Ladderane and lipid biomarker estimate of the sources and distributions of anammox activities in Laizhou Bay of the Bohai Sea, China

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Anaerobic ammonium oxidation (anammox) plays an important role in nitrogen removal in coastal seas, and ladderanes, as specific biomarkers of anammox bacteria, can be used to indicate the anammox activity. However, the origins of ladderanes and their controlling factors in the coastal seas influenced by anthropogenic activities are still not well constrained. To address this, we reported ladderanes, long-chain n-alkanols (terrestrial biomarker) and sterols (marine biomarker) in suspended particulates from the estuaries and inner area of Laizhou Bay in North China, to study ladderane sources and its distribution patterns. This study proposed a novel index, R_{lad}, using ladderane ratio of ladderane III to ladderane IV, and by correlating this index with other biomarker distributions to evaluate the source of ladderanes. High R_{lad} values (> 0.9) indicated biosynthesis by terrestrial anammox bacteria *Ca*. Brocadia and *Ca*. Kuenenia, while low R_{lad} values (< 0.9) indicated biosynthesis by marine anammox bacteria *Ca*. Scalindua. High R_{lad} values and high ladderane concentrations in particulates from the estuaries and nearshore area of Laizhou Bay revealed sources from the terrestrial input *via* riverine inflow as well as *in situ* production in oxygen-depleted estuaries, supported by high concentrations of terrestrial biomarkers; Low R_{lad} values and low ladderane concentrations in particulates from offshore area indicated sources from marine environment *via* the cold hypoxia water input by the Bohai circulation. Comparison of ladderane concentrations of our study with previously published results from a wide range of environments with human influences from Chinese coastal area revealed that high ladderane concentrations synthesized by terrestrial anammox bacteria could contribute significantly to coastal seas, and the anammox process in river-estuary-bay system might be underestimated. This study provides new understandings about the evaluation of the source and distribution of ladderanes under anthropogenic influences in coastal seas.

KEYWORDS

biomarker, ladderane, particulate, anammox, anthropogenic influence

1 Introduction

Dissolved inorganic nitrogen (DIN) is a key limiting nutrient in ocean primary production (Arrigo, 2005; Rush and Sinninghe Damsté, 2017). But in recent decades, enhanced DIN inputs by anthropogenic sewage and agricultural wastewater caused a series of environmental problems like eutrophication and hypoxia in coastal areas (e.g., Jickells, 1998; Conley et al., 2009; Altieri and Gedan, 2015; Wang et al., 2019b), which required a reduction of nutrient discharge for a healty ecostal ecosystem. Denitrification process was the predominant natural pathway for nitrogen removal in the marine environment. However, the imbalance in marine nitrogen budget revealed that the significance of other nitrogen loss pathways could have been underestimated, such as anaerobic ammonium oxidation (anammox) (Kuypers et al., 2003; Dalsgaard et al., 2012; Thamdrup, 2012). Anammox process was mediated by anammox bacteria, which could uniquely convert NH_4^+ directly to N_2 in anammoxosome (Van de Graaf et al., 1995). Anammox was first found in sewage treatment plants (Mulder et al., 1995), and in the past decades this process has been proved to be an effective nitrogen removal pathway in natural environment (Kuypers et al., 2003; Kuypers et al., 2005; Karthäuser et al., 2021). In marine nitrogen cycle, anammox even could be predominant in nitrogen loss in marine hypoxia zones and OMZs (Lam et al., 2009; Ward, 2013). For example, the contribution of anammox process to nitrogen loss was up to ~40% in the Black Sea OMZ (Kuypers et al., 2003), ~50% in the Peruvian OMZ (Lam et al., 2009) and ~67% in the Baltic-North Sea OMZ (Thamdrup and Dalsgaard, 2002).

Therefore, the evaluation of anammox activity in marine environments is important for a better understanding of nitrogen biogeochemical process and ecosystem functions (Peeters and van Niftrik, 2019). Nitrogen isotope labelling was the first technique applied to trace the biological origin of anammox (Van de Graaf et al., 1997), which remains the most frequently used method to quantify anammox activity (e.g., Engström et al., 2005; Boumann et al., 2009; Li et al., 2021; Teng et al., 2022). Molecular techniques in gene level, including qPCR and high-throughput sequencing, were used to locate the certain segment of functional genes to further investigate community structure and abundances of anammox bacteria (Klotz and Stein, 2008; Hirsch et al., 2011; Li et al., 2013; Li et al., 2022). Using the above techniques, seven chemolithoautotrophic microbial genera were found (Pereira et al., 2017 and references therein), known as *Ca.* Brocadia, *Ca.* Kuenenia, *Ca.* Scalindua, *Ca.* Jettenia, *Ca.* Anammoxoglobus, *Ca.* Brasilis and *Ca.* Anammoximicrobium. Among these genera, *Ca.* Brocadia, *Ca.* Kuenenia and *Ca.* Scalindua were confirmed as three dominating genera of anammox bacteria found in natural environments (e.g., Gonzalez-Silva et al., 2017; Zhang et al., 2018; Fozia et al., 2020). Most species of *Ca.* Brocadia and *Ca.* Kuenenia were found in terrestrial environments while species of *Ca.* Scalindua mainly occurred in marine environments (e.g., Risgaard-Petersen et al., 2004; Hou et al., 2013; Fozia et al., 2020). The presence of anammox bacteria can also be inferred from the detection and quantification of ladderane lipids, which are membrane lipids uniquely biosynthesized by anammox bacteria with the core structure of 3–5 concatenated cyclobutane moieties (Figure 1) (Sinninghe Damsté et al., 2002; Rush and Sinninghe Damsté, 2017).

Ladderane lipids have been proved to be the stable, specific, and easily-identified biomarkers to study the anammox activity (Brandsma et al., 2011; Pitcher et al., 2011; Rush et al., 2012a; Zhao et al., 2013; Rush and Sinninghe Damsté, 2017). Therefore, ladderanes provide a molecular indication for the nitrogen cycle. In the water columns, high ladderane concentrations and high anammox rates were simultaneously found in hypoxic zones with high nitrogen loss (e.g., Kuypers et al., 2005; Jaeschke et al., 2007). Besides, ladderane lipids in sedimentary records could be used to reconstruct past changes of anammox activity and to indicate the change of marine nitrogen cycle and hypoxia events (e.g., Jaeschke et al., 2009; Zhao et al., 2019).

The contributions of anammox to nitrogen loss in sediments have been well studied, e.g., up to 19% in the Jiaozhou Bay of the Yellow Sea (Wu et al., 2019), 22% in the Bohai Sea (Zhang et al., 2018) and 42% in southern New England Estuary and its shelf area (Brin et al., 2014). However, due to the difficulties of bacteria culture, studies of anammox were hardly carried out in particulates of the water column using isotope labelling or sequencing techniques. Ladderane biomarker could thus be a valuable proxy for anammox activity in water columns, but its distribution and origin in particulate were not well studied until now. The origin of ladderanes was generally considered as marine *in situ* production by anammox bacteria without allochthonous input in ocean (e.g., Jaeschke et al., 2007; Jaeschke et al., 2009; Rush et al., 2012a; Rush et al., 2012b; Rush and Sinninghe Damsté, 2017). However, ladderane sources in coastal waters might be more complicated due to the riverine input and anthropogenic influences, since anammox activities were universal in terrigenous environments such as rivers, sewage water discharge and soils (e.g., Hou et al., 2013; Shen et al., 2013; Zhang et al., 2017b). The origins of ladderanes in coastal seas influenced by the anthropogenic activities were still uncertain, limiting our understanding of the indication of ladderanes anammox studies.

