Blue-Green Systems

Historical exposure to wastewater disposal reinforces the stability of sediment bacterial community in response to future disturbance

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ABSTRACT

Given that long-term treated wastewater discharge may alter the microbial community of the recipient coast, it is important to evaluate whether and how the community's stability is impacted. We constructed microcosms using coastal sediments with (near-coast) and without (far-coast) a wastewater disposal history and compared the communities' responses to p-chloroaniline (PCAN, a typical organic pollutant) in low (10 mg/L) and high (100 mg/L) concentrations. Compared to the far-coast community, the near-coast community drove faster PCAN attenuation and nitrate generation. More significant negative correlations were observed between the alpha-diversity indices and PCAN concentrations in the far-coast communities than the near-coast ones. The community turnover rate, represented by the slopes of the time–decay curves, was slower for the near-coast community (-0.187) than that for the far-coast community (-0.233), but only when the PCAN was added in low concentration. Our study revealed that the long-term wastewater disposal may cause the sediment bacterial community to be less sensitive and more stable in response to a future disturbance, demonstrating a significant historical effect of environmental context on the coastal microbial community's stability.

Key words: coastal bacterial community, historical exposure, stability, time–decay relationship, wastewater disposal

HIGHLIGHTS

- Faster pollutant attenuation drove by microorganisms with a disturbance history.
- Slower turnover rate of the microbial community affected by the disposal of the wastewater treatment plants.
- Disturbance history slightly enhanced the microbial stability to future pollution.

