



Role of carbon and nutrient exports from different land uses in the aquatic carbon sequestration and eutrophication process



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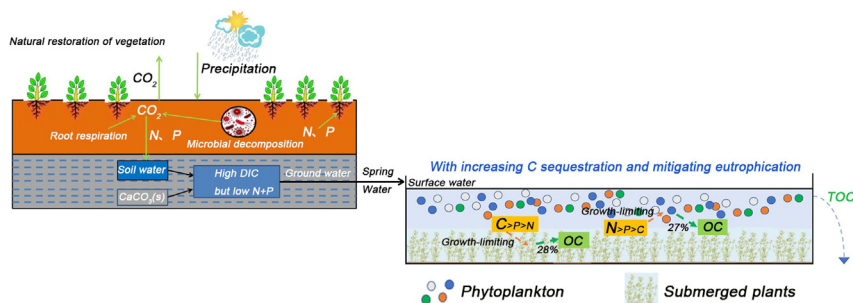
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HIGHLIGHTS

- Phytoplanktons are co-limited by N and P, but submerged plants are affected by C.
- Chlorophyta and Bacillariophyta have competitive advantages in waters with high C/N.
- Submerged plants in karst waters (with high pH) are prone to C limitation.
- Phytoplanktons and submerged plants contribute similar OC to the water.
- Natural vegetation restoration mitigates eutrophication and increases the carbon sink.

GRAPHICAL ABSTRACT



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ABSTRACT

The hydrochemical features affected by differing land uses play a key role in regulating both the primary production of aquatic photosynthetic organisms and the formation of autochthonous organic carbon (AOC); this impacts eutrophication and the global carbon cycle. In shallow water environments where phytoplankton and submerged plants coexist, the C-N-P limitations on the primary production of these aquatic organisms, and the mechanisms by which they promote the formation of AOC are poorly understood. In this study, over the hydrological year September 2018 to August 2019, a large-scale field simulation experiment at the Shawan Karst Test Site (SW China) with various types of land use was systematically conducted to investigate the C-N-P limitations on the primary production of phytoplankton and submerged plants. The results indicate that (1) phytoplankton are co-limited by nitrogen (N) and phosphorus (P) but with the N more important, while submerged plants are limited by carbon (C); (2) Chlorophyta and Bacillariophyta display a stronger competitive advantage than Cyanophyta in aqueous environments with high C but low N-P; (3) there is a seasonal difference in the contribution of phytoplankton and submerged plants to the formation of AOC, however, throughout the year, the contributions of phytoplankton (27%) and submerged plants biomass (28%) to AOC concentrations in the water were similar, combinedly accounting for approximately 17% of the formed AOC. It is concluded that natural restoration of vegetation, or injecting CO₂ into water, which results in higher C but lower N-P loadings, may simultaneously help to mitigate eutrophication (with changes in biological structure and species) and increase C sequestration in surface waters.

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1. Introduction

Eutrophication of inland waters has become a serious environmental problem worldwide (Lin et al., 2020). The increase in human activities promotes the discharge of a large quantity of nutrients (such as N and P) into the water, and blue algae blooming in the water causes eutrophication (Schindler, 1974; Lin et al., 2021). In the eutrophic waters, water transparency and dissolved oxygen are reduced, and algae residues decompose a large amount of greenhouse gas and toxic substances, seriously damaging human health when consumed and the survival of aquatic animals (Jiang et al., 2014; Li et al., 2021). Previous studies have found that carbon sinks in eutrophic lakes are increased, so it is believed that carbon sequestration and eutrophication are linked (Anderson et al., 2014; Huang et al., 2017). However, in the study of the eutrophication problem in Azores archipelago, the researchers proposed afforestation, but they did not integrate with carbon sinks (Cruz et al., 2015). Moreover, the researchers pointed out focusing river eutrophication management only on nutrients may not be effective if the interactions of nutrients, algae, and macrophyte with DIC are neglected (Kajiser et al., 2021).

Land use change is one of the main subjects of research on global change, and has important impacts on biogeochemical cycles (e.g., carbon-C, nitrogen-N and phosphorus-P) in watersheds (Luo et al., 2020; Tanaka et al., 2021). Changes of C, N and P in water have significant effects on the growth of aquatic photosynthetic organisms (Hayes et al., 2015; Wang et al., 2020). Many studies have contended that P is the principal factor limiting the growth of phytoplankton biomass (Schindler, 1974; Schindler et al., 2016), while others propose that N is more important (Howarth and Marino, 2006; Ko et al., 2020). Additionally, further studies consider the concentration of inorganic C to be extremely important (Riebesell et al., 1993; Zeng et al., 2019; Wang et al., 2020). For example, in the research on Sete Cidades volcanic lake, the researchers observed that the biomass of phytoplankton and macrophytes was greater in waters with higher CO₂ concentrations (Andrade et al., 2021). It thus appears that C, N, and P may all act as key factors limiting the growth of aquatic photosynthetic organisms, depending on differing environmental conditions (Maberly and Madsen, 2002; Bao et al., 2020). Human-induced increases in N and P production in watersheds has led to the harmful algal blooms dominated by cyanobacteria (Howarth and Marino, 2006; Jiang et al., 2014). Nevertheless, since Cyanophyta generally have a variety of pathways to use inorganic C, Chlorophyta or Bacillariophyta in water with higher dissolved inorganic carbon (DIC) concentrations may have relatively stronger competitive advantages (Reinfeldt, 2011; Low-Décarie et al., 2015). Furthermore, changes in the concentrations of inorganic C and nutrients in water also significantly impact the growth of submerged plants (Dülger et al., 2017). Overall, the changes in C, N, and P concentrations induced or mediated by differing land uses may affect the biomass of aquatic photosynthetic organisms (e.g., phytoplankton and submerged plants), and regulate the evolution of biological structure (Riebesell et al., 1993; Hayes et al., 2015; Su et al., 2016; Bao et al., 2020). Previous studies found that construction and cultivated lands were main factors that caused TN and TP increases in lakes and promoted the bloom of Cyanophyta, while the high-C, low-N and P aqueous environment formed by natural vegetation restoration is conducive for inhibiting the Cyanophyta growth (Bao et al., 2020; Peng et al., 2021). Moreover, natural vegetation restoration reduces the output of N and P and increases the output of DIC in the watershed, potentially promoting the growth of submerged plants and greatly improving water quality (Cruz et al., 2015; Dülger et al., 2017). Therefore, we believe that changes in the algae community structure and the restoration of submerged plants have the potential to improve the aqueous environment (Liu et al., 2018a; Bao et al., 2020).

The absorption of CO₂ from the atmosphere and soil to form DIC during the process of carbonate rock dissolution has long been regarded as a short-term carbon sink (Liu and Zhao, 2000). However, due to the

photosynthetic activity of aquatic organisms, DIC can be transformed into autochthonous organic C (AOC), forming a more stable and long-term carbon sink by OC sedimentation. This provides a new perspective in the search for the global missing carbon sink (Liu et al., 2018b). Previous studies show that increased concentrations of DIC in water promote planktonic algae and submerged plant biomass (Jones et al., 2002; Verspagen et al., 2014; Wang et al., 2020). Moreover, statistically significant positive correlations between AOC and DIC in karst waters have been confirmed in numerous investigations (Yang et al., 2016; Chen et al., 2017; Zeng et al., 2019). Therefore, we hypothesize that in karst areas, the output of C, N, and P can be regulated by different land uses, affecting the structure of phytoplankton in surface waters and OC formation, thus encouraging carbon sink enhancement and eutrophication mitigation (Zeng et al., 2019; Bao et al., 2020).

In the present study, we conducted monthly sampling from September 2018 to August 2019 in five simulated spring-pond systems with five different land uses, using five separate experimental tanks (artificial hydrological basins) at the Shawan Karst Test Site, Guizhou Province, China. The following questions were systematically investigated: (1) Are there significant differences in the C, N and P output under different land uses? (2) How does the biomass of phytoplankton and submerged plant biomass respond to these differences? (3) What are the possible causes of AOC formation in surface water under various types of land use? Combining the results obtained from addressing these three questions, we aim to verify our hypothesis that natural restoration of vegetation, which results in higher C and lower N-P loadings, may simultaneously help to mitigate eutrophication (by changing biological structure and species) and increase C sequestration in surface waters.

