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Negative effect of copper nanoparticles on the conjugation frequency of conjugative catabolic plasmids

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ABSTRACT

Due to their antimicrobial properties, copper nanoparticles (CuNPs) have been proposed to be used in agriculture for pest control. Pesticides removal is mainly done by microorganisms, whose genes usually are found in conjugative catabolic plasmids (CCP). The aim of this work was to evaluate if CuNPs at subinhibitory concentrations modify the conjugation frequency (CF) of two CCP (pJP4 and pADP1). CuNPs were characterized by scanning electron microscopy with an X-ray detector, dynamic light scattering and X-ray diffraction. Mating assays were done in LB broth supplemented with CuNPs (10, 20, 50 and 100 μg mL^{-1}) or equivalent concentrations of $CuSO_4$. Interestingly, we observed that in LB, Cu^{+2} release from CuNPs is fast as evaluated by atomic absorption spectrophotometry. Donor and recipient strains were able to grow in all copper concentrations assayed, but CF of mating pairs was reduced to 10% in the presence of copper at 20 or 50 μg Cu mL^{-1} compared to control. Thus, our results indicated that both copper forms, CuNPs or $CuSO_4$, negatively affected the transfer of catabolic plasmids by conjugation. Since dissemination of degradative genes by conjugation contribute to degradation of pesticides by microorganisms, this work improves our understanding of the risks of using copper in agriculture soils, which could affect the biodegradative potential of microbial communities.

1. Introduction

Pesticides are intensively used in agriculture to control of pest plants, and due to its chemical composition, most pesticides are persistent in the environment (Jacobsen and Hjelmsø, 2014). Natural attenuation of pesticides is mainly done by microbial communities, since that them are capable to biodegrade and use these compounds as a source of energy, carbon or nitrogen (Imfeld and Vuilleumier, 2012). Genes that encode the degradation of pesticide are often located in conjugative catabolic plasmids (CCP), which are mobilized and disseminated among the microbial community (Ochman et al., 2000). This process amplifies the reservoir of degradative genes, transforms receptor strains into new donor strains and increase the degradative potential of microbial communities (Wozniak and Waldor, 2010). Stimulants and inhibitors of conjugal transfer are poorly understood, however, it has been reported that some factors could affect the conjugation frequency (CF), such as temperature, soil composition and the organic matter content (Wang et al., 2014, 2018; Garbisu et al., 2017).

Have been demonstrated that several metal nanoparticles (MNps) present biocidal properties against diverse microorganisms (Kim et al., 2007; Hajipour et al., 2012). In comparison with their bulk structures, nanoparticles show high surface area to volume ratio, which causes significant changes on their physico-chemical properties (Nel et al., 2006). Copper nanoparticles (CuNPs) have been proposed as a tool to be used in the agriculture to control plant pathogens and reduce the amount of applied pesticides (Sekhon, 2014). A decreasing in natural pesticide attenuation has been reported when CuNPs were added to soil in combination with atrazine (Parada et al., 2019). However, there is still insufficient knowledge about the behavior and potential impacts of those materials on the microorganisms and environment health (Klaine et al., 2008; Rizwan et al., 2017).

Depending on the physical, chemical and biological environmental properties, MNps can undergo transformations such as disintegration, aggregation or sedimentation (Wang et al., 2016). In addition, its effects on bacterial cells depend on its composition, shape and size (Pal et al., 2007; Lemire et al., 2013). Mainly, their antibacterial activity is due to the liberation of ions, production of reactive oxygen species (ROS), stress by direct contact with cell surfaces, alteration of proteins,

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lipids and nucleic acids (Nowack and Bucheli, 2007; Rai et al., 2009).

Recently have been demonstrated that conjugation could be modulate by ionic metal at subinhibitory concentrations (Klümper et al., 2017). However, little information is provided on the possible effects that an emerging contaminant such as MNps could cause on the microbial process such as conjugation. Therefore, the aim of this work was to evaluate if subinhibitory concentrations of CuNPs affect the CF of two CCP, pJP4 and pADP1. This will provide useful information for the usage of nanomaterials on ecosystem and potential effects of them on environmental health.

2. Materials and methods

2.1. Pesticides

Analytical standard of 2,4-dichlorophenoxyacetic acid (2,4-D) (99% purity) was purchased from Sigma Aldrish (St. Louis, MO, USA) and the analytical standard of atrazine (ATZ) (99% purity) was purchased from Chem Service (West Chester, PA, USA). The stock solution of 2,4-D was prepared at 1 mg mL^{-1} in NaOH 0,1 N and the stock solution of ATZ was prepared at 1 mg mL^{-1} in methanol HPLC grade.

2.2. Copper nanoparticles

CuNPs (Zero valent copper nanopowder 40–60 nm nominal size, ≥99.5% trace metals basis) were purchased from Sigma Aldrich. CuNPs were suspended in Milli-Q water (5 mg mL^{-1}) and ultrasonicated in order to prevent agglomeration (20 °C, 250 W, 40 kHz) and subsequently stored in darkness at 4 °C. For all assays copper sulfate ($CuSO_4$) was used as a bulk form of copper for comparative purposes.

2.3. Bacterial strains

The bacterial strains evaluated in this study and their properties are shown in Table 1. *Pseudomonas sp*. strain ADP (DSMZ 11735) was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). This strain was used as donor of the plasmid pADP1 that gives the capacity to degrade ATZ (Mandelbaum et al., 1995), and *Cupriavidus pinatubonensis* JMP134, which is carrier of the plasmid pJP4 that provides the capacity to degrade 2,4-D (Don and Pemberton, 1981). In addition, both plasmids are conjugative and belong to the incompatibly group P (IncP) (Martínez et al., 2001).

Mutant strains resistant to rifampicin (Rif) of *Pseudomonas putida* KT2440 (Franklin et al., 1981) and *Pseudomonas sp*. RG8 (Barros et al., 2013), designated as *P. putida* KT2440Δr and *Pseudomonas sp*. RG8Δr, were used as recipient strains. Their selection was carry out in LB agar supplemented with Rif (50 μg mL^{-1}) incubated at 30 °C. Six colonies of each strain were selected and verified by phenotypic properties.

Table 1
Bacterial strains used and some of their properties related to the study.

