A New in Situ Rhizosphere Sediment Sampling Method for Emergent Aquatic Plants

Wenlin Wang¹, Wei Du¹, Wenjing Li¹, Zhou Fan¹, Fei He¹, Xiaoyan Tang¹, Bo Liu², Guoxiang Wang³,*

¹Nanjing Institute of Environmental Sciences, Ministry of Environmental Protection, Nanjing 210042, China
²School of Geography Science, Nantong University, Nantong 226007, China
³School of Environment, Nanjing Normal University, Nanjing 210046, China

*Corresponding author e-mail: wangguoxiang@njnu.edu.cn

Abstract. The rhizosphere provides a special environment for microorganisms. As such, emergent aquatic plants play an important role in wetlands. However, due to the sampling methods, it is difficult to obtain sediment samples attached to specific sections of roots. Here, we developed a sampling method for segmented acquisition of sediment and in situ detection of environmental factors from the rhizosphere. This method can be particularly useful for emergent aquatic plants with rhizomatic root systems.

1. Introduction

The roots of emergent plants provide a surface to which bacteria, fungi and other microbes attach [1, 2]. Due to the unique redox conditions and root exudate of rhizosphere, microorganism diversity and metabolic activity have been shown to be high in this area [3, 4]. This not only makes the emergent aquatic plant play an important role in adsorption and degradation of pollutants [5, 6, 7, 8] but also makes the microorganism community structure and functional diversity an important index regarding the evaluation of constructed wetlands [5, 9].

Previous studies have defined the sediment within a 4-mm distance around the root as the rhizosphere sediment [10, 11]. Most current sampling methods for separating rhizosphere sediment from saprophyte roots include sampling in a rhizobia [11, 12, 13, 14], shaking/washing the plants using sterile water or isolation buffer [8, 14, 15] and scraping the saprophyte root surfaces or the sediment nearby the root [5, 16]. However, sampling in a rhizobia usually cannot be performed at a 4-mm distance. In addition to this method, the rhizosphere condition is influenced by the whole plant root, and the contribution of a single root is difficult to estimate in this way. Furthermore, the sediment removed by shaking is generally referred to as sediment that is loosely attached to the roots; therefore, the detached sediment collected is not from the rhizosphere. Although the manual separation method, such as scraping with sterilized scraper or toothbrush, is more accurate than the other two methods, it is not only laborious and time consuming but also subjective, since results vary with the operator and the morphological type of host saprophyte.

Based on previous studies, different sections of the root are assumed to have a different influence on the rhizosphere environment, due to the different secretory capacity of the root sections [17, 18]. For example, the actual peak of rhizosphere oxygen saturation in sediment is in the middle of the root, and
the thickness of stably oxidized microzones is several times that of typical emergent aquatic macrophytes (Rejmankova* 2011). However, regarding the abovementioned methods, the sediment from specific sections of the rhizosphere along a single root is difficult to separate, and the environmental factors of these sections are also difficult to obtain.

A novel approach for separating rhizosphere sediment samples from the root surface of emergent aquatic plants as well as for in situ detection of the environmental conditions of different sections of the rhizosphere is presented here (Patent Number: 201510215791.4). The micro-optode system has been widely used as equipment for measuring the physical and chemical conditions in the rhizosphere and inside the roots. The Microx system can detect the environmental factors, e.g., dissolved oxygen (DO), pH, and total dissolved salt (TDS) as well as the concentration of some materials within a small radius, such as 4 mm around the root surface. We therefore applied a new device (Patent Number: ZL201520274136.1) to separate a single root from the whole root system of emergent aquatic macrophytes, to detect the environmental factors in the rhizosphere with the micro-optode system and to obtain sediment samples from specific sections of the root.

2. Materials and Methods

2.1. Sampling device
To separate a single root from the whole root system, a poly (methyl methacrylate) holder with a long infundibulate groove was used. The holder was constructed with detachable sections and a metal bar (Fig. 1). The diameter of the groove was selectable. During setup, the size of the sections was chosen based on the test root diameter, installed on the metal bar and filled with mud samples in advance.

![Figure 1. Schematic of poly (methyl methacrylate) growth groove](image)

2.2. Plant materials
Acorus calamus, a typical emergent aquatic plant with a rhizomatic root system, was chosen as object of this study. Acorus calamus plants were collected from the breeding conservation greenhouse at Nanjing Normal University. Three healthy plants were used in this study, which had an average height of 1.2 ± 0.2 m and 5 leaves total. The root length of these plant samples ranged from 15 to 40 cm. Six mature roots (average root length = 30 ± 2 cm, root diameters ranged from 3 ± 0.2 mm) of each plant sample were used as test roots, cleaned with sterile water, and softly scraped to remove the attached sediment.
2.3. Experimental procedure

In this study, we chose a 30-cm-long holder with 10 sections, whose grooves ranged from 9 to 10 mm. The test roots were set in the groove in line with the root growth and covered with homogenizing sediment so that the whole root was artificially divided into 10 sections, as follows: 3 cm base of root (T3), 3-6 cm (T6), 6-9 cm (T9), 9-12 cm (T12), 12-15 cm (T5), 15-18 cm (T18), 19-21 cm (T21), 21-24 cm (T24), 24-27 cm (T27), and 27-30 cm (T30).

