



## Factors influencing submerged macrophyte presence in fresh and brackish eutrophic waters and their impact on carbon emissions

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### ABSTRACT

In agricultural landscapes of North-Western Europe, the majority of water bodies do not meet the targets set by the European Water Framework Directive due to a lack of submerged macrophytes and associated biodiversity. These eutrophic waters can also be a substantial source of methane (CH<sub>4</sub>) and carbon dioxide (CO<sub>2</sub>) to the atmosphere. Here we present a two-year field experiment on the island of Goeree-Overflakkee (southwest Netherlands), conducted in six drainage ditches varying in salinity, where we monitored four permanent plots per ditch and varied the presence of both fish and macrophytes. We aimed to: 1) investigate factors limiting submerged macrophyte growth, focussing on exclusion of grazing pressure and bioturbation by fish; and 2) quantify the CO<sub>2</sub> and CH<sub>4</sub> emission under these conditions. Even in highly eutrophic, semi turbid ditches with fluctuating salinity levels and sulphide presence in the root zone, submerged macrophytes established successfully after introduction when the influence of grazing and bioturbation by fish was excluded. In the enclosures, diffusive CH<sub>4</sub> and CO<sub>2</sub> emissions, but not ebullitive CH<sub>4</sub> emissions were significantly reduced. The spontaneous development of submerged macrophytes in the enclosures without macrophyte introduction underlined the effect of grazing and bioturbation by fish and suggest that abiotic conditions did not hamper submerged macrophyte development. Our results provide important insights into the influential factors for submerged macrophyte development and potential for future management practices. Large-scale fish removal may stimulate submerged macrophyte growth and reduce methane emissions, albeit that the macrophyte diversity will likely stay low in our study region due to fluctuating salinity and eutrophic conditions.

### 1. Introduction

In agricultural landscapes of North-Western Europe, drainage ditches are a common but understudied aquatic habitat (Koschorreck et al., 2020). In the Netherlands the total length of ditches is estimated to be approximately 330,000 km. These water bodies are often heavily

influenced by their surroundings, receive high nutrient loads and are characterised by controlled water levels and high density of benthivorous fish. The majority of these water bodies do not meet the targets set by the European Water Framework Directive, often due to the low presence or even absence of submerged macrophytes and associated biodiversity (van Gaalen et al., 2020). Submerged macrophytes play an

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important role in aquatic ecosystems, providing primary production and a habitat for the aquatic fauna and flora (Scheffer, 1998). Recent work has shown that particularly unvegetated eutrophic waters can be substantial sources of methane (CH<sub>4</sub>) to the atmosphere (Davidson et al., 2018; Grasset et al., 2016).

The delta island of Goeree-Overflakkee, in the southwest of the Netherlands, is a landscape containing a large number of drainage ditches and canals primarily supporting agricultural functions resulting in high nutrient loads and strictly controlled water levels. Active water management, shore erosion and local brackish seepage result in fluctuations in surface water turbidity and salinity and a variable residence time of the surface waters. Under present conditions submerged macrophyte coverage is generally very low, or even zero (Postma et al., 2017). In the 1980's, however, submerged macrophyte coverage was very high which incentivised water-authorities to introduce Grass carp (*Ctenopharyngodon idella*) to mitigate nuisance aquatic macrophytes (Postma et al., 2017). It is presently not known which factors hamper the restoration of a healthy, submerged macrophyte community and associated biodiversity.

An earlier study conducted on these waterbodies suggested that a combination of factors may impede the development of a submerged macrophyte community: (1) high turbidity, (2) bioturbation and grazing pressure by fish and (3) fluctuating surface water salinity with reduced, sulphide-rich sediments (Postma et al., 2017). Turbidity has a direct effect on submerged macrophytes by partially absorbing incoming sunlight and subsequently inhibiting photosynthesis, favouring low light adapted primary producers such as cyanobacteria. High turbidity can be the result of runoff of suspended solids, shore erosion of agricultural fields (Ryan, 1991), resuspension of the aquatic sediment or phytoplankton blooms (Scheffer et al., 1993; van Donk and Gulati, 1995). Resuspension of sediments in shallow lakes, but also in ditches and canals, is often caused by bioturbating benthivorous fish, which may contribute substantially to water turbidity (Meijer et al., 1990). In addition, high densities of benthivorous and herbivorous fish are known stressors of submerged macrophytes due to grazing and uprooting (Lodge, 1991). The fluctuating salinity levels in the surface water are caused by the combination of brackish seepage (the island is partly surrounded by sea and contains brackish groundwater aquifers) and active flushing with fresh surface water during the growing season to reduce surface water salinity levels for agricultural purposes. Variation in flushing intensities and regional differences in brackish seepage pressure result in fluctuating surface water salinity levels (lower in summer, higher in winter) and consequential discrepancies between sediment and surface water salinity in summer. Fluctuating salinity levels can have several negative effects on the development and presence of submerged macrophytes. These effects include osmotic and physiological stresses as well as cationic imbalances, which in turn can lead to disturbed metabolism and growth inhibition (Duman et al., 2014; McGahee and Davis, 1971). Additionally, brackish aquatic sediments rich in organic matter often result in a reduced root environment containing free sulphide which can lead to physiological stress for submerged macrophytes (Lamers et al., 2013). The data analyses of Postma et al. (2017) combined with the recent mesocosm work in Velthuis et al. (2023) showed all the above-mentioned factors to potentially contribute to the near absence of submerged macrophytes but the relative contribution of these factors remained inconclusive.

Restoration of a submerged macrophyte community may not only restore the aquatic food web and enhance biodiversity but may also reduce greenhouse gas (GHG, CH<sub>4</sub> and CO<sub>2</sub>) emissions, although reports on the effects of macrophytes on CH<sub>4</sub> emissions differ (see e.g. Hilt et al., 2017). Aquatic CH<sub>4</sub> emissions to the atmosphere are governed by a myriad of biotic and abiotic processes and are therefore complex to predict. Studies often report increasing CH<sub>4</sub> emissions with increasing availability of dissolved organic carbon (Bastviken et al., 2004; Zhou et al., 2019), increased labile organic matter availability in the sediment (e.g. Kelly and Chynoweth, 1981) and increasing availability of

nutrients (Aben et al., 2022; Davidson et al., 2018). Elevated salinity levels and coinciding increased sulphate availability is reported to increase sulphate reduction, suppress methanogenesis and decrease CH<sub>4</sub> emissions (Marton et al., 2012; Weston et al., 2006). Bioturbation by fish may increase CH<sub>4</sub> emission through predation on effectively bio-irrigating macrofauna thereby likely reducing methane oxidation and enhancing methane production (Colina et al., 2021b). Fish bioturbation, however, has also been found to decrease CH<sub>4</sub> emissions (Oliveira junior et al., 2019), possibly related to less gas bubble build-up due to disturbance and an increase in dissolved methane oxidation. Submerged macrophytes may decrease CH<sub>4</sub> emissions as they provide a habitat and oxygen for CH<sub>4</sub> oxidising bacteria (Davidson et al., 2015; Heilman and Carlton, 2001). Others report increased CH<sub>4</sub> emissions under submerged macrophytes presence because of increased organic matter availability (Xing et al., 2006), or because of macrophyte-mediated CH<sub>4</sub> transport through the leaves (Sanders et al., 2007) or emerging flowers (Grasset et al., 2019; Schuette and Klug, 1995).

