Diversity and heavy metal tolerance of endophytic fungi from *Dysphania ambrosioides*, a hyperaccumulator from Pb–Zn contaminated soils

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**ABSTRACT**

The diversity and heavy metal (HM) tolerance of endophytic fungi (EF) associated with *Dysphania ambrosioides*, a hyperaccumulator from two Pb–Zn contaminated sites were investigated. A total of 237 culturable EF were isolated and identified to 43 taxa based on morphological characteristics and rDNA internal transcribed spacer analysis, of which 13 occurred as endophytes of both sites, while other taxa were only found in either site. The colonization rate, dominant genera, community structure of EF as well as the HM content in the plant from two sites were significantly different. We suggest that these differences may result from the difference in the soil HM content; lower HM content in the soil, more EF in the plant, which may enhance the plant HM accumulation and thus result higher HM in it. HM tolerance tests indicated that 50% of the isolates exhibited HM tolerance. Among them, two isolates exhibited better HM tolerance, of which FT2G59 could tolerate Pb, Zn, and Cd, and the minimum inhibitory concentration (MIC) of them were 30–50, > 680, 20–30 mmol/l, respectively. While, the isolate FT2G7 could tolerate Cd, and the MIC was 30–50 mmol/l. These isolates may have potential application in phytoremediation.

**Introduction**

The soil is an important life-supporting system and is central to essential planetary functions such as primary production, the regulation of biogenic gases and the earth’s climate, biogeochemical and water cycling, and the maintenance of biodiversity (Abhilash et al. 2012). However, continued worldwide industrialization, modern agriculture, and urbanization have introduced heavy metals (HMs) into the soil and caused serious environmental issues (Rajkumar et al. 2010). To overcome these problems, microbial-assisted phytoremediation is being explored. Microorganisms could promote plant growth by transformation of nutrient elements, production of phytohormones, or provide iron to reduce phytotoxicity, improve plant growth, and enhance the phytoremediation effectiveness of host plants (Ren et al. 2011; Zhang et al. 2012; Deng et al. 2014). *Dysphania ambrosioides* (L.) Mosyakin & Clemants before known as *Chenopodium ambrosioides* is an invasive plant in China, and previous studies have indicated that it is a Pb-hyperaccumulator (Wu et al. 2004). In our previous study (Li, Li, et al. 2012), we found that *D. ambrosioides* is the dominant plant species in some Pb–Zn contaminated sites in Huize County, Yunnan Province, Southwest China. To understand the community structure and the possible ecological role of fungal endophytes associated with *D. ambrosioides*, plants were collected from two HM contaminated sites and fungal endophytes were isolated, and the diversity, HM tolerance as well as the minimum inhibitory concentration (MIC) of endophytes were investigated.

**Materials and methods**

**Study site and sampling**

The sampling sites were located in Huize County, Yunnan Province, Southwest China (25°48′–27°04′N, 103°03′–103°55′E), where Pb–Zn mining has been carrying out for more than 300 years. There, many slag heaps and wastelands are covered with sparse vegetation; *D. ambrosioides* is one of the dominant plant species (Qin et al. 2013).
Plants of *D. ambrosioides* were collected from two sites which were about 1.5–2 km away from each other: one was a slag heap and the other was a wasteland. From each sampling site, 15 healthy *D. ambrosioides* plants (without any apparent disease symptoms) were collected at random (each plant at least 30 m away from another), and the soils adjacent to the plants (about 5–10 cm depth) were collected and mixed thoroughly. Each sample was placed in a plastic bag and transported to the lab and processed within 24 h in November 2014.

**Isolation and identification of fungal endophytes**

Two leaves, two stems, and two root segments (about 6 cm long) were selected randomly from each plant; total of 30 leaves, 30 stems, and 30 root segments of plants from one site were chosen. Plant pieces were washed in running tap water and processed as follows: the samples were cut into segments (about 5 × 5 mm) and surface-sterilized by sequentially dipping into 0.5% sodium hypochlorite (2 min) and 70% ethanol (2 min) (Li, Shen, et al. 2012). Then, 100 root segments and 100 shoot segments (50 stems and 50 leaves) (about 7 root pieces and 7 shoot pieces from each plant) of plants collected from each sampling site were placed in Petri dishes containing potato dextrose agar (PDA) medium amended with 0.5 g/l streptomycin sulfate. The plates were incubated at 25°C and checked every other day for 45 days; fungi growing out of the plant tissues were transferred to fresh PDA plates. The effectiveness of the surface sterilization was confirmed by making imprints of disinfected plant fragments on PDA plates from which no fungal growth was observed (Schulz et al. 1998). All of the isolates were deposited in the Faculty of Life Sciences and Technology, Kunming University of Science and Technology.

