Bull. Environ. Contam. Toxicol. (2005) 75:443–450
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DOI: 10.1007/s00128-005-0773-1

Bulletin of Environmental Contamination and Toxicology

# Biodegradation of Pyrene and Phenanthrene in Soil Using Immobilized Fungi *Fusarium sp.*

P. Li,[1] H. Li,[1] F. Stagnitti,[2] X. Wang,[3] H. Zhang,[1] Z. Gong,[1] W. Liu,[1] X. Xiong,[1] L. Li,[4] C. Austin,[2] D. A. Barry[5]

[1] Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, People's Republic of China
[2] School of Ecology and Environment, Deakin University, Warrnambool Campus, Post Office Box 423, Warrnambool, Vic 3280, Australia
[3] School of Science, Shenyang University of Technology, Shenyang 110023, People's Republic of China
[4] Center for Eco-Environmental Modeling, Hohai University, Nanjing 210098, People's Republic of China
[5] Institute for Infrastructure and Environment, School of Engineering and Electronics, The University of Edinburgh, Edinburgh EH9 3JL, United Kingdom

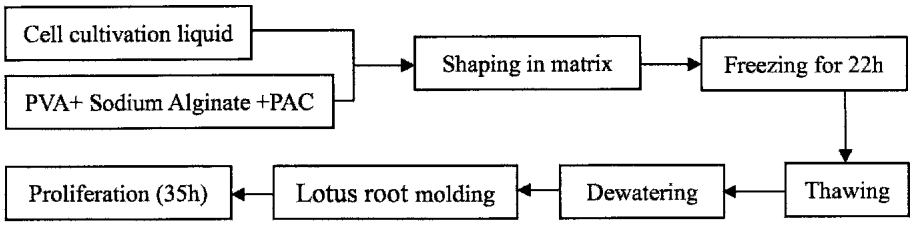
Received: 19 November 2004/Accepted: 16 July 2005

Phenanthrene ($C_{14}H_{10}$) and pyrene ($C_{16}H_{10}$) are components of polycyclic aromatic hydrocarbons (PAHs). Polycyclic aromatic hydrocarbons constitute the largest group of environmental contaminants released in the environment and some are suspected as being carcinogenic and mutagenic substances. Therefore the potential biodegradation of these compounds is of vital importance. The major sources of PAHs in soils are petroleum products such as coal tar, creosote, and vehicle emissions (Pinto and Moore 2000). In China, significant sources of PAHs found in soils also result from dumping or accidental spilling of crude oil from oil fields and the irrigation of wastewater from petroleum refineries. For example, in the Liaohe Oil Field, it was estimated that about 10,000 tons of crude oil entered the soil in 1998 (Jiang 2000). The composition of aromatic hydrocarbons in the crude oil in the soil was found to range from 14% to 20% (Jiang 2000; Li et al. 2002). In the suburb of Shenyang City, several thousand hectares of crops were irrigated with refinery effluent for more than 35 years. This irrigation resulted in severe contamination by petroleum hydrocarbons and PAHs at levels from 4 to 10 times higher than China's legislated safe levels (Wu et al. 1986).

Bioremediation is a desirable technology for removing high concentration of PAHs in soil. However, it is plagued with problems such as long treatment times and the difficulty in maintaining viable microbe populations (Li et al. 2002). Current research is dealing with improving the degradation rates of PAHs in soils, including preserving microorganism cells from mechanical damage and chemical toxicity. Among the techniques for increasing the biodegradation rates of hydrocarbons, microorganism immobilization seems to be a promising method (Ionata et al 2005; Liao et al. 1997). Immobilization can be defined as the fixation of the biocatalysts, including enzymes, microorganisms and organelles to insoluble solid supports (Dodor et al 2004; Ahn et al 2002; Kok et al. 2000). Immobilization technology attempts to maintain high populations of microorganism cells that are formed and sustained within biocarriers, and offers continuous biotreatment and prevention of the spread of microorganisms to neighboring field plots (Edwards 1994).

