Insights into autotrophic carbon fixation strategies through metagonomics in the sediments of seagrass beds

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A B S T R A C T

Seagrass beds contributes up to 10% ocean carbon storage. Carbon fixation in seagrass bed greatly affect global carbon cycle. Currently, six carbon fixation pathways are widely studied: Calvin, reductive tricarboxylic acid (rTCA), Wood-Ljungdahl (WL), 3-hydroxypropionate (3HP), 3-hydroxypropionate/4-hydroxybutyrate (3HP/4HB) and dicarboxylate/4-hydroxybutyrate (DC/4-HB). Despite the knowledges about carbon fixation increase, the carbon fixation strategies in seagrass bed sediment remain unexplored. We collected seagrass bed sediment samples from three sites with different characteristics in Weihai, a city in Shandong, China. The carbon fixation strategies were investigated through metagenomics. The results exhibited that five pathways were present, of which Calvin and WL were the most dominant. The community structure of microorganisms containing the key genes of these pathways were further analyzed, and those dominant microorganisms with carbon fixing potential were revealed. Phosphorus significantly negatively corelated with those microorganisms. This study provides an insight into the strategies of carbon fixation in seagrass bed sediments.

1. Introduction

The global warming crisis forces us to find ways to reduce CO_2 concentration in the atmosphere. At present, countries around the world are developing new energy sources to reduce CO_2 emissions. On the other hand, it is also important to vigorously improve the CO_2 assimilation during the autotrophic processes. It was estimated that more than half of Earth's carbon is captured by marine organisms (Balmaseda et al., 2013). In marine environment, more than 50% of carbon sinks occur in the coastal ecosystems, although these coastal zones account for a small fraction of the ocean's surface (Mao et al., 2016; Mcleod et al., 2011). Therefore, it is a key issue to improve the carbon sequestration potential of the coastal areas facing climate warming (Mao et al., 2016).

Chinese coastline is mainly composed of three types of ecosystems: mangroves, seagrass beds and salt marshes (Mao et al., 2016). The coastal zone of Shandong Peninsula is an important distribution area of seagrass beds in northern China (Zheng et al., 2014). In spite of estimated less than 0.2% cover of the world's ocean, seagrass beds contribute up to 10% of global ocean carbon storage, and most carbon is stored in sediment of seagrass beds (Duarte et al., 2005; Fourqurean et al., 2012; Liu et al., 2017a, 2017b). Among them, seagrass itself contributes half of the carbon fixation (Duarte and Chiscano, 1999). In addition to seagrass, autotrophs in sediments should also play an important role in carbon fixation in this ecosystem (Cúcio et al., 2018). However, the processes and mechanisms of autotrophic carbon fixation in seagrass bed sediments are still unknown.

Currently, seven biological CO_2 fixation pathways have been discovered, including the Calvin-Benson-Bassham (CBB) cycle, the reductive tricarboxylic acid (rTCA) cycle, the reductive acetyl-CoA Pathway or Wood-Ljungdahl (WL) pathway, 3-hydroxypropionate (3HP) bicycle, 3-hydroxypropionate/4-hydroxybutyrate (3HP/4HB) cycle, dicarboxylate/4-Hydroxybutyrate (DC/4-HB) Cycle and the reductive glycine (rGly) pathway (Alfreider et al., 2018; Jiang et al., 2022; Sanchez-Andrea et al., 2020; Song et al., 2020). In addition, TCA cycle was also found recently that it can be driven in the reverse under autotrophic conditions. But this pathway lacks the key enzyme (Nunoura et al., 2018; Mall et al., 2018). Besides, rGly pathway is a new discovery. So, it is difficult to make bioinformatical predictions for these

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two pathways. Therefore, this study focused on the six carbon fixation pathways (Calvin, rTCA, WL, 3HP, 3HP/4HB and DC/4-HB) that have sufficient reference gene sequences through metagenomic.

In nature, CBB cycle is the quantitatively most important pathway for autotrophic carbon fixation (Berg et al., 2010). CBB cycle had long been considered to be the only biochemical autotrophic CO_2 fixation pathway of significance in the ocean (Hügler and Sievert, 2011). However, in the past decade, the other autotrophic pathways have been discovered successively, and many pathways are shown to play important roles in a range of oceanic ecosystems (Hügler and Sievert, 2011). These alternative carbon fixation pathways are used mainly by chemolithoautotrophic bacteria and archaea (Berg et al., 2010; Martin et al., 2008; Sanchez-Andrea et al., 2020; Song et al., 2020). CBB cycle, 3-HP bicycle and 3-HP/4HB cycle occur under fully aerobic conditions. The aerobic autotrophs are energy-demanding. Unlike that, WL pathway, rTCA cycle and DC/4-HB cycle are used by anaerobic or microaerophilic microorganisms. These anaerobic chemolithoautotrophs require much less energy (Berg et al., 2010; Hügler and Sievert, 2011). CBB cycle is the only carbon fixation pathway running in eukaryotes (algae and plants). Besides, the CBB cycle also occurs in cyanobacteria and photo- and (aerobic) chemoautotrophic bacteria (Berg, 2011; Chi et al., 2018b). However, the other different carbon fixation pathways are not related to distinct taxonomic groups because they usually widespread among prokaryotes (Berg, 2011). Therefore, it is difficult to identify carbon fixation strategies by commonly used phylogenetic gene markers (e.g. 16S rRNA) for species taxonomy. Nevertheless, functional genes encoding key enzymes involved in different CO_2-assimilation pathways provides an excellent way to investigate specific CO_2 fixation strategy (Hügler and Sievert, 2011).

To date, the key and characteristic enzymes of each pathway have also been identified along with the elucidation of the six autotrophic CO_2 fixation mechanisms (Berg, 2011; Ruiz-Fernández et al., 2020). The gene encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO, EC:4.1.1.39) has been widely used as a molecular marker to indicate the autotrophs employing CBB cycle for carbon fixation (Alfreider et al., 2018; Berg, 2011; Chi et al., 2018b). For rTCA cycle, the gene of ATP citrate lyase (EC:2.3.3.8) catalyzing the cleavage of citrate to acetyl-CoA and oxaloacetate is often used as a molecular marker to study this cycle (Alfreider et al., 2018). The key enzyme genes of other pathways have also been successively used as molecular markers to study the autotrophic diversity and community structure in specific environment, e.g. the gene of acetyl-CoA synthase [EC:2.3.1.169] for WL pathway (Ruiz-Fernández et al., 2020), and the gene of 4-hydroxybutyryl-CoA dehydratase (EC:4.2.1.120) for 3-HP/4-HB cycle (Alfreider et al., 2018; Ruiz-Fernández et al., 2020). In the related studies, cloning library technology were used predominantly. Limited by this technology, it is very difficult to analyze simultaneously multiple pathways and evaluate their ecological niches in the specific environment. Ocean sediments are O_2-depleted, those chemoautotrophic pathways rather than the CBB cycle may also play an important role in carbon fixation. In such case, the niche determination of different CO_2-assimilation pathways is the first issue to be clarified. Metagenomic analysis based on high-throughput sequencing can not only analyze the six pathways simultaneously and identify their niches, but also obtain more knowledge about those rare species undetected by clone library. Currently, there are several reports about metagenomic studies of microbial carbon fixation pathways in some types of environments (Cúcio et al., 2016, 2018). Metagenomic studies of seagrass bed sediments can be used to analyze autotrophic biodiversity and community structure of all six carbon fixation pathways, and thus to determine their ecological niches.