Whether CO<sub>2</sub> emissions decrease or increase after restoration of a submerged macrophyte community is also difficult to predict. Water bodies that have a high primary producer biomass (macrophytes or algae) are often CO<sub>2</sub> under-saturated (Balmer and Downing, 2011; Kosten et al., 2010), and may function as carbon sinks (Hilt, 2015). On the other hand, carbon burial can be less efficient in submerged macrophyte-dominated waters compared to turbid algae-dominated waters due to higher oxygen availability in the benthic zone (Brothers et al., 2013), resulting in increased mineralisation rates.

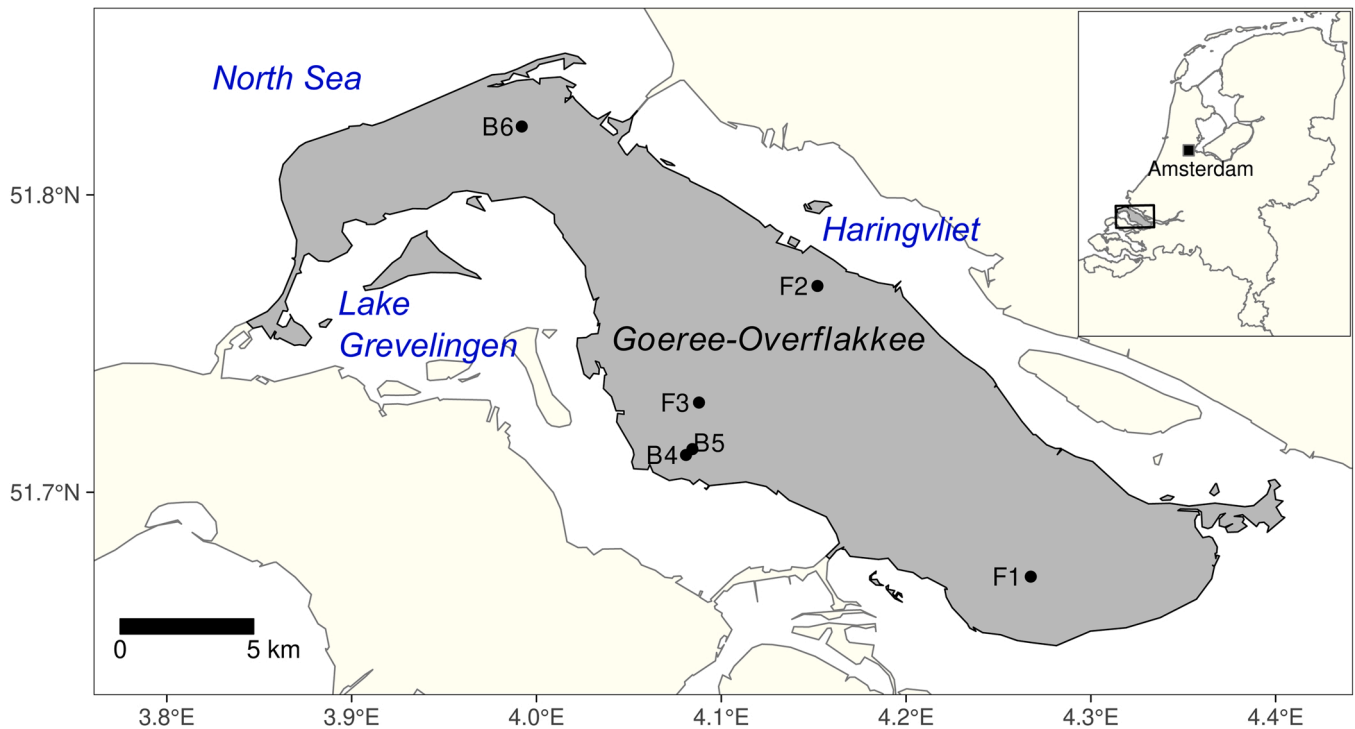
Here, we investigate the effects of exclusion of herbivory and bioturbation by fish, the introduction of submerged macrophytes, and abiotic conditions on macrophyte recovery and CH<sub>4</sub> and CO<sub>2</sub> emissions in fresh and brackish water agricultural ditches. We hypothesised that (1) the exclusion of herbivory and bioturbation by fish is insufficient to promote growth and development of submerged macrophytes (2) introduction of submerged macrophytes is needed for successful macrophyte settlement and development, (3) the exclusion of herbivory and bioturbation by fish and concomitant macrophyte development will result in lower CH<sub>4</sub> and CO<sub>2</sub> emissions, and (4) both macrophyte development and CH<sub>4</sub> emissions will be lower under higher salinity.

## 2. Methods

### 2.1. Experimental setup

In April 2018 four permanent plots (2 m<sup>2</sup>) were installed in six waterways spread over the island of Goeree-Overflakkee (southwest Netherlands, Fig. 1). These waterways were considered representative for the different shallow drainage ditches present on the island (the ditches varied in dimensions, from around 3–8 m wide and 0.7–1.5 m deep, photographs are included in Supplementary Fig. S8). Three were characterised as fresh (< 8.5 mmol l<sup>-1</sup> Cl) (locations F1, F2 and F3) and three as (slightly) brackish (8.5 – 85 mmol l<sup>-1</sup> Cl) (locations B4, B5 and B6). To exclude the limiting effect of turbidity on macrophyte growth in the experiment only semi-turbid locations were selected with a combination of water depth and turbidity that should not limit macrophyte development (Secchi depth to water depth ratio above 0.6 is optimal (Hosper, 1997; Scheffer, 1998), waters were included with average ratios > 0.4, based on earlier monitoring data of the local water management authority Waterschap Hollandse Delta). Water levels differed between locations (range of 50–110 cm in summer) but showed the same seasonal variation at all locations, with high water tables in summer, and low water tables in winter, as a consequence of active inlet of fresh surface water from the Haringvliet (Fig. 1) in summer. Turbidity varied strongly over time and between locations, with Secchi depth values ranging from 19 cm in June 2018 at location B5, to 105 cm in August 2019 at location F2.

At all six locations the set-up consisted of two plots with exclosures (“exclosure”) and two plots without exclosures (“open”). The treatments

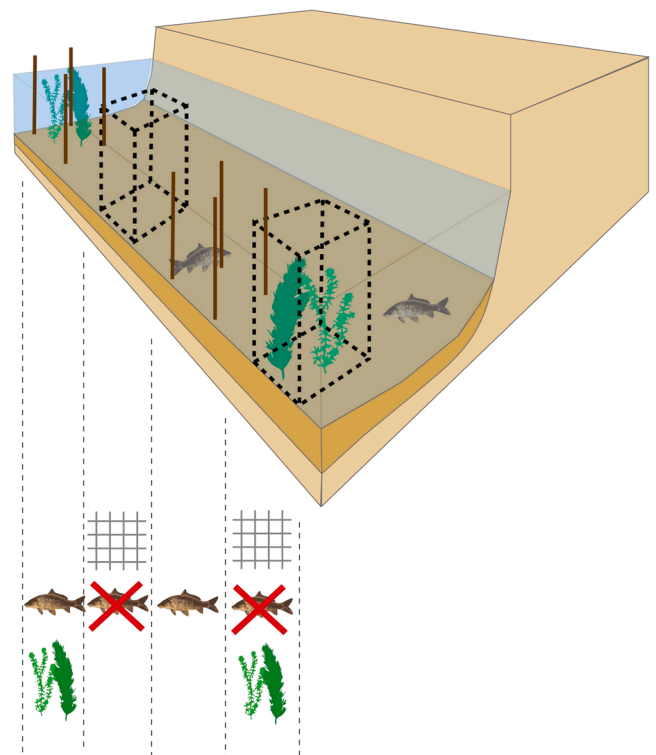


**Fig. 1.** The location of the island of Goeree-Overflakkee in the Netherlands (black square in inset) and the locations on Goeree-Overflakkee (coloured grey) where the experimental plots were placed. The fresh water/freshwater locations are marked by F1, F2 and F3, and the brackish locations are marked by B4, B5 and B6. The island is surrounded by waterbodies with different salinities. The freshwater of the Haringvliet is used to flush the ditches of Goeree-Overflakkee in spring. Seepage from the North Sea (salt) and Lake Grevelingen (brackish) result in increased groundwater salinity.