2. Materials and methods

2.1. Description of the study site

The Shawan Karst Test Site (26°14'–26°15'N, 105°42'–105°43'E, at 1200 m above sea level) is located in the Puding Comprehensive Karst Research and Experimental Station, Puding County, Guizhou Province, SW China (Fig. 1a). This area has a typical humid subtropical monsoonal climate with a mean annual air temperature of 15.2 °C, mean annual precipitation of 1341 mm (80% of which occurs during the rainy season, May–October) and a mean annual relative humidity of 78% (Hu et al., 2020). Five concrete tanks were installed for simulating natural watersheds, with identical hydrogeological conditions (bedrocks and watershed boundaries) but different land covers: bare-rock, bare-soil, cultivated, grass, and shrub lands (Fig. 1b). Each concrete tank was 20 m long, 5 m wide, 3 m deep, and coated with epoxy resin to avoid the influence of possible concrete dissolution on the tank hydrochemistry. Each tank was partially filled with 2 m of dolomitic limestone gravel, followed by 0.5 m of soil. Details of the chemical composition of the rock and soil materials can be found in Zeng et al. (2017). The first tank was filled with only gravel, to simulate strictly bare-rock conditions. The second tank contained 0.5 m of local soil added to simulate bare-soil (e.g., freshly plowed) conditions. The third tank contained the same gravel and soil, and was planted with corn. The corn was cultivated from April to August, with compound fertilizer (N + P + K ≥ 45%, 0.01 kg m⁻²) being applied during the growth. The remaining two tanks were planted with alfalfa and Roxburgh roses, respectively, in January 2014, simulating perennial grasslands and shrub lands, and maintained thereafter (Zeng and Liu, 2013). Each tank was drained using a tap at the end to simulate a natural karst spring (S1–S5, Fig. 1b, Fig. S1) feeding an artificial pond (P1–P5, Fig. 1c). The ponds, representing natural lakes or reservoirs, were 3 m long, 0.5 m wide, and 0.5 m deep (Fig. 1c). Each tank and its connected pond system (Fig. 1b, c) simulated a single land use catchment of precisely known, identical dimensions and the same climatic and hydrogeological conditions. At the end of August 2018, phytoplankton (4 L river water to each

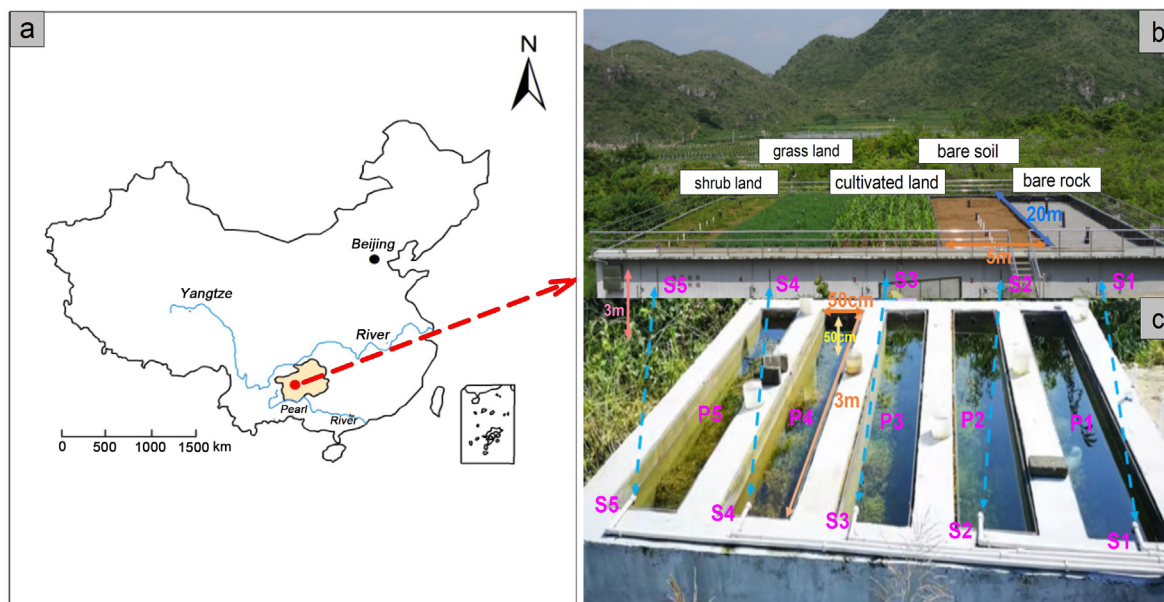


Fig. 1. (a) Location of the Shawan Karst Test Site; (b) The five tank watersheds with their different land uses: bare rock, bare soil, cultivated land (corn), grass land (alfalfa), and shrub land (Roxburgh roses); (c) The five associated spring-fed ponds (S1-P1 to S5-P5).

pond) and the aquatic plant *Chara* spp., a submerged plant (Van den Berg et al., 1997), from a nearby river were transplanted uniformly into the five sampling ponds (18 kg to each pond). Moreover, before planting submerged plants, each pond was evenly placed with 1 kg of sediment (from their native environment with the relevant nutritional information showed in Table S1) for promoting their growth. At the beginning of September 2018, we observed that the *Chara* spp. had survived and conducted our experiment.

2.2. Field monitoring

The fieldwork was carried out in the tanks on a sunny day at the end of each month, with the exception of July 2019 when there was no sampling due to instrument repair. T, pH, electrical conductivity (EC, 25 °C), and dissolved oxygen (DO) were monitored using a multi-parameter water quality probe (Wissenschaftlich-Technische Werkstaetten Technology Multiline 350i, Germany) (Bao et al., 2020). We calibrated the instrument before each test,

The meters were calibrated prior to deployment using pH (6.86 and 10), EC (1412 $\mu\text{S cm}^{-1}$), and DO (100%) standards. The measuring accuracies of the device for the pH, T, DO, and EC were 0.01, 0.01 °C, 0.01 mg L^{-1} , and 0.01 $\mu\text{S cm}^{-1}$, respectively. In addition, DIC (chiefly in the form of HCO_3^-) was titrated in the field, using an Aquamerck alkalinity test kit (Merck, Germany) with an estimated accuracy of 0.05 mmol L^{-1} (Zeng et al., 2017). Using the field measured temperature, pH and alkalinity, we used Phreeqc to calculate the dissolved CO_2 (aq) concentration in the water.

2.3. Sample collection and laboratory measurements

Each initial water sample was taken in washed 100 mL glass bottles for determining of total nitrogen (TN), total phosphorus (TP), and filtered through 0.45 μm filter membranes for determining NO_3^- and PO_4^{3-} and tested in three repetitions. The spring and pond water samples were obtained from the spring outlet, and from the outlet at the end of the pond, respectively. In the laboratory, samples were stored at 4 °C and all tests were completed within two days. The concentrations of TN, TP, and PO_4^{3-} were measured by colorimetry, and the NO_3^- concentrations were measured by ion chromatography (Van Veldhoven and Mannaerts, 1987; Bao et al., 2020). A total of

1.8 L water from the middle of each pond was drawn into a plastic bottle that was washed with pure water thrice; a 0.45 μm membrane was used for filtration. 95% solutions of ethanol heated to 80 °C were used for extraction to measure the concentration of Chl-a; with three repetitions (Sartory and Grobbelaar, 1984).

Phytoplankton samples for qualitative study were collected directly from the pond waters by using a plankton net (mesh size = 0.064 mm) in a ∞ shape, and then placed in 50 mL centrifuge tubes with two drops of Lugol's solution added. Individual specimens were then identified at the species/genus level using an inverted microscope. At the end of November 2018 and May 2019, we used 10 cm \times 10 cm aluminum sheets placed vertically down from the water surface for collecting all the stems and leaves of submerged plants in the pond (without roots); this process was repeated thrice. Each sample was then oven dried (110 °C for 2 h and 70 °C for 24 h) and the dry weight per unit area at the time of planting was subtracted for calculating plant biomass (the net dry weight per unit area) in each pond. In May 2019, the fresh leaves in each pond were collected, stored in liquid nitrogen, and then taken to the laboratory where 1.0 g of plant sample (enough for 3 repetitions) was placed in a pre-cooled mortar with liquid nitrogen to assist grinding; 5 mL of barbital buffer (20 mmol L^{-1} , pH = 8.3) was added and mixed well. The solution was centrifuged at 16,000 rpm for 15 min, and the supernatant was collected and refrigerated for testing. The pH meter method was used to detect the supernatant and calculate the Carbonic anhydrase (CA) activity; detailed steps are provided in Li et al. (2011). Simultaneously, another plant sample in each pond were collected, after drying was soaked in 2 mol L^{-1} hydrochloric acid, washed with ultrapure water until the pH was approximately 7, and freeze-dried, for measuring C/N and stable carbon isotope ratios. The C/N of submerged plants was measured using Elementar Vario Macro cube (Elementar, Germany). The stable carbon isotope ratios ($\delta^{13}\text{C}$) of submerged plants and DIC in the water were analyzed using MAT-252/253 (Thermo Fisher Scientific Inc., America) mass spectrometer (He et al., 2020).