Bacterial strain	Plasmid	Property	Reference
C. pinatubonensis JMP134	pJP4	*tfd* and *mer*	Don and Pemberton (1981)
Pseudomonas sp. ADP	pADP1	*atz* and *mer*	De Souza et al. (1998)
P. putida KT2440Δr	–	Rif^r	This study
Pseudomonas sp. RG8Δr	–	Rif^r	This study
*P.putida*KT2440ΔrpJP4	pJP4	*tfd* and *mer*	This study
Pseudomonas sp. RG8ΔrpJP4	pJP4	*tfd* and *mer*	This study
*P.putida*KT2440ΔrpADP1	pADP1	*atz* and *mer*	This study
Pseudomonas sp. RG8ΔrpADP1	pADP	*atz* and *mer*	This study

tfd: 2,4-D degradation; *atz*: atrazine degradation; *mer*: mercury resistance; Rif^r: rifampicin resistance.

2.4. Characterization of copper nanoparticles

Characterization of CuNPs was done by scanning electron microscopy with X-ray detector (SEM-EDS) by using a microscope JSM-6380 (Jeol; Tokyo, Japan), transmission electron microscopy (TEM) by using TEM JEOL-JEM 1200 EX II (Jeol Ltd., Tokyo, Japan) at an accelerating voltage of 80 kV, dynamic light scattering (DLS) by using a Zetasizer Nano-ZS90 (Malvern Instruments; Malvern, Worcestershire, UK) and X-ray diffraction (XRD) by using a Bruker D8 advance diffractometer (Cu Kα, 40 kV and 40 mA) (Billerica, Massachusetts, USA). The pattern was recorded in the 2θ region from 20° to 120°.

Releases of copper ions from CuNPs on liquid media were quantified by atomic absorption spectrophotometry (AAS) by using a SensAA (GBC; Melbourne, Australia). For this, LB broth and mineral saline medium (MSM) were supplemented with CuNPs (10 or 100 μg mL^{-1}) and incubated at 30 °C in constant shaking (120 rpm). Aliquots from each sample were obtained at 0.5, 3 and 6 h. Blank controls were prepared without CuNPs. MSM is a copper-free medium containing (mg mL^{-1}): Na_2HPO_4 H_2O 0.715, KH_2PO_4 0.365, $(NH_4)_2SO_4$ 0.5 and $MgSO_4$ $7H_2O$ 0.1 (Aranda et al., 2003), and prepared using Milli-Q water.

2.5. Minimum inhibitory concentrations (MICs) and bacterial growth under subinhibitory concentrations of copper

Both plasmids, pJP4 and pADP1, contain a functional mercury resistance operon (Don and Pemberton, 1981; Martínez et al., 2001); therefore, $HgCl_2$ and Rif were used together to select transconjugants strains on agar. The MICs of donors and receptors strains to $HgCl_2$ and Rif were determined by broth dilution method on LB supplemented with 2, 4, 8, 16, 32, 64, 128 and 256 μg mL^{-1} by triplicate. Bacterial cultures were inoculated at 1×10^5 CFU mL^{-1} and incubated at 30 °C in constant shaking (120 rpm) during 24 h to detect growth by visible turbidity (Wiegand et al., 2008).

The toxicity of CuNPs (and $CuSO_4$ as a control) on bacterial strains was evaluated on LB broth supplemented with 10, 20, 50, 100, 200, 400 and 500 μg Cu mL^{-1} by triplicate. Bacterial cultures were inoculated at 1×10^5 CFU mL^{-1}, and incubated at 30 °C during 24 h to detect growth by visible turbidity.

Viable bacterial count in presence of CuNPs at 0, 10, 20, 50 or 100, 200, 400 or 500 μg mL^{-1} was determined after 24 h of incubation at 30 °C by triplicate. Bacterial strains were inoculated at 1×10^5 CFU mL^{-1}. Volumes of 0.02 mL from diluted cultures were plated onto LB agar by triplicate and incubated 24 h at 30 °C for colony count.

2.6. Mating assays

Mating assays were carry out in liquid media during 6 h at 30 °C by triplicate. Erlenmeyer flasks containing 10 mL of LB broth, 0.5 mL of donor strains and 4.5 mL of recipient strains (cultures from 24 h in LB broth at 30 °C) (Schenzle et al., 1997) where supplemented with CuNPs at sub-inhibitory concentrations (10, 20, 50 or 100 μg mL^{-1}) and $CuSO_4$ as a control. Aliquots of 0.1 mL were disseminated on LB agar supplemented with Rif (50 μg mL^{-1}) and $HgCl_2$ (15 μg mL^{-1}) to quantify the transconjugant strains. Aliquots of 0.1 mL were disseminated on LB agar supplemented with Rif (50 μg mL^{-1}) to quantify the transconjugant and receptor strains together. After 24 h of incubation at 30 °C the colony count was performed, and the CF was determined through the ratio of transconjugants/receptor strains. The results obtained were analyzed and compared by Tukey test ($p \leq 0.05$), using SPSS software (SPSS Inc., trial version) ($n = 3$).

To evaluate the effect of diluted LB (25%, 50% and 75%) or MSM, using *C. pinatubonensis* JMP134 as donor and *P. putida* KT2440Δr as recipient of plasmid pJP4.

2.7. Pesticide biodegradation

Pesticide biodegradation was analyzed in 10 randomly selected transconjugants strains of each type. To evaluate the biodegradation of ATZ (50 μg mL^{-1}), MSM without nitrogen source was used and supplemented with glucose (5.56 mM), whereas the biodegradation of 2,4-D (50 μg mL^{-1}) was analyzed in MSM. In order to achieve this, the assays were performed in triplicate with their respective abiotic controls. The transconjugants strains were cultured in LB broth at 30 °C for 18 h, washed twice in MSM and inoculated at 10^5 UFC mL^{-1}. These cultures were incubated at 30 °C for 48 h under constant agitation (120 rpm) on rotary shaker. Subsequently, 1 mL aliquots of each sample were centrifuged (5000 rpm for 3 min at 4 °C) and the supernatant was filtered through a PTFE membrane (0.2 μm pore size, Millipore) Then, the pesticide concentration was determined on the supernatant by HPLC using a Merck Hitachi L-2130 pump equipped with a Rheodyne 7725 injector with a 20 μL loop and a Merck Hitachi L-2455 diode array detector. Separation was achieved using a C18 column (Chromolit RP-8e, 4.6 × 100 mm). Eluent A was phosphoric acid (0.1%) and eluent B was acetonitrile at 25/75% and 50/50% for ATZ and 2,4-D respectively. The flow rate was set at 1.0 mL min^{-1}, with 0–10 min in an isocratic mode. The column temperature was maintained at 30 °C. The detector was set at 222 and 228 nm for ATZ and 2,4-D respectively, for the data acquisition. Instrument calibrations and quantification were performed against pure pesticide reference standards (0.1–10 mg L^{-1}).

