

Quantitative gene monitoring of microbial tetracycline resistance using magnetic luminescent nanoparticles

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A magnetic/luminescent nanoparticles (MLNPs) based DNA hybridization method was developed for quantitative monitoring of antibiotic resistance genes and gene-expression in environmental samples. Manipulation of magnetic field enabled the separation of the MLNPs-DNA hybrids from the solution and the fluorescence of MLNPs normalized the quantity of target DNA. In our newly developed MLNPs-DNA assay, linear standard curves ($R^2 = 0.99$) of target gene was determined with the detection limit of 620 gene copies. The potential risk of increased bacterial antibiotic resistance was assessed by quantitative monitoring of tetracycline resistance (*i.e.*, *tetQ* gene) in wastewater microcosms. The gene abundance and its expression showed a significant increase of *tetQ* gene copies with the addition of tetracycline, triclosan (TCS), or triclocarban (TCC). A real-time PCR assay was employed to verify the quantification capability of the MLNPs-DNA assay and accordingly both assays have shown strong correlation ($R^2 = 0.93$). This non-PCR based MLNPs-DNA assay has demonstrated its potential for gene quantification *via* a rapid, simple, and high throughput platform and its novel use of internal calibration standards.

Introduction

Antibiotic-resistant organisms can be proliferated by the selection of corresponding antibiotics due to their tolerance. Rapid and accurate detection of antibiotic resistance can prevent a potential public health crisis in an event of antibiotic resistant pathogen outbreak. Furthermore, in water environments, where a number of antibiotics coexist with the chemicals from pharmaceutical/household products, the dynamics of microbial antibiotic resistance can be complicated and uncertain.

Advanced nanomaterials have brought evolutionary advantages to the detection of DNA, such as very large surface areas of nanoparticles that provide the binding sites for DNA, and size compatibility with DNA as a target. Various fluorescent nanoparticles such as quantum dots,¹⁻⁴ gold,⁵⁻⁷ and dye doped silica

nanoparticles^{8,9} have been adopted for DNA detection. Previously, multi-functional $\text{Fe}_3\text{O}_4/\text{Eu}:\text{Gd}_2\text{O}_3$ core/shell magnetic/luminescent nanoparticles (MLNPs) were synthesized and successfully applied for the quantitative detection of DNA.^{10,11} The magnetic property of the nanoparticles allows efficient magnetic separation of MLNP-DNA hybridization complex from a solution and it decreases the assay time. The stable and long-lasting fluorescence from the lanthanide shell serves as an internal calibration for the assay. The unique internal calibration method has enabled us to reduce the errors resulted from different numbers of nanoparticle in each reaction. Leveraging on the advantages of multi-functional MLNPs for DNA quantification, we have developed a DNA hybridization-in-solution assay using MLNPs.^{10,11} MLNPs are functionalized with Neutravidin *via* passive adsorption. Following the immobilization of a biotinylated probe DNA on the surface of MLNPs, both the target DNA and the signaling probe DNA are simultaneously hybridized with the probe DNA. Since hybridization is achieved on the surface of the MLNPs instead of on a chip as in DNA microarrays, the hybridization-in-solution format has both assay simplicity and flexibility.¹⁰

Tetracycline antibiotics are widely used in both human and animal care and are commonly detected in wastewater treatment

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Environmental impact

Owing to a number of antibiotics and household chemicals in the aquatic environment, the dynamics of microbial antibiotic resistance may be complicated and can result in multiple resistance patterns. A primary concern in this case will be the temporal uncertainty of increasing emergence in the number of pathogenic microorganisms that are antibiotic resistant. Unfortunately the water quality test, which is typically based on chemical analysis, is not able to quantify the microbial antibiotic resistance. Our novel microbial DNA sensing technique using advanced nanomaterials has shown its ability to quantify microbial antibiotic resistance as well as the effect of common household reagents on the microbial dynamics in the environment.

plants. Out of eight tetracycline-resistant genes coding for ribosomal protection proteins, *tetQ* is the most abundant gene in the environment as identified in waste lagoons and groundwater.^{12–14} *TetQ* gene was selected for our study as a representative antibiotic resistance gene for the purpose of the assay development and optimization.^{12,15} Triclosan (TCS) and triclocarban (TCC) are commonly added antimicrobial substances in many household products including toothpaste and soaps.^{16–18} For example, TCC is added in 84% of antibacterial soaps in the United States¹⁷ at levels of 0.5–5% (w/w).¹⁹ According to the U.S. Geological Survey, TCS and TCC are among the most frequently detected pharmaceutical and personal care product additives in the water resources.¹⁶ Several studies^{20,21} have demonstrated TCS or TCC are possibly linked with antibiotic resistance toward cross- or multiple resistance. Dr Levy's group at Tufts University indicated that low levels of TCS may encourage the survival of mutants that are resistant to multiple antibiotics.²⁰

The goal of our study was to demonstrate the proof of concept for a DNA quantification assay to monitor antibiotic resistance in a wastewater environment by using MLNPs in 96-well high-throughput hybridization-in-solution format. The proposed technology overcomes the issues encountered when standard microbiological (*i.e.*, clinical) methods are used with environmental samples. While the standard EPA method for the determination of bacterial antibiotic resistance is limited to microorganisms that can be cultured, our method is able to monitor the antibiotic resistance without involving a culturing step by targeting microbial community DNA extracted from the environmental samples. The gene encoding tetracycline resistance was quantitatively monitored in the microcosm reactors spiked with tetracycline using the MLNPs-DNA assay. To investigate the potential risk of increased antibiotic resistance associated with the use of antibiotics and antibacterial household agents, tetracycline resistance levels were measured in the presence of TCS or TCC. An established real-time PCR assay was employed to validate the newly developed MLNPs-DNA assay pertaining to assay sensitivity and range of quantification.

