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Fish Mediate Surface Soil Methane Oxidation in the Agriculture Heritage Rice–Fish System

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Abstract

Though the effects of soil microorganisms, plants, and their interaction on methane (CH_4) oxidation have been well documented, the roles of animals in this process are less known. We examined how a local common carp, *Cyprinus carpio*, affects CH_4 oxidation in surface soil (that is, soil–water interface) in a 1200-year-old agriculture heritage rice–fish system. A 5-year experiment (field experiment 1) showed that rice yield and soil nitrogen (N) were higher under rice–fish co-culture than rice monoculture. Fish presence did not change CH_4 emission but increased CH_4 production archaea (methanogens), and aerobic CH_4 oxidation bacteria (methanotrophs) and CH_4 oxidation. Food component analysis by $\delta^{13}C$ and $\delta^{15}N$ showed that fish foraged paddy-dwelling organisms (for example, duckweeds, algae, phytoplankton, zooplankton, and zoobenthos). A survey on zooplankton *Daphnia* showed that fish decreased *Daphnia* abundance. Mesocosm experiment 1 further indicated that the absence of *Daphnia* increased methanotrophs and CH_4 oxidation. Fish swimming and feeding activity in the paddy circulated the N they excreted and egested. ^{15}N tracing in field experiment 2 demonstrated that N from fish feed was enriched in rice, paddy-dwelling organisms, and fish, while N released by the fish accumulated in soil surface layer (0–1 cm). Mesocosm experiment 2 further indicated that fish-released N increased methanotrophs and CH_4 oxidation. ^{15}N labeled-deoxyribonucleic acid (DNA) stable-isotope probing disclosed that fish feces-N was used by methanotrophs. Our work reveals that the fish enhances surface soil CH_4 oxidation in the rice–fish system by increasing methanotrophs through feeding interactions that cause trophic cascades and drive N transfer.

Key words: common carp; feeding activity; food source; methanotroph; methane oxidation; nitrogen transfer; rice–fish system; trophic interaction.

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Author Contributions XC, LLH, and JJT conceived the work and designed the study. LFZ, RXD, TJZ, LG, QYL, JXC, SYZ, XCX, and LLH conducted the experiment and analyzed the data. XC, LLH, and JJT interpreted the results. All authors contributed critically to the drafts.

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Highlights

- We tested how fish affect surface soil methane (CH_4) oxidation in rice-fish system
- Fish presence in paddy field releases methan-

otrophs from predation by zooplankton
- Fish presence also mediates nitrogen transfer, separately increasing (CH_4) oxidation

Introduction

Methane (CH_4) is an important component of the total carbon (C) emissions of littoral, intertidal zone, paddy field, and other wetland ecosystems (Shine and Sturges 2007; Montzka and others 2011; IPCC 2022). Methane emission is affected by the rates of CH_4 production and oxidation and the modes of CH_4 transport (Conrad 2002; Le Mer and Roger 2001). Living organisms play critical roles in CH_4 emission, but prior research focused mainly on the contributions of microorganisms, plants, and their interactions to this process (Conrad 2009; Kao-Kniffin and others 2010; Laanbroek 2010; Robroek and others 2015). Methane-producing archaea and methane-oxidizing bacteria and archaea such as methanogens and methanotrophs are directly implicated in CH_4 production and oxidation (Chistoserdova and others 2005; Conrad 2007; Conrad 2009). Plants affect the methanogens and methanotrophs in their rhizospheres by mediating C and oxygen (O_2) allocation to them (Saarnio and others 2004; Cho and others 2012). Plant aerenchyma tissues are also crucial CH_4 efflux pathways (Van der Nat and Middelburg 2000; Ström and others 2003), and thus plant vascular system contributes most of the CH_4 emission in wetlands (Seiler and others 1984; Schutz and others 1989), Animals are now increasingly recognized to involve in CH_4 emission (Schmitz and others 2014; Schmitz and others 2018). They affect CH_4 emission through trophic interactions (Dingemans and others 2011; Devlin and others 2015) and by modulating nutrient availability and sediment aeration (Winton and Richardson 2017; Colina and others 2021).

In ecosystems, most of CH_4 produced by anoxic methanogens are oxidized by methane-oxidizing microorganisms (Chistoserdova and others 2005; Le Mer and Roger 2001; Steinsdóttir and others 2022). At the anoxic–oxic interface of wetland ecosystems, for example, 10–30% and 30–99% of the CH_4 are consumed by methanotrophs in rice paddy and in the water column, respectively (Oremland and Culbertson 1992; Neue and others 1997; Kruger and others 2002; Bastviken and others 2008). Hence, understanding methane oxidation process could help to effectively mitigate CH_4 emission. Soil microorganisms involved in CH_4 oxidation include aerobic methane-oxidizing bacteria and anaerobic methane-oxidizing archaea (Chistoserdova and others 2005; Steinsdóttir and others 2022). In paddy field ecosystem, anaerobic methane-oxidizing archaea were found in soil layer of 20–40 cm with relative lower abundances (Wang and others 2022; Fan and others 2020), while high abundances of aerobic methanotrophs were found in the oxygenated soil surface layer (that is, soil–water interface) (Conrad and Rothfuss 1991) and in the O_2 zone of rhizosphere (Cho and others 2012). Most of the total CH_4 produced in the paddy soils were mainly oxidized by aerobic methanotrophs in these oxygenated places (Kumaraswamy and others 1997; Le Mer and Roger 2001; Eller and Frenzel 2001; Horz and others 2001). Thus, the O_2 zone of rhizosphere and soil–water interface in wetland ecosystems are important places in which CH_4 was oxidized.

Animals can directly affect CH_4 oxidation in soil–water interface through their movements and indirectly affect CH_4 oxidation in the rhizosphere by influencing plant growth in wetland ecosystems (Kankaala and others 2006; Winton and Richardson 2017; Booth and others 2019). Animal swimming, muddying, digging, and burrowing can alter the abiotic conditions of the soil surface layer and, therefore, affect CH_4 oxidation (Kankaala and others 2006; Booth and others 2019). Trophic interactions involving animals may also modulate the properties of the soil surface layer wherein microorganisms, phytoplankton, zooplankton, and zoobenthos coexist. Bottom-up and top-down trophic interactions strongly affect C emission and uptake in an ecosystem and carbon cycling within and across habitats (Schindler and others 1997; Strickland and others 2013; Schmitz and others 2018). Trophic interactions involving animals also can drive nutrient recycling in ecosystems through excreta and egesta (Vanni and others 2002; Vanni and others 2013). However, how these trophic interactions mediated by animals affect CH_4 oxidation are less known. As soil surface layer (the soil–water interface) is the important place that the passage through CH_4 is oxidized before emitting (Conrad and Rothfuss 1991), understanding the mechanisms by which animal-mediated trophic interactions affect surface soil CH_4 oxidation would help to mitigate CH_4 emission.

