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Nitrogen-based fertilizers differentially affect protist community composition in paddy field soils

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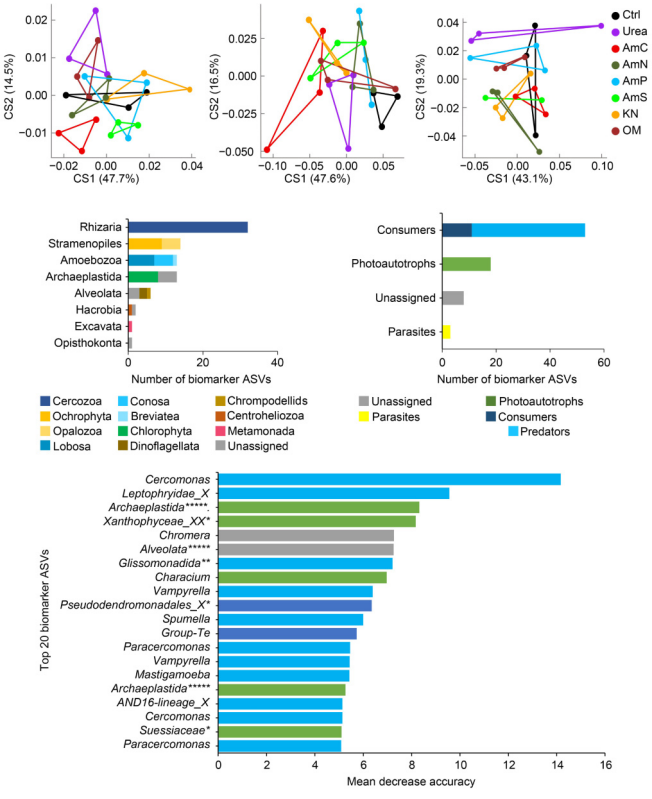
ABSTRACT

• Nitrogen fertilizers' effects on protists in three paddy field soils were analyzed.
• Different nitrogen fertilizers had distinctive effects on the protist communities.
• The effect of nitrogen fertilizers on protist communities slightly depended on the soil types.
• Predatory protists were the main groups that were affected by nitrogen fertilizers.

Protists are one of the most diverse and dominant microbial groups and they play critical roles in the soil ecosystem. Although nitrogen fertilizers have a profound impact on protist communities, still less is known about how different nitrogen fertilizer types affect protist community composition in different soil types. Here we investigated the effects of six inorganic nitrogen fertilizers (urea, ammonium nitrate, ammonium sulfate, potassium nitrate, ammonium chloride, and diammonium hydrogen phosphate) and an organic fertilizer (a mixture of rice husk and cow manure) on protist community composition in three paddy field soils using a high-throughput sequencing method. The effect of the fertilizers on the functional groups of protists, namely consumers (predators and decomposers), photoautotrophs, and parasites (plant pathogens and animal parasites) was also analyzed. The results showed that nitrogen fertilizers had distinctive effects on the beta diversity of the protists, while we also observed that the same fertilizer had slightly different effects depending on the soil type. Amoebozoa and Rhizaria were the most affected protist taxonomical groups, while predatory protists were the main functional groups that were affected by nitrogen fertilizers. Random forest analysis showed that most of the fertilizer-affected protists were predators, among which Cercozoa was the most affected taxa. In conclusion, our results provide important insights into the impact of nitrogen fertilizers on soil protist communities.



Keywords nitrogen fertilizer, bottom-up, paddy field, predatory protist, soil protist community

1 Introduction

Protists are one of the most dominant and diverse microbial groups in the soil ecosystem. They exhibit high taxonomic diversity, allowing them to play important functional roles as consumers (predators and decomposers), primary producers (photoautotrophs), and parasites (plant pathogens and animal /microbial parasites) (Geisen et al., 2018). Predatory protists that feed on bacteria, archaea, fungi, nematodes, and other protist species are the most dominant group of protists in the soil ecosystem (Gao et al., 2019; Murase and

Asiloglu, 2023). By feeding on microorganisms, predatory protists control microbial community composition, accelerate nutrient turnover, enhance bacterial activities, and increase agricultural productivity (Gao et al., 2019; Asiloglu et al., 2020; Murase and Asiloglu, 2023). Decomposers contribute to the nutrient cycle by decomposing dead organic materials, which results in increased microbial activities (Geisen et al., 2018). Photoautotrophic protists, mainly algae, dominate the surface soils of agricultural fields and have a significant impact on the global soil carbon balance through carbon fixation (Jassey et al., 2015, 2022). Not all protists are beneficial as parasitic protists cause diseases in animals and humans, while plant pathogenic protists cause serious harm to agricultural productivity (Hultberg et al., 2010; Van Buyten and Höfte, 2013). Despite their important roles in nutrient cycling and agricultural productivity, soil protists are the less-studied microbial group in the soil ecosystem (Caron et al., 2009; Sibbald and Archibald, 2017; Asiloglu, 2022).

Protist community composition responds differently to biotic and abiotic factors such as geography, climate, soil water content, pH, root exudates, and inorganic or organic fertilizers (Gao et al., 2019; Murase and Asiloglu, 2023). Among those factors, nitrogen fertilizer is one of the most important factors affecting protist diversity and community composition (Guo et al., 2018; Zhao et al., 2019, 2020; Fiore-Donno et al., 2022). According to Zhao et al. (2019), protist communities show higher responsiveness to nitrogen fertilization than bacteria and fungi. Nitrogen fertilizers have a strong negative impact on protists by reducing protist diversity (Guo et al., 2018; Zhao et al., 2019) and populations (Schnurer et al., 1986; Zhao et al., 2020). The effect of nitrogen fertilizers on protist communities can be stronger than those of water management or seasonality (Murase et al., 2015) and plant genotype (Picot et al., 2021). Among the functional groups of protists, predators are the most vulnerable group to nitrogen addition (Zhao et al., 2020; Sun et al., 2021; Li et al., 2021), which implies that nitrogen fertilizer is an important determinant of the microbial food web (Murase et al., 2015). Conflicting results were also observed in some studies. For instance, Lentendu et al. (2014) showed that protist communities were not affected by nitrogen fertilizers, and Krashevska et al. (2014) showed a positive impact of nitrogen fertilizers on testate amoeba populations. Since studies are done in different agricultural soil types using different amounts of nitrogen, it is difficult to evaluate the conflicting results. In addition, different types of nitrogen fertilizers are used in those studies: most of the studies are done with urea (Krashevska et al., 2014; Zhao et al., 2019, 2020; Sun et al., 2021; Li et al., 2021), while only a few used different fertilizer types such as ammonium nitrate (Picot et al., 2021) and ammonium sulfate (Murase et al., 2015). Therefore, although nitrogen fertilizers have a profound impact on protist communities, still less is known about how different nitrogen fertilizers affect protist community composition in different soil types.