Fungal morphological identification was based on the morphology of the colony as well as the mechanism of spore production and spore characteristics (Barnett & Hunter 1987; Ellis 1988). For frequently occurring morphotypes that were sterile or were sporulating but difficult to identify to genus, molecular identification was attempted using the internal transcribed spacer (ITS) region and a reference database. Two to four isolates were selected randomly from each morphotype and total 61 isolates from 12 morphotypes were chosen to conduct molecular analysis. To produce biomass of fungi growing out of the plant tissues were transferred to fresh PDA plates. The effectiveness of the surface sterilization was confirmed by making imprints of disinfected plant fragments on PDA plates from which no fungal growth was observed (Schulz et al. 1998). All of the isolates were deposited in the Faculty of Life Sciences and Technology, Kunming University of Science and Technology.

**Heavy mental content analysis**

Plant samples were washed with distilled water to remove surface element trace, then, were divided into roots and shoots and oven-dried at 65°C for 48 h until constant weight. Subsequently, the samples were crushed to fine powders with a mortar and pestle, and 0.2 g roots/shoots powders were digested with 5 ml HNO₃ (65% w/w) at 110°C for 2 h, then, cooled and added with 1 ml H₂O₂ (30% w/w) and boiled for 1 h. Finally, the digests were diluted to 50 ml with triple deionized water in a volumetric flask (Shen et al. 2013). Soil samples were dried at room temperature (25°C), then crushed and sieved with a 0.15 mm mesh to get fine powders. Then, 0.5 g soil powders were digested with 4 ml HCl–HNO₃ (3:1, v/v) mixture at 80°C for 30 min, then 100°C for 30 min, at last 120°C for 1 h. Then, cooled and added with 1 ml HClO₄ to continue digesting at 100°C for 20 min, and then 120°C for 1 h. Finally, the digests were diluted to 50 ml with triple deionized water in a volumetric flask; triplicates for each sample were prepared. The contents of Pb, Zn, and Cd in plant and soil digests were determined by flame atomic absorption spectrometry (Li et al. 2014).

**Heavy mental tolerance of fungal endophytes**

Approximately one-third of the isolates of each taxa were selected randomly for HM tolerance tests; a total of 61 isolates belonging to 18 taxa were chosen, and one isolate (*Xylaria berteri* from Dawei Mountain, Southwest of China) was used as the control isolate (Zhou et al. 2015). All 62 fungal endophytes were cultured on PDA plates for 7 days, then, 3 fungal disks (diameter 4.4 mm) of each isolate were cut down from the edge of the colony and then placed on another fresh PDA plate, which contained different kinds of HMs, including Pb(NO₃)₂, ZnSO₄·7H₂O or 3CdSO₄·8H₂O (HM-PDA) (Shen et al. 2013). The concentration of HM in PDA was as follows: Pb-PDA (9.66 mmol/l Pb), Zn-PDA (46.15 mmol/l Zn), Cd-PDA (1.00 mmol/l Cd), and PDA without HM. The inoculated plates were incubated at 25°C, and the diameter of the colony was measured every other day for 6 days. Replicates were in triplicate.

**Determination of MIC**

To determine the MIC of HMs (Xu et al. 2012), three mycelial pieces (diameter 4.4 mm) of the isolates FT2G59 (*Penicillium*) and FT2G7 (*Phomopsis columnaris*), which exhibited better HM tolerance, were cut from the edge of the 7 days old cultures and inoculated onto fresh PDA plates which contained serially increasing concentrations of Pb, Zn, or Cd, respectively. After 7 days incubation at 25°C, the MIC was recorded (Fazli et al. 2015).