In the present study, we investigated how fish affected surface soil CH_4 oxidation through biotic interactions in a rice–fish system that was listed as one of the globally important agriculture heritage systems (GIAHS) by the Food and Agriculture Organization (FAO) in 2005. The fish was a local population of common carp (*Cypinus carpio*) (Ren

and others 2018) that had been conserved in the rice–fish system by local farmers for over 1200 years. In this ancient rice–fish system, the fish remove rice weeds and insect pests by feeding on them (Xie and others 2011). They also feed on paddy-dwelling duckweeds, algae, phytoplankton, zoobenthos, and zooplankton. (Zhang and others 2017). They help improve nitrogen use efficiency by complementary use N between rice and fish (Xie and others 2011; Guo and others 2022). The roles of fish in these paddy field ecosystems suggest that they may also affect the soil microorganisms that generate and oxidize CH_4 in paddy field ecosystems.

The overall hypothesis of this study is that the fish may enhance aerobic CH_4 oxidation by promoting aerobic methane-oxidizing bacteria (methanotrophs) in the surface soil (the soil–water interface). We hypothesized that the fish could release methanotrophs from ingestion or predation by zooplankton such as *Daphnia* spp. because the fish feed on *Daphnia* (Figure 1A). We also hypothesized that fish could promote methanotrophs in surface soil through mediating nitrogen (N), that is, different forms of N (for example, N in the feed and in the paddy-dwelling organisms) are transformed and release through excreta and egesta by the fish. These fish-released N are more readily utilized by methanotrophs (Figure 1B). To address these hypotheses, we designed a 5-year field experiment to test whether the presence of fish affects CH_4 oxidation in surface soil. We also design field microplot experiments, mesocosm experiments and incubation experiments to test how the fish affects CH_4 oxidation. Aerobic methanotrophs and aerobic CH_4 oxidation are the focus in this study.

Materials and Methods

Study System

This study was conducted at the GIAHS rice–fish co-culture pilot site in southern Zhejiang Province, China (120°26′-121°41′E, 27°25′-28°57′N) *(SI-1)*. In the study area, local farmers culture rice and fish together, and they harvest rice and fish products from the rice–fish system (Xie and others 2011). The growing season for the rice–fish systems is usually from late May to early October. The fish remain in the rice field all year but they are temporarily moved to and kept in a corner of the field in May, when rice is transplanted, and in October, when rice is harvested. Annual fish yield in the rice–fish system is 0.30–1.5 t ha^{-1}. Sometimes local feed was applied by local farmers for obtaining high fish yield.

Experiments

Field, mesocosm and incubation experiments were designed in this study. Field experiments included a 5-year continuous experiment with treatments of rice–fish co-culture and rice monoculture, and a field micro-plot experiment with ^{15}N tracing.

Field Experiments

Field Experiment 1

Field experiment 1 was to test the effects of fish presence in the paddy ecosystem. The experiment was established in 2017 and had a completely randomized block design with two treatments (that is, rice monoculture, RM; and rice–fish co-culture, RF) and four replicates for each treatment. Details of the experimental procedures are described in *SI-2*.

During the rice growing periods of 2020 and 2021, the CH_4 emission and oxidation rates were measured in all experiment plots at the main rice growth stages, that is, transplanting (7 days after transplanting (DAT), tillering (30 DAT), panicle primordium differentiating (50 DAT), flowering (80 DAT), and ripening (110 DAT). CH_4 emission was measured by the static chamber-gas chromatography method (Figure S1; *SI-3*). The CH_4 emission was expressed in mg m^{-2} h^{-1}). The CH_4 oxidation rates were determined by incubating surface soil samples (0–1 cm depth) (*SI-4*). The CH_4 oxidation rate was expressed in $\mu mol \cdot g^{-1} \cdot h^{-1}$.

In 2021, soil methanogens and methanotrophs were measured for all experiment plots on the same dates as the CH_4 emission and oxidation measurements. Soil samples at the 0–1 cm and 5–10 cm depths were collected as described in *SI-4*. Genomic DNA was extracted from all samples (*SI-5*). The *mcrA* copy number was used to indicate methanogen abundance at the 5–10 cm depth and was quantified by real-time quantitative PCR (RT-qPCR) with MLfF/MLrR primers (Table S1; *SI-5*). The *pmoA* copy number was used to indicate methanotroph abundance at the 0–1 cm and 5–10 cm soil depth and was quantified by RT-qPCR with A189f/mb661r primers (Table S1; *SI-5*).

The food components were determined by analyzing the ^{13}C and ^{15}N contents in the paddy-dwelling organisms assumed to be foraged by the fish (Haines and Montague 1979). In 2021, weeds, duckweeds, macroalgae, phytoplankton, zoo-