were placed in a randomised order in the ditch parallel to the shore, at one meter distance from the shore bank. Treatments were placed 10 m apart to limit treatments influencing each other. The enclosures consisted of cages (2 \* 1 \* 1.5 m (length\*width\*height)) with a 10 mm mesh-size on all sides (including the top and bottom), installed some centimetres in the aquatic sediment. No macrophytes were growing in the plots at the start of the experiment. The mean water depth in the growing season differed per location depending on the depth of the ditch and ranged from an average of 52 cm at location B4, to an average of 109 cm at location F3. In June 2018 (two months after installation) submerged macrophytes were introduced at each location in one enclosure plot and one open plot, resulting in the following treatments: Open+Plants, Open, Excl.+Plants and Excl. (see Fig. 2 for a schematic overview of the treatments). To this end, four macrophyte species (*Elodea nuttallii*, *Ceratophyllum demersum*, *Potamogeton crispus* and *Potamogeton pectinatus*) commonly occurring on the island were selected and collected from 10 local populations. All macrophytes collected from the different populations were pooled per species before introduction. Fifteen individuals of each species were carefully planted in crates (0.5 \* 0.3 \* 0.15 m (l\*w\*h), with 1 cm mesh size on all sides) containing local sediment from the respective ditch. Two crates containing both two species (and two bricks serving as weights) were carefully lowered with ropes on the aquatic sediment. After placement the crates were fixed to the sediment by inserting plastic-coated metal sticks vertically through the mesh bottom of the crates (see Supplementary Fig. S9 for a top view of the crates just before installation).

Quantitative data on fish biomass in the waters studied is absent. Sporadic fish monitoring in the area and eDNA sampling in the studied waters, however, indicate high fish densities and the fish population to be dominated by benthivore fish (Common roach (*Rutilus rutilus*), European carp (*Cyprinus carpio*), Common bream (*Abramis brama*), Common rudd (*Scardinius erythrophthalmus*) and European perch (*Perca fluviatilis*) (van Dijk et al., 2020).

After two growing seasons (2018 & 2019) the total macrophyte



**Fig. 2.** Schematic overview of experimental setup. From left to right: Open+Plants, Enclosure, Open, Enclosure+Plants.

biomass was harvested in August 2019. The biomass was separated per species, after which it was rinsed, dried for four days at 70 °C and weighed. We were not able to differentiate between macrophytes that were introduced at the start of the experiment and macrophytes that established spontaneously.

## 2.2. Diffusive C flux and ebullitive CH<sub>4</sub> flux measurements

Diffusive CH<sub>4</sub> and CO<sub>2</sub> fluxes were measured monthly from May to August 2019 in each plot at all locations except location F3, where no measurements could be carried out due to accessibility issues with the required equipment. At location B4 no measurements were carried out in June 2019 due to the weather conditions. The fluxes were measured using a cylindrical-shaped semi-transparent (approximately 70% reduction of photosynthetic active radiation) polypropylene floating chamber (10.3 l) connected to a closed-loop GHG analyser (GGA) using Off-axis ICOS technology and a sampling frequency of 1 s (GGA-24r-EP, Los Gatos Research). Measurements were conducted in duplicate per plot, for approximately 3 min each. The flux of CO<sub>2</sub> and CH<sub>4</sub> was calculated based on the slope of the relationship between the concentration in the chamber head-space and time, as described in (Almeida et al., 2016):

$$F = \frac{V}{A} * slope * P * F_1 * F_2$$

$$R * T$$

Where  $F$  is the gas flux ( $\text{g m}^{-2} \text{d}^{-1}$ ),  $V$  is the chamber volume ( $\text{m}^3$ ),  $A$  is the chamber surface area ( $\text{m}^2$ ),  $slope$  is the slope of the relationship between the CH<sub>4</sub> or CO<sub>2</sub> concentration and the time ( $\text{ppm s}^{-1}$ ),  $P$  is atmospheric pressure (Pa),  $F_1$  is molar mass of CO<sub>2</sub> (44) or CH<sub>4</sub> (16) ( $\text{g mol}^{-1}$ ),  $F_2$  is the conversion factor of seconds to days,  $R$  is the gas constant  $8.3145 \text{ (m}^3 \text{ Pa K}^{-1} \text{ mol}^{-1}\text{)}$ , and  $T$  is temperature (K) (Bergen et al., 2019). The increase in CO<sub>2</sub> and CH<sub>4</sub> concentrations in the chamber were checked visually for linearity in the field to ensure ebullition was not present. All fluxes of CO<sub>2</sub> and CH<sub>4</sub> exceeded the minimum detectable flux (i.e.  $4.2 \text{ mg m}^{-2} \text{ d}^{-1}$  for CO<sub>2</sub> and  $10.3 \text{ } \mu\text{g m}^{-2} \text{ d}^{-1}$  for CH<sub>4</sub>) by a large margin. The duplicate fluxes were averaged to give fluxes for each plot. In the Enclosure and Enclosure+Plants plots at location B4, and the Enclosure+Plants plot at location B5, negative CO<sub>2</sub> were measured as a result of uptake by floating macrophytes. Because the focus of this study is on the effect of submerged macrophytes on carbon emissions the CO<sub>2</sub> fluxes from these plots were omitted (15% of the measurements) in further analysis.

The ebullitive CH<sub>4</sub> fluxes were also measured at each plot at all locations except F3. Also, in the Open plot at location B4 no samples could be taken because the bubble trap disappeared from the site two times. The flux was estimated by continuous collection of bubbles using bubble traps as described in [Aben et al. \(2017\)](#). The traps were installed in May 2019 and sampled in June and July 2019 and twice in August 2019, with respectively 43, 27, 35 and 5 days between sampling. At location B4 no samples were collected in June 2019, resulting in 70 days between sampling. The bubble traps consisted of inverted funnels ( $0.0314 \text{ m}^2$ ) directly connected to water-filled glass bottles (0.5 or 1 l) and were placed approximately 20 cm below the water surface. The bubble traps were fixated by polyethylene foam floats connected to a stick which was secured in the sediment. To minimise the risk of additional ebullition from the stick the floats were attached loosely to the stick with tie-wraps to allow the floats to move freely under windy conditions. Also, the funnels were located approximately 10–15 cm to the side of the stick so ebullition caused by the stick would not enter the bubble traps. Once per month the volume of displaced water was determined by measuring the volume of water needed to completely refill the bottle. Also, “fresh bubbles” from the local sediment at each location were collected once by disturbing the sediment and collecting the bubbles, of which a subsample was taken that was stored in a vacuum septum-capped glass vial (12 ml vials containing chlorobutyl septa, Labco exetainer®, High