Herein, we present ladderanes, marine and terrestrial biomarkers data in suspended particulates of the water column from Laizhou Bay to study the source and distribution of ladderanes and factors controlling anammox activities. Laizhou Bay is located at the south part of the Bohai Sea, China (Figure 2). As a typical bay with multiple small river inputs and complicated hydrological condition, the estuary-bay system here has been considered as an ideal study area influenced by anthropogenic activity. The main objectives of this study are: 1) to investigate the distribution pattern of ladderanes in the estuary-bay system of the Laizhou Bay, 2) to distinguish the origin of ladderanes by using biomarker index and 3) to evaluate the anammox activities in different coastal areas. This case study will be essential to understand the distribution and source of ladderanes in particulates from the coastal system, which can provide new insights into anammox activity and marine nitrogen cycle in coastal areas influenced by anthropogenic activities.

2 Material and methods

2.1 Study area and sample collection

The Laizhou bay is a semi-closed bay of the Bohai Sea, China, covering about ~6000 km^2 area with around ~320 km coastline
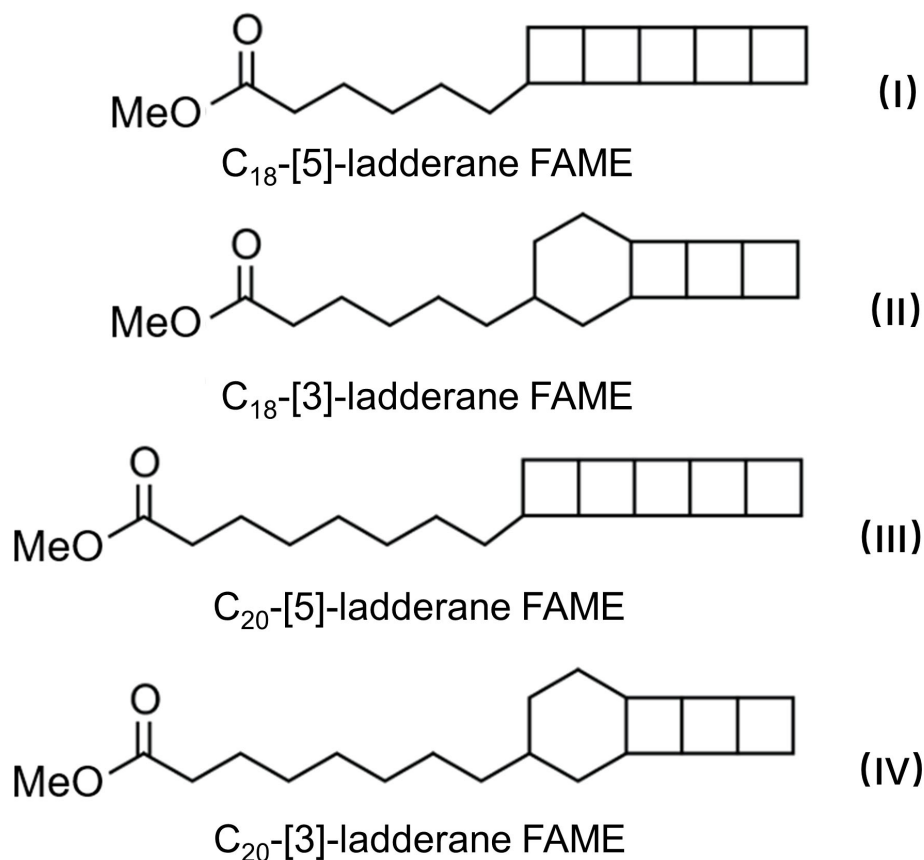
FIGURE 1
The molecular structure of ladderane fatty acid methyl esters (ladderane FAMEs) used in this study (modified after Sinninghe Damsté et al., 2002).

(Wang et al., 2020). The Laizhou Bay is generally shallow, with the average water depth of ~9 m (Jiang et al., 2018). Multiple rivers (Figure 2) discharge terrestrial materials and nutrients to the bay each year (Jiang et al., 2005; Guo et al., 2015; Wang et al., 2019b; Zhang et al., 2019; Peng et al., 2021). The highest nutrient concentrations normally occurred in summer due to the strong river runoff in wet season (Peng et al., 2021). Rivers flowing into Laizhou Bay, like the Xiaoqing River (XQR), were severely contaminated by industrial and agricultural sewage, which could further affect the coastal ecological environment and biogeochemical cycles in Laizhou Bay (He et al., 2018a; He et al., 2018b). These processes and nearshore marine aquaculture activities (Cui et al., 2005) have resulted in low subsurface dissolved oxygen (DO) concentration in the central Bohai Sea during summer (Song et al., 2016; Zhai et al., 2019; Zheng et al., 2020), which also influenced the distribution of hypoxic zone in adjacent regions *via* hydrodynamic processes (Wei et al., 2019). Hypoxia in sea water has been observed in the Xiaoqing River Estuary, which is strongly impacted by anthropogenic activities (He et al., 2018b). Moreover, the river discharge and the south Bohai anti-clock coastal current results in rather complicated hydrologic conditions for this small bay (Qiao et al., 2010; Zhou et al., 2017; Wei et al., 2019). The eutrophication area and hydrologic condition of Laizhou Bay were closely linked to the river input, which might reveal the potential differences of biogeochemical process in

nearshore area and offshore area (Jiang et al., 2018; Zhang et al., 2019; Peng et al., 2021).

We divided our study area into the sub-regions as 'estuary', 'nearshore areas' closed to river mouths, and 'offshore areas' to discuss the source of ladderanes. The estuarine area includes the small river estuaries along the south and east bank of Laizhou Bay. This area was dominated by riverine lateral transportation from upper stream, showing brackish condition between river freshwater discharge and sea water intrusion. The biogeochemical process in these small rivers were less studied comparing with the Yellow River although they were also impacted by strong anthropogenic activity with the development of agriculture and industry in the northern Shandong Peninsula (Zhang et al., 2015; He et al., 2018a). The nearshore 'area A' (Figure 2) along the coast was intensely influenced by river input (Zhang et al., 2015; Jiang et al., 2018). The offshore 'area B' (Figure 2) was impacted by the Yellow River discharge and Bohai Sea circulations. Although the Yellow River contributed significantly to the hydrology and ecology condition in the northwestern bay (Liu et al., 2012; Peng et al., 2021), anammox activity in the Yellow River Estuary was proved to be very weak comparing with that of the Bohai Sea (Zhang et al., 2017b; Zhang et al., 2018).

Twenty-one suspended particulate samples (6 m depth) from Laizhou Bay were collected in August 2018, and eight suspended particulate samples from estuaries were collected in August 2020
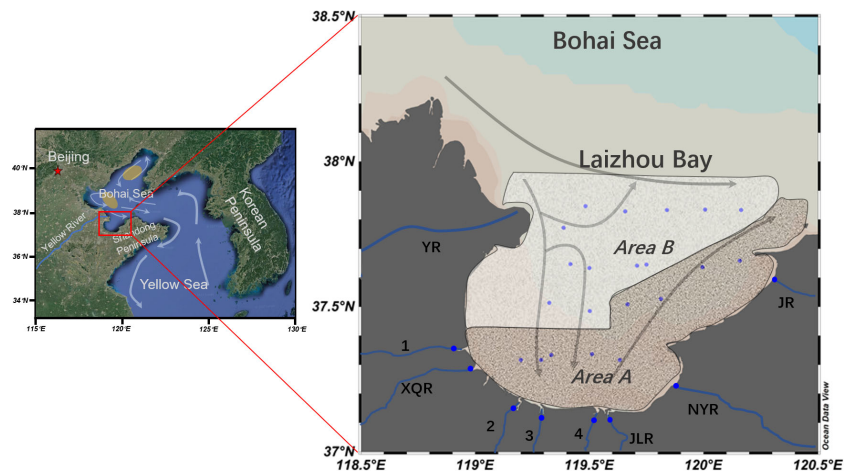
FIGURE 2
Sampling sites and circulations in Laizhou Bay, Bohai Sea. The red star on the left map is the city Beijing of China. Yellow shadows in the left map represent the summer hypoxia zones in the Bohai Sea, modified after Wei et al. (2019). The surface circulations displayed by gray arrows were modified after Guan (1994), Qiao et al. (2010), Wei et al. (2019) and Zhang et al. (2022). Area A in brown texture fill represents the nearshore area which is significantly influenced by small river inputs. Area B in white texture fill represents the offshore area which represented the central and north area of Laizhou Bay. Blue dots in the right map represent sampling sites including estuarine sites and bay sites. YR, Yellow River; XQR, Xiaoqing River; JLR, Jiaolai River; NYR, Nanyang River; JR, Jie River; 1, Zimai River; 2, Mi River; 3, Bailang River; 4, Wei River.

