b}	54.7 ± 5.6 ^{b}	36.4 ±2.4 ^{a}	1.93 ± 0.12 ^{a}

Non-significant (ns), Significance: *** ($p \leq 0.001$), ** ($p \leq 0.01$), * ($p \leq 0.05$). Different letters indicate statistical differences at $p \leq 0.05$ based on One-way ANOVA Student–Newman–Keuls method. Values represent mean ± SEM applied. The 100% N control (applied as CAN) consistently outperformed the 75% N (applied as CAN) with *p*-values of <0.0001 and 0.011 for loam and silt loam, respectively (Figures 1A and 1B). Grass yields from 75% N (applied as protected urea, PU) appeared to be lower when compared to 75% N CAN but the differences were not significant.

Co-application of PSI-362 at 1.0x and 1.5x the recommended rate at 75% N (applied as CAN) increased yield across both soil types compared to the 75% N (applied as CAN). These increases are significant at the recommended rate of 1.0x concentration for both soil types with greater yields of 15 and 16 % (1270 and 1672 kg of DM ha^{-1}) for loam and silt loam soils, respectively (Figures 1A and 1B). Although greater yields of 14 % and 8 % (1217 and 872 kg of DM ha^{-1}) for loam and silt loam soils, respectively were observed when PSI-362 was co-applied at the higher rate (1.5x the recommended rate), this increase in yield was only significant for loam soil when compared to the 75% N (applied as CAN) treatment (Figures 1A and 1B). In addition, grass yields under a fertilisation regime of CAN (75% N) + 1.0x PSI-362 were not significantly reduced when compared to 100% N CAN controls in both loam and silt loam soils.

Nitrogen use efficiency (NUE)

Nitrogen use efficiency (NUE) was significantly affected by both soil type and treatment, with a significant interaction between soil type and treatment detected (Table 1). NUE increased with the level of CAN nitrogen fertiliser applied, with increases from 25% to 46.3% when going from 75% N to 100% N. However, no significant differences were observed between CAN and PU at 75% N (Fig. 2). PSI-362 significantly improved the NUE over the 75% N (applied as CAN) and 100% N (applied as CAN) treatments in a concentration-dependent manner, in agreement with previous



	p-value
100% CAN vs 75% CAN	< 0.0001
100% CAN vs 75% PU	< 0.0001
100% CAN vs 75% CAN + 1x PSI-362	ns
100% CAN vs 75% CAN + 1.5x PSI-362	0.09
75% CAN vs 75% PU	ns
75% CAN vs 75% CAN + 1x PSI-362	0.03
75% CAN vs 75% CAN + 1.5x PSI-362	0.01
75% CAN + 1x PSI-362 vs 75% CAN + 1.5x PSI-362	ns

	p-value
100% CAN vs 75% CAN	0.011
100% CAN vs 75% PU	0.02
100% CAN vs 75% CAN + 1x PSI-362	ns
100% CAN vs 75% CAN + 1.5x PSI-362	ns
75% CAN vs 75% PU	ns
75% CAN vs 75% CAN + 1x PSI-362	0.03
75% CAN vs 75% CAN + 1.5x PSI-362	ns
75% CAN + 1x PSI-362 vs 75% CAN + 1.5x PSI-362	ns

Fig 1 Total grass yield data for (**A**) loam and (**B**) silt loam soils across 2021 and 2022 from the lysimeter trials. Maximum and minimum values are represented by the ends of each whisker, while the boxes represent the lower, median and upper quartile values (n = 12). Dark circles represent the 5th and 95th percentiles Significance via paired t-tests is displayed between treatments in the tables, with false discovery rate (FDR of 0.05) correction applied to presented *p*-values. ns = not significant