2.8. Presence of degradative genes in transconjugants strains

The presence of plasmids genes in the transconjugants strains was verified through the polymerase chain reaction (PCR). Markers for genes *atz*B (De Souza et al., 1998) and *tfd*B (Neilson et al., 1992) were used. DNA extraction was carried out by Ultra clean microbial DNA Isolation kit (Promega) according to the manufacturer protocols. PCR amplifications were performed using the SapphireAmp® Fast PCR Master Mix kit (Takara), according to the recommendations by the manufacturer. DNA visualization was performed after electrophoresis in 1% agarose gel.

3. Results

3.1. Characterization of copper nanoparticles

SEM-EDS and TEM images showed that CuNPs were spherical with approximate size between 40 and 100 nm (Fig. 1a and Fig S1) and correspond to 87.2% copper and 12.7% oxygen. XRD patterns showed the presence of three characteristic peaks for copper (Fig. 1b) and that were observed at 2θ = 43°, 50°, 74° that correspond to (111), (200), (220) crystallographic planes of face-centred cubic Cu crystals (JCPDS No. 04-0784) (Suresh et al., 2014). Moreover, peaks at 2θ between 35.5° and 38.7° corresponding (002) and (111) showed that Cu° present some oxide nanoparticles residues. The results obtained by DLS showed that the initial average hydrodynamic size of CuNPs was 190,5 nm in LB (Fig. 1c) and 239,4 in MSM (Fig. 1d). However, the hydrodynamic diameter is always greater than the size estimated by TEM (Huang et al., 2007).

Moreover, we observed through AAS that Cu^{+2} release from CuNPs in LB media was greater than in the MSM media as shown in Fig. 1e.

3.2. MICs and bacterial growth in presence of copper

Both donor strains, *C. pinatubonensis* JMP134 and *Pseudomonas sp*. ADP, exhibit a MIC of 32 μg mL^{-1} for $HgCl_2$ and 2 μg mL^{-1} for Rif. Meanwhile, both receptor strains, *P. putida* KT2440Δr and *Pseudomonas sp*. RG8Δr, exhibit a MIC of 4 μg mL^{-1} for $HgCl_2$ and 128 μg mL^{-1} for Rif (Table 2).

In the case of copper, C. pinatubonensis JMP134 presented a MIC of 200 μg Cu mL^{-1} for both copper forms. *Pseudomonas sp*. ADP presented a MIC of 400 μg Cu mL^{-1} for both copper forms. *Pseudomonas putida* KT2440 presented a MIC of 500 μg Cu mL^{-1} for CuNPs and 200 μg Cu mL^{-1} for $CuSO_4$. *Pseudomonas* sp. RG8 presented a MIC of 500 μg Cu mL^{-1} for CuNPs and 200 μg Cu mL^{-1} for $CuSO_4$ (Table 2).

CuNPs at 10, 20, 50 or 100 μg mL^{-1} do not affect bacterial viable count compared to control without CuNPs after 24 h of incubation at 30 °C (Fig. 2). Therefore, these concentrations of CuNPs were used for matting assays at subinhibitory concentrations.

3.3. Mating assays

Each transconjugant strain obtained, KT2440ΔrpJP4, RG8ΔrpJP4, KT2440ΔrpADP1 or RG8ΔrpADP1, represent each mating pair assessed. In presence of both copper forms at 10 μg Cu mL^{-1}, the CFs of mating pairs were similar to control (Table 3). However, as shown in Table 3, CFs was reduced to 10% and 1% at 20 or 50 μg Cu mL^{-1} and 100 μg Cu mL^{-1} respectively, in presence of both copper form evaluated.

The CF of plasmid pJP4 from *C. pinatubonensis* JMP134 to *P. putida* KT244Δr (represented by transconjugant strain KT2440ΔrpJP4 in Table 3) was 8 times lower than the CF of plasmid pJP4 from *C. pinatubonensis* JMP134 to *Pseudomonas* sp. RG8Δr (RG8ΔrpJP4). Whereas the CF of plasmid pADP1 from *Pseudomonas* ADP was similar in both receptor strains, represented by their transconjugants strains KT2440ΔrpADP1 and RG8ΔrpADP1 (Table 3).

The CFs of the plasmid pJP4 from *C. pinatubonensis* JMP134 to *P. putida* KT2440Δ under mating conditions in diluted LB (25%, 50% or 75%) or MSM are shown in Table 4. In LB 50% with both copper forms at 100 μg Cu mL^{-1}, the CFs decreased to 10%. In LB 75% with both copper forms at 50 μg Cu mL^{-1} the CFs decreased to 10%, and with 100 μg Cu mL^{-1} decreased to 1%. Meanwhile, the CFs in MSM and LB 25% in presence of both copper forms at all assessed concentrations, were the same that observed in control without copper.

3.4. Presence of degradative genes and pesticide degradation

The presence of degradative genes (*tfd*B and *atz*B) was confirmed in both donor strains and transconjugants strains; meanwhile, in both receptor strains was not detected (Fig. S2). Both transconjugants strains carrying plasmid pADP1 (KT2440ΔrpADP1 and RG8ΔrpADP1) degraded atrazine. However, in the case of plasmid pJP4, transconjugant strain RG8ΔrpJP4 degraded 2,4-D, but not transconjugant strain KT2440ΔrpJP4 (Table 5).