Materials for the biocarrier should have "ideal" physical, chemical, and economic parameters that optimizes cell populations and maintains viability, and in addition, must be non-toxic. Many types of polymers, such as polyurethane and alginate, have been successfully used in the entrapment and encapsulation processes of immobilized microbes (Hu et al. 1994). Liao et al. (1997) investigated the removal efficiencies of naphthalene, fluoranthene and benzopyrene by an immobilized white-rot fungus in a batch experiment. Their results showed that

*Correspondence to:* F. Stagnitti




**Schedule 1.** A flow diagram of the process for fungus cells immobilization.

mixed biocarriers composed of alginate and powdered activated carbon presented a satisfactory pore volume for the colonization and maintenance of viable microorganism populations. Many researchers have previously studied the effects of immobilized microbes on wastewater treatment. However, hitherto few studies have dealt with the utilization of introduced microorganisms for the bioremediation of contaminated soils. Therefore, the aims of this research were to investigate (a) new materials for biocarriers and immobilization methods that pose little stress on fungus cells, have high pore volume and low degradability; and (b) the ability of the immobilized fungus to biodegrade phenanthrene and pyrene in sterilized and unsterilized "natural" soils.

## MATERIALS AND METHODS

The fungus *Fusarium sp.* was separated and identified from crude-oil contaminated soils from the Liaohe Oil Field as reported in Li et al. (2002). A paddy soil from Panjin City, Xinglongtai District, which is adjacent to the Liaohe Oil Field, was selected as a control site to test the biodegradation of the phenanthrene and pyrene in a "natural", uncontaminated site. A homogenized soil sample was collected from the surface layer (0 to 20 cm below the soil surface). The soil is characterized by having typical soil properties of pH 7.33; organic matter content (OM) 2.12 %; total nitrogen content (TN) 0.117 %; total phosphorus (TP) 0.038 %; and potassium content (K) 0.687 %.

The study was divided into two parts; (a) the immobilization of the *Fusarium sp* cells; and (b) the biodegradation rates of phenanthrene and pyrene by immobilized fungus in sterilized and unsterilized ("natural") soil slurry reactors. The first part of the study, the immobilization of the *Fusarium sp* cells is shown diagrammatically in Schedule 1. The process mainly involved cell cultivation in the biocarrier, followed by freezing (22 h), thawing, dewatering, molding and proliferation (35 h). The *Fusarium sp.* growing in the exponential growth stage was mixed with the carrier gel (at 40 to 45 °C) under sterile conditions, and then injected into a 8.0 cm round matrix and frozen for 22 h at -15 °C. After which, the frozen carrier was thawed at room temperature, and then dewatered in a sterile room until all the pore moisture was vaporized. A lotus root matrix was then used to shape the biocarrier. These biocarriers were subsequently cultivated in a proliferation culture for 35 h. After compositions of different biocarriers (Li et al. 2002), the optimal composition was found to consist of polyvinyl alcohol (PVA) 100 g $L^{-1}$ with polymerization degree of 17-99; sodium alginate (SA) 5 g $L^{-1}$; and powdered activated carbon (PAC) 50 g $L^{-1}$. Basal and proliferation cultures were prepared in the following manner; The basal culture medium consisted of glucose 20 g $L^{-1}$ and potato juice 200 g $L^{-1}$ that were added to 1L deionized water (pH 7.0) and sterilized at 1.01325 Pa for 30 min. This solution was mixed with the test soil and the ratio of water to soil was 3:1. The proliferation culture medium was the same as basal culture medium with the addition of waste honey 20g $L^{-1}$.

The phenanthrene and pyrene are products of Fluca Co., Germany, and their purities were 90.6 % and 97% respectively. Phenanthrene and pyrene were identified and quantified using the method described by Song et al. (1995) on a HP 1090-II High Performance Liquid Chromatography with a DAD detector.

Three experimental designs were considered. The first experimental design examined the effect of inoculation ratio (5%, 10%, 15%, 20% and 25%) of introduced, immobilized *Fusarium sp.* on the PAHs biodegradation rate with initial concentrations of 100 mg $kg^{-1}$ phenanthrene and pyrene respectively. The second experimental design consisted of different initial concentrations of phenanthrene and pyrene in sterilized soil (treated to avoid the influences of free bacteria and fungi). Five treatment levels were adopted with phenanthrene and pyrene concentrations ranging from 0 to 200 mg $kg^{-1}$. For convenience the 5 treatment levels are summarized in Table 1. The third experiment was designed to compare and contrast the biodegradation of phenanthrene and pyrene in unsterilized "natural" soils containing indigenous bacterium and fungus species under three treatments: No treatment (control), unsterilized soil with immobilized *Fusarium sp*, and unsterilized soil with free *Fusarium sp.* In each case the initial concentrations of phenanthrene and pyrene added to the soil was 100 mg $kg^{-1}$. This experimental design is summarized in Table 2. The operating conditions for all three experimental designs were set at constant temperature of 25 °C, under 130 rpm with pH 7.0. Analysis of Variance (ANOVA) using SPSS for Windows Version 11 was used to compare and contrast the means of residue rates of phenanthrene and pyrene in each experiment. Homogeneity of variance was checked prior to analysis.

