[Original Article]

Continuous effects of winter flooding on soil fauna, as revealed by community structure of soil nematodes in a paddy field in northern Japan

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Beneficial effects of winter flooding in rice paddies have been proved for birds and other above-ground wildlife. However, there have been few studies that accounted for soil organisms. In this study the effects of winter flooding together with an organic amendment on soil fauna as represented by nematodes were examined. Soil samples were taken from experimental rice paddies in northern Japan with one of three treatments, *i.e.*, winter flooding with organic farming (WFO), no winter flooding with organic farming (NFO), and no winter flooding with conventional farming (CVN, only chemical fertilizers and chemicals applied). During this study period of over one year and a half, nematodes were extracted and counted to estimate their density. Then to evaluate nematode diversity, a polymerase chain reaction-denaturing gradient gel electrophoresis analysis of the nematode 18S rRNA gene was conducted, which found 34 operational taxonomic units with *Tobrilus* spp. and *Hirschmanniella* sp. as the three most dominant taxa. Statistical analysis showed the following trends: 1) nematode density in the 0–5 cm soil layer was WFO > NFO > CNV, and 2) nematode diversity, *H'*, in the 5–10 cm layer was WFO < CNV < NFO. The results of our nematode and soil physico-chemical analyses suggest, 1) winter flooding and organic farming could increase nematode density at least in the upper soil layer with increased fertility, and 2) on the contrary, winter flooding could be harmful to maintain nematode diversity in the deeper part of soil, probably due to an increased soil reduction level. Nematol. Res. 46(2), 45–58 (2016).

Key words: density, diversity, 18S rRNA gene, organic farming, polymerase chain reaction-denaturing gradient gel electrophoresis analysis

INTRODUCTION

Winter flooding is an agricultural practice in which rice paddies are flooded during the winter season after harvesting. Winter flooding practices have been conducted across the world for various intentions. One of the earliest records in Japan appears in *'Aidzu Nousho'* (agricultural handbook of Aidzu domain, currently a local area in the Tohoku District) documented by Yojiemon Saze in 1684, which is considered to have been used to supply organic debris for plant nutrition with drainage water (Fujiwara and Shoji, 1977). In the Albufera Wetland, Valencia, Spain, rice paddies had been winter flooded for bird hunting since the 13th century, with the additional intention of preventing seawater intrusion (Oficina de Gestión Técnica del Parc Natural de l'Albufera, 2002). In California winter flooding has been practiced to promote the decomposition of rice straw, since legislation of the State of California (Rice Straw Burning Act, AB 1378 1991) mandated growers to reduce the practice of burning rice stubble (*e.g.* Elphick *et al.*, 1998).

Recently, winter flooding has been re-evaluated in terms of its significant role in biological conservation; flooded rice paddies serve as alternative feeding or roosting sites for waterbirds besides natural wetlands as described later. Winter flooding was implemented by the European Union through the Common Agricultural Policy in 1998, and applied to rice paddies in Albufera and Ebro Delta, the two most important Ramsar-

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designated wetlands in Spain. This modern legislation increased the area of flooded rice paddies and the time during which they are flooded (Pernollet *et al.,* 2015). In Japan, the area under winter flooding has recently been expanding with the aim of protecting and enhancing the local biodiversity, in addition to farming reasons (Mineta *et al.,* 2004; Kurechi, 2007). Winter flooding changes the soil surface to be more porous and muddy (called the *toro-toro* layer in Japan) (Ito *et al.,* 2011), which physically inhibits the establishment of weed germlings (Kurihara, 1983), and allows the farmers to reduce herbicide applications.

Beneficial effects of winter flooding have been studied comprehensively for waterbirds in California, USA (Elphic *et al.,* 1998), Japan (Tajiri and Ohkawara, 2013) and Europe (Pernollet *et al.,* 2015). Mineta *et al.,* (2009) confirmed that winter flooding was beneficial in the conservation of swans, fishes, and an endangered plant species at a local area in northern Japan. Takada *et al.* (2014) verified that winter-flooded rice paddies harbored more diverse spiders more densely than conventional rice paddies. In contrast to such significant attention paid to the wildlife above ground, few studies have focused on soil organisms. Protozoan (Kameyama *et al.,* 2006), cyanobacterial communities (Ariosa *et al.*, 2006) and aquatic oligocaetes (Yachi *et al.,* 2012) were studied in winter-flooded rice paddies, but these studies did not include a paddy without winter flooding for comparison. The responses of soil biota to winter flooding have therefore remained largely unstudied, except for ecological and functional studies of aquatic oligocaetes (Ito *et al.,* 2011; 2015).

Soil biota contribute significantly to decomposition and nutrient cycling, soil structure modification, and affect the primary production in the food web directly and indirectly, as well as comprising a large part of the Earth's biodiversity (Barrios, 2007). Therefore, the diversity of soil organisms is worth considering for the sustainable management of a local ecosystem. Among soil organisms, nematodes are the most abundant metazoan taxa in number. Nematodes include a vast diversity of species that have a variety of feeding habits, *e.g.* bacterivorous, fungivorous, carnivorous, plant-parasitic, entomopathogenic, entomoparasitic, and animal parasitic habits, and thus affect the cycling of carbon and nutrients in soils (Yeates, 2003). Nematodes are commonly distributed in soils, wetland sediments, and other environmental media that contain moisture. Taking advantage of these ecological features of nematodes, their community structure can be used effectively as an indicator of environmental conditions (Yeates, 2003). In the context of biological conservation, the nematode community has also been used to assess the effects of agricultural practices on the biodiversity of soils in upland cropland (Okada and Harada, 2007).

Nematodes also inhabit rice paddies (Imamura, 1931; Mishra and Dash, 1981; Okada *et al*., 2011). Nematode taxa dominant or characteristic in rice paddies reported previously are mostly common to freshwater sediment (Traunspurger and Drews 1996; Heininger *et al.,* 2007) reflecting a well-developed ecosystem under water (Okada *et al.,* 2011). During the flooding period, nematode community index values (Yeates, 2003) suggest that bacteria dominate over fungi with slow decomposition due to anaerobic conditions (Okada *et al.,* 2011). Simultaneously, the overall density of nematodes declines (Mishra and Dash, 1981; Okada *et al.,* 2011). In this manner, community structure significantly changes in response to the cycle of flooding and drainage (Imamura, 1931; Okada *et al*., 2011).

