Supporting Information for

Assessing Biodegradation Processes of Atrazine in Constructed Wetland Using Compound-specific Stable Isotope Analysis

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Materials and Methods

Reagents: Atrazine (1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine, CAS: 1912-24-9) (97.7%, purity) ($\delta^{13}C$: -29.9‰; $\delta^{15}N$: -2.05‰) was provided by Zhongshan Chemical co. Ltd (www.zschem.com). Ethyl acetate (99.8%, ANPEL Scientific Instrument (Shanghai, China) Co., Ltd.) was used as the solvent for standard solutions. Acetonitrile, used as the HPLC eluent, was purchased from ANPEL Scientific Instrument (Shanghai) Co., Ltd., $KH_2PO_4 \cdot 12H_2O$, $K_2HPO_4 \cdot 2H_2O$, dichloromethane and other chemical reagents used in cultivated media were purchased from Sinopharm Chemical Reagent Co., Ltd.

PBS: (OLD# BS7008-500ml) was provided by BBI life Sciences, Shanghai, www.life-biotech.com), PBS composition of NaCl 136.89 mM, KCl 2.67 mM, Na_2PO4 8.10 mM, KH_2PO4 1.76 mM, pH 7.2-7.4. **Mineral salt medium (MSM)** comprised of 1.6 g/L K_2PO_4, 0.4 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 0.1 g/L NaCl, and 0.5 g/L sucrose, further containing a trace element solution of 5 mL, with 30mg/L atrazine as the sole nitrogen source. The trace elements solution contained 2.75 g/L of $FeSO_4 \cdot 7H_2O$, 0.33 g/L of $MnSO_4 \cdot 7H_2O$, 0.24 g/L of $CoCl_2 \cdot 6H_2O$, 1.15 g/L of $ZnSO_4 \cdot 7H_2O$, 0.24 g/L of $CuSO_4 \cdot 5H_2O$, and 0.17 g/L of Na_2MoO_4. **Synthetic wastewater** comprised of 1.6 g/L K_2PO_4, 0.4 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 0.1 g/L NaCl, and 150mg/L sucrose, and trace element solution of 5 mL, with different atrazine as the sole nitrogen source.

Details of Sample Preparation: At regular intervals, 2 mL medium were used for HPLC analysis immediately after passage through a 0.22 μm filter followed method

described below. Meanwhile, a certain volume of aquatic samples filter under passive pressure with 0.22 μm glass fibric membrane to remove the suspended particles and cells, then extracted by SPE (CNW, LC-C18 SPE tubes, 500 mg, ANPEL Laboratory Technologies (Shanghai) Inc.) to prepare the final injection concentration of approximately 200 mg/L following steps: SPE tubes were preconditioned with ethyl acetate, dichloromethane and methanol each for 60 s and with 60 s of air-dry time. Samples were loaded with a flow rate of 1 mL/min and 20 min for drying after loading. Elution was carried out once with ethyl acetate, followed by two replicates with dichloromethane. The eluates were evaporated entirely and resuspended in 300-500 μL of ethyl acetate to make the final concentration of atrazine at around 200 mg/L for GC-IRMS analysis. Sample preparation procedures of SPE did not result in significant isotope fractionation as described in our previous study[1].

Analysis of Atrazine and the Metabolites in Pore Water: Water samples were collected from different points of the compartment, including the inflow, the four sampling ports across the compartment, and the outflow, for the concentration analysis of atrazine and its metabolites. The HPLC-DAD system (LC-A10 series system, Shimadzu, Japan) was used to perform the analysis, following the method adapted from previous studies[1, 2]. The separation of target compounds was carried out by using an ACE Excle 5 C_{18}-AR column (5 μm, 250 × 4.6 mm, Advanced Chromatography Technologies Ltd) with a flow rate of 0.8 mL/min and 20 μL injection. The elution gradients started with 30% acetonitrile and 70% KH2PO4 buffer (1 mM) and followed a linear gradient to 55% acetonitrile within 8 min, then continued with another 17 min to allow the acetonitrile linearly increase to 90%, and was kept isocratic for 3 min with 90% acetonitrile. The system then returned to initial conditions within 4 min, and the conditions were kept for 3 min to equilibrate the column. During the analysis, atrazine and the metabolite 2-hydroxyatrazine were detected and quantified at 220 nm, and the metabolite cyanuric acid was detected and quantified at 213nm, respectively, using the external standard working line.