The total area of existing seagrass beds in China is about 87.65 km^2, which is divided into two major distribution areas: the South China and the Yellow-Bohai Sea seagrass, the latter mainly distributes in Weihai, Shandong provinces (Zheng et al., 2014). The seagrass beds in Weihai distributed in Yue Lake (Swan Lake), Sanggou Bay and Lidao Bay (Zheng

et al., 2014). These seagrass beds are dominated by *Zostera marina* (*Z. marina*), which grow mainly in the middle and upper parts of the intertidal silt (Zheng et al., 2014). This study aims to determine the ecological niches of different carbon fixation pathways in seagrass bed sediments of northern China, to analyze the biodiversity and community structure of the autotrophic microorganisms, and further reveal the impacts of environmental factors on the autotrophic microorganisms. The results will help us to understand the process and mechanism of autotrophic carbon fixation in seagrass beds, and give instructions to improve the carbon sequestration potential of seagrass beds.

2. Material and methods

2.1. Samples collection and processing

The sediment samples of seagrass beds were respectively collected from the Lidao Bay, Sanggou Bay and Swan Lake in Rongcheng, Weihai city in April 2021, when the *Z. marina* has emerged seedlings. The sampling sites were sequentially named as LDW, SGW and TEH. The samples name suffixed with the letter "s" indicate the sediment with growing *Z. marina*, and those with the letter "c" suffix indicate the control samples without *Z. marina*. The location of the three sampling sites are shown in Fig. 1. From each sampling site, three samples were taken as replicates, whose detailed latitude and longitude information are shown in Supplementary Material 1 Table S1. TEH is located in a Rongcheng Swan National Nature Reserve, where sea cucumbers or other seafood are sometimes cultivated. The sampling site LDW in Lidao Bay is close to the village and susceptible to more domestic pollution. SGW is far from the other two sampling points in Sanggou Bay. The sampling site SGW is not in the protected area, and is less affected by domestic pollution. Approximately 2 cm of sediment surface was removed during sampling, beneath it, the sediment with a depth of 2–15 cm was collected for the current study. For any sampling site, about 1000 g sediment dispersed in different locations of this site was collected and then homogenized. Then, about 100 g was separated from the homogenized sediment and stored at −20 °C for the subsequent DNA extraction. The rest were air-dried at room temperature for the detection of physical and chemical parameters. The pH, water content (WC), organic matter (OM), total nitrogen content (N) and total phosphorous content (P) were determined according to the previous reports (Chi et al., 2013, 2015, 2018a), and the results of these parameters were indicated in Supplementary Material 1 Table S2.

2.2. DNA extraction, library construction and metagenomic sequencing

0.5 g sediment of each sample was used to extract the total genomic DNA. Total genomic DNA was extracted by the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) following the manufacturer's instructions. DNA concentration and purity was detected using TBS-380 Mini-Fluorometer and NanoDrop 2000 Spectrophotometer, respectively. DNA extraction quality was determined by 1% agarose gel. The qualified DNA that was extracted was fragmented to an average length of about 400 bp using ultrasonic crusher Covaris M220 (Gene Company Limited, China) for the construction of paired-end library, which was constructed using NEXTFLEX® Rapid DNA-Seq Kit (Bioo Scientific, Austin, TX, USA). The blunt-end of fragments were ligated with the adapters containing the full complement of the sequencing primer hybridization sites. Paired-end sequencing was performed on Illumina NovaSeq platform (Illumina Inc., San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) using NovaSeq Reagent Kits according to the manufacturer's instructions (www.illumina.com). The sequence data has been deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive database (Accession Number: SRP383171).
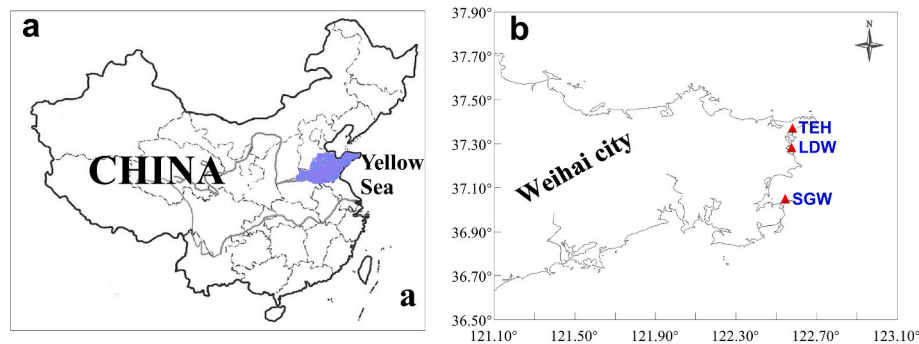
Fig. 1. a. The map of China. The location marked with purple is Shandong Province where the sampling sites of Weihai are located. b. Location of the sampling sites LDW, SGW and TEH in Weihai coastal seagrass beds. From each sampling site, three samples were taken as replicates. Swan Lake (TEH) is located in a national nature reserve, LDW is close to the village. SGW is in the natural sea area.

2.3. Sequence quality control and metagenomic assembly

The adapter sequences were stripped from the 3′ and 5′ end of Illumina reads using Fastp (https://github.com/OpenGene/fastp, version 0.20.0) (Chen et al., 2018). Low-quality reads with length less than 50 bp, average base mass value less than 20 and containing the N base were removed by Fastp (https://github.com/OpenGene/fastp, version 0.20.0), and other parameters were kept the default values (Chen et al., 2018). Then, the high-quality reads were assembled using MEGAHIT (https://github.com/voutcn/megahit , version 1.1.2) that is based on succinct de Bruijn graphs principle. The concatenation parameter iterates from small k-mer to large k-mer, and the other parameters were kept the default values (Li et al., 2015). The contigs equal to or longer than 300 bp were finally selected as assembly results, and then the contigs were used for further gene prediction and annotation.