**Table 1.** Different initial concentrations of phenanthrene and pyrene in the sterilized soil reactors.

| Treatment | PAH added (mg $kg^{-1}$) | |
|---|---|---|
| | Phenanthrene | Pyrene |
| Control | 0 | 0 |
| Treatment 1 | 10 | 10 |
| Treatment 2 | 50 | 50 |
| Treatment 3 | 100 | 100 |
| Treatment 4 | 200 | 200 |

**Table 2.** Biodegradation of phenanthrene and pyrene in the unsterilized "natural" soil reactors with free (indigenous) fungi and introduced immobilized *Fusarium sp.*

| PAH added (mg $kg^{-1}$) | Treatment | | |
|---|---|---|---|
| | S | S+IMF | S+FF |
| Phenanthrene | 100 | 100 | 100 |
| Pyrene | 100 | 100 | 100 |

[Key: S: unsterilized "natural" soil; S+IMF: soil with immobilized fungi, S+FF: soil with free fungi].

## RESULTS AND DISCUSSION

1. Bio-carrier shape. The hyphae of the *Fusarium sp* are relatively large and long compared to other fungus species. It therefore requires a comparatively larger existing space and more strictly aerobic conditions. The "lotus-root" shaped bio - carrier (Figure 1) not only provided excellent specific surface area for cell

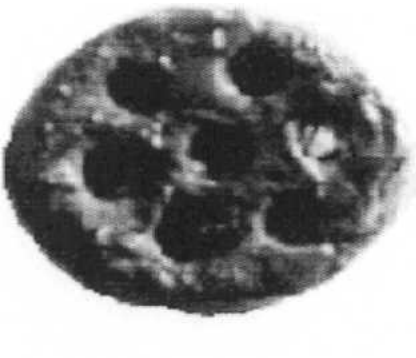


**Figure 1.** Example of the lotus root shape bio-carrier (diameter 1.8 cm, thickness 0.8 cm).

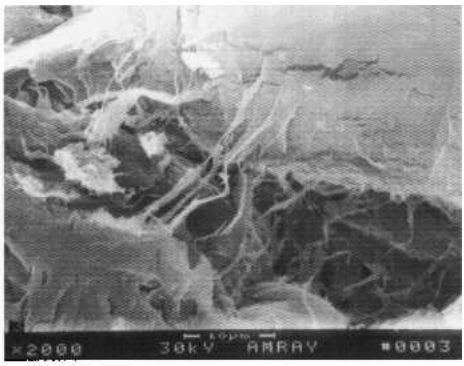



**Figure 2.** Scanning electron micrograph of immobilized fungus, *Fusarium sp.*

colonization, but also had highly mechanical intensity (on average higher than 20 kg $cm^{-2}$). When used in a slurry reactor with high agitation speed, the biocarriers would not be destroyed. The "lotus root" shape provided large contact space when the biocarriers in the slurry, and hence enhance the contact between fungus cells and pollutants (phenanthrene and pyrene). Another superior advantage of this kind of biocarrier is its large intrinsic porosity for oxygen and substrate diffusion compared to other biocarriers tested by Li et al. (2002). A scanning electron micrograph illustrating the immobilized *Fusarium sp.* in the biocarrier is shown in Figure 2.