Stable Carbon and Nitrogen Isotope Analysis: Carbon and nitrogen isotope were analysis by GC-IRMS adopted from the method described by Chen et al[1]. The GC-IRMS system consists of TRACE GC Ultra gas chromatograph (Thermo Fisher Scientific) equipped with a Trace Gold analytical column (30 m × 0.25 mm × 0.25 μm), which was coupled to a Finnigan MAT 253 isotope ratio mass spectrometer (IRMS) via

a Finnigan GC Combustion III interface (Thermo Fisher Scientific). Liquid samples were injected with a GC Pal auto sampler. The injection was conducted in split mode (split ratio 1:10) and kept at 250 °C with a helium (Grade 5.0) carrier flow rate of 1.0 mL/min. The GC oven temperature started at 40 °C (hold 1 min) and consequently increased to 100 °C (hold 5 min) at rate of 50 °C/min. Then at a ramp of 5 °C/min led to 250 °C (hold 5 min). During isotope analysis by GC-IRMS, analytes were measured against a laboratory standard gas (CO2) which was introduced into at the beginning and the end of each run. The laboratory gas was calibrated to Vienna Pee Dee Belemnite (V-PDB). Isotope values of carbon was reported as $\delta^{13}C$ values in ‰ relative to Vienna Pee Dee Belemnite (VPDB) according to equation (S1) [3], where R_{sample} and $R_{reference}$ are isotope ratios of the elements (E) of concern in the target compound and the international reference standard (e.g., VPDB for the carbon isotope).

$$\delta(E) = \frac{R_{sample} - R_{reference}}{R_{reference}} \quad \text{(S1)}$$

Soil Sample Collection and 16S rRNA Sequencing

To profile the microbial community structure changes in the wetland, soils were collected from the constructed wetland system on day 20, day 80, day 110, day 150, and day 200. Each sample was prepared by combining 5 soil cores from treatment cell A and B, respectively. Total bacterial DNA was extracted using the DNA®SPIN Kit for Soil (Mpbio, USA) according to the manufacturer's protocols. For the approximation of taxonomic affinity, the universal primers (338F 5′-ACTCCTACGGGAGGCAGCAG-3′ and 806R 5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify the 16S rRNA through Polymerase Chain Reaction (PCR). The PCR conditions were as follows: 98 °C for 30 seconds, 35 cycles of 98 °C for 10 seconds, 54 °C for 30 seconds, and 72 °C for 45 seconds, and a final step at 72 °C for 10 min. The PCR products were sequenced on an Illumina Sequencer MiseqTM platform (Lianchuan Biotech, Hangzhou, China).

Table S1. The δ ^{13}C and ^{15}N values of some s-triazines measured by EA-IRMS (a) and GC-IRMS after solid phase extraction (SPE)(b), the deviation of the results between two analytical procedures were less than 0.7‰.

S-triazines	Supplier	$\delta^{13}C$-a (‰)	$\delta^{13}C$-b (‰)	Deviation (‰)	$\delta^{15}N$ -a (‰)	$\delta^{15}N$-b (‰)	Deviation (‰)
Atrazine-Desisopropyl	Ehrensberger	-29.2 (n=3)	-29.2 (n=3)	0	-6.3 (n=3)	-6.1 (n=3)	0.1
Atrazine-Desethyl	Ehrensberger	-29.2 (n=5)	-29.3 (n=4)	0.39	-6.2 (n=4)	-5.6 (n=3)	0.47
Simazine	Ehrensberger	-32.7 (n=3)	-31.7 (n=3)	0.7	1.4 (n=3)	1.6 (n=3)	0.1
Cyanazine	Ehrensberger	-28.3 (n=3)	-28.0 (n=3)	0.2	-2.3 (n=3)	-2.0 (n=3)	0.2
Atrazine	Cerilliant	-26.4 (n=5)	-27.2 (n=5)	0.39	-2.9 (n=5)	-2.2 (n=5)	0.46

