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# Stimulants and donors promote megaplasmid pND6-2 horizontal gene transfer in activated sludge

*Shan Wang*[1], *Shanshan Li*[1,*], *Dan Du*[1], *Olusegun K. Abass*[2], *Muhammad Salman Nasir*[1,3], *Wei Yan*[1]

[1] *Department of Environmental Science and Engineering, Xi'an Key Laboratory of Solid Waste Recycling and Resource Recovery, Xi'an Jiaotong University, Xi'an 710049, China*

[2] *School of Civil and Environmental Engineering, Nanyang Technological University, Singapore 639798, Singapore*

[3] *Department of Structures and Environmental Engineering, University of Agriculture, Faisalabad 38040, Pakistan*

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ABSTRACT

The activated sludge process is characterized by high microbial density and diversity, both of which facilitate antibiotic resistance gene transfer. Many studies have suggested that antibiotic and non-antibiotic drugs at sub-inhibitory concentrations are major inducers of conjugative gene transfer. The self-transmissible plasmid pND6-2 is one of the endogenous plasmids harbored in *Pseudomonas putida* ND6, which can trigger the transfer of another co-occurring naphthalene-degrading plasmid pND6-1. Therefore, to illustrate the potential influence of stimulants on conjugative transfer of pND6-2, we evaluated the effects of four antibiotics (ampicillin, gentamycin, kanamycin, and tetracycline) and naphthalene, on the conjugal transfer efficiency of pND6-2 by filter-mating experiment. Our findings demonstrated that all stimulants within an optimal dose promoted conjugative transfer of pND6-2 from *Pseudomonas putida* GKND6 to *P. putida* KT2440, with tetracycline being the most effective (100 μg/L and 10 μg/L), as it enhanced pND6-2-mediated intra-genera transfer by approximately one hundred-fold. Subsequently, seven AS reactors were constructed with the addition of donors and different stimulants to further elucidate the conjugative behavior of pND6-2 in natural environment. The stimulants positively affected the conjugal process of pND6-2, while donors reshaped the host abundance in the sludge. This was likely because stimulant addition enhanced the expression levels of conjugation transfer-related genes. Furthermore, *Blastocatella* and *Chitinimonas* were identified as the potential receptors of plasmid pND6-2, which was not affected by donor types. These findings demonstrate the positive role of sub-inhibitory stimulant treatment on pND6-2 conjugal transfer and the function of donors in re-shaping the host spectrum of pND6-2.



## Introduction

Antibiotic resistance has become an emerging threat to public health worldwide. The spread of antibiotic resistance genes is greatly enhanced by horizontal gene transfer, par-

* Corresponding author.
*E-mail:* shanshan0320@xjtu.edu.cn (S. Li).

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ticularly plasmid-based conjugative transfer. Therefore, numerous studies have sought to characterize the factors that mediate conjugation in different natural environments (Ikuma et al., 2012; Wang et al., 2014; Jutkina et al., 2018). However, conventional evaluation methods that depend on cultivable microorganisms tend to underestimate the occurrence of conjugative plasmid transfer in nature, as only endpoint measurements are conducted (Li et al., 2020b). Therefore, due to paucity of suitable selection approaches to assess uncultivable transconjugants (Fu et al., 2017), the current knowledge on plasmid-mediated conjugative transfer specifically in activated sludge (AS) communities is still limited. Several studies have investigated the composition and mechanisms of the conjugation system, which is encoded by several genes in conjugative plasmids, and have revealed that the composition and structure of the conjugative system significantly affect the process of conjugative plasmid transfer (Kubori and Nagai, 2016; Mathioudaki et al., 2021). Kishida et al. (2017) characterized the conjugative process of the naphthalene-degrading plasmid NAH7 by analyzing the fundamental composition of its conjugative system and the *oriT* region for transfer initiation. Additionally, a few studies found that the conjugative transfer process partly relies on the characteristics of the plasmid donor and the stimulator in the environment. Although little is known about recipient recognition and the nature of the conjugative pore, some evidence indicates that different donor backgrounds may lead to discrepancies in the conjugative plasmid host range (De Gelder et al., 2005; Shintani et al., 2005). Moreover, sub-minimum inhibitory concentrations (sub-MICs) of antibiotics and antimicrobials may enhance conjugative plasmid transfer (Parra et al., 2019; Wang et al., 2019, 2020a), and the underlying mechanisms of this phenomenon are strongly linked to the SOS response and reactive oxygen species (ROS) response systems. These observations thus highlight the need for a more detailed analysis of the potential effects of plasmid donors on the host range of broad-host-range (BHR) plasmids in natural and microbial communities.

Plasmid pND6-2 is a self-transmissible megaplasmid harbored by the naphthalene-degrading bacterium *Pseudomonas putida* ND6, which enables another endogenous naphthalene-degrading plasmid pND6-1 to be transferred to the recipient (Li et al., 2013; Wang et al., 2020b). Although the pND6-1 transfer rate is extremely low (e.g., previous studies have reported rates as low as $10^{-9}$), conjugation facilitates the dissemination of degradative genes located on the mobilizable plasmid, which accelerates the elimination of naphthalene in culture. Furthermore, the transferability of pND6-2 from *P. putida* ND6 to *P. putida* KT2440 in different aquatic environments has been described (Wang et al., 2020b). Thus, additional studies are needed to further investigate the effect of plasmid donors and promoting factors on the transfer of plasmid pND6-2 in a natural microbial community.

Both cultivable and uncultivable microorganisms can be effectively elucidated via microbial diversity analysis based on a metagenomics approach, thus providing a more comprehensive and reliable overview of plasmids and a powerful means for host range identification (An et al., 2022; Tao et al., 2019). However, the host range of plasmid pND6-2 and its possible influencing factors have not been explored. Therefore, this study sought to explore the range of transconjugants that received the plasmid pND6-2 after its introduction into AS microbial community. Additionally, we investigated whether the donor strain delivering the plasmid affected the plasmid host range. The impact of different doses of antibiotics and naphthalene on the transfer of pND6-2 miscellaneous recipients was also evaluated.

## 1. Materials and methods

### 1.1. Activated sludge sampling and analysis of sludge characteristics

AS was sampled in a municipal wastewater treatment plant (Xi'an, China) in August 2019. Three-liter samples from the secondary sedimentation tank were collected into sterile polyethylene containers, which were transferred to the laboratory within 1 hr of sampling and kept at 4°C. Basic physicochemical properties were determined immediately and are listed in **Appendix A Table S1**. The concentration of cultivable kanamycin-resistant bacteria in the AS samples was 1000 CFU/mL. Concentrated sludge was precipitated overnight and centrifuged at 4000 ×*g* for 20 min, after which the pellet was resuspended in sterile water for subsequent experiments to eliminate kanamycin-resistant microbes from the AS samples. The microbial communities in the settled AS were used as recipients in the host-range exploration experiment.

### 1.2. Strains, growth media, antibiotics, and naphthalene

The gentamycin- and kanamycin-resistant *P. putida* strains GKND6 and KT2440GK carrying the pND6-1::Gm and pND6-2::Km plasmids (Wang et al., 2020b) were used as model donors in this study, whereas *P. putida* KT2440 was chosen as the model recipient in the solid filter mating assay. These strain stocks were cultured in LB medium supplemented with antibiotics. The transconjugants were screened using minimal salt medium plates containing 0.8 g/L *p*-coumaric acid with 25 μg/mL kanamycin or 15 μg/mL gentamicin as previously described (Wang et al., 2020b). The antibiotics, naphthalene (Nah), and *p*-coumaric acid were separately purchased from Sangon Biotech (China), Sinopharm (China), and Sigma-Aldrich (China). The ampicillin, gentamicin, and kanamycin stock solutions were prepared in sterilized Milli-Q water, whereas the tetracycline and naphthalene solutions were dissolved in ethanol and methanol, respectively.