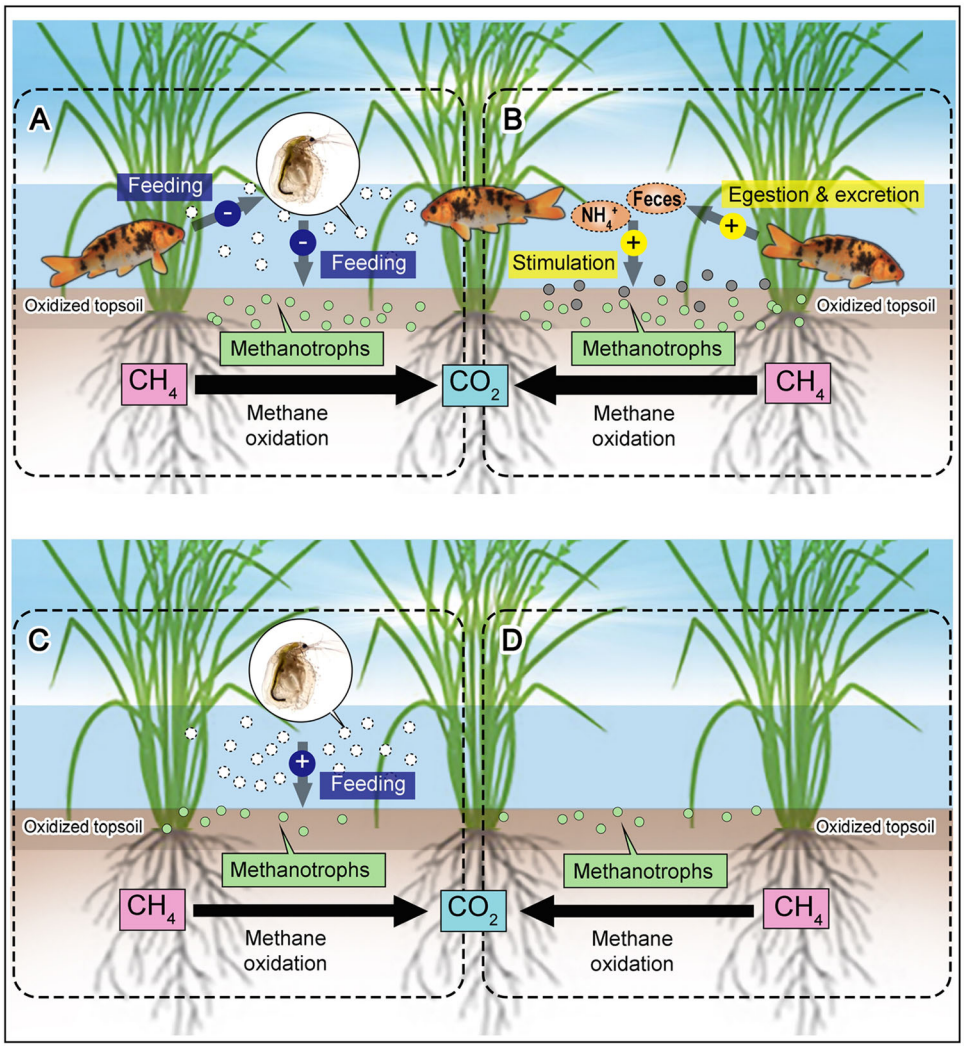


Figure 1. Conceptual model illustrating the possible ways that fish affect surface soil CH_4 oxidation in the agriculture heritage rice–fish system. Compared to rice monoculture without fish (C and D), fish presence in rice field may promote methanotrophs and thus enhance CH_4 oxidation through trophic cascading (A) and through mediating nitrogen (B). **A** These fish are omnivores that preferentially consume zooplanktons such as *Daphnia*. The latter are bacterivores that consume methanotrophs at surface soil (the soil–water interface). Hence, the fish release the methanotrophs from *Daphnia* by ingesting the latter. **B** The fish transform nitrogen (N) in the feed and the biomass of the paddy-dwelling organisms into fish biomass and manure (feces and urine). The fish disperse the manure N throughout the rice field as they swim and feed there. The N released by the fish accumulates in the surface soil where it is directly used by methanotrophs. **C** In rice monoculture, there will be higher abundance of *Daphnia* because of lacking predator (the fish) and lower abundance of methanotrophs. **D** In rice monoculture without fish, there is no fish-released N input, thus there will be lower abundance of methanotrophs. White dots represent *Daphnia*, green dots represent methanotrophs, and gray dots represent N released by fish. '' + '' represents enhancing, '' − '' represents reducing. The width of black arrow indicates the relative quantity of CH_4 oxidation rate.

plankton, and zoobenthos were collected in each RF plot every month during the rice growing period (*SI-6*). Each type of food resources from each month was kept separately. Fish were sampled at the beginning of experiment and at the rice harvest (*SI-7*), and the samples were also kept separately. Each food source from all months was homogenized together and was ground in a ball mill (RETSCH MM 400; Retsch GmbH, Haan, Germany). The ^{13}C and ^{15}N were analyzed with a ThermoFinnigan DELTA Plus continuous-flow isotope ratio MS (Thermo Fisher Scientific). Stable isotope values were reported using δ notation where $\delta^{13}C$ and $\delta^{15}N = ([R_{sample} / R_{standard}] - 1) \times 1000$. The ^{13}C:^{12}C and ^{15}N:^{14}N ratios were also calculated (Peterson and Fry 1987). The con-

tributions of the putative food sources in the field and the input feed were assessed by stable isotope analysis and dietary reconstruction using the linear mixing model (LMM) in Isosource v. 1.3.1 (Microsoft Corp., Redmond, WA, USA) (Phillips and Gregg 2003; Phillips and others 2005). Fish samples collected from different times were separately ground, and the ^{13}C and ^{15}N contents were analyzed. In the diet reconstruction, the ^{13}C and ^{15}N discrimination factors of fish were 2.73‰ and 1.71‰, respectively (Guo and others 2016).

At the same time as the food component analysis, samples of possible food sources were collected from each experimental plot and the biomass was measured (*SI-6*). The weeds, duckweeds, macroalgal, phytoplankton, zooplankton, and zoobenthos were collected, cleaned, oven-dried, and weighted (*SI-6*). *Daphnia* spp. density was measured by collecting field water from the RM and RF plots at the main rice growth stages in 2022. *Daphnia* spp. were identified and enumerated under a dissecting microscope (Nikon SMZ800, Nikon Corporation, Japan). *Daphnia* spp. density was expressed as numbers of individuals per liter water (ind. L^{-1}).

At the rice tillering stage, six quadrats (1.5 m × 1.5 m) were established per RF plot to monitor fish activity in the field. A video recording system was installed near each quadrat (Figure S2; *SI-8*). The fish-released N (excretion-N and feces-N) was measured at stages of rice tillering, heading, and maturing by culturing in an aquarium (*SI-9)*.

Each experimental year (2017–2021), the rice and fish yields were estimated by harvesting rice grain and fresh carp from entire plots. The rice grains were air-dried and weighed and the yield was expressed as air-dried weight of grain in per hectare (*t* air-dried grain ha^{-1}). The fish yield was expressed as fresh weight of carp in per hectare (*t* fresh carp ha^{-1}). Immediately after harvest, soil samples were collected from each plot at 0–20 cm depth and air-dried. The total N content and the soil organic carbon (SOC) were determined by the Kjeldahl and $K_2Cr_2O_7$ oxidation methods, respectively (Lu 1999).

Field Experiment 2

Field experiment 2 was designed to determine how the fish transfer N and how the N is recycled in the rice–fish system by using stable isotope N (^{15}N) as tracer. The experiment was conducted using field microplots (each plot 1.5 m × 3 m). The experiment was a completely randomized block design with two treatments and four replicates. The treatments included rice–fish co-cultures with (i) unlabeled feed as control (CK) and (ii) ^{15}N-labeled feed (^{15}N-feed). Details of the experimental procedures and preparation of ^{15}N-labeled feed were described in *SI-10* and *SI-11*.

