# Development of non-soil media capable of degrading organic nitrogen to nitrate, as in natural soil

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# Abstract

Artificial soil materials are unable to catalyse nitrification because added organic substances suppress nitrifying bacteria. We used a multiple parallel mineralization method, which enables the use of organic fertilizers in hydroponics, to support nitrification in non-soil materials. In this method, microorganisms immobilized on porous carriers produce inorganic nitrate from organic substances, as in natural soil. The carriers also released phosphate and potassium ions. Microorganisms produced nitrate from organic substances when immobilized on polyurethane resin, rockwool, vermiculite, oyster shell lime, and rice husk charcoal. The optimal amount of organic material added daily to 100 mL of carrier held 6 mg of organic N. Vegetable plants grew on inoculated materials but not on uninoculated materials. These results show that non-soil materials can be used to create artificial soils in which plants can be grown with the addition of organic fertilizer, as in natural soil.


# Introduction

Soil is used to grow 98.8% of food [1]. It is common to use organic fertilizers made from food residues and livestock manure. Organic fertilizer ploughed into the soil is degraded to inorganic nutrients such as nitrate, ammonium, and phosphate. However, if organic fertilizer is ploughed into sterile regolith, nitrate is not generated [2]. For this reason, Moon and Mars regolith would not be able to degrade organic fertilizer to produce nitrate, even if microorganisms populated the fertilizer. Many crop plants require nitrate N, and if nitrate is not produced, they will not grow well [3–5]. In soil-less hydroponic culture, too, it is not possible to grow plants with organic fertilizers [6–12], because nitrate is not easily generated in water[9]. Thus, organic substances can be used as fertilizers only in natural soils.

In natural soils, organic N is converted into nitrate N through a two-step degradation process of ammonification and nitrification [13,14]. Nitrification is carried out by nitrifying bacteria, ammonia-oxidizing bacteria such as *Nitrosomonas* spp., and nitrite-oxidizing bacteria such as *Nitrobacter* spp. [15–19] However, nitrifying bacteria tend to become inactivated when exposed to organic substances [20–22]. In artificial culture, nitrifying bacteria must be grown in an organic-free inorganic salt medium [23,24]. In the presence of organic substances, it is difficult to culture nitrifying bacteria in a medium other than natural soil.

Shinohara et al. (2011) developed a hydroponic technology that can use organic fertilizers by degrading organic fertilizers into inorganic nutrients in water [15]. This “multiple parallel mineralization method” allows co-culturing of nitrifying bacteria with heterotrophic microorganisms even in the presence of organic substances. By this method, it may be possible to add the function of degrading organic substances to nitrate in non-soil media. This would mean being able to grow crops in artificial soil using organic fertilizer. However, to now, this has not been successful in non-soil materials [2,15].

Here, we created soil-like media from non-soil media by using the technique of multiple parallel mineralization to immobilize microorganisms on a non-soil carrier in order to degrade organic N to nitrate

N, and tested plant growth in the media.

# Methods

**Porous carriers.** We tested 12 materials: rockwool (grain size 3–7 mm; Grodan, Roemond, Netherlands; bulk density [ρ] 91 g/L); vermiculite (Nitto Hiruishi, Ianabe, Japan; ρ 285 g/L); pumice (Setogahara Kaen, Midori, Japan; ρ 447 g/L); perlite (Katanagawa Heiwa Nouen, Kanuma, Japan; ρ 112 g/L); coconut husk (Yoshimoto Nousan, Nangoku, Japan; ρ 78 g/L); 5 polyurethane resins (7.5 mm, Inoac Corporation, Nagoya, Japan: AQ-20, ρ 22.7 g/L; AQ-15, ρ 18.5 g/L; AQ-14, ρ 21.3 g/L; Mixel GR, ρ 32 g/L; Mixel GP, ρ 41 g/L); oyster shell lime (Suzuki Yuukan Center, Miyagi, Japan; ρ 830 g/L); and rice husk charcoal (Komeri, Niigata, Japan; ρ 158 g/L). We packed 100 mL of each material into an open-bottom tube made from the inverted top half of a 350-mL soft-drink bottle (EBM, Tsubame, Japan) (Fig. S1).