Despite the number of protist studies being far behind that of bacterial and fungal studies, protists are relatively well studied in the paddy field ecosystems (Murase and Asiloglu, 2023). Due to irrigation, the paddy field is a unique environment with characteristic biogeochemical cycles (Kirk, 2004). Although activities of micro-eukaryotes, especially fungi and small soil animals are limited in paddy fields due to flooding and anoxia (Nakamura et al., 2003; Murase et al., 2015; Asiloglu et al., 2015; Asiloglu and Murase, 2016), protists are well-adapted to the paddy fields, allowing them to play important roles in the microbial food web in rice field soil (Murase and Asiloglu, 2023). In addition, the interface between the floodwater and soil (the oxic layer) in the paddy field soil is an optimal environment for photoautotrophic protists, contributing to the net primary production (Murase and Asiloglu, 2023). Therefore, paddy fields provide a model ecosystem to study protist community composition.

Here, we studied the short-term effects of six inorganic nitrogen fertilizers (urea, ammonium nitrate, ammonium sulfate, potassium nitrate, ammonium chloride, and diammonium hydrogen phosphate) and a commercial organic fertilizer (a mixture of rice husk and cow manure) on protist community composition in three paddy field soils with distinct soil physicochemical properties. In this study, we hypothesized that protist communities would be differentially affected by the nitrogen fertilizers. High throughput sequencing and bioinformatics were used to reveal the changes in protists' taxonomic and functional community composition.

2 Materials and methods

2.1 Soil samples, experimental set-up, and sampling

Soil samples were taken from the plow layer (0−10 cm) of three Japanese paddy fields. The fields were selected based on their distinct physicochemical properties (Fujino et al., 2023) to evaluate the effect of nitrogen fertilizers under various soil types. Field 1 sample (ACH) was collected on 18th April 2021 from Chita, Aichi (N34.98, E136.89); Field 2 sample (SHD) was collected on 25th March 2021 from Shindori Station in the Field Center for Sustainable Agriculture and Forestry, Niigata University, Niigata (N37.85, E138.96); and Field 3 sample (NAG) was collected on 23rd April 2021 from Hata, Matsumoto City, Nagano (N36.20, E137.87). Soil samples were obtained from at least five locations in each field and then combined. Each soil sample was air-dried, sieved (< 2 mm), individually mixed to be homogenized, and then stored (4°C). Physicochemical properties and classifications of the soils are previously published (Kononov et al., 2022). World Reference

Base for Soil Resources (WRB) was used to classify the soil types. The soil properties were briefly provided in Table 1.

Commonly used inorganic and organic fertilizers at field application doses were used in this study to mimic the paddy field conditions. The inorganic fertilizers were urea (CH_4N_2O), ammonium nitrate (NH_4NO_3), ammonium sulfate ($[NH_4]_2SO_4$), potassium nitrate (KNO_3), ammonium chloride (NH_4Cl), and diammonium hydrogen phosphate ($[NH_4]_2HPO_4$). The organic fertilizer (OF) was a mixture of rice husk and cow manure was obtained from a commercially available product (Akagi Engei, Isesaki, Gunma, Japan). The OF was autoclave-sterilized to eliminate the associated microbial community. The nutrient content of the OF that was determined after autoclaving was as follows: total nitrogen, 25 mg g^{-1}; carbon/nitrogen ratio, 15; available phosphate, 34 mg g^{-1}; available potassium, 31 mg g^{-1}. The amounts of inorganic and organic fertilizers were calculated so that they would include the same amount of nitrogen (0.1 mg-N g $soil^{-1}$). The inorganic fertilizers were dissolved in sterile water and then passed through a 0.45 µm filter. Microcosms contained 40 g of the paddy field soils and 60 mL of water to keep them under submerged conditions (2 cm water above topsoil). The microcosms were incubated at room temperature (24°C) in the dark for 3 weeks. During the incubation period, the water level was checked and additional water was added daily. Our previous studies showed that protist communities are affected by environmental factors in 3 weeks (Asiloglu et al., 2021c). By keeping the incubation period short, we could identify the direct effect of fertilizers, as the effect of fertilizers may decrease over time in longer periods. Soil sampling from each container was processed as described previously (Asiloglu et al., 2021a). Briefly, the top water was removed and then the soil in the microcosms was mixed thoroughly. The 0.5 g of the soil was sampled for molecular analysis.

2.2 Molecular analysis and bioinformatics

The ISOIL for Bead Beating (Nippon Gene, Tokyo, Japan) was used to extract DNA from 0.5 g of the soil samples according to the manufacturer's instruction and, then eluted in TE buffer (100 µL). The V9 region of the 18S rRNA gene in the extracted DNA was amplified using the universal eukaryotic primers (1389F/1510R) (Amaral-Zettler et al., 2009) tailed with the barcoded adapters (Illumina, San Diego, CA) (Caporaso et al., 2012). Primary polymerase chain reaction (PCR) was performed as described elsewhere (Amaral-Zettler et al., 2009). Then the PCR products were purified, followed with the index PCR for Illumina MiSeq, and then the Illumina MiSeq sequencing were performed as described previously (Asiloglu et al., 2021c).

The QIIME2 pipeline (Version 2021.11, available at qiime2.org) was used for the primary analysis of raw FASTQ data obtained from the high throughput sequencing. Error correction, removal of forward and reverse primers, quality filtering, singleton, and doubleton removal, and chimera removal of the sequences were done by the DADA2 (Callahan et al., 2016). Only the reads with over a quality score > 30 were used in the following processes. QIIME2's q2-feature-classifier plugin was used against the most recent Protist Ribosomal Reference (PR^2) database (5.0.1) (Guillou et al., 2012) for the taxonomy assignment of the protists (Burki et al., 2020). The non-protist sequences belonging to Fungi, Metazoa, unidentified Opisthokonta, Streptophyta, Rhodophyta, and unclassified eukaryotes were removed from all samples using the Qiime2 (taxa filter-table/seq) to obtain exclusive protist data. We then normalized the read numbers to the minimum sequence reads (4500) to compare the protist communities between the treatments. The beta diversities were analyzed with permutational multivariate analysis of variance (PERMANOVA) with 999 random permutations ($p < 0.05$) with the adonis function of the vegan package. Three major functional groups (consumers, photoautotrophs, and parasites) were used as described previously (Asiloglu et al., 2021c). Briefly, all organisms capable of photosynthesis were assigned as photoautotrophs, and all organisms using phagocytosis to uptake nutrients grouped as consumers, including decomposers and the predators. Any organisms showing a negative effect on its host were labeled as parasites, including microbial/animal/human parasites and plant pathogens. To distinguish predatory protists from consumers, all protists that feed on other microorganisms including mixotrophs, are labeled as predators. The R program (Version 4.2.2, 2022.10.31; available at r-project.org) was used for all of the statistical analyses unless otherwise specified. Random forest classifi-

Table 1 Physicochemical properties of the three paddy field soils used in the study.