Paddy-dwelling organisms were collected monthly from each microplot as described in *SI-6*. Samples of each type of organism were oven-dried and weighed as described in field experiment 1. The ^{15}N content of each type of paddy-dwelling organism was determined with a ThermoFinnigan DELTA Plus continuous-flow isotope ratio MS (Thermo Fisher Scientific) as described in field experiment 1.

At rice harvest, rice and fish samples were collected from each plot (*SI-12*). Soil profile samples (0–1 cm, 1–5 cm, 5–10 cm, and 10–15 cm) were collected from each plot and air-dried. The $\delta^{15}N$ values for all rice, fish, and soil samples were quantified with a ThermoFinnigan DELTA Plus continuous-flow isotope ratio MS (Thermo Fisher Scientific) as described in field experiment 1. The total N content was determined by the method as described in field experiment 1.

Mesocosm Experiments

The mesocosm experiments were designed to test how fish-mediated trophic cascade and fish-released N (excretion-N and feces-N) on methanotrophs and CH_4 oxidation.

Mesocosm Experiment 1

Mesocosm experiment 1 was conducted to test the effects of *Daphnia* density on methanotrophs and CH_4 oxidation. The dominant species *Daphnia magna* in the paddy field was used in the experiment. The experiment had three *Daphnia* densities and four replicates: The density treatments were: (i) CK (no *Daphnia*), (ii) LD (low *Daphnia* density; 5 ind. L^{-1}), and (iii) HD (high *Daphnia* density; 10 ind. L^{-1}). Details of the procedures are described in *SI-13.* Soil samples were collected 10 days apart before and after incubation. The *pmoA* abundances were determined by RT-qPCR (*SI-5*). CH_4 oxidation was measured using the same method applied in field experiment 1 (*SI-4*).

Mesocosm Experiment 2

Mesocosm experiment 2 was to test the effects of fish-released N (excretion-N and feces-N) on CH_4 emission and oxidation, and methanogens and methanotrophs. The experiment was a completely randomized block design with two treatments (simulating rice monoculture without fish-released

N vs simulating rice–fish system with fish-released N) and four replicates. Details of the procedures are described in *SI-14* and Table S3.

During the experiment, CH_4 emission and oxidation and methanogen and methanotroph abundance were measured at 7, 30, 50, 80, and 110 DAT as described in field experiment 1. The methods for measurements of CH_4 emission and oxidation, and methanogens and methanotrophs were the same as those applied in field experiment 1 (*SI-3, SI-4, and SI-5*).

Incubation Experiment

This microcosm incubation experiment was performed to test whether methanotrophs use the fecal ^{15}N from the fish by using the method of ^{15}N labeled DAN stable isotope probing (DAN-SIP). The experiment was set up in a 150-mL serum bottle with two treatments (that is, unlabeled feces, UL-feces, and ^{15}N-labeled feces, ^{15}N-feces) and eight replicates. The preparations of ^{15}N-labeled feces and unlabeled feces were described in *SI-15*. The details of the incubation experiment were described in *SI-16*.

After incubation for 10 days, soil samples were collected for total DNA extraction and ^{15}N DNA separation (*SI-17*). The unlabeled DNA and the 'light' and 'heavy' ^{15}N-DNA fractions were amplified using the A189F_mb661R primers targeting *pmoA* (Table S1). Purified ^{15}N-DNA samples were sequenced on an Illumina MiSeq PE3000 platform (Illumina) using the primers targeting *pmoA* as described in Table S1. The data were analyzed in the QIIME2 pipeline (https://qiime2.org) (Bolyen and others 2019) and on the Majorbio Cloud Platform (www.majorbio.com).

Statistical Analysis

The generalized linear model (GLM) in SPSS v. 20.0 (IBM Corp., Armonk, NY, USA) was used for the statistical analyses. A least significant difference (LSD) test at the 5% confidence level was used for pairwise comparisons.

For field experiment 1, ANOVA with a split-plot design comprising RM and RF as the main plots and sampling or measurement dates as the subplots was performed on rice yield, soil C and N, the CH_4 emission and oxidation rates, methanogen and methanotroph abundance, biomass of food sources, and *Daphnia* spp. density. For field experiment 2, the $\delta^{15}N$ values of the rice, fish, paddy-dwelling organisms, and soil samples at each soil profile layer from ^{15}N-feed labeled treatment were compared against those from the unlabeled control via *F*-tests.

For mesocosm experiment 1, one-way ANOVA was performed using treatment as the fixed effect to determine whether *pmoA* abundance and CH_4 oxidation rate significantly differed among the treatments (no *Daphnia*, low density of *Daphnia*, and high density of *Daphnia*).

For mesocosm experiment 2, ANOVA with a split-plot design with and without fish-released N (excretion-N and feces-N) treatments as the main factors and sampling dates as the subfactors was performed on CH_4 emission and oxidation as well as methanogens and methanotrophs.

Results

Rice and Fish Yields and Soil Nitrogen and Carbon Levels

Field experiment 1 showed that the rice yield was significantly higher under the rice–fish co-culture (RF) than the rice monoculture (RM) ($F_{1,6}$ = 11.724; P = 0.014). The average annual fish yield in RF treatment was 0.77 ± 0.03 t ha^{-1} over the 5-year experimental period (Figure 2A). Soil organic C did not significantly differ between RM and RF ($F_{1,6}$ = 0.122; P = 0.739; Figure 2B), while soil total N was significantly higher in RF plot than in RM plot over 5 years ($F_{1,6}$ = 6.311; P = 0.049; Figure 2C) although fish feeds were added in the corner of a RF plot, over the 5-year experimental period.

CH_4 Emission and Oxidation Rates, Methanogens, and Methanotrophs

The average for 1 year of measurements in field experiment 1 showed that CH_4 emission did not differ between RM and RF (2020: $F_{1,6}$ = 0.542, P = 0.489; 2021: $F_{1,6}$ = 1.317, P = 0.295; Figure 3A). During the rice growth period, both RM and RF had similar change trends of CH_4 oxidation rates that oxidation rates started to increase from 7 DAT and reached maximum at the flowering stage (80 DAT) and then decreased. However, RF had a higher oxidation rate than RM did at each rice growing stage ($P < 0.05$) except for ripening stage (110 DAT) in both 2020 and 2021 ($P > 0.05$, Figure 3B). The average for 1 year of measurements indicated that the RF plots had higher rates of CH_4 oxidation than the RM plots in 2020 ($F_{1,6}$ = 27.213; P = 0.001) and 2021 ($F_{1,6}$ = 25.122; P = 0.002).