**Chemical and microbiological analysis.** To measure the generation of inorganic nutrients in each carrier, we measured ammonium, nitrite, nitrate, potassium, and phosphate ions with RQ-flex test strips (Merck, Darmstadt, Germany); pH by pH meter (C-73, AZ-ONE, Osaka, Japan); and electrical conductivity (EC) by EC meter (Twin Cond, Horiba, Kyoto, Japan). To estimate microbial density, we added 1 g (fresh weight) of sample carrier to 9 mL of water and vortexed the mixture for 5 min; applied 100 µL of sample to 1/10 NA medium (0.8 g/L Difco nutrient broth (BD, Franklin Lakes, New Jersey, USA), 15 g/L agar (Fujifilm Wako, Osaka, Japan) or fish-based medium (1 g/L fish-based soluble fertilizer, Yaizu Suisankagaku Industry, Yaizu, Japan), 15 g/L agar) in the dilution plate technique; and incubated the plates at 25 °C for a week in the dark. We analysed the microbial phase in inoculated AQ-14, uninoculated AQ-14, and liquid culture solution from each carrier incubated with 1 g/L of fish fertilizer in a flask for 2 weeks at 25 °C and 120 rpm. For inoculation, three tubes filled with 100 mL of AQ-14 were washed with 100 mL of water; 1 g of bark compost (Sanyo Chip Kogyo, Shimonoseki, Japan) was added on the top as inoculum, 1 mL of fish based soluble fertilizer diluted 10 times with distilled water was added as the organic substance, and the tubes were incubated in the dark overnight at 25 °C. The pellets were washed with 100 mL of water the next day and the leachate was tested for nitrate ions. The addition of fish fertilizer, incubation, and washing were repeated until nitrate ions were detected in the leachate, and then the microbial phase in the AQ-14 was analysed [25] by the Bioengineering Lab. Co., Ltd. The microbial phase of uninoculated AQ-14 and the precipitate collected by centrifuging the liquid culture solution were also analysed.

**Nitrogen mineralization in each carrier.** To immobilize microorganisms on the carriers [26], we packed a weight equivalent to 100 mL of carrier in plastic tubes and added 1 g of bark compost as inoculum. The carriers were washed with 100 mL of distilled water, 0.1 g of fish fertilizer was added, and the tubes were incubated for 24 h in the dark at 25 °C. The next day, the carriers were rinsed with 100 mL of distilled water and the leachate was tested for nitrate ions. The addition of fish fertilizer, incubation, and washing were repeated until nitrate ions were detected in the leachate. Concentrations of inorganic N, ammonium, nitrite, and nitrate were measured.

**Addition of organic substances and generation of inorganic N.** We examined rates of N mineralization and efficiencies of conversion of organic N to inorganic N in the presence and absence of immobilized microbes. We packed 100 mL of rockwool into tubes and added 1 g of bark compost as inoculum. The rockwool was washed with 100 mL of distilled water, 0.1 g of fish fertilizer was added, and the tubes were incubated for 24 h in the dark at 25 °C. The next day, the rockwool was rinsed with 100 mL of distilled water, and inorganic N in the leachate was measured. The addition of fish fertilizer, incubation, and washing was repeated for 3 weeks. Then we added 0.1 g (6 mg N), 0.2 g (12 mg N), 0.5 g (30 mg N), or 1 g (60 mg N) of fish fertilizer per tube and incubated the tubes for 24 h in the dark at 25 °C. The next day, they were rinsed with 100 mL of distilled water and the above amounts of fish fertilizer were again added. The addition of fish fertilizer, incubation, and washing were repeated for over 30 days. We measured inorganic N, ammonium, nitrite, and nitrate in the leachates.

**Optimal conditions for N mineralization.** To compare conditions, new tubes packed with 100 mL of rockwool with nitrification ability were prepared by repeating the addition of 0.1 g of fish fertilizer, incubation at 25 °C overnight, and washing with 100 mL water for 2 to 3 weeks.

To compare the effects of different organic substances, we added fish fertilizer (6% N), corn steep liquor (CSL, OAT Agrio, Tokyo, Japan; 3.3% N), or rapeseed oil cake (Sun and Hope, Kitakyushu, Japan; 6% N) as sources of organic N in amounts equivalent to 6 mg N per tube and incubated the tubes for 24 h in the dark at 25 °C. The next day, the tubes were rinsed with 100 mL of distilled water and the above amounts of organic materials were again added. The addition of organic substances, incubation, and washing were repeated every day for 3 weeks. We measured inorganic N, ammonium, nitrite, and nitrate in the leachates in triplicate.