2.4. Gene prediction, taxonomy and functional annotation

The prediction of open reading frames (ORFs) in each assembled contig were used by MetaGene (http://metagene.cb.k.u-tokyo.ac.jp/) (Noguchi et al., 2006). The predicted ORFs with length being or over 100 bp were retrieved and translated into amino acid sequences using the NCBI translation table (transl_table = 11) (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/index.cgi?chapter=tgencodes#SG1). CD-HIT (http://www.bioinformatics.org/cd-hit/, version 4.6.1) was used to cluster the predicted genes with a 90% sequences identity (90% coverage) (Fu et al., 2012), the longest sequences of each cluster were selected as the representative sequences to construct the non-redundant gene catalog. The high-quality reads from each sample were mapped to the non-redundant gene catalog with 95% identity by SOAP aligner (Li et al., 2008) (http://soap.genomics.org.cn/, version 2.21), and gene abundance in each sample was calculated.

The amino acid sequences of the non-redundant gene catalog were aligned to NCBI Non-Redundant Protein Sequence Database (NR) with e-value cutoff of $1e^{-5}$ using BLASTP by Diamond (http://www.diamondsearch.org/index.php , version 0.8.35) (Huson and Buchfink, 2015). Taxonomic annotations about species were obtained from the corresponding taxonomic information in NCBI NR database, and then the species abundance was calculated using the sum of the corresponding gene abundance. For the functional annotation, the amino acid sequences of the non-redundant gene catalog were aligned to Kyoto Encyclopedia of Genes and Genomes database (KEGG, version 94.2) with e-value cutoff of $1e^{-5}$ using BLASTP by Diamond (http://www.diamondsearch.org/index.php , version 0.8.35) (Huson and Buchfink, 2015). The KEGG functions of the genes can be obtained. The abundances of corresponding functional categories were calculated using the sum of the gene abundances corresponding to the same KEGG Orthology (KO) and KEGG Module.

In KEGG, the KEGG modules of M00165, M00173, M00377,

M00376, M00375 and M00374 represent the CBB cycle, rTCA cycle, the WL pathway, 3HP bicycle, 3HP/4HB cycle and DC/4-HB Cycle, respectively. By comparing the abundance of different KEGG modules, the ecological niches of different carbon fixation pathways can be roughly concluded. Due to an enzyme may play a function in different modules, a module abundance may be overestimated. It is more accurate to use the abundance of key and characteristic enzymes in a pathway of carbon fixation to infer the role of this pathway. In KEGG Orthology (KO) database, the enzymes with highly similar sequences are grouped in the same KO number and considered to have the same function. A certain KO abundance in the sample represents the abundance of enzymes with this function. In this study, the abundance of K01601, K15230, K15023, K14470, K15019 and K14467 were used to quantify the sequences related to ribulose-bisphosphate carboxylase in CBB cycle, ATP-citrate lyase in rTCA cycle, 5-methyltetrahydrofolate corrinoid/iron sulfur protein methyltransferase in WL pathway, 2-methylfumaryl-CoA isomerase in 3HP bicycle, 3-hydroxypropionyl-coenzyme A dehydratase in 3HP/4HB cycle and 4-hydroxybutyrate—CoA ligase in DC/4-HB Cycle, respectively.

2.5. Multivariate statistical analyses

The abundance bubble graphics showing the abundance of dominant microbial species in each carbon fixation pathway were drawn by the online tool of Majorbio Cloud Platform (https://cloud.majorbio.com/page/tools/). In each bubble graphic, the sizes of those filled circles are proportional to the relative abundance of the gene sequences of key enzyme for each carbon fixation pathway per metagenome. In order to reveal the significant environmental parameters affecting the carbon fixation microorganisms in seagrass bed sediment, those multivariate statistical analysis including redundancy analysis (RDA), canonical correlation analysis (CCA) and correlation heatmap analysis were performed using vegan and pheatmap packages within the R statistical Package (version 3.3.3) (https://www.r-project.org/). The significance of the RDA/CCA models were examined by Monte Carlo permutation at the 5%. In correlation heatmap analysis, Spearman coefficient was used to calculate the relationship between the microorganisms at the genus level and environment factors detected in this study.

3. Results

The microbial diversity and community structure in the sampled sediment of seagrass bed were shown in Supplementary Figs. S1–2 at the levels of phylum and genus, respectively. The abundance of key enzyme in each pathway was used to reflect the contribution of this pathway in carbon fixation. The sequences abundance of key enzyme associated with their respective pathway was presented in Supplementary Material 2. In SGW and TEH samples, the sequence K01601 associated with ribulose-bisphosphate carboxylase in the CBB cycle was the most

abundant, followed by sequence K15023 (KO for 5-methyltetrahydrofolate corrinoid/iron sulfur protein methyltransferase) related to the WL pathway (Fig. 2), indicating that in the sediments, with or without seagrass *Z. marina*, it is the CBB cycle and the WL pathway that play the most important role in carbon fixation. But the main carbon fixation strategy is different in LDW with high phosphorus level. In LDW without seagrass *Z. marina*, the abundance of key genes of CBB cycle and 3HP bicycle were dominant (indicated by the sequence abundance of K14470 of 2-methylfumaryl-CoA isomerase); while the dominant carbon fixation pathway is only CBB cycle in LDW with *Z. Marina* (Fig. 2). A small number of sequences related to K15230 (KO for ATP-citrate lyase) in rTCA cycle and K15019 (KO for 3-hydroxypropionyl-coenzyme A dehydratase) in 3HP/4HB cycle were found in SGW and TEH. No sequences associated with K14467 (KO for 4-hydroxybutyrate—CoA ligase) in DC/HB cycle had been detected from all the sampling sites, suggesting that DC/HB cycle was absent in the sampled seagrass bed sediments. The abundance of microorganisms containing the key genes of these carbon fixation pathways accounted for 0.016%–0.042% of the total microbial abundance in the sediment (Supplementary Fig. S3). The abundance, diversity and community structure of these microorganisms were respectively investigated at different classification levels. For each pathway, the abundance of major species containing the key gene (top four) at the phylum or class levels (for *Proteobacteria*) was shown in Fig. 3 at each sampling site. It can be seen that *Proteobacteria* are the most abundant microorganism containing the key genes in any carbon fixation pathway in all samples (Fig. 3). If further subdivided, these *Proteobacteria* are distributed in different classes for different carbon fixation pathways (Fig. 3). Besides, the dominant environmental factors affecting the microbial species containing the key genes in these carbon-fixation pathways were revealed by the environmental factor correlation analysis. It must be pointed out that sample LDW_S2 was excluded in this study, because the DNA concentration extracted from LDW_S2 was an order of magnitude higher than other samples, and its analysis results were too different from other parallel samples. At the same sampling time, the LDW water depth is much deeper than the other two sampling sites of SGW and TEH. In LDW_S2, the sediment sampling depth may be not well controlled, resulting in the discrepancies in corresponding results.
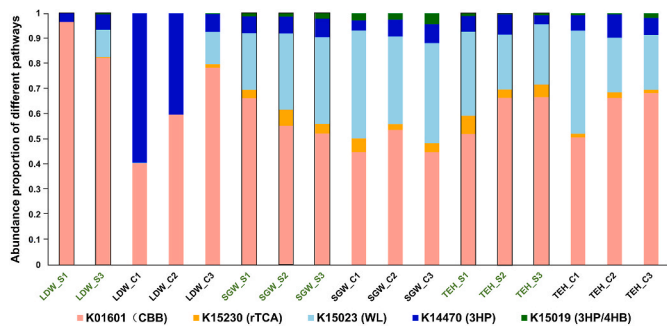