Soil type	Sampling site	Sampling date	Soil classification (WRB)	Sand (%)	Silt (%)	Clay (%)	pH	EC (mS cm^{-1})	Total carbon (mg g^{-1})	Total nitrogen (mg g^{-1})	Organic matter (mg g^{-1})	Cation excange capacity (meq g^{-1})
ACH	Agui, Aichi	29 April 2019	Stagnosol	37.0	33.8	29.0	5.17	0.08	15.0	1.5	51.8	9.5
NAG	Matsumoto, Nagano	24 April 2019	Silandic Andosol	33.9	24.4	41.5	5.66	0.80	45.7	4.0	68.2	13.2
SHD	Shindori, Niigata	3 October 2019	Gleyic Fluvisol	48.0	24.0	28.0	5.05	0.12	18.6	1.7	50.6	5.5

*Original data: Kononov et al., 2022.

cation offers sensitive and accurate identification of microorganisms in environmental samples (Roguet et al., 2018). Here we aimed to use random forest analysis to understand the effect of each nitrogen fertilizer type on protist ASVs. For this aim, random forest analysis was separately conducted for each treatment using the randomForest function in the randomForest package (version 4.7-1.1) (Breiman, 2001; Roguet et al., 2018). The significance of each predictor was evaluated by the rfPErmute package (v2.5.1) (Archer, 2013). The raw sequence data has been deposited in the NCBI database under the BioProject ID PRJNA1009153.

3 Results

3.1 Taxonomic and functional composition of protist communities

After removing the non-protist sequences, we obtained 543 970 sequences with 3 180 protist ASVs across the 72 samples (4 500 reads per sample). Four indices were used to evaluate the alpha diversity of protist communities, including evenness, Faith, observed ASVs, and Shannon (Figs. S1 and S2). The alpha diversity of protist communities was different statistically between the soils according to evenness and Shannon index (Fig. S1). While NAG showed the highest evenness and Shannon, ACH had the lowest (Fig. S2). Taxonomical classification showed that Archaeplastida, Rhizaria, Amoebozoa, and Stramenopiles were dominant in all soil types (Fig. 1A, B, C). A comparison of the protist communities in three soil types showed that each soil consisted of different protist communities. According to the functional grouping of protists, the protist community in all soils consists of 45.4%, 37.1%, and 11.8% of consumers, photoautotrophs, and parasites, respectively (Fig. 1D, E, F). In ACH, the most dominant group was Archaeplastida with an average of 47.4% (Fig. 1A), while in NAG, Rhizaria was the most dominant group with 38.1% (Fig. 1B). In SHD, Rhizaria and Archaeplastida were the most dominant groups with 26.0% and 24.7%, respectively (Fig. 1C). Additionally, in SHD, Alveolata was the third most dominant group, more dominant than the other two soils (Fig. 1C). ACH, NAG, and SHD included 35.4%, 59.0%, and 44.8% of consumers, respectively (Fig. 1D, E, and F). NAG and SHD were dominated by consumers, while ACH was dominated by photoautotrophs (52.4%). In SHD, parasites were relatively more abundant than in the other two soils (Fig. 1F).

3.2 Effect of the nitrogen fertilizers on protist community composition

The addition of nitrogen fertilizers did not affect evenness, ASVs, and Shannon Index, although some fluctuations were observed (Fig. S2). The observed difference was only significant between Faith's PD values of the AmC and AmN

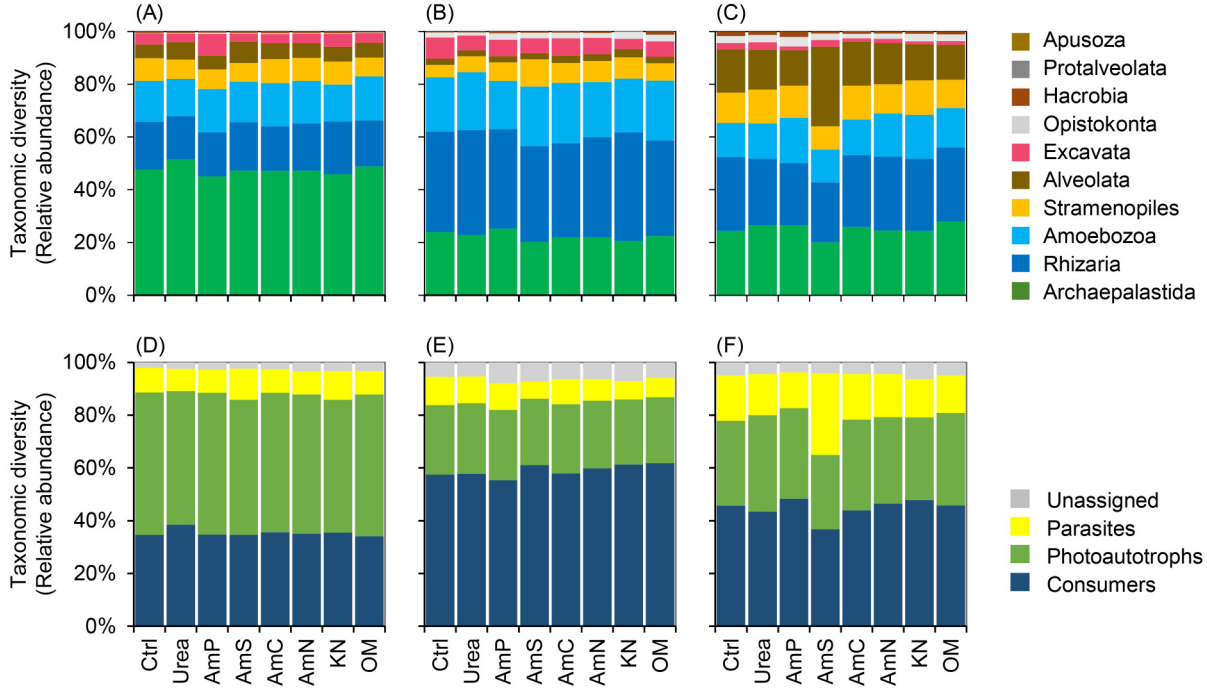


Fig. 1 Relative abundances of the taxonomic (A–C) and functional (D–F) diversity of protist communities in ACH (A and D), NAG (B and E), and SHD (C and F) soils. Ctrl, control treatment with no fertilizer addition; Urea, Urea treatment; AmP, Ammonium phosphate treatment; AmS, Ammonium sulfate treatment; AmC, Ammonium chloride treatment; AmN, Ammonium nitrate treatment; KN, Potassium nitrate treatment; and OM, Organic matter.

treatments in the Shindori soil. The effect of nitrogen fertilizers on the protist beta diversity in each soil was observed as shown in Fig. 2. The PERMANOVA analysis showed that the nitrogen fertilizers significantly affected the beta diversity in ACH (R^2: 0.34108, $p < 0.001$), NAG (R^2: 1.106, $p = 0.021$) and SHD (R^2: 1.1488, $p = 0.006$) soils (Fig. 2). In addition, different applications of nitrogen fertilizers had different effects on the protist community for each soil. The effects of Urea, AmC, and AmS were different from the control group in ACH (Fig. 2A), while AmC, AmS, Urea, and OM applications in NAG (Fig. 2B) and AmN application in SHD (Fig. 2C) had the most significant impact.