Wycimbe UK). The CH<sub>4</sub> concentration of the gas was analysed on a HP 5890 gas chromatograph equipped with a Porapak Q column (80/100 mesh) and a flame ionization detector (GC-FID, Hewlett Packard). The CH<sub>4</sub> concentration from each location was averaged ( $n = 5$ ) and then used to calculate the ebullitive CH<sub>4</sub> flux. The flux was calculated as follows:

$$F = \frac{C * V}{A * \Delta t}$$

Where  $F$  is the ebullitive CH<sub>4</sub> flux ( $\text{g m}^{-2} \text{ d}^{-1}$ ),  $C$  is the CH<sub>4</sub> concentration of the bubbles in  $\text{mg l}^{-1}$ ,  $A$  is the area of the funnel in  $\text{m}^2$ ,  $V$  is the displaced volume in the bottle and  $\Delta t$  is the number of days between sampling moments.

To correct for varying sampling frequency between measurements of diffusive CO<sub>2</sub> and CH<sub>4</sub>, a time-weighted mean was calculated per plot at each location. This was done by calculating the area under the curve (AUC) with time on the x-axis and the respective response variable (CO<sub>2</sub> and CH<sub>4</sub>) on the y-axis (AUC function with trapezoid method from the R package DescTools; [Signorell et al., 2022](#)). The weighted mean was obtained by dividing the AUC by the total number of days between the first and last measurement ( $\Delta x$ ). As the ebullition was measured continuously the time-weighted mean was calculated by summing the harvested bubbles per plot at each location and dividing it by the total numbers of days the bubble traps were in the field ( $\Delta x$ ).

To calculate total GHG emissions (CO<sub>2</sub> diffusive + CH<sub>4</sub> diffusive + CH<sub>4</sub> ebullitive) the CH<sub>4</sub> emissions were converted to CO<sub>2</sub> equivalents by multiplying with a factor of 27.2. This is the global warming potential of CH<sub>4</sub> over a 100-year period (GWP<sub>100</sub>) as defined by the IPCC (IPCC, AR6).

## 2.3. Abiotic conditions

Surface water samples were taken by hand (approximately 10 cm below the water surface) in July, August, September and November 2018, and February, April, May, June, July and August 2019. Sediment pore water samples were taken in July and November 2018, and once per month between April and August 2019. Also, both Secchi depth and water level were measured at every field visit. Porewater was sampled anaerobically in the top 15 cm of the aquatic sediment using MacroRhizons (9 cm, Eijkelpark Agrisearch Equipment). The pH was measured using a standard Ag/AgCl electrode (Orion Research Inc.) and total inorganic carbon (TIC) by injecting 0.2 ml sample in a N<sub>2</sub> (g) flushed chamber with 1 ml 0.4 M H<sub>3</sub>PO<sub>4</sub> connected to an Infrared Gas Analyser (IRGA; ABB Analytical). A 10 ml acidified subsample (65% HNO<sub>3</sub>) was stored at 4 °C until elemental analysis on an ICP-OES (ARCOS, Spectro Analytical, Kleve, Germany) for total phosphorus (TP), chloride (Cl) and sulphur (S). A 20 ml subsample was stored at – 20 °C until colorimetric analysis for nitrate (NO<sub>3</sub><sup>-</sup>) and ammonium (NH<sub>4</sub><sup>+</sup>) on an auto analyser system (Auto Analyser III, Bran and Luebbe GmbH).

Additional sediment porewater samples were collected for methane and sulphide analyses by connecting vacuumed 12 ml glass exetainers (Labco exetainer®, High Wycimbe, UK) with 0.5 ml of 1 M HCl to the same macro-rhizons as described above. Concentrations of CH<sub>4</sub> and sulphide were measured in the headspace of the exetainers (after removing the vacuum with N<sub>2</sub> gas) using gas chromatography and recalculated for the water volume using Henry's constants. CH<sub>4</sub> concentrations were measured using a Hewlett-Packard 5890 gas chromatograph (Avondale, California) equipped with a flame-ionization detector and a Porapak Q column (80/100 mesh) operated at 120 °C with N<sub>2</sub> as carrier gas. Sulphide concentrations were analysed using a 7890B gas chromatograph (Agilent Technologies, Santa Clara, USA) equipped with a Carbopack BHT100 glass column (2 m, ID 2 mm), flame ionization (FID) and flame photometric detector (FPD).



## 2.4. Statistics

All analyses were carried out using R (version 4.1.2, R Core Team, 2022). Enclosure, introduced macrophytes and salinity (brackish vs freshwater locations) effects on biomass yield were tested with a three-way ANOVA (*aov* function from the *stats* package; R Core Team, 2022), followed by a Tukey post-hoc test. Correlations between biomass and CH<sub>4</sub> and CO<sub>2</sub> emissions were analysed with linear random effects models (*lmer* function from the package *lme4*; Bates et al., 2015) with the time-weighted mean carbon emissions as the response variables and location as a random effect, followed by Analysis of Deviance (*Anova* function from the package *car*; Fox and Weisberg, 2018) with Type II Wald tests using a Kenward-Roger approximation to compute the F-statistic (see [Supplementary Table S2](#)).

Correlations between surface and pore water quality and CH<sub>4</sub> and CO<sub>2</sub> emissions were analysed with Pearson linear correlation (*stat\_cor* function in the package *ggpubr*; Kassambara, 2020) after taking the average per location for each point in time. It was assumed that the time in between measurements (4–6 weeks) was sufficient to assume independence. Because the effect of salinity on the C emission was tested directly with the aforementioned analysis a two-way ANOVA was used to test the effect of the enclosures and introduced macrophytes on the ebullitive and diffusive CH<sub>4</sub> emission, and diffusive CO<sub>2</sub> emission (*aov* function from the *stats* package). Residuals were visually checked for normality and heterogeneity of variances using histograms and qqplots. Data were ln-transformed to improve normality of the residuals when necessary. Correlations were compared using Akaike information criterion (AIC) values (R package *AICcmodavg*; Mazerolle, 2020). All graphs were created with the package *ggplot2* (Wickham et al., 2022).

## 3. Results

### 3.1. Abiotic environment

Surface water salinity levels fluctuated strongly over time both in the freshwater and brackish water locations ([supplementary Fig. S2](#)). Due to the active inlet of fresh surface water during the growing season, surface water salinity was generally lower during summer and higher during winter. For example, at locations B4 and B5 surface water chloride concentrations fluctuated from > 100 mmol l<sup>-1</sup> in November 2018, to < 6 mmol l<sup>-1</sup> in July 2019. Sediment porewater Cl concentrations were often higher than in the surface water and showed less fluctuations. The locations with a high surface water salinity were also more sulphate rich and often had high concentrations (100–1500 μmol l<sup>-1</sup>) of free sulphide in the sediment pore water ([Supplementary Fig. S2](#)). However, the sediment pore water of the freshwater locations contained free sulphide as well. Most notably at location F1, where in April 2019 a concentration of 123 ± 224 μmol l<sup>-1</sup> H<sub>2</sub>S (mean ± SD, n = 4) was measured.