To determine the optimal incubation temperature, tubes with nitrification ability were incubated at 15, 20, 25, 30, 37, 42, or 45 °C and washed with 100 mL of water, and the addition of 0.1 g of fish fertilizer, overnight incubation, and washing was repeated for 2 weeks. We measured inorganic N, ammonium, nitrite, and nitrate in the leachates in triplicate.

To determine the optimum humidity, tubes with nitrification ability were incubated in incubators at a relative humidity of 20% (WFO-600ND, EYELA, Tokyo, Japan), 51% (MLR-352, Panasonic, Kadoma, Japan), or 92% (CN-25C, Mitsubishi Denki, Tokyo, Japan). The addition of 0.1 g of fish fertilizer, overnight incubation, and washing was repeated for 2 weeks. We measured inorganic N, ammonium, nitrite, and nitrate in the leachates in triplicate.

**Test plants.** On inoculated rockwool we sowed 5 seeds of komatsuna (*Brassica rapa* var. *perviridis*, ATU121), added 0.1 g of fish fertilizer to each carrier, incubated the carriers in chamber MLR-352 (Panasonic) at 25 °C in 12-h light/dark, and then added 100 mL of water the next day. The addition of fish fertilizer, incubation, and washing was repeated for 11 days. We also grew lettuce (*Lactuca sativa* var. *capitata*), saradana (*L. sativa*, Melbourne MT), radish (*Raphanus sativus* var. *sativus*, Akamaru Hatsuka Daikon), and turnip (*Brassica rapa* var. *glabra*, Shogoin Kabu) on rockwool and nursery soil

(Naeichiban; Sumirin Agro-Products, Aichi, Japan) in a greenhouse at Tsu, Mie Prefecture, from September to December, 2013. During these experiments, light and temperature were not controlled.

# Results

**Nitrogen mineralization on inoculated carriers.** The chemical properties of leachates from rockwool differed significantly between inoculated and uninoculated materials (Fig. 1). The generation of nitrate was stable for 2 weeks after the start of generation (Figs. S2, S3 online). By 30 days, 1.7 mg of K and 1.4 mg of phosphate were generated (Fig. S4).

**Chemical, physical, and microbiological analyses.** Oyster shell lime, rice husk charcoal, and rockwool were effective at producing inorganic nutrients and large amounts of nitrate (Fig. 2). Oyster shell lime achieved an efficiency of 69% in net N mineralization. However, phosphate couldn't be detected. Physical properties of carriers are shown in Table S1.

**Optimum amount of organic substance to add and generation of inorganic N.** The generation of inorganic N increased as the amount of organic N applied increased, but the quantity of nitrate and the efficiency of conversion of organic N to inorganic N decreased (Fig. S5 online).

**Optimal conditions for N mineralization.** Fish fertilizer was better mineralized than CSL and rapeseed oil cake (Fig. 3). The generation of nitrate in rockwool was influenced by temperature (Fig. S6 online). The production of inorganic N was low at 15 and 20 °C, and very little nitrite and nitrate were produced at 15 °C. The production of nitrate was high at 25–42 °C but poor at 45 °C. No nitrate was generated at 20% RH, and the production of inorganic N was much lower at 20% than at 51% and 92% RH (Fig. S7 online).

**Analysis of microbial phase.** The proportion of *Bacillus* was large in the inoculated AQ-14 (42.1%), the uninoculated AQ-14 (39.6%), and the liquid culture (77.5%). The inoculated AQ-14 was dominated by *Actinobacteria* (14.6%), *Achromobacter* (7.9%), *Pseudomonas* (5.3%), and *Rhodococcus* (3%; Fig. S8 online). The uninoculated AQ-14, in contrast, had high proportions of *Aminobacter* (7.1%), *Brevibacillus* (8%), *Cupriavidus* (8.9%), and *Stenotrophomonas* (17.9%), which comprised only 0.1%, 0.1%, 0.2%, and 0.3%, respectively, in the inoculated carrier. The liquid culture solution was characterized by *Sediminibacterium* (0.8%), *Streptomyces* (0.1%), *Parachlamydia* (0.5%), *Planctomyces* (0.3%), *Hyphomicrobium* (0.1%), *Rhodoplanes* (0.1%), *Legionella* (0.6%), and *Lysobacter* (0.1%).

**Plant growth.** Komatsuna seedlings grew well in inoculated rockwool but not in uninoculated rockwool (Fig. 4). Lettuce, saradana, radish, and turnip grew as well in inoculated carrier as in nursery soil (Fig. S9 online). These results suggest that the inoculated carriers acquired the microbial function of degrading organic N into nitrate suitable for plants.