The effect of nitrogen fertilizers on the protist community composition was identified by random forest analysis and the significance of the results was analyzed with the rfPermute package in R (Fig. 3), which indicates only the significantly affected taxa. Although the effect of each nitrogen fertilizer slightly differed in each soil, the random forest analysis enabled us to detect the protist ASVs that were commonly affected in all soil types. In Fig. 3A, the specific ASVs associated with each respective nitrogen fertilizer application consistently exhibit the highest relative abundance within their corresponding treatment, as indicated by the green color. We did not observe similar effects of the application between AmC, AmN, AmS, and AmP, all of which release ammonium ions, nor between KN and AmN, which release nitrate ions. For instance, while the specific ASVs associated with the AmS treatment were observed in the highest abundance in OM and their lowest abundance was observed in AmC, the specific ASVs related to the AmC treatment were observed in the highest abundance in AmN, and their lowest abundance was found in KN.

The random forest detected ASVs were taxonomically classified, and they were dominated by Rhizaria, Stramenopiles, Amoebozoa, Archaeplastida, Alveolata, Excavata, Opisthokonta, and Hacrobia respectively (Fig. 3B), while their relative abundances were dominated by Amoebozoa, Opisthokonta, Stramenopiles, Rhizaria, Archaeplastida, Excavata, Alveolata, and Hacrobia respectively (Fig. 3C). The Urea application exhibited the highest number of ASVs, followed by AmN, KN, AmC, AmP, OM, AmS, and Ctrl, which affected the least number of ASVs. Rhizaria showed the highest number of ASVs in all treatments, while Stramenopiles were more abundant in the fertilized conditions than in Ctrl (Fig. 3B). As shown in Fig. 3C, Amoebozoa exhibited the highest relative abundance except Ctrl, where Stramenopiles displayed the highest relative abundance.

When categorizing the number of ASVs based on their functional classification, consumers had the highest number of ASVs, followed by photoautotrophs and parasites in this order. The relative abundance also showed the same pattern. Consumers accounted for 59.2% of random forest detected ASVs and the relative abundance was 56.2% (Figs. 3D and E). Among all treatment groups, as indicated by mean decrease Gini (MDG), Cercozoa demonstrated the highest level of susceptibility (Fig. S3).

3.3 Effect of the nitrogen fertilizers on the predatory protists

Using biomarker ASVs that were detected by random forest analysis, we identified the protist ASVs most affected by the nitrogen fertilizer application. Figure 4A shows the number of protist biomarker ASVs in supergroups and phyla of protists for all soil types and treatments. Rhizaria, Stramenopiles, Amoebozoa, and Archaeplastida were the most abundant supergroups affected by the nitrogen fertilizer application (Fig. 4A). At the phylum level, Cercozoa (Rhizaria), Ochrophyta (Stramenopiles), Chlorophyta (Archaeplastida) and Lobosa (Amoebozoa) were the most affected. Figure 4B shows the number in functional groups affected. Nearly two-thirds of the biomarker ASVs, 64.6%,

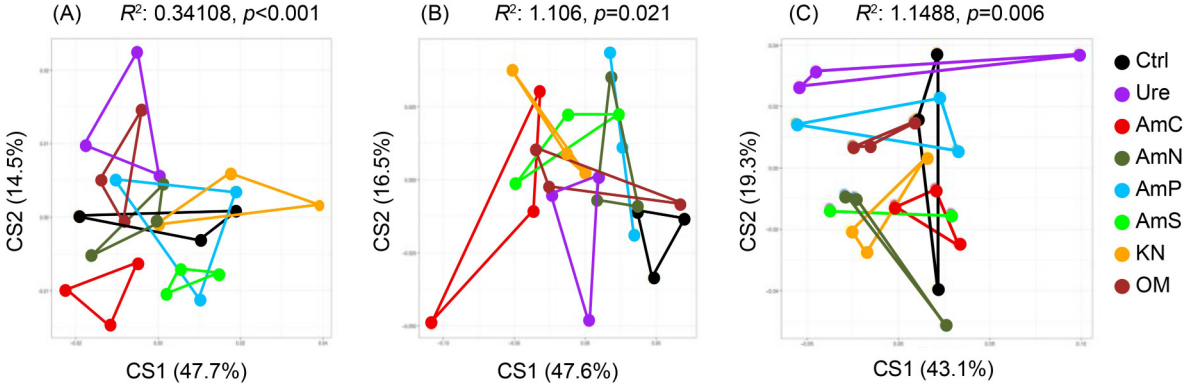


Fig. 2 Principal coordinates analysis (PCoA) was performed at the Amplicon Sequence Variant (ASV) level based on the Bray-Curtis dissimilarity index for Aichi (A), Nagano (B), and Shindori (C) soil samples. Ctrl, control treatment with no fertilizer addition; Urea, urea treatment; AmP, Ammonium phosphate treatment; AmS, Ammonium sulfate treatment; AmC, Ammonium chloride treatment; AmN, Ammonium nitrate treatment; KN, Potassium nitrate treatment; and OM, Organic matter. The R^2 and p values represent the results of PERMANOVA analysis for the beta diversity.