On the basis of the results of these studies, we hypothesized that winter flooding can alter the community profiles of soil nematodes. The long flooding period in winter-flooded rice paddies can make the soil environment more reductive than conventionally managed ones, and possibly leads to the loss of nematode abundance and diversity. To test this hypothesis, we compared the community structure, density, and biological diversity of soil nematodes and their seasonal changes over a period of one and a half years in rice paddies under three different cultivation regimes. Structural equation modeling (SEM) was also applied to quantify direct and indirect effects of environment on nematode density and community composition.

MATERIALS AND METHODS

Site description:

The study site was previously used by Liu *et al.* (2012). The site lies on a gentle slope facing southward, located at the Integrated Terrestrial Field Station (38° 44'N, 140° 45'E, altitude 175 m), Field Science Center, Graduate School of Agricultural Science, Tohoku University, Osaki, Miyagi Prefecture, northern Japan. The mean monthly temperature ranged between 0.3 and 26.2°C, and the annual precipitation was 1255 mm in 2009 and 1307 mm in 2010 at the nearest automated meteorological station in Furukawa (38° 36'N, 140° 55'E, altitude 28 m). The soil is non-allophanic Andisol (Andosol). Before the experimental plots were established, the site had been continuously cropped with rice and managed conventionally for several years, *i.e.*, chemical fertilizer and pesticides applied, organic fertilizer not applied, summer flooding with mid-season drainage conducted, winter flooding not conducted. Experimental plots (300–400 m^2) were established in 2008, which were arranged randomly in a paddy field with two replications. Three treatments were imposed: CNV (conventionally managed as described before), NFO (no winter flooding and organically managed), and WFO (winter flooding and organically managed). The main objective of this study was to compare nematode profiles of NFO and WFO. However, CNV was included in the study as a reference site. The NFO and WFO plots were flooded in the cropping season without mid-season drainage, and organic fertilizers were applied without chemical pesticides used in these plots. The WFO plots were flooded again in winter. For management details, see Table 1.

Soil sampling, nematode collection and chemical analyses:

Five soil cores 5 cm in diameter and 10 cm in height as subsamples were extracted from each plot of WFO, NFO, and CNV on 4 Aug 2009, 9 Oct 2009, 12 May 2010, 10 Aug 2010, and 29 Oct 2010 for nematode analysis. The cores were separated into top (0–5 cm) and second (5–10 cm) layers, and then pooled in two corresponding plastic bags. Nematodes were extracted from 150 g fresh soil of each sample using a double-layer centrifugation (2,000 rpm, 5 min) method followed by a Baermann tray

Table 1. Management practices applied to the study fields.

Management practice	2009		2010	
	WFO and NFO	CNV	WFO and NFO	CNV
Compost application (1 t/10 a)	2008/12/15 (−164 – −163)	2008/12/15 (−164 – −163)	2009/12/8–9 (−176 – −175)	2009/12/8–9 (−176 – −175)
Start of winter flooding (only for WFO)	12/17 (−162)	NA*	12/11 (−173)	NA
Seeding	2009/4/17 (−41)	4/17/2009 (−41)	2010/4/16 (−46)	4/16/2010 (−46)
Fungicide and insecticide application to nursery box (fipronil and orysastrobin)	NA	4/17 (−41)	NA	4/16 (−46)
Basal dressing (Organic Agret 666 (Asahi Industries, Tokyo), 4 kgN/10 a)	4/24 (−34)	NA	5/6 (−27)	NA
Tilling	5/1–2 (−27 – −26)	5/1–2 (−27 – −26)	5/8 (−25)	5/8 (−25)
Flooding	5/3 (−25)	5/23 (−5)	5/8 (−25)	5/26 (−7)
First puddling	5/5 (−23)	5/24 (−4)	5/11 (−22)	5/28 (−5)
Final padding	5/26 (−2)	5/26 (−2)	5/31 (−2)	5/31 (−2)
Transplanting	5/28 (0)	5/28 (0)	6/2 (0)	6/2 (0)
Side dressing (Toruzou-kun (Co-op Chemical, Tokyo) 7 kgN/10 a)	NA	5/28 (0)	NA	6/2 (0)
Pelletized rice bran application (80 kg/10 a, 2009: 1.52 kgN/10 a; 2010: 1.53 kgN/10 a)	6/1 (4)	NA	6/4 (2)	NA
Herbicide application (Top Gun 1-kg Granule (Kumiai Chemical Industry))	NA	6/12 (15)	NA	6/7 (5)
Mid-summer drainage	NA	7/21–26 (54 – 59)	NA	7/23–8/2 (51 – 61)
Firtilizing (Organic Agret 666, 2009: 1.48 kgN/10 a; 2010: 1.47 kgN/10 a)	7/31 (64)	NA	7/27 (55)	
Fungicide application ((Pyroquilone granule; Syngenta Japan, Tokyo))	NA	7/31 (64)	NA	8/3 (62)
Pesticide application (Starkle granules, anti-hemipteran; Mitsui Chemicals Agro, Tokyo)	NA	8/21 (85)	NA	8/18 (77)
Draining	9/15 (110)	9/15 (110)	9/4 (94)	9/4 (94)
Harvest	WFO: 10/19 (144) NFO: 10/13 (138)	10/13 (138)	10/9 (129)	9/21 (111)

Values are month/day with days after transplanting indicated in parentheses.
WFO, NFO and CNV stand for winter-flooded and organic, non-winter-flooded and organic and conventional managements, respectively.
*NA stands for not applied.

extraction (Whitehead and Hemming, 1965) for 48 h. We counted the extracted nematodes under a stereomicroscope. A portion of each composite soil sample was oven-dried at 105°C for 2 days or longer to determine water content. The numbers of nematodes were converted to density per 100 g dry soil.

Two soil samples were also taken from the plow layer (0–10 cm) biweekly during the growing season to analyze the soil physical and chemical properties. Concentrations of NH_4^+, NO_3^-, available P_2O_5 (by the modified Bray No. 2 method), and Fe^{2+} of each soil sample were measured. The oxidation-reduction potential (Eh) at 5-cm depth was also measured. All physico-chemical measurements followed the procedures of the Committee for Analytical Methods of the Soil Environment (1997).