# Discussion

Non-soil carriers usually do not allow organic substances to be used as fertilizer (Fig. 4). Rather, the presence of organic substances suppresses nitrifying bacteria [15, 27, 29] (Fig. S4 online), perhaps because heterotrophic microorganisms cannot completely degrade the substances that inactivate nitrifying bacteria. If we want to grow crops with organic fertilizer in non-soil media, materials capable of degrading organic N to nitrate are needed. Here we achieved nitrification in non-soil media by immobilization of microbes.

Nitrate production stabilized by 3 weeks of incubation (Fig. S2 online). The nitrification ability of inoculated carriers was high at 25–42 °C (Fig. S6 online). In natural soils, the conversion of organic N to inorganic N increases from 5 °C to 35 °C [29–31] and is highest at 35 °C [32]. Our results indicate that the N mineralization ability of the inoculated carriers is similar to that of soils. The amount of inorganic N is influenced more by soil temperature than by soil humidity [33], but our results show that the N mineralization ability decreased in drier air. It will be necessary to investigate how the temperature and moisture content of carriers affect N mineralization. The NA medium that we used to determine bacterial density is frequently used to culture common soil microorganisms (Fig. S10 online)[34]. However, our assay probably excluded nitrifying bacteria, the growth of which is significantly inhibited on media containing organic substances [28]. We tested artificial resins (polyurethane), mineral-derived materials (vermiculite and rockwool), biological materials (oyster shell lime and charcoal), and natural materials (coconut husk). Even on these non-soil carriers, it proved possible to induce the same inorganic nutrient–producing ability as in natural soil. This shows the possibility of creating artificial soil from materials with intentionally designed pore size, particle size, water retention, and ion exchange capacity. The oyster shell lime produced a high amount of inorganic N (Fig. 2), but the effluent did not contain phosphate (data not shown). Since the main component of the oyster shell is calcium carbonate, it is possible that phosphate was immobilized.

We used bark compost as the inoculum, but many bark composts are likely to be free of nitrifying bacteria on account of being composted on concrete. We confirmed that the bark compost we used was aged on soil. We recommend that future studies use soil from organic farms, where nitrifying bacteria are sure to live.

An inoculated carrier could be used to produced inorganic fertilizers from organic substances. Adding an organic substance such as fish fertilizer to an inoculated carrier and flushing it with water the next day could allow the inorganic nutrient solution to be collected daily. Inorganic fertilizers, in general, are chemically synthesized from minerals or air through the use of large amounts of energy. Our technique offers a new method for producing inorganic fertilizers with little energy input.

*Bacillus* was the most abundant genus in the inoculated carrier (42.1%), uninoculated material (39.6%), and culture solution (77.5%). Among plant growth–promoting rhizobacteria, *Bacillus* is one of the most extensively studied genera and promotes plant growth and development [34]. Out test plants did not grow on uninoculated material (Fig. 4), suggesting that they cannot grow without nitrifying bacteria, even in the presence of *Bacillus*.

In summary, this study showed the ability of inoculated carriers to mineralize organic N to nitrate and their potential to replace natural soils. Technology to create artificial soils will help clarify the ideal physical and chemical properties of soils and contribute to increased food production.

# Declarations

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**Author contributions** Shinohara M. conceived the project. Iwai T. performed the main experiments and analysed the data. Fujiwara, K. conducted experiments in collaboration with Iwai, T., Nishida, R. and Meeboon, J. performed the supporting experiments. Meeboon, J., Nishida, R., Takano, M., and Shinohara, M. wrote the manuscript. All authors approved the manuscript.

