



International Journal of Research in Agronomy

E-ISSN: 2618-0618
P-ISSN: 2618-060X
© Agronomy
NAAS Rating (2026): 5.20
www.agronomyjournals.com
2026; 9(8): 1219-1221
Received: 24-06-2026
Accepted: 28-07-2026

Pradeep D
Centre for Agricultural
Nanotechnology, Tamil Nadu
Agricultural University,
Coimbatore, Tamil Nadu, India

Subramanian KS
Retired Professor, Centre for
Agricultural Nanotechnology,
Tamil Nadu Agricultural
University, Coimbatore, Tamil
Nadu, India

Marimuthu S
Professor and Head, Centre for
Agricultural Nanotechnology,
Tamil Nadu Agricultural
University, Coimbatore, Tamil
Nadu, India

Corresponding Author:
Pradeep D
Centre for Agricultural
Nanotechnology, Tamil Nadu
Agricultural University,
Coimbatore, Tamil Nadu, India

***In vitro* cytotoxicity and microbial safety assessment of a novel nano-nitrogen fertilizer formulation**

Pradeep D, Subramanian KS and Marimuthu S

DOI: <https://www.doi.org/10.33545/2618060X.2026.v9.i8o.6409>

Abstract

The increasing use of nano-enabled fertilizers in agriculture necessitates comprehensive evaluation of their potential effects on beneficial soil microorganisms. The present study assessed the microbial safety of a novel nano-nitrogen (Nano-N) fertilizer formulation using *Methylobacterium* spp. (PPFM) as a test microorganism. Bacterial viability and cellular integrity were evaluated using neutral red uptake (NRU) and lactate dehydrogenase (LDH) assays following exposure to different concentrations of the formulation (200, 400, 1200, and 1600 ppm). The NRU assay showed a concentration-dependent reduction in PPFM viability, with viability decreasing from 100% in the untreated control to 99.5%, 98.7%, 94.9%, and 92.9% at 200, 400, 1200, and 1600 ppm, respectively. The LDH assay showed a corresponding increase in cellular damage, with cell death increasing from 0.8% in the control to 8.9% at 1600 ppm. Despite the concentration-dependent responses, PPFM viability remained above 90% and cell death remained below 10% even at the highest tested concentration. The agreement between NRU and LDH responses indicates only limited cellular stress and membrane damage at higher concentrations. Overall, the Nano-N formulation exhibited low microbial toxicity and good compatibility with PPFM, suggesting a favourable microbial safety profile under the tested conditions. These findings support the potential suitability of the formulation for agricultural application, although further studies under soil and field conditions are recommended to confirm its long-term effects on beneficial microbial communities.

Keywords: Nano fertilizer, cytotoxicity, microbes and PPFM

Introduction

The rising global demand for food has led to increased use of nitrogen fertilizers in modern agriculture. Although conventional nitrogen fertilizers are essential for achieving high crop productivity, their nitrogen use efficiency (NUE) is often relatively low, resulting in substantial nitrogen losses through ammonia volatilization, nitrate leaching, runoff, and denitrification (Galloway *et al.*, 2008; Guo *et al.*, 2010; Saurabh *et al.*, 2024) ^[18, 10]. These losses not only reduce nitrogen availability to crops but also contribute to groundwater contamination, eutrophication, and greenhouse-gas emissions, particularly nitrous oxide (N₂O) emissions (Galloway *et al.*, 2008; Lassaletta *et al.*, 2014; Channab *et al.*, 2024) ^[18, 20, 1]. In recent years, nanotechnology has emerged as a promising approach for improving nutrient-use efficiency and developing nano-enabled fertilizers with enhanced nutrient delivery characteristics (DeRosa *et al.*, 2010; Dimkpa and Bindraban, 2018; Zulfiqar *et al.*, 2019) ^[4, 17, 27].

Nano-nitrogen (Nano-N) fertilizers are specially designed formulations intended to improve nitrogen delivery, nutrient uptake, and nitrogen-use efficiency through controlled nutrient release and enhanced interaction with plant surfaces and tissues. Their physicochemical characteristics, including particle size, surface properties, composition, and nutrient-release behaviour, can influence nutrient availability and plant uptake (DeRosa *et al.*, 2010; Liu and Lal, 2015; Dimkpa and Bindraban, 2018) ^[16, 21, 17]. Several studies have reported that nano-enabled fertilizers can improve crop growth and nutrient-use efficiency while potentially reducing the quantity of fertilizer required compared with conventional formulations (Kah *et al.*, 2018; Zulfiqar *et al.*, 2019) ^[19, 27]. However, the increasing application of nanoscale materials in agriculture has also raised concerns regarding their potential effects on soil microorganisms, particularly beneficial microorganisms that are essential for nutrient cycling, soil fertility, and plant health (Simonin and Richaume, 2015; Kah *et al.*, 2018) ^[24, 19].

