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Use of legacy nitrogen as a resource: Unfertilized lotus fields contribute to water quality improvement and biodiversity conservation

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ABSTRACT

Managing legacy nitrogen (N) that has accumulated over decades of intensive agriculture is necessary to balance agricultural production and environmental conservation. Using legacy N as a resource can reduce extant legacy N. The uplands of Japan's Lake Inba watershed are dominated by agroecosystems, and spring waters in the lowlands contain high NO_3-N concentrations. We focused on an unfertilized, commercial lotus (*Nelumbo nucifera*) farm where paddy fields are irrigated with spring waters. We hypothesized that the lotus field could reduce the export of legacy N and provide habitat for fish (co-benefit). Dating of spring waters using sulfur hexafluoride revealed that the residence time of water at the study site was 12.5 years and suggested that the spring waters contained legacy N. We found large reductions of NO_3-N and total nitrogen (TN) concentrations in water that passed through the lotus fields. A meta-analysis revealed that the reductions of NO_3-N concentrations were highest in unfertilized lotus fields, and reductions of TN concentrations were higher in unfertilized paddy and lotus fields than in fertilized fields. The N removal by harvesting lotus roots 38.7 kg N/ha/year was smaller than the literature-based rate of N removal by denitrification. We used environmental DNA metabarcoding to identify the fish fauna in the lotus fields and an adjacent stream. The native fish richness was a little lower in the lotus fields than in the stream, but the presence of two endangered fish species in the lotus field suggested a moderate biodiversity conservation function. Our case study could be a good example of a nature-based solution to harness ecosystem functions to reduce legacy N.

Introduction

Although the food produced by agriculture systems is essential to human well-being [1], striking the proper balance between agricultural production and environmental integrity is important [1–3]. In recent decades, the dramatic increase of anthropogenic nitrogen (N) inputs from fertilizers associated with rising populations, developing supply chains, and changing diets has deteriorated water quality and eroded biodiversity [4–6]. In areas of intensive agriculture, decades of fertilizer and manure applications have led to the accumulation of surplus N from cropland and livestock production, referred to as legacy N, in numerous landscape elements, including agricultural soils, groundwater, riparian zones, and stream and lake sediments [4,7]. Legacy N can continue to increase N concentrations in streams and lakes for many years after inputs decline, causing persistent water quality issues, such as harmful algae blooms and hypoxia [6]. More seriously, as the climate warms, extreme precipitation events are expected to increase and mobilize stored legacy N [4,8], highlighting the urgent need to find creative strategies to manage legacy N.

The importance of preventing future legacy N build-up has been recognized, but drawing-down existing legacy N through capture and recycling is also important to accelerate water quality improvement [4, 9]. Basu, et al. [4] proposed the use of legacy N as a resource. Crop yields can be maintained with low fertilizer application rates by utilizing legacy N in soils and groundwaters [10]. The effective use of legacy N may also be applicable to waterbodies where groundwater discharges onto the surface (i.e., spring water). Legacy N in spring waters can be removed effectively before it reaches downstream waters by harnessing the denitrification and plant uptake functions of wetlands and riparian buffers [5,11]. Although wetland and riparian restoration is an effective way to capture legacy N, these projects do not exploit legacy N as a resource. Cultivating crops using legacy N in waterbodies may save

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money, provide environmental benefits, and create opportunities for local livelihoods. This strategy might generate social, economic, and environmental co-benefits related to ecosystem maintenance, biodiversity conservation, food safety and security, revitalization of local economies, and human health. In fact, this approach is part of the nature-based solution (NbS) for water proposed by IUCN [12,13].

The watershed of Lake Inba, a hypereutrophic lake in Chiba Prefecture, is dominated by crop and livestock production, and Chiba ranks among the top- five Japanese prefectures in terms of agricultural productions. In this watershed, legacy N is a consequence of decades of intense agriculture, and N runoff continues to degrade downstream water quality and the surface water quality of Lake Inba [14,15]. In Lake Inba, both phosphorus (P) and N limit phytoplankton growth, and N limitation is especially strong during the summer [16]. There is a salient need to balance agricultural production and environmental-quality goals in the watershed. The Kanto Plain, including the Lake Inba watershed, consists of flat, low-elevation uplands and lowlands with many narrow, shallow, branching valleys [17–19]. The uplands are dominated by croplands, orchards, and livestock facilities, and in the many springs that discharge in lowlands along the edge of the valley the nitrate concentrations are often high (> 10 mgL^{-1} NO_3-N). Rice paddies have traditionally been sited in valley bottoms and irrigated with spring water, and the paddies have removed much of the N from the spring waters [20,21]. Since the 1960s, however, many paddy fields have been converted to modern irrigation systems, wherein water is supplied through underground pipes and is drained into deep, concrete-sided ditches [22–24]. In this modern system, spring water is not used for irrigation; N-rich waters flow directly to concrete ditches, from which they are quickly discharged to streams and lakes without N removal by the paddies. These drastic changes in agricultural landscapes have adversely affected wetland- and/or spring-dependent amphibians, fishes, and birds [18,19,24,25]. Thus, a multi-objective watershed restoration plan is needed to manage legacy N and sustain biodiversity.

The focus of this study was a lotus (*Nelumbo nucifera*) farm in the watershed of Lake Inba. The farm is irrigated with spring water and produces lotus without the use of fertilizers. This farming method is unique because lotus is conventionally produced with high fertilizer inputs and irrigation water from rivers or lakes. In this region, spring waters are considered to contain legacy N, and we hypothesized that the unfertilized lotus fields reduce the export of legacy N. We estimated the age of spring water by measuring its sulfur hexafluoride (SF_6) concentration to examine the presence of legacy N. As metrics of N removal effectiveness, we measured the changes of dissolved and particulate N concentrations between the inlet and outlet of the lotus fields. We then performed a meta-analysis to compare the N removal effectiveness of the unfertilized lotus fields with that of previous studied paddies and lotus fields. We also hypothesized that the unfertilized lotus fields provide a suitable habitat for native fishes as a co-benefit. We assessed fish species composition of the lotus fields and the adjacent stream using environmental DNA (eDNA) metabarcoding, which is a sensitive, efficient, and non-invasive tool for detecting the presence of rare, threatened, and invasive species that are often present in low abundance [26,27].

Methods

Study site

We studied an unfertilized lotus farm (Satoyama-Renkon Farm) in the valley bottom of the Lake Inba watershed. Orchards (pears), croplands (Japanese yams and peanuts), and a cattle ranch are situated in the surrounding uplands (Fig. 1A). The farm converted abandoned paddy fields to lotus fields in 2019, and the lotus fields have been irrigated with spring water that discharges from the lowest elevation of the valley bottom (Fig. 1B). The farm has cultivated lotus without fertilizers, manures, pesticides, and any other chemicals.