**Competing interests** The authors declare no competing interests.

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# Figures

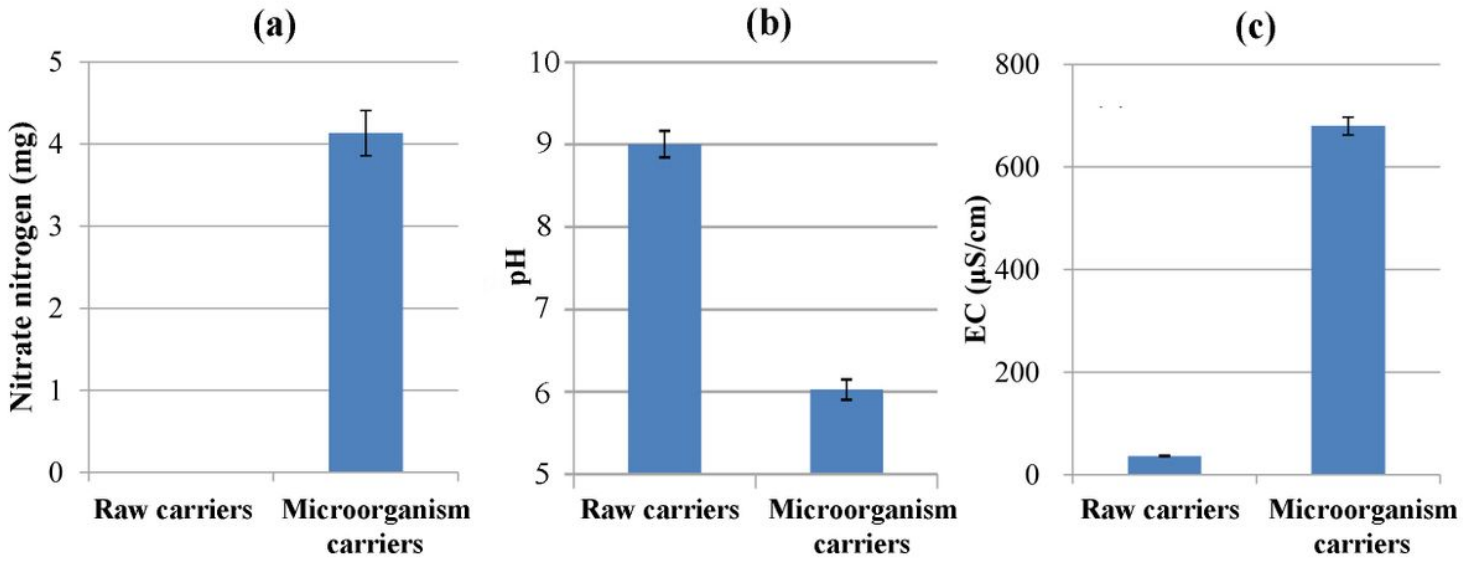


**Figure 1**

Comparisons between inoculated rockwool carrier and uninoculated material. Comparisons of (a) nitrate N, (b) pH, and (c) electrical conductivity (EC) of leachate solutions from 100 mL of rockwool. The inoculated carrier received 1 g of bark compost as inoculum. All tubes received 0.1 g of fish fertilizer every day and were washed with 100 mL of water the next day. Error bars show SD of 3 iterations.

