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## Perspective

# Novel stable isotope concepts to track antibiotics in wetland systems

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## ABSTRACT

Antibiotics, their transformation products, and the translocation of antibiotic-resistant genes in the environment pose significant health risks to humans, animals, and ecosystems, aligning with the One Health concept. Constructed wetlands hold substantial yet underutilized potential for treating wastewater from agricultural, domestic sewage, or contaminated effluents from wastewater treatment plants, with the goal of eliminating antibiotics. However, the comprehensive understanding of the distribution, persistence, and dissipation processes of antibiotics within constructed wetlands remains largely unexplored. In this context, we provide an overview of the current application of stable isotope analysis at natural abundance to antibiotics. We explore the opportunities of an advanced multiple stable isotope approach, where isotope concepts could be effectively applied to examine the fate of antibiotics in wetlands. The development of a conceptual framework to study antibiotics in wetlands using multi-element stable isotopes introduces a new paradigm, offering enhanced insights into the identification and quantification of natural attenuation of antibiotics within wetland systems. This perspective has the potential to inspire the general public, governmental bodies, and the broader research community, fostering an emphasis on the utilization of stable isotope analysis for studying antibiotics and other emerging micropollutants in wetland systems.

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Antibiotics play an essential role in enhancing livestock and aquaculture production as well as promoting human

health. Nevertheless, their excessive use and improper application can facilitate the evolution and dissemination of antibiotic resistance genes (ARGs), thereby diminishing their effectiveness against human and animal pathogens. This represents a significant threat to both ecosystems and

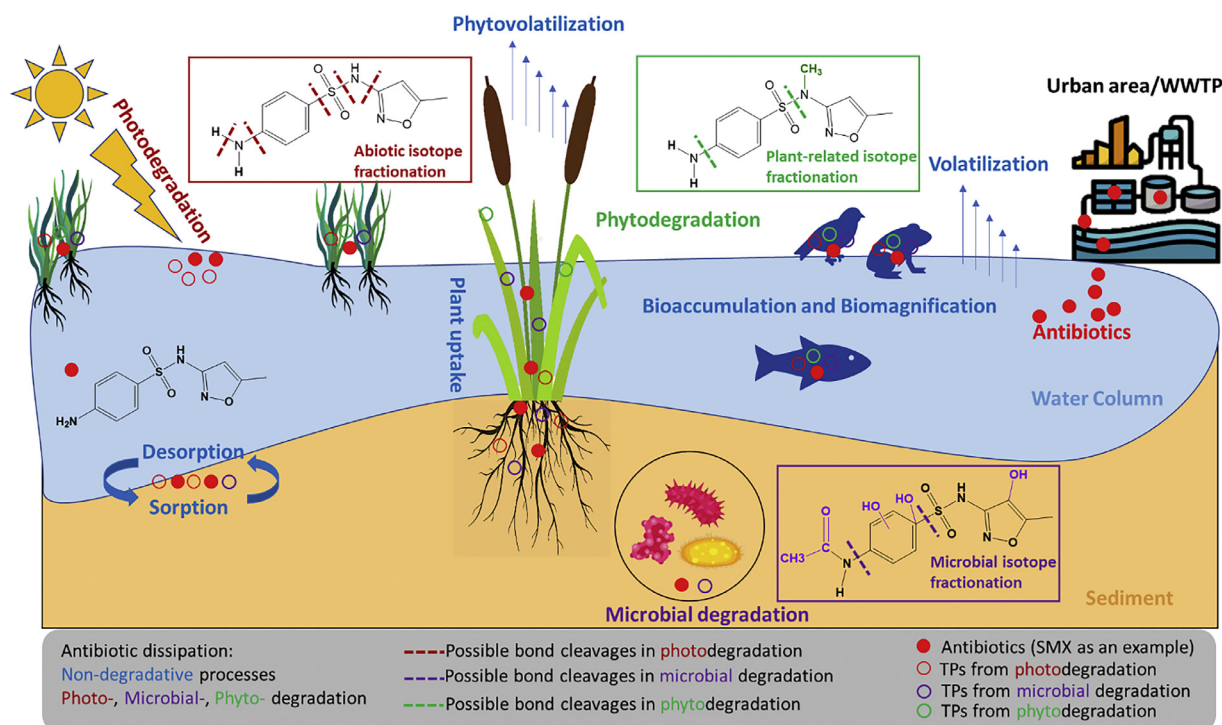
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**Fig. 1 – Dissipation and bioaccumulation processes of antibiotics and related stable isotope fractionation in constructed wetlands using sulfamethoxazole (SMX) as model antibiotic. TPs: transformation products.**