All locations were generally rich in nutrients, both in the surface water and pore water, and can be characterised as eutrophic ([Supplementary Fig. S2](#)). Nutrient levels strongly fluctuated over time. Surface water nitrate concentrations ranged from ~10 μmol l<sup>-1</sup> to ~400 μmol l<sup>-1</sup>, with a peak concentration of 877 μmol l<sup>-1</sup> at location F3 on February 20 2019. Surface water ammonium (NH<sub>4</sub>) concentrations ranged from ~10 μmol l<sup>-1</sup> to 150 μmol l<sup>-1</sup>. The NH<sub>4</sub> concentration was much higher in the pore water compared to the surface water and ranged from ~30 μmol l<sup>-1</sup> at location B5 to 8.8 mmol l<sup>-1</sup> at location B6. Total dissolved phosphorus (TP) concentrations in the surface water were lower in the freshwater locations (all < 10 μmol l<sup>-1</sup>) than at the brackish water locations. In particular, high surface water TP concentrations (> 20 μmol l<sup>-1</sup>) were observed at location B6, with a peak concentration of 46 μmol l<sup>-1</sup>. Pore water TP concentrations were higher compared to the surface water at all locations. No clear difference between brackish and freshwater locations was observed ([Supplementary Fig. S2](#)). As the factor of turbidity was ruled out via site selection only minor attention was paid to surface water turbidity. Field Secchi depth measurements

(ranging from 19 to 105 cm during the growing season in cases where high macrophyte biomass did not impede Secchi depth measurements) did indeed indicate turbidity not to be an influential factor in the study sites selected. Differences in turbidity between treatments could not be assessed with sufficient accuracy due to the high number of missing values related to the high macrophyte biomass in several plots.

### 3.2. Submerged macrophyte development

The enclosures had a highly significant positive effect on submerged macrophyte development (Three-way ANOVA, [Supplementary Table S1](#)). During the two growing seasons of the experiment submerged macrophytes were able to establish at all six locations and reached high biomass in the enclosures, especially in those in which macrophytes were introduced (~ 100–350 g dw m<sup>-2</sup>). At four of the six locations spontaneous macrophyte development was observed in enclosures in which no macrophytes were introduced.

Introduction of macrophytes without enclosure did not have a significant effect on biomass production, but the interaction between enclosure and introduced macrophytes did have a significant positive effect on macrophyte biomass production ([Supplementary Table S1](#)). A Tukey-HSD post-hoc test revealed that the harvested biomass was significantly higher in the Enclosure+Plants treatment compared to both the Open (p = 0.0002) and Open+Plants (p = 0.0004) treatment and the Enclosure treatment (p = 0.039) ([Fig. 3](#)). No significant differences were found between the Open and Open+Plants treatment. Also, no significant effect of salinity (brackish vs fresh water locations) on the harvested biomass was found ([Supplementary Table S1](#)).

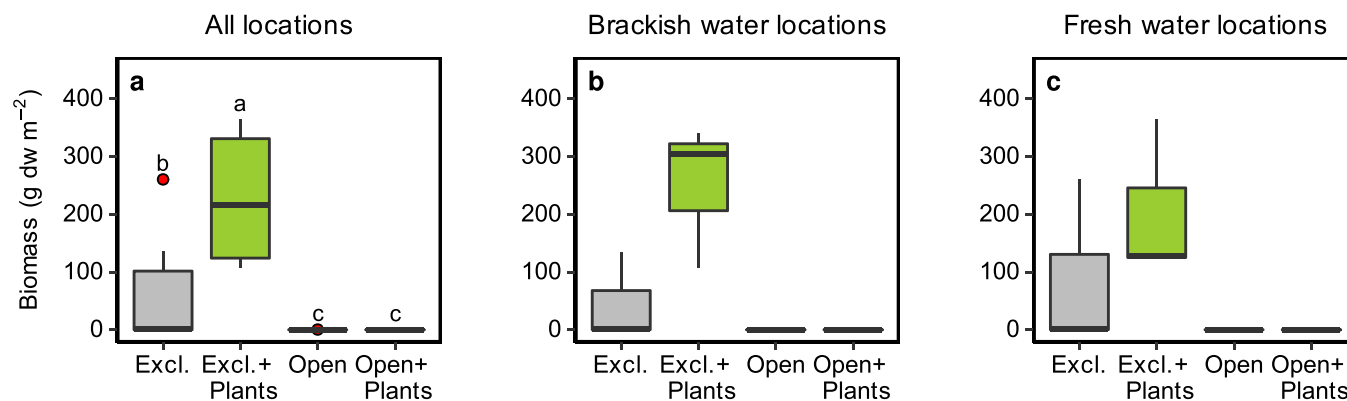
At four locations, macrophytes developed spontaneously inside the enclosures without introduced macrophytes. At two of those locations high biomass was reached as well. Most notably at location F1 where the total submerged macrophyte biomass did not differ substantially from the enclosure in which macrophytes were introduced (261 and 295 g dw m<sup>-2</sup>, respectively). No submerged macrophytes developed outside the enclosures ([Fig. 3](#)) and macrophytes that were introduced outside the enclosures were completely absent after the second growing season.

The macrophyte biomass was generally dominated by *E. nuttallii* at all locations, both in enclosures with and without introduced macrophytes. Coverage percentage of *E. nuttallii* reached ~100% at the moment of harvest (August 2019) in the Enclosure+Plants treatment of location F1, B4 and B5 ([Supplementary Fig. S3](#)). Of the other three introduced species *C. demersum* was also present at locations F1, F2 and B6, but biomass per plot was < 10 g dw m<sup>-2</sup> except in the Enclosure+Plants plot at location F1 (70 g dw m<sup>-2</sup>). *Potamogeton crispus* was not present anymore at any location except F2, where a very small amount (0.7 g dw m<sup>-2</sup>) was found in the Enclosure treatment. The fourth species introduced, *P. pectinatus*, disappeared at all locations. Some species that were not introduced established spontaneously, of which *Callitriche* sp. produced the most biomass (25 g dw m<sup>-2</sup> in the B4 enclosure treatment, [Supplementary Fig. S3](#)).

The enclosures successfully excluded large fish, but smaller fauna was able to enter. During harvest, at the end of the second growing season, several juvenile fish (e.g. *Cyprinus carpio* and *Sander lucioperca*) and crayfish (*Faxonius limosus*) were found in the enclosures ([Supplementary Table S2](#)). On average the number of individuals found in the Enclosure+Plants treatment (34 ± 33 SD) tended to be higher compared to the Enclosure treatment (12 ± 13 SD), but differences were not significant (two-sided t-test, t = -1.525, df = 10, p = 0.158) ([Supplementary Fig. S4](#)).

### 3.3. Diffusive C fluxes and ebullitive CH<sub>4</sub> fluxes

The enclosures had a significant effect on the diffusive CH<sub>4</sub> flux (Two-way ANOVA, [Supplementary Table S1](#)) and the diffusive CO<sub>2</sub> flux (Two-way ANOVA, [Supplementary Table S1](#)), but not on the ebullitive CH<sub>4</sub> flux (Two-way ANOVA, [Supplementary Table S1](#)). The introduction



**Fig. 3.** Total biomass per treatment on August 19 2019 for all locations (a,  $n = 6$ ), the locations with brackish water (b,  $n = 3$ ), and the locations with fresh water (c,  $n = 3$ ). Letters in panel a indicate significant differences ( $p < 0.05$ , Tukey-HSD post-hoc on three-way ANOVA).