GRAPHICAL ABSTRACT
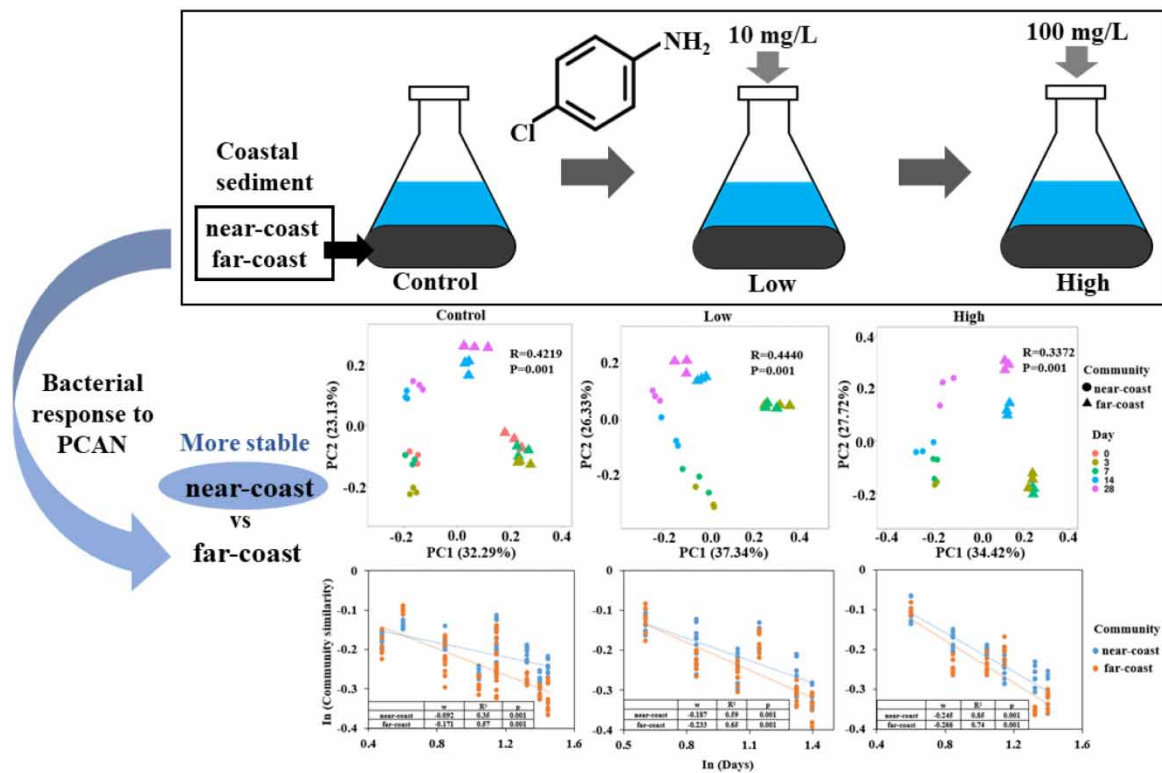
INTRODUCTION

The abundant and diverse microorganisms in coastal ecosystems play important roles in land-sourced pollutants' transformation, water purification, and element cycling (Harley *et al.* 2006; Lidbury *et al.* 2012; Ali *et al.* 2016; Bryson *et al.* 2017). However, as the microbial communities can be affected by various disturbances, increasing anthropogenic activities in coastal areas, such as wastewater discharge, aquaculture, shipping, and port construction, usually influence negatively on microbial communities and cause significant health risk to human beings (Balmford & Bond 2005; Stewart *et al.* 2008).

Although advanced technologies have been applied in many wastewater treatment plants (WWTPs), a certain amount of complex pollutants still remained in the effluent (Martí *et al.* 2011; Tao *et al.* 2017). Therefore, discharging WWTP's effluent often poses a threat to the coastal waterbody (Moon *et al.* 2008; Zhang *et al.* 2013; Rizzo *et al.* 2019; Su *et al.* 2020). For example, polluted by the treated wastewater from the pulp and paper industry, the coastal sediments of the Gulf of Finland contained 120–620 μg/g (dry weight) of adsorbable organic halogens (AOXs), which are refractory and toxic to microorganisms (Kankaanpää & Tissari 1994). In China, Hangzhou Bay receives at least 645.4 t of AOXs from the surrounding WWTPs every year (Xie *et al.* 2018; Zheng *et al.* 2019). A number of studies evidenced that WWTP's effluent promoted the shifts of microbial communities in both structure and function (Chahinian *et al.* 2012; Zhang *et al.* 2014; Atashgahi *et al.* 2015; Saarenheimo *et al.* 2017; Mansfeldt *et al.* 2020), among which reduced community diversity and enriched pollution-tolerant species were the vital changes (Drury *et al.* 2013; García-Armisen *et al.* 2014). Here, we further considered the following question: if both microbial communities, i.e., the pristine one and the shifted one with historical exposure to WWTP's effluent, are affected by a future disturbance, which one would be more sensitive and faster to respond to the disturbance? Unfortunately, no investigation was made into this question.

In this study, we constructed microcosms using coastal sediments with different environmental histories from Hangzhou Bay and tracked the responses of sediment bacterial communities to an additional organic pollutant, p-chloroaniline (PCAN), with different concentrations using high-throughput sequencing of 16S rRNA genes. We hypothesized that the pre-exposed bacterial community would be more tolerant to the additional disturbance, since its past environmental condition may leave an imprint on the adaptation and stress tolerance (Székely &

Langenheder 2017). PCAN is one of the typical AOX species frequently detected in water and soil environments, because it is a commonly used compound and an organic intermediate in dyestuff, pharmaceutical, pesticide, and herbicide industries. Therefore, we used PCAN as a representative of AOXs to evaluate their impacts on coastal ecosystems. Understanding the sensitivity of coastal bacterial communities to future anthropogenic disturbance would improve our abilities of environmental forecast and decision-making.