4. Discussion

CuNPs are toxic for a wide diversity of bacteria (Hajipour et al., 2012). Under the conditions evaluated in this study, both copper forms (CuNPs as well as $CuSO_4$) presented similar minimum inhibitory concentrations against the bacterial strains evaluated. On the contrary, Kaweeteerawat et al. (2015) reported that CuNPs exerts bacterial toxicity higher than ionic analogues. However, were employed *Escherichia coli* and *Lactobacillus brevis* as model microorganisms.

The results obtained demonstrated a reduction in the CF in presence of low subinhibitory concentrations (20 μg Cu mL^{-1}) of both copper forms in LB broth. Moreover, was observed a fast ion release from CuNPs (< 3 h) in LB medium under the conditions evaluated. Therefore, the reduction on plasmid transfer by conjugation would have occurred by the effect of soluble ions release as well as by CuNPs themselves. However, this must be evaluated in details in future studies. Besides, we observed that reduction of CF is greater in a culture medium rich in organic matter (LB) more than a poor medium (MSM). In this sense, Vale et al. (2016) demonstrated that CuNPs undergo a fast dissolution in a media with a higher level of organic matter compared with lower level of organic matter. Meanwhile, Gunawan et al. (2011) suggested that complexation-mediated leaching of copper oxide

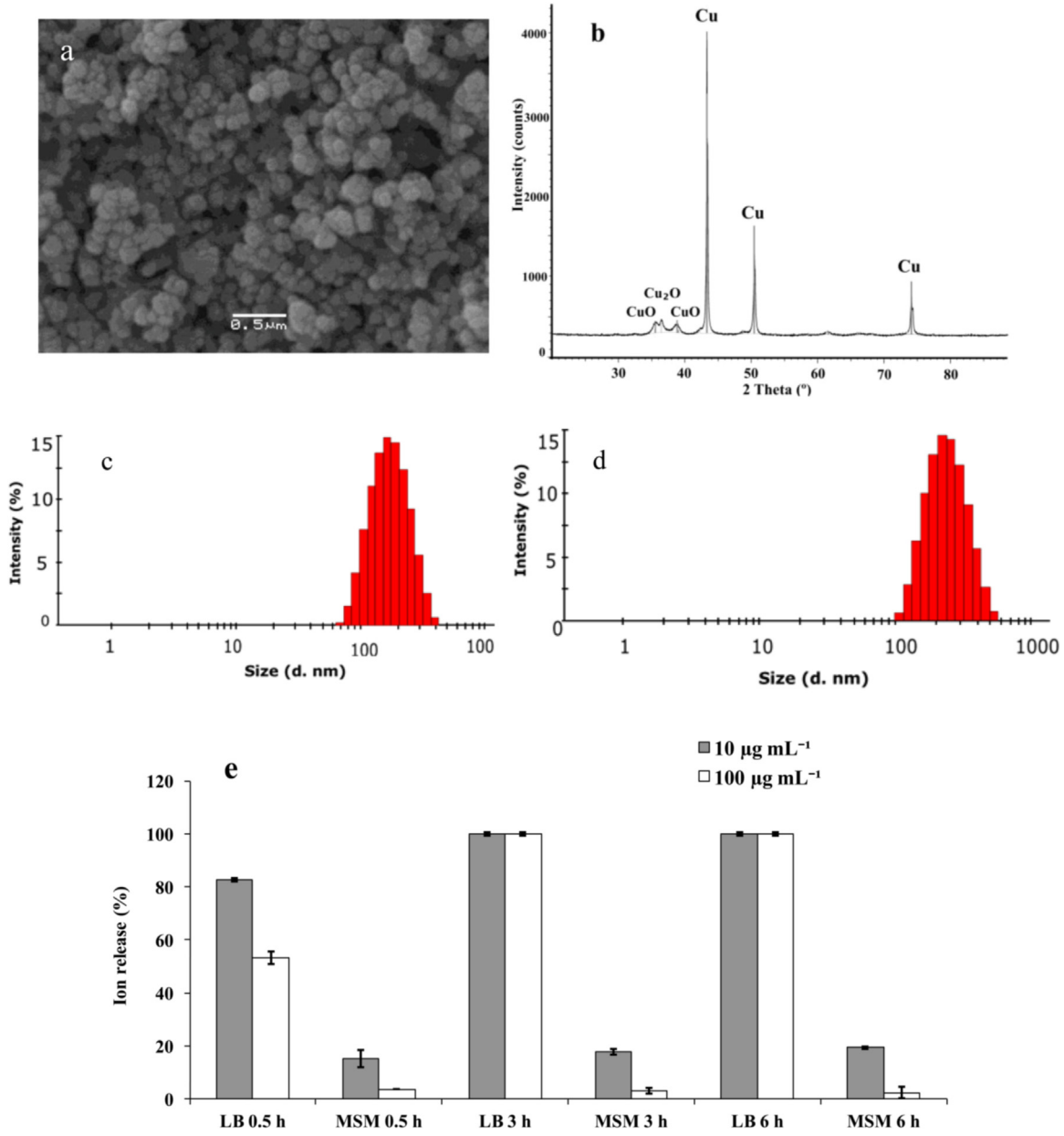

Fig. 1. Characterization of copper nanoparticles used in this study. (a) Scanning electron microscopy of dry copper nanoparticles, (b) XRD pattern of CuNPs, (c and d) Hydrodynamic diameter of CuNPs in LB broth and MSM respectively determined by DLS, (e) Copper ion (Cu^{+2}) release from CuNPs (10 or 100 µg mL^{-1}) into LB or MSM quantified by AAS. The data in panel are shown as means of percent values of three replicate determinations ($n = 3$) ± standard deviation.

Table 2
Minimum inhibitory concentrations (µg mL^{-1}) estimated by broth dilution method.

Bacterial strains	$HgCl_2$	Rifampicin	CuNPs	$CuSO_4$
C. pinatubonensis JMP134 (D)	32	2	200	200
Pseudomonas sp. ADP (D)	32	2	400	400
P. putida KT2440Δr (R)	4	128	500	200
Pseudomonas sp. RG8Δr (R)	4	128	500	200

D: donor strain; R: receptor strain. Values correspond to three replicates.

nanoparticles (CuONPs) by amino acids is their source of toxicity toward E. coli.