**Data analyses**

The colonization rate (CR) was calculated as the total number of plant tissue fragments infected by one or more fungi divided by the total number of fragments incubated (Sun et al. 2011). The relative frequency (RF) was calculated as the number of isolates of one species divided by the total number of isolates (Yuan et al. 2011). The EF diversity was evaluated using the Shannon index, which has two main components, evenness and the number of species (Spellerberg & Fedor 2003). The Shannon index (H') was calculated according to the following formula: 

\[ H' = - \sum_{i=1}^{k} P_i \times \ln P_i, \]

where \( k \) is the total species number of one plot and \( P_i \) is the relative abundance of endophytic fungal species in one plot. To evaluate the degree of community similarity of the EF between the
two treatments, Sorensen’s coefficient similarity index (CS) was employed and calculated according to the following formula: \( CS = \frac{2j}{(a + b)} \), where \( j \) is the number of endophytic fungal species co-existing in two treatments, \( a \) is the total number of endophytic fungal species in one treatment and \( b \) is the total number of endophytic fungal species in another treatment (Southwood 1978). The heavy metal tolerance index (MTI) of the isolate was calculated as the mean diameter on PDA with HMs divided by the mean diameter on PDA without HMs. Isolate with MTI more than 50% at the 6th day was designated as HM-tolerant isolate.

SPSS software ver. 17.0 was used for statistical analysis. Chi-squared test was used to compare the differences in the CR of endophytes from two sites. The averages and standard deviation of the HMs content were calculated using three replicates of a mixed plant and soil samples from two sites, respectively. A t-test was used to determine the differences in mean HMs content between samples from two sites; \( P \) was set at < .05.

**Results**

**Colonization rate**

A total of 237 EF were isolated from 368 tissue segments, of which 83 from \( D. \) ambrosioides growing in the slag heap and 154 from \( D. \) ambrosioides growing in the wasteland (Table 1). The total CR of EF of plants growing in the slag heap (35.20%) was significantly lower than that of plants growing in the wasteland (57.67%) (\( P < .05 \), chi-squared test). The CR of EF in roots of plants growing in the slag heap was 57.29%, it was significantly higher than that in shoots (9.64%) (\( P < .05 \), chi-squared test). Contrary to this, there was no significant difference in the CR of endophytes between roots and shoots of plants growing in the wasteland (48.91% and 65.98%) (\( P > .05 \), chi-squared test).

**Composition of fungal endophytes**

The fungal endophytes were identified to 43 taxa based on morphological characteristics and ITS sequence analysis (Table 2), and the accession number of representative isolates as well as their most closely related species in Genbank were presented in Table 3. Among all 43 taxa, there were only 13 taxa which occurred as endophytes on both sites, while other taxa were only found in either site (Table 2). The CS of fungal endophytes of plants from two sites was 0.46. The dominant genera of \( D. \) ambrosioides growing in the slag heap were \( Plectosphaerella \), \( Cladosporium \), and \( Verticillium \), and their RFs were 18.07%, 10.84%, and 8.43%, respectively; while, the dominant genera of fungi from the wasteland were \( Phoma \), \( Peyronellaea \), and \( Alternaria \), and their RFs were 20.13%, 12.34%, and 11.69%, respectively (Table 2). The \( H' \) of EF of plants from the slag heap and wasteland were 2.92 and 2.82, respectively (Table 2).

**HM content analysis**

The HM content of soils and plants from two sites are shown in Table 4. Compared with the ‘Environmental Quality Standard for Soils’ of China (GB15618-1995, grade 3), the soils from both two sites were heavily polluted by Pb, Zn, and Cd. Similarly, the content of Pb, Zn, and Cd in plants from both two sites have largely exceeded the limiting value (Pb and Cd compared with GB2762-2012; Zn compared with GB13106-1991). In total, the HM content of soils from the slag heap was higher than that from the wasteland. Contrary to this, the HM content of plants from the slag heap was lower than that from the wasteland.