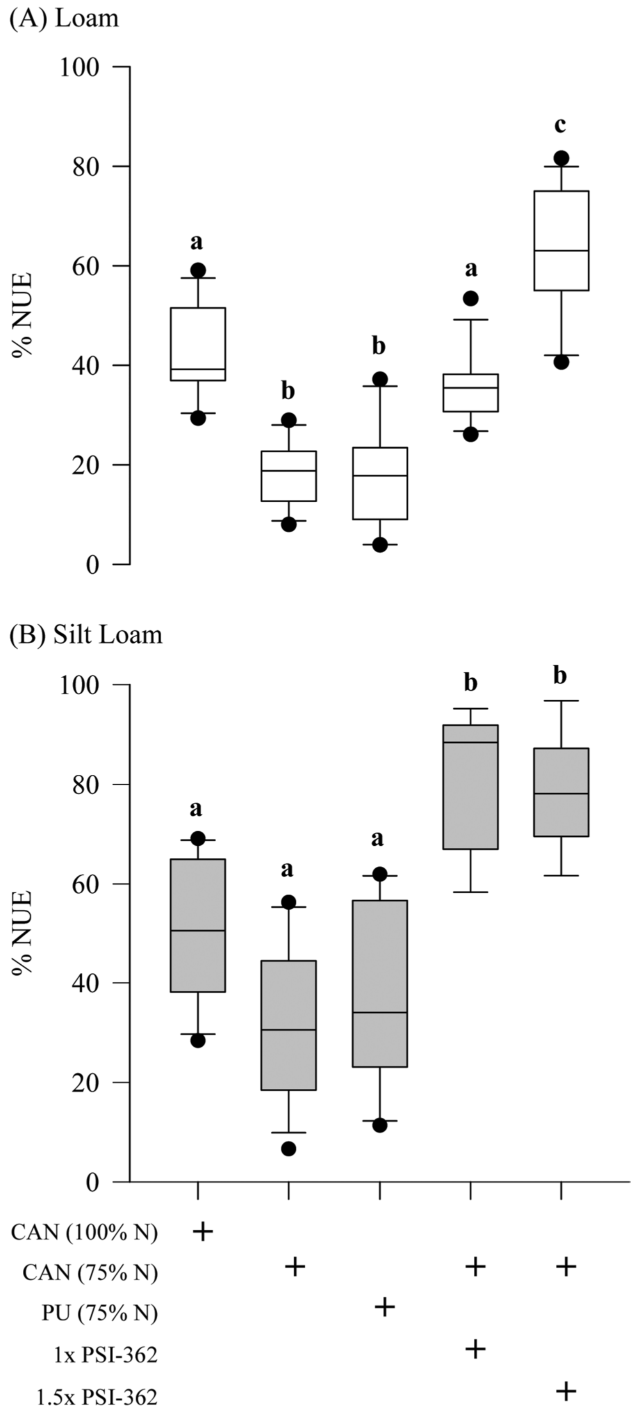


Fig. 2 The nitrogen use efficiency for all treatments for (A) loam and (B) silt loam soils. Maximum and minimum values are represented by the ends of each whisker, while the boxes represent the lower, median and upper quartile values (n = 6). Dark circles represent the 5th and 95th percentiles. Significance is indicated by different letter(s) (Tukey HSD test) within soil types ($p \leq 0.05$)

work for this biostimulant (Quille et al. 2022). PSI-362 co-application with 75% N (applied as CAN) also demonstrated a positive effect on the quality of the biomass, resulting in increased crude protein per hectare compared to the 75% N (applied as CAN) treatment for both tested concentrations (Table 1).

Nitrous oxide emissions

To assess the environmental footprint associated with use of PSI-362 application, nitrous oxide (N_2O) emissions were measured during the growing season from April to September at three different timepoints; pre-fertiliser application (24 h before fertiliser application) and two post fertiliser applications (24 h post and 48 h post fertiliser application; Figures 3A and 3B). Background N_2O emissions from the soils (0% N) ranged from 20 to 48 g N ha^{-1}day $^{-1}$ and 10 to 24 g N ha^{-1}day $^{-1}$ for silt loam and loam soils, respectively.