(Figure 2). The sea waters for particulates collection were obtained by submerged pump to large High-Density Polyethylene (HDPE) bottles on board aboard a small vessel of LuChangYu 60003, and about 25 L of water at each station was filtered through pre-combusted glass fiber filters (Whatman GF/F, 150 mm) to get the suspended particulates. The collection of particulate samples from the inner bay in 2018 and from the estuaries in 2020 were both collected in summer, representing similar hydrodynamic conditions in wet season. Specifically, mean water temperature in Laizhou Bay were 27.2 °C and 26.9 °C in the summer of 2018 and 2020, respectively, and water salinity in Laizhou Bay were 30.9 and 30.0 in the summer of 2018 and 2020, respectively (Lu, 2022). Higher temperature and lower salinity both occurred in the western bay in 2018 and 2020 (Lu, 2022). Moreover, according to the monitoring data of water quality, biodiversity and pollution discharge in the Laizhou Bay, the river flows and sea water environments of 2018 and 2020 were similar, e.g., highest DIN concentration both occurred near the XQR estuary at 38.2 and 33.5 μmol/L in 2018 and 2020, respectively (Lu, 2022). We herein suggest our data of particulate samples in estuaries and embayment were comparable.

2.2 Sea water parameter determination

Water temperature and salinity were measured by a portable multiparameter probe (WTW Multi 3420). Dissolved oxygen (DO) was measured by an automatic potentiometric titrator (Metrohm 848 Titrino plus) after *in situ* water was additionally collected by Polymethyl Methacrylate (PMMA) hydrophore. Water samples for nutrient analysis were filtrated through GF/F filters (Whatman) and immediately frozen at −20℃. Dissolved inorganic nitrogen (DIN) in seawater including NH_4^+, NO_2^- and NO_3^- were quantified using flow injection analysis (QuAAtro, Seal, Germany) after thawing the

samples at 4 °C. Precision of measurements above were 0.1 °C for water temperature, 0.1 for salinity, 0.1 mg/L for DO and 0.01 μmol/L for nutrients. The detection limits were 0.03 μmol/L for NH_4^+, 0.02 μmol/L for NO_2^-, 0.05 μmol/L for NO_3^-. All these parameters were used as the average value after triple measurement.

2.3 Bulk carbon analysis

Measurement of bulk organic carbon in particulate samples followed the procedure of Hu et al. (2016). Suspended particulates on filters were first freeze-dried and then treated with 4 mol/L HCl to remove inorganic carbon. After drying in an oven at 55℃ for 24 hours to volatilize the excess HCl, filters were wrapped in tin boat. Particulate organic carbon (POC) was analyzed with a CHN element analyzer (Thermo Flash EA2000). Atropine Standard (OC = 70.50 ± 0.24%) and Low Organic Content Soil Standard OAS (OC = 1.61 ± 0.09%) were used as standard samples, and the relative deviation was less than 5% (*n* = 6). The POC contents were reported as μg/L in seawater of this study, with the detection limit of 3 μg/L.

2.4 Biomarker analysis

2.4.1 Sterols and *n*-alkanols

Sterol and *n*-alkanol extraction and purification followed the procedure of Zhao et al. (2006). Freeze-dried suspended particulate samples were extracted using the mixture of dichloromethane (DCM) and methanol (MeOH) (3:1, v/v) with internal standard C_{19} *n*-alkanol added. The extracts were dried under gentle nitrogen flow and then were hydrolyzed using 6% KOH in MeOH for 12 hours. The hydrolyzed extracts were further extracted with hexane, concentrated under gentle nitrogen flow, and subsequently separated using silica

gel column chromatography. The hydrocarbon fraction was eluted with hexane. The alcohol fraction (containing sterols and *n*-alkanols) was eluted with DCM: MeOH (95:5 v/v) after the hydrocarbon fraction. Finally, the alcohol fraction was silylated with 80 µL BSTFA (N, O-bis(trimethylsilyl)-trifluoroacetamide) for further analysis. Agilent 7890N GC with flame ionization detector and HP-1 capillary column (50 m) was used for detecting the sterols and long-chain *n*-alkanols. H_2 was applied as the carrier gas at 1.3 mL/min. The oven was kept initially at 80 °C for 1 min, programmed to 200°C at 25°C/min, followed at 4°C/min to 250°C, 1.8°C/min to 300°C, and finally holding at 300°C for 5 mins. The detection limits of sterols and *n*-alkanols were 10 ng/g particulate (about 1 ng/L water volume). The average standard deviation was below 10%.

2.4.2 Ladderanes

Ladderane extraction and purification were modified after the procedure of Zhao et al. (2019). Freeze-dried suspended particulate samples were extracted using mixture of DCM and MeOH (2:1, v/v). The extracts were dried under gentle nitrogen flow and added with copper to remove the sulfur. The total extracts were hydrolyzed with 6% KOH in MeOH for 12 hours. Subsequently, the hydrolyzed extracts were further extracted with hexane. The residual liquids were acidified to pH of 1 and then were extracted with hexane for fatty acids fraction (containing ladderane lipids). After drying with anhydrous sodium sulfate, fatty acids were converted to FAMEs using BF_3/MeOH solution. 2-hydroxy-4-methoxy benzophenone was spiked into the extracts as internal standard before instrumental analysis.

High-performance liquid chromatography-mass spectrometer (HPLC-MS) with Zorbax Eclipse XDB-C8 column (3 mm × 250 mm, 5 µm, Agilent) was used for detecting ladderane FAMEs. The mobile phased was eluted with MeOH (0.3 mL/min, 15 mins), then brought to the mixture of MeOH: H_2O (v/v=85%:15%, 2 mins) and held for 3mins. For MS parameters, corona was set up to 3 µA, cone voltage to 35 V, source temperature to 120°C and APCI probe temperature to 550°C. The cone gas flow was at 120 L/h, desolvation gas flow at 400 L/h. Ladderane FAMEs were measured under Selected Ion Recording (SIR) mode at $[M+H]^+$ m/z = 289, 291, 317, and 319 for C_{18}-[5]-ladderane FAME (I), C_{18}-[3]-ladderane FAME (II), C_{20}-[5]-ladderane FAME (III), and C_{20}-[3]-ladderane FAME (IV), respectively. The chromatogram traces and MS spectra of ladderane core lipids were shown in Figure S1. The concentration of ladderanes were given as relative amounts because ladderane lipid standards are not available commercially, and ladderane concentration data were normalized by comparing the signals of ladderanes with the internal standard. The detection limit of ladderane lipids was 30 ng/g particulate (about 0.003 µg/L water volume). The average standard deviation was below 10%.

2.5 Biomarker ratio indices

Terrestrial and marine biomarkers ratio, abbreviated as TMBR, has been used to evaluate the relative contribution of terrestrial

organic carbon to the total organic carbon (Xing et al., 2011). TMBR′ has also been used by using *n*-alkanols in the calculation to replace the *n*-alkanes which are susceptible to pollution (Yuan et al., 2013). TMBR′ is calculated using the following equation:

$$TMBR' = \frac{\sum (C26 + C28 + C30\ n\text{-alkanols})}{\sum (C26 + C28 + C30\ n\text{-alkanols}) + \sum (B + D + A)}$$

where $\Sigma(C_{26} + C_{28} + C_{30}$ *n*-alkanols) represents the terrestrial input. $\Sigma(B + D + A)$ is the sum of marine biomarker concentrations, referring to brassicasterol (B), dinosterol (D) and C_{37} alkenones (A), respectively. Alkenones were below detection limit in all samples from Laizhou Bay, thus the sum of marine biomarker concentrations in this study was represented as $\Sigma(B + D)$.