The farm has six lotus fields, with surface areas that range from 0.06 to 0.17 ha (mean 0.11 ha; Fig. 1A). A small stream flows at the center of the fields complex, and outflows from the lotus fields discharge into this stream. The stream flows into the Kashima River 500 m downstream (Fig. S1A), which flows into Lake Inba (approximately 10 km further downstream).

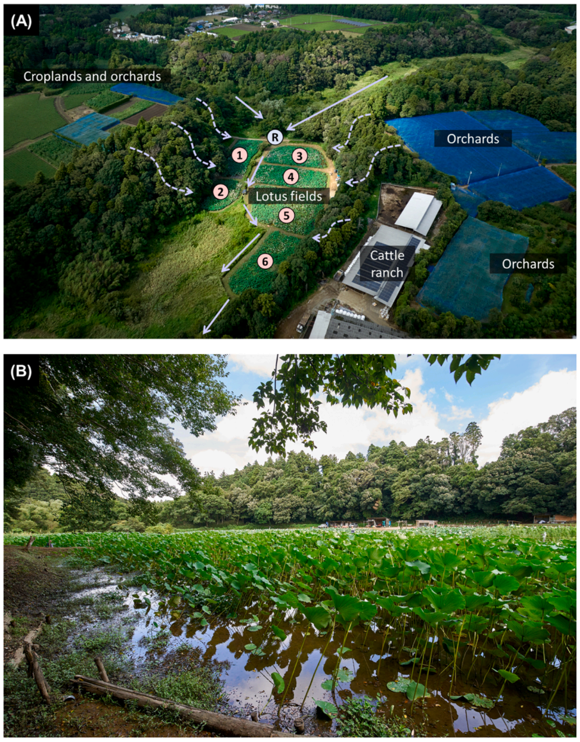

Fig. 1. (A) A bird's-eye view of the study area with location of unfertilized lotus fields in Sakura City, Chiba Prefecture, Japan. Six lotus fields (encircled numbers) are located in the valley. The uplands are occupied by orchards, croplands, and a livestock facility (cattle ranch). The light-blue broken arrows indicate potential groundwater flows from uplands to the bottom of the valley. The light-blue arrows show the direction of flow of stream that discharges into the center of the lotus fields. The encircled R is the location where the eDNA sample of the small stream was collected as a reference. (B) Photo of the lotus field No. 2. Spring waters discharge slowly from the left side.

Spring water dating

To investigate the presence and time-lag effect of spring water containing legacy N, we measured the concentration of SF_6 as an age metric. Significant industrial production of SF_6 began in the 1960s, and its atmosphere concentration has increased linearly from 0.03 parts per trillion (pptv) in 1970 to 6.8 pptv in 2009 because of its long persistence (3200 years) in the atmosphere [28]. SF_6 is a particularly effective dating tool for post-1993 young groundwater compared to tracers such as chlorofluorocarbons [29].

We collected duplicate water samples from three places where spring water was discharging on 31 August 2022. We inserted a 30-cm-long stainless-steel tube with 10 slits (0.2-mm width) into each spring. The stainless-steel tube was connected to a peristaltic pump (model Wpx-1000, Welco) with a nylon tube. The water was first poured into a 5-L stainless-steel bucket. To avoid contact of the water sample with the atmosphere, we lowered a 550-mL glass bottle with an ethylene propylene rubber seal liner screw cap into the bucket. Sample bottles were slowly filled with spring water and capped underwater while overflowing.

We measured dissolved SF_6 concentrations in the water samples using a purge-and-trap gas chromatograph with an electron-capture detector (GC-ECD) at the Geo-Science Laboratory Co., Ltd., Nagoya, Japan. The procedure involved 400-mL of sample water that was stripped of SF_6 with ultra-pure N_2 gas. The extracted SF_6 was purified and concentrated by two cold traps and finally injected into the GC-ECD. The precision and the detection limit of the analysis were less than 3% and 0.05 fmol L^{-1}, respectively. The SF_6 concentrations in spring waters were converted to equivalent air concentrations (EACs) at the time of recharge based on Henry's Law. Annual mean temperature (14.6 ℃) and average upland elevation (30 m) were used to estimate EAC.

We estimated spring water ages by comparing the atmospheric-converted SF_6 concentrations with historical average atmospheric SF_6 concentrations of the Japanese archipelago [30]. Groundwaters usually contain excess air because of the forcible dissolution of air bubbles that inevitably occurs during recharge. To consider the effect of excess air, we measured dissolved argon (Ar) simultaneously with SF_6 with a GC-ECD and corrected the residence time for excess air [29].

Concentration changes of N species through the lotus fields

We sampled spring water entering the lotus fields and water exiting the fields on 23 August, 1 September, and 24 November 2021, and 31 August 2022. Spring water slowly discharged from multiple points and did not always seep from the same points during the study period. We identified and sampled all spring waters entering the lotus fields on each sampling date (Fig. S1B) and averaged the concentrations of each N species in those spring waters. We assumed that the inlet concentrations of N species were the same for all the lotus fields. Outlet waters did not always discharge from all lotus fields (Fig. S1B), but we were able to sample them from more than three fields on all sampling dates. When there were two outlets from a lotus fields, we collected two outlet water samples and calculated the average concentration.

Water temperatures and chlorophyll-a concentrations were measured using a YSI ProDSS multi-parameter meter (YSI, Inc., Yellow Springs, OH, USA). Spring waters and outlet waters were collected using a disposable syringe and immediately filtered through 0.45-µm filters (Chromatodisc 25 AI; GL Sciences Inc., Tokyo, Japan). For particulate organic N (PON), water samples were filtered through a pre-combusted 47-mm Whatman GF/F glass fiber filter (nominal pore size: 0.7 µm). In the laboratory, the water samples were frozen at –30 °C for nutrient analyses, and GF/F filters were dried at 60 ℃ for 2 days.

We measured the concentrations of NO_3-N, NH_4-N, dissolved organic nitrogen (DON), and total dissolved nitrogen (TDN) using a colorimetric auto-analyzer (TRAACS-800, Bran-Luebbe, Tokyo, Japan). The detection limits for NO_3-N, NH_4-N, DON, and TDN were 2.1, 2.4, 2.1, and 2.1 µg L^{-1}, respectively. The PON concentration were analyzed with a CHN analyzer (model MT-6; Yanako, Tokyo, Japan). The total nitrogen (TN) concentrations were calculated as the total of TDN and PON. To consider the effects of dilution and evaporation in the lotus fields, we used the concentration of sodium ions (Na^+) as a conservative tracer [31]. The concentrations of Na^+ is affected by water gains and losses but is unaffected by biological uptake. We measured Na^+ concentrations using an ion chromatograph (DX-320 with an eluent generator, EG40; DIONEX, Tokyo, Japan). The detection limit was 1.9 µg L^{-1} for Na^+.