global health, in accordance with the One Health concept (Hernando-Amado et al., 2019). The application of manure in agriculture can result in antibiotic accumulation and persistence in the soil, establishing it as a reservoir (Chen et al., 2022). Subsequently, agricultural runoff transports antibiotics into aquatic ecosystems, including wetlands, where they can sorb, accumulate and degrade in water, sediment, and organisms, including the vegetation. Constructed wetlands (CW) are recognized as ecologically friendly, cost-effective and highly efficient natural-based solutions for water treatment (Wu et al., 2023). CW find extensive application in the treatment of wastewater from agriculture, domestic sewage, and contaminated water effluents from wastewater treatment plants (WWTP) with the primary goal of removing micropollutants (Wu et al., 2023), including antibiotics (He et al., 2021; Lv et al., 2022). The removal of antibiotics in CW relies on a myriad of co-occurring removal processes, encompassing sorption, hydrolysis, photodegradation, aerobic/anaerobic microbial degradation, as well as phytoremediation.

Here we use sulfamethoxazole as a model compound to illustrate the fate of antibiotics in CW (Fig. 1). In CW, SMX photodegradation in the water column includes both direct and indirect processes. Direct photodegradation induces several bond cleavages, resulting in the formation of dominant transformation products (TPs) such as sulfanilic acid (Boreen et al., 2004). In contrast, indirect photodegradation, occurring in the presence of dissolved organic matter or nitrate, may be particularly relevant in wetlands, leading to the hydroxylation of SMX as dominant TPs (Boreen et al., 2004). In addition, mi-

crobial degradation can occur under both aerobic and anaerobic conditions, and include transformation reactions such as acetylation (Achermann et al., 2018), hydroxylation (Liang and Hu, 2019), and ipso-hydroxylation (Kim et al., 2019) of SMX. Plant degradation may result in methylation and several bond cleavages of SMX (Ruan et al., 2022). Nevertheless, the comprehensive understanding of SMX distribution and persistence, and the contribution of diverse processes and mechanisms to the overall antibiotic dissipation in CW compartments often remains elusive with classical monitoring approaches.

Traditionally, monitoring of changes in the concentration of parent compounds and the generation of TPs in CW systems to establish inlet-outlet balances has conventionally served to evaluate the antibiotics dissipation. While this approach may in some cases aid in distinguishing dissipation processes, non-degradative processes such as sorption, evaporation, and dilution also affect antibiotic concentrations in CW and remain challenging to distinguish from degradative processes. Moreover, relying solely on TPs to differentiate degradation processes, especially in field conditions, poses challenges. Indeed, TPs may also degrade and the potential observation of similar transformation products in various and possibly co-occurring degradation pathways may complicate their identification (Fig. 1). Despite the challenges involved, differentiating between the various processes contributing to overall antibiotic dissipation in CW is crucial for informing risk assessments, quantifying the extent of degradation in CW, and estimating potential antibiotic exposure to higher organisms. This distinction can be instrumental in guiding remediation strategies by identifying the dominant dissipation pro-