Polymerase chain reaction-denaturing gradient gel electrophoresis analysis:

Part of the nematode suspension estimated to contain 300 individuals was taken for each soil sample and applied to DNA extraction. The Wizard SV genomic DNA purification System® (Promega, Madison, WI, USA) was used to prepare nematode DNA templates from the nematodes, following Okada and Oba (2008). Polymerase chain reaction-denaturing gradient gel electrophoresis analysis (PCR-DGGE) was performed with the primers SSU9R/GC (5’- CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GAG CTG GAA TTA CCG CGG CTG-3’) and SSU18A (5’-AAA GAT TAA GCC ATG CAT G-3’). Reaction mixtures were prepared containing Prime Star Polymerase HS (TaKaRa, Otsu, Japan) in 25-µl volumes. The PCR was carried out under the following conditions: 98°C for 3 min, 27 cycles at 98°C for 10 sec, 52°C for 15 sec, 72°C for 40 sec, and 72°C for 10 min. DGGE was performed with the DCode™ System (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were immediately loaded onto 6% (w/v) polyacrylamide gels (acrylamide : bisacrylamide = 37.5 : 1; denaturant gradient of 30–45%) at 10 µl/well in 1 × Tris-acetate-EDTA (TAE) buffer and were electrophoresed at 75 V and 60°C for 16 h. The gels were stained with GelRed™ (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and then Molecular Imager FX software (Bio-Rad) was used to analyze the image. Bands were considered to be of the same operational taxonomic unit (OTU) if they had the same mobility in the gels. We amended a molecular marker, DGGE Marker V (Nippon Gene, Tokyo, Japan), with three additional marker bands and used this to compare DGGE patterns between gels. OTUs were sequentially numbered in ascending order according to the corresponding DGGE mobility. Sample bands that moved more slowly than the fourth least mobile marker bands were omitted because the majority of such bands are not nematodes (Okada and Oba, 2008).

Sequencing and homology search:

Small pieces (*ca.* 5 × 1 × 1 mm) of the gels corresponding to the major DGGE bands were excised with a disposable surgical blade. Each piece was immersed in 100 µl of sterilized distilled water, frozen, and then thawed at 95°C for 5 min. Thawed fluid (2 µl) was used as templates for PCR as described above, except the number of cycles was 25. The PCR products (2 µl) were treated with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and then used as templates for the sequencing reaction with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and each of the primers. An ABI 3100 genetic analyzer (Life Technologies) was used to collect the sequence data. To determine the taxonomic placement of the species of the respective bands, BLAST (http://www.ncbi.nlm.nih.gov/) was used to search the homology of the partial sequences of the 18S rRNA gene in the public database.

Statistical analyses:

The Shannon-Wiener’s diversity index H' was calculated for the nematode community of each soil sample. The structure of the nematode communities was determined as the composition of nematode OTUs weighed by their band intensity in the DGGE gel images. The intensity of a band was considered to be a measure of the abundance of OTUs, as it reflects their biomass, though this is not always proportional (Okada and Oba, 2008). H' values were calculated using the following equation:

$$H' = \sum_{i=1}^{n} \{p_i \cdot \ln(p_i)\},$$

where p_i is a fraction of the intensity of i-th band in the total intensity in a DGGE lane. To demonstrate the similarity between the community samples and the OTUs characteristic to each treatment, principal component analysis (PCA) was applied to the set of all community data. The Euclidian distance was used as the distance measure in the PCA.

To examine the effects of the treatments, soil depth, sampling date, and their interactions on the log-transformed number of nematodes per 100 g dry soil, the H' value and the first principal component (PC1) of the communities, generalized linear models (GLMs) were applied, including analyses of variance (ANOVAs). Because random effects of the paddy field sections reduced the goodness of model fit, the random effect was omitted from all analyses. When the effect of sampling date or its interaction with another variable was significant, a cosine transformation with a cycle of 365 days was introduced to the unit time t to detect the seasonal trend (Sasaki, 1978). The cosine transformation is expressed as:

$$A \cos(\omega t - \varphi),$$

where ω is the angular velocity of $2\pi/365$ rad/day, t is time in days beginning at 1 January, and φ is phase lag in rad. The formula is expanded as:

$$A\cos\varphi\cos\omega t + A\sin\varphi\sin\omega t.$$

If the terms $\cos\omega t$ and $\sin\omega t$ are introduced, the coefficients A and φ can be determined as:

$$A = \sqrt{(a_1^2 + a_2^2)} \text{ and}$$
$$\varphi = \tan^{-1}(a_2 / a_1),$$

where a_1 and a_2 are the unstandardized coefficients of $\cos\omega t$ and $\sin\omega t$ in the GLMs, respectively. In this approach, seasonal trends can be drawn from the total variance, saving degrees of freedom. More informative models were selected on the basis of Akaike's information criterion (AIC; Akaike, 1973). Tukey's honestly significant difference was used as a post hoc test to control family-wise errors.

Further analysis was conducted with structural equation models (SEMs) after determining an acceptable independent graph by covariance selection (Dempster, 1972). Two response variables were included, namely log-transformed density and PC1, and water content of the soils as an indicator of soil porosity in the endogenous variables in the SEMs. The water content should not be included in GLM analyses, as it was considered to be confounded by agricultural practices and other exogenous variables mentioned below; to test the indirect effect of the exogenous variables to the nematodes via soil water content, therefore, SEM was needed. Winter flooding practice (0 or 1), application of organic matter (0 or 1), soil depth (top layer: 1; second layer: 0), and cosine-transformed values of sampling date were included as exogenous variables. The transformations had phase modulation with φ values if it was determined in the GLM analyses. Improvements to the models were tested by stepwise addition or deletion of a path from the feasible full model (Fig. 1a) on the basis of AIC. The R software package version 2.15.1 (R Core Team, 2015) was used to perform all statistical analyses.