The holder was placed into a plastic container (50×30×20 cm) (Fig. 2), which was almost the same size of the rhizobox used in previous studies [14, 19], and covered with a piece of cellulose acetate membrane (2 μm pore size). The membrane distinguished the rhizosphere from the external environment as well as from additional sediment so that H₂O, nutrients and microorganisms could exchange freely on both sides. In addition, sediment inside and outside the membrane was used as an inner-control (ICK) and outer-control (OCK), respectively. The plastic container and plant were placed into an illumination incubator, in which the daily illumination time was from 6:00 to 18:00, the illumination intensity was 3000 lux, and the culture temperature was 25 °C.

![Figure 2. Schematic of rhizosphere sediment sampling processes](image_url)

After 3 days, the devices were carefully removed from the container. Because cellulose acetate membranes were very thin and capable of being directly pierced by the micro-optodes, the rhizosphere oxygen saturation of three roots was determined with a Micro-optode (Microx TX3 System, Presens, Regensburg, Germany) as Wang et al. described in 2014 [18]. The other three roots were removed from the membrane and separated into 10 sections with a sterilized scalpel at each section interval. The sections were then separated, and the sediment in the groove and/or attached to the root was removed with an ultrasonic washer. In addition, root information, such as diameter, length, and biomass, was also recorded.

2.4. DNA extraction

These sediment samples were kept in 5 mL sterilized centrifuge tubes and stored at -20 °C until used. The total DNA extraction was carried out with a PowerSoil® DNA Isolation Kit (MoBio Laboratories, Inc., USA) following the standard protocol. The concentration and purity were detected with a Nanodrop 2000 Micro-Ultraviolet Spectrophotometer (Nanodrop Technology, Wilmington, DE, USA).
2.5. **T-RFLP procedure**

Following DNA extraction, the terminal restriction fragment length polymorphism (T-RFLP) method was used to estimate the bacterial community diversity. The primers used were 27F/1492R, and the 3’ tip of the forward primer was marked with FAM fluorescence labelling. For each sample, three reactions were conducted: each contained 12.5 μL of 2x TransTaq-T PCR Super Mix (TransGen Biotech, Beijing, China), 1 μL of each primer, and 1 μL of total DNA, with sterile deionized H₂O added to a final volume of 25 μL. The PCR reaction programme was 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, ending at 72 °C for 10 min. The PCR products were electrophoresed on 1.5% (wt/vol) agarose gel and kept at -20 °C for enzyme digestion.

The samples were enzyme digested with MspI restriction enzymes in a 30 μL reaction system, which consisted of 1 μL of MspI (Thermo Scientific), 2 μL of buffer Tango (Thermo Scientific), and 10 μL of PCR product, with sterile deionized H₂O to add to a final volume 30 μL. The digestion progress was performed at 37 °C for 6 h, followed by 80 °C for 20 min to deactivate the enzymes.

The analysis of the sequence reads was carried out by Sangon Biotech Co., Ltd. (Shanghai, China). Sequence data were manually calibrated with Gene Marker V 2.8.0; only peaks from 50 to 600 bp and larger than a threshold of 100 (peak height, arbitrary units) were considered. The sum of all peak areas in each sample was calculated as an indication of the total DNA quantity with the following formula:

Relative amount of total bacteria = total peak area × total DNA amount (mol) / (dosage of DNA (mol) × sediment sample amount (g)).

The relative abundance of each T-RF was also estimated (relative T-RF abundance % = single peak area/total peak area). Peaks representing >3% of the total in all replicates and those > 5% occasionally were analysed individually. The other peaks were combined and designated “Other T-RFs”.

2.6. **Statistical analysis**

The Shannon-Wiener index was used to estimate the bacterial community diversity, and statistical analysis was also performed with SPSS 13.0. Classify hierarchical cluster analysis in SPSS was used to present a cluster trend diagram. One-way ANOVA and the Tukey multiple comparison tests were used to analyse significant differences of T-RFs and dissolved oxygen concentration data of the rhizosphere.