MATERIALS AND METHODS

Collection of original sediment samples

As one of the most urbanized and industrialized areas in east China, the Hangzhou Bay is heavily affected by the discharge of municipal and industrial wastewaters. Coastal sediments were collected from Hangzhou Bay at two sites (Supplementary Material, Fig. S1): (1) the near-coast site (30°13′12″N, 120°51′36″E), which is suffering from long-term AOX-contained WWTP's effluent discharge near the south bank of the bay (Zhang et al. 2014) and (2) the far-coast site (30°25′48″N, 121°2′24″E), which is free from direct anthropogenic disturbances. At each site, about 2 kg of surface (0–10 cm) sediment samples were collected by a grab sampler (Van Veen, Hydro-Bios, Germany), stored in aseptic plastic bags, and transported to our laboratory at 4 °C for the microcosm experiment. Intense hydrodynamic flows enhance the dissolved oxygen (DO) transportation via large fluxes of oxygenated seawater down to the sediments in the Hangzhou Bay (Xie et al. 2009). The DO concentrations in the sediment samples overlying water in the Hangzhou Bay were >8.0 mg/L, as suggested by in situ measurements (Dai et al. 2020). Accordingly, the microcosms were kept in aerobic conditions during the incubation.

Microcosm setup and collection of samples

A synthetic seawater stock solution was prepared with NaCl (22 g/L), Na_2SO_4 (3.7 g/L), $NaHCO_3$ (0.2 g/L), $NaNO_3$ (0.06 g/L), $MgCl_2·6H_2O$ (9.7 g/L), KCl (0.65 g/L), $CaCl_2$ (1 g/L), and H_3BO_3 (0.023 g/L) and then filtrated by a 0.2-mm membrane to remove bacteria and was diluted five times by sterile water before use (Dai et al. 2020). The original coastal sediments were stabilized with the synthetic seawater (volume ratio 1:2) at 4 °C in dark for 48 h before the experiment.

Microcosms were constructed with 50 mL of synthetic seawater and 25 g of the stabilized coastal sediment in a series of 100 mL sterile Erlenmeyer flasks. Low (10 mg/L) or high concentration (100 mg/L) of PCAN was spiked in the microcosms to represent moderate or strong disturbance of a typical AOX input, while no PCAN was added in the control microcosms (Dai et al. 2020). The microcosms were sealed with a sterile sealing film, incubated at 25 °C, and shaken twice per day to maintain the aerobic environment.

The initial stabilized sediments were sampled and labeled as day 0. Triplicate samples were prepared from each microcosm during the incubation at days 3, 7, 14, and 28. All the sediment samples were stored at −80 °C before DNA extraction and 16S rRNA gene sequencing, and all the water samples were used for PCAN and nitrate analyses immediately after sampling.

Chemical analysis

The water sample from each microcosm was filtered by 0.45 mm membranes. The concentration of PCAN was measured by high-performance liquid chromatography (Shimadzu, LC20AT, Japan) using a C18 chromatographic column (5 μm), and the UV detector was set at 240 nm. The mobile phase was a mixture of 70% methanol and 30% water with a flow rate of 1 mL/min. The concentration of nitrate was measured by the ultraviolet spectrophotometric screening method (Li et al. 2020).

DNA extraction and bacterial 16S rRNA high-throughput sequencing

Total DNA was extracted from 0.25 g of the sediment sample by a Power Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). The purity and concentration of the extracted DNA were measured by a NanoDrop UV-Vis spectrophotometer (Thermo Fisher, Waltham, MA, USA). All the extracted DNA were diluted to 2 ng/μL before the PCR. The V4 hypervariable region of bacterial 16S rRNA gene was amplified using the primer pairs of 515F (5′-GTGCCAGCMG CCGCGGTAA-3′) and 806R (50′-GGA CTA CHVGGG TWT CTA AT-30′) (Caporaso et al. 2011) using BioRad S1000 (Bio-Rad Laboratories, Irvine, CA, USA). The PCR program was as follows: initial denaturation at 94 °C for 5 min; followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 52 °C for 30 s, then renaturation at 72 °C for 30 s; and extension at 72 °C for 10 min at last. The

PCR products were purified by EZNA Gel Extraction Kits (Omega, GA, USA) and then sequenced on Illumina MiSeq 2500 platform at Magigene Co., Ltd (Guangzhou, China).