Fig. 2. The relative abundance of sequences related to the carbon fixation pathways in each sampling site of LDW, SGW and TEH. The samples name in green suffixed with the letter "s" indicate the sediment with growing *Z. marina*, and their bar graphs are framed in black. The samples name with the letter "c" suffix indicate the control samples without *Z. marina*, and their bar graphs are not framed by black edges. K01601 (KO number in the KEGG database) is the key enzyme of ribulose-bisphosphate carboxylase involving in CBB cycle. K15230 is the key enzyme of ATP-citrate lyase in the rTCA cycle. K15023 is the key enzyme of 5-methyltetrahydrofolate corrinoid/iron sulfur protein methyltransferase in the WL pathway. K14470 is the key enzyme of 2-methylfumaryl-CoA isomerase in the 3HP bicycle. K15019 is the key enzyme of 3-hydroxypropionyl-coenzyme A dehydratase in the 3HP/4HB cycle.
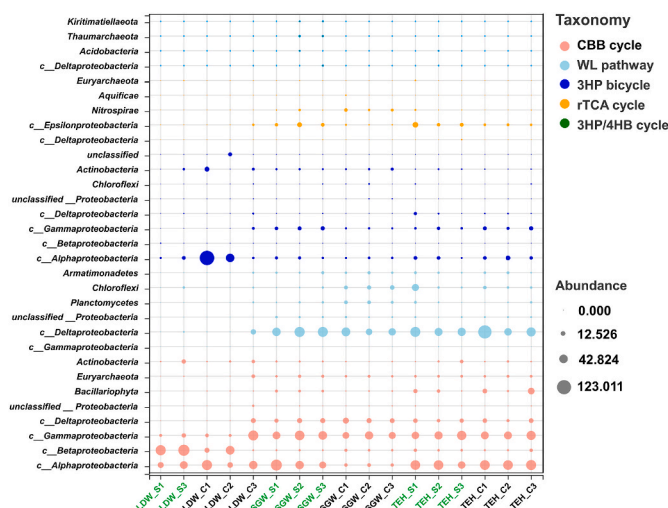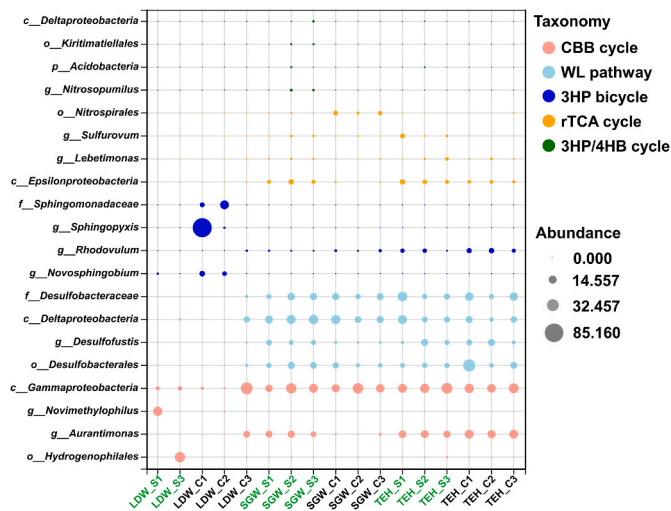

Fig. 3. Abundances of the top four taxonomic autotrophs (at the phylum level) using different carbon fixation pathways in the seagrass bed sediments from LDW, SGW and TEH. The sizes of these filled circles are proportional to the relative abundance of the gene sequences of key enzyme for each carbon fixation pathway per metagenome. *Proteobacteria* are subclassified from the class level, and the prefix letter "c" represents "class". The samples name suffixed with the letter "s" indicate the sediment with growing *Z. marina*, and these names are shown in green. The samples name with the letter "c" suffix indicate the control samples without *Z. Marina*, and they are shown in black.

3.1. Microbial diversity and community structure in CBB cycle and their influencing factors

The sequence abundance of K01601 (KO for ribulose-bisphosphate carboxylase large chain) belong to the CBB cycle is the highest, indicating that CBB cycle is the most dominant carbon fixation pathway in all sediment samples (Fig. 2 and Supplementary Material 2). In CBB cycle, the phylum of *Proteobacteria* is the absolute most dominant carbon-fixing autotrophs, with an approximate abundance of 85.7% ± 9.92% in LDW , 74.17% ± 3.56% in SGW, and 69.82% ± 3.94% in TEH, respectively. *Proteobacteria* in CBB cycle mainly distributes in the classes of *α-Proteobacteria*, *β-proteobacteria* and *γ-proteobacteria* (Fig. 3). In addition to *Proteobacteria*, the autotrophic species that utilize the CBB cycle for carbon fixation also include *Bacillariophyta*, *Euryarchaeota*, *Actinobacteria* and so on at the phylum level (Fig. 3). The barplot analysis of autotrophic species at the phylum level (except *Proteobacteria*) in CBB cycle are shown in Supplementary Fig. S4. Genus-based heatmap analysis of carbon-fixing autotrophs using CBB cycle were shown in Supplementary Fig. S5. Among them, the four most abundant carbon-fixing organisms at the genus level are *Aurantimonas*, *Novimethylophilus*, unclassified *Hydrogenophilales* order (can't be further classified) and unclassified *γ-proteobacteria* class (Fig. 4). It is evident that the autotrophic diversity and community structure in CBB cycle in LDW were different from those in SGW and TEH (Supplementary Figs. S4–5). From both the phylum and genus levels, the diversity of autotrophic microorganisms in LDW were remarkably lower than those of SGW and TEH (Supplementary Figs. S4–5). The relationship of these autotrophic species to the sedimentary physicochemical parameters are indicated by the Canonical Correlation Analysis (CCA) in Fig. 5a. It showed that nitrogen content had the greatest impact on the autotrophic community structure ($P = 0.001$, $P < 0.05$), followed by phosphorus content ($P = 0.004$, $P < 0.05$) (Fig. 5a). The other environmental factors did not significantly affect the autotrophic community structure. Phosphorus is significantly negatively correlated with a few autotrophic species, as is indicated by the heatmap correlation analysis of the relationship between the autotrophs in CBB cycle at the genus level and the environmental factors (Supplementary Fig. S6). It is shown that high phosphorus in LDW indeed reduced the