Soil microorganisms, including beneficial bacteria and fungi such as *Azotobacter*, *Rhizobium*, *Bacillus*, and *Trichoderma* species, perform important functions in agricultural soils, including biological nitrogen fixation, nutrient solubilization, phytohormone production, pathogen suppression, organic-matter decomposition, and maintenance of soil health (Vessey, 2003; Bhattacharyya and Jha, 2012; Woo and Pepe, 2018) [25, 14, 26]. Consequently, adverse effects of nano-fertilizers on beneficial microorganisms could potentially alter microbial community structure and soil biochemical processes, thereby affecting soil ecological balance and long-term agricultural sustainability (Simonin and Richaume, 2015; Rajput *et al.*, 2018) [24, 23]. Model microorganisms such as *Escherichia coli* are also widely used in nanoparticle toxicity studies to investigate cellular responses, oxidative stress, membrane damage, and changes in bacterial viability (Auffan *et al.*, 2009; Bondarenko *et al.*, 2013) [13, 15].

Nanoparticles can exert toxic effects on microorganisms through several mechanisms, including the generation of reactive oxygen species (ROS), oxidative stress, interaction with and disruption of cell membranes, release of toxic ions, intracellular accumulation, and interference with essential metabolic processes (Nel *et al.*, 2006; Simonin and Richaume, 2015; Bondarenko *et al.*, 2013) [22, 24, 15]. Their small size and high specific surface area can increase their interaction with microbial cell surfaces and biological components, potentially resulting in membrane damage, altered membrane permeability, enzyme inhibition, and impaired cellular metabolism (Nel *et al.*, 2006; Bondarenko *et al.*, 2013) [22, 15]. Although numerous studies have reported beneficial effects of nano-fertilizers on crop growth and nutrient-use efficiency, comprehensive evaluations of their potential effects on beneficial soil microorganisms remain comparatively limited (Kah *et al.*, 2018; Rajput *et al.*, 2018) [19, 23]. Therefore, controlled laboratory investigations are necessary to evaluate microbial responses to nano-fertilizer exposure, including concentration-dependent effects on microbial growth, viability, oxidative stress, membrane integrity, and cellular morphology. Such assessments are important for establishing the biosafety profile of nano-enabled fertilizers and supporting the development of appropriate application strategies for sustainable agriculture.

The present study therefore aimed to evaluate the microbial safety of a novel nano-nitrogen fertilizer formulation using standardized microbiological and cellular assays. The study investigated concentration-dependent effects on microbial viability and growth, colony-forming ability, oxidative stress responses, membrane integrity, and cellular morphology in selected beneficial microorganisms. These assessments were undertaken to determine the compatibility of the Nano-N formulation with beneficial soil microbiota and to provide evidence for its biosafety and potential suitability for sustainable agricultural application.

Materials and Methods

Microbial Toxicity

The study aimed to visualise the toxicity in different microorganisms. PPFM subjected to different ppm of nanofertilizer from 200ppm to 1600 ppm.

Lactose DeHydrogenase (LDH) assay

The lactose dehydrogenase assay determines the number of non-viable cells by detecting the lactate dehydrogenase enzyme produced by the cells under adverse conditions. Lactate dehydrogenase is an enzyme that converts the stored form of

energy (lactate) into active energy (pyruvate). During this conversion, NAD⁺ is reduced to NADH. As a result of the proton generated by the oxidation of NADH to NAD⁺, the exogenously supplied diaphorase enzyme can transform the INT tetrazolium salt into a red-colored formazan product. The red-colored formazan product was measured at 490 nm. The absorbance increases with an increase in the number of non-viable cells and vice versa. The substance-mediated cytotoxicity assay on *Bacillus subtilis* and *Azotobacter chroococcum* bacteria was determined using the LDH assay (EZcount TM Lactose dehydrogenase cell assay kit).