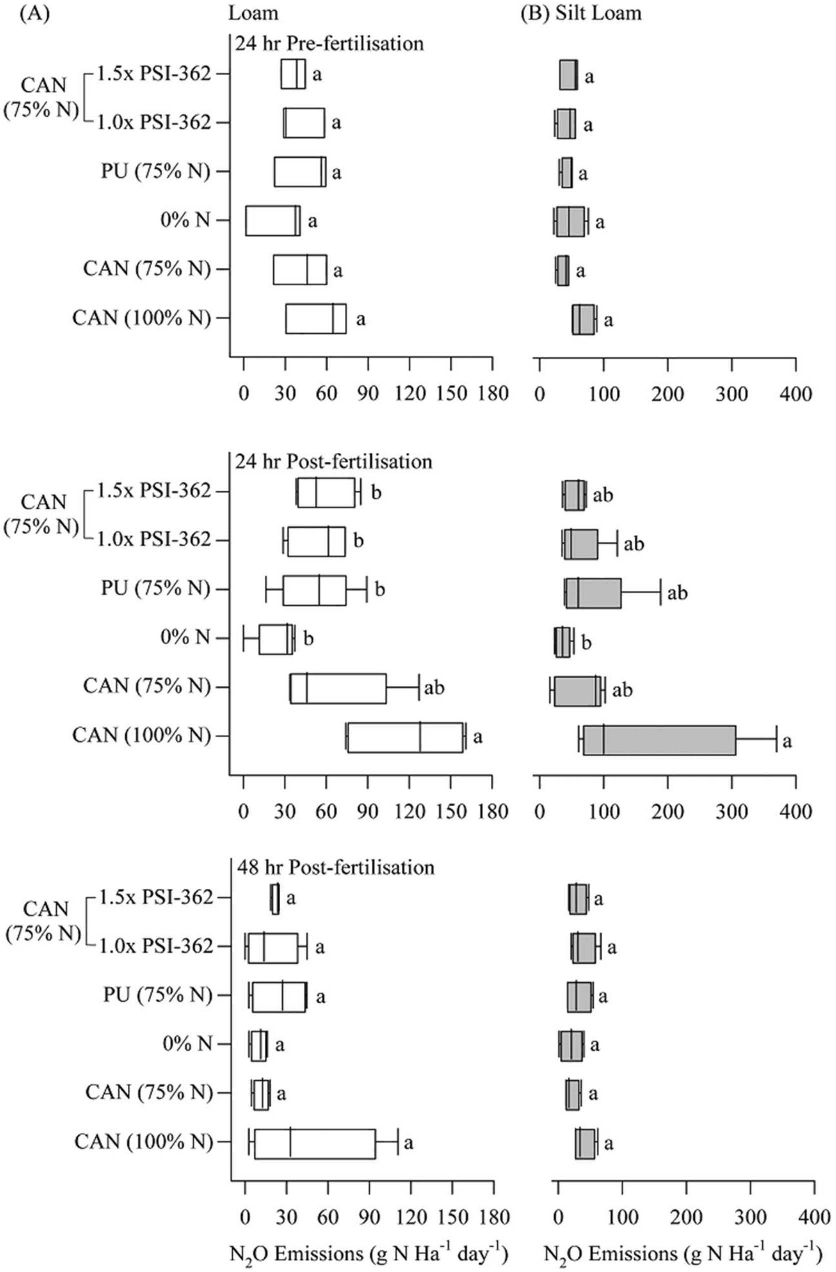
We observed a significant effect ($p \leq 0.001$) on N_2O emissions caused by soil type with higher emissions coming from the silt loam soils (Table 1). There was no effect from PSI-362 co-application on the overall N_2O emissions when compared to the 75% N CAN treatment, although there was a slight reduction in the emissions 24 h after fertiliser addition (Fig. 3A and 3B). For the loam soil, there was a significant reduction in N_2O emissions between both the 1.0x and 1.5x PSI-362 formulations when compared to the 100% N CAN control. This significant difference was not seen with the 100% N CAN control and the 75% N CAN treatment.