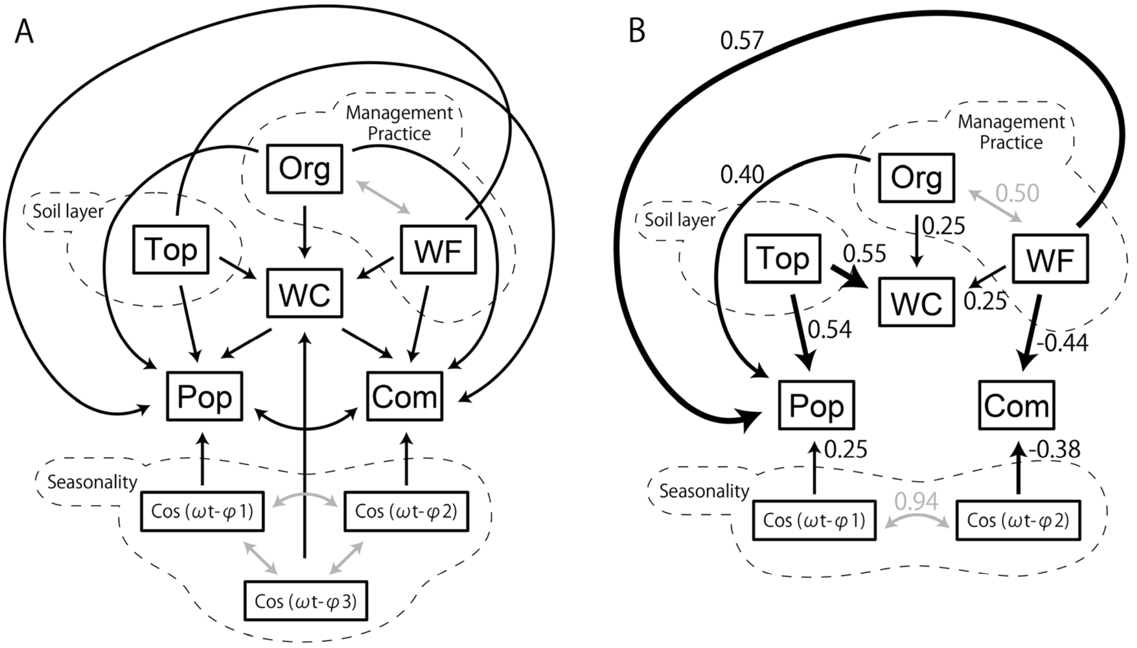

Fig. 1. Path diagrams of (A) all plausible interaction pathways and (B) those inferred by the analysis of structural equation models in the study system. Squares are selected variables in the study system: Org, WF, and Top: dummy variables for organic farming, winter-flooding, and soil top layer; WC: water content of soil samples; Pop: log-transformed density of nematodes; Com: community profile as represented by the first principal component of nematode community; Cos $(\omega t - \varphi 1)$, Cos $(\omega t - \varphi 2)$ and Cos $(\omega t - \varphi 3)$ are seasonal trends with phase lags. Single-headed arrows with digits indicate putative direction of causality with standardized partial correlation coefficients $\hat{\beta}$. Double-headed arrows indicate putative correlation with correlation coefficients.

RESULTS

Temporal variability of soil NH_4^+, NO_3^-, P_2O_5, Fe^{2+}, and Eh:

The soil chemical properties, NH_4^+, NO_3^-, P_2O_5 and Fe^{2+}, showed similar temporal variability during cropping periods in 2009 and 2010. NH_4^+ increased from April to June, just after flooding, and decreased from July to September (Fig. 2). NH_4^+ was higher in WFO and NFO than in CNV during the cropping periods. NO_3^- was