The NL_5 ratio has been widely used to infer ladderane synthesis temperature. In order to keep the fluidity of anammoxosome membrane in different environment, the membrane lipid chain length of anammox bacteria is temperature dependent. NL_5 is calculated using following equations (Rattray et al., 2010):

$$NL_5 = \frac{\text{ladderane III}}{\text{ladderane I + ladderane III}}$$

$$NL_5 = 0.2 + \frac{0.7}{1 + e^{-\left(\frac{Temp - 16.3}{1.5}\right)}}$$
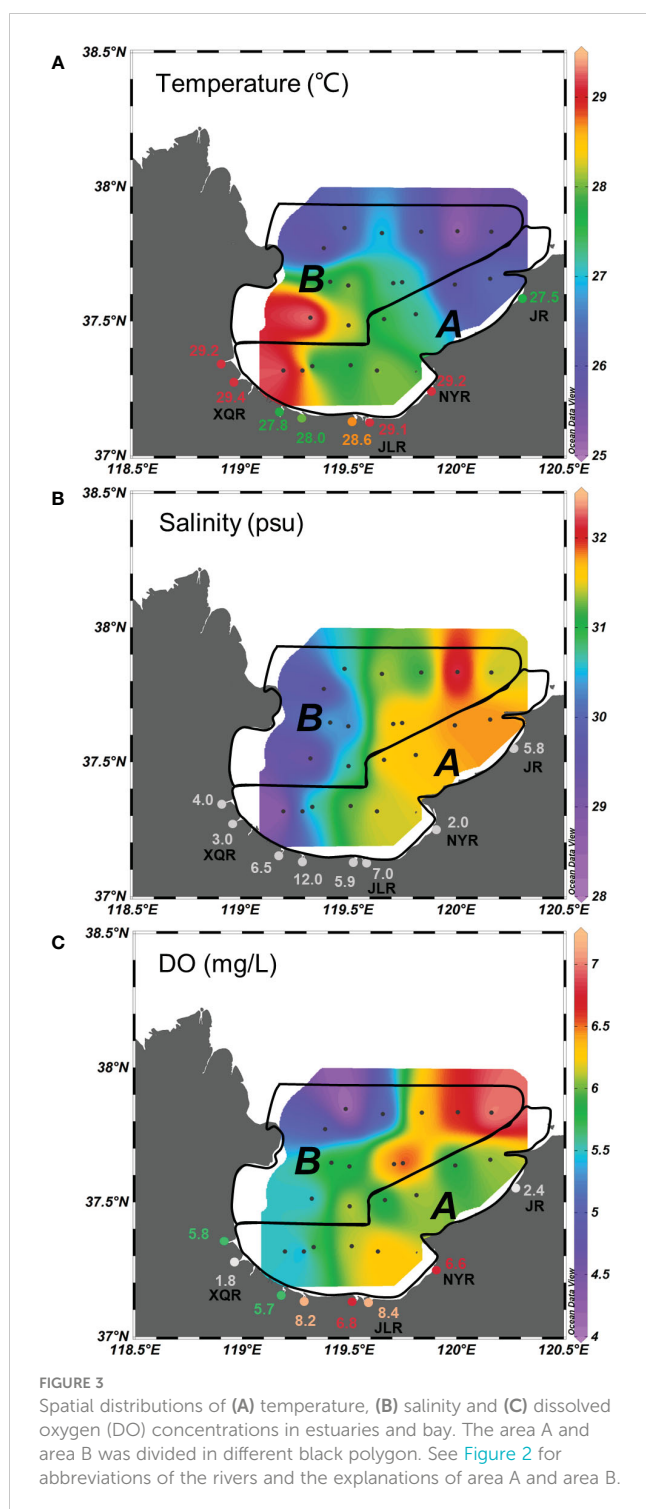
2.6 Statistical analysis

Distribution maps of sea water temperature, salinity, DO, DIN, POC, biomarker concentrations and biomarker indices were generated using software Ocean Data View (Schlitzer, 2021). Maps were created using weight-average gridding loaded by Ocean Data View version 5.6.3.

Statistical tests including t test and Pearson's correlation analysis were performed with OriginPro 2018C software. A probability (p) of 0.05 was used to judge the statistical significance.

3 Results

3.1 Hydrological and chemical parameters

The water temperature, salinity and DO were measured both in estuaries and bay. The water temperature in estuaries was in the range of 27.5–29.2°C, slightly higher than that from the area A (26.0–29.2°C) and area B (25.2–29.1°C) (Figure 3A). Water temperature in the bay was the highest in the most southwestern site of area A nearby the XQR estuary (Figure 3A). The salinity ranged from 2.0 to 12.0 in waters from the estuaries (Figure 3B), showing a brackish condition between river freshwater discharge and sea water intrusion. In the aera A and area B, the salinity varied from 28.4–31.8 and 29.3–32.2, respectively, with lower salinity in the western bay (Figure 3B). DO concentrations in estuaries varied from 1.8 to 8.4 mg/L, with the lowest DO concentration in the XQR Estuary (1.8 mg/L) located at the southwest bank (Figure 3C). In the

FIGURE 3
Spatial distributions of **(A)** temperature, **(B)** salinity and **(C)** dissolved oxygen (DO) concentrations in estuaries and bay. The area A and area B was divided in different black polygon. See Figure 2 for abbreviations of the rivers and the explanations of area A and area B.

area A, the lowest DO concentration (5.3 mg/L) was found in the southwestern bay nearby the XQR estuary, while the highest value (6.3 mg/L) occurred in the southwestern bay nearby the Nanyang River (NYR) estuary and Jiaolai River (JLR) estuary. In the area B, the lowest DO concentration (4.1 mg/L) was found in the northwestern bay in the adjacent area of the Yellow River Estuary, while the highest value (7.0 mg/L) occurred in the most northeast site (Figure 3C). DIN was measured in the bottom water

in Laizhou Bay. Generally, Total DIN and individual DIN (NH_4^+, NO_2^- and NO_3^-) concentrations were generally higher in the western bay of both area A and B, especially in the southwestern sites of area A nearby the XQR Estuary (Figure S2).

3.2 POC and biomarkers

POC concentrations varied from 897 to 4279 µg/L in estuaries, which were much higher than those from the area A (260–767 µg/L) and area B (148–560 µg/L). Maximum POC concentrations were found in the JLR Estuary located at the southeast bank of Laizhou Bay (Figures 4A, S3A). In Laizhou Bay, the highest POC concentrations of 767 µg/L and 638 µg/L were detected at two nearshore sites in the area A (Figure 4A).

Concentrations of terrestrial biomarker long-chain ($C_{26}+C_{28}+C_{30}$) *n*-alkanols were 80–930 ng/L in estuaries, 12–80 ng/L in the area A and 20–180 ng/L in the area B (Figures 4B, S3B). Concentrations of marine biomarker sterols (sum of brassicasterol and dinosterol) were 215–1500 ng/L in estuaries, 74–412 ng/L in the area A and 155–409 ng/L in the area B (Figures 4C, S3C). Long-chain *n*-alkanols concentrations were generally high in the western bay (Figure 4B), while marine sterols concentrations displayed relatively high values in the central bay (Figure 4C). Terrestrial and marine biomarker ratio TMBR′ values were higher in estuaries (0.19–0.61, average 0.33) than that in the area A (0.06–0.41, average 0.20) and area B (0.05–0.41, average 0.17) (Figure 4D), which displayed similar distribution pattern with that of *n*-alkanols (Figures 4B–D). Although average TMBR′ values were close between area A (0.20) and area B (0.17), OC sources were quite different. For terrestrial OC, area A was mainly influenced by small river inputs, while area B influenced by Yellow River input (Figure 4B). Besides, higher primary productivity as indicated by high sterols concentrations (Figure 4C) in offshore area B, resulting in the relative low TMBR′ values in the central and northern of Laizhou Bay.