2.4. Data analyses

OriginPro2018 was used for correlation analysis and the figure drawing. For comparing the differences in C, N, and P concentrations in leachate associated with various types of land use and the biomass

of aquatic photosynthetic organisms in the corresponding sample ponds, we used the IBM SPSS Statistics 24 to perform one-way ANOVA with LSD test, after passing the normal distribution test and homogeneity test of variances ($P > 0.05$). For response data with just one variable (AOC), only the linear model is applicable. Therefore, we used redundancy analysis (RDA) to analyze the variation of the biomass of phytoplankton and submerged plants (explanatory variables) on OC formation (response variable). Since there is no order of magnitude difference in data of biomass and AOC, we did not perform logarithmic conversion on biomass data. Then, variables (including Chl-a, plant biomass) were used as explanatory variables, and OC formed in pond water were used as response variables. Moreover, we performed collinearity and inflation factor analysis. A forward selection procedure (499 permutations) was performed to select biomass parameters and the ordination axis eigenvalues and variation of total explanation results of RDA analysis showed in Table S2. Then, the variation partitioning method was used to evaluate the relative influence of biomass variables. Both RDA analysis and variance decomposition were performed using Canoco 5.0 software (Peng et al., 2021).

3. Results

3.1. Spatial and seasonal physiochemical changes of the spring waters and pond waters

The physiochemical features of the five springs displayed both monthly and seasonal variations (Fig. 2). The HCO_3^- concentrations ranged between 91.50 mg L^{-1} and 305.00 mg L^{-1} (Fig. 2 a1–e1), with the minimum in winter, and the maximum in summer. The CO_2 (aq) concentrations varied from 0.04 mg L^{-1} to 18.21 mg L^{-1} with the maximum in autumn, and the minimum in spring (Fig. 2 a1–e1). This is because the biomass of the overlying vegetation reaches a maximum value in autumn and more CO_2 produced in the soil enters the groundwater, further promoting the dissolution of the bedrock (Zeng et al., 2017). TN concentrations ranged between 0.37 mg L^{-1} and 4.97 mg L^{-1} , being generally highest in S3 (Fig. 2 a2–e2). The NO_3^- concentrations showed the same trend as TN, with the maximum and minimum values in winter and spring, respectively. The TP concentrations varied from 0.0031 mg L^{-1} to 0.0116 mg L^{-1} , being generally higher in S2 and S3 (Fig. 2 a3–e3). The PO_4^{3-} concentrations showed the same behavior as TP, with the maximum and minimum values in winter and spring, respectively.

The physiochemical features of the five pond waters also indicated monthly and seasonal variations (Fig. 2). The concentrations of HCO_3^- , CO_2 (aq), TN, TP, NO_3^- , PO_4^{3-} in pond waters were generally lower than those in the springs. Overall, there were significant differences ($P < 0.05$) in the C, N and P concentrations of the output water under different land uses: the shrubs and grasslands produced aqueous outputs with high C and low N and P concentrations, the cultivated land produced an output with medium C and high N and P, while the bare soil and bare rock produced low C and medium N and P concentrations (Fig. S2).

There were significant differences in the temperatures, pH, DO, and EC between the springs and the pond waters (Fig. S3). The temperatures of the spring waters were more stable than those of the pond waters throughout the year. The pH in each pond was generally higher than that in the corresponding spring (Fig. S3 a1–e1). DO showed the same trend as pH (Fig. S3 a2–e2). EC displayed the opposite trend to pH and DO, the pond waters being generally lower than spring waters (Fig. S3 a3–e3), especially in summer due to their photosynthetic consumption of DIC (Chen et al., 2017).

3.2. The dynamics of Chl-a and phytoplankton species composition in the pond waters

The Chl-a concentration ranged between $0.04 \mu\text{g L}^{-1}$ and $4.45 \mu\text{g L}^{-1}$, and showed significant monthly changes (Fig. 3a). From September to October 2018, the phytoplankton showed a declining trend, with the Chl-a concentration in the P2 and P3 pond waters relatively high, and the lowest observed in P4. From November 2018 to February 2019, the phytoplankton was dormant and the Chl-a concentration was generally low. From March to August 2019, the algae grew rapidly, with Chl-a concentration gradually increasing each month. P3 displayed the highest Chl-a concentration at all times in the study except August 2019, when P2 surpassed it.

The changes of phytoplankton species composition in the five ponds are shown in Fig. 3b. Bacillariophyta, and Chlorophyta, especially Bacillariophyta, became the dominant group in all five ponds. From January to March 2019, the proportion of Cyanophyta increased, but remained low in P4 and P5. Moreover, in P3 (with medium C, high N and P) and P1 (low C, medium N and P), the proportion of Cyanophyta (21%) evidently increased.

3.3. Biomass, CA activity, and C/N and carbon isotope of submerged plant-Chara spp.

During September to November 2018 there were significant differences in the plant biomass of *Chara* spp. growth among the five ponds. Biomasses in P4, P5, and P3 were significantly higher than in P1 and P2 (Fig. 4a). There were also significant differences in plant biomass among the five ponds from March to May 2019, with that in P4 significantly exceeding those in P5 and P3, which significantly presented higher values than those in P1 and P2. It is noteworthy that the concentrations of DIC in S4, S3 and S5 were found to be generally higher than those in S2 and S1.

In May 2019, the CA activity of the new leaves of submerged *Chara* spp. in the five ponds also showed significant differences (Fig. 4b). The highest CA activity was $4.62 \text{ (U (g mL}^{-1}))$ in P1 and the lowest was $0.16 \text{ (U (g mL}^{-1}))$ in P4. CA activities in P1, P2 and P3 were significantly higher than in P4 and P5.