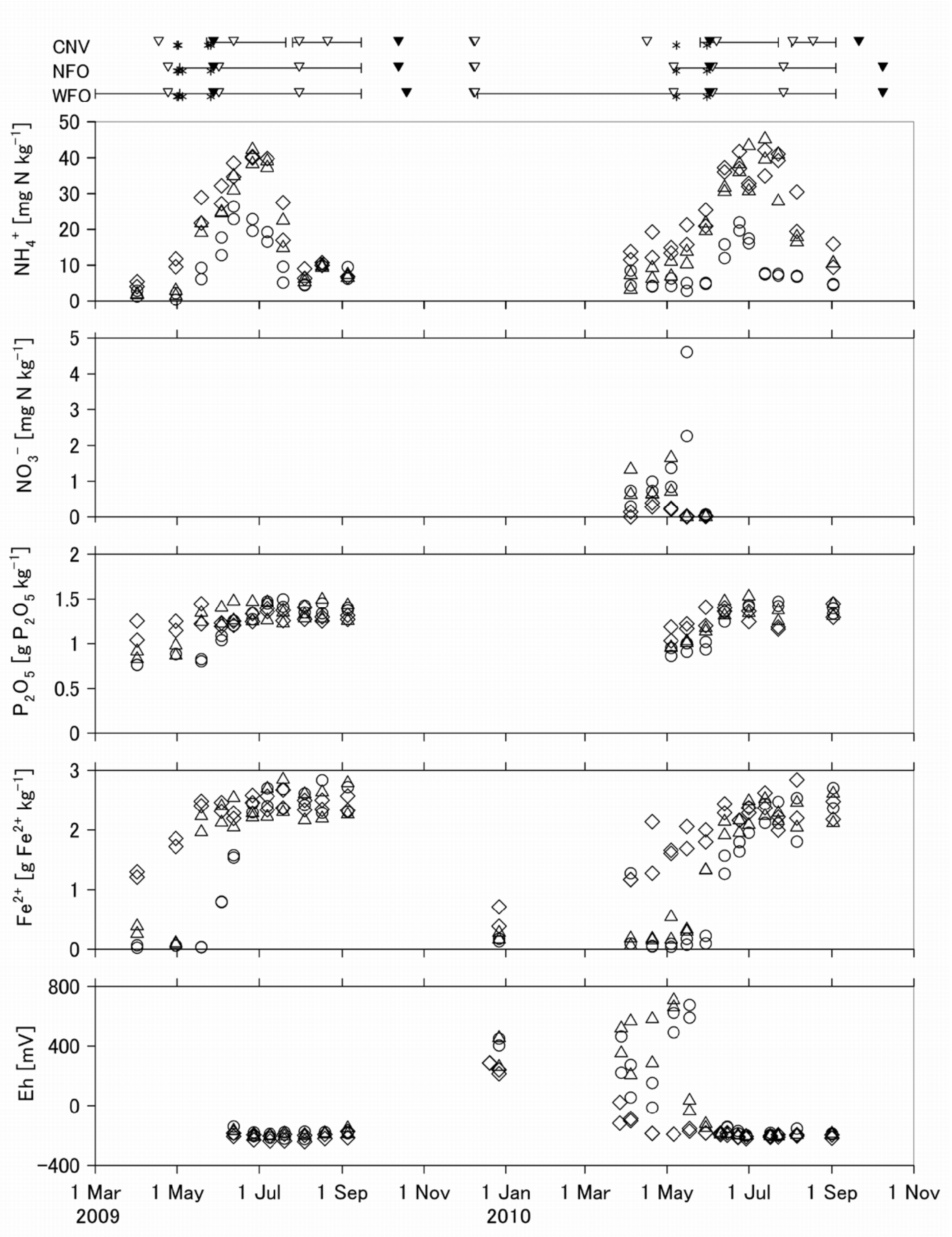

Fig. 2. Temporal variability of NH_4^+, P_2O_5, Fe^{2+}, and Eh in 2009 and 2010, and of NO_3^- in 2010. All measurements were obtained from the plow layer (0–10 cm), except Eh, which was measured at 5-cm depth. In each graph, Diamonds, triangles, and circles indicate single replicate plots of winter flooding organic (WFO), no flooding organic (NFO), and conventional (CNV) treatments. The line above the graphs shows the flooding period for each treatment. White downward triangles (▽) indicate timing of fertilizer, manure or herbicide input, black downward triangles (▼) indicate transplanting or harvest timing, and asterisks (*) indicate tilling or puddling timing. Details on these practices are given in Table 1.

measured only in 2010. NO_3^- was lower in WFO than in CNV and NFO plots just after irrigation. P_2O_5 and Fe^{2+} increased from April to June and showed constant values thereafter, and these concentrations in the WFO plots were higher than and similar to those in the NFO and CNV plots during the same periods, respectively. There was no significant difference in Eh among the treatments in 2009; after flooding, however, Eh values in WFO were markedly lower than those in CNV and NFO during all months until June. Only Fe^{2+} and Eh were measured during the winter period. Reducible iron, Fe^{2+}, was higher in the WFO than in the CNV and NFO plots. Similarly, Eh was lower in WFO than in CNV and NFO.

Nematode density:

The stepwise selection of GLMs yielded a model that included treatment, soil depth, sampling date, and the interaction between soil depth and sampling date. As the interaction was significant (ANOVA, $P = 0.03$), the data set was divided for further analyses. The independent effect of soil depth was shown in the GLM as a coefficient of –1.00 ($P < 0.01$) for the second layer compared with the top layer of the soil. The means (with SD ranges) of density were 478 (235–971) and 190 (88–407) per 100 g dry soil in the top and second layers, respectively.

In the top layer (Fig. 3a), nematode density was explained by seasonality and treatment as independent factors (Table 2). The coefficients were estimated as 0.90 ($P < 0.01$) and 0.53 ($P = 0.04$) for WFO and NFO, respectively, compared with CNV, and as 0.54 ($P < 0.01$) for seasonal trend: nematode density reached its maximum in late September and minimum value in late March ($\varphi = -0.36$). The means (with SD ranges) of density were 732 (407–1317), 501 (245–1023), and 297 (170–518) per 100 g dry soil for WFO, NFO, and CNV, respectively. Nematode density was significantly higher for WFO than for CNV ($|Z| = 3.84$, $P_{adj} < 0.01$). The difference between NFO and CNV was marginally significant ($|Z| = 2.22$, $P_{adj} = 0.07$).

Nematode density in the second soil layer (Fig. 3b) was best explained by sampling date as the nominal variable (ANOVA, $F = 6.74$, $P < 0.01$), along with treatment (ANOVA, $F = 30.40$, $P < 0.01$) and their interaction (ANOVA, $F = 3.61$, $P = 0.02$; Table 2). Nematode density was higher for WFO than for CNV ($|Z| = 2.78$), and for NFO than for CNV ($|Z| = 1.57$); however, these main effects were mitigated by the significant interaction.

Diversity index:

The stepwise selection of the GLMs yielded a model that included treatment, soil depth, sampling date, and their interactions. The independent effect of soil depth was marginally significant (ANOVA, $P = 0.05$), but the interactions of soil depth with treatment and with sampling date were both significant (ANOVA, $P = 0.01$ and $P < 0.01$, respectively). Therefore, the dataset was divided by soil depth for further analyses.

In the top soil layer (Fig. 4a), the H' value was

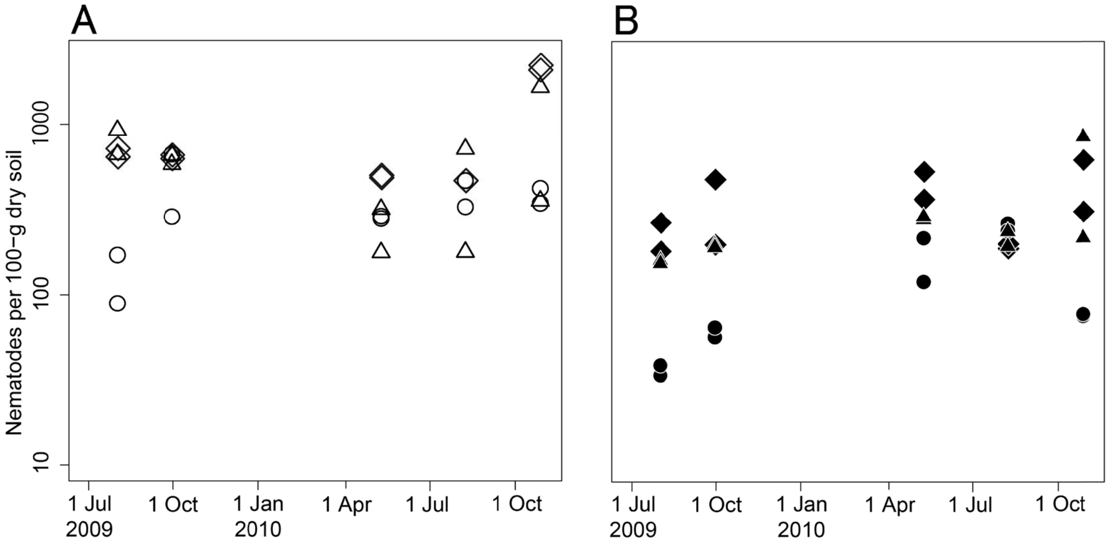

Fig. 3. Temporal change in the density of nematodes in (A) top and (B) second soil layers of rice paddies under different treatments. Diamonds, triangles and circles indicate single replicate plots of winter flooding organic, no winter flooding organic, and conventional treatments, respectively. The top and second soil layers are indicated with open and solid symbols, respectively. Abbreviations are the same as those used elsewhere in this manuscript.

explained by sampling date as an independent nominal variable (Table 2; ANOVA, $P < 0.01$). The mean H' value was highest in August 2009 and decreased to its minimum value in October 2010.