Anammox biomarker ladderane lipids were detected in all suspended particulates from the estuaries and bay. In estuaries, total ladderane concentrations varied from 1.1 to 6.0 µg/L, with ladderane III and ladderane IV as the two dominating compositions (accounting for 50% and 45% of total ladderanes, respectively) (Figure 4E). For the bay, total ladderane concentrations varied from 0.01 to 2.5 µg/L, showing high values in the area A (Figure 4F). Ladderane III and ladderane IV were also the two most abundant ladderane in Laizhou Bay, accounting for 43% and 42% of the total ladderanes, respectively (Figure S4).

4 Discussion

The distinct spatial differences of ladderane lipid concentrations and compositions were combined with the spatial characteristics of terrestrial and marine lipid biomarkers to discuss ladderane sources and processes for these spatial distributions. Our study area comprised Laizhou Bay and the estuaries of the surrounding small rivers (Figure 2).

4.1 Biomarker proxy estimates of ladderane sources

Ladderanes in particulates and sediments from the open ocean were commonly considered as *in situ* production (Jaeschke et al., 2007; Jaeschke et al., 2009; Rush et al., 2012a; Rush et al., 2012b; Zhao et al., 2019), however, ladderane sources in coastal seas are more complicated due to riverine inputs and anthropogenic influences (Hou et al., 2013; Hu et al., 2016; Zhang et al., 2017b). In this study, we interpret the distinct differences of ladderane lipid compositions and concentrations from the estuaries (average 2.6 μg/L) to area A (average 0.9 μg/L) and then to area B (average 0.1 μg/L) as broadly reflecting ladderane sources. However, the biosynthetic origins of ladderanes from terrestrial and marine anammox bacteria were not well constrained, which limited our understanding of the controlling mechanism of ladderane sources in coastal area. We herein used a ladderane compound ratio in particulates, and the correlation between ladderanes and terrestrial-marine biomarkers (e.g., Hu et al., 2016; Zhao et al., 2019) to semi-quantitatively estimate the origins of ladderanes in Laizhou Bay. The proportion of individual ladderane compounds varied in different anammox bacteria genera (Rattray et al., 2008). The ladderane components were broadly similar in two terrestrial anammox bacteria (*Ca.* Brocadia and *Ca.* Kuenenia) while the marine anammox bacteria *Ca.* Scalindua showed a notably different ladderane proportion (Rattray et al., 2008). The absolute values of ladderane concentrations from different publications were difficult to be compared due to analytical different methods used. However, the relative proportion of each individual compounds in different environments were comparable according to the Rattray et al. (2010), in which another ladderane ratio index 'NL$_5$' was proposed and been widely used in the following studies. We herein proposed a new index in terms of ladderane ratios, Rlad, to distinguish the biosynthetic origin of ladderanes:

$$R_{lad} = \frac{[\text{ladderane III}]}{[\text{ladderane IV}]}$$

R_{lad} was the ratio of ladderane lipids (ladderane III: C_{20}-[5]-ladderane FAME and ladderane IV: C_{20}-[3]-ladderane FAME), both occurred widely with higher concentration than lad I and lad II in natural environments (e.g. Hu et al., 2016; Zhao et al., 2019). In a few culture experiments, R_{lad} value was 1.1 ± 0.2 in terrestrial anammox bacteria *Ca.* Brocadia and *Ca.* Kuenenia, and 0.6 in marine anammox bacteria *Ca.* Scalindua (single measurement) (Rattray et al., 2008). By summarizing published ladderane data (41 samples) of both particulates and sediments, we found R_{lad} values varied significantly in different environments. R_{lad} values were generally above 0.9 in coastal areas, for example 1.0–1.8 (average 1.4) in the Senegal River Estuary (Jaeschke et al., 2010), 0.9–9.0 (average 2.3) in the Pearl River Estuary (Zhu, 2014) and 0.9–2.1 (average 1.5) in the Changjiang River Estuary (Hu et al., 2016). On the other, R_{lad} values were lower than 0.9 in more open ocean environments, such as 0.6–0.8 (average 0.7) in the Celtic Sea (Rattray, 2008; Jaeschke et al., 2009), 0.2–0.3 (average 0.3) in the Arabian Sea (Jaeschke et al., 2007; Rattray, 2008) and 0.1–0.4

(average 0.3) in the Black Sea (Kuypers et al., 2003; Rattray, 2008). Though the absolute ladderane contents might be different in these studies due to the different methods used, the ratio of individual ladderanes were more comparable in the application of R_{lad} ratio. Thus, we propose that the R_{lad} values could semi-quantitatively distinguish ladderane from terrestrial or marine anammox bacteria, with R_{lad} values higher than 0.9 indicating terrestrial source and R_{lad} values lower than 0.9 indicating marine source.

4.1.1 Ladderane sources and concentrations in estuaries

In estuaries, the highest ladderane concentrations and highest TMBR′ values were found in the XQR and Jie River (JR) estuaries (Figures 4D, E, S3D). Thus, ladderane concentrations showed significant positive correlation with terrestrial TMBR′ index (Figure 5A, r = 0.82, p< 0.05), indicating terrestrial sources for ladderanes (Hu et al., 2016; Shi et al., 2021). The R_{lad} values were in the range of 0.9–1.9 in estuaries (Figures 6A, B) and provided strong evidence that ladderanes in these small river estuaries were produced by terrestrial anammox bacteria *Ca.* Brocadia and *Ca.* Kuenenia. By using molecular techniques, the abundances of anammox bacteria were found to be negatively correlated with salinity in Laizhou Bay (Zhang et al., 2014), which proved that brackish estuarine water was favorable for the growth of terrestrial anammox bacteria.

