Supplementary Material

(1) Ecological response of enzyme activities in watershed sediments to the reintroduction of antibiotics

Materials and Methods

Statistical analysis

Enzyme activity-based index calculation

(2) Urease activity

(3) To measure the urease activity (UA), 5g of soil sample was weighed into a 50 mL triangular flask and 1 mL of toluene added. After adding 10 mL of 10% urea solution and 20 mL of pH 6.70 citrate buffer solution for 15 minutes, the solution was shaken well and incubated at 37°C for 24 hours. After filtration, 1.00 mL of filtrate was added to a 25.00 mL volumetric flask, and 2.00 mL of sodium phenol solution and 1.50 mL of sodium hypochlorite solution were added and shaken well. After 20 minutes, the color was developed and the volume fixed [25, 26]. Finally, the absorbance at 578 nm was measured within 1 h of the reaction and UA was calculated as follows:

(4) $\mathrm{UA} = \dfrac{(\text{A-B-C}) \times V \times \text{ts}}{m}$

(5) where UA is the urease activity measured in milligrams of NH_4^+-N per gram of soil after 24 hours of cultivation (mg/(kg·24 h)), A is the absorbance value of the sample derived from the standard curve for NH_4^+-N, B is the NH_4^+-N mg from the standard curve of the soil-less control absorbance value, C is the absorbance value of the substrate-free control derived from the standard curve in milligrams of NH_4^+-N, V is the volume of the color developing liquid, t is the fractionation factor, equal to volume of leachate / volume of filtrate aspirated, and m indicates the weight of the dried soil.

(2) Alkaline phosphatase activity

(6) To measure the alkaline phosphatase activity (APA), 5.00 g of soil was weighed into a 50 mL volumetric flask. To this, 1.00 mL of toluene was added, and the flask was stoppered and shaken gently for 15 minutes. Then, 5.00 mL of sodium benzene phosphate and 5.00 mL of buffer were added. A control was set up with water instead of substrate. This was shaken well and incubated in a thermostat at 37°C for 24 hours. The mixture was diluted in the volumetric flask to the mark with distilled water heated to 38°C and then filtered. To this, 0.25 mL of the filtrate was added to a 25.00 mL volumetric flask, with 1.25 mL of pH 9.00 borate buffer, 0.75 mL of 2.5% potassium ferricyanide, and 0.75 mL of 0.5% 4-aminoantipyrine solution. The solution was carefully mixed and water added to reach the desired volume. After the color stabilized (20-30 minutes), the absorbance of each sample was measured at 570 nm [26]. Finally, the APA was calculated using the following formula:

APA=(A-B-C)×D

(7) where APA is the Alkaline phosphatase activity measured in milligrams of NH_4^+-N per gram of soil after 24 h of cultivation (mg/(g·24 h)), A is the absorbance value of the sample derived from the standard curve for NH_4^+-N, B is the NH_4^+-N mg from the standard curve of the soil-less control absorbance value, C is the absorbance value of the substrate-free control derived from the standard curve in milligrams of NH_4^+-N, D is the fractionation factor (if expressed as milligrams of P, the result is multiplied by 0.32; if expressed as milligrams of P_2O_5, multiply by 2.29).

(3) Peroxidase activity

To measure the peroxidase activity (POA) using the potassium permanganate titration method, 40.00 mL of distilled water and 5.00 mL of a 0.30% H_2O_2 solution were added to a triangular flask. The flask was immediately sealed and shaken for 20 minutes. Then, 1.00 mL of saturated aluminum potassium was added and filtered into another triangular flask containing 5.00 mL of 1.50 mol sulfuric acid. The solution was filtered until it was dry, and 25.00 mL of the filtrate was drawn up. The filtrate was titrated with 0.02 mol/L potassium permanganate until a purplish-red color was achieved. Note that the solution may initially turn purple, but this is not the end point of the titration. The titration should continue until the purplish color remains for 30 seconds [26]. Finally, the POA was calculated using the following formula:

$$POA = \frac{(V - Vs) \times C \times 51 \times 17}{Vo \times W}$$

(8) where catalase activity is expressed as milligrams of hydrogen peroxide decomposed per gram of soil in 20 minutes (mg/(g·20min)). V represents the volume of $KMnO_4$ used for titration of the blank, VS represents the volume of $KMnO_4$ used for titration of the sample, C is the concentration of $KMnO_4$, V_o is the titration volume (25.00 mL), and W is the weight of the soil.

(9) (4) Dehydrogenase activity

(10) To measure the dehydrogenase activity (DHA), 5.00 g of soil sample was weighed and passed through a 2.00 mm sieve into a stoppered test tube. To this, 5.00 mL of TTC solution was added and the result was mixed thoroughly. The tubes were incubated in the dark at 30 °C for 6 hours. After incubation, 50.00 mL of methanol was added in parts. The mixture was transferred to a stoppered triangular flask and shaken for 1 hour on a shaker. The mixture was filtered and the filtrate measured using a spectrophotometer at a wavelength of 485 nm, with methanol as a blank [26]. The DHA was calculated as follows:

(11) $DHA = \frac{c \times V \times 150.35}{m}$

where c is the concentration of triphenyl filth in the filtrate (mg/mL), V is the volume of the filtrate, 150.35 is the coefficient for converting the amount of triphenyl filth into volume (μL) of hydrogen, and m is the dry soil weight.
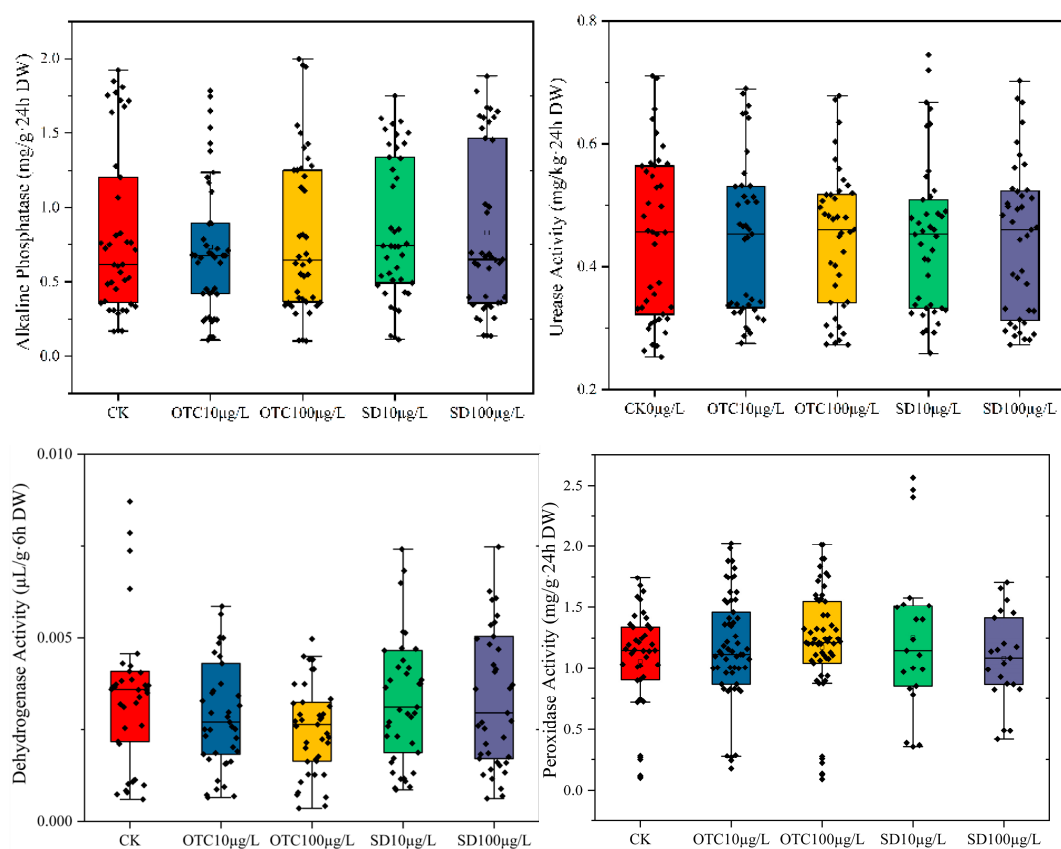
Figure S1. Box plots of sediment urease activities (UA), alkaline phosphatase activities (APA)，dehydrogenase activities (DHA), and peroxidase activities (POA) under different antibiotics.

The CK group represents the control treatment without the reintroduction of any antibiotics. The OTC10μg/L, OTC100μg/L, SD10μg/L, and SD100μg/L groups refer to the overlying water to sediment containing oxytetracycline (OTC) or sulfadiazine (SD) at concentrations of 10 μg/L and 100 μg/L.
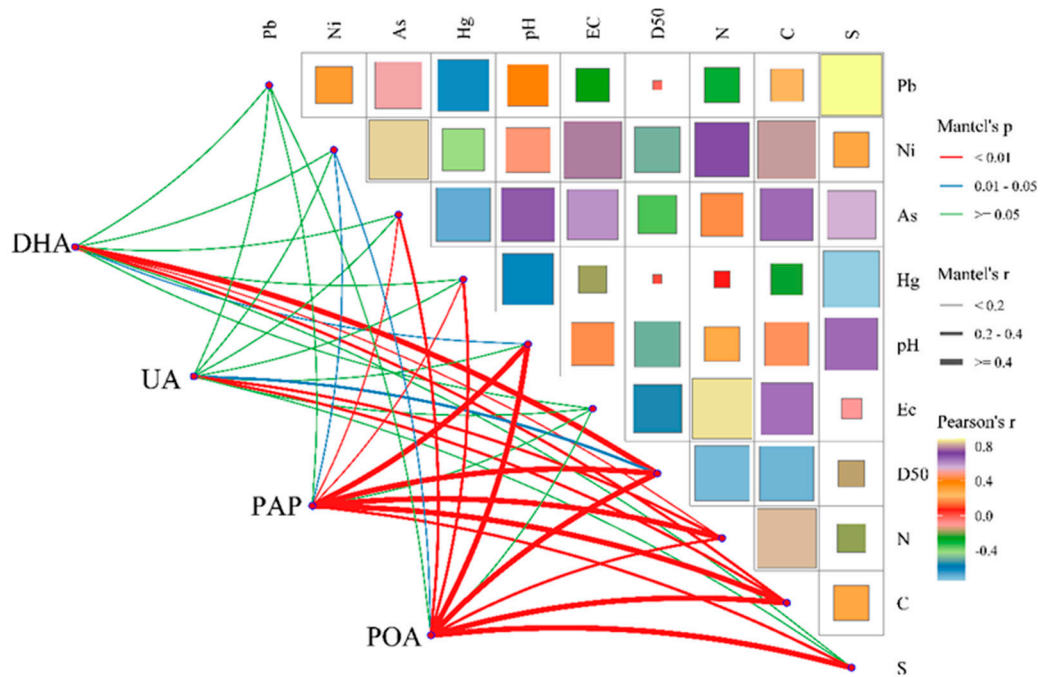
Figure S2. Mantel analysis between enzyme activities and sediment properties.

UA is urease activities; APA is alkaline phosphatase activities; DHA is dehydrogenase activities; POA is peroxidase activities; EC is electric conductivity; D50 is vol. weighted median value of particle diameter; N is total nitrogen content; C is total carbon content; S is total sulfur content