Raw sequencing reads were filtrated by Trimmomatic (V0.33, http://www.usadellab.org/cms/?page=trimmomatic). The obtained clean reads were merged by FLASH (V1.2.11, https://ccb.jhu.edu/software/FLASH/). Sequences with a similarity of >97% were clustered into the same operational taxonomic units (OTUs) using UPARSE (Edgar 2013). The OTUs were annotated by the RDP classifier (https://sourceforge.net/projects/rdp-classifier/) with 50% confidence cutoff (Dai *et al.* 2020).

Statistical analysis

All statistical analyses were performed using R (V4.0.3, http://www.r-project.org). OTUs' rarefaction curves and the communities' alpha-diversity indices (Richness, Shannon-Wiener and Pielou evenness) were calculated by the function 'diversity' in the *vegan* package. To screen out the bacterial OTUs that responded significantly to PCAN, Pearson's correlation test was performed between OTUs' abundances and PCAN concentrations using the function *cor.test* in the *stats* package. Principal coordinates analysis (PCoA) based on the Bray–Curtis distance was used to visualize bacterial communities' dynamics over time by the function *pcoa* in the *ape* package. Bacterial communities' turnover rates were explored by the time–decay relationships based on the Bray–Curtis distance (Liang *et al.* 2015) using a power law model, $S = CT^w$; where S is the pairwise similarity of community composition, T is the time interval, and w is the regression slope of bacterial community similarity (Dai *et al.* 2020). To achieve the significance comparison of regression slope (w-value), the observed slope was compared with the distribution of slopes generated by 999 times permutation of the original community matrix (Zhou *et al.* 2008; Dai *et al.* 2020).

RESULTS

Changes of chemical factors

PCAN was not detected in the water samples after 14 days of incubation in all microcosms (Figure 1(a)). During the process of PCAN biodegradation, the hydrogen bonding between amidogen and benzene ring can be broken under the aerobic condition and then the NH_2^- is oxidized to nitrate via nitrification (Latorre *et al.* 1984). In the present study, nitrate concentrations decreased in the beginning 3 days and later increased gradually (Figure 1(b)). At the end of the incubation, nitrate concentrations in microcosms with high PCAN loading were much higher
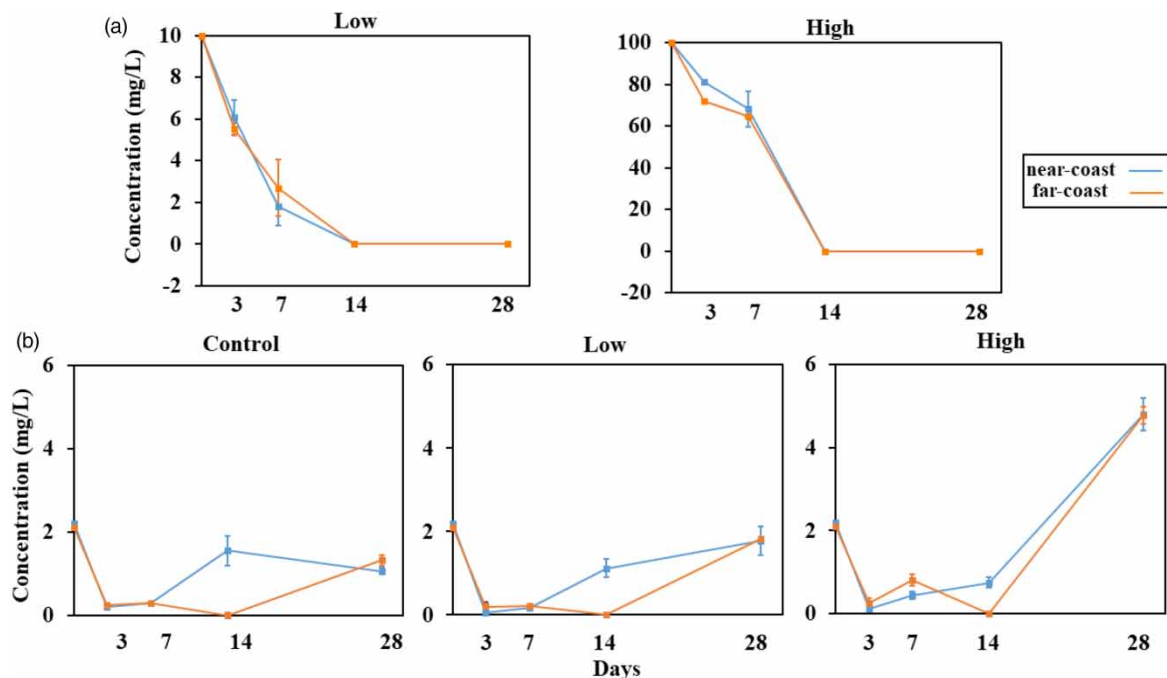