### 1.3. Antimicrobial susceptibility testing

The donor *P. putida* GKND6 is resistant to ampicillin, gentamycin, and kanamycin, and the genes that confer resistance to these antibiotics are located in genome, pND6-1 and pND6-2 plasmid, respectively. Furthermore, this strain also possesses a slight natural tolerance to low doses of tetracycline. These four antibiotics were thus selected to explore their possible effects on conjugal transfer. The resistance of *P. putida* GKND6 and *P. putida* KT2440 towards antibiotics was determined via agar dilution susceptibility screening

**Table 1 – Characteristics of activated sludge mixture samples collected in this study.**

| Sample | Stimulant (+/-) | Donor | Activated sludge sterilized (+/-) | Sample collection date (day) |
|---|---|---|---|---|
| SND1 | + | *P. putida* GKND6 | - | 1 |
| SKT1 | + | *P. putida* KT2440GK | - | 1 |
| SAS1 | + | / | - | 1 |
| nSND1 | - | *P. putida* GKND6 | - | 1 |
| nSKT1 | - | *P. putida* KT2440GK | - | 1 |
| nSAS1 | - | / | - | 1 |
| SND7 | + | *P. putida* GKND6 | - | 7 |
| SKT7 | + | *P. putida* KT2440GK | - | 7 |
| SAS7 | + | / | - | 7 |
| nSND7 | - | *P. putida* GKND6 | - | 7 |
| nSKT7 | - | *P. putida* KT2440GK | - | 7 |
| nSAS7 | - | / | - | 7 |
| SNDU1 | + | *P. putida* GKND6 | + | 1 |
| SKTU1 | + | *P. putida* KT2440GK | + | 1 |
| nSNDU1 | - | *P. putida* GKND6 | + | 1 |
| nSKTU1 | - | *P. putida* KT2440GK | + | 1 |
| SNDU7 | + | *P. putida* GKND6 | + | 7 |
| SKTU7 | + | *P. putida* KT2440GK | + | 7 |
| nSNDU7 | - | *P. putida* GKND6 | + | 7 |
| nSKTU7 | - | *P. putida* KT2440GK | + | 7 |

+: containing; -: none; /: none.

(Ferreira et al., 2003). Briefly, Luria-Bertani (LB) agar plates containing serial doses of antibiotics or naphthalene were prepared in advance. After culturing single colonies in LB medium overnight, the cultures were washed twice and then adjusted to approximately $10^5$ CFU/mL with fresh LB medium. Approximately 100 µL of each of the diluted mixtures were separately transferred onto each plate and resistance to each antibiotic was confirmed following the plate count method after incubation at 30°C for 1 day. Each trial was conducted in triplicate.

### 1.4. In vitro *conjugation*

Conjugation assays were conducted as described in a previous study (Wang et al., 2020b). Here, these experiments were conducted in the presence of an antibiotic and naphthalene concentration gradient (**Appendix A Table S2**). The antibiotics and naphthalene in 1.2 mg/L $MgSO_4$ solution were added to a pellet (total volume of 200 µL) that contained $10^8$ CFU/mL of both the donor and recipient strains. This solution was also used in the mating plate with the mixed pellet spotted on a filter in an LB agar plate without antibiotics, which was used as a negative control. All plates were incubated at 30°C for 1 day and all mating systems were evaluated in triplicate (i.e., biological replicates). Afterward, serial ten-fold dilutions of each sample were spread onto transconjugant selection plates containing *p*-coumaric acid, gentamycin, and kanamycin. Intraspecific transfer frequency was determined by calculating the ratio of transconjugants to recipient cells (*T*/*R*, transconjugant/recipient).

### 1.5. *Activated sludge microcosms set up*

Given that minimal stimulant (gentamicin, kanamycin, tetracycline, and naphthalene) doses remarkably enhanced conjugal intraspecies transfer from *P. putida* GKND6 to *P. putida* KT2440, activated sludge microcosms were set up to investigate the potential influence of stimulant mixtures and donor on the conjugal transfer behavior of pND6-2. Briefly, a stimulant combination consisting of gentamicin (5 µg/L) and kanamycin (5 µg/L), tetracycline (10 µg/L), or naphthalene (1 µg/L) was added to the mixtures of donor and indigenous AS microorganisms.

#### 1.5.1. *Experimental procedures*

Approximately 150 mL aliquots of treated AS were added to 500 mL beakers, then separately inoculated with equal numbers of GKND6 and KT2440GK donor bacteria cells (approximately $10^7$ CFU/L). Two flasks containing identical volumes of AS microbes were treated as negative controls. The beakers were stirred at 100 r/min to ensure sufficient aeration and incubated for 7 days at room temperature (26-35°C). The beakers were then monitored and labeled as SND, SKT, SAS, nSND, nSKT, and nSAS depending on whether they were stimulated or non-stimulated (Table 1). Here, "(n)S" meant (not) added stimulants, while ND(KT) meant the mixture of donor bacteria GKND6 (KT2440GK) and AS. And "AS" stood for AS without donor. Given that the precise conjugation characteristics of high-abundance AS recipients remained unknown, further experiments were conducted to confirm conjugation, and sample collection was conducted on days 1 and 7. The samples were labeled SND1, SND7, SKT1, SKT7, SAS1, SAS7, nSND1, nSND7, nSKT1, nSKT7, nSAS1, and nSAS7 (Table 1) and were used for high-throughput sequencing analysis, after which gene expression analysis was conducted. Specifically, *dotG* (i.e., a channel protein-coding gene associated with the conjugative transfer system) and *kan* (i.e., a kanamycin-coding gene) were located on pND6-2, whereas the key naphthalene degradation genes *nahG* and *aacC1* (gentamycin-coding gene) were located on pND6-1. The variations in the transcription

level of the four aforementioned genes were then explored in *P. putida* GKND6 and *P. putida* KT2440GK, and 150 mL of sterile sludge (121°C, 20 min) was used as a negative control. Following the above-described procedures, additional stimulated and unstimulated AS mixture samples were generated and collected at day 1 and 7. The resulting samples were labeled SNDU1, SNDU7, SKTU1, SKTU7, nSNDU1, nSNDU7, nSKTU1, and nSKTU7. Table 1 summarizes the details of the aforementioned samples. The "U" represented sterilized AS and "1(7)" means the collected days. All beakers were transferred to a rotary shaker at 100 r/min at ambient temperature (30 ± 2°C) to mimic the conditions of the sewage plant. Each sample was evaluated in duplicate (i.e., two biological replicates).

#### 1.5.2. *Sample collection*

The kanamycin tolerance of the cultivable microbes in sludge (after sedimentation) was evaluated at a 50 mg/mL kanamycin exposure level without disturbing the growth of the donor bacteria in the previous experiment. An aliquot of 10 mL of each tested sample was collected on days 1 and 7. After transferring the aliquots into sterile, cold-resistant polyethylene tubes, all samples were treated with kanamycin (50 mg/mL) for 3 hr to eliminate most of the kanamycin-sensitive cells. Finally, the samples were stored at −80°C until needed for downstream analyses.

### 1.6. *Total RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*

Total RNA was extracted using the RNeasy® PowerSoil® Total RNA Kit (Qiagen, Germany) according to the manufacturer's instructions. The quality of the obtained RNA samples was assessed via electrophoresis, after which the samples were reverse-transcribed using the FastKing RT Kit (TIANGEN, China). The resulting cDNA was stored at −20°C until required for subsequent analyses. RT-qPCR was conducted to identify changes in the expression of *nahG* (naphthalene-degrading gene), *aacC1* (gentamycin-coding gene), *dotG* (key gene of the conjugative transfer system), and *kan* (kanamycin-coding gene). These experiments were conducted in triplicate in a LightCycler® 96 Instrument (Roche, Germany) using the SuperReal preMix Plus kit (SYBR Green) (TIANGEN, China). The 16S rRNA gene was selected as a house-keeping gene, and all primer pairs involved in this study were summarized in **Appendix A Table S3**. All experiments were conducted in triplicate for each sample.

### 1.7. *High throughput analysis of 16S rRNA and statistical analysis*

Samples were shipped on dry ice to Personal Biotechnology Co., Ltd, (Shanghai, China) for microbial diversity analysis via high-throughput sequencing. Briefly, PCR amplification of the tag-encoded 16S rRNA gene (V3–V4 region) was performed using the 338F (5'-ACT CCT ACG GGA GGC AGC A-3') and 806R (5'-GGA CTA CHV GGG TWT CTA AT-3') universal primer pair. After purification and quantification, the PCR products were sequenced on an Illumina MiSeq 2500 sequencer by Personal Bio (Shanghai, China). Data quality and sequence analyses were carried out using the Quantitative Insights into Microbial Ecology (QIIME, v1.8.0) pipeline (Schloss et al., 2009). Alpha diversity (Shannon and Chao indices) was calculated using QIIME with the flattening method of the whole OTUs abundance matrix. Cluster analysis of relatively high-abundance genera and beta-diversity-based principal component analysis (PCA) were conducted using R software (version 3.6.1) (White et al., 2009). The 16S rRNA OTU data was used for Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt, v.1.0.1) to predict gene functions.