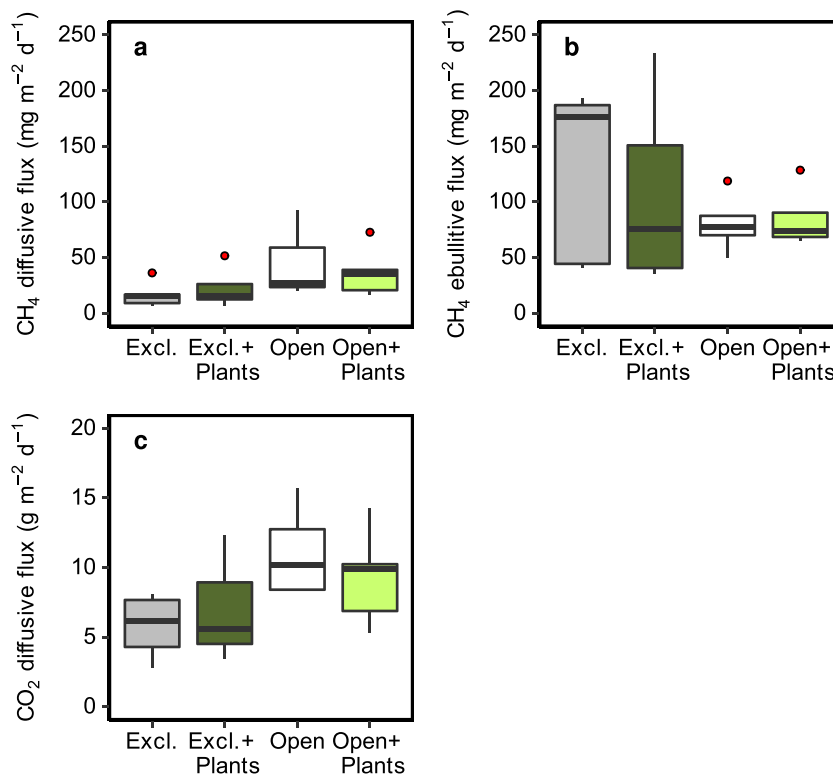
of macrophytes did not have a significant effect on the C fluxes. Also, the interaction effect between enclosure placement and the introduction of macrophytes was not significant. On average ( $\pm$  SD) the diffusive CH<sub>4</sub> fluxes were higher in the open treatments ( $44.5 \pm 30.8$  and  $36.9 \pm 22.0$  mg m<sup>-2</sup> d<sup>-1</sup>) compared to the enclosure treatments ( $16.9 \pm 13.4$  and  $26.4 \pm 21.9$  mg m<sup>-2</sup> d<sup>-1</sup>) (Fig. 4). The average ( $\pm$  SD) diffusive CO<sub>2</sub> fluxes were also higher in the open treatments ( $11.1 \pm 3.14$  and  $9.32 \pm 3.44$  g m<sup>-2</sup> d<sup>-1</sup>) compared to the enclosure treatments ( $5.79 \pm 2.45$  and  $7.10 \pm 4.62$  g m<sup>-2</sup> d<sup>-1</sup>).

Although no significant treatment-effect of macrophyte introduction was found, we did find a significant negative correlation between biomass yield and the time-weighted mean diffusive CH<sub>4</sub> and CO<sub>2</sub> flux (Fig. 5, Supplementary Table S2). The time-weighted mean ebullitive CH<sub>4</sub> flux did not correlate significantly with biomass yield (Fig. 5, Supplementary Table S2).

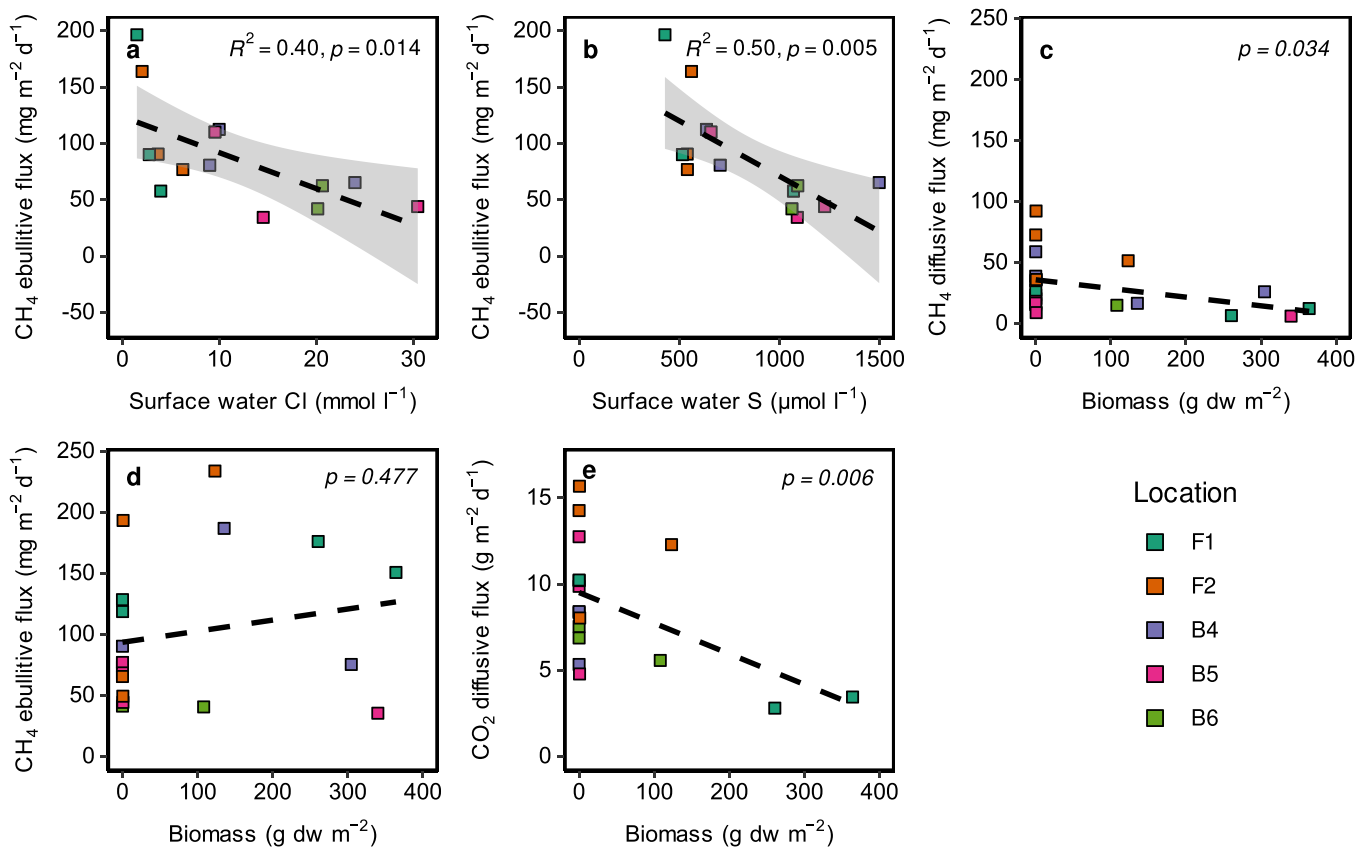
Analysing all locations together (averaging per location), we found a

significant negative linear correlation between the surface water Cl concentration and ebullitive CH<sub>4</sub> flux ( $R^2 = 0.40$ ,  $p = 0.014$ , Fig. 5). No correlations were found between surface water Cl and the diffusive CH<sub>4</sub> and CO<sub>2</sub> flux, or between pore water Cl and the ebullitive CH<sub>4</sub> flux, diffusive CH<sub>4</sub> flux, or diffusive CO<sub>2</sub> flux (Supplementary Fig. S5). Also, we found a significant negative linear correlation between the surface water S concentration and the ebullitive CH<sub>4</sub> flux ( $R^2 = 0.50$ ,  $p = 0.005$ ). No relationship was found between the C fluxes and pore water sulphide, pore water S (Supplementary Fig. S5), or surface water and pore water nutrient concentrations (data not shown).