We estimated N removal effectiveness of unfertilized lotus fields following a modified method of Sabater, et al. [32]. We calculated the concentration changes of NO_3-N, NH_4-N, TDN, PON, and TN between inlet and outlet waters of each lotus field as a percentage using the following equation:

$$\text{N concentration change } (\%) = \left[\left(N_{O-obs} - N_{O-exp}\right) / N_{O-exp}\right] \times 100,$$

where N is each nitrogen species, $N_{O\text{-}obs}$ is the observed N concentration of outlet waters, and $N_{O\text{-}exp}$ is the expected N concentration of outlet waters when the only significant effects are water gain (dilution) and/or loss (evaporation) because depletion by denitrification and bio-assimilation are negligible. $N_{O\text{-}exp}$ was calculated with the following equation:

$N_{O-exp} = N_{I-obs} \times \left(Na^+_O / Na^+_I\right)$, where $N_{I\text{-}obs}$ is the observed N concentration of inlet waters (i.e., average concentration of spring waters), Na^+_O / Na^+_I is the dilution factor, and Na^+_O and Na^+_I are the observed Na^+ concentrations of outlet and inlet waters, respectively. The more negative the N concentration changes, the greater the reduction of the N concentration in the fields. We also tested whether $N_{O\text{-}obs}$ was significantly different from $N_{O\text{-}exp}$ using a paired *t*-test.

N removal by harvesting lotus

We quantified the N removal by harvesting lotus and compared it with the rates of N removal by paddy fields reported in previous studies. We randomly harvested and dried samples of lotus roots (edible part) at 45 °C for 1 day and ground them to a fine powder. We then dried the fine powders at 60 °C for 1 day. We measured lotus N content ($N = 25$) with a CN elemental analyzer (JM10, J-Science Lab Co., Ltd., Kyoto, Japan). To calculate the annual N removal by harvesting, we multiplied the average N content by the annual total lotus yield in 2022.

Meta-analysis

We searched for peer-reviewed papers published before November 2022 that reported the concentrations of NO_3-N and TN of both inlet and outlet waters in paddy fields and lotus fields/ponds. We used both Google Scholar and J-Stage (https://www.jstage.jst.go.jp/browse/-char/ja). The latter is a platform for scholarly publications in Japan, and many paddy and lotus field studies conducted in Japan are written in Japanese. To find candidate publications, we searched with different combinations of the following English and Japanese keywords: nitrogen, nitrate, removal, denitrification, uptake, concentration change, paddy field, rice field, lotus field, and lotus. We translated this search string into Japanese for J-Stage.

Sixty-four datasets from 27 published papers met our criteria for analyses (Appendix S1). We calculated N removal effectiveness using the following equation:

$$\text{N concentration change } (\%) = \left[\left(N_{Outlet} - N_{Inlet}\right) / N_{Inlet}\right] \times 100,$$

where N_{Outlet} and N_{Inlet} are the average N concentrations of outlet and inlet waters, respectively. Because of lack of information about the concentrations of conservative tracers, we did not consider dilution and evaporation effects on these concentration changes. We classified fields into four categories: unfertilized or fertilized paddy fields and unfertilized or fertilized lotus fields. We compared changes of NO_3-N and TN concentrations among these four categories using a generalized linear model (GLM) with a Gaussian distribution.

eDNA sampling and metabarcoding analysis

We conducted water sampling at six lotus fields and one adjacent stream (Fig. 1A) on 1 September 2021. At each site, we poured two 1-L water samples into hypochlorous acid-treated-polypropylene containers. For each 1-L sample, 250 mL of water was taken from four different points within the site. We immediately added a 10% benzalkonium chloride solution (OSVAN S, Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) to suppress DNA degradation [33]. The water samples were immediately stored on ice and transferred to a laboratory, where they were stored at 4 ℃ until filtration.

We filtered the water samples under negative pressure using 47-mm-diameter Whatman GF/F glass microfiber filters (Cytiva, Tokyo, Japan) within 20 h of collection. Because the filters clogged easily, we used up to four filters to filter each 1-L sample. Two 1-L of pure water were filtered concurrently with the same type of filter to serve as negative controls. We wrapped the filters in aluminum foil and stored them at −28 °C until DNA extraction. We extracted DNA from the filters using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). Two DNA extraction negative controls were processed to test for cross contamination during DNA extraction. The reactions were performed with Buffer AL and Proteinase K for each filter. A spin column was then applied to the reaction solutions and eluted with 100 mL of AE buffer to obtain a template for PCR. If there were multiple filters per replicate, we mixed the reaction solutions of up to two filters when applying the spin column and mixed equal amounts of eluted solutions from the same sample.

We used the universal primer set MiFish-U [26], which amplifies a hypervariable region of the 12S rRNA gene. The volume of the first PCR reaction solution was 12 μL, containing 2 μL of template DNA, 6 μL of 2× PCR buffer, 0.3 μM of MiFish-U-F and -R primers with Illumina adapter sequences, 0.4 mM of dNTPs, and 0.24 U of KOD FX Neo (TOYOBO, Osaka, Japan). Eight reactions were performed for each eDNA sample. One replicate of a no-template negative control was included to detect cross contamination during the PCR step. The cycling of the first PCR consisted of 94 °C for 2 min of initial denaturing, followed by 35 cycles of 98 °C for 10 s, 61 °C for 30 s, 70 °C for 30 s, and 5 min at 70 °C for the final extension. Equal volumes of eight replicates of first PCR products were mixed. The merged products and negative controls were purified using AMPure XP (Beckman Coulter Inc., Brea, CA, USA) according to the manufacturer's instructions. The second PCR was performed with the same reaction solution, except that 1 μL of the first purified PCR product was used as a template for addition of adapters and indices to identify samples. Cycling conditions were as follows: initial denaturation at 94 °C for 2 min, 10 cycles of 98 °C for 10 s, 70 °C for 30 s, and final elongation at 68 °C for 5 min.

We mixed an equal volume of the products of the second PCR of each replicate to obtain a sequence library. We purified the mixture using a QIAquick PCR Purification Kit (QIAGEN) and size-selected samples using E-Gel SizeSelect II (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of the library was measured using an Agilent Bioanalyzer system with a high sensitivity DNA kit (Agilent, Santa Clara, CA, USA). After an alkaline denaturation, the library was paired-end sequenced in MiSeq (Illumina, San Diego, CA, USA) using the MiSeq Reagent Kit v2 for 300 cycles with 10% PhiX (Illumina). The nucleotide sequences obtained in this study were registered with the DNA Data Bank of Japan (accession no. DRA015652).