Nevertheless, the level of applied nitrogen had the strongest effect ($p \leq 0.001$) on nitrous oxide emissions (Table 1). This was most evident in the 24 h post fertiliser application timepoint where the level of N_2O emissions for the 100% CAN control was more than doubled the emissions for the 75% N treatment (on silt loam soils there is 170 g N_2O-N ha^{-1}day $^{-1}$ for the 100 % N control vs. 65 g N_2O-N ha^{-1}day $^{-1}$ for the 75% N treatment and 36 g N_2O-N ha^{-1}day $^{-1}$ for the 0% N control at the same timepoint; Figure 3B). At the two other timepoints, there was very little difference between each N level. There was also no significant difference in N_2O emissions due to fertiliser choice at the lower dose rate (75% N CAN vs. 75% N protected urea).

Nitrate leachate

Leaching of nitrate is a significant problem associated with almost all intensive agriculture worldwide. Figure 4 shows the cumulative (April to December) leachate of nitrate for both soil types and all treatments. In terms of total leachate, little difference was observed between soil textural classes and fertiliser type (PU vs CAN), with the biggest effect being the level of nitrogen applied. Table 1 shows a trend in nitrate leaching as the level of applied nitrogen increased; 23.9, 31.9 and 41.8 kg ha^{-1} for the 0% N, 75% N treatments and 100% N control, respectively, but these increases were not significantly different, while Fig. 4 shows a similar pattern across each soil type

Fig. 3 Nitrous oxide emissions for lysimeter trials using (**A**) loam and (**B**) silt loam soils. Maximum and minimum values are represented by the ends of each whisker, while the boxes represent the lower quartile, median and upper quartile values (n = 4). Significance is indicated by different letter(s) (Tukey HSD test) between treatments across the same timepoint ($p \leq 0.05$).



with the rate of N fertilisation affecting the rates of nitrate leaching, where a decline was seen from 100% N to 75% N to 0% N. The effect was more pronounced in the loam soil with the 0% N rate leaching almost a third of the nitrate of the 100% N rate - with the silt loam soil, this value was 60 %. Overall, the application of PSI-362 did not significantly affect nitrate leaching when compared to either the 75 % N treatment or 100 % N control.

Discussion

The application of PSI-362 at 1.0x and 1.5x the recommended rate at 75% N CAN increased yield across both soil types compared to the 75 % CAN treatment. Importantly, the results showed that grass yields under a fertilisation regime of CAN (75% N) + 1.0x PSI-362 were not

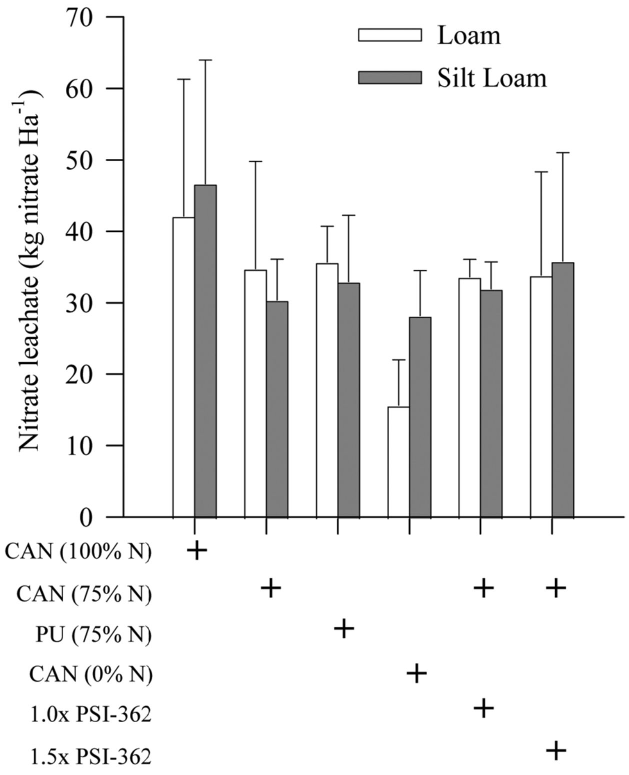


Fig. 4 Cumulative nitrate leachate values for lysimeter trials using loam and silt loam soils from April to December in 2022. No significance was seen between treatments. Values represent mean ± SEM (n = 3)

significantly reduced when compared to the 100% CAN controls in both loam and silt loam soils (Fig. 1A and 1B), in agreement with our previous work (Quille et al. 2022).