At almost all (17 out of 19) plots more than 50% of the total carbon flux (CH<sub>4</sub> ebullitive + CH<sub>4</sub> diffusive + CO<sub>2</sub> diffusive) in CO<sub>2</sub>-eq consisted of the diffusive CO<sub>2</sub> flux. Only at location F1, in the Exclusion and Exclusion+Plants treatments, this was not the case (35% and 43%, respectively, Supplementary Table S4).



**Fig. 4.** Diffusive (a) and ebullitive (b) CH<sub>4</sub> fluxes and diffusive CO<sub>2</sub> fluxes (c) per treatment. The plotted values are time-weighted means per plot at location F1, F2, F3, B4 and B5 ( $n = 5$ ).



**Fig. 5.** Correlation between the average chloride concentration in the surface water per location and the average ebullitive methane flux per location (a), and the average sulphur concentration in the surface water per location and the average ebullitive methane flux per location (b). The regression lines in panels a and b represent a Pearson linear correlation, with the confidence interval (95%) in gray. Panels c, d and e show the correlation between biomass yield and the time-weighted mean carbon fluxes. The regression lines at panels c, d and e are simple linear models to point out the trend. Statistical significance was tested using random linear models with location (ditch) as the random factor (see Table S2).

## 4. Discussion

### 4.1. Factors influencing submerged macrophyte presence and development

In the aquatic systems studied, many factors could potentially hamper submerged macrophyte development, such as the eutrophic conditions, fluctuating salinity and presence of sulphide in the root-zone. However, our results clearly indicate that herbivory and/or bioturbation by fish played the strongest role, overruling the above-mentioned factors. The strong effect of herbivory and bioturbation by fish is evident through the absence of macrophytes outside the exclosures and the disappearance of introduced macrophytes outside the exclosures at all locations. The strong effect of bioturbation and herbivory by fish is in line with previous studies (e.g. Bakker et al., 2016; Lodge, 1991), and had large negative effects on submerged macrophyte biomass. In our experimental set-up only larger fish were excluded. Juvenile fish and crayfish were able to enter the exclosures during the two growing seasons and potentially used the exclosures as refugia and/or feeding grounds. Although we cannot exclude a negative influence of small fauna on macrophyte development, we observed high macrophyte biomass yield in the exclosures and no correlation was found between the number of fish in the exclosures and macrophyte biomass (supplementary Fig. S7). As Spiny-cheek crayfish (*Faxonius limosus*) were also found in the exclosures during harvest, a negative effect of crayfish on submerged macrophytes (e.g. Soes and Koese, 2010) cannot be excluded. However, our data clearly show that the effects of presence or absence of fish overrules the influence of crayfish on submerged macrophytes as crayfish presence was often found in combination with high macrophyte biomass in the exclosures. This is in line with

an earlier study that indicates that *Faxonius limosus* has only minor effects on submerged macrophytes—especially when compared to other crayfish (Soes and Koese, 2010). Monitoring data indicate low crayfish densities on the island (Koese, 2021), hence we suspect that the effect of crayfish on macrophytes on Goeree-Overflakkee is currently limited.

A recent study on the same study sites showed plant germination on all locations (based on a laboratory germination experiment), indicating that dispersal limitation is probably not a major limiting factor for plant development in the region (Velthuis et al., 2023). However, macrophyte species richness was low throughout the study period. This is likely related to the combination of high turbidity, fluctuating and high salinity levels, differences between surface water and sediment salinity, free sulphide presence in the sediment and high ammonium and phosphorus concentrations. The surface water turbidity was generally high (Secchi depths generally < 50 cm, Supplementary Fig. S1), but we assume that turbidity did not limit macrophyte growth as the ditches are shallow (average  $81 \pm 21$  (SD) cm). Notably, however, macrophytes were found to be covered with silt days after temporarily strong currents (caused by the pumping station that regulates the water level in the ditches on the island). This may be detrimental for species less adapted to (periodic) semi-turbid conditions. The studied ditches were selected to cover a wide range of salinities. These salinity levels were found to fluctuate over time (Supplementary Fig. S2) and regularly surpassed thresholds that can limit macrophyte development (Hinojosa-Garro et al., 2008; Kaijser et al., 2019). High and fluctuating salinity levels are known to negatively impact submerged macrophytes, due to direct osmotic stress, delayed growth and or a disturbed metabolism (e.g. McGahee and Davis, 1971; Velthuis et al., 2023). In addition, elemental imbalances can cause physiological stress and internal toxicity, which

can lead to mortality (e.g. Masood et al., 2006; Rout and Shaw, 2001). Particularly oligohaline species such as *C. demersum* and *P. crispus*—with salinity tolerances up to 43 mmol l<sup>-1</sup> Cl in the field (Kajiser et al., 2019 and references therein) and toxicity effects at ~100 mmol l<sup>-1</sup> Cl under laboratory conditions (Hinojosa-Garro et al., 2008)—did not thrive in our brackish water ditches. The total biomass of the submerged vegetation, however, seemed unaffected by salinity as indicated by the absence of a significant difference between the freshwater ditches (F1, F2 & F3) and the brackish ditches (B4, B5 & B6). This can be explained by the fact that the vegetation in the enclosures in the brackish water ditches was dominated by one generalist species: *E. nuttallii*. In the freshwater ditches *E. nuttallii* was co-dominating with *C. demersum*. *E. nuttallii* is known to tolerate salinity levels up to 14‰ (≈ 240 mmol l<sup>-1</sup> NaCl) (Josefsson, 2011), relatively low light availability (Abernethy et al., 1996; Angelstein and Schubert, 2009) and high nutrient concentrations (Melzer, 1999; Zehnsdorf et al., 2015).

The macrophyte species *Potamogeton crispus* and *P. pectinatus* were not able to settle after introduction or were outcompeted at all locations. This may be explained by the fact that *E. nuttallii* is able to quickly colonize the water column—when nutrients are not limiting—through rapid stem elongation, forming dense canopies which reduces growth of other slower growing macrophytes because of light limitation (Barrat-Segretain, 2004; Zehnsdorf et al., 2015). Salinity stress is less likely to have been a growth-inhibiting factor for *P. pectinatus*, as it is known to grow in brackish water up to sea water salinity (Van Wijk, 1988).