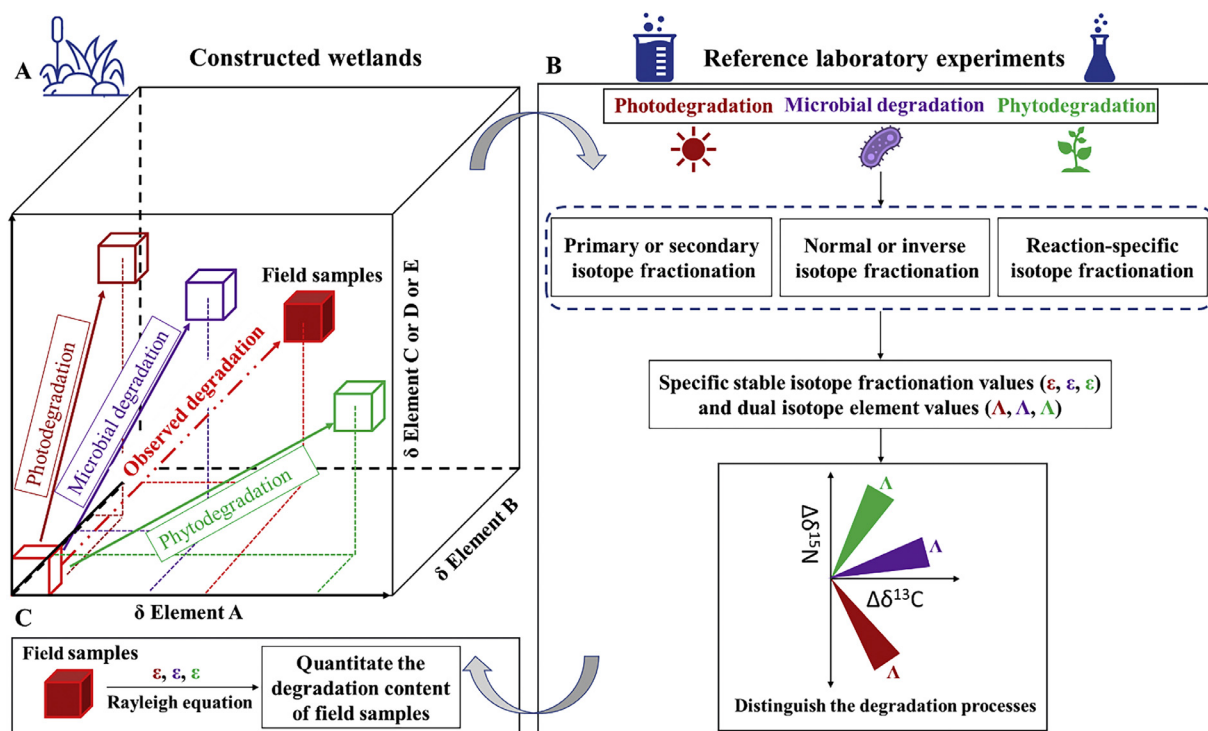
cess of antibiotics and monitoring their overall dissipation in various CW compartments over time.

In this context, an emerging approach for distinguishing dissipation processes and monitoring the degradation of antibiotics in complex environmental systems is multi-element compound-specific isotope analysis (ME-CSIA) (Nijenhuis and Richnow, 2016; Vogt et al., 2016). ME-CSIA entails the evaluation of several stable isotope ratios in individual organic compounds at natural isotopic abundances (see **Box 1** for definitions). Principally, CSIA can be employed to distinguish between degradative and non-degradative processes. Non-degradative, mostly physical processes generally do not alter the isotopic signature of contaminants or have a negligible effect compared to degradative processes. Degradative processes, involving bond cleavage, typically result in kinetic stable isotope fractionation. Therefore, the application of isotopic concepts allows for differentiation between non-degradative and degradative processes. In the context of distinguishing various antibiotic degradation processes through ME-CSIA, this goal can be achieved as distinct degradation processes exhibit specific isotope pattern. These include different directions of isotope fractionation, which can manifest as normal or inverse isotope fractionation, and variation in the extent of isotope fractionation denoted by the isotope fractionation factor ( $\epsilon$ ), along with the relationship of stable isotope fractionation of different elements represented by dual-element isotope values ( $\delta$ ) (Fig. 2). For example, an inverse isotope fractionation and mass-independent fractionation of sulfur isotopes has been commonly observed in photodegradation of several pollutants. Furthermore, the quantification of the antibiotic transformation presents another advantage of ME-CSIA in tracking the fate of antibiotics in CW compared to traditional concepts. Antibiotic ME-CSIA holds the potential to facilitate the assessment of in situ antibiotic transformation in CW, and may enable the estimation of the degradation extent using the Rayleigh concept and  $\epsilon$  derived from laboratory reference experiments (Fig. 2).