Each of R1 and R2 of the resulting sequences was filtered and denoised after primer removal with Cutadapt 2.8 [34]. ASV-R1 and ASV-R2 were concatenated, and chimeric removal with DADA2 [35] and filtering by length (135–210 bp) were performed. The taxonomic assignment was conducted using the database "animals_mt_species" and blast through the command "clidentseq" in Claident 2 [36]. Taxa were identified using the top three criteria with 97% or higher identity. We also performed a rarefaction curve analysis to evaluate whether our sequence depth adequately captured the fish species present in each sample using the R package iNEXT (Hsieh and others 2016).

We classified the detected fish species as native, translocated, or exotic based on the Red Lists of the Ministry Environment of Japan and Chiba Prefecture.

Results and discussion

Legacy N in spring waters

The NO_3-N concentrations of spring waters that we sampled for SF6 analysis exceeded 10 mg L^{-1} (mean 12.1 ± 1.8 mg L^{-1}, Table 1). The SF_6 EACs of these spring waters averaged 7.95 pptv, and the variabilities among replicates and among sites were small (Table 1). The average SF_6 concentration corresponded to atmospheric concentrations around the year 2010, and thus the average estimated residence time was 12.5 years (range: 9 – 14 years). These results clearly indicated that the legacy N in spring waters was high as a result of N inputs from decades of upland agricultural activity. Our findings suggest that legacy N may influence downstream water quality for decades but could also maintain lotus production under unfertilized conditions.

Table 1

Results of spring water dating using SF_6. Mean (±SD) values calculated from duplicate measurements for each spring water sample.

Springs	NO_3-N (mg/L)	SF_6 equivalent air concentration of spring waters (pptv)	Recharge year	Residence time (year)
No. 1 lotus field	10.4	8.56 ± 0.67	2011.5 ± 2.1	10.5 ± 2.1
No. 2 lotus field	11.9	7.64 ± 0.11	2008.5 ± 0.7	13.5 ± 0.7
No. 3 lotus field	13.9	7.65 ± 0.29	2008.5 ± 0.7	13.5 ± 0.7
Mean	12.1 ± 1.8	7.95 ± 0.58	2009.5 ± 1.9	12.5 ± 1.9

N removal effectiveness in lotus fields

The small, negative changes of Na^+ concentrations on all sampling dates (Fig. 2A), suggested dilution by rainfall. The dominant form of dissolved N in spring waters was NO_3-N (mean 83.2%, Fig. S2), and the NO_3-N concentration in spring water was high on all sampling dates (mean 7.64 mg L^{-1}, range: 0.14–15.4 mg L^{-1}). The NO_3-$N_{O\text{-}obs}$ was significantly lower than NO_3-$N_{O\text{-}exp}$ on all sampling dates (Table S1). The change of NO_3-N concentrations ranged from −59.3% to −99.3% (mean ± SD: −84.9 ± 12.2%) (Fig. 2B), indicating that most NO_3-N was removed even after dilution was considered. The reduction of the NO_3-N concentrations was larger in summer than in autumn. The temperature differences between spring waters and outlet waters were significantly greater in summer (Fig. 3A). NH_4-N concentration changes varied among the sampling dates (Fig. 2C). On the last date, the NH_4-N concentration at the outlet increased, but the TDN concentration decreased (Fig. 2D) because NO_3-N was the dominant form of dissolved N, and the fractions of NH_4-N, NO_2—N, and DON in TDN were small (Fig. S2).

Although we measured PON only twice, its concentration increased in the lotus fields (Fig. 2E), and the significantly higher $PON_{O\text{-}obs}$ than $PON_{O\text{-}exp}$ (Table S1) may have been due to increased phytoplankton biomass because chlorophyll-a concentrations at the outlet increased significantly (Fig. 3B). An increase of soil particles in autumn due to increased lotus harvesting might also have contributed to an increase of PON in the lotus fields, but the PON was a small fraction of the TN (Fig. S2). On both sampling dates, changes of TN concentrations were large and negative. They ranged from −52.9% to −84.0% (Fig. 2F, mean: −71.7 ± 12.6 SD%). $TN_{O\text{-}obs.}$ was significantly lower than $TN_{O\text{-}exp}$ (Table S1). These results suggested that the unfertilized lotus fields effectively reduced export of legacy N, mainly by decreasing NO_3-N.

Two mechanisms primarily explain the reductions of NO_3-N and TN concentrations in the lotus fields: denitrification and plant uptake. Denitrification in paddies and lotus fields can very effectively remove N [20,21,37–39]. Toda, et al. [40] used ^{15}N as a tracer to show that denitrification accounts for most N removal in paddy fields. In fertilized rice and lotus fields, the relative contributions of denitrification and plant uptake are ~80% and ~20%, respectively [38,41]. With regard to plant uptake, both rice and lotus absorb large amounts of N, but the uptake capacity of lotus is higher [42], and N in lotus roots is ultimately removed by harvesting. Lotus roots can also facilitate denitrification by sediment microbes and enhance N uptake by microbes in the rhizosphere [43]. Both denitrification and N uptake may decrease NO_3-N concentrations at the study site. Because water temperature strongly affects both processes [44], N removal effectiveness may increase in summer and decrease in autumn and winter.

Our meta-analysis revealed that NO_3-N concentrations decreased in all studies (Fig. 4A, Table S2). The decreases of the NO_3-N