Fig. 4. Relative abundances of the top four taxonomic autotrophs at the taxonomic level that can not be furhter subdivided using different carbon fixation pathways in the seagrass bed sediments from LDW, SGW and TEH. The sizes of these filled circles are proportional to the relative abundance of the gene sequences of key enzyme for each carbon fixation pathway per metagenome. The prefix letters "p", "c", "o", "f" and "g" of the taxonomic autotrophs represent "phylum", "class", "order", "family" and "genus", respectively. The samples name suffixed with the letter "s" indicate the sediment with growing *Z. marina*, and these names are shown in green. The samples name with the letter "c" suffix indicate the control samples without *Z. Marina,* and they are shown in black.

autotrophic biodiversity associated with CBB cycle. However, seagrass (*Z. Marina* in this study) may increase nitrogen concentration of sediment, and increase the autotrophic biodiversity in high phosphorus environments. Two species of the genus *Novimethylophilus* and the order *Hydrogenophilales* are only found from the seagrass sediments of LDW (Fig. 4). It is emphasized that the existence of seagrass does not seem to affect obviously on the CBB cycle in SGW and TEH (Fig. 5a).

3.2. Microbial diversity and community structure in WL pathway and their influencing factors

The abundance of sequences associated with K15023 (KO for 5-methyltetrahydrofolate corrinoid/iron sulfur protein methyltransferase) in WL pathway is approximately 20–40% of all detected sequences assigned to be the key genes of carbon fixation in SGW and TEH (Fig. 2), where the WL pathway is the second most important carbon fixation pathway after the CBB cycle. However, autotrophs in this pathway are rare in LDW sediments. The most dominant species that use WL pathway to fix carbon are δ-Proteobacteria (Fig. 3). In addition, *Chloroflexi*, *Planctomycetes* and *Armatimonadetes* also played an important role in carbon fixation in WL pathway (Fig. 3). At the genus level, the most dominant species mainly distributed in unclassified *Desulfobacterales* order, unclassified *Desulfobacteraceae* family and *Desulfofustis* genus (Fig. 4). The detailed analysis of phylum-level barplot (except *Proteobacteria*) and the genus-level heatmap for these active species are exhibited in Supplementary Figs. S7–8. Among the environmental factors, the Redundancy analysis (RDA) suggests that OM significantly ($P = 0.045$, $P < 0.05$) affects autotrophic community structure in WL pathway (Fig. 5b). Indicated by correlation heatmap analysis between the autotrophs in WL pathway at the genus level and the environmental factors (Supplementary Fig. S9), OM had a significant positive effect on many species using the WL pathway for carbon fixation, in contrast, phosphorus is significantly negatively correlated with many autotrophic species. In addition, the seagrass of *Z. Marina* obviously affected carbon fixation biodiversity and community structure in WL pathway in SGW (Fig. 5b).
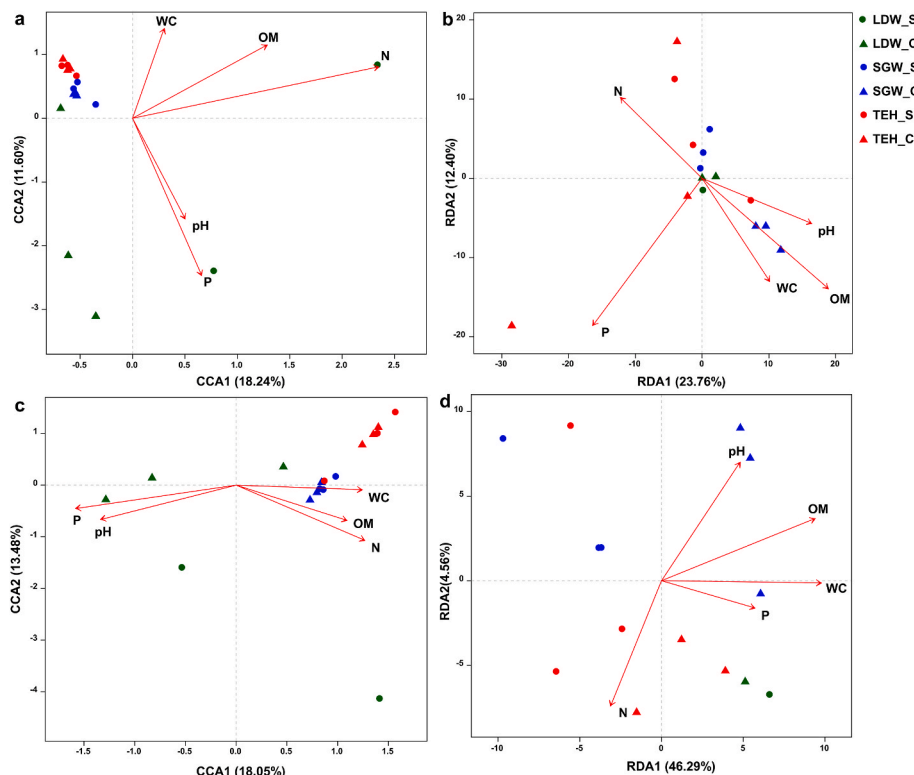
Fig. 5. Ordination graphs from the Canonical Correlation Analysis (CCA) and Redundancy Analysis (RDA) based on the abundance of sequences associated with corresponding carbon fixation pathway and environmental factors from the seagrass bed sediments from LDW, SGW and TEH. a. CBB cycle; b. WL pathway; c. 3HP bicycle; d. rTCA cycle. Solid lines with arrows represent different environmental variables. The symbols of circles, triangels, squares, and so on represent the samples collected from different sediment sites of LDW, SGW and TEH. The samples name suffixed with the letter "s" indicate the sediment with growing *Z. marina*, and those with the letter "c" suffix indicate the control samples without *Z. marina*. The lengths of the solid lines are related to the effects of environmental factors on the different samples. The longer the lines, the more they effect the samples, and vice versa. The distance among samples represents the similarity of autotrophic community structure, the closer the distance, the greater the similarity. The projected distance from the sample point to the quantitative environmental factor vector represents the degree to which the sample is affected by the environmental factor, that is, the two samples draw a vertical line to the same environmental factor vector, and the closer the projection line is, the more similar the influence of this environmental factor on the two samples is.