In the deeper layer (Fig. 4b), the H' value was explained by treatment (ANOVA, $P < 0.01$) and sampling date (ANOVA, $P < 0.01$) as the only independent nominal variables, and their interaction was not included in the best model (AIC = 38.97). The effects of WFO and NFO were negative and positive, respectively, contrasted with the effect of the CNV (Table 2). The H′ values were significantly lower for WFO than those for NFO ($|Z| = 5.09$, $P_{adj} < 0.01$) and for CNV ($|Z| = 3.05$, $P_{adj} < 0.01$). When seasonal trend was considered, the GLMs yielded a minimum AIC value of 44.62. This model was inferior to the previous one and, therefore, it was not regarded as acceptable.

Community structure:

In total, 34 OTUs were distinguished in this study. *Tobrilus* sp. 1, the most dominant OTU, represented 46.3% of the relative abundance. The top 10 OTUs

Table 2. Factors affecting density, diversity index and community structure of nematodes in paddy field soil analyzed using generalized linear models (GLMs) including analyses of variance (ANOVAs).

Response variables	AIC	Intercept[a]	Treatment		Sampling date		Interaction
			WFO[a]	NFO[a]	As nominal variable[b]	As seasonality[a] (φ value)	between treatment and sampling date[b]
Density[c]							
in top layers	52.22	5.79*	0.90*	0.53*	NS	0.54* (−0.36)	NS
in second layers	34.85	5.09*	1.00*	0.56	0.93*	NS	0.68*
Diversity index H'							
in top layers	50.6	1.88*	NS	NS	1.83*	NS	NS
in second layers	38.97	2.61*	−0.55*	0.36*	1.49*	NS	NS
Community structure[d]							
in top layers	7.92	−0.13	NS	NS	NS	−0.34* (0.22)	NS
in second layers	5.84	0.19*	−0.52*	−0.19	NS	−0.15* (−0.39)	NS

[a] Values are coefficients estimated in GLMs.
[b] Values are root mean square in ANOVAs.
[c] Log-transformed number of nematodes per g dry soil.
[d] Represented by the first principal component in the communities.
* The factors caused significant deviation from 0 ($P < 0.05$).
NS stands for not selected as a significant factor in model selection.

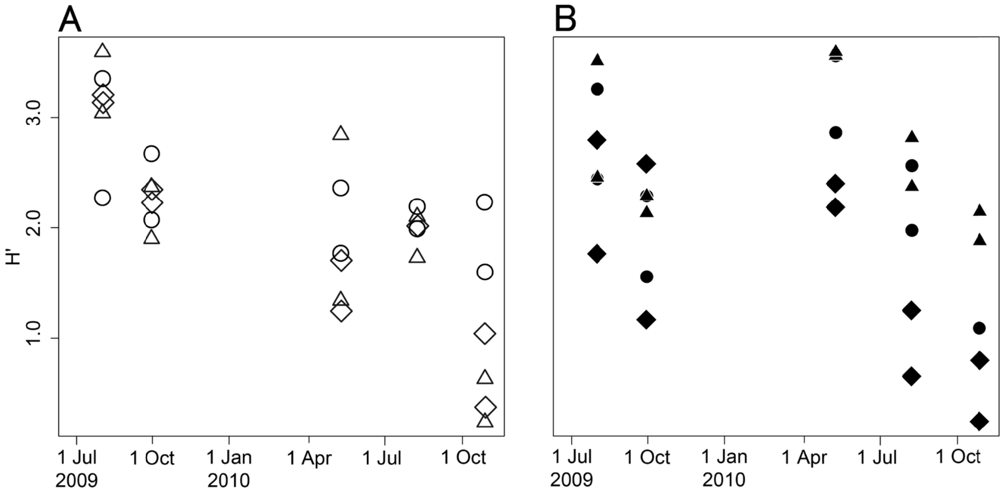

Fig. 4. Temporal change in nematode diversity as indicated by Shannon-Weaver index H' in (A) top and (B) second soil layers of the three paddy field management treatments. Diamonds, triangles, and circles indicate single replicate plots of winter flooding organic, no winter flooding organic, and conventional treatments, respectively. The top and second soil layers are indicated with open and filled symbols, respectively. Abbreviations are the same as those used elsewhere in this manuscript.

comprised 90.5% of the relative abundance, and the top 17, 95.5%. Several OTUs were assigned to nematode taxa obtained from the BLAST searches (Table 3; Fig. 5). The major bands in the DGGE patterns were regarded as single OTUs, because they had single genotypes when sequenced.

The PC1 of the nematode communities explained 70.0% of the total variance; it was positively loaded with *Tobrilus* sp. 2 and negatively with *Tobrilus* sp. 1. These two OTUs comprised 98.7% of the total variance in the PC1. The PC2 of the nematode communities explained 14.9% of the total variance; it was positively loaded with *Tobrilus* sp. 1 and sp. 2 and negatively loaded with *Hirschmanniella* sp. These three OTUs comprised 90.3% of the total variance in the PC2. As shown in Fig. 6, the nematode communities of WFO were mainly distributed around the origin to the left-hand side, although they overlapped with those of CNV and NFO. This implies the specific dominance of *Tobrilus* sp. 1 over sp. 2 for WFO.

As the best model for PC1 included the interaction between treatment and soil depth, the dataset was divided by soil depth for further analyses.

In the top soil layer, PC1 was explained by seasonal trend as the only independent variable (Table 2; ANOVA, $F = 10.36$), with a maximum in mid-May and a minimum in early November ($\varphi = 0.22$).

In the second soil layer, PC1 was explained by treatment (ANOVA, $F = 11.87$, $P < 0.01$) and seasonal trend (ANOVA, $F = 4.50$, $P = 0.04$) as independent variables, with a maximum in early April and a minimum in early October ($\varphi = -0.39$) (Table 2). The PC1 was significantly influenced by flooding and organic treatment ($|Z| = 3.06$, $P_{adj} < 0.01$; $|Z| = 4.81$, $P_{adj} < 0.01$).