Nevertheless, the main challenge in implementing ME-CSIA for antibiotics in CW lies in the low concentration and high polarity of antibiotics, along with the limited availability of reference laboratory degradation studies to infer transformation processes in wetland systems. The high polarity of antibiotics poses challenges in GC measurements. Mass enrichment of sufficient analyte, typically a few mg for multiple injections, for isotope analysis involves the extraction from large amounts of water (Elsner and Imfeld, 2016). Solid phase extraction (SPE) is commonly employed for the extraction and enrichment of antibiotics from water. However, during SPE, non-volatile matrix components are also enriched along with antibiotics. This co-enrichment of matrix components can potentially hinder chromatographic separation and the accurate identification of target antibiotics, a phenomenon referred to as the “matrix effect” (Elsner and Imfeld, 2016; Höhener et al., 2022). In addition, the polarity of antibiotics poses challenges for direct GC measurement, often necessitating a derivatization step before GC separation (Blessing and Baran, 2022). Hence, the upcoming tasks involve the development of reliable, non-fractionating extraction and separation methods, as well as adapted instrumental approaches. Currently, the existing literature on isotope anal-

ysis of antibiotics predominantly centers on carbon isotope fractionation, utilizing Liquid Chromatography Isotope Ratio Mass Spectrometry (LC-IRMS) in photodegradation and microbial degradation (Birkigt et al., 2015; Willach et al., 2017; Willach et al., 2018) (Table 1). Nevertheless, there are limitations in utilizing LC-IRMS for the separation of analytes in environmental extracts, and this technique is restricted to carbon isotope analysis. LC-IRMS systems utilize online wet-chemical oxidation to convert all carbon within the compounds to  $\text{CO}_2$ . This  $\text{CO}_2$  is then extracted from the mobile phase and directed to the mass spectrometer for carbon isotope ratio analysis (Perini and Bontempo, 2022). However, LC-IRMS is currently limited to the measurement of carbon isotope ratios. The current separation unit is designed to work exclusively with  $\text{CO}_2$ , making it challenging to strip other elements (Perini and Bontempo, 2022). Recent advancements have successfully extended antibiotics CSIA to carbon and hydrogen stable isotope analysis using Gas Chromatography-IRMS (GC-IRMS) (Ouyang et al., 2023), thereby shedding new light on the applicability of ME-CSIA. Furthermore, previous studies have harnessed dual-element isotope analysis (e.g., carbon and chlorine) to differentiate between various degradation pathways of chlorinated organic pollutants in soil-plant and food web systems (Liu et al., 2021, 2020). In the future, with the establishment of suitable derivatization procedures for various types of antibiotics, it may become feasible to measure nitrogen, chlorine, and sulfur isotopes by GC-IRMS and Gas Chromatograph Coupled with a Multiple-collector Inductively Coupled Plasma Mass Spectrometer (GC-MC-ICP-MS). For instance, a derivatization method of antibiotics is currently developed for SMX using trimethylsilyldiazomethane (TMSD), involving methylation on the nitrogen atom in the S-N bond (data not published). Additionally, there have been recent developments in universal chlorine and sulfur isotope analysis (Horst et al., 2017; Kümmel et al., 2020), presenting an opportunity to develop and validate these methods for antibiotics, with the perspective to follow-up their stable isotope composition in CW.

Given the widespread use of antibiotics and the potential health risks they pose, it is also imperative to develop innovative frameworks for assessing the extent of degradation and elucidating the pathways of antibiotics within CW. These ubiquitous artificial ecosystems, renowned for their adaptability in bioremediation of micropollutant contaminated water, can serve as a focal point for this endeavor. Newly developed isotope concepts offer significant potential to address multiple challenges. Primarily, these concepts can facilitate the distinction between dissipation processes, encompassing both non-degradative and degradative pathways, as well as among various transformation pathways in CW. This capability can aid in prioritizing degradation pathways within CW, thereby improving risk assessment and the implementation of remediation strategies. These advancements can provide guidance for formulating risk management approaches by improving predictions of transformation processes within CW. Furthermore, integrating isotope concepts into the toolkit for evaluating the kinetics and processes of antibiotic removal in wetland systems will enhance the establishment of a more universal framework. This framework can generate standardized concepts that go beyond the realm of CW research, uti-