2. Effect of inoculation ratio of immobilized *Fusarium sp.* The effect of different inoculation ratios on the biodegradation of phenanthrene and pyrene are showed in Figures 3 and 4. After 360 h, the percent biodegradation of phenanthrene was 88.6, 87.5, 89.7, 87.0 and 88.8% for the inoculation ratios of 5, 10, 15, 20 and 25% respectively. The residual quantity of phenanthrene remaining after 360 h were not significantly different, however the speed of degradation prior to the completion of the experiment differed significantly. Generally, higher initial inoculation ratios led to higher rates of phenanthrene biodegradation. This trend, however, is not always the case (e.g. 10 % inoculation ratio of the biocarriers had the highest degradation efficiency than compared to other inoculation ratio at 150 h). Very similar results and trends for biodegradation of pyrene were also found (Figure 4). The biodegradation percent of pyrene were 73.4, 76.6, 78.7, 77.2 and 74.8 % for 5, 10, 15, 20 and 25 % initial inoculations respectively after 360 h. Generally, the biodegradation rate of phenanthrene was higher than pyrene.

3. Effect of different initial concentrations of phenanthrene and pyrene added into soil. For the second experimental design, the inoculation ratio of introduced, immobilized fungus, *Fusarium sp* to sterilized soil samples was 5% and 4 treatments of increasing concentrations of phenanthrene and pyrene (10, 50, 100 and 200 mg $kg^{-1}$ respectively) were applied to the soils. The results of this experiment are showed in Figures 5 and 6. It was very interesting that soils with higher initial phenanthrene concentrations of 100 mg $kg^{-1}$ and 200 mg $kg^{-1}$ had higher percentage of biodegradation (83.7 and 70.0 % respectively at 350 h) than soil samples with low initial phenanthrene concentrations. In the case of pyrene, the highest biodegradation efficiency (74.6 %) was found when the pyrene concentration was 100 mg $kg^{-1}$, while the lowest biodegradation efficiency (32.1%)

occurred at concentration of 200 mg $kg^{-1}$. Phenanthrene is a 3-ring polycyclic aromatic hydrocarbons with a molecular weight of 178.23. In contrast, pyrene is a 4-ring PAH with a higher molecular weight of 202.26. The soil solutions contained a sufficiently high organic matter (2.12%) which provided sufficient substrate for the cometablism and consequently the high initial concentration of phenanthrene was not a limiting factor affecting the rate of biodegradation (Figure 5). However, for pyrene there may have been insufficient organic matter in the soil sample to degrade the 200 mg $kg^{-1}$ treatment. This observation suggests that the support of a cometabolic substrate is important to increase the degradation of PAHs. At low initial concentrations, the PAHs demonstrated strong adsorption onto soil particles, and therefore reflected in the lower removal percentages.