**HM tolerance and MIC of fungal endophytes**

HM tolerance tests indicated that 50% of the tested isolates (31 isolates) exhibited HM tolerance capacity (Figure 1). Among them, 4.8% (3 isolates), 4.8% (3 isolates), and 46.8% (29 isolates) could tolerate Pb, Zn, and Cd, respectively. However, only one isolate (FT2G59) could tolerate three HMs. In addition, it was found that the isolates of different taxa showed different metal tolerance capacities. For example, under Cd stress, there were 71.43% and 60% of \( Peyronellaea \) and \( Phoma \) isolates which exhibited tolerance capacity, respectively. However, none of the isolate of \( Alternaria \) showed tolerance. Among all active isolates, FT2G59 (\( Pencillium \)) and FT2G7 (\( P. \) columnaris) exhibited better metal tolerance. FT2G59 could tolerate Pb, Zn, and Cd simultaneously, and their MICs were 30–50, > 680, 20–30 mmol/l, respectively. However, FT2G7 could only tolerate Cd, and its MIC was 30–50 mmol/l.

**Discussion**

**Soil HM content effects on endophytes**

CR is an indication of the number of endophytes in host plants and is known to vary with the plant community, host plant species, altitude, air humidity, precipitation, and air temperature (both annual average temperature and temperature seasonal oscillations) (Arnold & Lutzoni 2007; Hashizume et al. 2010; Li, Shen, et al. 2012). In the present study, the two sampling sites were only 1.5–2 km away from each other and some environmental conditions were similar, such as the altitude, air humidity, precipitation, and air temperature. In a previous study, Okane and Nakagiri (2015) proposed that the CRs of a particular plant species in similar environments should be comparable. However, we found that the CRs of EF of \( D. \) ambrosioides from two sites showed significant differences (\( P < .05 \), chi-squared test), and the

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**Table 1. Number and CR of EF of \( D. \) ambrosioides from two sites.**

| Origin of plants | No. of segments plated (no. of segments colonized by EF) | No. of EF isolated | CR (%) |
|-----------------|--------------------------------------------------------|--------------------|--------|
|                 | Root Shoot Total                                      | Root Shoot Total   |        |
| Slag heap        | 96 (55) 83 (8) 179 (63)                                | 75 8 83            | 57.29 a 6.94 a 35.20 a |
| Wasteland        | 92 (45) 97 (64) 189 (109)                              | 61 93 154          | 48.91 a 65.98 b 57.67 b |
| Total            | 188 (100) 180 (72) 368 (172)                           | 136 101 237        |        |

Note: Different letters in the same column indicate a significant difference at \( P < .05 \).
composition as well as the dominant genera were different, too (Table 2). It was supposed that these differences may result from the difference in the soil HM content in two sites. Chan et al. (2013) found the same phenomenon, where arbuscular mycorrhizal fungi CRs decreased with the increase of arsenic levels in soils. In addition, we found that the HM content of plants from the lower HM content soils (the wasteland) was higher than that of plants from the higher HM content soils (the slag heap) (Table 4). We suggest that this may result from the difference of EF in *D. ambrosioides* from two sites. Previous studies have demonstrated that endophytes can enhance host plants HMs accumulation. For example, Khan et al. (2015) found that endophytic fungal isolate *Penicillium janthinellum* LK5 symbiosis improved