Figure 1 | The concentrations of (a) PCAN and (b) nitrate in the overlying water during 28 days' incubation of two communities in control, low, and high PCAN groups. Error bars represent the standard error of triplicate microcosms.

than the low loading and control microcosms, suggesting the generation of nitrate via nitrification during PCAN transformation. Noticeably, the nitrate was generated at higher rates in microcosms with the near-coast sediment than those with the far-coast sediment (Figure 1(b)), suggesting a higher PCAN transformation efficiency of the near-coast microbial community.

Alpha-diversity of the bacterial community

A total of 17,466 bacterial OTUs at a 97% similarity level were observed in all the sediment samples. We equalized the sequence depth to 38,231 per sample for downstream analysis and got 17,049 OTUs. The Goods coverage of the sequence libraries ranged from 95 to 97%, suggesting that the present sequencing depth was satisfied to reveal most of the bacterial community diversity. The rarefaction curves (Supplementary Material, Fig. S2) generally indicated that more bacterial species were in the control groups than those in the low and high PCAN groups. Based on the Richness, Shannon–Wiener, and Pielou indices (Supplementary Material, Fig. S3), the bacterial alpha-diversity for both the near-coast and far-coast communities decreased at the beginning 3 days in three groups and then recovered gradually till the end of the incubation. Although the alpha-diversity of two bacterial communities had a similar trend with and without PCAN loading, the alpha-diversity in the control groups better recovered than those with the PCAN loading. Moreover, in both the low and high PCAN groups, the recovery of the near-coast communities was slightly faster than that of the far-coast communities, suggesting a higher resilience of the near-coast communities to PCAN loading.

The effect of different PCAN loads on bacterial alpha-diversity is further analyzed in Table 1. More significant negative correlations were observed between an alpha-diversity index and a PCAN load in the far-coast communities than the near-coast ones. For the far-coast communities, the correlation became stronger with increasing PCAN load, while for the near-coast communities, stronger correlation was only reflected by the Pielou index, demonstrating a higher sensitivity of the far-coast community to the PCAN load.

Succession patterns of bacterial communities

PCoA based on the Bray–Curtis similarity was used to explore the overall succession patterns of the bacterial communities in response to PCAN load during the incubation of microcosms.

The near-coast and far-coast communities separated clearly along PC1 in the control, low, and high PCAN groups, but approached convergence along PC2 at the end of the incubation (Figure 2(a)). Despite similar succession trajectories along time, the near-coast communities changed rapidly at the beginning, but the far-coast ones were dilatory, suggesting the near-coast communities were more responsive than the far-coast ones. The Adonis test revealed that the initial bacterial community structure was the most influential factor to the community successions ($F = 23.35$, $P = 0.001$), followed by incubation time ($F = 17.97$, $P = 0.001$) and PCAN concentration ($F = 2.88$, $P = 0.002$) (Table 2).