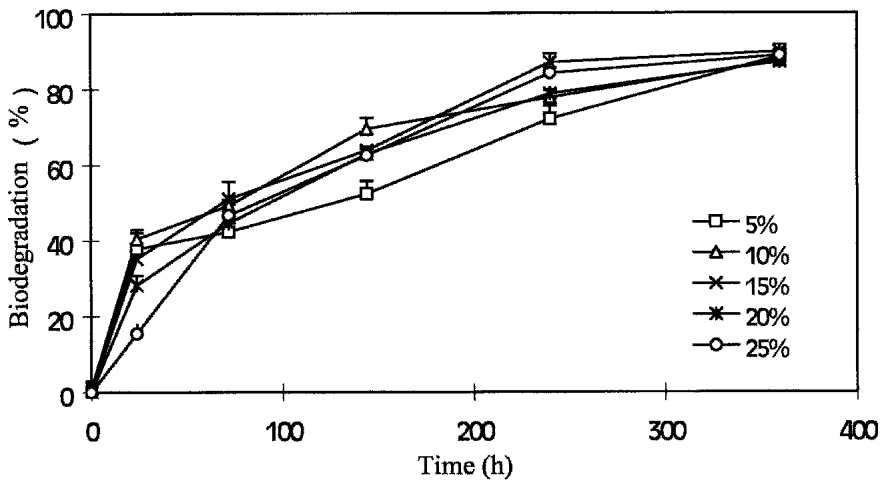



**Figure 3.** Effect of inoculation ratio of immobilized *Fusarium sp.* On biodegradation of phenanthrene.

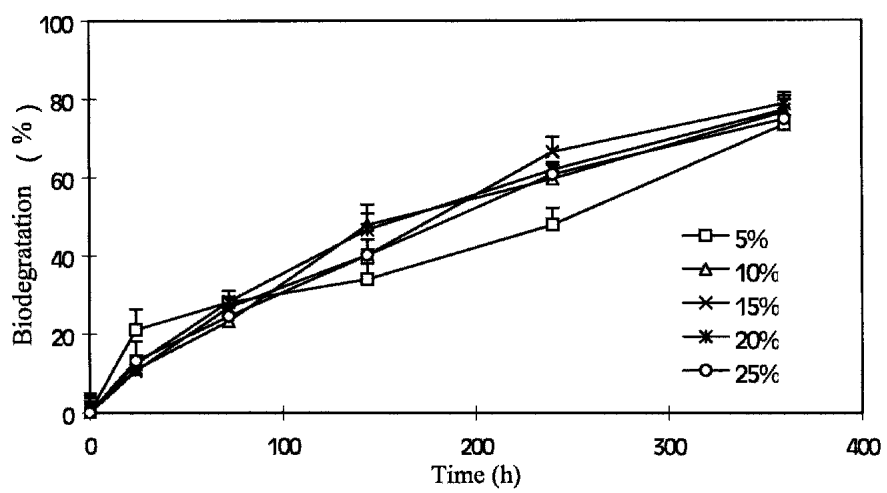



**Figure 4.** Effect of inoculation ratio of immobilized *Fusarium sp.* on biodegradation of pyrene.

4. Biodegradation of phenanthrene and pyrene in non-sterilized "natural" soils. Unlike sterilized soils, the newly introduced, immobilized fungi will be in competition with indigenous soil microbes in natural soil samples and consequently may impact on degrading potentials. The biodegradation of phenanthrene and pyrene in "natural" soils with and without inoculation with immobilized and free fungus was therefore also investigated. For this experiment, 3 treatments were examined: a control treatment with no added *Fusarium sp;* a treatment with introduced and mobile Fusarium sp, and another treatment with introduced, immobilized *Fusarium sp.* The inoculation ratio for the latter two treatments was 5% *Fusarium sp* (w/w).