## 2. Results

### 2.1. *Dose-dependent effects of sub-MICs of stimulants significantly promote pND6-2-mediated horizontal transfer*

The sensitivity of *P. putida* GKND6 and *P. putida* KT2440 to ampicillin, gentamycin, kanamycin, and tetracycline was determined via the agar plate dilution method (**Appendix A Table S4**). *P. putida* GKND6 had multidrug resistance against ampicillin, gentamycin, and kanamycin, whereas it exhibited resistance to low doses of tetracycline. The MIC values for kanamycin and gentamycin in *P. putida* GKND6 were higher than 2.2 mg/mL, whereas the MIC values for ampicillin and tetracycline were 1.5 mg/mL and 40 μg/mL, respectively. *P. putida* KT2440 was naturally resistant to low doses of ampicillin but was sensitive to gentamycin, kanamycin, and tetracycline.

To investigate the effects of stimulants on pND6-2 conjugative transfer, the intra-genera transfer from *P. putida* GKND6 to *P. putida* KT2440 was evaluated upon exposure to different stimulant doses from sub-MIC concentrations to high screening concentrations (Fig. 1). Our findings elucidated the impact of these stimulant concentrations on gene transfer frequency, with 100 and 10 μg/L of tetracycline having the strongest effect compared to other stimulants, which resulted in an approximately one hundred-fold increase. As expected, other stimulants exhibited moderate effects when compared to the control, with 31.8-, 2.9-, 5.4-, and 5.5-fold increases for 1 μg/L naphthalene, 100 μg/L ampicillin, 1000 μg/L gentamycin, and 5 μg/L kanamycin, respectively.

Moreover, all analyzed tetracycline concentrations promoted gene transfer; however, growth rate and survival were steadily decreased with increasing antibiotic doses, which was likely due to the adverse effects of high antibiotic concentrations on protein function (Schuurmans et al., 2014; Aslam et al., 2018). Therefore, 5 μg/L gentamycin, which was linked to a 4.3-fold increase in gene transfer, was selected in subsequent experiments, in addition to 10 μg/L tetracycline, 1 μg/L naphthalene, and 5 μg/L kanamycin.

### 2.2. *Response of microbial communities to different stimulants and donors in the activated sludge matrix*

The results of intra-genera transfer indicated that optimal contributor mixtures could potentially facilitate the inter-genera transfer from *P. putida* to AS indigenous bacteria. To confirm this, we analyzed the microbial diversity of the AS in the presence and absence of stimulants. The species accumu-

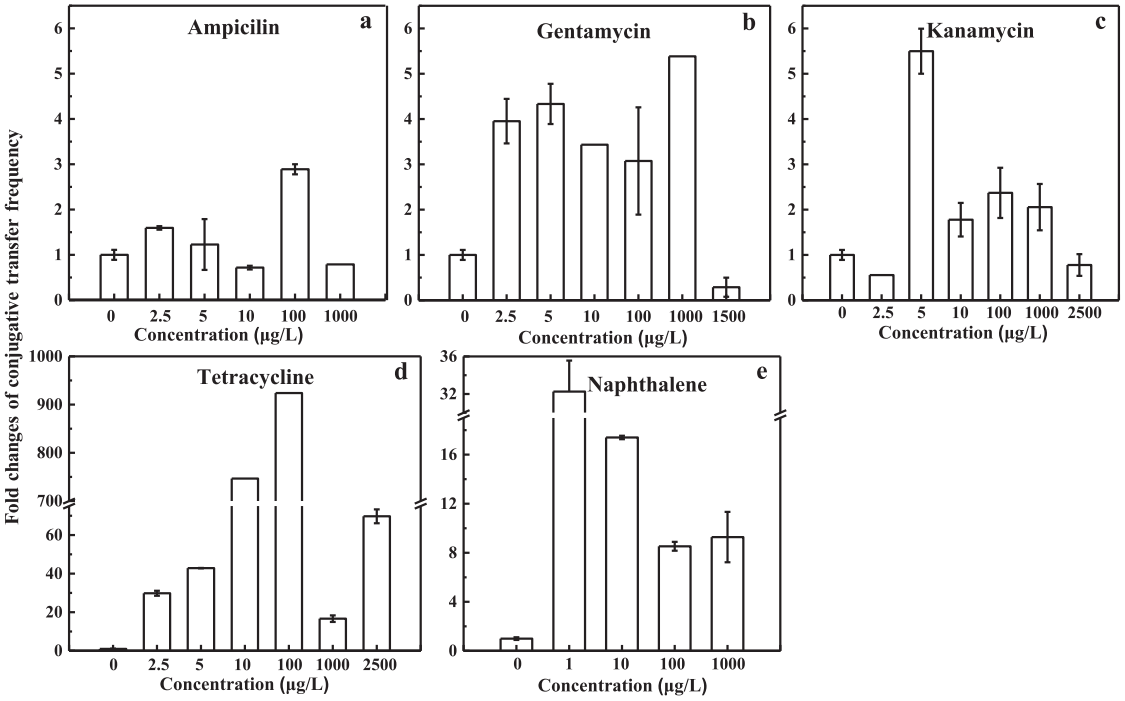


**Fig. 1 – Effects of different stimulants on the conjugative transfer efficiency of pND6-2 between *P. putida* GKND6 and *P. putida* KT2440. (a) Ampicillin; (b) gentamycin; (c) kanamycin; (d) tetracycline; (e) naphthalene. μg/L, parts per million; error bars indicate mean ± standard deviation (SD), *n*=3.**

lation curves of all sequenced sludge mixture samples tended to be saturated (**Appendix A Fig. S1**), indicating that the sequencing depth was sufficient to reflect the microbial diversity in AS (Beagrie et al., 2017) .

*2.2.1. Stimulants induce variations in the diversity of indigenous activated sludge bacterial communities*

Upon comparing the results of the AS samples with the original (*P. putida* GKND6) and secondary (*P. putida* KT2440GK) donor bacteria as shown in Fig. 2a and b, both the SKT and nSKT groups exhibited slight decreases in richness and diversity on the first day; however, these values increased significantly on the seventh day, with the SKT values being consistently higher than those of nSKT. The diversity of SND1, nSND1, SND7 and nSND7 remained largely stable throughout the experiment and a relatively large number of species (Fig. 2b) was identified on the first day. However, we inferred from the results that *P. putida* GKND6, a donor, adapted quickly to the sludge environment and complete the mating process than that of the AS samples inoculated with *P. putida* KT2440GK. Moreover, the proportion of stable transconjugants decreased in the *P. putida* GKND6-containing population. Regardless of which donor bacteria were inoculated in the mating samples, low stimulant dosage romoted conjugation and gene transfer compared with the non-stimulated group, which increased transconjugant richness while decreasing microbial diversity (Ghoul and Mitri, 2016).

As shown in Fig. 2c, the PCA indicated that the dominant modulator of the microbial community structure was the donor (62.94%). Additionally, *P. putida* KT2440GK had a more dominant influence on the community structure of the AS than *P. putida* GKND6. Previous studies have demonstrated that the original plasmid donor and its genetic background affect plasmid transfer (De Gelder et al., 2005). After the donor, culturing time was another important factor that altered bacterial richness (16.30%).

The top 50 most abundant genera were selected to characterize the effect of the interactions between stimuli, contact time, and donor types on the microbiome structure diversity of AS samples (Fig. 3). Interestingly, the microbial community structure was divided into two distinct groups over time, with the abundance of Group 1 containing 30 phylotypes from *Haliangium* to *Lautropia*, being higher on day 1, whereas the richness of Group 2, with another 20 phylotypes from *SM1A02* to *Roseomonas*, markedly increased on day 7. Furthermore, *Pseudomonas* accounted for less than one-thousandth of the bacterial abundance in raw sludge; however, the variations in the number of donors indicated that the abundances of *P. putida* KT2440GK and GKND6 were respectively reduced to 2% and less than 1% on day 7 of cultivation. This indicates that the adaptive capacity of *P. putida* GKND6 in AS was lower than that of *P. putida* KT2400GK on day 7, although the abundances of both strains were substantially decreased over time. In contrast, the abundance of *Rhodoplanes* and *Tetrasphaera* increased in the presence of stimulants on day 1, whereas the *Haliangium*, and *Kouleothrix* genera in all samples were strikingly enriched at day 7 when donors were added. Association networks were constructed for these dominant genera with rho $> 0.6$ and $p$-value $< 0.01$.