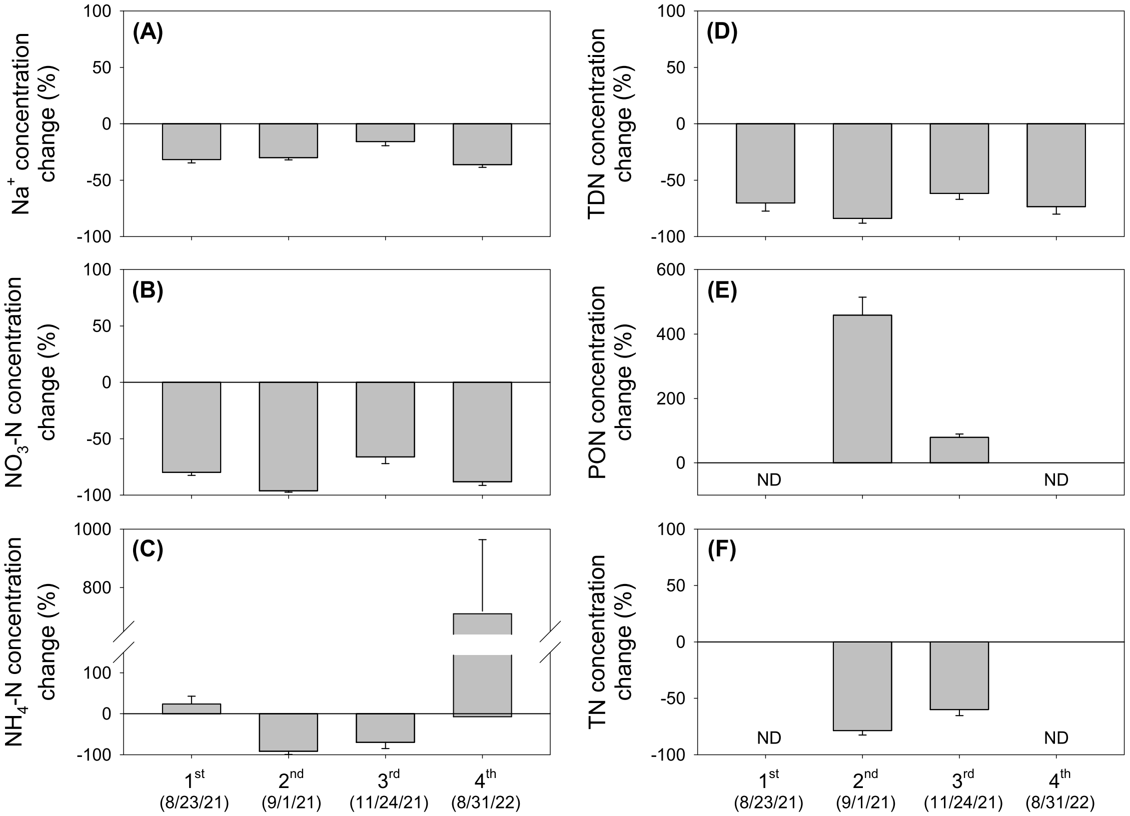

Fig. 2. The concentration changes of (A) Na$^+$, (B) NO_3-N, (C) NH_4-N, (D) total dissolved nitrogen (TDN), (E) particulate organic nitrogen (PON), and (F) total nitrogen (TN) through the unfertilized lotus fields. The calculated changes of the concentrations of NO_3-N, NH_4-N, TDN, PON, and TN took into consideration a dilution effect. Negative values mean a reduction of the concentration in outlet waters, whereas positive values mean the concentration was higher in the outlet waters. ND, no sampling data.

concentrations between the inlet and outlet were significantly greater in the unfertilized lotus fields (only data from this study) than in the fertilized and unfertilized paddy fields or the fertilized lotus field. Changes of NO_3-N concentrations in paddies did not differ between fertilized and unfertilized conditions. The decline of TN concentrations was significantly greater in unfertilized than fertilized paddy and lotus fields (Fig. 4B, Table S3). The cause of the different patterns between NO_3-N and TN could be the application of N fertilizer increases PON by stimulating phytoplankton. Alternatively, the resuspension and flushing of sediments with high N loads during paddling and transplanting could increase TN. Our meta-analysis suggested that fertilization could reduce the N removal effectiveness of paddy and lotus fields. The use of fertilizer in addition to spring water containing legacy N may therefore adversely affect N removal effectiveness.

Several possible explanations could account for the high N removal effectiveness observed in our study. First, the water residence time was longer in the lotus fields than in paddy fields supplied with water via pipeline. Long water residence times can enhance denitrification and increase water temperature [45]. Second, spring waters have high NO_3-N concentrations. Increasing NO_3-N loading facilitates denitrification [32,46]. Third, the organic matter content of surface soil in lotus fields is high (7.9 – 17.0%, Nagashima, et al. [47]; >20%, Ibaraki Kasumigaura Environmental Science Center [48]). These values are comparable to or higher than the organic matter content of paddy fields (5.5 – 14.6% [49] and 7.3% [50]). Higher soil organic matter content enhances the denitrification potential of wetlands, riparian zones, and stream channels [51,52]. Because the establishment of deep-rooted vegetation can increase the accumulation of soil organic matter [52], lotus roots may greatly enhance N removal efficiency. Although our meta-analysis might be biased because of differences in retention times, waterbody areas, water depths, and sampling designs among the studies, our results suggest that unfertilized lotus fields have an excellent ability to mitigate the export of legacy N.

The total root harvest during 2022 and the associated amount of N removal were 10.5 Mg-fresh wight (16.2 Mg/ha/year) and 38.6 kg N/ha/year, respectively (Fig. 5). The harvest amount was within the range reported for lotus fields fertilized by conventional methods in neighboring Ibaraki Prefecture (13.3 – 17.2 t/ha/year, [53]), suggesting that unfertilized fields could provide farmers with stable yields equal to those of fertilized fields and could perform better environmentally. In contrast, the N removed by harvesting was smaller than that of fertilized lotus fields (38–53 kg N/ha/year, [53]), probably because the N content of the lotus was smaller. The rate of N removal from unfertilized, non-vegetated paddy fields occurs mainly via denitrification and in Ibaraki Prefecture has been reported to range from 989 to 2235 kg N/ha/year ([21,41,54,55]). The N input via precipitation around Sakura City, Chiba Prefecture, was 5.1 kg N/ha/year in 2011 [56]. At our study site, denitrification rather than nitrate uptake could account for most of the N removal. The N loadings of similar nearby watershed systems have been reported as 419 kg N/ha/year [57] and 755 kg N/ha/year [39]. The order of magnitude of the N removal by denitrification and harvesting might be similar to that of the N loading (Fig. 5). Furthermore, in Chiba Prefecture, 260 kg N/ha/year is recommended as the standard rate of N application to lotus fields to achieve a yield of 20 Mg/ha/year. The harvest of lotus roots at our study site (16.2 Mg/ha/year) suggests that approximately 200 kg N/ha/year would have been supplied from legacy N. Although we did not measure hydrological data to estimate the N balance of the unfertilized lotus fields, our rough estimation suggests that the unfertilized lotus fields could contribute to the reduction of not only $NO_3^- N$ concentrations but also of the N discharges to downstream and to Lake Inba.