Methanogen abundance based on the *mcrA* copy number was significantly higher in the 5–10-cm

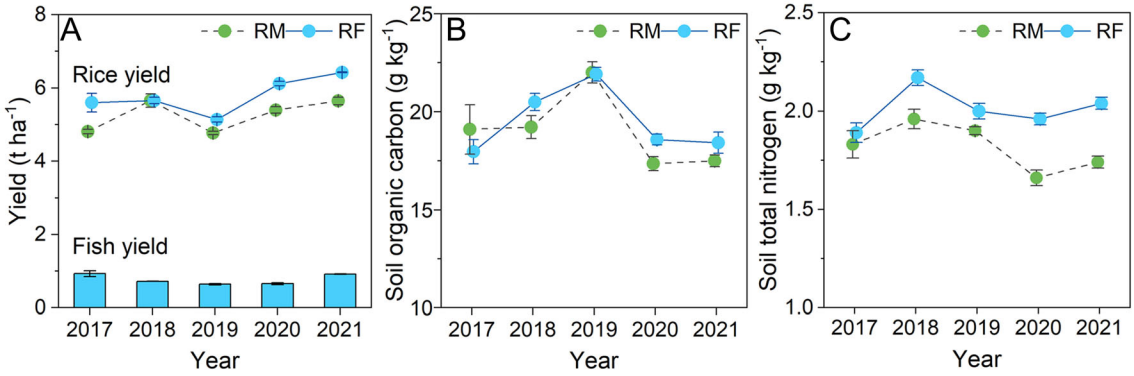


Figure 2. Yield, soil organic carbon, and total nitrogen in field experiment 1. **A** Rice and fish yields. **B** Soil organic carbon. **C** total nitrogen. RM: rice monoculture; RF: rice–fish system. In **A**, points indicate rice yield, and bars indicate fish yield. Values are mean ± SE.

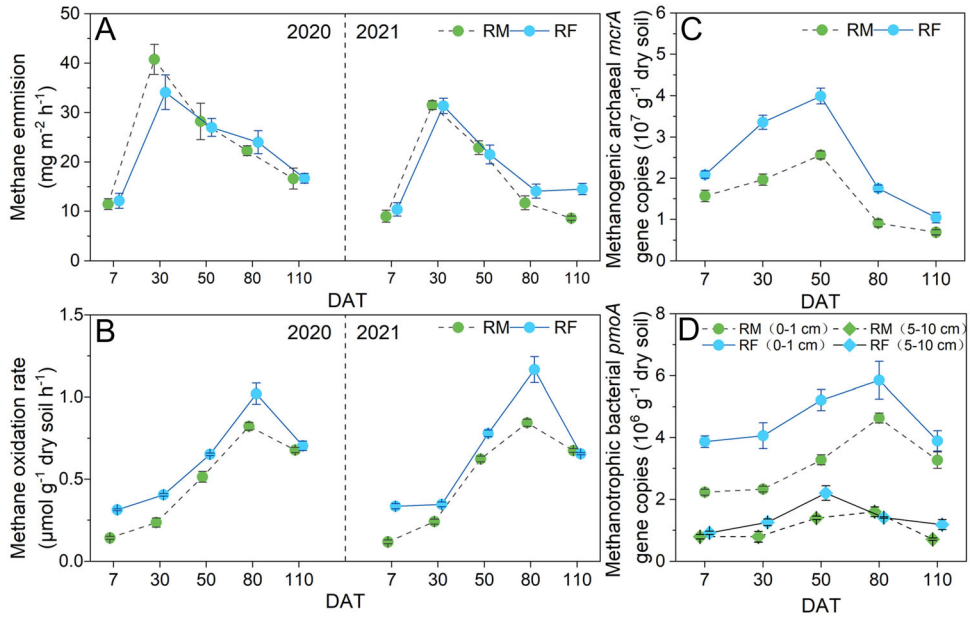


Figure 3. Methane emission and oxidation, and soil methanogens and methanotrophs in field experiment 1. **A** CH_4 emission. **B** CH_4 oxidation rate in surface soil (0–1 cm). **C** Methanogens in 5–10-cm soil layer. **D** Methanotrophs in 0–1-cm and 5–10-cm soil layers. RM: rice monoculture; RF: rice–fish system. DAT: days after transplanting; Values are mean ± SE.

soil layer of RF than that of RM ($F_{1,6}$ = 27.213; P = 0.002; Figure 3C). Methanotroph abundance based on the number of *pmoA* copies was significantly higher in the 0–1 cm ($F_{1,6}$ = 66.865; $P < 0.001$; Figure 3D) and 5–10 cm ($F_{1,6}$ = 32.738; P = 0.001; Figure 3D) soil layers of RF than those of RM. Under both RM and RF, methanotroph abundance was significantly higher in the 0–1-cm soil layer than the 5–10-cm soil layer (RM: $F_{1,6}$ = 412.354, $P < 0.001$; RF: $F_{1,6}$ = 433.809; $P < 0.001$; Figure 3D).

Food Components and the Effects of Feeding Interactions

A food source survey (field experiment 1) disclosed that weeds, duckweeds, macroalgae, phytoplankton, zooplankton, and zoobenthos such as tubifex worms and snails resided in both the RM and RF plots (Figure S3 and Figure S4). Dietary reconstruction using stable isotope (δ^{13}C and δ^{15}N) data revealed that the fish foraged the paddy-dwelling organisms even though receiving input feed throughout the experiment. A food component

analysis indicated that 22.8% ± 11.6% consisted of input feed while 77.2% ± 5.9% of it comprised weeds, duckweeds, macroalgae, phytoplankton, zooplankton, and zoobenthos. In zooplankton, *Daphnia* made up 15.1% ± 6.8% while other zooplankton 2.2% ± 2.0% of the food component (Figure 4A).

In field experiment 1, the duckweeds, macroalgal, and snail biomass quantities were similar under both RM and RF Figure S4; Table S5). Nevertheless, the weeds, phytoplankton, zooplankton, and tubifex worm biomass quantities were significantly lower under RF than RM (Figure S4; Table S5). The population density of zooplankton *Daphnia* spp., which are the preferred food of fish fry, was significantly lower under RF than RM ($F_{1,6}$ = 168.540; P = 0.001; Figure 4B).

Mesocosm experiment 1 showed that the *Daphnia* density significantly affected the *pmoA* copy number ($F_{2,14}$ = 147.606; $P < 0.001$; Figure 4C) and the CH_4 oxidation rate ($F_{2,14}$ = 65.072; $P < 0.001$; Figure 4C). High *Daphnia* density reduced the *pmoA* copy number and the CH_4 oxidation rate to a significantly greater extent than the low- and zero-density treatments ($P < 0.001$; Figure 4C).