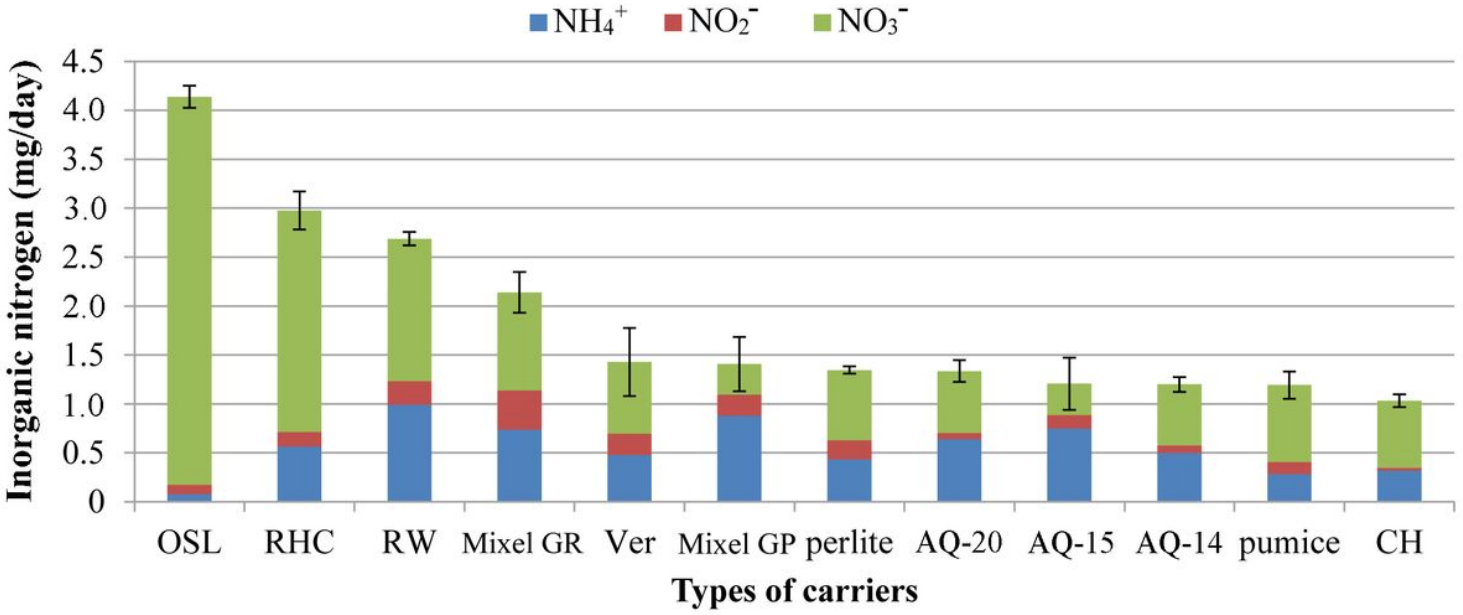


**Figure 2**

Nitrogen mineralization in different carriers. OSL, oyster shell lime; RHC, rice husk charcoal; RW, rockwool; Mixel GR, polyurethane Mixel GR; Ver, vermiculite; Mixel GP, polyurethane Mixel GP; AQ-20, polyurethane AQ-20; AQ-15, polyurethane AQ-15; AQ-14, polyurethane AQ-14; CH, coconut husk. Error bars show SD of 3 iterations.

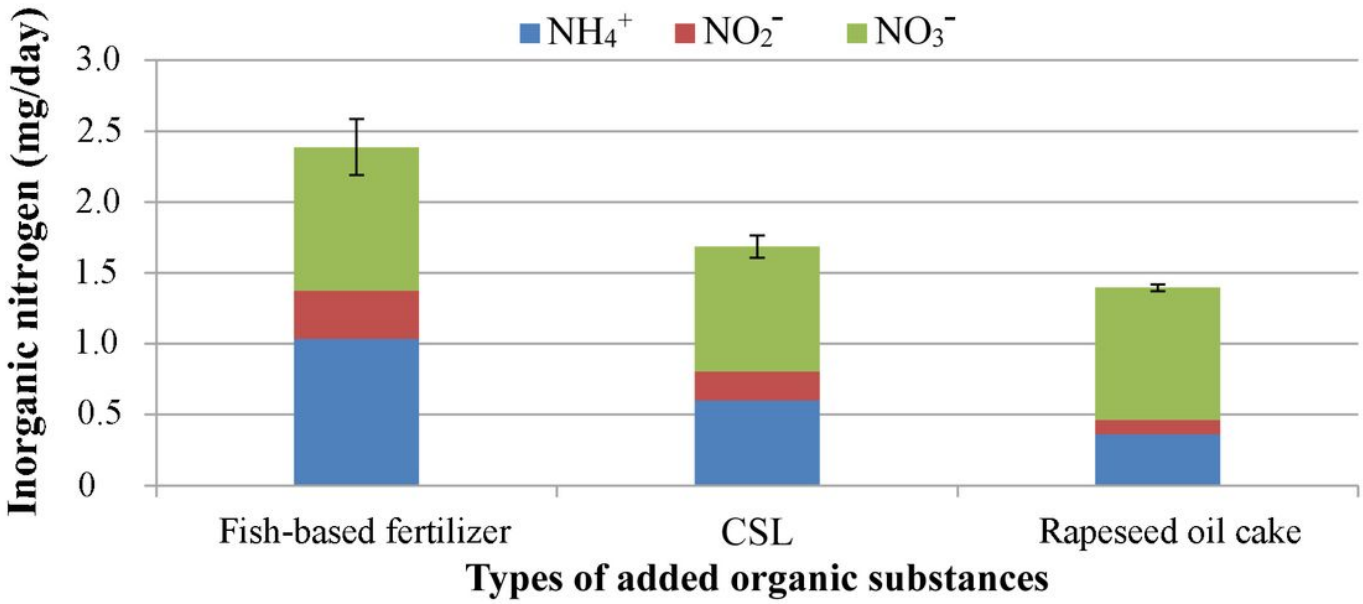


**Figure 3**

Effect of organic substance type on N mineralization. Composition of inorganic N in the leachate from rockwool treated with different organic substances. CSL, corn steep liquor. Error bars show SD of 3 iterations.

## Supplementary Files

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