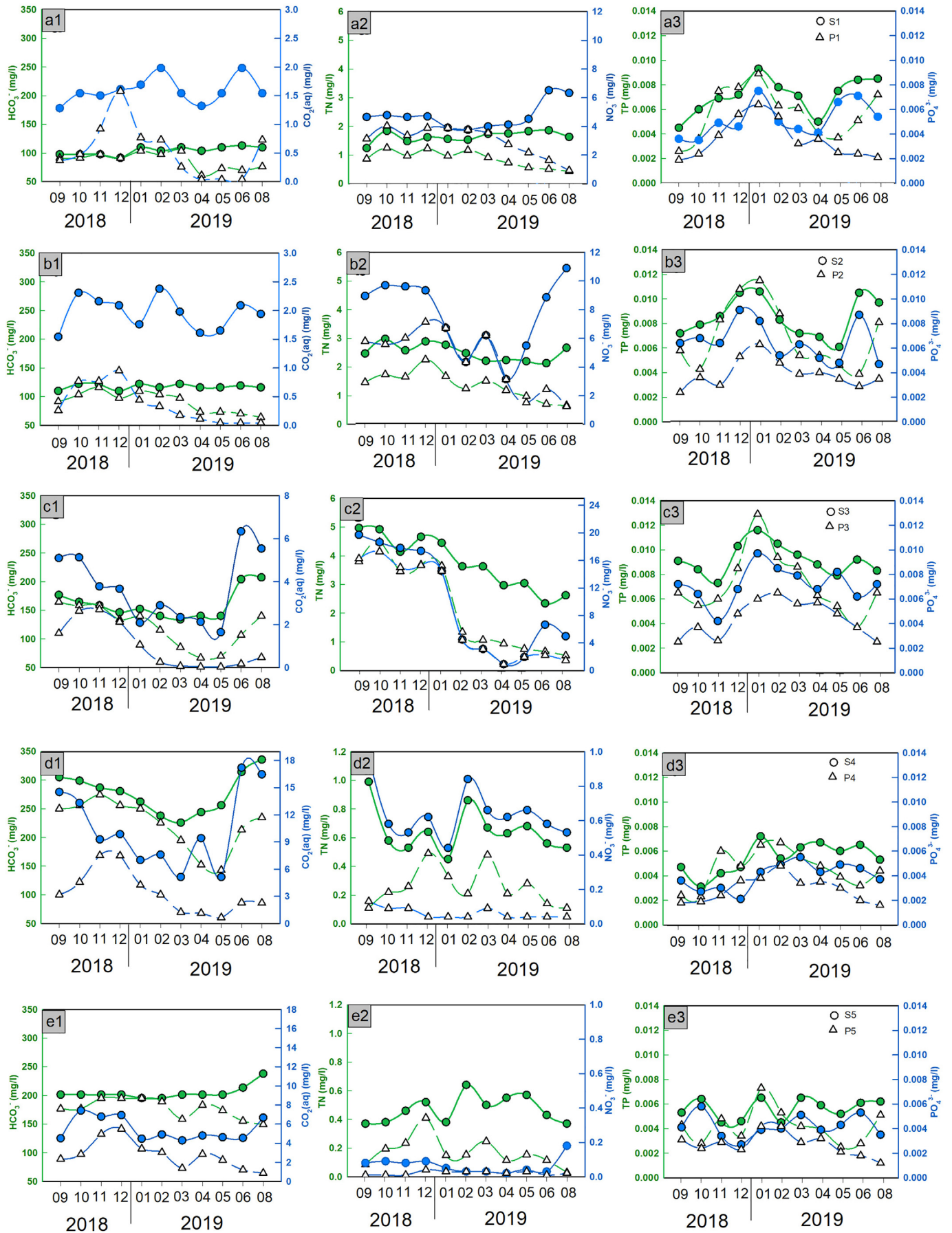
The C/N and carbon isotope data for new leaves are shown in Fig. 4c. The C/N ratios in November 2018 and May 2019 show the same trend, ranging from 13.93 to 25.68. The C/N ratios showed a trend of $\text{P5} > \text{P4} > \text{P1} > \text{P2} > \text{P3}$ in November 2018 and $\text{P5} > \text{P4} > \text{P2} > \text{P3} > \text{P1}$ in May 2019. The carbon isotope ratios ranged between -24.93% and -12.77% and displayed the opposite trend to that of C/N; i.e., the lower the C/N of the new leaves, the more negative their carbon isotope ratios.

To summarize, with higher DIC concentrations in their springs, and P4 and P5 pond waters the plant biomass of submerged plant *Chara* spp. was higher, CA activity was extremely low, C/N was higher and the C isotope ratios were more negative. In P1 and P2, with lower DIC concentrations in their spring waters, the plant biomass and C/N were lower, the CA activity was higher, and C isotope ratios were more positive.

3.4. Temporal and spatial variations of TOC concentrations in spring and pond waters

The variations in TOC concentrations in the five spring and pond waters are shown in Fig. 5a. TOC concentrations in the spring and pond waters ranged from 0.72 mg L^{-1} to 3.37 mg L^{-1} and 1.61 mg L^{-1} to 6.69 mg L^{-1} , respectively. The average values of TOC concentrations exhibited the trend $\text{S4} > \text{S3} > \text{S5} > \text{S1} > \text{S2}$. This is mainly caused by highest plant litter in the grassland (S4), and thus resulted in higher

Fig. 2. Monthly variations in the concentrations of C, N, and P in the five springs and their ponds during the experimental period. (a – bare rock; b – bare soil; c – cultivated (corn) cover; d – grass (alfalfa) cover; e – shrub (Roxburgh roses) cover). 1: HCO_3^- and dissolved carbon dioxide (CO_2 (aq)). 2: TN and NO_3^- . 3: TP and PO_4^{3-} . Note: The circles represent spring waters and the triangles represent pond waters.



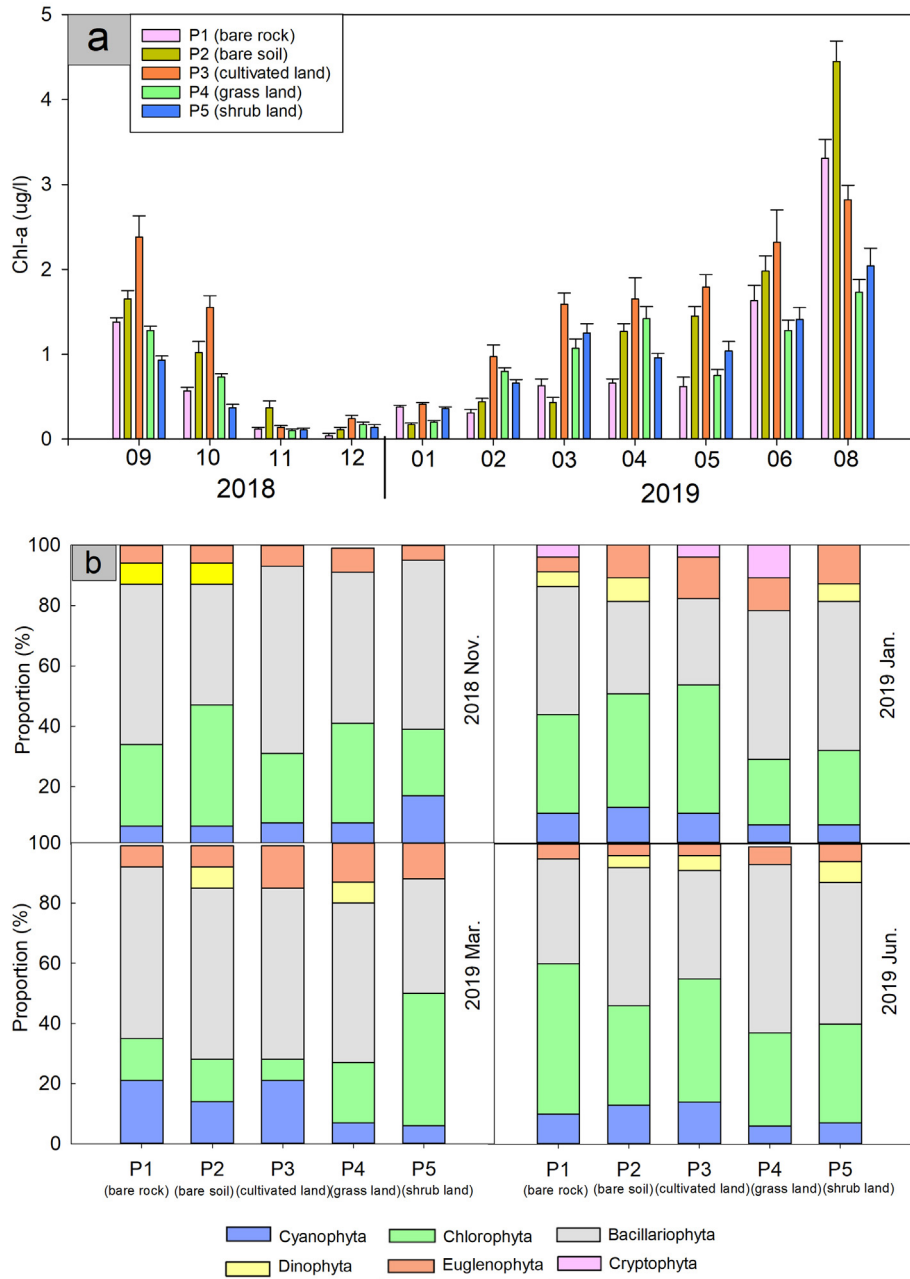


Fig. 3. (a) Monthly variations of phytoplankton biomass (Chl-a); (b) Phytoplankton proportional composition in the five ponds in different seasons.