Fish Behavior, Nitrogen Release, and Effects of Released N by Fish

Video recordings (field experiment 1) showed that the total daily fish activity levels were similar for all six quadrats ($F_{5,23}$ = 1.307; P = 0.096). The fish activity levels at 6:00–7:00, 9:00–10:00, 11:00–12:00, and 15:00–16:00 were similar across all six quadrats ($F_{5,23}$ = 0.456; P = 0.716; Figure 5A). The fish activities were swimming and foraging mainly in the paddy field (Figure 5B). The proportions of foraging on rice stems (I), in the water (II), and on the bottom mud (III) were 53.9% ± 1.0%, 36.9% ± 2.4%, and 9.2% ± 1.9%, respectively (Figure 5B).

Observation in field experiment 1 indicated that fish-releasing N through egestion and excretion occurred continuously throughout the day (Figure 5C). The rates of both feces-N and excretion-N released by fish in 1 day increased with fish body weight increase during the experiment (Figure 5D).

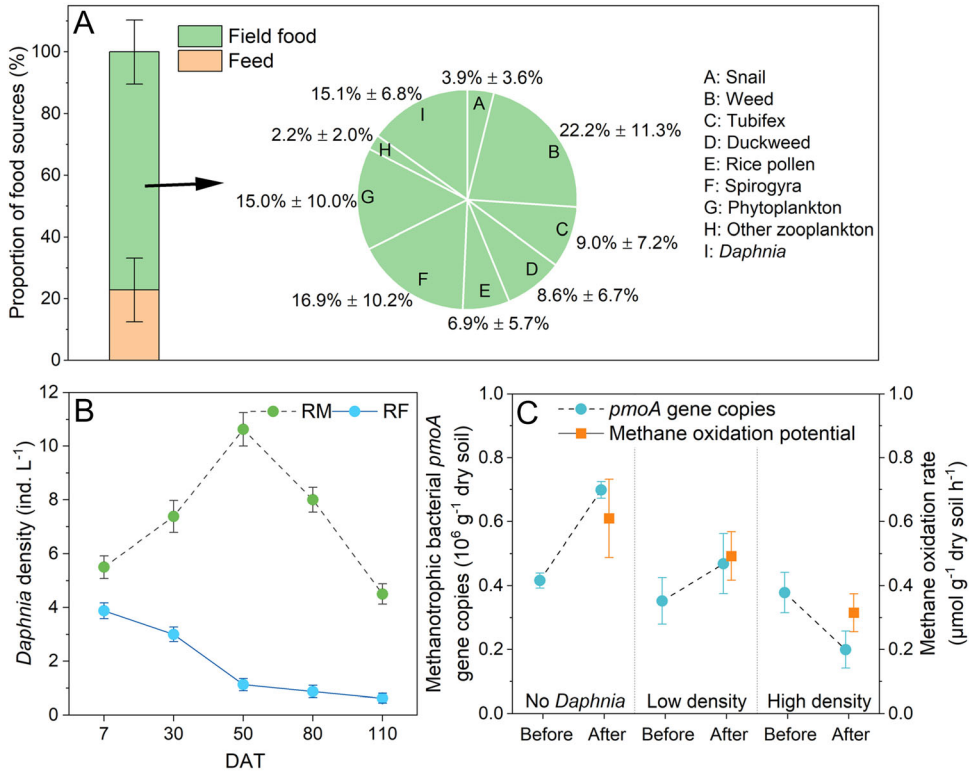
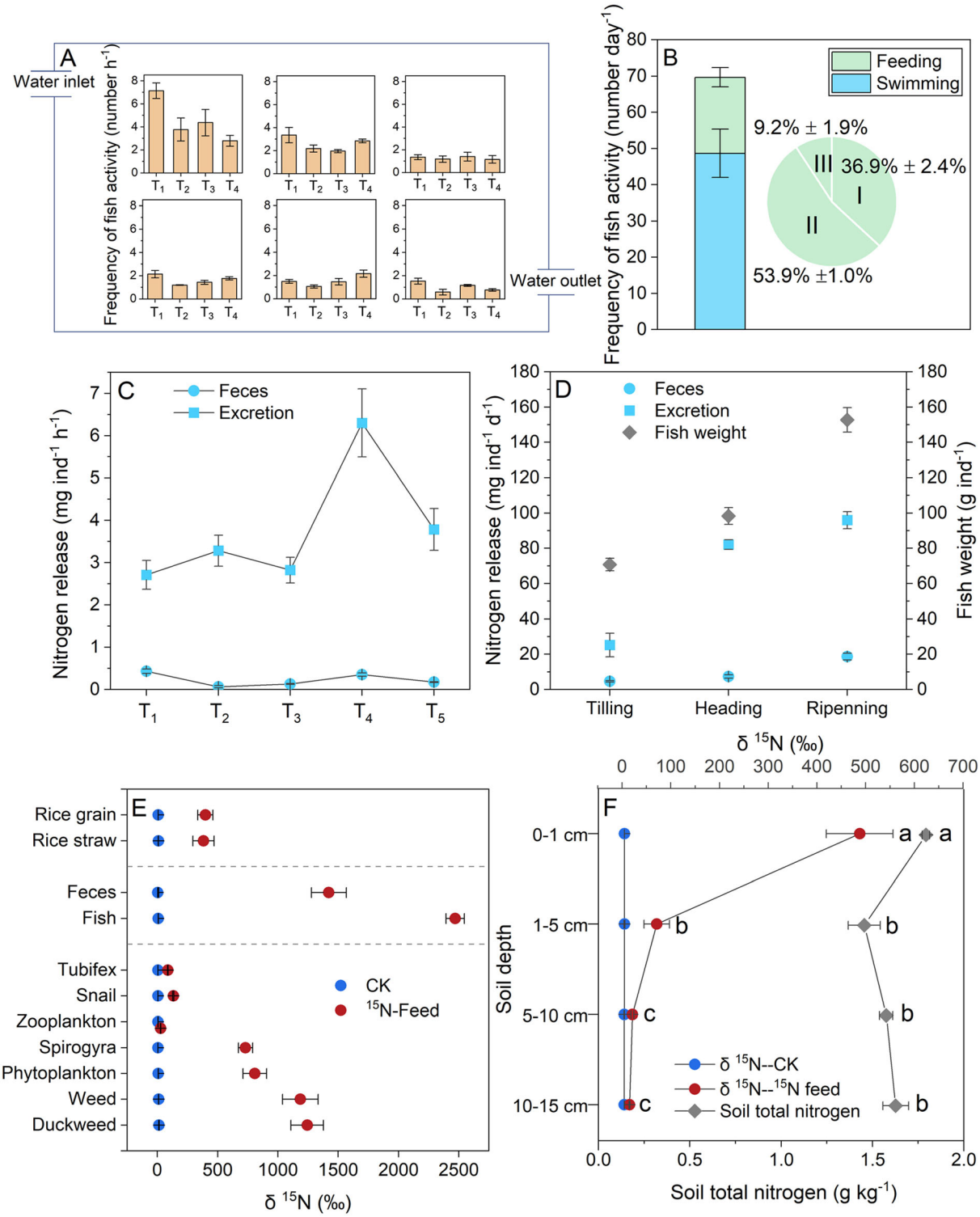