| Taxa                        | Plants from the slag heap | Plants from the wasteland |
|-----------------------------|---------------------------|---------------------------|
|                            | Root | Shoot | Total | Root    | Shoot | Total | Total |
| Alternaria sp.              | –    | 2     | 2.241 | –       | 18    | 19.35 | 20    |
| Alternaria tenuissima       | –    | –     | –     | –       | 2     | 2.15  | 2     |
| Ascochyta sp.               | –    | 1     | 1.20  | –       | –     | –     | 1     |
| Ascomycota sp.              | –    | –     | –     | –       | 1     | 1.08  | 1     |
| Cephalosporium sp. 1        | 3    | 4     | 3.61  | –       | –     | –     | 3     |
| Chactomium globosum         | 2    | 2.67  | –     | 2       | 1.20  | 3.47  | 3     |
| Colletotrichum sp. 1        | 1    | 1.33  | –     | 1       | 1.20  | 2.53  | 2     |
| Dendryphion sp. 1           | 2    | 2.67  | –     | 2       | 1.20  | 3.87  | 3     |
| Diplodia sp.                | –    | –     | –     | –       | 2     | 1.30  | 2     |
| Discosia sp.                | 1    | 1.33  | –     | 1       | 1.20  | –     | 1     |
| Epipoccum nigrum            | 5    | 6.67  | –     | 5       | 6.02  | –     | 5     |
| Fusarium sp. 1              | 3    | 4.00  | 3.61  | 2       | 3.28  | 5.58  | 5     |
| Fusarium sp. 2              | 1    | 1.33  | –     | 1       | 1.20  | 2.53  | 2     |
| Fusarium sp. 3              | –    | –     | –     | 2       | 3.28  | 4.56  | 4     |
| Fusarium sp. 4              | 1    | 1.33  | –     | 1       | 1.20  | –     | 1     |
| Fusarium sp. 5              | 1    | 1.33  | –     | 1       | 1.20  | –     | 1     |
| Fusarium sp. 6              | 1    | 1.33  | –     | 1       | 1.20  | –     | 1     |
| Gilmanella sp.              | –    | 1     | 12.5  | 1       | 1.20  | –     | 1     |
| Hainesia sp.                | –    | –     | –     | 1       | 1.64  | 1     | 1     |
| Humicola fuscoatra           | 4    | 6.56  | –     | 6       | 5.62  | –     | 6     |
| Ilyonectria radicola        | 9    | 14.75 | –     | 9       | 8.74  | –     | 9     |
| Macrospora sp.              | 2    | 2.67  | 2     | 2       | 2.41  | 4.88  | 4     |
| Microspora sp.              | –    | –     | –     | –       | 1     | 1.64  | 1     |
| Monilia sp.                 | 2    | 2.67  | 2     | 2       | 2.41  | 1     | 1     |
| Monocillium sp.             | 1    | 1.33  | –     | 1       | 1.20  | –     | 1     |
| Mucor sp.                   | 3    | 4.00  | 3.61  | 5       | 8.20  | 1.08  | 6     |
| Nodulisporium sp.           | –    | 1     | 12.50 | 1       | 1.20  | –     | 1     |
| Penicillium sp.             | –    | –     | –     | 4       | 6.56  | –     | 4     |
| Peyronellaea sp.            | 2    | 2.67  | 2     | 2       | 2.41  | 4     | 2     |
| Phoma sp.                   | 4    | 5.33  | 1     | 1       | 1.20  | 5     | 4     |
| P. columnaris               | –    | –     | –     | 2       | 3.28  | 1.08  | 6     |
| Plectosphaerella sp.        | 14   | 18.67 | 1     | 12.50   | 15     | 18.07 | 18    |
| Rhytchophoma sp.            | –    | –     | –     | 1       | 1.64  | 2     | 1.69  |
| Septoria sp.                | –    | –     | –     | 3       | 3.23  | 3     | 3.27  |
| Verticillium sp.            | 7    | 9.33  | –     | 7       | 8.43  | –     | 7     |
| Unidentified 1              | –    | –     | –     | 2       | 2.15  | 2     | 2.15  |
| Unidentified 2              | 3    | 4.00  | 3.61  | 3       | 3.61  | –     | 3     |
| Total                       | 75   | 8     | 100   | 83      | 100   | 93    | 100   |

| H                       | 2.82 | 1.73 | 2.92 | 2.72 | 2.14 | 2.82 |

Note: ‘–’ indicates the taxa were not found in plants.

Table 2. Number, taxa, RF, and Shannon index (H’) of EF of *D. ambrosioides* from two sites.

| Accession no. of isolate | Isolate obtained in the present study | Morphotype | Identity (%) | Query coverage (%) | Most closely related species (accession no.) |
|--------------------------|--------------------------------------|------------|--------------|-------------------|--------------------------------------------|
| FT2Y30                   | KT291415                             | A. tenuissima | 100          | 97               | A. tenuissima (KF308837.1)                 |
| FT2J22                   | KT291423                             | C. globosum  | 99           | 97               | C. globosum (GQ966953.1)                   |
| FT2G74                   | KT291428                             | C. lobatum   | 100          | 94               | C. lobatum (KF308838.1)                    |
| FXY14                    | KT291414                             | Colletotrichum sp. 2 | 99        | 98               | Colletotrichum sp. (KF308837.1)            |
| FX40                     | KT291419                             | Dendryphion sp. | 96           | 89               | Dendryphion sp. (KJ769501.1)               |
| FT1G55                   | KT291413                             | E. nigrum    | 99           | 98               | E. nigrum (HM467833.1)                     |
| FT2G52                   | KT291422                             | H. fuscoatra  | 88           | 76               | H. fuscoatra (KF308830.1)                  |
| FT2G11                   | KT291420                             | I. radicola  | 99           | 96               | I. radicola (KF308832.1)                   |
| FXZ44                    | KT291418                             | Peyronellaea sp. | 99        | 97               | Peyronellaea sp. (KP117270.1)              |
| FT2G7                    | KT291432                             | P. columnaris | 99           | 96               | P. columnaris (KF308830.1)                 |
| FT1G11                   | KT291416                             | Plectosphaerella sp. | 99        | 99               | Plectosphaerella sp. (KF308830.1)          |
| FMV23                    | KT291426                             | Septoria sp. | 100          | 96               | Septoria sp. (KF308830.1)                  |