Known as the time–decay relationships in community similarity, the significant negative linear correlations between the community Bray–Curtis distance and time interval were observed with varied regression slopes (w value) (Figure 2(b)). For both the near-coast and far-coast communities, the regression slopes were steeper in the low and high PCAN groups (-0.187 to -0.266) than those in the control groups (-0.092 to -0.171),

Table 1 | Relationship between alpha-diversity indices of bacterial communities and PCAN concentration

		Near-coast			Far-coast		
		Coefficient	R^2	P	Coefficient	R^2	P
Richness-PCAN	Low	−0.873	0.762	0.000***	−0.062	0.004	0.849
	High	0.579	0.335	0.049*	−0.703	0.494	0.011**
Shannon-PCAN	Low	−0.448	0.201	0.144	−0.567	0.321	0.055•
	High	−0.463	0.214	0.129	−0.899	0.808	0.000***
Pielou-PCAN	Low	−0.133	0.177	0.680	−0.595	0.354	0.041•
	High	−0.732	0.536	0.007**	−0.903	0.815	0.000***

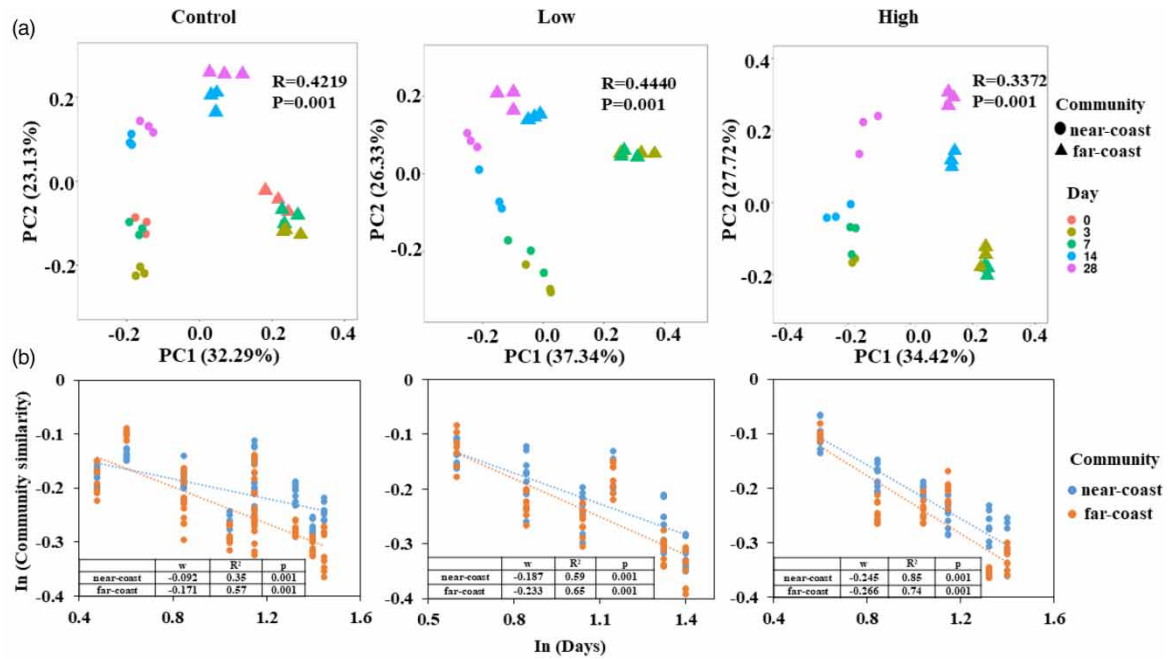•$P < 0.1$.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

Figure 2 | (a) PCoA based on the Bray–Curtis distance, showing the changes in the microbial community composition in control, low, and high PCAN groups and (b) time–decay curves for bacterial communities of two sites. Embedded s are the statistic of linear regression models. The significance of regression slope (w value) was tested by comparing the observed slope and the distribution of 999 times permutated slopes by randomizing the original data matrix.