To adequately characterize the changes in AS microbial community structure after stimulant exposure, 20 groups at

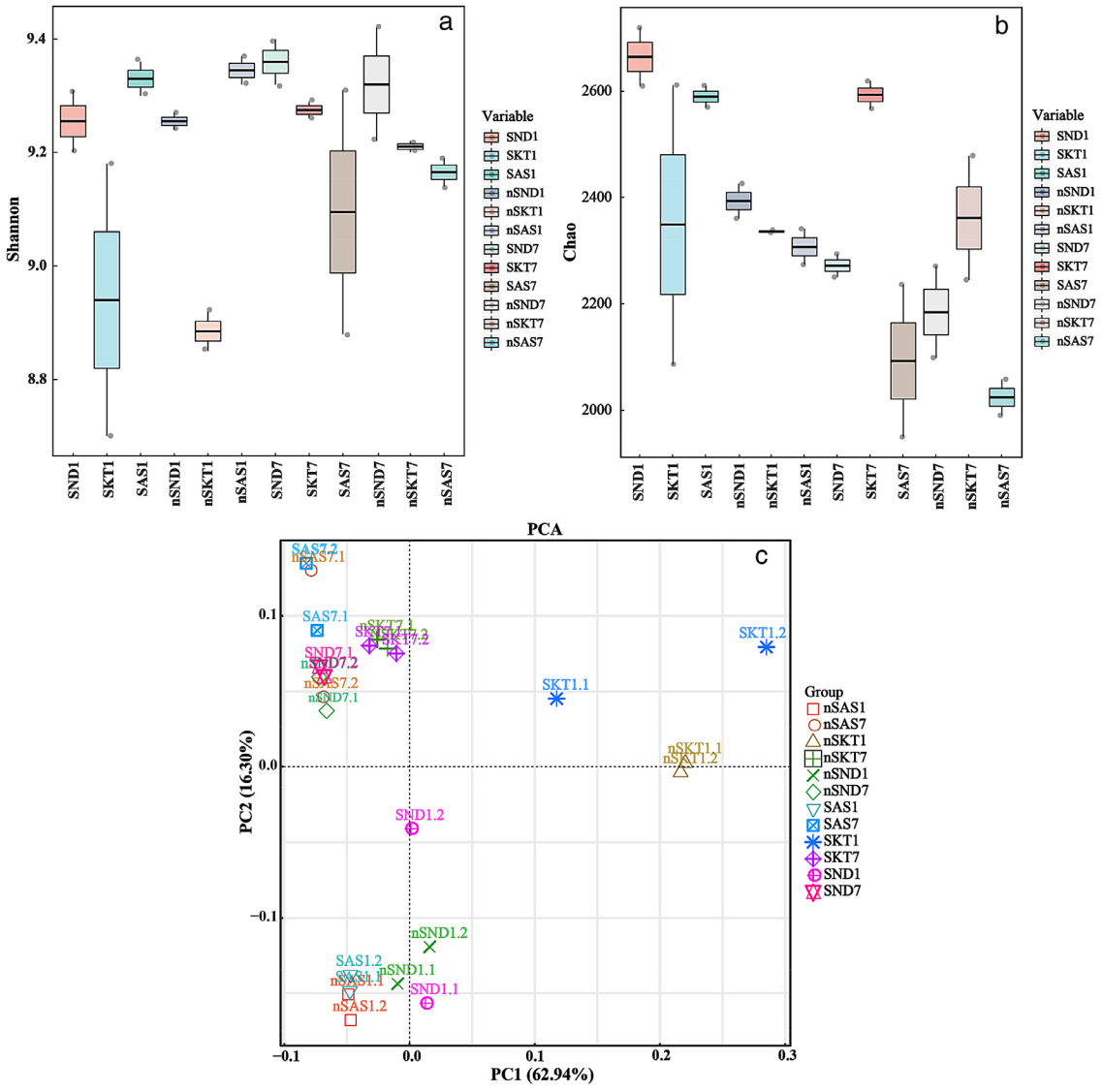


**Fig. 2 – Microbial community profiling of all AS (activated sludge)-related samples. Microbial alpha diversity analysis: (a) Shannon index; (b) Chao index; (c) PCA (principal component analysis) of community structure.**

the genus level with a remarkable difference between the groups were selected (Fig. 4). Consistent with the results in Fig. 3, the abundance of several phylotypes decreased following the incubation time, including genus *Brachumonas*, *Clostridium*, *Ferribacterium* and *Hydrogenophaga*. In contrast, the population of *Blastocatella*, *Chitinimonas*, *Klebsiella*, *Azovibrio* and HdN1 group increased on day 7 by 1-20 fold when compared with the corresponding group on day 1. Among them, the abundance of *Blastocatella* increased with the adding of *P. putida* GKND6 in SND7 than that in SAS7, similar shift was observed in nSND7 than that in nSAS7. Besides adding GKND6, the addition of KT2440GK also leaded to the increasing abundance of *Chitinimonas* genus. These above results indicated that *Blastocatella* and *Chitinimonas* genus may be the potential recipients of plasmid pND6-2 in AS. Furthermore, the addition of stimulants caused a shift in the abundance of several genus. The abundance of *Blastocatella* and *Citobacter* increased significantly with the addition of stimulants in SAS7 than that in nSAS7, whereas The abundance of *Crenothrix*, *Hydrogenophaga* and HdN1 group decreased.

### 2.2.2. *Relative abundance of microbial functional groups in response to inter-genera conjugation with activated sludge communities*

The top 50 dominant functional groups were selected by cluster analysis (**Appendix A Fig. S2**) and the functional annotation information corresponding to the KEGG object identifier is described in **Appendix A Table S5**. A total of 12 classified groups were divided into two groups based on prominent abundance differences. Functional group shifts in the case of cell-to-cell contact were consistent with the results of the differences in community abundances. The functional gene levels of all treated samples were linked to the participation of *P. putida* GKND6, and the abundance values exhibited the

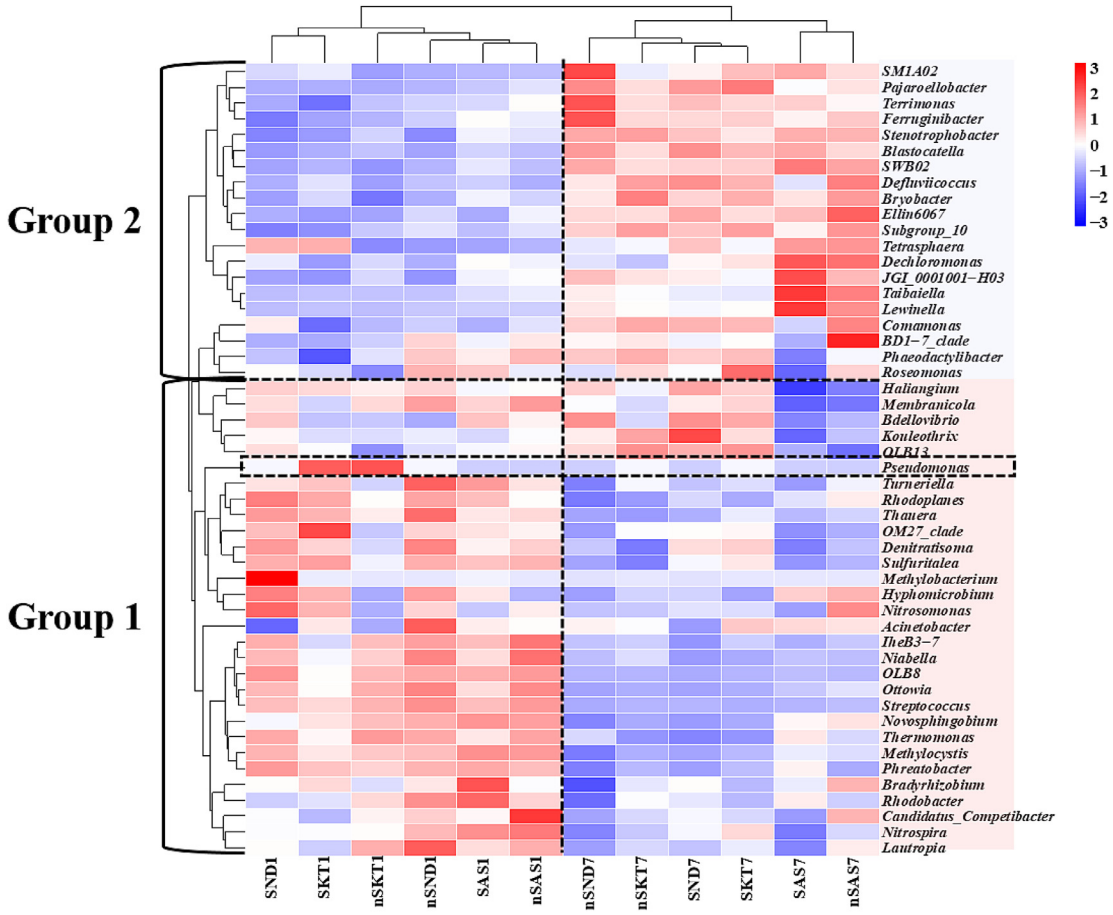


**Fig. 3 – The heat map of tope 50 genera microbial consortia shift analysis of AS samples under different treatments. High abundances are indicated in red and low abundances are indicated in dark blue.**