One of the major claims a product can make to be classed as a biostimulant is to improve the NUE of a crop (Ricci et al. 2019). Application of seaweed extracts can enhance root growth and root biomass, thus improving plant nutrient uptake (Rouphael et al. 2020). Under reduced and optimal nitrogen regimes seaweed extracts have been shown to increase NUE in baby spinach and lambs lettuce (Rouphael and Colla 2020), which the authors attributed to improved chlorophyll synthesis, along with the accumulation of antioxidant metabolites that can enhance photosynthetic activity and overall agronomic performance. Another brown seaweed extract significantly improved the NUE of a tomato crop by 23% (Cozzolino et al. 2021) in addition to increased antioxidant activity.

PSI-362 has previously been shown to increase NUE in barley (Goñi et al. 2021), wheat (Łangowski et al. 2022), and grass (Quille et al. 2022). In the studies involving wheat and barley, they found that PSI-362 dysregulated genes in nitrate transport and assimilation which also had downstream effects on the amount of nitrate taken up by the plant, thus significantly improving NUE in these cereals (Goñi et al. 2021; Łangowski et al. 2022). Łangowski et al. (2022) showed that PSI-362 significantly enhanced nitrate uptake in wheat seedlings, an effect that was found to be more pronounced under nitrogen limiting conditions. This effect was also seen in barley where a significant increase in the levels of the enzymes nitrate reductase and glutamine synthase was reported (Goñi et al. 2021). The same study also found increased expression of genes responsible for the uptake and utilisation of nitrogen (*NRT1.1, NRT 2.1* and *NRT1.5*). From our work, PSI-362 also had a positive effect on the quality of produced biomass, resulting in increased crude protein per hectare compared to the 75% N CAN treatment for both tested concentrations. In fact, the crude protein output per hectare for PSI-362 applied at 1.5x rate significantly exceeded that observed for the 100% N CAN control (292 kg ha^{-1} vs. 251 kg ha^{-1}).

This increased NUE and reduced nitrogen regime permitted by the application of PSI-362 also has environmental benefits with PSI-362 displaying a significant reduction in nitrous oxide emissions compared to the 100 % N controls (however it did not differ significantly from the 75 % N treatment; Table 1). Xu et al. (2022) found that three different biostimulant types (humic acid, chitosan and an algae extract) all slightly increase N_2O production when growing cauliflowers and tomatoes. However, these same biostimulants reduced nitrogen losses via leaching and ammonia volatilisation (Xu et al. 2022). Other studies investigated nitrogen losses from soil (N_2O emissions and nitrate leaching) after application of N-fixing microbes (another class of biostimulants) reported an increase in nitrous oxide emissions in combination with urea, relative to urea alone (Souza et al. 2019, 2021). Another study (with a bacterial and enzymatic biostimulant) did not show any significant effect on N_2O emissions from a potato crop over two growing seasons (Thilakarathna et al. 2023). From our analysis, the main benefit of PSI-362 in terms of nitrous oxide reductions is through the reduced application of nitrogen fertiliser, however this outcome does point towards the need for a more integrated approach to the application of this type of biostimulant (an *A. nodosum* extract) in terms of reduced levels of nitrogen application to obtain maximum benefits from an environmental and yield point of view.