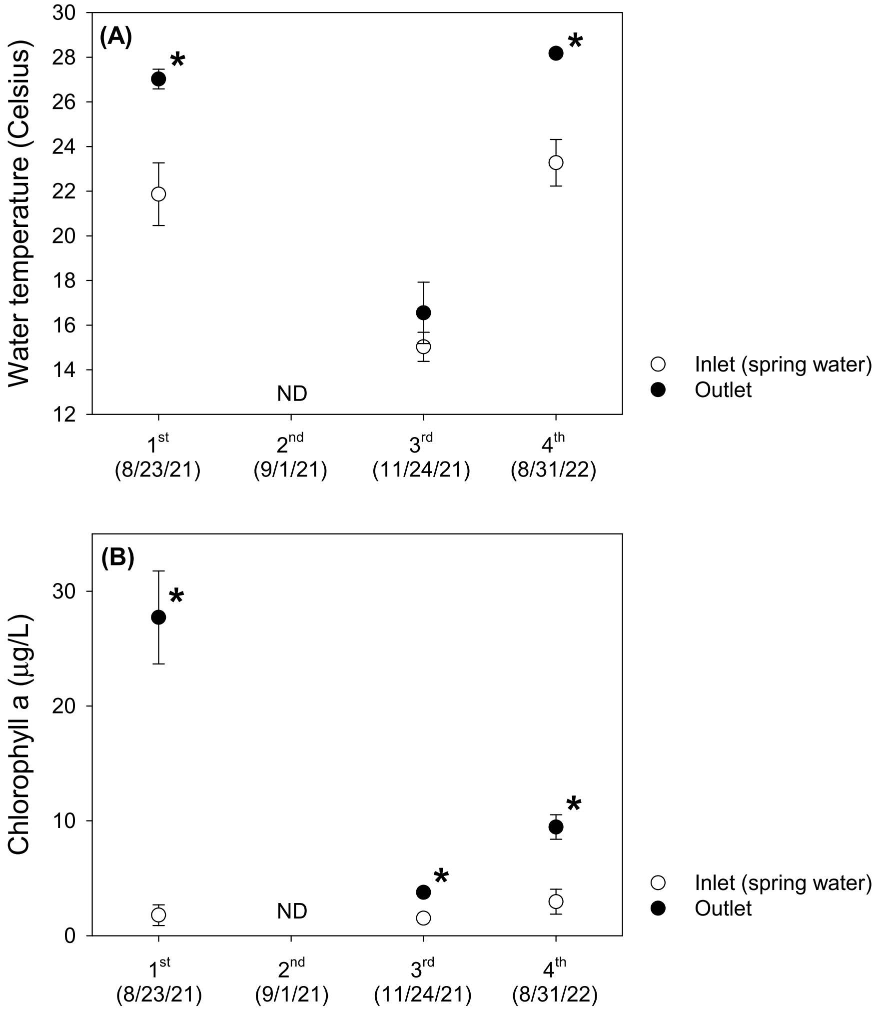

Fig. 3. (A) Surface water temperature and (B) chlorophyll-a concentration of inlet and outlet waters of the lotus fields. ND, no data. An asterisk indicates that outlet water is significantly different from inlet water (t-test, $P < 0.05$).

Fish diversity in lotus fields

Following sequencing and data filtering, a total of 6340,068 reads (mean ± SD: 520,097 ± 401,794) of 13 samples were assigned as fish (Table S4). The 1422 reads (mean ± SD: 284 ± 414) of all negative controls were approximately 0.022% of the total number of reads. We therefore judged that a species with more than 0.022% of the total number of reads per sample was positively detected by eDNA metabarcoding. Finally, the eDNA metabarcoding detected 13 fish species: 9 native species, 3 translocated species, and 1 exotic species (Table 2). Rarefaction analysis indicated that our sequencing depth was sufficient to identify all or nearly all of the amplified fish species present in the study area (Fig. S3).

We detected nine native fish species in the adjacent stream (Table 2). The richness of native fish species in the unfertilized lotus fields ranged from two to six species, but *Pseudorasbora parva*, which is a near-threatened species of the Chiba Prefectural Red List, and *Misgurnus anguillicaudatus* sp1 and sp2, which are listed as near-threatened species in the National Red List, appeared in almost all lotus fields. Two other Red List Cobitidae species, *Misgurnus* sp. Clade A and *Cobitis* sp. BIWAE type C, were detected in all the unfertilized lotus fields. These results suggest that lotus fields as well as rice paddies may be suitable habitats for Cobitidae. One remarkable result was that two endangered species, *Lefua echigonia* and *Oryzias latipes*, appeared in one and two unfertilized lotus fields, respectively. *Lefua echigonia* is known to be spring-dependent and to prefer shallow water with low and constant flow velocities [19]. *Oryzias latipes* requires both spawning/nursery habitats (such as paddy fields) and overwintering habitat (such as shallow earth-banked ditches), and is considered to be vulnerable to recent modernization and abandonment of paddy fields [58]. On the same date, we observed spring-dependent Japanese freshwater crab, *Geothelphusa dehaani*, and common kingfisher, *Alcedo atthis bengalensis*, which are registered as vulnerable species in the Chiba Prefectural Red List. Our results suggest that the unfertilized lotus fields may have a moderate biodiversity conservation function as well as a N removal function.

In lowland paddy fields, modern irrigation/drainage systems to increase production efficiency have serious impacts on fish diversity [22, 23,59,60]. The loss of connectivity especially between paddy fields and ditches or streams has seriously prevented upstream migration of fish from ditches to paddy fields and decreased their abundance, because paddies provide spawning habitats and supply some unique food sources [23,60]. Small gaps exist between lotus fields and streams, meaning that fish may move between them only during heavy rains or flooding. Nevertheless, the native fish fauna in unfertilized lotus fields and adjacent streams were similar. Abandonment of rice paddies has reduced fish and amphibian abundances through reduction of wet paddy area and vegetation succession [61]. Irrigation of the unfertilized lotus fields with spring water throughout the year maintains wet conditions. The lotus fields may therefore provide temporary but complementary foraging, spawning, and refuge habitats for fishes.

One concern was that two translocated fish species (*Candidia sieboldii, Gnathopogon elongatus*) and one exotic species (*Gambusia affinis*)