Figure 4. Contributions of food sources to fish diet components in field experiment 1 and the effects of feeding interactions on methanotrophs and CH_4 oxidation in microcosm experiment 1. **A** The fish diet components. **B** *Daphnia* density in paddy field. **C** Effects of *Daphnia* density on soil methanotrophs and CH_4 oxidation rate. RM: rice monoculture; RF: rice–fish system. DAT: days after transplanting; values are mean ± SE.



◀**Figure 5.** Fish behavior and N flux among paddy-dwelling organisms in field experiments 1 and 2. **A** Indicating an experiment plot that water inlet and outlet, and quadrats are placed. The bars indicate fish swimming frequency and feeding behavior in each quadrat in a day (that is, T_1:6:00–7:00, T_2:9:00–10:00, T_3:10:00–11:00, T_4:15:00–16:00). **B** Daily fish swimming frequency and feeding behavior. In the pie chart, I indicates that fish foraged in the water surface, II indicates that fish foraged on rice leaves and stem bases, and III indicates that fish foraged on bottom mud. **C** N released by the fish at 7:00–10:00 (T_1), 10:00–13:00 (T_2), 13:00–16:00 (T_3), 16:00–19:00, (T_4) and 19:00 -7:00 (T_5) in the same day. **D** N released from the fish in 1 d during the rice growth period. **E** δ^{15}N values for fish, rice, and paddy-dwelling organisms under ^{15}N-labeled fish feed treatment. **F** Soil profile δ^{15}N values and total N under ^{15}N-labeled fish feed treatment. Values are mean ± SE.

Feed-^{15}N tracing (field experiment 2) showed that the δ^{15}N content was significantly higher in the rice straw, rice grain, paddy-dwelling organisms, and fish under the ^{15}N treatment than in those under the control treatment (Figure 5E; Table S6), indicating that feed-N for fish was also used by rice and paddy-dwelling organisms. The soil profile of the feed-^{15}N treatment disclosed that both the δ^{15}N value and total N content were significantly different among soil layers (δ^{15}N: $F_{3,12}$ = 38.447; P = 0.001; total N: $F_{3,12}$ = 5.456; P = 0.013; Figure 5F). The δ^{15}N value and total N content in soil surface layer (0-1 cm) were significantly higher than in the other soil layers ($P < 0.001$, Figure 5F).

In Mesocosm experiment 2, treatment with fish-released N did not significantly affect CH_4 emission ($F_{1,6}$ = 3.168; P = 0.106; Figure 6A), but significantly increased CH_4 oxidation rate ($F_{1,6}$ = 35.622; P = 0.001; Figure 6B) and methanogens ($F_{1,6}$ = 48.246; P = 0.001; Figure 6C) and methanotrophs ($F_{1,6}$ = 32.731, P = 0.012; Figure 6D).

By using the method of DNA-SIP in the Incubation experiment, ^{15}N-DNA was found in methanotrophs in the soil sample with ^{15}N-labeled fish fecal compared to the soil sample with unlabeled fish fecal, indicating that fecal N was used by methanotrophs (Figure S5A, Figure S5B). The Illumina Hiseq3000 platform identified methanotroph *pmoA* from the ^{15}N-DNA and indicated that *Methylocystis*-affiliated Type II methanotrophs predominated (Figure S6C).

Discussion

The present study reveals the mechanisms by which a local common carp affects aerobic CH_4 oxidation in surface soil (the soil–water interface) in the rice–fish system. Multiple interactions among fish, rice, paddy-dwelling organisms, and soil microorganisms occur in soil surface layer of this system. In the multiple species interaction, the fish participated in feeding interactions and caused trophic cascades, drove N-transfer, enriched N in soil surface layer and affected methanotrophs and CH_4 oxidation (Figure 1).

Although anaerobic methane-oxidizing archaea were found in the paddy soil, high abundance of aerobic methanotrophs were detected in the O_2 zone of rice rhizospheres and the oxygenated soil surface layer in rice paddy (Le Mer and Roger 2001; Wang and others 2022; Fan and others 2020). Here, field experiment 1 revealed that fish decreased zooplanktons (for example, *Daphnia*) and increased aerobic methanotroph abundance as the *pmoA* copy number was elevated in the 0–1-cm and 5–10-cm soil layers. Nevertheless, aerobic methanotroph abundance was twice as high in the former than the latter (Figure 3D). Aerobic CH_4 oxidation rate in the 0–1-cm soil layer was also higher in the treatment with fish than that without fish (Figure 3C). These results suggested that fish directly affected the aerobic CH_4 oxidation. Unlike waterfowl, geese, and crab, etc. aerate and resuspend the wetland sediment by moving, digging, muddying, burrowing, grazing, and so on (Vanni 2002; Estes and others 2011; Vanni and others 2013; Schmitz and others 2014), fish swimming and feeding in this study did not have a strong impact on the surface soil with fish only spent 9% of their foraging on the bottom (Figure 5B). Thus, the fish affected the methanotrophs mainly through biotic interactions (Figure 1).