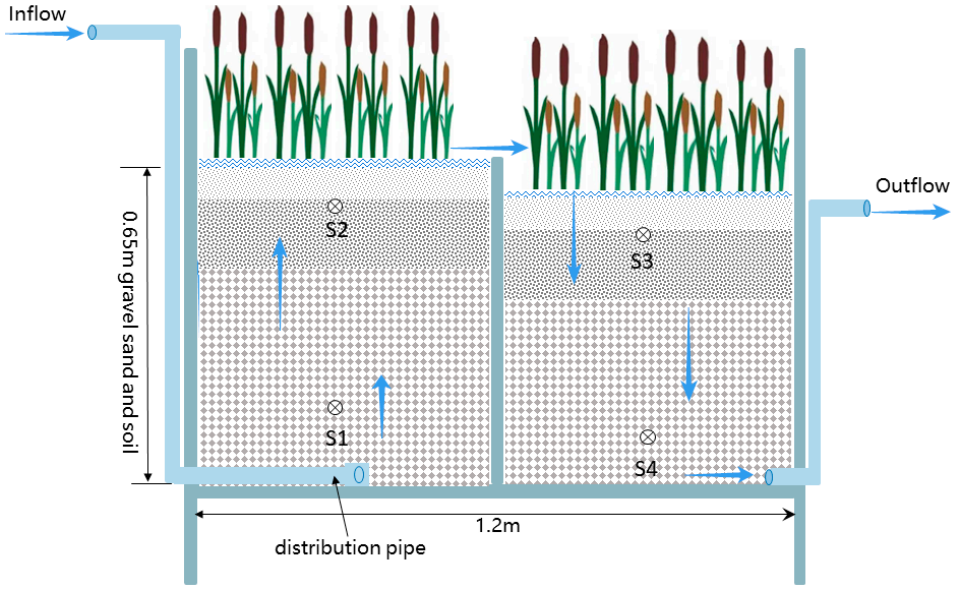

Figure S1. Diagram of the two-stage vertical flow constructed wetland, wherein A is the primary treatment cell and B is the secondary treatment cell. The arrows illustrate the direction of the water flow. S1, S2 and S3, S4 are the sampling ports in both processing cells, respectively.

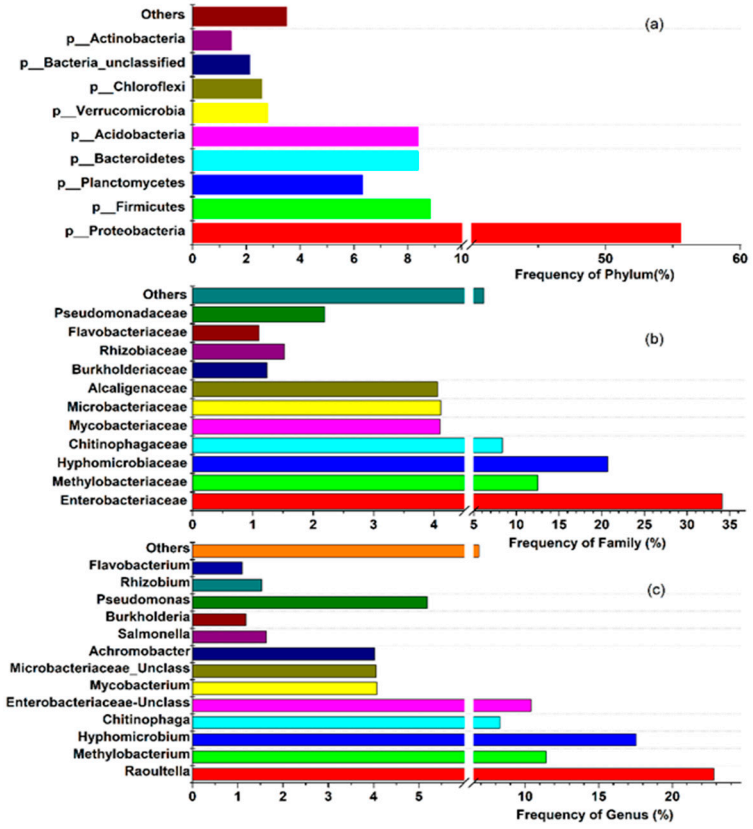

Figure S2. The microbial community structure of the enrichment mix culture. (a) represents the composition of the community at the phylum level, (b) family level and (c) genus level.