**Fig. 2 – Conceptual framework of Multiple Element Compound-Specific Stable Isotope Analysis (ME-CSIA) applied to antibiotics in constructed wetlands (CW). (A) The footprint of antibiotic degradation in CW: Stable isotope composition of antibiotics in CW may vary due to kinetic isotope effects when antibiotics undergo degradation processes such as photo-, phyto-, and microbial degradation, as shown for Element A, B, and C. (B) Degradation mechanisms and pathway identification from reference laboratory experiments: The direction and magnitude of isotope fractionation may indicate the underlying reaction chemistry. Stable isotope fractionation values ( $\epsilon$ ) for individual elements and dual isotope element values ( $\Delta$ ) (represented by  $\delta^{15}\text{N}$  versus  $\delta^{13}\text{C}$  values) are retrieved, serving for the identification and differentiation of pathways within CW. (C) Quantitation of the extent of antibiotic degradation in field samples: The degradation extent can be quantified using the Rayleigh equation, employing reference stable isotope fractionation values (Vogt et al., 2016).**

**Table 1 – Overview of antibiotic CSIA studies and associated conditions.**

Antibiotics	Method	Stable isotope	Conditions
Sulfamethoxazole (SMX)	LC-IRMS <sup>a</sup>	$\delta^{13}\text{C}$	Direct photodegradation (Birkigt et al., 2015; Willach et al., 2018)
SMX	LC-IRMS <sup>a</sup>	$\delta^{13}\text{C}$	Indirect photodegradation with ozone and chlorine dioxide (Willach et al., 2017)
SMX	LC-IRMS <sup>a</sup>	$\delta^{13}\text{C}$	Aerobic Microbial degradation- <i>Microbacterium</i> sp. strain BR1 (Birkigt et al., 2015)
SMX	GC-IRMS <sup>b</sup>	$\delta^{13}\text{C}$ , $\delta^{\text{b}}\text{H}$	Anaerobic Microbial degradation- <i>Desulfovibrio vulgaris</i> Hildenborough (Ouyang et al., 2023)

<sup>a</sup> LC-IRMS: Liquid Chromatography Isotope Ratio Mass Spectrometry.  
<sup>b</sup> GC-IRMS: Gas Chromatography Isotope Ratio Mass Spectrometry.

lizing them as a prototype for complex and dynamic systems. The potential of such a generic framework may also extend to broader applications, encompassing various micropollutants and a range of environmental scenarios at urban, territorial, or watershed scales (Hofstetter et al., 2024).

To advance the development and implementation of antibiotic CSIA in wetlands, collaborative efforts among research, local and territorial monitoring programs, and government entities are crucial to addressing key milestones. These include the validation and standardization of the antibiotic isotope methodology, encompassing  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^2\text{H}$ ,  $^{37}\text{Cl}$ , and  $^{33/34}\text{S}$ . This involves ensuring the consistency and reliability

of sampling, extraction, measurements, and interpretation of isotopic data. Moreover, expanding reference antibiotic laboratory studies to encompass a broader range of wetland-specific processes, such as indirect photodegradation or bioaccumulation and biomagnification in food chains, is essential to enhance the applicability of antibiotic CSIA in field studies. This expansion will pave the way for future applications of antibiotic CSIA in CW, including source apportionment, bioaccumulation in trophic levels, biodegradation, and phytoremediation concepts. In turn, such applications will contribute to the development of a framework for risk assessment and regulatory evolution through the application of antibiotic

CSIA, ensuring a robust foundation for addressing environmental concerns associated with antibiotic contamination in wetlands. Finally, the potential and contribution of antibiotic CSIA in wetlands may align with the Global Health concept, promoting a holistic approach to addressing health-related challenges associated with antibiotics in engineered and nature-based wastewater treatment solutions worldwide.

#### Box 1: Classic Definitions

**Isotopes** are naturally occurring variations of elements that have different atomic masses due to a different number of neutrons but identical numbers of protons (e.g.  $^{13}\text{C}$ ,  $^{12}\text{C}$ )

**Stable isotopes** are non-radioactive naturally occurring forms of elements

**ME-CSIA:** Multi-element Compound-specific Isotope Analysis

**Isotope fractionation/ Isotope effect (IE)** is a change in the isotopic composition due to a biological, chemical or physical process

**Delta value ( $\delta$ )** describes the difference in the isotopic ratio (e.g.  $^{13}\text{C}/^{12}\text{C}$ ) of a sample relative to an international standard in ‰

**Isotope Fractionation factor ( $\epsilon$ )** describes the change in the isotopic composition of a substrate over the course of a reaction

**Dual element isotope analysis** is the slope of changes of  $\delta$  values of two elements (e.g. C–H) compared to the source, which is represented by Lambda value ( $\Lambda$ ).

## Declaration of Competing Interest

All authors of the article disclose no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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