3.3. Diversity and community structure of those microorganisms containing the key K14470 gene of 2-methylfumaryl-CoA isomerase in 3HP bicycle and their influencing factors

As analyzed in 3.1 and 3.2, the autotrophs associated with CBB cycle and WL pathway are ubiquitous and dominate in marine sediments. The microbial species contain the key K14470 gene of 3HP bicycle are also found to exist in small amounts in the sediments, except for LDW sediment without the seagrass of *Z. marina*. In LDW seagrass-free sediment, the abundance of key genes involved in 3HP bicycle together with the CBB cycle were dominated (Fig. 2). The microbial species containing the key K14470 gene of 3HP bicycle overwhelmingly belonged to the two phyla of *Proteobacteria* and *Actinobacteria* (Fig. 3). *Proteobacteria* are mainly distributed in *α-Proteobacteria* and *γ-Proteobacteria* at the class level (Fig. 3). Besides, a small number of sequences associated with K14470 in 3HP bicycle were also found in *Chloroflexi* phylum in SGW and TEH (Fig. 3). The detailed analysis of class-level barplot and the genus-level heatmap for these microorganisms containing the key gene in 3HP bicycle are shown in Supplementary Figs. S10–11. The four most abundant microorganisms at the genus level are shown in Fig. 4. Among them, *Sphingopyxis*, *Novosphingobium* and unclassified *Sphingomonadaceae* family were only found in LDW without seagrass of *Z. marina*. Another predominant genus *Rhodovulum* was almost only found in SGW and TEH (Fig. 4). CCA analysis indicated that the microorganisms containing the key K14470 gene in 3HP bicycle are significantly influenced by nitrogen ($P < 0.05$, $P = 0.002$), phosphorus ($P < 0.05$, $P = 0.007$) content and pH ($P < 0.05$, $P = 0.025$) (Fig. 5c). In all samples collected, the nitrogen concentration in the seagrass sediments was higher than that in the sediments without seagrass. The sequences belonging to the key K14470 gene of 3HP bicycle are more abundant in LDW seagrass-free sediments than in LDW seagrass sediments, suggesting that microorganisms containing the key gene in 3HP bicycle may occupy a niche in a high phosphorus and low nitrogen environment. So, seagrass may alter the nitrogen and phosphorus content in sediments, thereby altering the ecological niches of microorganisms containing the key genes of carbon fixation pathways (Figs. 2 and 5c). The correlation heatmap analysis among the species containing the key genes of carbon fixation at the genus level and environmental factors were shown in Supplementary Fig. S12.

3.4. Microbial diversity and community structure related to rTCA and 3HP/4HB cycle and their influencing factors

As shown in Fig. 2, a small number of sequences related to K15230 (KO for ATP-citrate lyase) in rTCA cycle and K15019 (KO for 3-hydroxypropionyl-coenzyme A dehydratase) to 3HP/4HB cycle were found in SGW and TEH, indicating that rTCA cycle and 3HP/4HB cycle also fix carbon in SGW and TEH. The major autotrophic microorganisms in rTCA cycle are *ε-Proteobacteria* (class level), *Nitrospirae*, *Aquificae* and *Euryarchaeota* at the phylum level (Figs. 3–4). At the genus level, the dominant species are unclassified *ε-Proteobacteria* class, *Lebetimonas*, *Sulfurovum* and unclassified *Nitrospirales* order (Fig. 4). The detailed analysis of family-level barplot and the genus-level heatmap for these autotrophic microorganisms in rTCA cycle are shown in Supplementary Figs. S13–14. The abundance of unclassified *ε-Proteobacteria*, *Sulfurovum* and *Lebetimonas* in SGW seagrass-free samples were remarkably lower than those in other samples in SGW and TEH. Unlike this, *Nitrospirales* abundance in SGW seagrass-free samples were remarkably higher than that in SGW seagrass samples (Fig. 4 and Supplementary Fig. S15). It means that the seagrass *Z. marina* does alter the autotrophic community structure in rTCA cycle. This inference can be confirmed by the RDA analysis that the samples with and without seagrass are clearly on either side of RDA1 axis, which explained 46.29% of the total variance (Fig. 5d). The other environmental factor had no significant effect on the autotrophic community structure in rTCA cycle (Fig. 5d). The correlation heatmap analysis among the autotrophic species at the genus level

and environmental factors were shown in Supplementary Fig. S15. One of the dominant autotrophic species, *Nitrospirales* order, is positively significantly affected by pH ($P < 0.05$, $P = 0.019$) (Supplementary Fig. S15).

The sequence abundance related to the K15019 in 3HP/4HB cycle was extremely low. For 3HP/4HB cycle, the dominant species for carbon fixation were *δ-Proteobacteria* class, *Acidobacteria*, *Thaumarchaeota* and *Kiritimatiellaeota* at the phylum level (Figs. 3–4), and *Nitrosopumilus*, unclassified *Kiritimatiellales* order, unclassified *δ-Proteobacteria* class, unclassified *Acidobacteria* phylum at the level of genus (Fig. 4). The barplot analysis of autotrophic microorganisms in 3HP/4HB cycle at the genus level is shown in Supplementary Fig. S16.

4. Discussion

4.1. Overview of carbon fixation strategies of seagrass bed sediment

This study determined the niches of six carbon fixation pathways, revealed the community structure of the microorganisms containing the key gene of each pathway, and analyzed the environmental factors that affected the carbon fixation processes in the seagrass bed sediments. The results indicate that CBB cycle is the most predominant pathway of carbon fixation, and WL pathway also plays an important role in carbon fixation in the O_2-deplted sediments. A small number of microorganisms containing the key genes of the rTCA, 3HP and 3HP/4HB cycles were also detected there. However, these chemoautotrophic pathways of WL, rTCA, and 3HP/4HB seemed to be negatively correlated with high phosphorus, as they are barely detectable in LDW sediments with high phosphorus probably from domestic pollution near villages. Furthermore, the autotrophic biodiversity in LDW is also obviously lower than at other sites of SGW and TEH (Figs. 3–4), indicating that high phosphorus reduced autotrophic biodiversity and largely affect the ecological niche of different carbon fixation pathways. While the microorganisms containing the key gene of 3HP bicycle seemed to be insensitive to phosphorous, even occupied a niche and became another important carbon fixation pathway besides CBB cycle in seagrass-free LDW sediments under high phosphorus and low nitrogen environment (Fig. 2). The presence of seagrass could alter nitrogen and phosphorus content in sediments, thereby affecting autotrophic biodiversity and community structure, creating new ecological niches for carbon fixation pathways.