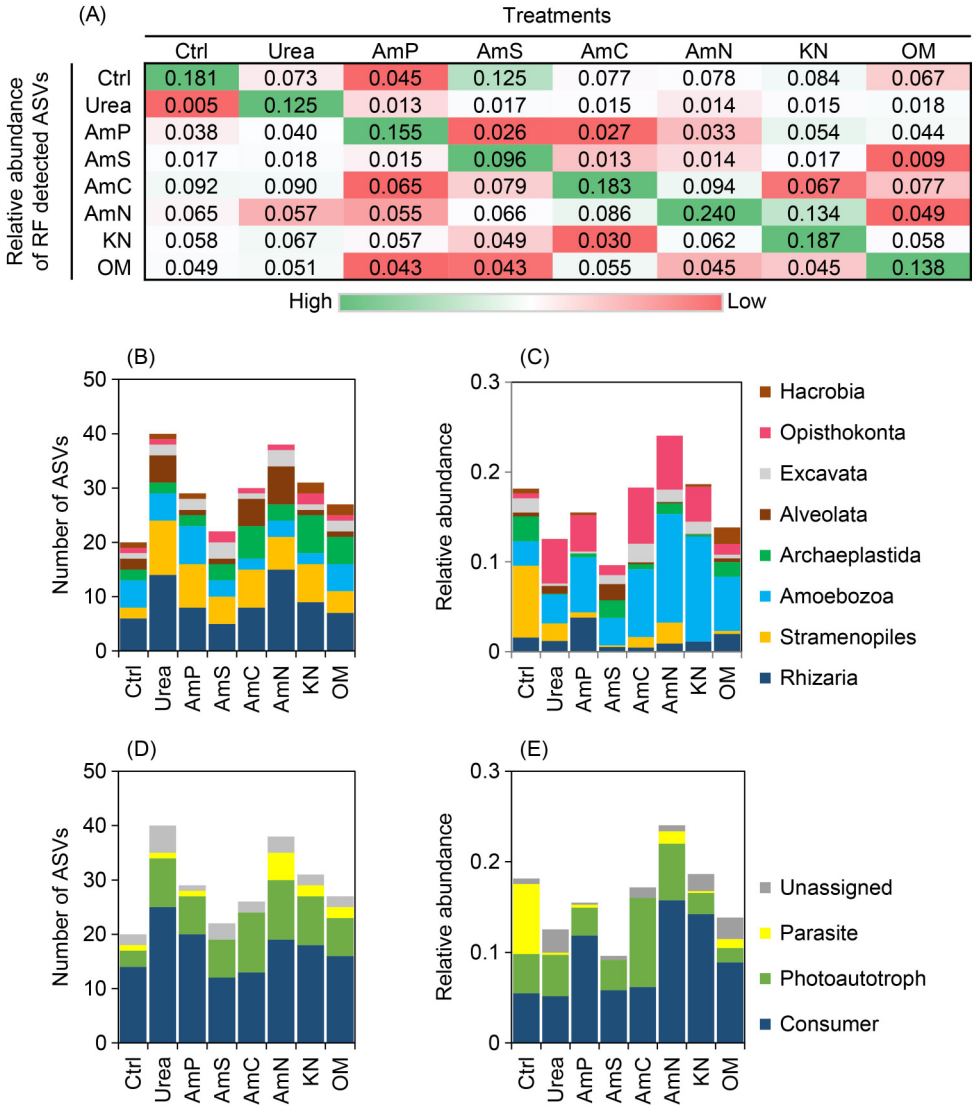


Fig. 3 Total relative abundance of significantly affected ASVs as detected by random forest analysis (A). Rows represent treatments and columns represent the relative abundances of specific ASVs to each treatment. Bar plots show the number of ASVs (B and D) and their relative abundances (C and E) both taxonomically (B and C) and functionally (D and E). Colors represent the supergroups and functional groups. Ctrl, control treatment with no fertilizer addition; Ure, Urea treatment; AmP, Ammonium phosphate treatment; AmS, Ammonium sulfate treatment; AmC, Ammonium chloride treatment; AmN, Ammonium nitrate treatment; KN, Potassium nitrate treatment; and OM, Organic matter.

were consumers, 49.5% of which belonged to Cercozoa (Fig. 4B). The top 20 protist sequences based on MDG are shown in Fig. 4C. Of 20 protist genera, 11 were consumers, 5 were photoautotrophs, and 2 were decomposers. Notably, *Cercomonas* sp. emerged as the most prominently affected protist species. Most of the protist species affected by the nitrogen fertilizer applications were bacterial predators, although some fungivorous protists were detected (i.e., *Vampyrella* sp.).

4 Discussion

Although the effect of nitrogen fertilizers on protist communities is well-studied (Geisen et al., 2018; Guo et al., 2018; Zhao et al., 2019; Fiore-Donno et al., 2022; Murase and Asiloglu, 2023), only a few types of nitrogen sources have been examined, including urea (Krashevska et al., 2014; Zhao et al., 2019, 2020; Sun et al., 2021; Li et al., 2021), ammonium nitrate (Picot et al., 2021) and ammonium sulfate (Murase et al., 2015). In addition, most of the studies did not evaluate the effect of nitrogen fertilizers in different soil types. Here we investigated the effects of six inorganic nitrogen fertilizers and one organic fertilizer on protist communities in three types of paddy field soils and found that the application of different fertilizers affected soil protist communities differently (Figs. 2 and 3). Among the functional groups, predatory protist communities were the most