Bondarenko et al. (2012) mentioned that CuONPs induces negative effects on *E. coli* already at very low sub-toxic levels (0.1 µg Cu mL^{-1}), and that the dissolution of nanoparticles is the key factor to trigger their toxic effects. For this part, Wang et al. (2016) using the aquatic bacteria *Photobacterium phosphoreum* as model, described that CuONPs disturbed the bacterial cells by a combined effect between ions released and nanoparticles. However, these authors mentioned that the release of ions from CuONPs depends on the equilibrium between dissolution and adsorption, which in turn depends on their concentration. With a low nanoparticle concentration, the dissolution is greater than adsorption capacity, meanwhile with a high concentration this phenomenon is reversed.

The presence of degradative genes in transconjugants strains demonstrated the transfer of DNA by conjugation. However, despite the presence of *tfd*B on *P. putida* KT2440Δr, 2,4D was not degraded by these transconjugant strains. This effect has been described previously (DiGiovanni et al., 1996; Goris et al., 2002), and a possible reason is that transconjugants does not have the enough copies of plasmid required for the degradation, since although this plasmid has two tfd operons, at least 5 copies per cell are required for the metabolism of 2,4-D (Pérez-Pantoja et al., 2008). On the other hand, both CCP present

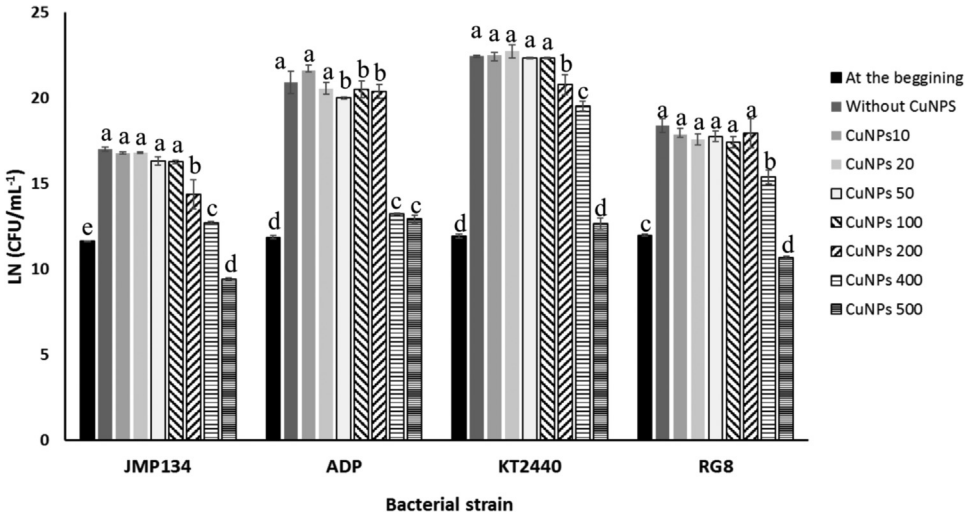

Fig. 2. Viable count in presence of CuNPs after 24 h of incubation at 30 °C in LB broth. CuNPs were added at 10 μg mL^{-1} (CuNPs 10), 20 μg mL^{-1} (CuNPs 20), 50 μg mL^{-1} (CuNPs 50), 100 μg mL^{-1} (CuNPs 100), 200 μg mL^{-1} (CuNPs 200), 400 μg mL^{-1} (CuNPs 400) or 500 μg mL^{-1} (CuNPs 500). Control without CuNPs and viable count at the beginning of experiment are shown. Error bars represent standard deviation of three replicates. Different letters show significant differences among values, determined with $n = 3$ and Tukey test ($p < 0.05$).

Table 3
Conjugative frequencies of the mating pairs represented by each transconjugant strain, KT2440ΔrpJP4, RG8ΔrpJP4, KT2440ΔrpADP1 or RG8ΔrpADP1, in LB broth supplemented with CuNPs or $CuSO_4$ at 10, 20, 50 or 100 μg Cu mL^{-1}.

		KT2440ΔrpJP4	RG8ΔrpJP4	KT2440ΔrpADP1	RG8ΔrpADP1
Without Cu		$1{,}1 \times 10^{-4} \pm -5{,}1a$	$8{,}4 \times 10^{-4} \pm -4{,}3a$	$1{,}0 \times 10^{-4} \pm -5{,}2b$	$1{,}2 \times 10^{-4} \pm -5{,}0a$
$CuSO_4$	10	$1{,}1 \times 10^{-4} \pm -5{,}1a$	$8{,}4 \times 10^{-4} \pm -4{,}4a$	$1{,}0 \times 10^{-4} \pm -5{,}1a$	$1{,}2 \times 10^{-4} \pm -5{,}2a$
	20	$1{,}1 \times 10^{-5} \pm -5{,}8b$	$8{,}4 \times 10^{-5} \pm -5{,}2b$	$1{,}2 \times 10^{-5} \pm -6{,}3b$	$1{,}2 \times 10^{-5} \pm -5{,}8b$
	50	$1{,}1 \times 10^{-5} \pm -5{,}9b$	$8{,}4 \times 10^{-5} \pm -5{,}1b$	$1{,}0 \times 10^{-5} \pm -5{,}7b$	$1{,}2 \times 10^{-5} \pm -5{,}7b$
	100	$5{,}0 \times 10^{-6} \pm -5{,}8b$	$1{,}4 \times 10^{-6} \pm -5{,}9c$	$5{,}1 \times 10^{-6} \pm -5{,}9b$	$4{,}8 \times 10^{-6} \pm -6{,}6b$
CuNPs	10	$1{,}2 \times 10^{-4} \pm -5{,}3a$	$8{,}5 \times 10^{-4} \pm -4{,}6a$	$1{,}1 \times 10^{-4} \pm -5{,}9a$	$1{,}3 \times 10^{-4} \pm -5{,}1a$
	20	$1{,}2 \times 10^{-5} \pm -5{,}7b$	$8{,}3 \times 10^{-5} \pm -5{,}3b$	$1{,}1 \times 10^{-5} \pm -5{,}8b$	$1{,}3 \times 10^{-5} \pm -5{,}3b$
	50	$1{,}1 \times 10^{-5} \pm -5{,}7b$	$8{,}4 \times 10^{-5} \pm -5{,}4b$	$1{,}0 \times 10^{-5} \pm -5{,}6b$	$1{,}2 \times 10^{-5} \pm -5{,}5b$
	100	$5{,}1 \times 10^{-6} \pm -6{,}0b$	$1{,}4 \times 10^{-6} \pm -5{,}9c$	$5{,}1 \times 10^{-6} \pm -5{,}6b$	$4{,}8 \times 10^{-6} \pm -5{,}9b$