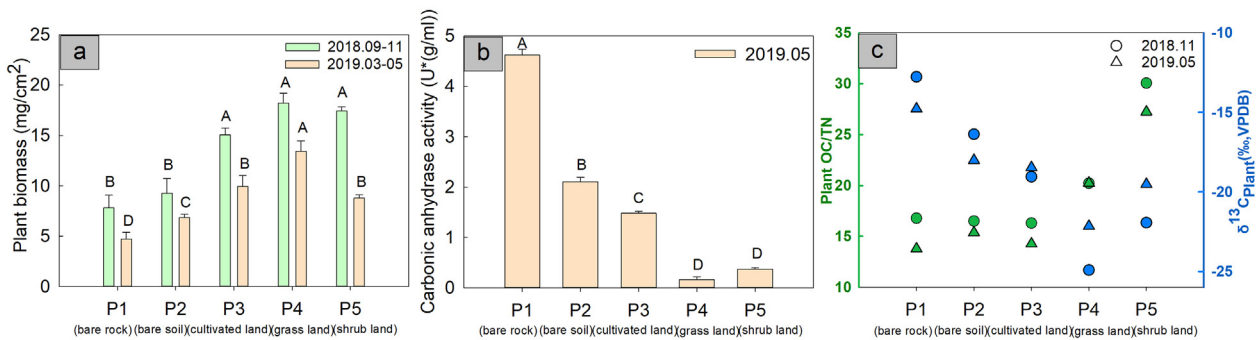


Fig. 4. (a) The Plant biomass; (b) Carbonic anhydrase (CA) activity; (c) C/N (molar ratio) and Carbon isotope of submerged *Chara* spp. in November 2018 and May 2019. The different uppercase letters above the bars (in a, b) represent significant differences among the five ponds (one-way ANOVA with LSD test, $p < 0.05$).

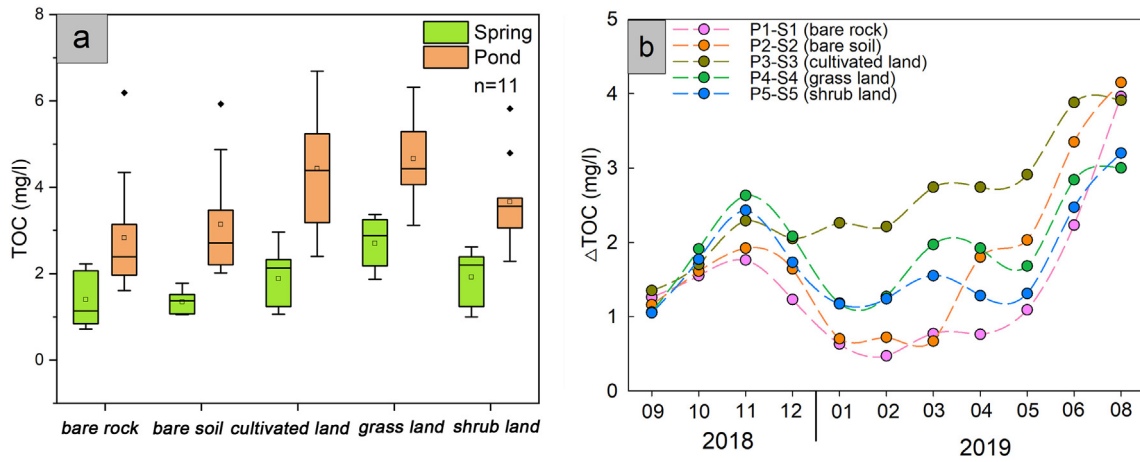


Fig. 5. (a) The TOC concentrations in the five spring and pond waters; (b) the monthly change of TOC produced in pond waters (pond water minus spring water (Δ TOC)) during the study.

soil organic matter than that in the cultivated land (S3) and shrub land (S5). In the ponds, TOC concentrations in P4 and P3 were higher than in P5, and those in P1 and P2 were the lowest. We use TOC difference (Δ TOC) between pond water and spring water to characterize the self-generated TOC; i.e., the AOC that was created in the pond waters. From September to December 2018, Δ TOC gradually changed from being highest in P3 (1.35 mg L^{-1}) to being highest in P4 (2.08 mg L^{-1}). P1 was always the lowest (Fig. 5b). From January to August 2019, all of the Δ TOC concentrations showed evident increases.

4. Discussion

4.1. Effects of nutrient concentrations and their ratios on phytoplankton

The different land uses in this study lead to different concentrations of C, N and P in the spring and pond waters (Fig. 2). Consequently, the

phytoplankton biomass (as Chl-a) and compositional structure in the pond water showed differences (Fig. 3). This indicates that the nutrient supplies mediated by differing land uses had important impacts on the growth of the algae and on the community succession in the open waters (Jiang et al., 2014; Hayes et al., 2015). Based on the measured concentrations of Chl-a, our study was divided into periods of growth and dormancy and the relationships between Chl-a and concentrations of C, N, or P were examined. The results are shown in Fig. S4. In the growth period, the concentrations of Chl-a were mainly influenced by nitrogen and phosphorus output via the land use mediation. Furthermore, we analyzed the relationship between Chl-a and the elemental ratio (molar ratio) of $\text{CO}_2(\text{aq})$, NO_3^- , and PO_4^{3-} during the growth period (Fig. 6a–c). The results show that the growth of algae was primarily limited by N. This is similar to our previous study (Bao et al., 2020). Therefore, N may be the main limiting factor for algal growth in karst watersheds, although N and P co-limit the outbreak and growth of

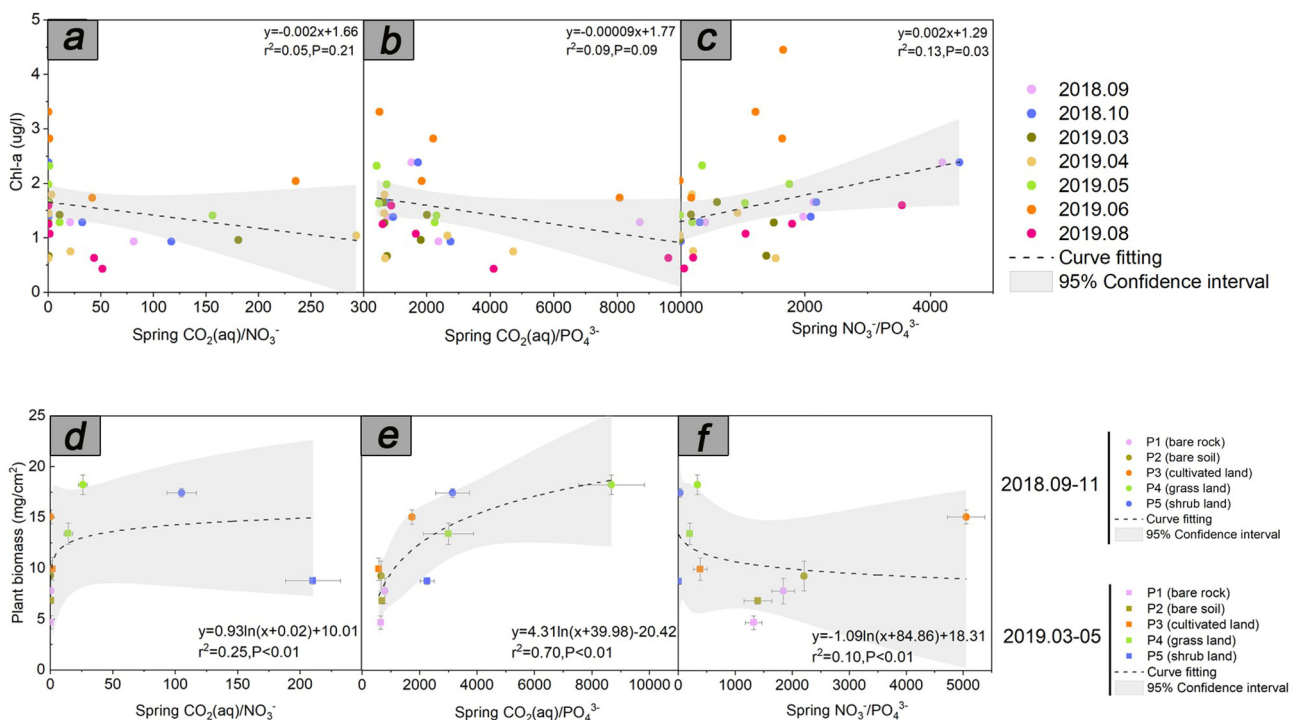


Fig. 6. The relationships between phytoplankton biomass (Chl-a, $\mu\text{g L}^{-1}$), the plant biomass of *Chara* spp. (mg/cm^2) and the nutrient ratios (molar ratios) of $\text{CO}_2(\text{aq})/\text{NO}_3^-$ (a, d), $\text{CO}_2(\text{aq})/\text{PO}_4^{3-}$ (b, e) or $\text{NO}_3^-/\text{PO}_4^{3-}$ (c, f) in the spring waters during the growth periods, respectively.

algae in other environments (Howarth and Marino, 2006; Paerl et al., 2011). However, we need also to consider the synergistic effects of temperature, N and P. The results of this study are similar to those of Hayes et al. (2015) which shows that temperature had a significant effect on Chl-a ($r^2 = 0.65$, $P < 0.01$) (Fig. S5a).