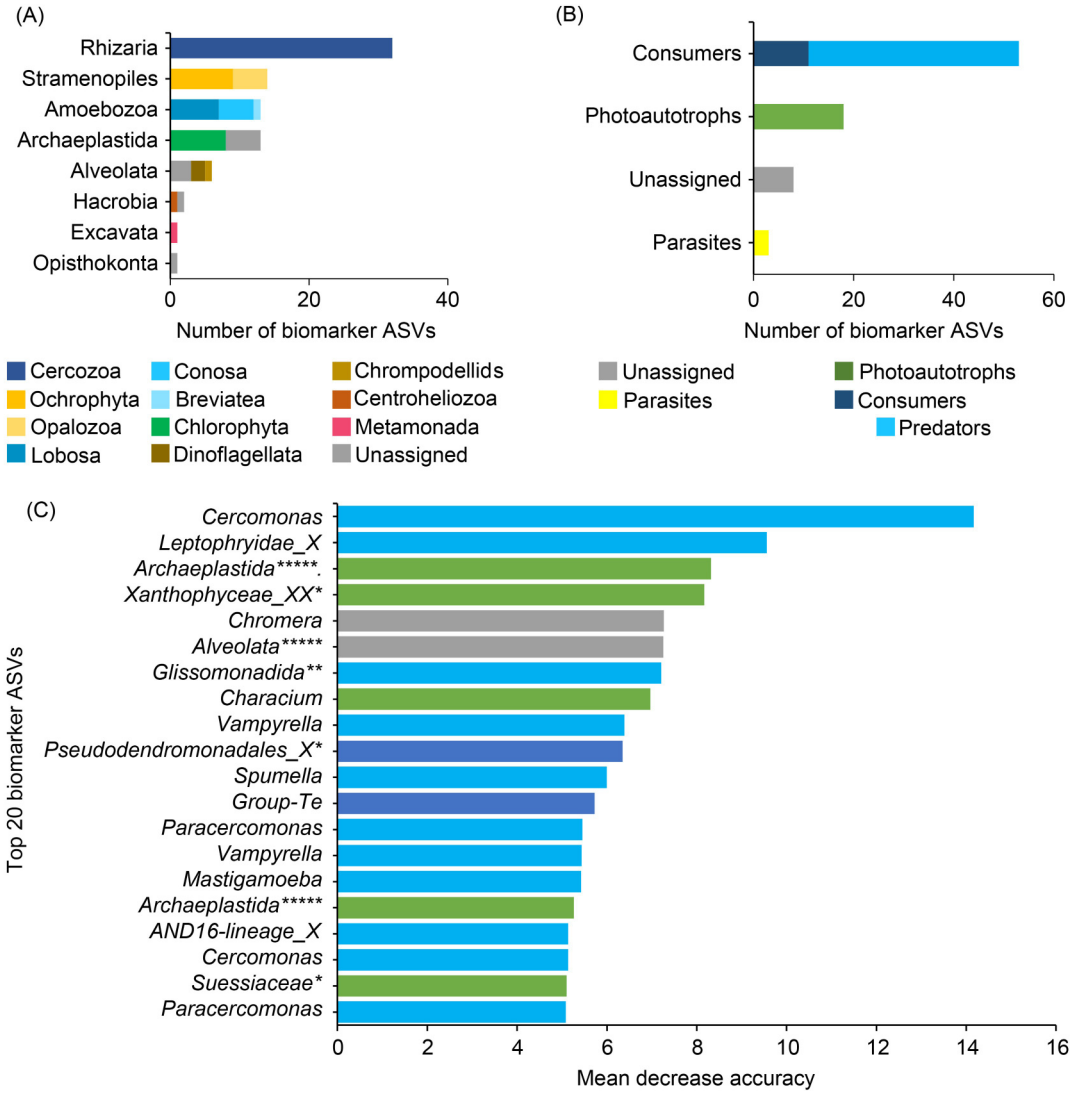


Fig. 4 The number of biomarker ASVs detected by random forest analysis for each supergroup and phylum levels (A) and their functionalities (B). The bar plot in C shows the top 20 biomarker ASVs with taxonomic information at species level (colors indicate functional groups). *, unassigned taxonomic information. Light blue, predators; blue, consumers; green, photoautotrophs; yellow, parasites; gray, unassigned.

sensitive group to nitrogen addition (Fig. 3D−E and Fig. 4B−C). Comparison of protist communities in different soil types under field conditions is often challenging due to the geographical differences including climate and soil water content, which are major factors affecting protist communities (Geisen et al., 2018; Murase and Asiloglu, 2023). To minimize the effects of the geographical differences, we conducted a short-term microcosm study to focus on the effects of several nitrogen fertilizers on soil protist communities. This approach, which was also used in our previous studies (Asiloglu et al., 2021c, 2021b), enabled us to directly evaluate how nitrogen fertilizers shape protist communities in different soil types.

In our study, the effect of nitrogen fertilizers on protist community composition was evaluated using both alpha and beta diversity measures. Only a few significant changes were observed in alpha diversity indices (Supplementary Fig. S2), which may have resulted from the short experimental period in our study. Beta diversity analysis revealed significant differences in protist communities between different fertilizer applications in each soil type in this study (Figs. 2 and 3). The results are in line with previous studies showing that long-term nitrogen fertilization significantly changed the community composition of protists rather than alpha diversity (Li et al., 2021; Zhang et al., 2022).

The random forest analysis provided further insight into the effect of nitrogen fertilizers on protists. The identification of specific ASVs associated with each fertilizer application highlighted the potential for nitrogen fertilization to affect certain protist taxa. Moreover, the taxonomic classification of ASVs revealed the sensitivity of Rhizaria, Stramenopiles, Amoebozoa, and Archaeplastida to nitrogen applications

(Figs. 3 and 4). The relative abundances of the protist ASVs affected by nitrogen applications showed that Amoebozoa and Stramenopiles were particularly sensitive. After the application of ammonium-based fertilizers, the affected protist ASVs differed depending on the type of fertilizers used, suggesting that not only nitrogen sources but also accompanying ions may alter protist community composition in the soil. Although nitrogen fertilizers are among the major factors affecting protists, other fertilizers such as phosphorus and potassium are also known to alter protist communities, including predatory protists and green algae (Murase et al., 2015). We also observed some changes in the effect of the same fertilization in different soil types, which is in line with previous reports (Zhao et al., 2019; Murase and Asiloglu, 2023). Therefore, both soil type and applied fertilizer would be essential determinants of the composition and function of protists in paddy field soils (Murase et al., 2015) as demonstrated in upland fields (Xiong et al., 2018).

Predators are the major functional group of protists across soil ecosystems (Gao et al., 2019), including paddy fields (Asiloglu et al., 2021c, 2021b; Murase and Asiloglu, 2023). Compared to the upland soils, the paddy soils include higher amounts of microbial biomass (Wei et al., 2022), which would benefit predatory protists. Predatory protists potentially impact microbial biomass more in rice field soil than in upland soil (Murase and Asiloglu, 2023). Our previous study in paddy fields showed that the top-down effects of predatory protists are more significant than the bottom-up effects of fertilizers on the bacterial communities (Asiloglu et al., 2021a). Therefore, fertilizer-induced changes in the predatory protist communities are likely to affect bacterial community composition in soil. Predatory protists not only affect bacterial community composition and function but also modulate fungal communities (Huang et al., 2021). In this study, we found several fungivorous protist ASVs (*i.e.*, *Vampyrella* sp.) were affected by nitrogen fertilizer applications. This suggests that fertilizer-induced changes in predatory protist community composition are likely to affect microbial food webs and, then, alter microbial communities and functions in paddy fields. However, still less is known about whether nitrogen fertilizers directly affect predatory protists or not. Although ammonium can be toxic to predatory protists (Puigagut et al. 2005), it is unlikely that toxicity of ammonium appeared in this study because only a small amount of nitrogen fertilizers (0.1 mg-N g soil^{-1}) was added to the soil. A possible indirect effect of nitrogen fertilizers on predatory protists could be due to changes in the prey (bacterial and fungal) communities (Xiong et al., 2018).

Although each nitrogen fertilizer affected different protist groups, Cercozoa was the most affected taxonomic class in all of the treatments. According to random forest analysis, the biomarker ASVs with the highest MDG belonged to *Cercomonas* sp. (Cercozoa). *Cercomonas* sp. is one of the most common protists in the soil ecosystem and is highly sensitive to changes in the soil nutrient status (Geisen et al., 2018; Gao et al., 2019). According to Guo et al. (2021), Cercozoan protists including *Cercomonas* sp. alter bacterial communities and activities, which may have a positive impact on plant growth. In addition, increases in *Cercomonas* sp. population after fertilizer application play a crucial role in plant health by suppressing a plant pathogen (Guo et al., 2022). Although *Cercomonas* sp. has commonly been known to be bacterivorous, it was shown that they also feed on fungi, including plant-pathogenic *Fusarium* sp. (Geisen et al., 2016). Taking together, it could be concluded that nitrogen fertilizer application has an important effect on predatory protist species in the paddy field soil, which in turn affects microbiome dynamics, nutrient turnover, and plant production.