Add 10 µl of 10% neutral red reagent to all treatments, which are then incubated in an orbital shaker at 37°C, 80 rpm for 2-4 hours. All the samples were centrifuged and the pellet was used for the assay. To the collected pellet, add 100 µl of neutral red solubilization solution and incubate the 96-well plate at room temperature for 10 minutes, then observe OD at 540 nm. The cell viability percentage was calculated using the formula after background correction.

$$\text{Cell viability\%} = \frac{\text{control OD} - \text{Test sample OD}}{\text{Control OD}} \times 100$$

Results and Discussion

The neutral red uptake (NRU) assay was used to assess the viability and functional integrity of PPFM cells following treatment with the nano-fertilizer formulation (Table 1). The NRU assay is based on the uptake and accumulation of neutral red within the lysosomes of viable cells and is widely used as an indicator of cellular viability (Repetto *et al.*, 2008; Grizzle and McNally, 2021; Khalef *et al.*, 2024) [9, 6, 7]. In PPFM, the highest cell viability was observed in the untreated control (100%), followed by 200 ppm (99.5%), 400 ppm (98.7%), and 1200 ppm (94.9%). At the highest concentration of 1600 ppm, cell viability decreased to 92.9%. The progressive reduction in NRU response at higher concentrations may indicate alterations in lysosomal function and cellular integrity (Repetto *et al.*, 2008; Godoy *et al.*, 2021) [9, 5]. However, the maintenance of more than 90% cell viability even at 1600 ppm indicates that the formulation exerted only a limited effect on PPFM viability.

The lactate dehydrogenase (LDH) assay was employed to evaluate cellular damage based on the release of intracellular LDH following disruption of the plasma membrane (Table 1). LDH release is an established indicator of loss of membrane integrity and cellular damage (Decker and Lohmann-Matthes, 1988; Kumar *et al.*, 2018; Cummings *et al.*, 2021) [3, 8, 2]. An increase in LDH activity, reflected by increased colour development and absorbance, indicates greater membrane damage and cell death. In PPFM, the percentage of dead cells increased with increasing treatment concentration, with the highest mortality recorded at 1600 ppm (8.9%), whereas the control showed only 0.8% cell death. The concentration-dependent increase in LDH response indicates a gradual increase in membrane damage at higher concentrations. Nevertheless, cell death remained below 10% even at the highest tested concentration, demonstrating only a limited toxic effect on PPFM. The LDH results corroborate the NRU findings and, together with the high NRU viability, indicate that the nano-fertilizer formulation caused minimal cellular damage under the tested conditions (Zipperer *et al.*, 2023; Khalef *et al.*, 2024) [12, 7].

Table 1: Neutral Red and LDH to assess the toxicity of Nano nitrogen fertilizer on PPFM

Treatment	PPFM LDH	PPFM Neutral Red
Control	0.8	100
200 ppm Pickering emulsion	2.9	99.5
400 ppm Pickering emulsion	3.4	98.7
600 ppm Pickering emulsion	3.3	98.1
800 ppm Pickering emulsion	4.8	97.4
1000 ppm Pickering emulsion	5.3	96.2
1200 ppm Pickering emulsion	6.4	94.9
1400 ppm Pickering emulsion	6.6	94.8
1600 ppm Pickering emulsion	8.9	92.9

Conclusion

Overall, the NRU and LDH assays demonstrated that the nano-nitrogen fertilizer formulation had minimal adverse effects on PPFM viability and cellular integrity under the tested conditions. PPFM maintained 92.9% viability even at the highest concentration of 1600 ppm, while LDH analysis showed only 8.9% cell death at the same concentration. The concentration-dependent decline in NRU response and corresponding increase in LDH release indicate a mild cellular stress response at higher concentrations; however, the overall effect remained limited. The consistent results obtained from both assays indicate low microbial toxicity and good compatibility of the nano-nitrogen formulation with PPFM, supporting its potential microbial safety for agricultural application.