3. **Results and Discussion**

The diameter, thickness, and weight of the sediment collected from different sections of *A. calamus* in this study are shown in Table 1. Since the groove of the holder was infundibulate and gradually narrowed following the growth trend of the root, the total amount of sediment collected from the different sections of the rhizosphere in the experiment was almost the same.

| Section | Diameter of root (mm) | Thickness of rhizosphere sediment obtained (mm) | Weight of rhizosphere sediment obtained (g) |
|---------|-----------------------|-----------------------------------------------|--------------------------------------------|
| T3      | 3.0±0.1               | 3.8±0.1                                       | 0.223±0.011                                |
| T6      | 2.9±0.1               | 3.7±0.1                                       | 0.228±0.016                                |
| T9      | 2.7±0.1               | 4.0±0                                          | 0.245±0.021                                |
| T12     | 2.5±0.1               | 4.1±0                                          | 0.256±0.012                                |
| T15     | 2.2±0                 | 3.9±0.1                                       | 0.274±0.008                                |
| T18     | 1.9±0                 | 4.1±0.1                                       | 0.291±0.006                                |
| T21     | 1.6±0                 | 4.0±0.1                                       | 0.305±0.002                                |
| T24     | 1.4±0                 | 4.2±0                                          | 0.312±0.004                                |
| T27     | 1.2±0                 | 4.1±0                                          | 0.318±0.006                                |
| T30     | 1.0±0                 | 4.2±0.1                                       | 0.325±0.006                                |
As shown in Fig. 3, the dissolved oxygen concentrations of OCK and ICK were 0, and oxygen diffusion was detected in all rhizosphere sections. However, the average oxygen concentrations significant differed between sections, and the typical unimodal trend suggested that the oxygen production capacity of the middle portion of the root was higher than that of the root base and root tip. This result verified the conclusion of Wang et al. (2014) [18].

![Figure 3. Spatial distribution of dissolved oxygen of the rhizosphere of *A. calamus*](image)

Based on the results using T-RFLPs, no significant difference was discovered between the bacterial community of OCK and ICK. Five main T-RFs were obtained in the OCK and ICK (Fig. 4), and the relative abundance of these T-RFs was quite similar between the OCK and ICK.

![Figure 4. Structural feature of microbial communities at various root positions of *A. calamus* (T-RF relative abundance > 3%)](image)

In addition, the total bacterial abundance of the OCK and ICK was $4.98 \times 10^4$ and $4.87 \times 10^4$ (Fig. 5) and the Shannon-Wiener diversity index 1.64 and 1.65 (Fig. 6), respectively, which means that the cellulose acetate membrane had no influence on the bacterial community.
At the same time, the total peak area (Fig. 5) and the number of T-RFs (relative abundance greater than 3%) obtained in the rhizosphere sediment samples (Fig. 4) were also higher in the rhizosphere. However, all rhizosphere samples have T-RFs of 61 bp (T-RF_1), 160 bp (T-RF_4), 462 bp (T-RF_5), 485 bp (T-RF_8), and 507 bp (T-RF_11), which are also the main T-RFs in OCK and ICK, but the relative peak area exhibits obvious difference among samples. For example, the T-RFs of 485 bp (T-RF_8) occupied 21.0% and 20.7% in OCK and ICK, respectively, but only 7.2% (T21) to 14% (T6) were in the rhizosphere. This may be attributed to the more complex community structure of the rhizosphere bacteria [20, 21]. In addition, the Shannon-Wiener diversity index also proved the hypothesis that a higher diversity index would be obtained in the rhizosphere than in the OCK and ICK. The ratios of diversity between both the OCK and ICK and T3 were 1.48 and 1.47, and the ratio was even higher in other sections (Fig. 6). The diversity difference obtained in this study was much higher than that reported in previous studies [22].

Along the growth of the root, the highest diversity was found in the middle sections, the second-highest was in the root tip, and the lowest was in the root base. The same variation trend was also obtained in the total peak area of the rhizosphere bacterial community. The highest total peak area (T18 section) obtained in this study was 5.63 times higher than that of the lowest section (T3). This variation trend was the same as the trend of the dissolved oxygen concentrations. From the classify hierarchical cluster analysis, the bacterial communities were classified into three clusters (Fig. 7), which means there are similarities in the bacterial community at the base of the root (T3 and T6) and in the OCK and ICK.
The ability to secrete oxygen from different sections of the root may significantly influence bacterial community structure [19, 20].

**Figure 7.** Cluster analysis of the rhizosphere microbial communities of *A. calamus*

In this study, we have demonstrated a new sampling method and device applicable to the emergent aquatic plant rhizosphere, and we have shown that sediment samples as well as *in situ* environment data can be collected from specific sections of the root. The device can be particularly useful for emergent plants with a rhizomatic root system.

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