Results and Discussion

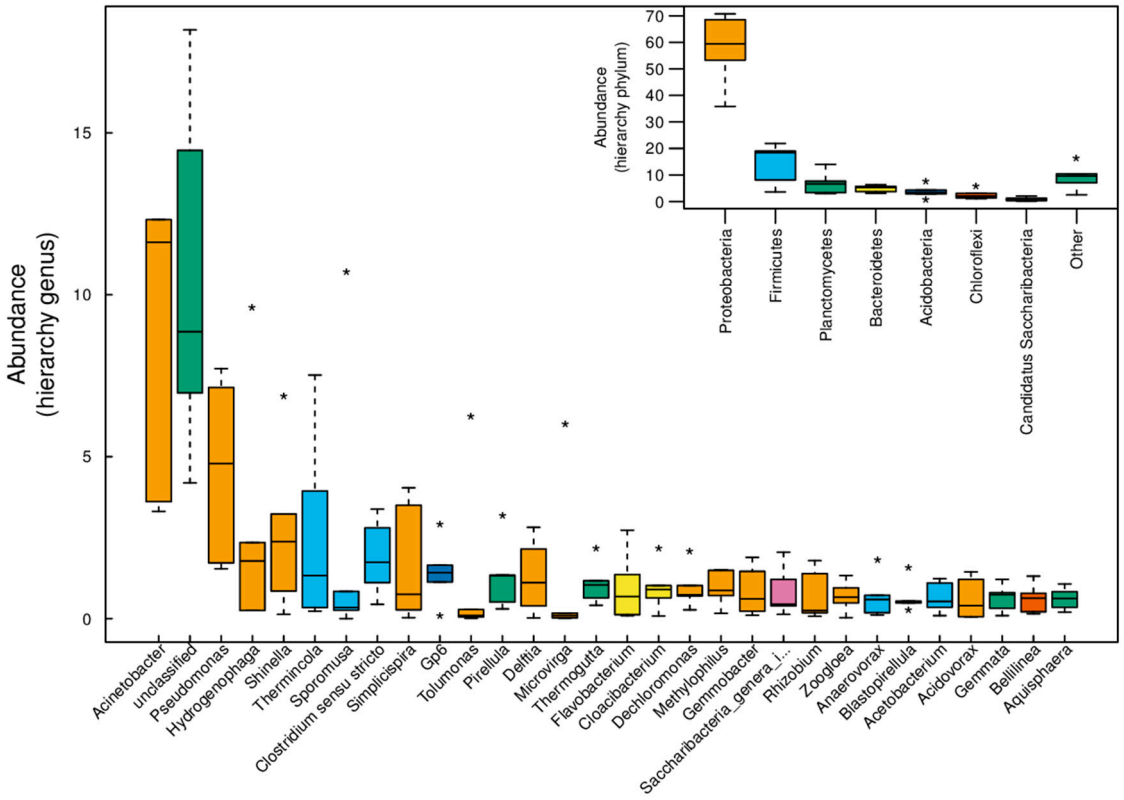

Figure S3. Relative abundance of major bacteria in the constructed wetland displays in hierarchy genus and hierarchy phylum (insert)