Values correspond to the means of three replicates ± standard deviation in logarithmic expression. Different letters show significant differences among values in a column determined with $n = 3$ and Tukey test ($p \leq 0.05$).

insertion sequences that flank the degradative genes (Don and Pemberton, 1985; Martínez et al., 2001), which facilitates the loss of fragments (Wackett et al., 2002; Juhas et al., 2009). In fact, it has been shown that C. pinatubonensis JPM134 can lose fragments of the plasmid pJP4 by successive transfers, being generated strains unable to metabolize 2,4-D (Clément et al., 2001). Meanwhile, both transconjugant strains of *Pseudomonas sp*. RG8Δr (RG8ΔrpJP4 and RG8ΔrpADP1) were able to metabolize 2,4-D and ATZ, respectively. This indicates that *Pseudomonas sp*. RG8Δr acquire and express these genes.

Graves et al. (2015) proposed that bacteria quickly become resistant to ionic and nanosilver (a metal similar to copper), due to the appearance of mutant strains resistant to the metal. Then, is possible speculate that after a certain exposure time of copper, mutants may appear (donor or receptor) able to conjugate under these conditions.

Table 5
Pesticide biodegradation by bacterial strains after 24 h of incubation.

Bacterial strain	2,4-D	ATZ
C. pinatubonensis JMP134 (D)	+	−
Pseudomonas sp. ADP1 (D)	−	+
P. putida KT2440Δr (R)	−	−
Pseudomonas sp. RG8Δr (R)	−	−
*P. putida*KT2440ΔrpJP4 (T)	−	−
*P. putida*KT2440ΔrpADP1 (T)	−	+
Pseudomonas sp. RG8ΔrpJP4 (T)	+	−
Pseudomonas sp. RG8ΔrpADP1 (T)	−	+

D: donor strain, R: receptor strain, T: transconjugant strains, 2,4-D: 2,4- dichlorophenoxyacetic acid, ATZ: atrazine.

Table 4
Conjugative frequencies of plasmid pJP4 from *Cupriavidus pinatubonensis* JMP134 to *Pseudomonas putida* KT2440Δr in diluted LB broth (25%, 50% or 75%) or MSM supplemented with of CuNPs or $CuSO_4$ at 10, 20, 50 or 100 μg Cu mL^{-1}.

		MSM	LB 25%	LB 50%	LB 75%
Without Cu		$1{,}1 \times 10^{-4} \pm -5{,}1a$	$1{,}0 \times 10^{-4} \pm -5{,}8a$	$1{,}0 \times 10^{-4} \pm -5{,}1a$	$1{,}0 \times 10^{-4} \pm -4{,}8a$
$CuSO_4$	10	$1{,}0 \times 10^{-4} \pm -5{,}3a$	$1{,}0 \times 10^{-4} \pm -5{,}4a$	$1{,}2 \times 10^{-4} \pm -4{,}9a$	$1{,}2 \times 10^{-4} \pm -5{,}2a$
	20	$1{,}1 \times 10^{-4} \pm -5{,}1a$	$1{,}0 \times 10^{-4} \pm -5{,}9a$	$1{,}0 \times 10^{-4} \pm -4{,}9a$	$1{,}0 \times 10^{-4} \pm -5{,}4a$
	50	$1{,}1 \times 10^{-4} \pm -5{,}2a$	$1{,}1 \times 10^{-4} \pm -5{,}2a$	$1{,}1 \times 10^{-4} \pm -4{,}8a$	$1{,}0 \times 10^{-5} \pm -5{,}0a$
	100	$1{,}1 \times 10^{-4} \pm -5{,}3a$	$1{,}0 \times 10^{-4} \pm -4{,}8a$	$1{,}0 \times 10^{-5} \pm -5{,}0a$	$5{,}0 \times 10^{-6} \pm -5{,}3b$
CuNPs	10	$1{,}1 \times 10^{-4} \pm -5{,}4a$	$1{,}1 \times 10^{-4} \pm -5{,}3a$	$1{,}1 \times 10^{-4} \pm -5{,}5a$	$1{,}1 \times 10^{-4} \pm -5{,}1a$
	20	$1{,}0 \times 10^{-4} \pm -5{,}1a$	$1{,}0 \times 10^{-4} \pm -5{,}1a$	$1{,}1 \times 10^{-4} \pm -4{,}7a$	$1{,}1 \times 10^{-4} \pm -5{,}1a$
	50	$1{,}1 \times 10^{-4} \pm -5{,}0a$	$1{,}1 \times 10^{-4} \pm -5{,}0a$	$1{,}0 \times 10^{-4} \pm -4{,}8a$	$1{,}0 \times 10^{-5} \pm -4{,}7a$
	100	$1{,}1 \times 10^{-4} \pm -5{,}0a$	$1{,}0 \times 10^{-4} \pm -5{,}3a$	$1{,}1 \times 10^{-5} \pm -5{,}4a$	$5{,}0 \times 10^{-6} \pm -4{,}9b$

Values correspond to the means of three replicates ± standard deviation in logarithmic expression. Different letters show significant differences among values in a column determined with $n = 3$ and Tukey test ($p \leq 0.05$).

It has been described that copper modulate genetic expression of some genes in bacteria (Guo et al., 2017). Moreover, it is known that zinc modified the fertility of donors (Ou and Anderson, 1972) and receptors strains (Ou, 1973) on conjugation process. Therefore, according to our results, we hypothesized that the effect of copper on the CF of plasmids pJP4 and pADP1 could be due to the action of copper ions released from copper nanoparticles at: (a) gene expression level of plasmids (b) effect on proteins of donor or receptor cells involved in conjugation.