5 Conclusion

Here we showed that nitrogen fertilizer application affected protist community composition based on fertilizer types and soil types. Although this was a short-term experiment using the amount of nitrogen typically applied in paddy fields, we were able to detect important changes in the soil protist communities. The findings highlight the importance of fertilizer application on the protist communities, particularly on the predatory protists, in the paddy soil. Considering that the predatory protists are one of the most important determinants controlling microbial (bacterial and fungal) communities and functions in paddy field soils, future studies should focus on trophic interaction between soil microorganisms that may be modified by fertilizer application.

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Data availability

The raw sequence data obtained in this study have been deposited in the NCBI database under the BioProject ID PRJNA1009153.

Ethics approval

Not applicable.

Conflict of Interest

The authors declare no competing or financial interests.

Authors contributions

RA and SBO designed the study with the advice of KS and NH. SBO and SOS performed the laboratory work and high-throughput sequencing and bioinformatics. SBO and RA interpreted the results and prepared the manuscript. KS and NH provided feedback and comments. All authors read and approved the final manuscript.

Electronic supplementary material

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References

Amaral-Zettler, L.A., McCliment, E.A., Ducklow, H.W., Huse, S.M., 2009. A method for studying protistan diversity using massively parallel sequencing of v9 hypervariable regions of small-subunit ribosomal RNA genes. PLoS One 4, e6372.

Archer, E., 2013. rfPermute: Estimate Permutation p-Values for Random Forest Importance Metrics. *R package version*. **1.5.2**.

Asiloglu, R., 2022. Biochar–microbe interaction: more protist research is needed. Biochar 4, 72.

Asiloglu, R., Honjo, H., Saka, N., Asakawa, S., Murase, J., 2015. Community structure of microeukaryotes in a rice rhizosphere revealed by DNA-based PCR-DGGE. Soil Science and Plant Nutrition 61, 761–768.

Asiloglu, R., Kenya, K., Samuel, S.O., Sevilir, B., Murase, J., Suzuki, K., Harada, N., 2021a. Top-down effects of protists are greater than bottom-up effects of fertilisers on the formation of bacterial communities in a paddy field soil. Soil Biology & Biochemistry 156, 108186.

Asiloglu, R., Murase, J., 2016. Active community structure of microeukaryotes in a rice (*Oryza sativa* L.) rhizosphere revealed by RNA-based PCR-DGGE. Soil Science and Plant Nutrition 62, 440–446.

Asiloglu, R., Samuel, S.O., Sevilir, B., Akca, M.O., Acar Bozkurt, P., Suzuki, K., Murase, J., Turgay, O.C., Harada, N., 2021b. Biochar affects taxonomic and functional community composition of protists. Biology and Fertility of Soils 57, 15–29.

Asiloglu, R., Shiroishi, K., Suzuki, K., Turgay, O.C., Harada, N., 2021c. Soil properties have more significant effects on the community composition of protists than the rhizosphere effect of rice plants in alkaline paddy field soils. Soil Biology & Biochemistry 161, 108397.

Asiloglu, R., Shiroishi, K., Suzuki, K., Turgay, O.C., Murase, J., Harada, N., 2020. Protist-enhanced survival of a plant growth promoting rhizobacteria, *Azospirillum* sp. B510, and the growth of rice (*Oryza sativa* L.) plants. Applied Soil Ecology 154, 103599.

Breiman, L., 2001. Random forests. Machine Learning 45, 5–32.

Burki, F., Roger, A.J., Brown, M.W., Simpson, A.G.B., 2020. The new tree of eukaryotes. Trends in Ecology & Evolution 35, 43–55.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods 13, 581–583.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME Journal 6, 1621–1624.

Caron, D.A., Worden, A.Z., Countway, P.D., Demir, E., Heidelberg, K.B., 2009. Protists are microbes too: a perspective. ISME Journal 3, 4–12.

Fiore-Donno, A.M., Human, Z.R., Štursová, M., Mundra, S., Morgado, L., Kauserud, H., Baldrian, P., Bonkowski, M., 2022. Soil compartments (bulk soil, litter, root and rhizosphere) as main drivers of soil protistan communities distribution in forests with different nitrogen deposition. Soil Biology & Biochemistry 168, 108628.

Fujino, M., Suzuki, K., Harada, N., Asiloglu, R., 2023. Protists modulate active bacterial community composition in paddy field soils. Biology and Fertility of Soils 59, 709–721.

Gao, Z., Karlsson, I., Geisen, S., Kowalchuk, G., Jousset, A., 2019. Protists: Puppet masters of the rhizosphere microbiome. Trends in Plant Science 24, 165–176.

Geisen, S., Koller, R., Hünninghaus, M., Dumack, K., Urich, T., Bonkowski, M., 2016. The soil food web revisited: Diverse and widespread mycophagous soil protists. Soil Biology & Biochemistry 94, 10–18.

Geisen, S., Mitchell, E.A.D., Adl, S., Bonkowski, M., Dunthorn, M., Ekelund, F., Fernández, L.D., Jousset, A., Krashevska, V., Singer, D., Spiegel, F.W., Walochnik, J., Lara, E., 2018. Soil protists: A fertile frontier in soil biology research. FEMS Microbiology Reviews 42, 293–323.

Guillou, L., Bachar, D., Audic, S., Bass, D., Berney, C., Bittner, L., Boutte, C., Burgaud, G., de Vargas, C., Decelle, J., del Campo, J., Dolan, J.R., Dunthorn, M., Edvardsen, B., Holzmann, M., Kooistra, W.H.C.F., Lara, E., Le Bescot, N., Logares, R., Mahé, F., Massana, R., Montresor, M., Morard, R., Not, F., Pawlowski, J., Probert, I., Sauvadet, A.L., Siano, R., Stoeck, T., Vaulot, D., Zimmermann, P., Christen, R., 2012. The Protist Ribosomal Reference database (PR2): a catalog of unicellular eukaryote small sub-unit rRNA sequences with curated taxonomy. Nucleic Acids Research 41, D597–D604.

Guo, S., Tao, C., Jousset, A., Xiong, W., Wang, Z., Shen, Z., Wang, B., Xu, Z., Gao, Z., Liu, S., Li, R., Ruan, Y., Shen, Q., Kowalchuk, G.A., Geisen, S., 2022. Trophic interactions between predatory protists and pathogen-suppressive bacteria impact plant health. ISME Journal 16, 1932–1943.