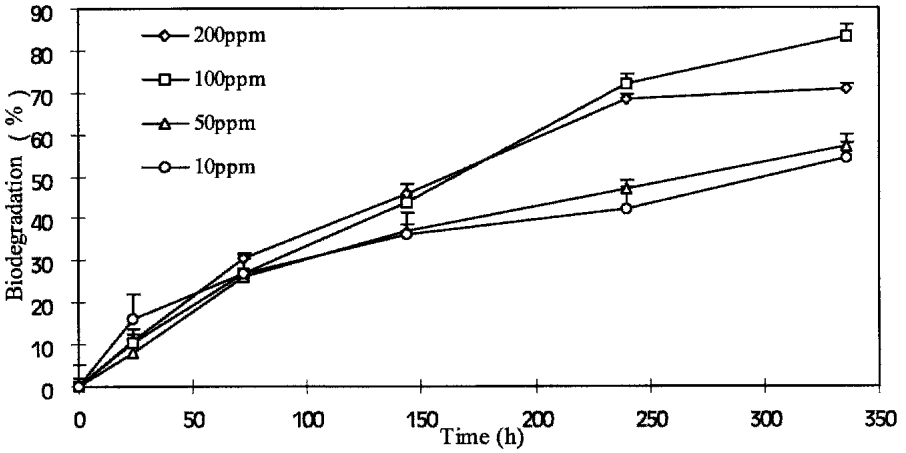



**Figure 5.** Biodegradation of phenanthrene with different initial concentrations in soil samples.

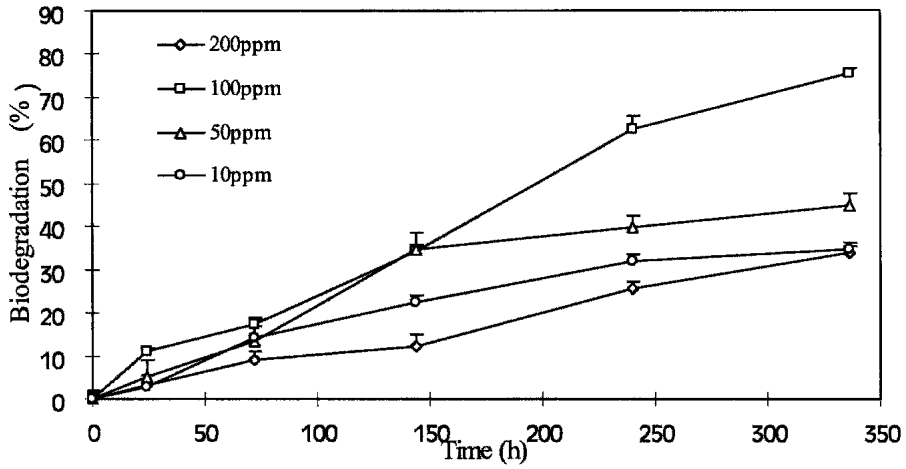



**Figure 6.** Biodegradation rate of pyrene with different initial concentrations in soil samples.

**Table 3.** Percent biodegradation of phenanthrene and pyrene in natural (unsterilized) soils.

| Time h | Phenanthrene | | | Pyrene | | |
|---|---|---|---|---|---|---|
| | S | S+IMF | S+FF | S | S+IMF | S+FF |
| 0 | $0^{A}$ (1.79) | $0^{A}$ (2.03) | $0^{A}$ (0.98) | $0^{a}$ (0.55) | $0^{a}$ (1.77) | $0^{a}$ (1.13) |
| 24 | $3.36^{A}$ (0.44) | $7.27^{A}$ (2.71) | $5.23^{A}$ (1.20) | $1.07^{a}$ (0.42) | $2.21^{a}$ (1.41) | $4.75^{a}$ (0.97) |
| 72 | $5.64^{A}$ (0.86) | $15.06^{A}$ (1.61) | $11.89^{A}$ (1.34) | $3.17^{a}$ (0.91) | $12.87^{b}$ (2.32) | $6.99^{a}$ (1.02) |
| 144 | $8.73^{A}$ (2.61) | $45.89^{B}$ (1.54) | $20.52^{C}$ (1.92) | $7.16^{a}$ (1.67) | $26.54^{b}$ (1.76) | $11.95^{a}$ (1.89) |
| 240 | $15.47^{A}$ (1.90) | $67.30^{B}$ (2.63) | $24.52^{C}$ (2.27) | $12.10^{a}$ (2.65) | $42.29^{b}$ (2.53) | $16.10^{a}$ (1.57) |
| 360 | $22.66^{A}$ (4.46) | $77.13^{B}$ (2.66) | $33.80^{C}$ (4.13) | $16.47^{a}$ (2.61) | $52.70^{b}$ (2.03) | $22.72^{c}$ (1.68) |

[The figures in the parentheses indicate standard deviations. S: unsterilized soil S+IMZ: sterilized soil with immobilized *Fusarium*; S+FF: sterilized soil with free *Fusarium sp*].

The initial concentrations of phenanthrene and pyrene were set to 100 mg $kg^{-1}$ (dry wt soil) for all samples. The biodegradation rates of immobilized *Fusarium sp.* in unsterilized "natural" soils showed satisfactory abilities to degrade phenanthrene and pyrene as shown in Table 3. The degradation of PAHs with immobilized fungus was generally greater than samples with either free fungus or control samples with no inoculation ($p < 0.01$). In Table 3, cells with the same superscripted alphabetic letter indicate no pairwise statistical difference. Cells that have any different letters indicate a statistically significant difference, e.g. cell with $^{B}$ is different from cell with $^{A}$, etc. Pairwise differences were conducted using LSDs at p=0.01 following a significant ANOVA.

The percent of biodegraded phenanthrene by immobilized *Fusarium sp.*, free *Fusarium sp.* and the control (without fungus inoculation) was 77.1, 33.8, and 22.7 % at 360 h respectively. For pyrene the biodegraded percentages were 52.7, 33.8 and 16.5% respectively. Durham et al. (1994) reported that immobilization protected the microorganisms from physical and chemical shocks. From this research, immobilization enhances the competitive ability of the introduced microorganisms to biodegrade PAHs in unsterilized soils and they are likely to remain the dominant strain during biodegradation.

*Acknowledgments.* This research was supported by the National Basic Research program of China (973 Program 2004CB418506); the National Developing Project of Research on Advanced Technologies 2004AA649060; the Key Project of Chinese National Natural Science Foundation Grants 20337010; and the Project of Chinese National Natural Science Foundation Grants 20277040 and 20377043 and the Australian Research Council Linkage Grant (LP0455383). Australian Research Council Linkage International Grant (LX0211202).

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