SEM:

The best SEM explained the variance and covariance adequately, with a chi-squared value of 8.57, df = 17, $P = 0.87$, and AIC 46.57. The coefficients of determination for the endogenous variables were 0.49, 0.61, and 0.33 for water content, density, and the PC1 of the nematode communities, respectively (Fig. 1b). Seasonal trend was significant in both nematode density (standardized partial correlation coefficient $\hat{\beta} = 0.25$, $P < 0.01$) and PC1 ($\hat{\beta} = -0.38$, $P < 0.01$). In addition to seasonality, nematode density was positively influenced by the application of organic farming ($\hat{\beta} = 0.40$, $P < 0.01$), winter flooding ($\hat{\beta} = 0.57$, $P < 0.01$), and the vertical position of the soil layer ($\hat{\beta} = 0.54$, $P < 0.01$). In addition to seasonality, the PC1 was influenced only by winter flooding ($\hat{\beta} = -0.44$, $P < 0.01$). The effect of the exogenous variables was all direct to the density and PC1, and there was no indirect path via soil water content.

DISCUSSION

Effect of winter flooding and organic farming on nematode density:

In the top soil layer, organic farming was associated with an increase in the nematode density, and it was increased further with winter flooding. This result

Table 3. Closest relatives for the DGGE bands with the greatest similarities in BLAST database search.

Band in Figure 5	OTU #	Family	Species inferred	Accession# of reference	Similarity (%)	Length (bp)
A	29	Tripylidae	*Tobrilus gracilis* (*Tobrilus* sp. 1)	AJ966506	100	510
B	31	Tripylidae	*Tobrilus gracilis* (*Tobrilus* sp. 2)	AJ966506	99	509
C	23	Tripylidae	*Tobrilus gracilis*	EU910599	98	119
D	30	Tripylidae	*Tobrilus gracilis*	AJ966506	99	505
E	22	Tubificidae (non nematode)	*Ilyodrilus templetoni*	HM460032	98	362
F	7	Pratylenchidae	*Hirschmanniella* cf. *belli*	EF029856	100	511
G	24	Tripylidae	*Tobrilus gracilis*	AJ966506	98	510
H	30	Tripylidae	*Tobrilus gracilis*	AJ966506	100	480
I	23	Mononchidae	*Mononchus truncatus*	AB36145	99	462
J	29	Tripylidae	*Tobrilus gracilis*	AJ966506	100	486
K	31	Tripylidae	*Tobrilus gracilis*	AJ966506	99	498
L	33	Tripylidae	*Tripyla* sp.	GQ503067	98	360
M	34	Tripylidae	*Tobrilus gracilis*	AJ966506	99	450
N	24	Tripylidae	*Tobrilus gracilis*	AJ966506	98	428
O	19	Tripylidae	*Tobrilus* sp.	GQ503073	97	111

partially contradicts our hypothesis expecting lower nematode density with flooding. The increase in density may be due to the enrichment of the microbial biomass by organic amendment and winter flooding, which should satisfy the nutrient demands of the nematodes. The absence of pesticide applications might also be beneficial. Similar to our results, a winter-flooded paddy field sustained populations of bacterivorous ciliates in paddy water, which was ascribed to increased decomposition of rice stubble prior to the cropping season (Kameyama *et al.*, 2006). Nematodes can also respond to the amendment of organic matter (Freckman and Ettema, 1993; Okada and Harada, 2007). In a citrus orchard soil, bacterivorous nematodes flourished immediately after the occurrence of a spike in easily decomposable organic matter, followed by an increase in fungivorous nematodes after a time lag (Porazinska *et al.*, 1999).

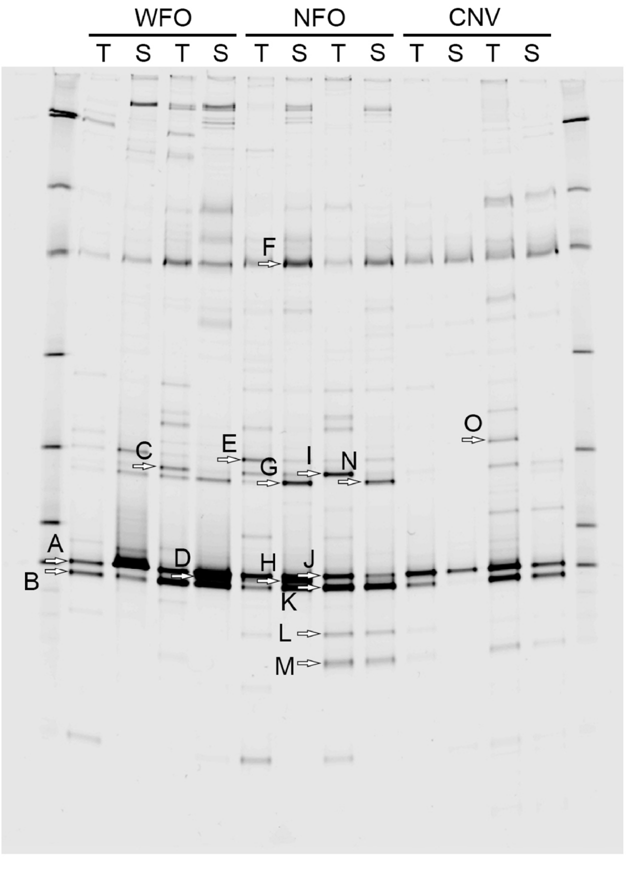

Fig. 5. Representative patterns of DGGE banding of soil nematodes under three paddy field management treatments. The specimens were collected in September 2009. WFO, NFO, and CNV are winter-flooded and organic, non-winter-flooded and organic, and conventional managements, respectively. T and S indicate top (0–5 cm) and second (5–10 cm) soil layers, respectively. We applied DGGE marker (DGGE Marker V; Nippon Gene) to the outermost lanes. We sequenced bands with white arrows and alphabets, and used the BLAST database search to infer taxonomic affiliation. See also Table 3 for details on the annotated bands.

The nutrient status in the study fields was partially indicated by the NH_4^+ levels. NH_4^+ concentrations were highest in WFO fields, followed by NFO fields, and lowest in CVN fields, although the difference gradually disappeared in the cropping season. Nitrogen enrichment usually enhances plant or microbial activity and increases the abundance of soil nematodes, which feed on the microbes (Yeates *et al.*, 1993; Vestergård, 2004; Liang *et al.*, 2009; Zhao *et al.*, 2014).