following order: SND1 > nSND1 > nSAS1 or SND7 > nSND7 > nSAS7. In the sludge samples where *P. putida* KT2440GK was added, the overall abundance values were ranked as SKT7 > nSKT7 > SAS1 > SKT1 > nSKT1 > nSAS1 > SAS7 > nSAS7. With the donor as the variable, the abundance values of *P. putida* GKND6 as the donor is much higher than that of *P. putida* KT2440 as the donor on the first day, and vice versa for day 7. Therefore, the following conclusions were drawn from the above-described results: (1) the stimulant was the principal driving force that increased the abundance of functional genes in all tested samples; (2) the addition of different bacteria harboring pND6-2 as the donors showed different levels of increases in conjugation; (3) microbial abundance was positively correlated with the microbial gene levels.

Next, the potential functions of all genes were predicted and classified using PICRUSt coupled with a biological metabolic pathway analysis database. The abundance matrix classification of the predicted functional groups is presented in **Appendix A Fig. S3**. The most abundant genes belong to the functions of amino acid metabolism, membrane transport, carbohydrate metabolism, replication and repair, and energy metabolism, the abundance distribution of these functional groups accounting for 10.6%, 10.3%, 10.1%, 7.3%, and 6.1%, respectively.

### 2.3. *Sub-MICs of stimulants and donors affect the expression of key conjugative genes in the activated sludge matrix*

Sub-MICs of stimulants can affect bacterial susceptibility by regulating key metabolic functions (Shun-Mei et al., 2018). In this study, plasmid pND6-1 and pND6-2 were labeled with a gentamycin resistance gene (*aac*C1) and a kanamycin resistance gene (*kan*), respectively. Previous studies (Kim et al., 2014; Zhang et al., 2017) have reported that sub-MICs of antibiotics strongly stimulate the spread of antibiotic-resistance genes in microbes. Therefore, the transcript levels of *aac*C1 and *kan* were quantified before and after the addition of stimulant mixtures. Furthermore, the essential salicylate hydroxylase gene *nahG* was chosen to analyze the possible impact on naphthalene degradation ability by HGT. To confirm the conjugation transfer pattern in AS protist, the expression of the essential conjugation related genes *dotG* which was the core component of the type IVB secretion system were analyzed simultaneously as shown in Fig. 5.

The four tested gene (*aacC1, kan, nahG* and *dotG*) transcriptional levels of the sludge sample control groups (nSAS1) were 5-fold higher than those in SAS1 after antibiotic treatment. These increases in gene expression were also observed on day 7, although overall transcription levels decreased dramat-

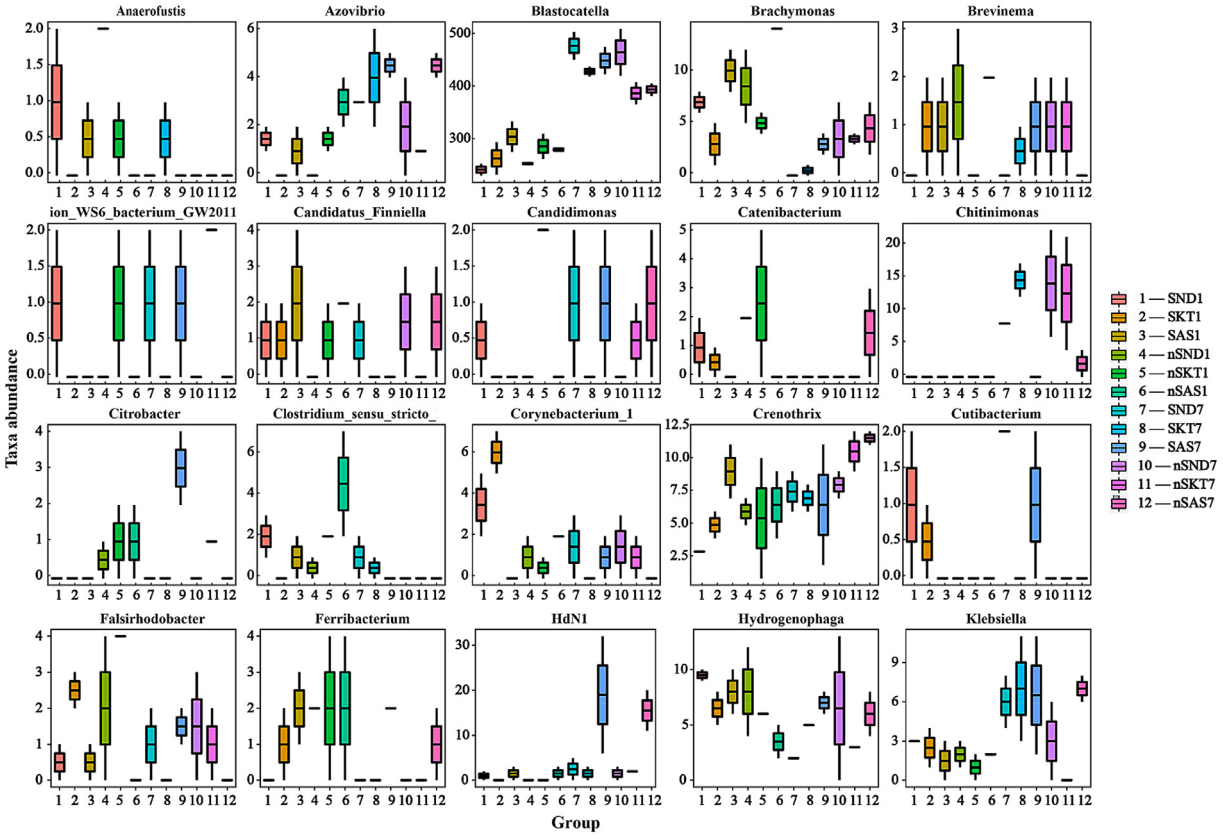


**Fig. 4 – Top 20 maximum differences in OTUs at the genus level. Each color indicates a different AS sample group.**

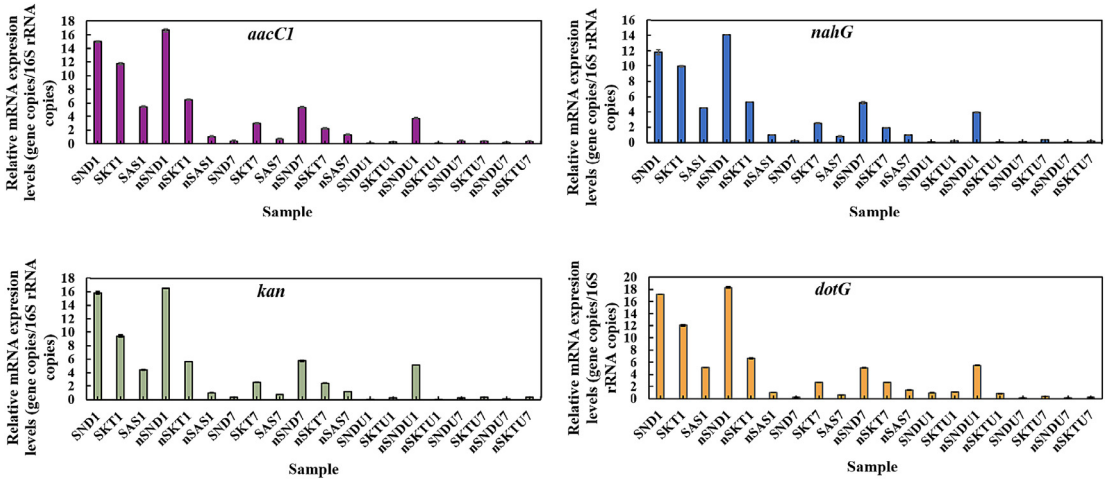


**Fig. 5 – Expression of key genes located on the pND6-1 and pND6-2 plasmids upon treatment with minimal-dose stimulant mixtures.**

ically. The transcription levels of genes *aacC1*, *nahG*, and *kan* in the SAS7 and nSAS7 samples markedly decreased to one-tenth. Particularly, the mRNA levels of *dotG* were the same as those in nSAS1 but were lower than in nSAS7.