Guo, S., Xiong, W., Hang, X., Gao, Z., Jiao, Z., Liu, H., Mo, Y., Zhang, N., Kowalchuk, G.A., Li, R., Shen, Q., Geisen, S., 2021. Protists as main indicators and determinants of plant performance. Microbiome 9, 64.

Guo, S., Xiong, W., Xu, H., Hang, X., Liu, H., Xun, W., Li, R., Shen, Q., 2018. Continuous application of different fertilizers induces distinct bulk and rhizosphere soil protist communities. European

Journal of Soil Biology 88, 8–14.

Huang, X., Wang, J., Dumack, K., Liu, W., Zhang, Q., He, Y., Di, H., Bonkowski, M., Xu, J., Li, Y., 2021. Protists modulate fungal community assembly in paddy soils across climatic zones at the continental scale. Soil Biology & Biochemistry 160, 108358.

Hultberg, M., Alsberg, T., Khalil, S., Alsanius, B., 2010. Suppression of disease in tomato infected by *Pythium ultimum* with a biosurfactant produced by *Pseudomonas koreensis*. BioControl 55, 435–444.

Jassey, V.E.J., Signarbieux, C., Hättenschwiler, S., Bragazza, L., Buttler, A., Delarue, F., Fournier, B., Gilbert, D., Laggoun-Défarge, F., Lara, E., Mills, R.T.E., Mitchell, E.A.D., Payne, R.J., Robroek, B.J.M., 2015. An unexpected role for mixotrophs in the response of peatland carbon cycling to climate warming. Scientific Reports 5, 16931.

Jassey, V.E.J., Walcker, R., Kardol, P., Geisen, S., Heger, T., Lamentowicz, M., Hamard, S., Lara, E., 2022. Contribution of soil algae to the global carbon cycle. New Phytologist 234, 64–76.

Kirk, G., 2004. The biogeochemistry of submerged soils. Wiley, Chichester.

Kononov, A., Hishida, M., Suzuki, K., Harada, N., 2022. Microplastic extraction from agricultural soils using canola oil and unsaturated sodium chloride solution and evaluation by incineration method. Soil Systems 6, 54.

Krashevska, V., Sandmann, D., Maraun, M., Scheu, S., 2014. Moderate changes in nutrient input alter tropical microbial and protist communities and belowground linkages. ISME Journal 8, 1126–1134.

Lentendu, G., Wubet, T., Chatzinotas, A., Wilhelm, C., Buscot, F., Schlegel, M., 2014. Effects of long-term differential fertilization on eukaryotic microbial communities in an arable soil: a multiple barcoding approach. Molecular Ecology 23, 3341–3355.

Li, F., Sun, A., Jiao, X., Bi, L., Zheng, Y., He, J.Z., Hu, H.W., 2021. Specific protistan consumers and parasites are responsive to inorganic fertilization in rhizosphere and bulk soils. Journal of Soils and Sediments 21, 3801–3812.

Murase, J., Asiloglu, R., 2023. Protists: the hidden ecosystem players in a wetland rice field soil. Biology and Fertility of Soils. https://doi.org/10.1007/s00374-023-01705-9.

Murase, J., Hida, A., Ogawa, K., Nonoyama, T., Yoshikawa, N., Imai, K., 2015. Impact of long-term fertilizer treatment on the microeukaryotic community structure of a rice field soil. Soil Biology & Biochemistry 80, 237–243.

Nakamura, A., Tun, C.C., Asakawa, S., Kimura, M., 2003. Microbial community responsible for the decomposition of rice straw in a paddy field: Estimation by phospholipid fatty acid analysis. Biology and Fertility of Soils 38, 288–295.

Picot, E., Hale, C.C., Hilton, S., Teakle, G., Schäfer, H., Huang, Y. J., Perryman, S., West, J.S., Bending, G.D., 2021. Contrasting responses of rhizosphere bacterial, fungal, protist, and nematode communities to nitrogen fertilization and crop genotype in field grown oilseed rape (*Brassica napus*). Frontiers in Sustainable Food Systems 5, 613269.

Roguet, A., Eren, A.M., Newton, R.J., McLellan, S.L., 2018. Fecal source identification using random forest. Microbiome 6, 185.

Schnurer, J., Clarholm, M., Rosswall, T., 1986. Fungi, bacteria and protozoa in soil from four arable cropping systems. Biology and Fertility of Soils 2, 119–126.

Sibbald SJ, Archibald JM., 2017. More protist genomes needed. Nature Ecology & Evolution 1, 0145.

Sun, A., Jiao, X.Y., Chen, Q., Trivedi, P., Li, Z., Li, F., Zheng, Y., Lin, Y., Hu, H.W., He, J.Z., 2021. Fertilization alters protistan consumers and parasites in crop-associated microbiomes. Environmental Microbiology 23, 2169–2183.

Van Buyten, E., Höfte, M., 2013. Pythium species from rice roots differ in virulence, host colonization and nutritional profile. BMC Plant Biology 13, 203.

Wei, L., Ge, T., Zhu, Z., Ye, R., Peñuelas, J., Li, Y., Lynn, T.M., Jones, D.L., Wu, J., Kuzyakov, Y., 2022. Paddy soils have a much higher microbial biomass content than upland soils: A review of the origin, mechanisms, and drivers. Agriculture, Ecosystems & Environment 326, 107798.

Xiong, W., Jousset, A., Guo, S., Karlsson, I., Zhao, Q., Wu, H., Kowalchuk, G.A., Shen, Q., Li, R., Geisen, S., 2018. Soil protist communities form a dynamic hub in the soil microbiome. ISME Journal 12, 634–638.

Zhang, S., Zhang, H., Liu, H., Wang, H., Xiu, W., Li, G., Zhang, G., Zhou, Z., Jiang, N., Zhang, H., Zhao, J., Yang, D., 2022. Fertilization drives distinct biotic and abiotic factors in regulating functional groups of protists in a 5-year fertilization system. Frontiers in Microbiology 13, 1036362.

Zhao, Z.B., He, J.Z., Geisen, S., Han, L.L., Wang, J.T., Shen, J.P., Wei, W.X., Fang, Y.T., Li, P.P., Zhang, L.M., 2019. Protist communities are more sensitive to nitrogen fertilization than other microorganisms in diverse agricultural soils. Microbiome 7, 33.

Zhao, Z.B., He, J.Z., Quan, Z., Wu, C.F., Sheng, R., Zhang, L.M., Geisen, S., 2020. Fertilization changes soil microbiome functioning, especially phagotrophic protists. Soil Biology & Biochemistry 148, 107863.