It is emphasized that all the analyses and conclusions depend on the correct choices of the key enzymes of each carbon fixation pathway. In fact, the functional genes encoding key enzymes involved in different CO_2-assimilation pathways have been widely used to investigate the autotrophic process and mechanism in different environments, such as freshwater lakes (Alfreider et al., 2018), oceanic waters (Alfreider et al., 2018; Bergauer et al., 2013), stratified lakes (Alfreider et al., 2017), etc. On one hand, we analyzed and referenced the key enzymes that the previous studies selected as molecular markers for each pathway of carbon fixation. On the other hand, we also verified whether the selected enzyme is only in the analyzed carbon-fixing pathway through the KEGG database, to prevent the overestimation of abundance in case the selected enzymes also exist in other non-carbon-fixing pathways. Eventually, these key proteins with KEGG Orthology (KO) numbers of K01601, K15230, K15023, K14470, K15019 and K14467 were used as molecular markers in this study to quantify the sequences related to key genes of ribulose-bisphosphate carboxylase in CBB cycle, ATP-citrate lyase in rTCA cycle, 5-methyltetrahydrofolate corrinoid/iron sulfur protein methyltransferase in WL pathway, 2-methylfumaryl-CoA isomerase in 3HP bicycle, 3-hydroxypropionyl-coenzyme A dehydratase in 3HP/4HB cycle and 4-hydroxybutyrate—CoA ligase in DC/4-HB Cycle, respectively. It is necessary to point out that only one key gene of a designated pathway was used to be molecular marker, which may lead to overestimation. Because some microorganisms may only contain the key gene of a pathway, but may lack a complete pathway of carbon

fixation. This study preliminary and roughly attempted to explore the dominant carbon fixation pathways, so, it is needed to ensure the minimal detection of each pathway. So only one key gene was used in this current study to represent the pathway which it is located. In the future, in-depth analysis of metatranscriptomic and carbon isotope labeling could be used to reveal active autotrophic microorganisms that greatly influence the rate of carbon fixation in seagrass beds (Ruiz-Fernández et al., 2020; Zhao et al., 2020).

4.2. Comparison of carbon fixation strategies in seagrass bed sediment and other anoxic environments

Ocean sediments of seagrass bed are O_2-depleted, so we compare the carbon fixation strategies in the sediment of this study with those in previous related studies of O_2-depleted oceanic waters and an anoxic-oxic biofilm-membrane bioreactor (Ruiz-Fernández et al., 2020; Zhou et al., 2022). Despite the environments of the seagrass sediment, oxygen-depleted oceanic water and anoxic-oxic biofilm-membrane bioreactor are qutie different, the dominant carbon fixation pathways are similar, which are CBB and WL pathways. In addition, the dominant autotrophic microorganisms driving carbon fixation in each pathway in two oxygen-deficient environments of sediment and oxygen-depleted oceanic water are similar except for CBB pathway, where δ-Proteobacteria, Planctomycetes and Chloroflexi were dominant autotrophic species in WL pathway (Ruiz-Fernández et al., 2020); α-Proteobacteria, γ-Proteobacteria, Actinobacteria and Chloroflexi predominated among the microorganisms contained the key gene in 3HP bicycle. Even for a small number of rTCA cycle and 3HP/4HB cycle in the seagrass sediments of this study, there are also some common autotrophic microorganisms similar to those in oxygen-depleted oceanic waters, e.g. ε-Proteobacteria, Nitrospirae in rTCA cycle and Thaumarchaeota, Acidobacteria in 3HP/4HB cycle. Although the sediment, waters and bioreactor are very different environments, the similarity of major carbon fixation pathways and autotrophic diversity indicate that oxygen may be a very critical factor in determining the autotrophic distribution and their ecological niches. It has been reported that the oxygen concentration is indeed one of the most important ecological factors in carbon fixation in the modern biosphere (Berg, 2011). The key enzymes of these carbon fixation pathways of the aerobic (CBB, 3-HP and 3-HP/4-HB) and anaerobic (WL and rTCA) pathways are O_2 sensitivity or tolerance, making these microorganisms using these pathways have the ability to cope with fluctuating O_2 concentrations, and can shape similar ecological niches in oxygen-depleted environments (Berg, 2011).

It has been widely accepted that CBB cycle is the quantitatively most important mechanism of autotrophic carbon fixation in nature. The autotrophic species using CBB cycle widely distribute in plants, algae, cyanobacteria, many aerobic or facultative aerobic Eubacteria, and some archaea (Berg, 2011). In oxygen-depleted sediment of this study, CBB cycle operates in bacteria and archaea, while Cyanobacteria were not found, despite Cyanobacteria are usually found from seawater even in oxygen-depleted oceanic water. It is indicated that CBB cycle is the most important pathway of carbon fixation in marine sediment, but their autotrophic community structure may be characteristic and distinct from other hypoxic environments. WL pathway occurs in strictly anoxic conditions since its key enzymes of CO dehydrogenase/acetyl-CoA synthase are highly oxygen sensitive. This pathway is usually found from environments with fluctuating oxygen tensions like soils or sediments. The microorganisms containing the key genes of 3HP, rTCA and 3HP/4HB cycles are present in small amounts. 3HP bicycle is reported to assimilate numerous compounds like acetate, propionate, and succinate, which makes the pathway best suitable for mixotrophy (Berg, 2011). All the enzymes related to 3HP bicycle do not contain oxygen-sensitive steps, therefore functionating under aerobic conditions. Its limited distribution may be due to insufficient tolerance to O_2- methylmalonyl-CoA mutase. Therefore, the 3HP bicycle can occupy a certain niche in anoxic sediment. rTCA cycle is less energy-consuming than the CBB cycle (Berg

et al., 2010). This cycle is reported to be widespread among diverse anaerobic or aerobic bacteria growing at low O_2 tensions, due to some key enzymes in this cycle are sensitive to O_2 (Berg, 2011). 3HP/4HB cycle was described as the most energy efficient aerobic carbon fixation cycle. The enzymes of 3HP/4HB cycle are tolerant to O_2, so this cycle can function in different concentration gradients of oxygen. 3HP/4HB cycle has long been thought to be distributed in archaea (Alfreider et al., 2018). It is increasingly found that bacteria can also fix carbon through this pathway (Berg et al., 2010), which was also confirmed in this study.

4.3. Autotrophic microorganisms' community that played important roles in each carbon pathway

This study showed that Proteobacteria are the most important autotrophic microorganisms in sediments and can widely utilize various carbon fixation pathways. Multiple classes of Proteobacteria, e.g., α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria and δ-Proteobacteria contain key genes of CBB cycle and 3HP cycle for carbon fixation (Fig. 3). Among, δ-Proteobacteria can also fix carbon through WL pathway, rTCA cycle and 3HP/4HB cycle. Notably, ε-Proteobacteria can utilize the rTCA cycle for carbon fixation. ε-Proteobacteria was originally known to fix carbon via the rTCA pathway in anaerobic environments because the key enzymes in this cycle are oxygen-sensitive (Alfreider et al., 2018). In summary, Proteobacteria play the most important roles in all five carbon fixation pathways identified in this study. Some unclassified Proteobacteria were also detected in the carbon fixation pathways, indicating that there may be still a large number of unknown autotrophic microorganisms yet to be discovered. It needs to be pointed out that α-Proteobacteria contained the key K14470 gene of 2-methylfumaryl--CoA isomerase involved in 3HP bicycle, but sometimes they are found to lack the complete 3HP bicycle in diverse organisms that also happen to accumulate polyphosphate (Jäckle et al., 2019; Shih et al., 2017; Zarzycki and Fuchs, 2011). It is suggested that only a single K14470 gene may be not adequate to be used for indicating the 3HP bicycle. Multiple key genes in the 3HP bicycle need to be used together as the molecular marker for further exploration of its ecological niches in future studies.