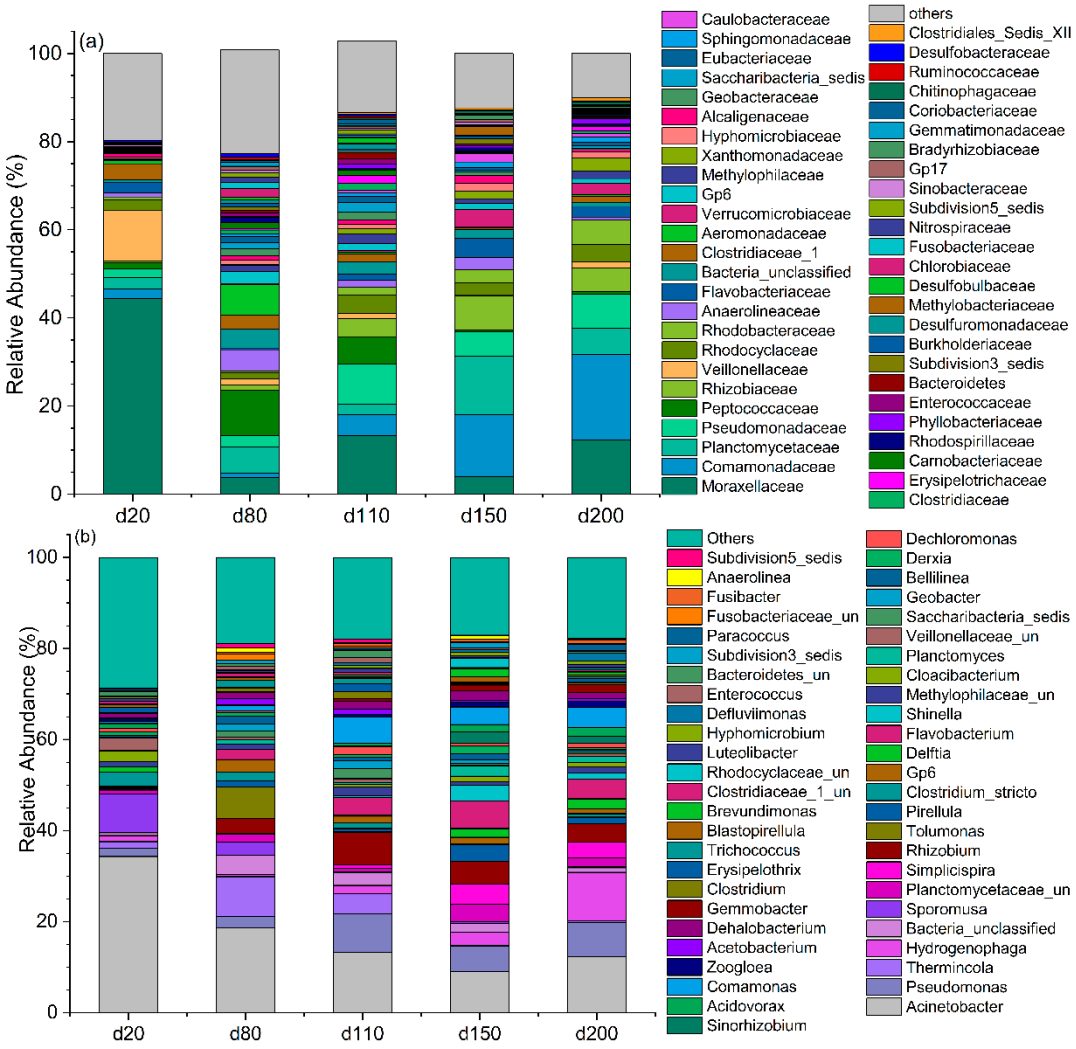

Figure S4. The microbial community structure of the samples taken at day 20, day 80 day 110, day 150 and day 200. (a) shows the frequency of family level and (b) displays the frequency of genus level.

Reference:

1. Chen, S., et al., *Isotope fractionation in atrazine degradation reveals rate-limiting, energy-dependent transport across the cell membrane of gram-negative rhizobium sp. CX-Z.* Environmental Pollution, 2019. **248**: p. 857-864.

2. Cantu, R., et al., *An HPLC method with UV detection, pH control, and reductive ascorbic acid for cyanuric acid analysis in water.* Analytical Chemistry, 2000. **72**(23): p. 5820-5828.

3. Coplen, T.B., *Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results*. Rapid Communications in Mass Spectrometry, 2011. **25**(17): p. 2538-2560.