In terms of background emissions (from a 0 % N control), our results tend to be higher than other studies (10 to 48 g N ha^{-1}day $^{-1}$ depending on soil texture). For example, Harty et al. (2016) reported emissions values ranging from 1.0 to 2.7 g ha^{-1} day $^{-1}$ across 2 years on three separate sites. However, these are 12-month values, and included the winter months when the soil temperatures were lower, thus reducing microbial activity and N mineralisation. In contrast, our results were based on emissions from April to September, when the soil temperatures would be at their highest (Supplementary Table 3). Increased soil temperatures are a contributing factor to increased N_2O emissions (Rafique et al. 2011). Rafique et al. (2011) also showed a strong correlation between water filled pore space and nitrous oxide production, where peak N_2O production was found between 60 and 80 % WFPS. They hypothesised that this level of WFPS resulted in maximal levels of nitrification and denitrification. The lysimeters in this study were irrigated throughout this sampling period to ensure WFPS was consistently kept between 70% and 80%. Gebremichael et al. (2021) looked at the nitrous oxide emissions from grass plots over two grass rotations from in a similar timeframe to this work - June until August and the reported N_2O fluxes were similar to the background control and ranged from −2.33 to 17.64 g N_2O-N $ha^{-1}day^{-1}$.

The biggest effect on N_2O emissions across both soil types was the level of applied nitrogen. As applied N increases, N_2O emissions also increase (from an average of 29.3 g ha^{-1} day^{-1} for the 0 % N control to 41.7 g ha^{-1} day^{-1} for the 75 % CAN control, to finally 84.4 g ha^{-1} day^{-1} for the 100 % CAN control). Similar reductions in emissions due to reduced N input was seen by Rahman et al. (2021) across 3 sites over two years of grass growth. In two of the sites, there were relatively strong correlations (R^2 values of 0.84 to 0.96) when correlating N_2O emissions with increasing additions of CAN (from 100 kg to 500 kg ha^{-1}) (Rahman et al. 2021)).

The level of nitrate leaching will be affected by plant type and growth stage, fertiliser type and rate, climate (in particular rainfall), as well as soil type including texture and structure (Bibi et al. 2016). In terms of nitrate leaching there was no significant difference between the soil textures used here. Sandy soils are more freer draining, while soils with a higher clay content will restrict and hold water movement. Van Es et al. (2002) found significant differences between the loamy sand and clay loam soils in terms of groundwater nitrate across two crop types in a lysimeter. In a study looking at N fertiliser rates and nitrate leaching on an arable system, Vogeler et al. (2021) observed an increase in leaching as the N rates increased from 0 kg ha^{-1} to 225 kg ha^{-1}. We also observed an increase in leaching as the level of nitrogen increased, however this was not significant in either soil type.

Conclusion

We have demonstrated that reduced nitrogen application leads to lower N_2O emissions, but also lower yields in pasture system. However, the co-application of PSI-362 with reduced nitrogen (75% N) eliminated this yield penalty without causing significant increases in N_2O emissions. A full life cycle assessment (along with associated emission factors) is necessary to assess the overall impact of the application of PSI-362 on total GHG emissions in the future. Further, PSI-362 significantly improved the crude protein output per hectare and had a positive impact on the overall NUE. This highlights its potential to contribute to modern farming practices, as improving the NUE is one of the cornerstones of sustainable agriculture, essential to meet the quality and quantity of food production needed to support the global population growth. The increased availability and application of nitrogenous fertilisers as prescribed by the original green revolution is no longer tenable, given the deleterious effects on the environment and overall biodiversity. A reduction in nitrogen usage, coupled with methods to enhance the efficiency of this nitrogen is required to both feed this growing population and do so in a more environmentally sustainable way. In recent years, biostimulants have gained more attention in general, and for their potential ability to improve NUE specifically. It is imperative that future studies investigating biostimulants role in NUE should incorporate the potential positive environmental impacts of using such products, especially under a reduced fertiliser regime.

However, the challenge remains to incorporate these technologies into mainstream agronomy. These include mode of application that will depend on farming system and crop type, consistency of formulation, and timing of application, again dictated by crop type and farming system. This research convincingly demonstrated the potential of an engineered biostimulant, that can easily be incorporated into conventional agronomic practice in pasture-based systems, to maintain crop yields while reducing N inputs, thus benefitting the environment.

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Data availability The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

Declarations SO is employed by the company Brandon Bioscience. The remaining authors (PQ, JK and CKYN) declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The funder (MTU Kerry) and Brandon Bioscience had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. The authors declare no conflict of interest.

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