Table 2 | Quantitative effects of community structure, incubation time, PCAN concentration, and their interactions on the microbial community variance by permutational multivariate analysis of variance (Adonis) based on the Bray–Curtis distance

Source of variation	Levels	F	R^2	P
Community	Near-coast, far-coast	24.347	0.243	0.001***
Time (day)	0, 3, 7, 14, 28	17.793	0.190	0.001***
Treatment	Control, low, high	2.878	0.071	0.002**
Community * time		9.684	0.066	0.001***
Community * Treatment		2.130	0.038	0.014*
Time * treatment		1.361	0.026	0.150
Community * time * Treatment		2.175	0.022	0.011*

*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

suggesting that their bacterial community successions were accelerated by the PCAN load. Comparing the low and high PCAN groups, the absolute values of w were higher for the far-coast communities than those for the near-coast ones, indicating more sensitive responses of the far-coast community to the PCAN load.

Bacterial OTUs related with PCAN transformation

During the incubation of microcosms, besides the sediment adsorption, PCAN transformation was carried out by the bacterial communities, which were also in constant changes. Here, we hypothesized that the microbes somehow functioning PCAN transformation were quantitatively and positively correlated with the concentration of PCAN. The bacterial OTUs that were potentially involved in PCAN transformation were identified according to Pearson's correlation coefficient between the OTUs abundance of each sample and the corresponding PCAN concentration. In total, 1,168 of 14,451 OTUs ($r = 0.316$–0.582, $P < 0.05$) in the near-coast communities and 860 of 14,114 OTUs ($r = 0.316$–0.578, $P < 0.05$) in the far-coast communities showed significant positive

correlations with PCAN; in other words, a larger proportion of OTUs in the near-coast communities (8%) were potentially involved in PCAN transformation than the far-coast ones (6%) (Figure 3(a)). Of all these identified OTUs, only 88 were shared by the two communities (Figure 3(b)), implying that the bacterial community responses to PCAN pollutant were heavily site-dependent. Calculating the relative abundance of the PCAN transformation-involved OTUs (Supplementary Material, Fig. S4), the identified OTUs experienced a steeper abundance decrease in the far-coast communities than the near-coast ones, indicating more stable community structures in the near-coast area under the PCAN disturbance.

DISCUSSION

Microbial community can respond to environmental disturbances, resulting in shifts in the community composition (Rodriguez-r et al. 2015). Whether and to what extent the microbial community would be changed depends on the disturbance type, intensity, and frequency (Bressan et al. 2008; Berga et al. 2012). Our study further indicated that the microbial response was 'hatched' partially by its historical environmental context; in other words, the historical environmental condition predetermined microbial resistance and resilience to future disturbances. This is in accordance with previous studies, showing that the resistance and resilience of microbial community are combined effects of the community itself and environmental characteristics (Allison & Martiny 2008; Shade et al. 2012; Griffiths & Philippot 2013).

Long-term WWTP's effluent disposal not only affects the sediment characteristics of the recipient environment, but also influences the structures and behaviors of the microbial communities inhabited there (Pasquini et al. 2013; Masson & Tercier-Waeber 2014; Miao et al. 2020). The WWTP's effluent contains complex chemical residues, which could be a selective pressure that filters out vulnerable species and enriches tolerant ones (van der Meer 2006). What's more, the effluent also contains microbial species that are resistant or efficient in pollutants' transformation (Mansfeldt et al. 2020).

In this study, we added PCAN in the microcosms to mimic a disturbance scenario caused by a single organic pollutant. In the near-coast site, the sediment has long been disturbed by the AOX-contained effluent from a nearby WWTP, where the sediment microbial communities may have developed resistance and resilience to PCAN disturbance. When the resilience of microbial community is higher, the sensitivity is lower, that means the stability of microbial community is higher (Griffiths & Philippot 2013; Leizeaga et al. 2021). On the contrary, the sediment microbial communities of the less-disturbed far-coast site were more sensitive to PCAN because of lacking 'pre-training' of environmental disturbances (Dıaz-Ravina & Bååth 2001).