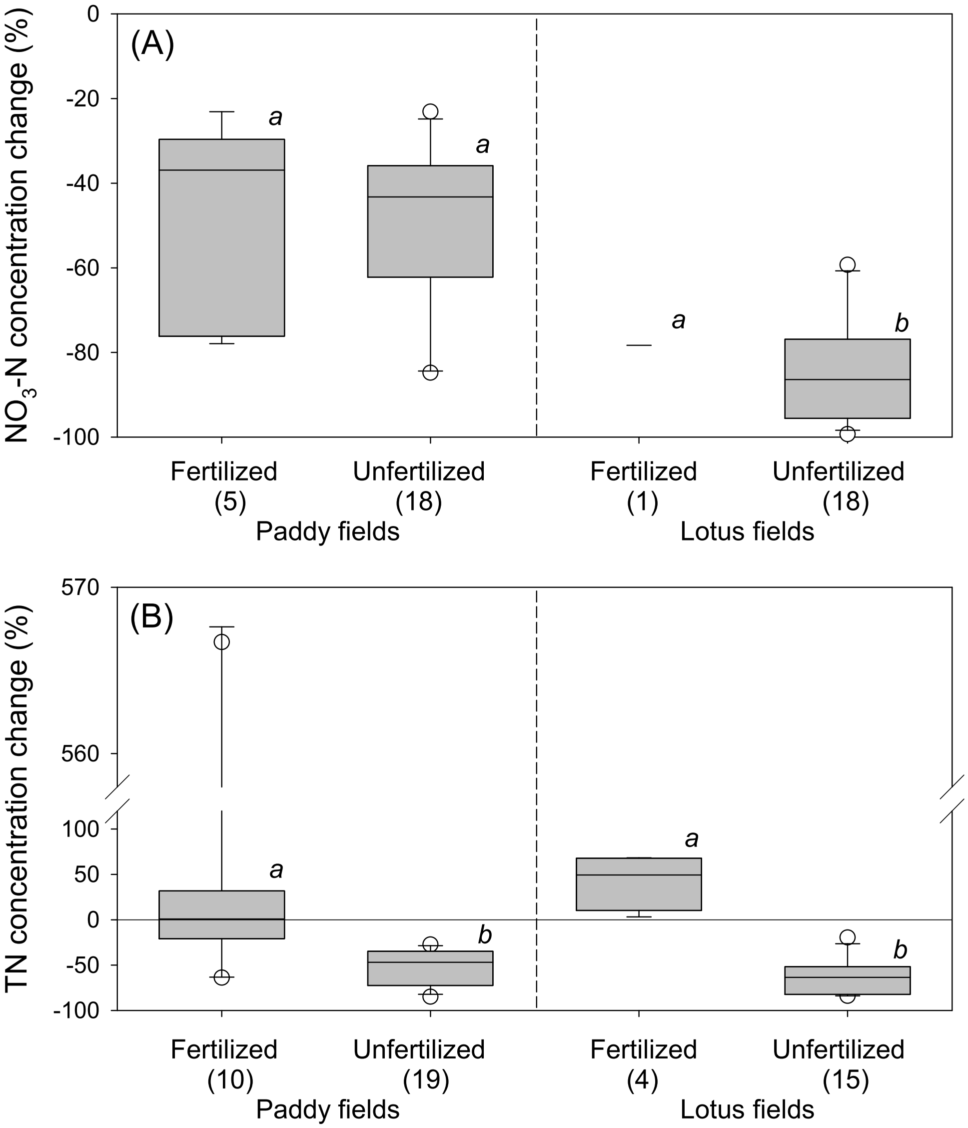

Fig. 4. Results of the meta-analysis and our study showing the average concentration changes of NO_3-N (A) and total nitrogen (TN) (B) through the fertilized and unfertilized rice paddy fields and lotus fields or ponds. Numbers in parentheses indicate sample size. The results of this study are included among the results from the unfertilized lotus fields (NO_3-N, n = 18; TN, n = 8). Please note that the concentration changes of NO_3-N in a unfertilized lotus fields are from only our study. Different letters above bars indicate significant differences ($P <$ 0.05).

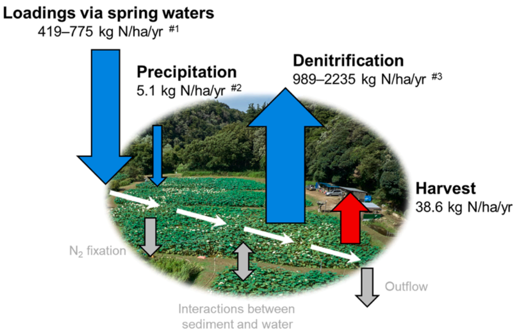

Fig. 5. N budget of the unfertilized lotus fields based on our measurements (red arrow) and the estimates from the literature (blue arrows, #1, Refs. 39 and 57; #2, Ref. 56; #3, Refs. 21,41,54, and 55). Gray arrows indicate fluxes that were not evaluated in this study.

were detected in the lotus fields but not in the stream (Table 2). Although these species are not considered to directly affect native species and louts through consumption, *G. affinis* can negatively affect *O. latipes* through predation, aggression, and competition for food [62]. Our findings suggest that there is reason for concern that unfertilized lotus fields might become a hotbed of translocated and exotic species.

Limitations

There are at least three limitations of our work. First, our findings are based on snapshot surveys. Future studies should investigate water quality at least monthly, especially during late spring when puddling and lotus planting can maximize nutrient loading, as well as during rainy seasons when runoff is high [37,63]. A single eDNA survey is also insufficient to elucidate the importance of lotus fields as temporary or permanent habitats. Repeated and seasonal eDNA sampling that includes a greater variety of organisms is needed to assess whether unfertilized lotus fields are priority sites for conservation of local biodiversity. Second, we lacked hydrological data and did not quantify the N budget from the lotus fields. Because extreme precipitation events are becoming more frequent and intense [8], future studies should calculate monthly and annual N fluxes to provide more useful and quantitative information for integrated watershed managements. Such studies will require high-frequency water sampling (e.g., hourly) with an autosampler and hydrological data collected with a data logger. Third, revealing the importance and uniqueness of lotus fields in watersheds will require further comparisons with other lotus and paddy fields as well as restored wetlands. Although our meta-analysis suggests

Table 2
Results of the metabarcoding of eDNA samples collected from the six lotus fields and the adjacent stream. X denotes presence. IB or B, endangered; II or C, vulerable; NT or D, near treatened; DD, data deficient.

	Family	Species name	Red List status		Lotus field						Stream
			Ministry of Environment (ver. 2020)	Chiba Prefecture (ver.2019)	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	
Native species	Cyprinidae	*Pseudorasbora parva*		D	X	X	X	X	X	X	X
		Tanakia lanceolata	NT	B							X
	Cobitinae	*Misgurnus anguillicaudatus* sp1	NT		X	X	X	X	X	X	X
		Misgurnus anguillicaudatus sp2	NT			X	X	X	X	X	X
		Misgrunus sp. Clade A	DD			X		X		X	X
		Cobitis sp. BIWAE type C		C						X	X
	Noemacheilidae	*Lefua echigonia*	IB	C		X					X
	Adrianichthyidae	*Oryzias latipes*	II	B		X				X	X
	Gobiidae	*Rhinogobius* sp.									X
Translocated species	Cyprinidae	*Candidia sieboldii*					X				
		Gnathopogon elongatus			X		X	X	X	X	
		Cyprinus carpio / Carassius sp.						X			X
Exotic species	Poeciliidae	*Gambusia affinis*			X	X		X	X	X	

a difference of N removal effectiveness between paddy fields and lotus fields (Fig. 4), further research is necessary to quantitatively and simultaneously compare the functions of N removal and biodiversity conservation as well as other functions (e.g., flood control) among those fields.