To further investigate the plasmid transfer characteristics of the two donors in AS, the plasmid transfer patterns of donor bacteria were investigated in sterilized sludge. The expression of all evaluated genes in all donor-containing samples tended to increase except for *dotG*, which decreased at day 7 compared to day 1. The stability of plasmids pND6-1 and pND6-2 in *P. putida* KT2440 was weaker than that of wild-type host bacteria (Wang et al., 2020b), which was likely due to a loss of plasmids. Upon comparing the sludge samples with equal amounts of *P. putida* GKND6 and *P. putida* KT2440GK inoculation, the mRNA levels of the samples with *P. putida* GKND6 were one-tenth of those in the *P. putida* KT2440GK samples

on day 1. This decrease in mRNA levels was further accentuated on day 7. The total amount of *P. putida* GKND6 cells varied throughout the experiments but bacterial activity decreased steadily with contact time. These results indicated that *P. putida* KT2440, a model strain of *P. putida*, can better adapt to complex bacterial communities (Molina et al., 2000).

For the mating reactions, AS samples with *P. putida* GKND6 showed a severe decrease in abundance, suggesting the presence of a high proportion of transconjugants involved in gene transcription. In contrast, the expression levels in nSKT1 did not increase after adding *P. putida* KT2440GK to the sludge, whereas the expression in SKT1 increased steadily with a weaker stimulation than that in the SND1 group. The overall gene expression of nSNDU7, nSAS7, SNDU7 with SAS7 exhibited a disparate decline. Specifically, the expression levels of the antibiotic-treated group decreased to one-sixth and the non-treated group was a few tenths of the original level. Moreover, the plasmid receptors involved in the nSKT7 and SKT7 samples contribute to the gene expression level enhancement and the former highly surpassed the latter. It was presumably speculated that the type and the number of strains in the sludge decreased after the addition of stimulants, resulting in a decline in the richness of recipients. This indicated that the donors played a major role in the conjugation process (Alderliesten et al., 2020).

## 3. Discussion

### 3.1. *Sub-MICs of stimulants promote the conjugative transfer of pND6-2 within genera*

Several studies have reported that the conjugative frequencies of bacteria are increased by sub-MICs of antibiotics (Andersson and Hughes, 2014), other antimicrobial agents (Zhang et al., 2017), and heavy metals (Guo and Tian, 2019; Parra et al., 2019). This phenomenon could be attributed to increased cellular energy, excess ROS production, and enhancement of the SOS response and cell permeability (Wang et al., 2019; Li et al., 2020a).

Nevertheless, most related studies have focused on the effects of specific drugs on the conjugative transfer efficiency of antibiotic resistance genes under laboratory conditions (Shun-Mei et al., 2018; Parra et al., 2019; Wang et al., 2019). Thus, our study sought to investigate the effects of different doses of ampicillin, gentamicin, kanamycin, tetracycline, and naphthalene on the conjugative transfer capacity of pND6-2 within species. *P. putida* GKND6 has ampicillin, gentamicin, kanamycin resistance, and naphthalene degradation ability. Therefore, we speculated that these three antibiotics and naphthalene might promote the pND6-2 transfer efficiency by influencing the conjugation process. In addition, tetracycline has shown a unique function in various types of studies, such as enhancement on hydrogen production performance (Zheng et al., 2019) and enhancing antibacterial potential of silver conjugated tetracycline (Hameed and Hussein, 2021). However, the introduction of tetracycline below the minimum inhibitory concentration did not show a negative effect on the growth of host strain *P. putida* GKND6. Our results confirmed that the appropriate doses of ampicillin, gentamycin, kanamycin, tetracycline, and naphthalene could increase the conjugative transfer efficiency of pND6-2 within species (Fig. 1). In previous studies, 0.3 ppm gentamycin resulted in a 5-fold increase in the conjugation rate of the gentamycin-resistance pUCP24T plasmid from *E. coli* SM10$\lambda\pi$ to *P. aeruginosa* PAO1 (Lu et al., 2017). Further, 1/2 MIC ampicillin increased the transfer of ESBL plasmids in *E. coli* from 1.0- to 11.3-fold (Liu et al., 2019), and previous studies had not reported similar effects of antibiotics at the μg/L level. Here, the addition of sub-inhibitory concentrations of five different drugs (100 μg/L tetracycline, 1 μg/L naphthalene, 100 μg/L ampicillin, 1000 μg/L gentamycin, and 5 μg/L kanamycin) greatly enhanced pND6-2 conjugative transfer, with tetracycline and naphthalene being the largest contributors to within-genera plasmid pND6-2 spread.

### 3.2. *Stimulants and donor types affect the conjugative transfer of pND6-2 in the AS matrix*

Next, we investigated the effect of different donors and stimulants on the transfer of pND6-2 in AS, as the complex bacterial community of AS could closely mimic natural environmental conditions. The diversity of microbial community members obtained from all samples remains consistent, meaning that the host types of plasmids are conservative and cannot be affected by stimulants and donor bacteria. However, the richness was improved by the addition of stimulants. This could be attributed to (1) the existence of transconjugants threatening the survival of the AS bacterial flora by competition (Ghoul and Mitri, 2016); (2) increases in horizontal gene transfer by producing reactive oxygen species (ROS) due to chemical stimulation (Ren et al., 2021). Multiple levels of antibiotics potentially induced structural changes in bacterial communities by affecting bacterial physiology (Martinez, 2017). Normally, population richness is positively correlated with low abiotic stress (Bowker et al., 2010), and collateral sensitivity is easily enhanced by antibiotic-antibiotic co-susceptibility (Baym et al., 2016; (3) plasmid persistence in transconjugants may be strengthened by kanamycin after additional stimulation of mating behavior. Spearman's correlation analyses indicated that *Pseudomonas* had a significant negative correlation with *Taibaiella* and *Lewinella*, whereas other dominant bacterial groups had a symbiotic relationship with *Taibaiella* and *Lewinella* on day 7, which explained the sharp reduction of donor bacteria by interspecific competition (**Appendix A Fig. S4**).

Although the bacterial information obtained by metagenomic analysis was limited, the potential host can be inferred by the variation of genus abundance between different treatments. The significantly increasing abundance of *Blastocatella* and *Chitinimonas* genus in the groups with *P. putida* GKND6 or *P. putida* KT2440GK on day 7 indicated that they are the potential recipients of pND6-2 in AS. Genus *Blastocatella*, as the potential receptor of pND6-2 from donor GKND6, was detected abundantly in diverse natural environments (Chalupa et al., 2019; Abena et al., 2020; Li et al., 2021). Nevertheless, genus *Chitinimonas* was regarded as the recipients of pND6-2 with both GKND6 and KT2440GK as the donor. Our results revealed the influence of donors on the recipients range even though the used donors belonged to the same species *P. putida*. Sim-

ilar results have been reported by Heß et al. (2021), they investigated the changes in the community composition as well as the uptake of the plasmid RP4 by the particular community members with three different *E. coli* strains as donors. The results revealed that the spread of the plasmid RP4 in a synthetic bacterial community is dependent on the particular donor strain. Phylogenetic relationships between donor and recipient strains were considered to influence conjugative transfer efficiency (Garbisu et al., 2017; Ren et al., 2018). A phylogenetic analysis of microbial genomes using a network approaches suggested that horizontal gene transfer occurs frequently between phylogenetically related species. This may be attributed to the interactions between the plasmid and the recipient chromosome during the transcriptional regulation of transfer genes (Rehman et al., 2019; Li and Christie, 2020). The plasmid stability and genotype might be affected by the chromosome modification system of the host, such as methylation, restriction endonuclease system, and clustered regularly interspaced short palindromic repeat (CRISPR)-Cas system (Seronick et al., 2022). The phylogenetically related species may share similar modification system, which could diminish the degradation of heterogenous DNA leading to an increase in conjugative transfer efficiency. Considering that only the relatively abundant bacterial communities in AS could be detected, high-throughput 16S rRNA gene sequencing analysis may underestimate the potential conjugative recipients of pND6-2 in AS.