5. Conclusions

It has been demonstrated that copper cause harmful effects on soil and water microbial communities. Nevertheless, little information exists about the effects of copper nanoparticles on relevant ecological processes of natural pesticide attenuation mediated by bacteria, and their mechanisms of gene dissemination. In this work we reported the first evidence that demonstrate that the transfer of catabolic plasmids by conjugation could be negatively affected by copper ions released from copper nanoparticles at sub inhibitory concentrations. However, the effect of copper nanoparticles also could be related to the capacity of medium for the dissolution of nanoparticles and ions release. We suggest that the use of copper nanoparticles in agricultural practices could inhibit the transfer of CCP between microbial communities by conjugation, generating a decrease in the pesticide attenuation. Therefore, more comprehensive studies to evaluate how copper ions interfere on bacterial conjugation are needed.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.11.057.

References

Aranda, C., Godoy, F., Becerra, J., Barra, R., Martínez, M., 2003. Aerobic secondary utilization of a non-growth and inhibitory substrate 2,4,6-trichlorophenol by *Sphingopyxis chilensis* S37 and *Sphingopyxis*-like strain S32. Biodegradation 14, 265–274..

Barros, J., Becerra, J., González, C., Martínez, M., 2013. Antibacterial metabolites synthesized by psychrotrophic bacteria isolated from cold-freshwater environments. Folia Microbiol. 58 (2), 127–133.

Bondarenko, O., Ivask, A., Käkinen, A., Kahru, A., 2012. Sub-toxic effects of CuO nanoparticles on bacteria: kinetics, role of Cu ions and possible mechanisms of action. Environ. Poll. 169, 81–89.

Clément, P., Pieper, D.H., González, B., 2001. Molecular characterization of a deletion/duplication rearrangement in tfd genes from Ralstonia eutropha JMP134 (pJP4) that improves growth on 3-chlorobenzoic acid but abolishes growth on 2, 4-dichlorophenoxyacetic acid. Microbiology 147 (8), 2141–2148.

De Souza, M.L., Seffernick, J., Martínez, B., Sadowsky, M.J., Wackett, L.P., 1998. The atrazine catabolism genes *atz*ABC are widespread and highly conserved. J. Bacteriol. 180 (7), 1951–1954.

DiGiovanni, G.D., Neilson, J.W., Pepper, I.L., Sinclair, N.A., 1996. Gene transfer of *Alcaligenes eutrophus* JMP134 plasmid pJP4 to indigenous soil recipients. Appl. Environ. Microbiol. 62 (7), 2521–2526.

Don, R.H., Pemberton, J.M., 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J. Bacteriol. 145 (2), 681–686.

Don, R.H., Pemberton, J.M., 1985. Genetic and physical map of the 2,4- dichlorophenoxyacetic acid degradative plasmid pJP4. J. Bacteriol. 161 (1), 466–468.

Franklin, F.C., Bagdasarian, M., Bagdasarian, M.M., Timmis, K.N., 1981. Molecular and functional analysis of the TOL plasmid pWWO from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring meta cleavage pathway. Proc. Natl. Acad. Sci. USA 78 (12), 7458–7462.

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

Goris, J., Dejonghe, W., Falsen, E., De Clerck, E., Geeraerts, B., Willems, A., Top, E.M., Vandamme, P., De Vos, P., 2002. Diversity of transconjugants that acquired plasmid pJP4 or pEMT1 after inoculation of a donor strain in the A-and B-horizon of an agricultural soil and description of *Burkholderia hospita* sp. nov. and *Burkholderia terricola* sp. nov. Syst. Appl. Microbiol. 25 (3), 340–352.

Graves Jr., J.L., Tajkarimi, M., Cunningham, Q., Campbell, A., Nonga, H., Harrison, S.H., Barrick, J.E., 2015. Rapid evolution of silver nanoparticle resistance in *Escherichia coli*. Front. Genet. 6, 42.

Gunawan, C., Teoh, W.Y., Marquis, C.P., Amal, R., 2011. Cytotoxic origin of copper (II) oxide nanoparticles: comparative studies with micron-sized particles, leachate, and metal salts. ACS Nano 5 (9), 7214–7225.

Guo, J., Gao, S.H., Lu, J., Bond, P.L., Verstraete, W., Yuan, Z., 2017. Copper oxide nanoparticles induce lysogenic bacteriophage and metal-resistance genes in *Pseudomonas aeruginosa* PAO1. ACS Appl. Mater. Interfaces 9 (27), 22298–22307.

Hajipour, M.J., Fromm, K.M., Ashkarran, A.A., de Aberasturi, D.J., de Larramendi, I.R., Rojo, T., Serpooshan, V., Parak, W.J., Mahmoudi, M., 2012. Antibacterial properties of nanoparticles. Trends Biotechnol. 30 (10), 499–511.

Huang, J., Li, Q., Sun, D., Lu, Y., Su, Y., Yang, X., Wang, H., Wang, Y., Shao, W., He, N., Hong, J., Chen, C., 2007. Biosynthesis of silver and gold nanoparticles by novel sundried *Cinnamomum camphora* leaf. Nanotechnology 18, 105104–105114.

Imfeld, G., Vuilleumier, S., 2012. Measuring the effects of pesticides on bacterial communities in soil: a critical review. Eur. J. Soil Biol. 49, 22–30.

Jacobsen, C., Hjelmsø, M., 2014. Agricultural soils, pesticides and microbial diversity. Curr. Opin. Biotechnol. 27, 15–20.

Juhas, M., van der Meer, J.R., Gaillard, M., Harding, R.M., Hood, D.W., Crook, D.W., 2009. Genomic islands: tools of bacterial horizontal gene transfer and evolution. FEMS Microbiol. Rev. 33 (2), 376–393.

Kaweeteerawat, C., Chang, C.H., Roy, K.R., Liu, R., Li, R., Toso, D., Fischer, H., Ivask, A., Ji, Z., Zink, J.I., Zhou, Z.H., Chanfreau, G.F., Telesca, D., Cohen, Y., Holden, P.A., Nel, A.E., Godwin, H.A., 2015. Cu nanoparticles have different impacts in *Escherichia coli* and *Lactobacillus brevis* than their microsized and ionic analogues. ACS Nano 9 (7), 7215–7225.

Kim, J.S., Kuk, E., Yu, K.N., Kim, J.H., Park, S.J., Lee, H.J., Kim, S.H., Park, Y.K., Park, Y.H., Hwang, C.Y., Kim, Y.K., Lee, Y.S., Jeong, D.H., Cho, M.H., 2007. Antimicrobial effects of silver nanoparticles. Nanomedicine: NBM 3 (1), 95–101.