Specific river and estuarine conditions caused the different ladderane concentrations and compositions in the estuaries. Although the highest *n*-alkanols concentrations were found in the JLR Estuary (Figures 4B, S3B), its total ladderane concentration was lower than those of the JR Estuary and the XQR Estuary (Figure 4E). Therefore, additional factors could have contributed to the source and distribution of ladderanes in estuaries. Consistent with previous studies (e.g., Brandsma et al., 2011; Sáenz et al., 2012; Hu et al., 2016; Zhao et al., 2019), we found a significant negative correlation between ladderane and DO concentrations in estuaries (Figure 5B, r = − 0.89, p< 0.05). The DO concentrations were significantly low (under t-test, p< 0.05) in the XQR Estuary (1.8 mg/L) and the JR Estuary (2.4 mg/L). Thus, DO was an important factor for ladderane distribution. Besides, the relative contribution of anammox to N_2 production were higher in low DO environment of estuaries (Zhang et al., 2014), further proved that hypoxia caused by anthropogenic influence could promote the activity of anammox in estuaries of Laizhou Bay, which was in accordance with our high ladderane concentrations at XQR and JR estuaries with hypoxic condition (Figure 7). We suggested that high ladderane concentration in the XQR was the combined contribution of *in situ* production under lower DO concentration and the lateral transportation from upper river reach (Figure 7). In contrast, the small JR received much less terrestrial input (Figure 4B), and *in situ* ladderane production under low DO conditions would be the main source of ladderanes in the estuary (Figure 7). As to the JLR Estuary, although terrestrial input was high as revealed by high *n*-alkanols concentrations (Figure 4B), high DO concentration (> 8mg/L; Figure 3C) might restrict the annamox activity and the *in situ*
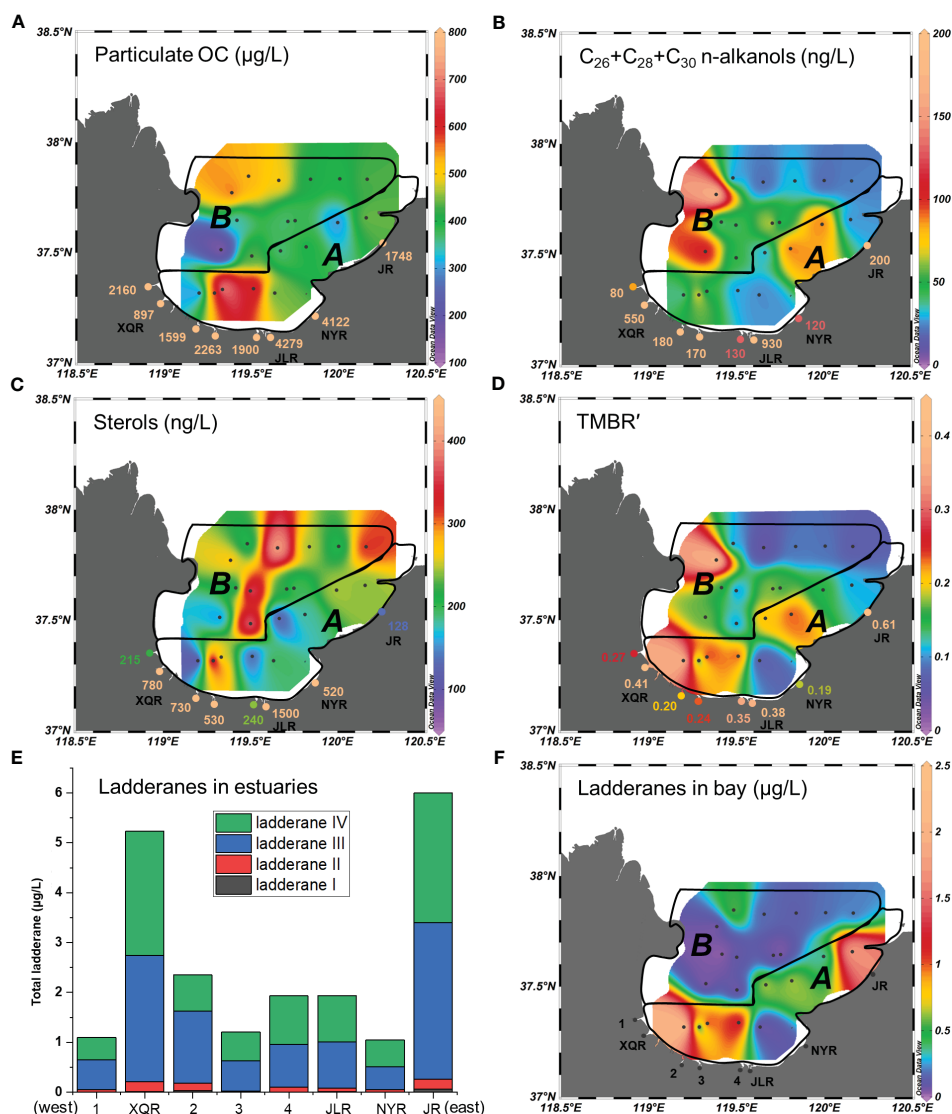
FIGURE 4
Spatial distributions of **(A)** particulate organic carbon (POC) concentrations in all sites, **(B)** long chain *n*-alkanol concentrations in all sites, **(C)** sterols (sum of brassicasterol and dinosterol) concentrations in all sites, **(D)** TMBR′ values in suspended particulates in all sites, **(E)** ladderane concentrations in estuaries, and **(F)** ladderane concentrations in bay. Concentrations of POC and biomarkers were measured from suspended particulates of each sampling site. See Figure 2 for abbreviations of the rivers and the explanations of area A and area B.

production of ladderanes (Liu et al., 2008). Therefore, ladderane concentration was just in moderate level in the JLR (Figures 4E, 6). In other small river estuaries of Laizhou Bay, the combination of low terrestrial input and high DO conditions resulted in lowed ladderane concentrations (Figure 7). Although all these river estuaries in Laizhou Bay were easily influenced by saltwater intrusion (Shi and Jiao, 2014; Zhang et al., 2017a), saltwater intrusion was not likely be the main origin of ladderanes because ladderanes in estuaries were mainly originated by freshwater anammox bacteria revealed by high R_{lad} values (Figure 6A). In addition, a study in the Changjiang River showed that dam could enhanced the anammox role in nitrogen loss (Chen et al., 2019), thus might influence the origin of ladderanes. However, comparing with the Changjiang River, river flows were much lower at the east and south bank of Laizhou Bay, with the highest runoff at 8.78×10⁸

m³/a from the Xiaoqing River. The influence of front-dam to the source of ladderanes remained uncertain in Laizhou Bay and should be further explored in the future.

4.1.2 Ladderane sources and concentrations in area A

The compositions of ladderanes were similar between estuaries and area A, with high proportions of Ladderane III and IV contributing 95% and 85% to total ladderanes in estuaries and area A, respectively (Figures 4E, S3). The spatial distributions of total ladderanes and individual ladderane in area A were broadly consistent with the corresponding estuaries. For example, high ladderane concentrations at the southwest and northeast sites from area A agreed with the high ladderane concentrations in the XQR and JR estuaries (Figures 4E, F). The extremely low ladderane
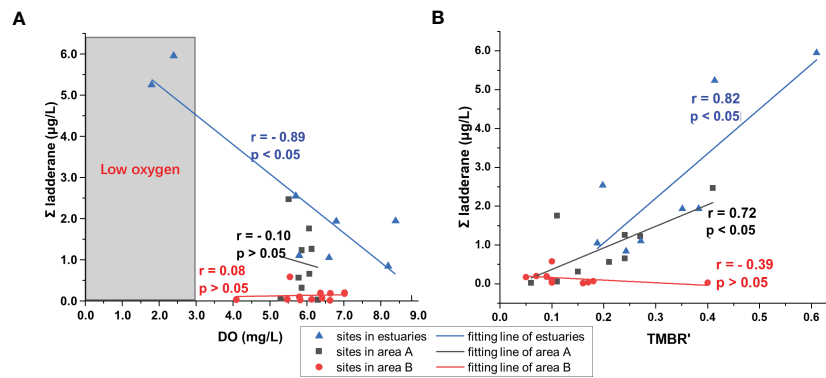
FIGURE 5
Relationships of ladderane concentrations with **(A)** TMBR′ values and with **(B)** DO concentrations in suspended particulates from the Laizhou Bay. Blue triangles in the plots represent sites in estuaries; Black squares and red dots in the plots represent sites in the area A and the area B of the inner bay, respectively.

concentration (< 0.01 μg/L) occurred at one station from the southeast of area A (Figure 4F), which was adjacent of the NYR Estuary with lowest ladderane concentration among all the estuaries (Figure 4E). These qualitative correlations suggested that particulate ladderane in area A were mainly biosynthesized in terrestrial environment and consequently transported to the bay by river discharges. This was further supported by the positive linear correlation between ladderane concentration and the TMBR′ index (r = 0.72, p< 0.05, Figure 5A). Furthermore, the average

value of R_{lad} (1.1) in area A was comparable with those from estuaries (Figure 6B), confirming the terrestrial biosynthesis origin of ladderanes. It should be noted that the R_{lad} value in the southeast station in area A was much lower than that of the nearby NYR Estuary (Figure 6A), likely a result of less river influence in the southeast Laizhou Bay by the low river flow (in Figures 4B–D). The poor correlation between the ladderane and DO concentrations (r = − 0.10, p > 0.05) (Figure 5B) suggested that the ladderanes were not likely produced *in situ* in area A. The measured DO concentrations