Table 3. ITS BLAST matches using Genbank database for morphotypes that were difficult to identify to genus.
aluminum phytoextraction in tolerant *Solanum lycopersicum*; Chen, Shen, et al. (2014) and Chen, Zhang, et al. (2014) found that the inoculation of endophyte *Sphingomonas SaMR12* improved the accumulation of cadmium and zinc in host plants. In our previous study, we also found that some isolates of *Peyronellaea* can improve host plants lead, zinc, or cadmium accumulation (Shen et al. 2013). In the present study, the plant from the wasteland harbored more endophytes (higher CR) and more *Peyronellaea* isolates (dominant EF, Table 2), which may enhance its HM accumulation and thus result higher HM in it.

The Shannon index ($H'$) estimates of EF from both sites were not lower than those of other environments (Suryanarayanan et al. 2011; Li, Shen, et al. 2012). Wężowicz et al. (2014) also found that $H'$ values of EF of *Verbascum lycnitis* from Pb–Zn mine tailings were higher in comparison to non-tailings sites. The dominant genera of fungi from the wasteland were *Phoma*, *Peyronellaea*, and *Alternaria*, and their

### Table 4. HM content of plants and soils (Mean ± SD).

| Sample site       | Plants (mg kg$^{-1}$, dry weight) | Soils (mg kg$^{-1}$, dry weight) |
|-------------------|-----------------------------------|-----------------------------------|
|                   | Pb                                | Zn                                | Cd                               | Pb                                | Zn                                | Cd                               |
| Slag heap         | 52.95 ± 0.68 a                     | 1648.93 ± 24.65 a                  | 27.12 ± 0.67 a                    | 4276.18 ± 61.27 a                  | 18496.89 ± 1357.70 a               | 8.89 ± 0.10 a                     |
| Wasteland         | 264.13 ± 5.88 b                    | 21436.25 ± 388.98 b                | 48.49 ± 1.55 b                    | 4126.37 ± 57.16 a                  | 7127.27 ± 143.62 b                 | 7.45 ± 0.14 b                     |
| Standard          | 20$\degree$                       | 0.2$\degree$                      | 0.2$\degree$                      | 500$\degree$                       | 500$\degree$                      | 1$\degree$                       |

*Notes:* Different letters in the same column indicate a significant difference at $P < .05$.
1. The standard of ‘national food safety standards’ (GB2762-2012).
2. The standard of ‘tolerance limit of zinc in foods’ (GB13106-1991).
3. The standard of ‘environment quality standard for soils’ (GB 15618-1995), grade III, pH > 6.5.

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**Figure 1.** HM tolerance capacity of EF. The concentrations of Pb, Zn, and Cd were 9.66, 46.15, and 1.00 mmol/l, respectively. The color represents different heavy metal tolerance index (MTI) and its gradient change from blue to red represents the MTI gradient increases from 0% to 100%.
RFs were 20.13%, 12.34%, and 11.69%, respectively (Table 2).

In our previous study, we also found that the dominant genera from six plant species (Arabis hirsute, Acacia decurrens, Symlocos paniculata, Rabbiosa eriocalyx, Arenaria serpulifolia, and Rosa longicuspis) collected from the same area but different sampling sites were Phoma, Peyronellaea, and Alternaria, and their RFs were 39.6%, 20.4% and 19.0%, respectively (Li, Li, et al. 2012). In addition, it was found that the isolates of dominant genera always exhibited better HM tolerance than the isolates of other taxa. The same phenomenon was found by Xu et al. (2015).

**Discipline statement**

No potential conflict of interest was reported by the authors.

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