Klaine, S.J., Alvarez, P.J., Batley, G.E., Fernandes, T.F., Handy, R.D., Lyon, D.Y., Mahendra, S., McLaughlin, M.J., Lead, J.R., 2008. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. Environ. Toxicol. Chem. 27 (9), 1825–1851.

Klümper, U., Dechesne, A., Riber, L., Brandt, K.K., Gulay, A., Sorensen, S.J., Smets, B.F., 2017. Metal stressors consistently modulate bacterial conjugal plasmid uptake potential in a phylogenetically conserved manner. ISME J. 11 (1), 152–165.

Lemire, J.A., Harrison, J.J., Turner, R.J., 2013. Antimicrobial activity of metals: mechanisms, molecular targets and applications. Nat. Rev. Microbiol. 11 (6), 371–384.

Mandelbaum, R.T., Allan, D.L., Wackett, L.P., 1995. Isolation and characterization of a *Pseudomonas* sp. that mineralizes the s-triazine herbicide atrazine. Appl. Environ. Microbiol. 61 (4), 1451–1457.

Martínez, B., Tomkins, J., Wackett, L.P., Wing, R., Sadowsky, M.J., 2001. Complete nucleotide sequence and organization of the atrazine catabolic plasmid pADP-1 from *Pseudomonas* sp. strain ADP. J. Bacteriol. 183 (19), 5684–5697.

Neilson, J.W., Josephson, K.L., Pillai, S.D., Pepper, I.L., 1992. Polymerase chain reaction and gene probe detection of the 2, 4-dichlorophenoxyacetic acid degradation plasmid, pJP4. Appl. Environ. Microbiol. 58 (4), 1271–1275.

Nel, A., Xia, T., Mädler, L., Li, N., 2006. Toxic potential of materials at the nanolevel. Science 311 (5761), 622–627.

Nowack, B., Bucheli, T.D., 2007. Occurrence, behavior and effects of nanoparticles in the environment. Environ. Pollut. 150 (1), 5–22.

Ochman, H., Lawrence, J.G., Groisman, E.A., 2000. Lateral gene transfer and the nature of bacterial innovation. Nature 405 (6784), 299–304.

Ou, J.T., Anderson, T.F., 1972. Effect of Zn2+ on bacterial conjugation: inhibition of mating pair formation. J. Bacteriol. 111 (1), 177–185.

Ou, J.T., 1973. Effect of Zn2+ on bacterial conjugation: increase in ability of F− cells to form mating pairs. J. Bacteriol. 115 (2), 648–654.

Pal, S., Tak, Y.K., Song, J.M., 2007. Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium *Escherichia coli*. Appl. Environ. Microbiol. 73 (6), 1712–1720.

Parada, J., Rubilar, O., Diez, M.C., Cea, M., Sant`Ana da Silva, A., Rodriguez-Rodriguez, C.E., Tortella, G.R., 2019. Combined pollution of copper nanoparticles and atrazine in soil: effects on dissipation of the pesticide and on microbiological community profiles. J. Hazard Mater. 361, 228–236.

Pérez-Pantoja, D., De la Iglesia, R., Pieper, D.H., González, B., 2008. Metabolic reconstruction of aromatic compounds degradation from the genome of the amazing pollutant-degrading bacterium *Cupriavidus necator* JMP134. FEMS Microbiol. Rev. 32 (5), 736–794.

Rai, M., Yadav, A., Gade, A., 2009. Silver nanoparticles as a new generation of antimicrobials. Biotechnol. Adv. 27 (1), 76–83..

Rizwan, M., Ali, S., Qayyum, M.F., Ok, Y.S., Adrees, M., Ibrahim, M., Zia-ur-Rehman, M., Farid, M., Abbas, F., 2017. Effect of metal and metal oxide nanoparticles on growth and physiology of globally impostant food crops: A critial review. J. Hazard Mater. 322, 2–16.

Schenzle, A., Lenke, H., Fischer, P., Williams, P.A., Knackmuss, H., 1997. Catabolism of 3-nitrophenol by *Ralstonia eutropha* JMP 134. Appl. Environ. Microbiol. 63 (4), 1421–1427.

Sekhon, B.S., 2014. Nanotechnology in agri-food production: an overview. Nanotechnol. Sci. Appl. 7, 31.

Suresh, Y., Annapurna, S., Singh, A.K., Bhikshamaiah, G., 2014. Green synthesis and characterization of tea decoction stabilized copper nanoparticles. Int. J. Innov. Res.

Sci. Eng. Technol. 3, 11265–11270.
Vale, G., Mehennaoui, K., Cambier, S., Libralato, G., Jomini, S., Domingos, R.F., 2016. Manufactured nanoparticles in the aquatic environment-biochemical responses on freshwater organisms: a critical overview. Aquat. Toxicol. 170, 162–174.
Wackett, L., Sadowsky, M., Martínez, B., Shapir, N., 2002. Biodegradation of atrazine and related s-triazine compounds: from enzymes to field studies. Appl. Microbiol. Biotechnol. 58 (1), 39.
Wang, Y., Kou, S., Jiang, Q., Xu, B., Liu, X., Xiao, J., Tian, Y., Zhou, Ch, Zhang, Ch, Xiao, M., 2014. Factors affecting transfer of degradative plasmids between bacteria in soils. Appl. Soil Ecol. 84, 254–261.
Wang, D., Lin, Z., Wang, T., Yao, Z., Qin, M., Zheng, S., Lu, W., 2016. Where does the toxicity of metal oxide nanoparticles come from: the nanoparticles, the ions, or a combination of both? J. Hazard Mater. 308, 328–334.
Wang, X., Yang, F., Zhao, J., Xu, Y., Mao, D., Zhu, X., Luo, Y., Alvarez, P.J.J., 2018. Bacterial exposure to ZnO nanoparticles facilitates horizontal transfer of antibiotic resistance genes. NanoImpact 10, 61–67.
Wiegand, I., Hilpert, K., Hancock, R.E., 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Prot. 3 (2), 163.
Wozniak, R.A., Waldor, M.K., 2010. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. Nat. Rev. Microbiol. 8 (8), 552.