Although this study focused on the N removal function of unfertilized lotus fields and their role in mitigating the effects of legacy N, it is worth testing the hypothesis that the unfertilized lotus fields can remove P, another key element that causes eutrophication, as a co-benefit of the removal of N. While PO_4-P concentrations decreased in outlet waters on all sampling dates, DOP concentrations increased in outlet waters (Appendix S2). The conversion of dissolved, inorganic to organic P results from the use of PO_4-P by phytoplankton, periphyton, and lotus. However, concentrations of dissolved total P (DTP) (i.e., PO_4-P + DOP) decreased between the inlet and outlet during three of four samplings (Appendix S2). The reduction or a slight increase of DTP suggests that the unfertilized lotus fields might not be a significant source of P, at least during spring and summer base flow. Considering that DOP is generally less bioavailable than PO_4-P, reduction of PO_4-P could mitigate the effect of P on downstream water quality. However, because our study was based on snapshot samples and we did not analyze particulate P, a more intensive and detailed survey of P is obviously required.

Management implications and conclusions

Our results suggest novel management perspectives for drawing-down existing legacy N and facilitating organic farming. Unlike wetlands, lotus fields provide farmers with an opportunity to produce a crop while reducing legacy N and sustaining biodiversity. In 2020, the Satoyama-Renkon Farm yielded ~6 tonnes of lotus, corresponding to 1.1 kg m^{-2}, which is ~65% of the average lotus yield by conventional farming methods (1.7 kg m^{-2}, Ibaraki Kasumigaura Environmental Science Center [64]). However, the Satoyama-Renkon Farm sells organic lotus for a premium price based on the added value associated with food safety and environmental conservation, and such premium prices could compensate for lower yields. Exploring alternative crops may be needed to diversify farmer income and incentives. For example, Uezono [65] reported that taro (*Colocasia esculenta*) can be cultivated in a wetland and is capable of removing N through uptake.

Our study highlights problems with modern irrigation systems. Water is typically supplied through underground pipes via taps and spring waters is directly drained into irrigation ditches. Legacy N thus flows downstream, and the high N removal function of paddy fields is not exploited. Although transition to unfertilized lotus cultivation is a practical option, management should encourage discharging spring water to modern and abandoned rice paddies to mitigate the effects of legacy N at a larger scale. Such a policy could help to restore local freshwater biodiversity, because many aquatic organisms have suffered from agricultural modernization and abandonment [25,66]. Broad-scale dating of spring water may help to identify and prioritize sites to manage legacy N. The sites with longer residence times and higher nitrate concentration should be given high propriety for controlling the export of legacy N.

Although we detected several exotic and translocated fish species in the unfertilized lotus field, more nuisance species, including red swamp crayfish (*Procambarus clarkii*), red-eared slider turtles (*Trachemys scripta elegans*), and common snapping turtles (*Chelydra serpentina*) have invaded. Red swamp crayfish and red-eared slider turtles are known to consume lotus directly and adversely impact macroinvertebrates, which are important prey for fishes [67]. Common snapping turtles pose a risk of injury to humans and are a threat to native biodiversity. These invasions are obstacles to maintaining lotus production without chemicals. To sustain lotus production in unfertilized and pesticide-free conditions, management strategies that continuously remove these invasive species (e.g., occasional drying, setting up fences) should be incorporated.

Sowińska-Świerkosz and García [68] have proposed that NbS actions should be interventions that: (1) are inspired and powered by nature; (2) address (societal) challenges or resolve problems; (3) provide multiple services/benefits, including increased biodiversity; and (4) are highly effective and economically efficient. Here, we discuss how our example fits the four fundamental criteria. Because unfertilized lotus fields improve water quality and maintain fish diversity by harnessing wetland's ecosystem functions, our study site met criteria 1 and 3 (Fig. 6). These benefits could be extended to adjacent waterbodies by restoring and maintaining connectivity. The unfertilized lotus fields could provide other ecosystem services, including flood control and provision of habitat for other aquatic species. The IUCN Global Standard for NbS concerns seven societal challenges [69], and the unfertilized lotus fields provided services that addressed at least three of the seven−water security, food security, and human health−, because they could mitigate the tradeoff between crop production and water quality, and the lotus were cultivated without fertilizers and pesticides. Because the unfertilized lotus is currently sold at a premium price, the unfertilized lotus

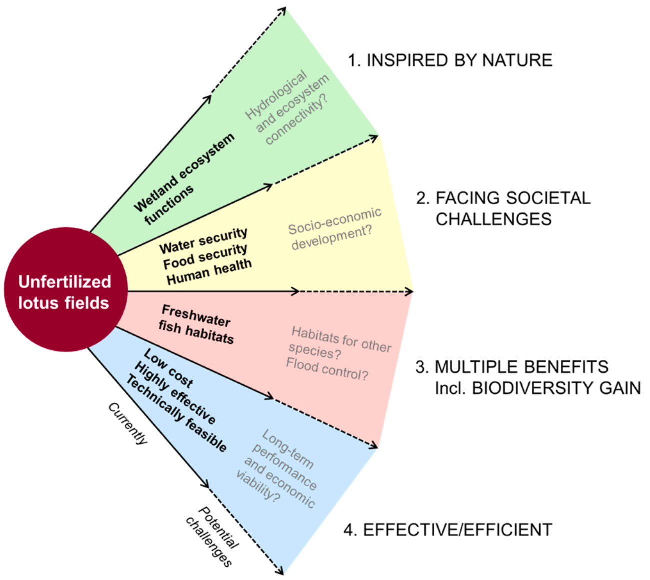

Fig. 6. Schematic overview of the current and potential benefits from the unfertilized lotus fields relative to the four fundamental criteria of NbS [Ref. 68].

fields could potentially stimulate socioeconomic developments, and our study site therefore met criterion 2 (Fig. 6). Our study suggested that the unfertilized lotus fields were highly effective in reducing the legacy N discharges downstream. A conversion from abandoned paddy fields to lotus fields is technically feasible, and the initial cost would be relatively low. Although challenges must be met to ensure long-term ecosystem performance and economic viability, our study site fulfilled criteria 4 (Fig. 6). This assessment suggests that although this was a small case study, our study could be a good example of NbS to harness ecosystem functions. Our findings might generate more creative options that fundamentally change the relationship between agriculture and the environment and inspire novel scenarios that could serve as radical alternative visions for a positive and sustainable future world [70].

Author contributions

S.S.M., A.K., M.W., and A.T. designed this study and conducted the field sampling. A.K. and M.W. analyzed water chemistry and N.I.K. conducted eDNA analyses. S.S.M conducted meta-analysis and drafted the manuscript. All the authors discussed the results and edited the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nbsj.2023.100080.

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