Compared to *P. putida* GKND6, the secondary donor *P. putida* KT2440GK enhanced the community diversity of conjugants, which may be due to its better adaptability, as demonstrated in our experiments. Previous studies have demonstrated that the original plasmid donor and its inherited characteristics show a crucial effect on the diversity of transconjugants. The ratio of transconjugant species number to the number of transconjugants was different in donor *S. meliloti* (0.10), *P. putida* (0.07), and *R. eutropha* (0.05) (De Gelder et al., 2005). Samples treated with mixed stimulants showed an increase in bacterial diversity compared to untreated samples. Further, the stimulant was the principal driving force that increased the abundance of the functional genes in all tested samples. Furthermore, the promotion of low concentration naphthalene on conjugation plays an important role in bioaugmentation as a selective pressure. In addition to conjugation systems, the conjugation process may be related to other functional systems in bacteria, including membrane transport, replication and repair, and energy metabolism, suggesting that the conjugation process may be accompanied by an upregulation of the aforementioned genes. Plasmids produce fitness cost in hosts because of the costs of replication, repair, and expression of plasmid genes (Millan and MacLean, 2017). Moreover, the transcription level of *dotG* was higher than those of the *nahG, aacC1*, and *kan* genes, which suggested that a high proportion of conjugation system-related genes existed in the indigenous microorganisms, and therefore a certain proportion of cells with mobile genetic elements should be present in the AS microbial flora. These results also suggest that horizontal gene transfer is widespread in AS, and further evidence indicated that antibiotic-resistance genes coding for multidrug resistance, particularly tetracycline resistance, were highly abundant in the AS, as suggested in previous studies (Zhang et al., 2011; Che et al., 2019). The transcript levels in samples containing the GKND6 group decreased sharply after seven days, indicating that *P. putida* KT2440, a model strain of *Pseudomonas putida*, drive a superior ability to better adapt to the complex bacterial community (Molina et al., 2000). Moreover, the stimulants enhanced intraspecific transfer efficiency when *P. putida* GKND6 was the donor, and therefore a similar promotion of inter-genera transfer should occur when an optimal stimulation mixture is supplied to *P. putida* GKND6 (*P. putida* KT2440GK) and the AS mixture.

## 4. Conclusions

Our findings suggested that sub-MICs of ampicillin, gentamycin, kanamycin, tetracycline, and naphthalene significantly enhanced intra- and inter-genera pND6-2 horizontal transfer efficiency. Moreover, the introduction of a plasmid donor played a dominant role in the diversity of the indigenous AS community. Thus, the correlation in the increase observed between the transcription levels of crucial genes coded in pND6-1, pND6-2 and conjugative transfer demonstrated the occurrence of inter-genera conjugation behavior between *P. putida* KT2440 or *P. putida* GKND6 with the complex AS community. Additionally, pND6-1 was a superior donor compared to pND6-2. Further, this study suggested that *Blastocatella* and *Chitinimonas* were the potential recipients of conjugative transfer, and the stimulants significantly increased conjugation in the complex AS fauna. Generally, the strains with a close phylogenetic relationship from the donor bacteria are more likely to be the plasmid receptor (Ren et al., 2018). However, we infer that there are great differences between the plasmid hosts even at the phylum level. Nevertheless, additional studies are required to precisely determine the plasmid recipient, as transconjugants that can replicate and stabilize expression are recommended as donors containing the self-transmissible plasmid. Further exploration is also required to identify the mechanisms by which plasmid donors affect native bacterial communities, as well as to identify accurate host ranges.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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## Appendix A Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jes.2022.03.011.

## REFERENCES


Abena, M.T.B., Chen, G.Q., Chen, Z.Y., Zheng, X.C., Li, S.S., Li, T.T., et al., 2020. Microbial diversity changes and enrichment of potential petroleum hydrocarbon degraders in crude oil-, diesel-, and gasoline-contaminated soil. 3 Biotech 10 (2), 42.

Alderliesten, J.B., Duxbury, S.J.N., Zwart, M.P., de Visser, J.A.G.M., Stegeman, A., Fischer, E.A.J., 2020. Effect of donor-recipient relatedness on the plasmid conjugation frequency: a meta-analysis. BMC Microbiol. 20 (1), 135.

Andersson, D.I., Hughes, D., 2014. Microbiological effects of sublethal levels of antibiotics. Nat. Rev. Microbiol. 12 (7), 465–478.

An, X.L., Abass, O.K., Zhao, C.X., Xu, M.R., Pan, T., Pu, Q., et al., 2022. Nanopore sequencing analysis of integron gene cassettes in sewages and soils. Sci. Total Environ. 817, 152766.

Aslam, B., Wang, W., Arshad, M.I., Khurshid, M., Muzammil, S., Rasool, M.H., et al., 2018. Antibiotic resistance: a rundown of a global crisis. Infect. Drug Resist. 11, 1645–1658.

Baym, M., Stone, L.K., Kishony, R., 2016. Multidrug evolutionary strategies to reverse antibiotic resistance. Science 351 (6268), aad3292.

Beagrie, R.A., Scialdone, A., Schueler, M., Kraemer, D.C., Chotalia, M., Xie, S.Q., et al., 2017. Complex multi-enhancer contacts captured by genome architecture mapping. Nature 543 (7646), 519–524.

Bowker, M.A., Soliveres, S., Maestre, F.T., 2010. Competition increases with abiotic stress and regulates the diversity of biological soil crusts. J. Ecol. 98 (3), 551–560.

Chalupa, J., Poík, O., Halecky, M., Kozliak, E., 2019. Thermophilic waste air treatment of an airborne ethyl acetate/toluene mixture in a bubble column reactor: stability towards temperature changes. J. Hazard. Mater. 384 (7), 120744.

Che, Y., Xia, Y., Liu, L., Li, A.D., Yang, Y., Zhang, T., 2019. Mobile antibiotic resistome in wastewater treatment plants revealed by Nanopore metagenomic sequencing. Microbiome 7 (1), 44.

De Gelder, L., Vandecasteele, F.P., Brown, C.J., Forney, L.J., Top, E.M., 2005. Plasmid donor affects host range of promiscuous IncP-1$\beta$ plasmid pB10 in an activated-sludge microbial community. Appl. Environ. Microbiol. 71 (9), 5309–5317.

Ferreira, R.B.R., Iorio, N.L.P., Malvar, K.L., Nunes, A.P.F., Fonseca, L.S., Bastos, C.C.R., et al., 2003. Coagulase-negative staphylococci: comparison of phenotypic and genotypic oxacillin susceptibility tests and evaluation of the agar screening test by using different concentrations of oxacillin. J. Clin. Microbiol. 41 (8), 3609–3614.

Fu, J.L., Yang, D., Jin, M., Liu, W.L., Zhao, X., Li, C.Y., et al., 2017. Aquatic animals promote antibiotic resistance gene dissemination in water via conjugation: role of different regions within the zebra fish intestinal tract, and impact on fish intestinal microbiota. Mol. Ecol. 26 (19), 5318–5333.

Garbisu, C., Garaiyurrebaso, O., Epelde, L., Grohmann, E., Alkorta, I., 2017. Plasmid-mediated bioaugmentation for the bioremediation of contaminated soils. Front. Microbiol. 8, 1966.

Ghoul, M., Mitri, S., 2016. The ecology and evolution of microbial competition. Trends Microbiol. 24 (10), 833–845.

Guo, M.T., Tian, X.B., 2019. Impacts on antibiotic-resistant bacteria and their horizontal gene transfer by graphene-based $TiO_2$&Ag composite photocatalysts under solar irradiation. J. Hazard. Mater. 380, 120877.

Hameed, A.Z., Hussein, N.N., 2021. Detection of antibiofilm formation by sliver nanoparticles created by tetracycline antibiotic. J. Phys. Conf. Ser. 1879 (2), 022035.

Heß, S., Kneis, D., Virta, M., Hiltumen, T., 2021. The spread of the plasmid RP4 in a synthetic bacterial community is dependent on the particular donor strain. FEMS Microbiol. Ecol. 97 (12), fiab147.

Ikuma, K., Holzem, R.M., Gunsch, C.K., 2012. Impacts of organic carbon availability and recipient bacteria characteristics on the potential for TOL plasmid genetic bioaugmentation in soil slurries. Chemosphere 89 (2), 158–163.