FIGURE 6
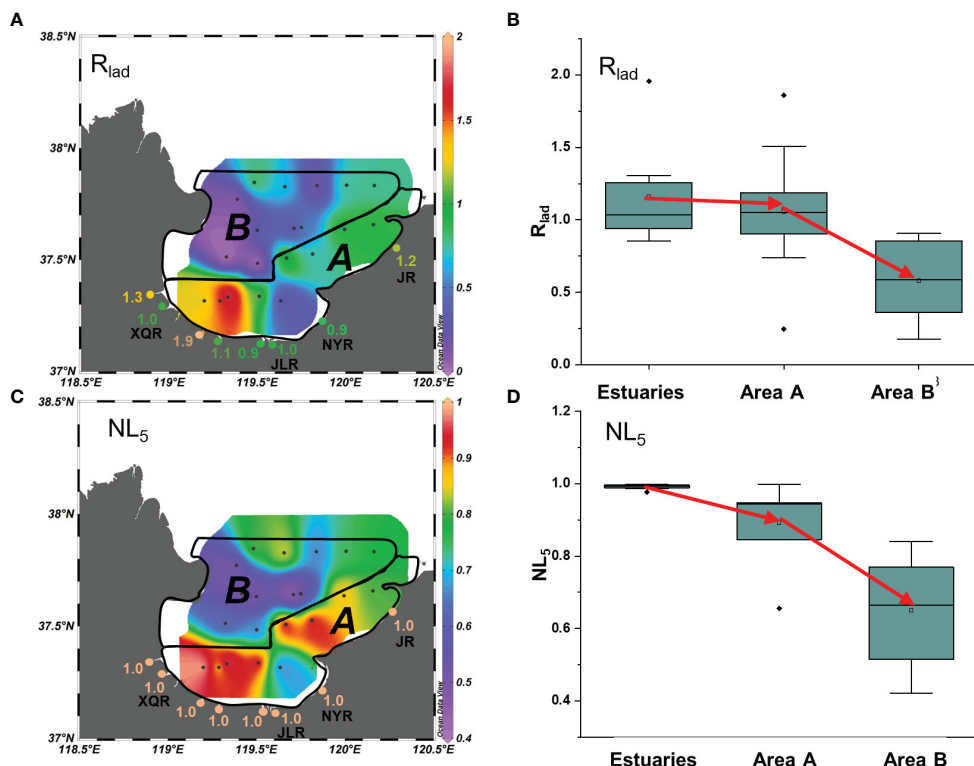**(A)** Spatial distribution and **(B)** box-and-whisker plot of R_{lad} values in suspended particulates. **(C)** Spatial distribution and **(D)** box-and-whisker plot of NL_5 values of in suspended particulates. Black dots in the box plots represent the outliers of R_{lad} or NL_5 values in different areas. See Figure 2 for abbreviations of the rivers and the explanations of area A and area B.
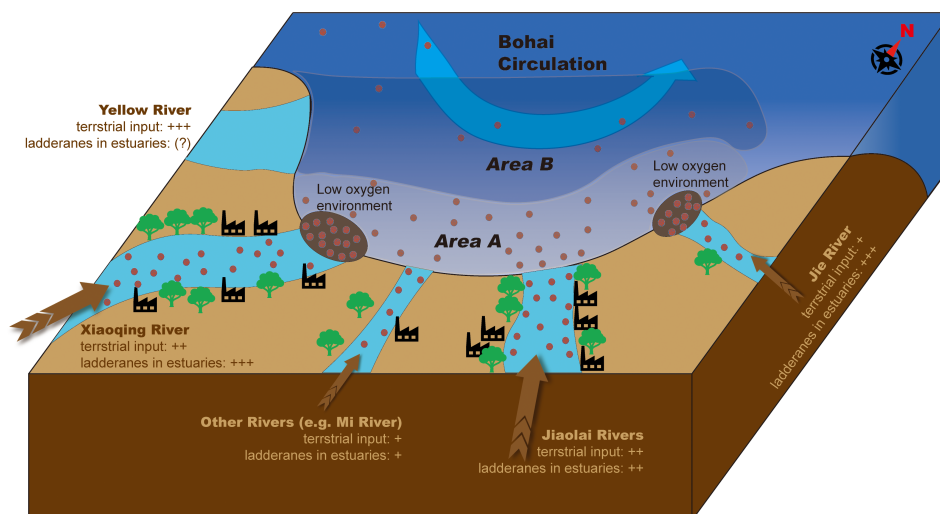
FIGURE 7
Schematic illustrations of ladderane distributions in Laizhou Bay. In the Xiaoqing River (XQR) Estuary, high terrestrial input and *in situ* production of ladderanes in the hypoxic water resulted in high ladderane concentrations. In the Jie River (JR) Estuary, low terrestrial input and *in situ* biosynthesis in the hypoxic water column also caused high ladderane concentrations. In the Jiaolai River (JLR) Estuary, ladderanes showed moderate values, accompanied with the abundant terrestrial input and the suppression of saturated DO (> 8mg/L). In other small river estuaries of Laizhou Bay, low terrestrial input and sufficient oxygen resulted in the relatively low ladderane concentrations. Although the Yellow River discharge was high, anammox rates in the Yellow River were very low (Zhang et al., 2017b), thus made very limited contribution to the ladderane concentrations in Laizhou Bay. Sewage plants and outlets were shown as factory icons along the rivers. Inside the bay, Area A and Area B was distinguished by different colors inside the bay, and ladderane concentrations in area A was higher than in area B because of the southern and eastern small river inputs. Ladderanes/anammox bacteria in area B could be transported *via* exogenous Bohai circulation.

in the area A were all above 3 mg/L (the threshold of hypoxia), not favorable for the anammox growth (Kuypers et al., 2005; Hamersley et al., 2007; Dalsgaard et al., 2012) and supporting the above deduction. Overall, ladderanes area A originated from the similar terrestrial sources as in estuaries, with limited contribution from *in situ* production.

4.1.3 Ladderane sources and concentrations in area B

Ladderane concentrations had poor relationship with TMBR′ values (Figure 5A), indicating terrestrial input from rivers might not be the main source of ladderanes in area B. However, long-chain *n*-alkanol concentrations and TMBR′ values were both high in the northwestern bay (Figures 4B–D), because the Yellow River could transport huge amount of terrestrial OC to area B (Qiao et al., 2010). The anammox activity was proved to be very weak in the Yellow River (Zhang et al., 2017b) comparing with that of the Bohai Sea (Zhang et al., 2018), and ladderanes from the Yellow River were unlikely the main sources of ladderanes in Laizhou Bay (Figure 7). This conclusion was supported by the lower R_{lad} values in the area B (0.2–0.9, Figure 6B), which indicated a marine biosynthetic origin of ladderanes. On the other hand, the NL_5 values (0.42–0.82) from the area B corresponded to a range of 14.8–19.8 °C for ladderane biosynthesis temperature, much lower than the *in situ* sea water temperature (25.2–29.4°C) in the area B (Figure 3A). Thus, ladderanes were not likely produced within area B, but could have originated from a lower temperature environment and then be transported to area B. One site at the boundary of the area B and the Bohai had relatively high ladderane concentration (0.58 μmol/L,

Figure 4F), and this site was easily influenced by the anti-clockwise Bohai current which resulted in lower water temperature (Qiao et al., 2010; Zhou et al., 2017; Wei et al., 2019), suggesting a potential source of ladderane from this region. The DO concentration was also low in this site (4.1 mg/L, Figure 3C), providing a suitable environment for anammox bacteria. However, we suggest that *in situ* production here was not the main origin of ladderane lipids since the ladderane biosynthesis temperature revealed by NL_5 as 19.2 °C was much lower than *in situ* temperature 26.3 °C. Indeed, previous studies had reported that there were bottom hypoxic zones in the northwest of Laizhou Bay (e.g., Zhou et al., 2017; Wei et al., 2019; Figure 2) with significant anammox rates (Zhang et al., 2018). We thus propose that ladderanes produced in the Bohai Sea and near the Bohai Sea-Laizhou Bay boundary could be transported to area B in summer by the anti-clockwise Bohai circulation (Wei et al., 2003; Qiao et al., 2010; Hu et al., 2013; Hu and Wang, 2015).