Archaea Euryarchaeota was found in the CBB and rTCA cycles (Figs. 3–4). It is reported that autotrophic Euryarchaeota are strictly confined to anoxic conditions, generally specialized in metabolizing C1 compounds and/or acetate (Berg et al., 2010). The archaea Euryarchaeota is generally found in the WL pathway, which is considered to be the most ancient autotrophic carbon fixation pathway (Berg et al., 2010). In this study, the Euryarchaeota found in CBB cycle all belong to the class Halobacteria, and distributed in order Haloferacales and Natrialbales, all related to halophilic archaea. Previous studies did not consider Halobacteriales to be autotrophic members (Berg et al., 2010). Thus, the possibility of Halobacteriales utilizing the CBB cycle for carbon fixation needs to be further confirmed. Besides Euryarchaeota, archaea Thaumarchaeota was also found to fix carbon through 3HP/4HB cycle in this study. It is reported that Thaumarchaeota are nitrifiers and use 3HP/4HB cycle for CO_2 fixation in oligotrophic environment (Alfreider et al., 2018). Planctomycetes prevalent in aquatic systems are also an important nitrogen-fixing population (Delmont et al., 2018). Nitrifiers belong to the chemolithoautotrophs, which use reduced inorganic nitrogen compounds as an energy source and inorganic carbon as carbon source (Alfreider et al., 2018). Similarly, Nitrospirae are also nitrifiers and fix carbon by rTCA cycle. This cycle may be widespread among anaerobic or microaerobic bacteria (Berg, 2011). Previously, rTCA cycle was not found in the archaeal domain (Berg, 2011), however, this study found that archaea Euryarchaeota can fix carbon by the rTCA cycle. At present, archaea have been found in all the carbon fixation pathways that have been discovered so far.

Carbon fixation pathways are not related to distinct taxonomic groups, because the autotrophs at the same phylum may use different carbon-fixing pathways. Actinobacteria was found not only in CBB cycle

but also in WL pathway. In addition, *Chloroflexi* is also found to contain the key genes of WL pathway and 3HP bicycle. It is reported that 3HP bicycle seems to have been "built up" in the *Chloroflexi* by horizontal gene transfer of genes from different sources (Shih et al., 2017). Two autotrophic species of genus *Novimethylophilus* and the order *Hydrogenophilales* in CBB cycle may be adapted to high nitrogen and phosphorus, because they are only found in seagrass sediments in LDW with high levels of nitrogen and phosphorus. In SGW, the abundance of some autotrophic microorganisms in seagrass sediment are remarkably higher than seagrass-free sediment, such as *Aurantimonas* for the CBB cycle, *Desulfofustis* for the WL pathway, *ε-Proteobacteria* for the rTCA cycle. Even, all major autotrophic microorganisms belonging to the 3HP/4HB cycle were found only from seagrass sediments. The results suggested that the seagrass have great potential for maintaining and enhancing the capacity of autotrophic carbon fixation in offshore sediment environments. *Bacillariophyta* and *Chloroflexi* found in this study are important autotrophs commonly found in marine environments (Chi et al., 2018b). *Armatimonadetes* found in the WL pathway could not be further subdivided, implying that it may be a new autotrophic species. An *Armatimonadetes* sp. has been found to degrade aniline (Liu et al., 2021). *Kiritimatiellaeota* found in the 3HP/4HB cycle can only be classified into the order, and only distributed in the same species. *Kiritimatiellaeota* is a newly identified bacterial phylum in recent years. This species is globally distributed and found in anaerobic or microaerobic environments such as vertebrate intestines, soils and marine environments (Sackett et al., 2019). Related studies have found that this species is involved in the biosynthesis of amino acids and fatty acids in the animal gut (Guo et al., 2021). The dominant autotrophs in the WL pathway at the genus level all belong to the order *Desulfobacterales* under the class *δ-Proteobacteria* (Fig. 4). *Desulfobacterales* are an order of sulfate-reducing bacteria and also play a role in promoting nitrate reduction in the nitrogen cycle. Collectively, *Desulfobacterales* may be an ancient bacterium that plays a role in all carbon, nitrogen and sulfur cycles. It needs to be added that the large number of autotrophic microorganisms are unclassified, which means that the seagrass bed environment has a large number of autotrophic biological resources to be discovered.

Overall, there is large amount of carbon sources in the sediment of seagrass bed. There also exists multiple ways to fix carbon. The multiple ways and rich diversity of autotrophic microorganisms can contribute to carbon fixation activities as much as possible in response to complex changes in the environment. The exploration of the processes and mechanisms of carbon fixation in seagrass bed sediment is indispensable to better evaluate the potential of offshore carbon sequestration. Additionally, this study also provided guidance for the control and improvement of the carbon sequestration capacity in this ecosystem. For example, this study found that nutrients may have a significant negative effect on carbon fixation, reminding us to drastically reduce the inputs of coastal nutrients. The seagrass may absorb nutrients to reduce their concentrations in sediment, thereby increasing the carbon sequestration potential of seagrass beds. Under the current situation of globally declining seagrass beds, the results of this study have emphasized the importance of protecting seagrass beds.

5. Conclusions

In the micro-aerobic or anaerobic seagrass bed sediment, there are diverse autotrophic pathways of carbon fixation. The autotrophic microorganisms at the same phlym level have multiple carbon fixation pathways. This study also found a large number of unknown autotrophic microorganisms. It implied the possibility of the existence of novel autotrophic metabolisms. The rich diversity of autotrophic microorganisms could help them to cope with complex changes in the environment. Anthropogenic activities lead to discharge of high nitrogen and phosphorous sewage into the ocean, which can reduce autotrophic biodiversity and affect autotrophic process. The presence of seagrasses have the potential to improve autotrophic biodiversity and enhance

carbon sequestration capacity. However, global seagrass beds are disappearing at a rate of nearly 5% per year. Seagrass bed ecosystems not only have extremely high productivity, but also have important ecological service functions, such as stabilizing the substrate, purifying pollutants, and so on. Therefore, we need to pay more attention to the protection of seagrass beds.

Conceptualization

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2023.106002.

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