Jutkina, J., Marathe, N.P., Flach, C.F., Larsson, D.G.J., 2018. Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low concentrations. Sci. Total Environ. 616-617, 172–178.

Kim, S., Yun, Z., Ha, U.H., Lee, S., Park, H., Kwon, E.E., et al., 2014. Transfer of antibiotic resistance plasmids in pure and activated sludge cultures in the presence of environmentally representative micro-contaminant concentrations. Sci. Total Environ. 468-469, 813–820.

Kishida, K., Inoue, K., Ohtsubo, Y., Nagata, Y., Tsuda, M., 2017. Host range of the conjugative transfer system of IncP-9 naphthalene-catabolic plasmid NAH7 and characterization of its *oriT* region and relaxase. Appl. Environ. Microbiol. 83 (1), 02319–02356.

Kubori, T., Nagai, H., 2016. The Type IVB secretion system: an enigmatic chimera. Curr. Opin. Microbiol. 29, 22–29.

Li, G.Y., Chen, X.F., Yin, H.L., Wang, W.J., Wong, P.K., An, T.C., 2020a. Natural sphalerite nanoparticles can accelerate horizontal transfer of plasmid-mediated antibiotic-resistance genes. Environ. Int. 136, 105497.

Li, L.G., Dechesne, A., Madsen, J.S., Nesme, J., Sørensen, S.J., Smets, B.F., 2020b. Plasmids persist in a microbial community by providing fitness benefit to multiple phylotypes. ISME J. 14 (5), 1170–1181.

Li, S.S., Zhao, H.B., Li, Y.X., Niu, S.M., Cai, B.L., 2013. Complete nucleotide sequence of plasmid pND6-2 from *Pseudomonas putida* ND6 and characterization of conjugative genes. Gene 512 (1), 148–156.

Li, Y.G., Christie, P.J., 2020. The TraK accessory factor activates substrate transfer through the pKM101 type IV secretion system independently of its role in relaxosome assembly. Mol. Microbiol. 114 (2), 214–229.

Li, Y.J., Fu, H., Zhang, J.Y., Zhang, Z.X., Li, J.K., 2021. Study of pollutant accumulation characteristics and microbial community impact at three bioretention facilities. Environ. Sci. Pollut. Res. 28 (32), 44389–44407.

Liu, G., Bogaj, K., Bortolaia, V., Olsen, J.E., Thomsen, L.E., 2019. Antibiotic-induced, increased conjugative transfer is common to diverse naturally occurring ESBL plasmids in *Escherichia coli*. Front. Microbiol. 10, 2119.

Lu, Y., Zeng, J.M., Wang, L.J., Lan, K., Shunmei, E., Wang, L., et al., 2017. Antibiotics promote *Escherichia coli-Pseudomonas aeruginosa* conjugation through inhibiting quorum sensing. Antimicrob. Agents Chemother. 61 (12), e01214–e01287.

Martínez, J.L., 2017. Effect of antibiotics on bacterial populations: a multi-hierachical selection process. F1000 Res. 6, 51.

Mathioudaki, E., Arvaniti, K., Munke, C., Drakonaki, A., Vranakis, I., Koutantou, M., et al., 2021. Expression, purification and characterization of the IcmG and IcmK proteins of the Type IVB secretion system from *Coxiella burnetii*. Protein Expr. Purif. 186, 105905.

Millan, A.S., MacLean, R.C., 2017. Fitness costs of plasmids: a limit to plasmid transmission. Microbiol. Spectr. 5 (5), 1–12.

Molina, L., Ramos, C., Duque, E., Ronchel, M.C., Garcıá, J.M., Wyke, L., et al., 2000. Survival of *Pseudomonas putida* KT2440 in soil and in the rhizosphere of plants under greenhouse and environmental conditions. Soil Biol. Biochem. 32 (3), 315–321.

Parra, B., Tortella, G.R., Cuozzo, S., Martínez, M., 2019. Negative effect of copper nanoparticles on the conjugation frequency of conjugative catabolic plasmids. Ecotoxicol. Environ. Saf. 169, 662–668.

Rehman, S., Li, Y.G., Schmitt, A., Lassinantti, L., Christie, P.J., Berntsson, R.P.A., 2019. Enterococcal PcfF is a Ribbon-Helix-Helix protein that recruits the relaxase PcfG through binding and bending of the *oriT* sequence. Front. Microbiol. 10, 958.

Ren, C.Y., Wang, Y.Y., Tian, L.L., Chen, M., Sun, J., Li, L., 2018. Genetic bioaugmentation of activated sludge with dioxin-catabolic plasmids harbored by *Rhodococcus* sp. strain p52. Environ. Sci. Technol. 52 (9), 5339–5348.

Ren, C.Y., Wu, E.L., Hartmann, E.M., Zhao, H.P., 2021. Biological mitigation of antibiotic resistance gene dissemination by antioxidant-producing microorganisms in activated sludge systems. Environ. Sci. Technol. 55 (23), 15831–15842.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., et al., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75 (23), 7537–7541.

Schuurmans, J.M., van Hijum, S.A., Piet, J.R., Händel, N., Smelt, J., Brul, S., et al., 2014. Effect of growth rate and selection pressure on rates of transfer of an antibiotic resistance plasmid between *E. coli* strains. Plasmid 72, 1–8.

Seronick, E., Son, J., Michael, C., Fogg, H., Gromley, Z., Gromley, A., 2022. CRISPR/Cas9 genome editing system confirms centriolin's role in cytokinesis. BMC Res. Notes 15 (1), 8.

Shintani, M., Habe, H., Tsuda, M., Omori, T., Yamane, H., Nojiri, H., 2005. Recipient range of IncP-7 conjugative plasmid pCAR2 from *Pseudomonas putida* HS01 is broader than from other *Pseudomonas* strains. Biotechnol. Lett. 27 (23-24), 1847–1853.

Shun-Mei, E., Zeng, J.M., Yuan, H., Lu, Y., Cai, R.X., Chen, C., 2018. Sub-inhibitory concentrations of fluoroquinolones increase conjugation frequency. Microb. Pathog. 114, 57–62.

Tao, K.Y., Zhang, X.Y., Chen, X.P., Liu, X.Y., Hu, X.X., Yuan, X.Y., 2019. Response of soil bacterial community to bioaugmentation with a plant residue-immobilized bacterial consortium for crude oil removal. Chemosphere 222, 831–838.

Wang, Q.Z., Gu, J., Wang, X.J., Ma, J.Y., Hu, T., Peng, H.L., et al., 2020a. Effects of nano-zerovalent iron on antibiotic resistance genes and mobile genetic elements during swine manure composting. Environ. Pollut. 258, 113654.

Wang, S., Li, S.S., Du, D., Wang, D., Yan, W., 2020b. Conjugative transfer of megaplasmids pND6-1 and pND6-2 enhancing naphthalene degradation in aqueous environment: characterization and bioaugmentation prospects. Appl. Microbiol. Biotechnol. 104 (2), 861–871.

Wang, Y.J., Kou, S.M., Jiang, Q.Y., Xu, B., Liu, X.X., Xiao, J., et al., 2014. Factors affecting transfer of degradative plasmids between bacteria in soils. Appl. Soil Ecol. 84, 254–261.

Wang, Y., Lu, J., Mao, L.K., Li, J., Yuan, Z.G., Bond, P.L., et al., 2019. Antiepileptic drug carbamazepine promotes horizontal transfer of plasmid-borne multi-antibiotic resistance genes within and across bacterial genera. ISME J. 13 (2), 509–522.

White, J.R., Nagarajan, N., Pop, M., 2009. Statistical methods for detecting differentially abundant features in clinical metagenomic samples. PLOS Comput. Biol. 5 (4), e1000352.

Zhang, T., Zhang, X.X., Ye, L., 2011. Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge. PLoS One 6 (10), e26041.

Zhang, Y., Gu, A.Z., He, M., Li, D., Chen, J.M., 2017. Subinhibitory concentrations of disinfectants promote the horizontal transfer of multidrug resistance genes within and across genera. Environ. Sci. Technol. 51 (1), 570–580.

Zheng, X.J., Ma, H.Y., Zhang, J., Yan, W., Yang, H.H., 2019. Improvement of photo-fermentative hydrogen production performance by expressing tetracycline resistance genes *tetAR* in *Rhodobacter sphaeroides* HY01. Int. J. Hydrog. Energy 44 (30), 15823–15832.