4.2 An indicator of human influences on anammox activity

Our study of the sources and spatial distributions of ladderanes in Laizhou Bay region provides an example of using ladderanes as an indicator to evaluate human influences on anammox activities in the river-coastal water environments. Laizhou Bay has been considered as a typical eutrophic bay since the 1980s (Jiang et al., 2005), with DIN concentration increased of 10 folds from the 1980s to the 2000s followed by gradual reduction since 2010 (Zhu et al., 2019).

Nutrients and OC into Laizhou Bay were transported by rivers mostly flow through the Shandong Province, where the population and economy had been growing fast in North China (National Bureau of Statistics of China, 1999–2020). According to the official statistical results of pollution source published by the Shandong Provincial Department of Ecological Environment (http://117.73.254.13:8801/wryfb/MapMainT.html), ~ 100 key sewage outlets and ~ 50 key sewage plants existed in the drainage area of rivers entering Laizhou Bay. Most of these outlets were located in the Xiaoqing River basin and the Jiaolai River basin (Figure 7). The Xiaoqing River, for example, was severely contaminated by anthropogenic discharge especially in wet season. A recent study reported that the Xiaoqing River displayed high discharge flux to Laizhou Bay with 2649.2 t/a for DIN, 89.94 t/a for phosphate and 4495.6 t/a for COD (chemical oxygen demand) (Lu, 2022). Besides, multiple wastewater outlets and sewage treatment plants were located along the river as reported by Shandong Provincial Department of Ecological Environment (Figure 7). The eutrophication and organic matter pollution triggered oxygen depletion along the river and in the estuary (Meng et al., 2005; He et al., 2018a; He et al., 2018b), providing favorable conditions for anammox growth (e.g., Dang et al., 2010; Zhao et al., 2019; Li et al., 2021). The point sources of sewage discharge could be also an important origin of ladderanes in estuaries because anammox bacteria and ladderanes were generally detected in sewage water/sludge (e.g., Ren et al., 2022) and nearby the wastewater outlets (e.g., Shi et al., 2021). Therefore, the ladderane contribution to the river estuaries was closely linked to the contaminated condition of rivers under anthropogenic influences. Inside Laizhou Bay, the distribution of ladderanes was less influenced by the *in situ* water condition but rather being mostly affected by river inputs or by bay circulation transport.

In order to further evaluate the anammox activity affected by the terrestrial and anthropogenic influences in a wider context, we compared the ladderane concentrations in particulates from Laizhou Bay with published data (Figure 8). Since that ladderane concentrations could reflected the intense of anammox activity (e.g., Pitcher et al., 2011; Rush et al., 2012a; Zhao et al., 2019), we could compare the anammox activity under different source mechanisms in different sea area. It should be noted that the previous studies only focused on the anammox process in sediments of Chinese marginal seas by using molecular techniques and isotope labelling technique (e.g., Dang et al., 2010; Hou et al., 2013; Zhang et al., 2019; Teng et al., 2022), but did not include the potential anammox activity in water particulates. We only compared ladderane concentrations from the China marginal seas because these data were analyzed using same procedures including ladderane extraction, purification, detection and quantification (Zhu, 2014; Hu, 2016; Hu et al., 2016; Shi et al., 2021). Generally, ladderane concentrations at hypoxia sites were higher than other sites in the same study area (Figure 8), which further proved that oxygen level (human influences) was the main controlling mechanism of anammox activity. By comparing ladderane data between different studies, the highest ladderane concentrations occurred in the estuaries of Laizhou Bay (average 2.6 µg/L, this study, Figure 8), where ladderanes mainly originated from terrestrial environment under severe anthropogenic influence. Highest ladderane concentrations in Laizhou Bay estuaries indicated the more intense anammox activity in small river estuaries comparing with other coastal areas. Ladderane concentrations in Jiaozhou Bay were also high (average 1.5 µg/L) though no obvious hypoxia occurred (Shi et al., 2021). As a semi-closed small bay in North China, Jiaozhou Bay was also strongly affected by anthropogenic discharge. Ladderanes distribution in Jiaozhou Bay was affected by river flow and wastewater discharge and their concentrations could reflect the bottom water quality condition (Shi et al., 2021). However, comparing with these small-scale systems above, ladderane concentrations were low in large rivers such as the Changjiang River and the Pearl River (Figure 8), which are two of the biggest rivers in China with large terrestrial input (Gao et al., 2002; Sun et al., 2020;

FIGURE 8
Comparisons of ladderane concentrations in suspended particulates from China marginal seas. Black bars in the plot represent the average ladderane concentrations. The red stars represent the ladderane concentrations at oxygen-depleted sites in each region. The ladderane data were obtained from Shi et al. (2021) in the Jiaozhou Bay, Hu et al. (2016) in the Changjiang Estuary, Hu (2016) in the East China Sea, and Zhu (2014) in the Pearl River Estuary.

Zhou et al., 2022) and occasional hypoxia events in estuaries (Dai et al., 2006; Li et al., 2020). Moreover, anammox activities had been observed in both two rivers and provided evidence for the terrestrial input of ladderanes in estuaries (Hou et al., 2018; Wang et al., 2019a), we thus deduced that the concentrations of terrestrial ladderanes might be diluted by the huge riverine flow. As to the East China Sea (ECS) adjacent to the Changjiang Estuary, ladderanes in the bottom water were proved to be produced *in situ* rather than terrestrial input (Hu et al., 2016), generally lower than in bays and most estuaries (Figure 8). However, ladderane concentrations in the hypoxic area of the ECS were much higher than other sites of the ECS and comparable with the hypoxic area of the Changjiang Estuary (Figure 8). The bottom hypoxia in the ECS has been occurring since the 1950s (Wei et al., 2007; Zhu et al., 2011; Liu et al., 2020). Increasing DIN discharge from the Changjiang River was the main reason of the occurrence and increasing of hypoxia zone in the East China Sea (Zhang et al., 2007; Wang et al., 2016). In conclusion, high ladderane concentrations in particulates were more likely occurred in small bays and hypoxic areas, furthermore indicated that the anammox activity could be enhanced by anthropogenic influence like sewage discharge, eutrophication and hypoxia. Nitrogen loss by anammox process might be underestimated in such small-scale system. In addition, the main terrestrial source of ladderanes in estuaries could prove key evidence for the importance of terrestrial contribution to coastal anammox process. Therefore, by identifying the sources of the ladderanes in human-influenced bays, we can better evaluate the intensity of anammox activity in these small but vital river and estuarine systems and further estimate the anammox contribution in the nitrogen cycle from coastal seas.

5 Conclusion

This study reported new data of anammox biomarker ladderanes and other lipid biomarkers in suspended particulates from Laizhou Bay and surrounding river estuaries, Bohai Sea in North China. Based on ladderane distributions, compositions, and ratios, as well as correlations between ladderanes and terrestrial index and DO concentrations, our conclusions were as follows: (1) The ladderane ratio of R_{lad} was proposed to distinguish ladderane biosynthetic origins by terrestrial anammox bacteria ($R_{lad} > 0.9$) and marine anammox bacteria ($R_{lad} < 0.9$) of ladderanes. (2) Ladderanes in particulates from the estuaries and nearshore area of Laizhou Bay mainly originated from the terrestrial input *via* riverine inflow as well as *in situ* production in oxygen-depleted estuaries, while ladderanes in offshore area were possibly from ex situ marine environment, such as the cold hypoxia water input by the Bohai circulation. (3) By comparing our ladderane data in particulates with previously published data from China marginal seas under different human influences, we found that high ladderane concentrations synthesized by terrestrial anammox bacteria could contribute significantly in coastal seas. In bays and estuaries, the role of anammox in nitrogen cycle, like the rate and contribution of N_2 production, should be further evaluated for the nitrogen budget influenced by enhanced anthropogenic activities.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MS: Conceptualization, Investigation, Writing - Original Draft. XX: Conceptualization, Writing - Review & Editing, Supervision. HM: Resources, Investigation. LL: Validation, Resources. YW: Validation, Resources. CD: Validation, Resources. YD: Validation, Resources. MZ: Conceptualization, Writing - Review & Editing, Funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1215883/full#supplementary-material

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