Physico-chemical data indicated that, in general, winter flooding had altered the paddy soil environment to be more reductive, *e.g.* having lower Eh value, higher methane emissions, and higher Fe^{2+} content. This reductive tendency is possibly more marked in the second compared to the top layer (Takai *et al.*, 1974), and consequently has a negative effect on the propagation and survival of many nematodes, which could have offset the positive effect of organic fertilization in our study. The reductive conditions and accompanying decrease in food source can suppress the density of marine benthic nematodes (Pfannkuche, 1985). In a freshwater lake, similar to our findings, benthic nematodes inhabited mostly the top 5 cm of the sediment, and a relationship

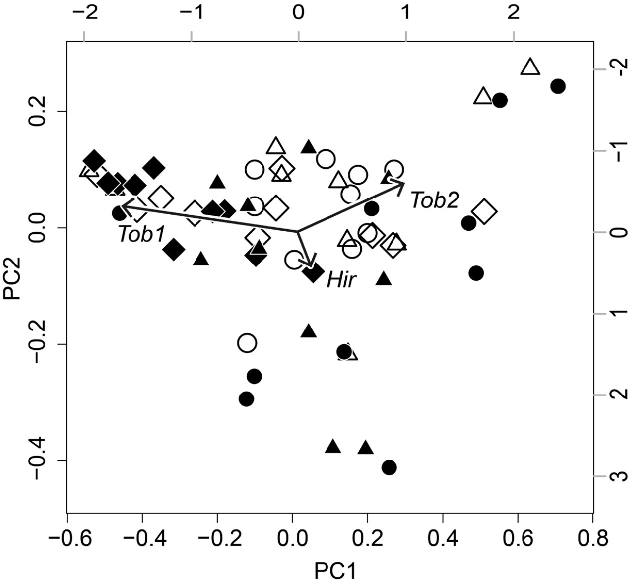

Fig. 6. Nematode communities by principal component analysis. Diamonds, triangles, and circles indicate single replicate plots of winter flooding organic, no winter flooding organic, and conventional treatments, respectively. The top and second soil layers are indicated with open and solid symbols, respectively. *Tob1*, *Tob2*, and *Hir* indicate *Tobrilus* sp. 1, *Tobrilus* sp. 2, and *Hirschmanniella* sp., respectively. Abbreviations are the same as those used elsewhere in this manuscript.

between the low density of nematodes and reductive conditions of the sediment below 5 cm, which was generally gray in color, was proposed (Traunspurger and Drews, 1996). Additionally, in farmland ponds, eutrophication and associated anaerobic conditions were suggested as the main factors explaining the low density and diversity of sediment nematodes (Bert *et al.*, 2007).

Effect of winter flooding and organic fertilizer on diversity and community structure:

The diversity of nematodes was high in the second soil layer in the NFO fields; however, in the WFO fields, the diversity was significantly reduced to a level even below that of the CNV fields. Especially in the WFO fields, the low diversity correlated with low PC1, which indicated the dominance of *Tobrilus* sp.1, resulting in lower values for the diversity index. This may also reflect the balance between the positive effect of organic fertilization and the negative effect of reductive conditions.

The structure of the nematode communities at our research site was influenced mostly by *Tobrilus* spp., aquatic algivorous and carnivorous nematodes. In the second layer of the WFO plots, as noted above, one of the dominant *Tobrilus* OTUs increased over the other, which led to the observed change in community structure and decrease in diversity before early cropping season. In the top layer of the same plots, on the other hand, the community structure showed only seasonal changes, with no difference among the treatments, which may explain why nematode diversity in the top layer varied only by sampling date. *Tobrilus*, equipped with an anaerobic respiration mechanism, is a genus that is well adapted to reductive environments (Schiemer and Duncan, 1974); however, the reductive environment at greater depths in winter-flooded paddies might be sufficiently severe to limit the distribution of some of the *Tobrilus* species. A recent molecular study uncovered cryptic species and genetic divergence in *Tobrilus gracilis* caused by geographic isolation, and reported a possible underestimation of the variation in single morphospecies of aquatic nematodes (Ristau *et al.*, 2013). Therefore, our study may reveal cryptic niche segregation of nematodes within a local site. Before testing this possibility, it is necessary to elucidate in detail the competitiveness of each *Tobrilus* OTU under reductive conditions in combination with their phylogenetic relationships.

The results of SEM were in agreement with those indicated by the GLM analyses mentioned above. It is noteworthy that the nematodes were influenced by winter flooding and organic farming, not via soil porosity but via other factors undefined in the present SEM. The porous and muddy soil layer, called *toro-toro* layer, is one of the well-known characteristics of winter-flooded rice paddies in Japan (Ito *et al.,* 2011), but its physical property itself does not apparently affect nematode community profiles. Winter flooding combined with organic farming altered the soil to be more fertile and reductive in our site, which might in turn have altered nematode communities. However, we could not include the chemical properties in statistical analyses because of the lack of correspondence to the data of nematodes.

In the same way, the relationship between the density and community structure of the nematodes was considered to be a superficial correlation, confounded by seasonality and treatment. SEM allows us to avoid such superficial correlations, and is therefore preferable for the cause-effect analysis of environmental impacts.

Conclusions:

This study is the first to demonstrate the effects of changes in the soil environment and soil fauna due to winter flooding, as indicated by nematodes. Flooding in the fallow season affected the nematode density and community structure in the subsequent cropping season, suggesting that the accumulated impact of environmental stress could be detected as changes in the nematode community profiles. Winter flooding in rice paddies has generally been regarded as “environmentally friendly” as mentioned in the introduction. However, our study demonstrated that winter flooding could have detrimental effects on soil fauna and biodiversity, as represented by nematodes. Further studies in various soil types and climate conditions are necessary to investigate the effects of winter flooding on paddy soil fauna in more detail. Niwa *et al.* (2011) studied the functional relationships between nematodes and physical environments in a deciduous cool-temperate forest, and these relationships also need to be clarified in rice paddies to better guide the ecological management of rice paddies as alternative wetlands.

Our results suggest that nematodes can be beneficial bio-indicators to detect an environmental stress in paddy soil, as previously indicated in upland fields (Porazinska *et al.*, 1999; Yeates, 2003). Actually, we have reported that the density of several nematode taxa inhabiting rice paddies could be reduced by future global warming (Okada *et al.*, 2014). We suggest that nematodes could further be used to assess the ecological risks to soil fauna in other wetland habitats than rice paddies. Molecular analyses will aid in elucidating the cryptic structure of nematode communities, hitherto identified on the basis of their morphology, to detect the detailed response of nematodes to ecological risks.

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