Cyanophyta primarily cause algal blooms during eutrophication (Paerl, 2008). After growth and death, some Cyanophyta release toxic substances into the host water bodies, worsening the water quality. Chlorophyta and Bacillariophyta do less damage to water quality (Van de Waal et al., 2011). Our research classified Cyanophyta (Cya.) as harmful algae and Chlorophyta (Chl.) and Bacillariophyta (Bac.) as harmless algae, and compared the ratio between them (Cya./ (Chl. + Bac)) with the ratios between the principal nutrients in the spring waters (Fig. 7). The results show that waters with high N and P increase the proportion of Cyanophyta, while waters with high CO_2 reduces it. This finding is consistent with that of Low-Décarie et al. (2015), but significantly different from that of Verspagen et al. (2014) who found that high CO_2 concentrations are beneficial for increased cyanobacteria biomass. This may be attributed to different species in the Cyanophyta having different sensitivity to CO_2 (Bao et al., 2020) and to the inhibition of cyanobacterial growth by the lush growth and secretion of organic matter from submerged plants in pond waters with high DIC concentrations (Jones et al., 2002). Overall, Chlorophyta and Bacillariophyta accounted for a higher proportion of mass than Cyanophyta in aqueous environments with high C, low N and P, and they have a more competitive advantage, particularly in P4 (grass land) and P5 (shrub land).

4.2. Effects of nutrient concentrations and ratios on the submerged plant *Chara* spp.

In most cases, submerged plants rely on their roots to absorb N, P, and trace elements from bottom sediments and on their branches and leaves to absorb calcium and magnesium from water (Waisel and Shapira, 1971; Barko et al., 1991). However, studies have also found that in relatively rich nutrient environments, submerged plants can rely on their branches and leaves alone to absorb N and P (Madsen and Cedergreen, 2002). Further, Van den Berg et al. (1999) have shown that the P content in the water is the chief limiting factor on the growth of *Chara* spp. Conversely, due to the substantial resistance of dissolved CO_2 to readily diffuse in water, inorganic carbon may also

become a limiting factor for the growth of submerged plants (Maberly and Madsen, 2002). We conducted a correlation analysis between the dry weights of *Chara* spp. and the inorganic C, N or P concentrations in the spring waters. The results are shown in Fig. S6. The plant biomass had a very significant positive correlation with CO_2 (aq) concentrations in the spring waters ($r^2 = 0.76$, $P < 0.01$), the data from the two growing seasons being well replicated (Fig. S6a). However, the plant biomass did not show significant relationships with the NO_3^- (Fig. S6b) and PO_4^{3-} (Fig. S6c) concentrations in the spring waters. In addition, we analyzed the relationship between the nutrient ratios (molar) of CO_2 (aq), NO_3^- , or PO_4^{3-} and plant biomass during the two periods (Fig. 6d–f). The results show that there were significant logarithmic correlations between the plant biomass and CO_2 (aq)/ NO_3^- ($r^2 = 0.25$, $P < 0.01$), CO_2 (aq)/ PO_4^{3-} ($r^2 = 0.70$, $P < 0.01$), or $\text{NO}_3^-/\text{PO}_4^{3-}$ ($r^2 = 0.10$, $P < 0.01$). Overall, the plant biomass of *Chara* spp. was primarily affected by C in the spring waters, with $C > P$ and P over N.

4.3. Carbon limitation on submerged plants and inorganic carbon utilization

The photosynthesis of submerged plants during the day consumes a lot of free CO_2 in water. However, the diffusion rate of CO_2 in water is only one ten thousandth of that in the air, therefore, submerged plants may be subject to carbon limitation conditions in many cases (Raven, 1970; Maberly and Madsen, 2002). Consequently, about half of all submerged plant species have developed a mechanism to use HCO_3^- as an additional carbon source (Dülger et al., 2017). However, the process of adjusting the potential to utilize HCO_3^- is energy-consuming and not a priority for plants (Maberly and Gontero, 2018). Our study found that the plant biomass of submerged plants had a very significant positive correlation with the CO_2 (aq) concentration in spring waters ($r^2 = 0.76$, $p < 0.01$) (Fig. S6a). Therefore, we speculate that the submerged plants we studied may have carbon limitation. Carbonic anhydrase activity is one of the chief ways to study HCO_3^- usage in plants (Huang et al., 2020). We compared the carbonic anhydrase activity in the new leaves in the pond waters with the concentrations of inorganic carbon (HCO_3^- and CO_2 (aq)) in the spring waters. The results showed that HCO_3^- , CO_2 (aq), and the carbonic anhydrase activity were best described by power function models (Fig. 8). In addition, the statistical correlation between CO_2 (aq) and

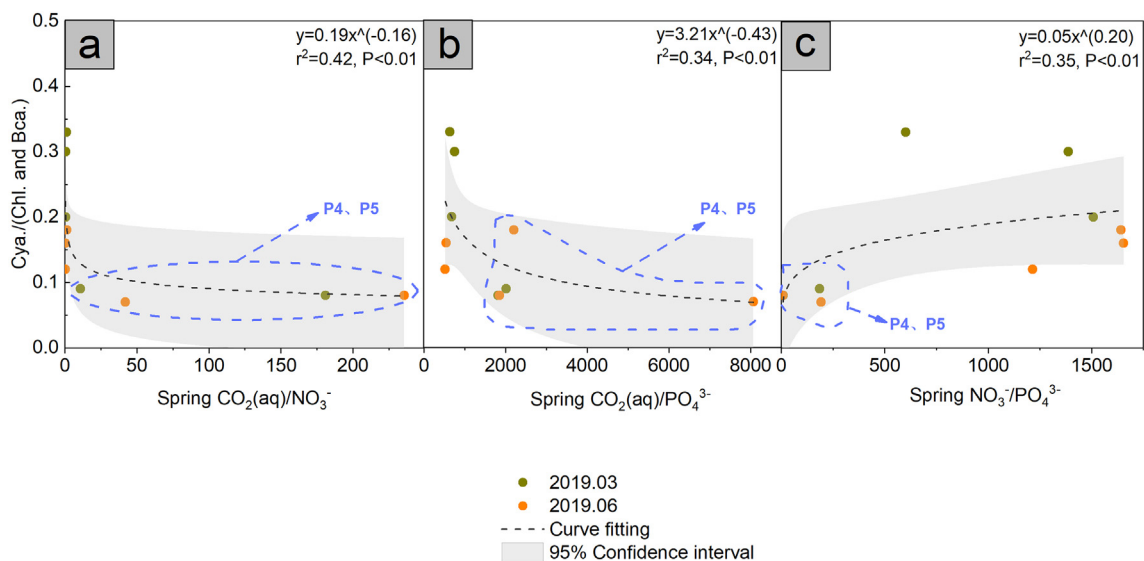


Fig. 7. The relationships between algae composition ratios Cyanophyta/(Chlorophyta and Bacillariophyta) (Cya./ (Chl. and Bac.)) and nutrient ratios (molar ratios) of CO_2 (aq)/ NO_3^- (a), CO_2 (aq)/ PO_4^{3-} (b) or $\text{NO}_3^-/\text{PO}_4^{3-}$ (c) in the spring waters in March and June 2019.