On the one hand, fish promote methanotrophs via trophic cascading (Figure 1A). These fish are omnivores (Ren and others 2018). The food source survey disclosed that the rice–fish system harbored various paddy-dwelling organisms (Figures S3 and S4). The food source analysis based on ^{13}C and ^{15}N data revealed that the fish diet included weeds, duckweeds, macroalgae, phytoplankton, zoobenthos, and zooplankton. *Daphnia* spp. were the major zooplanktons that contribute to fish diet (Figure 4A). In our study area, *Daphnia magna* and

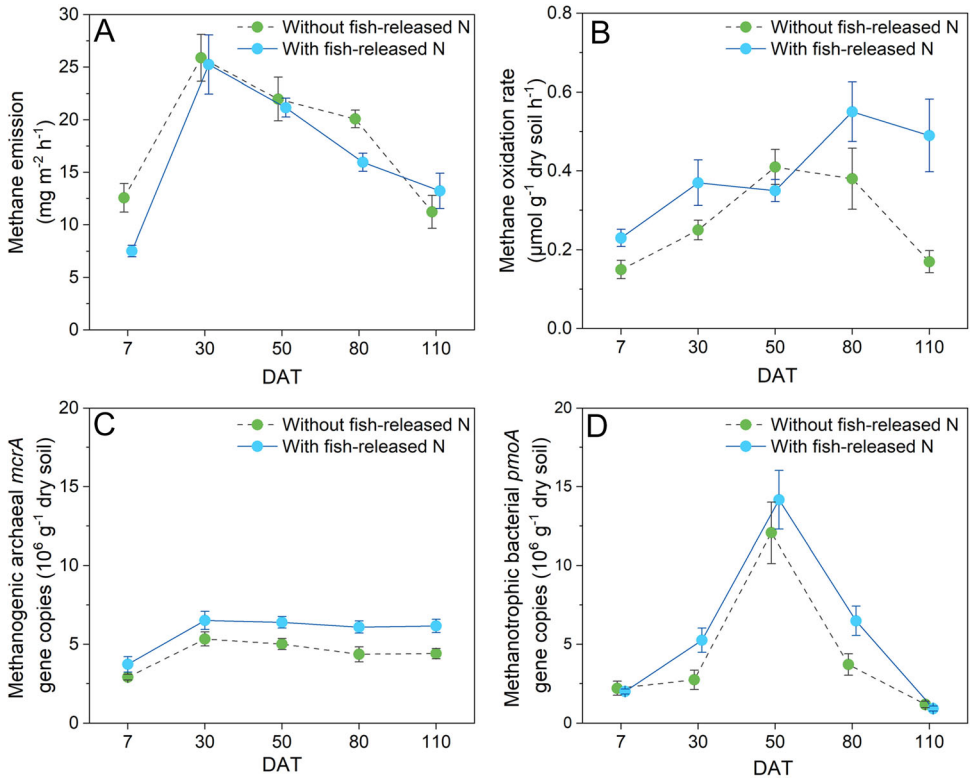


Figure 6. Effects of fish-released N on CH_4 emission and oxidation, and methanogens and methanotrophs in mesocosm experiment. **A** CH_4 emission. **B** CH_4 oxidation rate in 0–1-cm soil layers. **C** Methanogens in 5–10-cm soil layer. **D** Methanotrophs in 0–1-cm soil layers. DAT: days after transplanting; values are mean ± SE.

D. carinata commonly occur in paddy fields (Fan 2020; Wang 2011) and are used by local farmers to feed carp fry (Yang and Chen 2010). Our field experiment 1 further indicated that the density of *Daphnia* spp. was reduced by fish presence (Figure 4B). Studies have showed that *Daphnia* spp. are bacterivores and consume methanotrophs in lakes and wetlands (Kankaala and others 2006; Taipale and others 2009; Devlin and others 2015). Our mesocosm experiment that excluded other zooplankton also showed that methanotroph abundance and CH_4 oxidation rate decreased with increasing *Daphnia* density (Figure 4C). Taken together, the preceding results suggest that the fish in the paddy field increased methanotrophs by consuming *Daphnia*, thereby promoting CH_4 oxidation.

On the other hand, the feeding activities of fish make them the N-transfer vectors and eventually promotes methanotrophs in the surface soil (Figure 1B). Field experiment 2 showed that $\delta^{15}N$ was significantly higher in rice plants and paddy-dwelling organisms under the ^{15}N-feed treatment than under the control (Figure 5E). Field experiment 2 also showed both $\delta^{15}N$ and total N accumulated in the surface soil (0–1 cm) under the fish ^{15}N-feed treatment (Figure 5F). These results suggest that the enhanced ^{15}N was released from the fish because they were fed every morning at the corner of the field (Figure S2), moved around the field (Figure 5A), and egested and excreted during the day (Figs. 5C and 5D). Thus, the fish were important N transfer and carriers in the rice–fish system. Incubation experiment of ^{15}N-DNA-SIP disclosed that the surface soil methanotrophs directly utilized the egested and excreted- N (Figure S5). Mesocosm experiment further demonstrated that the N derived from fish excretion and feces enhanced methanotrophs and CH_4 oxidation (Figs. 6B and 6D). Some studies showed that both ammonium and nitrate fertilizers increased methanotroph abundance and CH_4 oxidation in the rhizosphere (Bodelier and others 2000; Krüger and Frenzel 2003; Li and others 2022), while other showed that ammonium fertilizer lowered the abundance of methanotrophs in the rice plant rhizosphere (Shrestha and others 2010). Our results described above indicated the fish in paddy field promote methanotrophs and CH_4 oxidation through N mediation.

The growth status of rice plants could affect CH_4 production and oxidation (Seiler and others 1984). Our previous studies showed that the presence of

fish in rice paddy promoted rice growth and extended tiller development period (Zhang and others 2017; Guo and others 2022). In the current field experiment 1, the presence of fish in the paddy increased soil total N and rice yield (Figure 2), suggesting that the fish promoted rice plant growth. Field experiment 1 indicated that fish increased methanogen abundance at the 5–10 cm soil depth (Figure 3C). One possible explanation is that fish promote the growth of rice plants, and the rhizospheres of the latter attract and support various microorganisms. However, the increase in methanogen abundance did not augment CH_4 emission in rice–fish co-culture (Figure 3A). Nevertheless, field experiment 1 indicated that the presence of fish accelerated CH_4 oxidation in the surface soil (Figure 3B), and this enhancement of oxidation rate by fish was highest at the rice flowering stage when CH_4 emission has started to decrease (Figure 3A). Taken together, the preceding results suggest that CH_4 oxidation induced by fish might help mitigate paddy CH_4 emissions. However, direct empirical evidence for this mechanism in rice–fish ecosystems is lacking at this time.

It was reported that the methanotrophs in the soil surface layer oxidized ~ 80% of the CH_4 that pass through (Conrad and Rothfuss 1991). In natural wetland and rice–fish system, the soil surface layer (that is, soil–water interface) is also an important place for animal-mediated interactions among multiple species (Winton and Richardson 2017, Figure 1). These biotic interactions can affect the surface soil methanotrophs and CH_4 oxidation. Thus, the surface soil in wetland ecosystems could be an important biological CH_4 filter that suppresses the escape of soil methane into the atmosphere. Our study proposed a putative mechanism by which fish and other animals affect CH_4 oxidation and increased our understanding of their roles in wetland and rice paddy ecosystems.

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DATA AVAILABILITY

The data that support the findings of this study are available from the authors upon reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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