High salinity levels often coincide with free sulphide presence and high ammonium levels (Herbert et al., 2015; van Dijk et al., 2019) as shown in the present study as well. Sulphate reduction in saline sediments produces free sulphide which can already be toxic for organisms at concentrations of several μmol per litre (Lamers et al., 2013). Additionally, the high concentration of positively charged ions in brackish waters can reduce the binding capacity of ammonium in the sediment inducing ammonium release to the water column (Herbert et al., 2015; van Dijk et al., 2015). Fluctuating salinity can influence nitrogen and phosphorus availability and thereby alter nutrient balances (Herbert et al., 2015; van Dijk et al., 2019). Due to the eutrophic nature of the surface water and sediments in the region it is not expected that nutrient limitations are a major issue. High ammonium concentrations can however be toxic and at (temporal) high pH also ammonia can be formed which is toxic already at low concentrations (e.g. Clarke and Baldwin, 2002). The multi-stressor environment to which the submerged macrophytes are exposed likely explains the small number of submerged macrophyte species that established themselves in the enclosures and that were able to thrive.

#### 4.2. Consequences for diffusive C fluxes and ebullitive CH<sub>4</sub> fluxes

Restoring macrophyte communities may not only improve biodiversity (see review by Hilt et al., 2017), but also reduce greenhouse gas emissions (Aben et al., 2022; Colina et al., 2021a; Davidson et al., 2015). Our findings confirm this as we found significant lower CH<sub>4</sub> and CO<sub>2</sub> diffusive emissions in enclosures (Fig. 4). Arguably, other variables—e.g. flow velocity and bioturbation—likely also differed inside and outside the enclosures which may also have impacted C emissions. Zooming in on the relationship between submerged biomass and diffusive CH<sub>4</sub> and CO<sub>2</sub> fluxes, however, substantiates the impact of submerged macrophytes on diffusive emissions (Fig. 5c and e). However, the ebullitive CH<sub>4</sub> flux did not significantly differ between the enclosures and open plots (Fig. 4) and was not significantly correlated to submerged macrophyte biomass (Fig. 5). This is in contrast with earlier experimental studies and field observations (Aben et al., 2022; Colina et al., 2021a; Davidson et al., 2018). Different processes may underlie this apparent discrepancy. For one, it may be related to differences in species traits that were studied. The abundant species present in the mesocosms of Davidson et al. (2018) (*Potamogeton crispus*, see Davidson et al., 2015) and those in the Uruguayan lakes (*Egeria densa*) studied by Colina et al.

(2021a) have well developed root systems and exhibit radial oxygen loss which may reduce methanogenesis. The dominant species in the enclosures in our experiment (*E. nuttallii*), however, is known to reduce investment in root development under eutrophic conditions—as it can take up nutrients through stems and leaves (Zehnsdorf et al., 2015). While this likely explains why this species is tolerant to the unfavourable sediment conditions in our study ditches it may also imply that radial oxygen loss is limited and hence little associated methanotrophy takes place in the sediment. Methanotrophy in the water column populated by submerged macrophytes may however be high (Davidson et al., 2015), possibly explaining the observed negative relationship between biomass and diffusive CH<sub>4</sub> emission. Beside the different effects submerged macrophytes may have on ebullitive and diffusive emissions in our ditches (see Bodmer et al., 2021 for a review on all possible macrophyte effects), also fish and other bioturbators may have played a role. The higher fish bioturbation outside the enclosures may have reduced not only submerged macrophyte growth, but also ebullition (as found by Oliveira Junior et al., 2019).

In line with our hypothesis and other studies (Marton et al., 2012; Weston et al., 2006), we found a negative relationship between surface water S and Cl concentrations and ebullitive CH<sub>4</sub> emissions. This could be explained by reduced methanogenic activity under more saline and sulphate rich environments due to sulphate reducing bacteria outcompeting the methanogenic bacteria as well as due to sulphide and/or osmotic stress for methanogenic bacteria (Herbert et al., 2015). Most probably, sulphate intrusion from the surface water to the sediment and consequential sulphate reduction had the strongest effect on reduced methanogenesis, as surface water sulphate concentrations had a stronger negative correlation with CH<sub>4</sub> emissions as surface water chloride concentrations (Akaike weight of 0.77 and 0.23, respectively). Enhanced anaerobic CH<sub>4</sub> oxidation coupled to sulphate reduction under sulphate rich conditions may have also played a role in reducing CH<sub>4</sub> emissions (Nauhaus et al., 2002; Milucka et al., 2012). Further studies are needed to disentangle the effects of salinity, interactions with nutrient and carbon availability on net CH<sub>4</sub> emissions.

A large share of the carbon emissions from our study ditches to the atmosphere took place in the form of CO<sub>2</sub> (Supplementary Table S4). The CO<sub>2</sub> emission intensities in our study ditches were similar to those measured in ditches in the North of the Netherlands (Hendriks et al., In prep) but higher than the—also high—ditch emissions reported by Deng et al. (2020) and Schrier-Uijl et al. (2011). The relatively high CO<sub>2</sub> emissions from ditches can likely be explained by both high within-ditch decomposition rates linked to the thick layer of organic matter on the bottom of these shallow waters as well as to the runoff and infiltration of water from the surrounding agricultural fields containing CO<sub>2</sub>. This degassing of terrestrial derived CO<sub>2</sub> from ditches has been suggested to substantially contribute to ditch CO<sub>2</sub> emissions (Evans et al., 2016). Within-ditch primary production may decrease aquatic CO<sub>2</sub> emissions or even lead to CO<sub>2</sub> uptake (e.g. Balmer and Downing, 2011; Hilt, 2015; Kosten et al., 2010). This corresponds with our finding that the CO<sub>2</sub> diffusive emission significantly decreased with plant biomass (Fig. 5).

#### 4.3. Implications for future management of fresh and brackish eutrophic waters

Our work shows that herbivory and bioturbation by large fish strongly impacts the presence and development of submerged vegetation in the fresh and brackish eutrophic study ditches. In addition, our results also indicate that a restored submerged macrophyte vegetation can reduce diffusive CH<sub>4</sub> and CO<sub>2</sub> emissions. We argue that longer monitoring following the effect of dense vegetation development—with possibly a more extensive rhizosphere development but possibly also an increase in sedimentation due to increased primary production and decreased flow rates—is needed to determine the overall effect of vegetation development on GHG emission in these ditches.

While the exclusion of large fish clearly enhanced macrophyte



growth, local conditions such as high nutrient concentrations, fluctuating and or high salinity levels as well as high turbidity will make it challenging to restore a diverse macrophyte community on the long term. In our study, fast growing ubiquitous species such as *E. nuttallii* and *C. demersum* became dominant once herbivory and bioturbation by fish was excluded, as was shown in previous studies (Reinertsen and Olsen, 1984). Under present conditions these ubiquitous species will reach high densities, out-compete specialist species and will limit the development of a more diverse aquatic ecosystem.

For future water management, our study showed fish removal or exclusion of fish to be a potentially successful measure to restore submerged macrophyte growth, potentially resulting in decreased GHG emissions as an added benefit. The eutrophic nature of the study area will likely result in highly productive submerged macrophyte communities—as was the case in the 1980's before benthivorous fish were introduced—and may require vegetation management in the future to ensure sufficient supply and discharge of water in these agricultural waterways. The results of the present study will guide future water management decisions, especially with the current need to restore aquatic biodiversity and reduce aquatic greenhouse gas emissions.

### 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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### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.aquabot.2023.103645](https://doi.org/10.1016/j.aquabot.2023.103645).

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