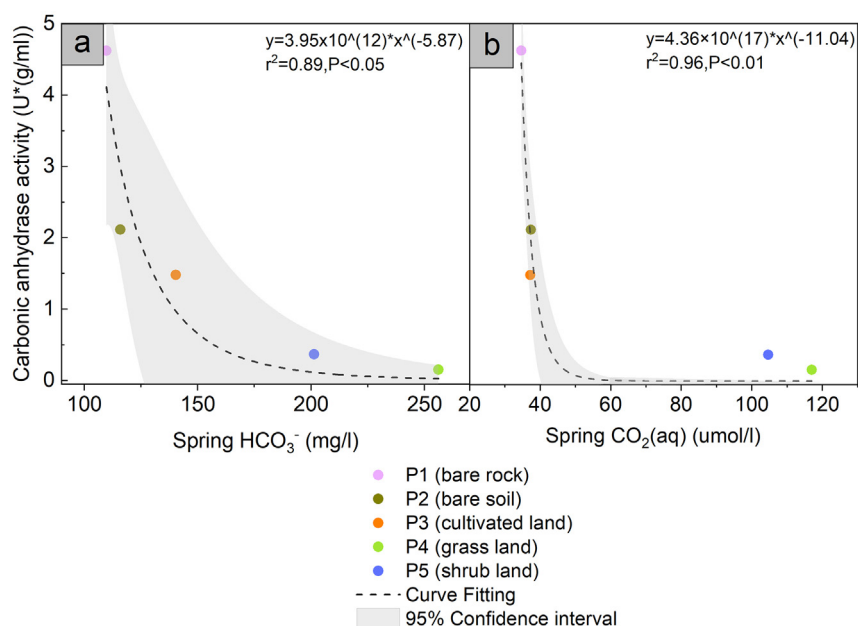


Fig. 8. The relationships between the carbonic anhydrase activity of new leaf of submerged *Chara* spp. and the concentrations of HCO_3^- (mg L^{-1}) (a) or CO_2 (aq) ($\mu\text{mol L}^{-1}$) (b) in spring waters.

CA ($r^2 = 0.96, P < 0.01$) is better than between HCO_3^- and CA ($r^2 = 0.89, P < 0.05$), indicating that the expression of CA is mainly affected by the concentration of free CO_2 in water (James and Larkum, 1996; Beer and Rehnberg, 1997). Fig. 8 also shows that a CO_2 (aq) concentration of approximately $50 \mu\text{mol L}^{-1}$ is the threshold for *Chara* spp. to use CA to convert HCO_3^- as an inorganic source of carbon. This differs from the results of Huang et al. (2020) who found that *Ottelia alismoides* uses CA to convert HCO_3^- at CO_2 (aq) concentrations of only $5\text{--}30 \mu\text{mol L}^{-1}$. This supports the premise that different submerged plants begin to use CA to extract C from inorganic HCO_3^- at different threshold concentrations of CO_2 (aq) (Maberly and Gontero, 2017; Iversen et al., 2019). Further analysis showed that the CO_2 (aq) in the spring and pond waters in this study was generally lower than $50 \mu\text{mol L}^{-1}$ during the *Chara* spp. growth period, especially in the water derived from bare rock, bare soil, and cultivated land (corn) tanks. This indicates that the inorganic carbon source of *Chara* spp. in these pond waters of our study came chiefly from HCO_3^- .

Measuring stable carbon isotope ratios is one of the principal ways to study carbon biogeochemical processes (Hobson and Wassenaar, 1999). The sources of inorganic carbon can be determined by the relationships between the isotope ratios in aquatic organisms and water (Pronin et al., 2016; Pronin et al., 2019). We conducted a correlation analysis of the carbon isotope ratios in the spring waters and in the new plant leaves and obtained a very significant linear positive correlation ($r^2 = 0.77, P < 0.01$) displayed in Fig. S7a. It shows that the inorganic carbon source for submerged plants in this study comes mainly from DIC in the spring waters, especially HCO_3^- . Comparing the OC/TN of the leaves with the DIC/TN in the spring waters also yielded a very significant linear positive correlation ($r^2 = 0.76, P < 0.01$) (Fig. S7b). This indicates that the submerged *Chara* spp. absorbed a certain proportion of nutrients in the spring waters (Su et al., 2016).

4.4. Influence of phytoplankton and submerged plants on OC generated in pond waters

The TOC in water is one of the main components of the carbon cycle in terrestrial ecosystems, mainly coming from terrestrial sources, photosynthesis of aquatic organisms, and inputs from human activities (Yang et al., 2016). In aquatic photosynthetic organisms, phytoplankton will

produce OC during growth and decay, which is one of the main sources of OC in water (Song et al., 2018; Wen et al., 2020). Moreover, submerged macrophytes can also release large amounts of organic carbon by excreting matter into the water (Gao et al., 2015; Li et al., 2020). Primary reasons for this release are growth inhibition of competitors and cell lysis caused by high temperature (Kogan et al., 1978; Powers et al., 2019). As shown emphatically in Fig. 9a–c, phytoplankton biomass (Chl-a) exhibited different relationships with the autochthonous OC (ΔTOC), namely AOC, over different periods in the growth cycle. From September to November 2018, the phytoplankton were in a period of decline and the biomass v AOC showed a very significant binomial distribution relationship ($r^2 = 0.68, P < 0.01$). Interestingly, when the content of Chl-a was high, the concentration of AOC was low (Fig. 9a). However, the plant biomass of submerged plants during this period had a very significant positive linear correlation with AOC ($r^2 = 0.93, P < 0.01$; Fig. 9d). This indicates that, during the algae decline period, the formation of AOC may mainly come from the release of submerged plants. From December 2018 to February 2019, the algae were in their dormant period, and there was no correlation between Chl-a and AOC (Fig. 9b). In this period the *Chara* spp. died, and the leaves generally withered and therefore contributed AOC. Thus, the AOC was primarily derived from cell decomposition after the death of the algae and the release of organic matter by microorganisms (Carvalho et al., 2015; Danhiez et al., 2017). A linear regression analysis between phytoplankton biomass (Chl-a), plant biomass of *Chara* spp., and AOC in their growth period in 2019 shows that there was a strong positive correlation between ΔTOC and Chl-a ($r^2 = 0.78, P < 0.01$) but no significant relationship between ΔTOC and the plant biomass (Fig. 9c; e). This indicates that the AOC during this period may be chiefly from the algae, the contribution of *Chara* spp. to OC formation being relatively small. This may be due to the increase in the phytoplankton biomass affecting the light conditions in the pond water and simultaneously intensifying the competition for nutrients, which makes algae contribute more to OC formation (Jones et al., 2002; Su et al., 2019).

To further study the influence of phytoplankton and submerged plants, variation partitioning analysis of AOC was undertaken (based on the results of RDA analysis - Fig. S8) by using Chl-a and the plant biomass as the explanatory variables. The Chl-a and the plant biomass explained about 85% of spatial variations in AOC during the September to

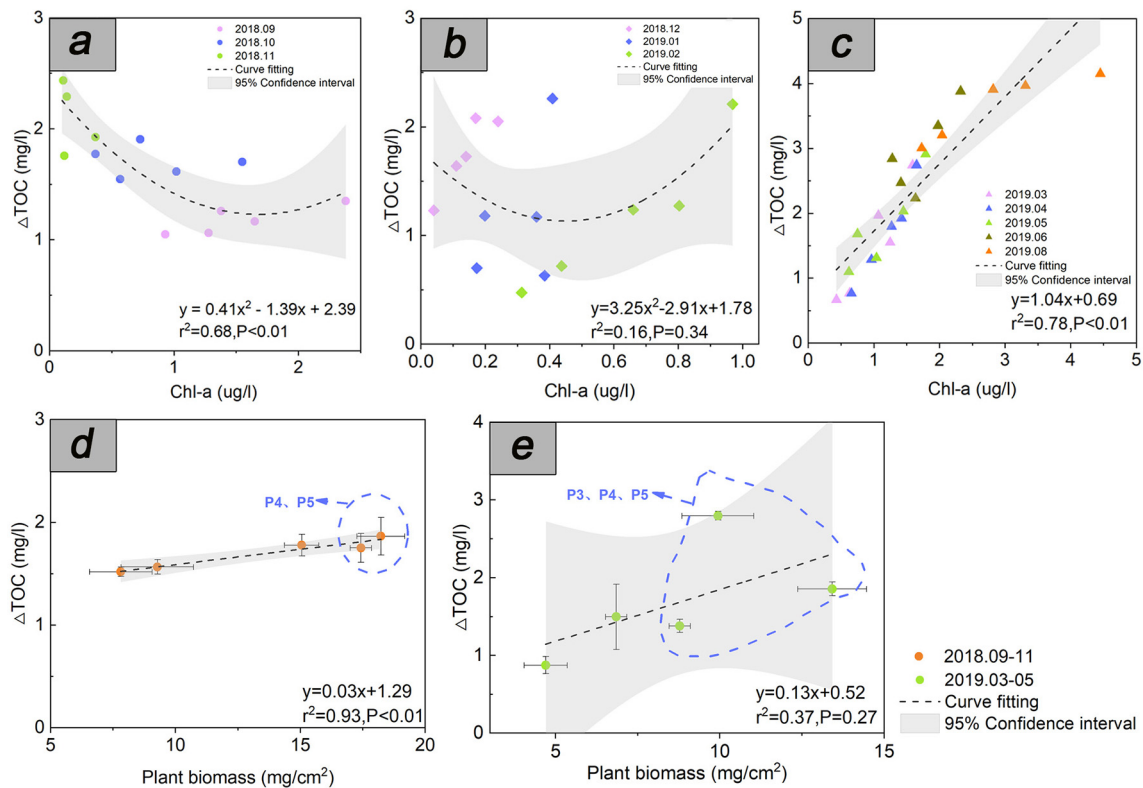


Fig. 9. The relationships between the autochthonous OC (Δ TOC) in pond waters and the biomass (Chl-a, $\mu\text{g L}^{-1}$) of phytoplankton in the decline period (a), dormant period (b), and growth period (c), and the relationships between the autochthonous OC (Δ TOC) in the pond waters and the plant biomass of *Chara* spp. in September–November 2018 (d), or in March–May 2019 (e).