Although such pre-exposure to wastewater disposal could enhance the stability of microbial community to future disturbances, our study indicated that the improvement was limited. Previous study also demonstrated
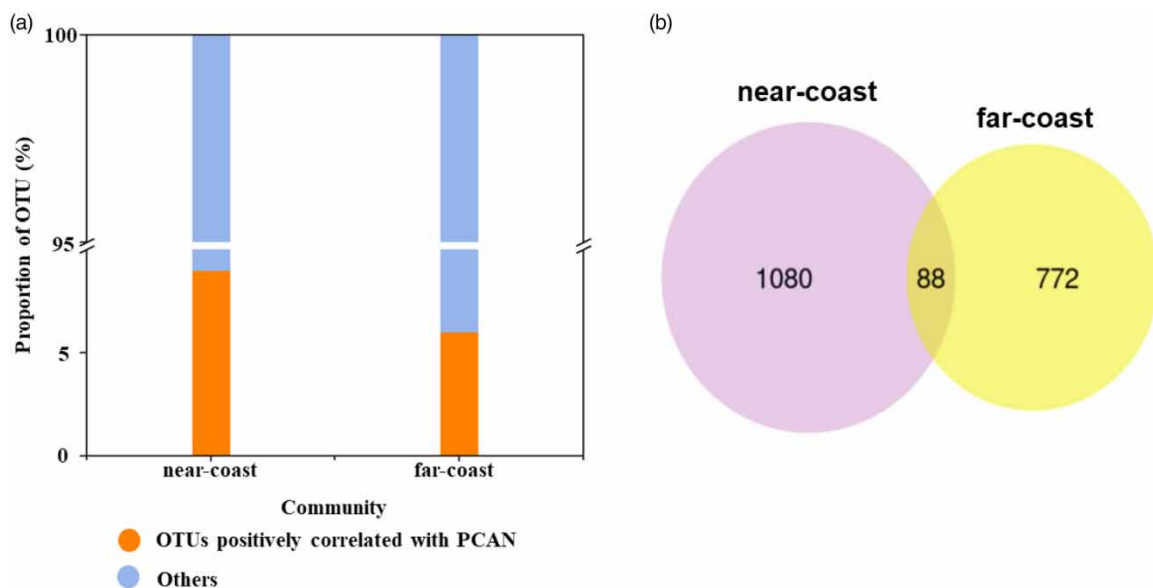

Figure 3 | (a) The proportion of the bacterial OTUs that positively correlated with PCAN transformation in two communities ($P < 0.05$) and (b) the number of the bacterial OTUs that positively correlated with PCAN transformation in two communities ($P < 0.05$).

that the buffering capacity of bacterial community to pollutants' input was limited (Xuan *et al.* 2019), because there exists a threshold for disturbance intensity, beyond which the community's stability may change permanently (Sobolev & Begonia 2008; Shade *et al.* 2012). Based on our results, high PCAN load might approach or exceed the threshold, resulting in a similar time–decay rates for the two communities with different environmental histories.

In the real environment, though it is difficult to predict the responses of local microbial communities to the future pollution event (Azarbad *et al.* 2013), our study suggested that the microbial community with historical exposure to a broad spectrum of pollutants might have a higher stability, but only when the disturbance intensity is low.

CONCLUSION

In this study, by constructing a series of microcosms, we evaluated whether and how long-term wastewater disposal may affect the coastal bacterial community's stability in response to a future disturbance. Our results showed that the wastewater exposure history may improve the microbial community's stability to a future pollutant disturbance, since less diversity changes occurred for the near-coast community after PCAN disturbances. Therefore, strict wastewater treatment and disposal management should always be necessary for conserving the stability and eco-functioning of the microbial community in the recipient water bodies.

ACKNOWLEDGEMENTS

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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