References

- Channab BE, Idrissi AE, Ammar A, Dardari O, Marrane SE, El Gharrah A, *et al.* Recent advances in nano-fertilizers: synthesis, crop yield impact, and economic analysis. *Nanoscale*. 2024;16(9):4484-4513.
- Cummings BS, Wills LP, Schnellmann RG. Measurement of cell death in mammalian cells. *Current Protocols*. 2021;1:e210.
- Decker T, Lohmann-Matthes ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *Journal of Immunological Methods*. 1988;115:61-69.
- DeRosa MC, Monreal C, Schnitzer M, Walsh R, Sultan Y. Nanotechnology in fertilizers. *Nature Nanotechnology*. 2010;5(2):91.
- Godoy P, Hewitt NJ, Albrecht U, *et al.* Recent approaches in *in vitro* liver toxicity testing and interpretation of cellular viability assays. *International Journal of Molecular Sciences*. 2021;22:5038.
- Grizzle WE, McNally LR. The neutral red assay can be used to evaluate cell viability during autophagy or in an acidic microenvironment *in vitro*. *Biotechnic & Histochemistry*. 2021;96:302-310.
- Khalef L, Lydia R, Filicia K, Moussa B. Cell viability and cytotoxicity assays: biochemical elements and cellular compartments. *Cell Biochemistry and Function*. 2024;42:e4007.
- Kumar P, Nagarajan A, Uchil PD. Analysis of cell viability by the lactate dehydrogenase assay. *Cold Spring Harbor Protocols*. 2018;2018:pdb.proT095497.
- Repetto G, del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature Protocols*. 2008;3:1125-1131.
- Saurabh K, Prakash V, Dubey AK, Ghosh S, Kumari A, Sundaram PK, *et al.* Enhancing sustainability in agriculture with nanofertilizers. *Discover Applied Sciences*. 2024;6(11):559.
- Wills LP, Schnellmann RG. Measurement of cell death in mammalian cells. *Current Protocols*. 2021;1:e210.
- Zipperer A, Scheurer J, Kretschmer D. Cytotoxicity assays as predictors of the safety and efficacy of antimicrobial agents. *Methods in Molecular Biology*. 2023;2601:153-167.
- Auffan M, Rose J, Bottero JY, Lowry GV, Jolivet JP, Wiesner MR. Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective. *Nature Nanotechnology*. 2009;4:634-641.
- Bhattacharyya PN, Jha DK. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology and Biotechnology*. 2012;28:1327-1350.
- Bondarenko O, Juganson K, Ivask A, Kasemets K, Mortimer M, Kahru A. Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells. *International Journal of Environmental Research and Public Health*. 2013;10:1081-1094.
- DeRosa MC, Monreal C, Schnitzer M, Walsh R, Sultan Y. Nanotechnology in fertilizers. *Nature Nanotechnology*. 2010;5:91.
- Dimkpa CO, Bindraban PS. Nanofertilizers: new products for the industry? *Journal of Agricultural and Food Chemistry*. 2018;66:6462-6473.
- Galloway JN, Townsend AR, Erisman JW, *et al.* Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. *Science*. 2008;320:889-892.
- Kah M, Kookana RS, Gogos A, Bucheli TD. A critical evaluation of nanopesticides and nanofertilizers against their conventional analogues. *Nature Nanotechnology*. 2018;13:677-684.
- Lassaletta L, Billen G, Grizzetti B, Anglade J, Garnier J. 50 year trends in nitrogen use efficiency of world cropping systems: the relationship between yield and nitrogen input to cropland. *Environmental Research Letters*. 2014;9:105011.
- Liu R, Lal R. Potentials of engineered nanoparticles as fertilizers for increasing agronomic productions. *Science of the Total Environment*. 2015;514:131-139.
- Nel A, Xia T, Mädler L, Li N. Toxic potential of materials at the nanolevel. *Science*. 2006;311:622-627.
- Rajput VD, Minkina T, Sushkova S, Tsitsuashvili V, Mandzhieva S, Fedorenko A, *et al.* Effects of nanoparticles on crops and soil microorganisms. *Environmental Science and Pollution Research*. 2018;25:21949-21958.
- Simonin M, Richaume A. Impact of engineered nanoparticles on the activity, abundance, and diversity of soil microbial communities: a review. *Environmental Science and Pollution Research*. 2015;22:13710-13723.
- Vessey JK. Plant growth promoting rhizobacteria as biofertilizers. *Plant and Soil*. 2003;255:571-586.
- Woo SL, Pepe O. Microbial consortia: promising probiotics as plant biostimulants for sustainable agriculture. *Frontiers in Plant Science*. 2018;9:1801.
- Zulfiqar F, Navarro M, Ashraf M, Akram NA, Munne-Bosch S. Nanofertilizer use for sustainable agriculture: advantages and limitations. *Plant Science*. 2019;289:110270.