November 2018 period (Fig. 10a). Chl-a had a trivial effect on AOC (0.21%), but the plant biomass of *Chara* spp. was greater, explaining about 38% of the variability in Δ TOC. In addition, the two types of explanatory variables had stronger combined effects on AOC, explaining about 47% of the variability, suggesting that the planktonic and the submerged plants jointly regulated the spatial variations of AOC, especially the submerged plants. However, during March–August 2019, Chl-a had a greater effect on AOC (approximately 46%) and the plant biomass of *Chara* spp. explained approximately 6% of the variability (Fig. 10b). The two types together explain approximately 32% and also indicate that the phytoplankton and the submerged plants together play an important role in the formation of OC, with the algae making a greater impact than *Chara* spp. in this period. Overall, Chl-a and plant biomass explained approximately 72% of spatial variations in AOC throughout the study period (Fig. 10c). The individual contributions of the algal biomass (approximately 27%) and the biomass of submerged plants (approximately 28%) to AOC in water were similar, and combinedly, they explain a further 17.36%. The Approximately 29% of the undetermined

variation may be primarily attributed to the OC fixed by microorganisms or formed by their decomposition of dead algae (Paerl, 1978).

4.5. Implications

Land uses changed the loads of C, N, and P in the model watersheds, thereby affecting the phytoplankton biomass in the corresponding pond waters (Bao et al., 2020; this study). Our studies (Bao et al., 2020; this study) have shown that depleting nutrients in the soil by natural restoration of vegetation controlled the algae biomass in water. It also shows that the vegetation restoration promoted the increase of OC concentration in water and regulated the composition structure of the phytoplankton. The proportion of Cyanophyta in the water with higher DIC concentrations was lower than that in the water with lower DIC concentrations, indicating that Chlorophyta and Bacillariophyta have greater opportunities to grow in high DIC water, this is, due to Cyanophyta having more ways to use DIC when it is at low concentrations (Reinfelder, 2011). Therefore, we conclude that the increase of DIC concentration

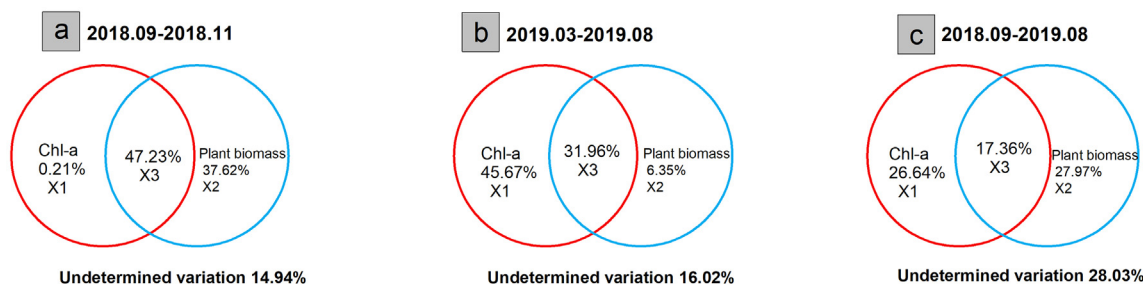


Fig. 10. The results of variation partitioning analysis of the autochthonous OC (AOC or Δ TOC) in the pond waters. The analysis distinguishes three fractions: X1–the biomass of algae, X2–the biomass of *Chara* spp., and X3–the combined effects during the decline period (a), growth period (b) and the whole year (c).

and the reduction of nutrients through land use management (e.g., natural restoration of vegetation) can have certain mitigating effects on Cyanobacteria bloom in the early stage of eutrophication.

The missing carbon sink has been one of the major topics in the research on the global carbon cycle. It is suspected that the sink may be observed in terrestrial systems (Wigley, 1993; Regnier et al., 2013). Liu et al. (2018b) showed that carbonate weathering coupled with aquatic photosynthesis may be an important contributor to the sink. Aquatic photosynthetic organisms use DIC to create an OC sink and bury some of that in sediments in karst surface water bodies (e.g., lakes, especially marl lakes) (Pentecost, 2009; He et al., 2020). As previously discussed, we found that the submerged plants may contribute a considerable part of the OC to the water, and in some cases, it may even be higher than that of algae. Moreover, due to the high pH of the water in karst areas and the subsequent long-term low levels of CO₂ (aq) in them, submerged plants may be better suited to evolve through the carbonic anhydrase, related carbon-concentrating mechanism (CCM) under carbon-limited conditions, more HCO₃⁻ is used. Therefore, we believe that it is possible to achieve a win-win situation of alleviating eutrophication and increasing the carbon sequestration, by regulating the concentrations of DIC in karst surface waters, e.g., by natural restoration of vegetation in the watersheds, or injecting CO₂ into water, thereby, creating aqueous environments with high C but low N-P loading.

5. Conclusions

This paper studied the effects of different model land uses on C, N, and P concentrations and the resulting impacts on the growth of phytoplankton and submerged *Chara* spp. in model outlet ponds. Affected by the differing vegetation and biomass of aquatic photosynthetic organism in ponds, the hydrochemistry and nutrients in the outlet spring and pond waters showed large temporal and spatial differences. Agricultural farming has formed a lower C, but high N-P loadings. In the aqueous environments, the biomass of phytoplankton is extremely high, and the proportion of cyanobacteria is large, and the submerged biomass is general. However, natural vegetation restoration (shrub and grass lands) increased the C in the leached surface water, but reduced N-P loadings. In the aqueous environments, the biomass of phytoplankton is low, and the proportion of cyanobacteria also is low, but the submerged biomass is extremely large. Correlation analysis shows that phytoplankton and submerged plants in the growth period are mainly limited by N and P (particularly N), and C, respectively. Further, Chlorophyta and Bacillariophyta account for a higher proportion than Cyanophyta in the aqueous environments with high C, but low N-P, and have a more competitive advantage. Due to the higher pH values found in karst areas, submerged *Chara* spp. is often in a carbon-limited state during the growth period, as indicated by the activation of the CA to utilize HCO₃⁻. In autumn, when the algae growth enters a period of decline, AOC is mainly determined by the submerged biomass in spring and summer when algae are in the growing period, AOC is mainly determined by the algal biomass. Throughout the study period, the contribution of the phytoplankton biomass (27%) and of the submerged plants (28%) to AOC in the pond waters was similar and, combinedly, they explain 17% of its formation. To conclude, by leading to high C, but low N-P loadings, the natural restoration of vegetation, or injecting CO₂ into water, may simultaneously help to mitigate eutrophication (with changes in biological structure and species) and increase carbon sequestration in pond waters or open lakes. This study may further have implications for the global inland water carbon sequestration estimation and solutions to the eutrophication on watershed scales.

CRedit authorship contribution statement

Qian Bao: Writing - original draft, review & editing, Software, Visualization, Data curation. **Zaihua Liu:** Conceptualization, Methodology,

Supervision, Writing - review & editing. **Min Zhao:** Data curation, Supervision, Formal analysis, Software, Writing - review & editing. **Yundi Hu:** Data curation, Investigation. **Dong Li:** Data curation, Investigation. **Cuihong Han:** Data curation, Investigation. **Cheng Zeng:** Data curation, Investigation. **Bo Chen:** Data curation, Investigation. **Yu Wei:** Data curation, Investigation. **Song Ma:** Data curation, Investigation. **Yang Wu:** Data curation, Investigation. **Yi Zhang:** Data curation, Investigation.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2021.151917>.

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