Materials and methods

Nanoparticles

Fe₃O₄/Eu : Gd₂O₃ core/shell MLNPs used in this work were synthesized by spray pyrolysis process.²² Magnetic cores (Fe₃O₄) synthesized *via* a co-precipitation method,²³ were dispersed in a precursor solution of 20% Eu(NO₃)₃ and 80% Gd(NO₃)₃ in methanol and the solution was then sprayed through a hydrogen flame. Consequently, Eu : Gd₂O₃ formed the luminescent layer on the surface of the magnetic core. Nanoparticles were functionalized by the passive adsorption of 50 µg neutravidin (Pierce, Rockford, IL) per mg MLNPs, which was determined as the optimum amount in a previous study.¹¹

Microcosm batch reactors

Mixed microbial community was obtained from an activated sludge wastewater treatment plant. Activated sludge mixed liquor was obtained from the aeration tank at University of California Davis wastewater treatment plant (Davis, CA). The

initial total suspended solid (TSS) of filtered and washed mixed liquor was 1400 mg L⁻¹. For microcosm reactor incubations, a synthetic media was prepared to simulate a wastewater environment based on the EPA/600/4-85/014 method. Glucose (46 mg L⁻¹) and sodium acetate (60 mg L⁻¹) were used as carbon sources; NH₄Cl (20 mg L⁻¹) as a nitrogen source; a combination of KH₂PO₄ (100 mg L⁻¹) and K₂HPO₄ (100 mg L⁻¹) as a phosphorous source as well as a pH buffer (pH 7) were added along with MgSO₄·2H₂O (10 mg L⁻¹) as a sulfur source and NaHCO₃ to control the alkalinity.

150 mL of synthetic wastewater microcosm batch reactors were aerobically prepared in 250 mL glass Erlenmeyer flasks and inoculated with activated sludge culture to maintain 500 mg L⁻¹ of microbial community. Tetracycline (500 µg L⁻¹), triclosan (TCS; 2,4,4'-trichloro-2'-hydroxydiphenyl ether, 300 µg L⁻¹), and triclocarban (TCC; 3,4,4'-trichloro-carbanilide, 100 µg L⁻¹) were spiked in the following microcosm reactors: (1) control (2) tetracycline (3) TCS (4) TCC in triplicates. The concentrations of chemicals added were chosen based on their respective reported concentrations in wastewater environments: 4–25 µg L⁻¹ for tetracycline in wastewater and groundwater;^{24,25} 0.07–14,000 µg L⁻¹ for TCS in wastewater;²⁶ EC₅₀ (rainbow trout) of TCS = 350 µg L⁻¹;²⁷ TCC was shown to be present from 2 µg L⁻¹ to ppm levels.^{19,28} Since TCS and TCC are sparingly soluble in water, they were instead prepared and diluted in dimethyl sulfoxide (DMSO). Triplicate microcosm reactors were aerobically incubated (at 26 °C and 150 rpm) for 28 days and samples were removed weekly for the extraction of nucleic acids.

Extraction of gDNA and RNA, cDNA synthesis

Genomic DNAs (gDNA) from microcosms were manually extracted in accordance with a protocol reported by Wilson.²⁹ A 10 mL sample taken from reactors was harvested and centrifuged at 5000 rpm for 5 min. The pellet, re-suspended in sucrose buffer (40 mM EDTA, 50 mM Tris-HCl, and 750 mM sucrose), was incubated at 37 °C with lysozyme for 30 min, and subsequently with 10% SDS and proteinase-K for 2 h. The cells were then disrupted using a FP 120 Savant bead beater for 3 min at a speed of 5.5. The collected genomic DNA was purified by standard phenol and chloroform extractions, and ethanol precipitation methods. The DNA concentration and purity of the gDNA samples were measured *via* a Shimadzu UV-VIS spectrophotometer at λ = 260 nm and 280 nm.

25 mL of mixed cultures that were pooled from microcosms (0 through 4 weeks) were treated with RNeasy Protect™ bacteria reagent (Qiagen, Valencia, CA) to stabilize RNA in a ratio of 1 part culture to 1 part reagent. RNA was extracted from the cells using a Qiagen RNeasy mini kit and RNase-free DNase according to the manufacturer's protocol. Eluted RNA was stored at -70 °C until cDNA synthesis. The concentration of RNA was determined by the spectrophotometer at λ = 260 nm and 280 nm. Complementary DNA (cDNA) was synthesized by SuperScript III First-strand synthesis SuperMix (Invitrogen, Carlsbad, CA) in accordance with manufacturer's protocol. RNase H was used to digest the RNA after the cDNA synthesis.

Table 1 DNA sequences of probes and primers used in this work^a

Name		Sequences (5' → 3')	Quantification platform	Source or references
<i>tetQ</i> (170 bp)	Probe	biotin -CACTGGCAAA CAGCAGATTCT	MLNPs-assay	This study
	Signaling probe	Cy3 -CGGAGTGTCAA TGATATTGCA	MLNPs-assay	This study
	F. primer	AGAATCTGCTG TTTGCCAGTG	SYBR real-time PCR	13
	R. primer	CGGAGTGTCAA TGATATTGCA	SYBR real-time PCR	
16S rRNA (123 bp)	F. primer: (BACT1369F)	CGGTGAATAC GTTTCYCGG	Taqman real-time PCR	32
	R. primer: (PROK1492R)	GGWTACCTTG TTACGACTT	Taqman real-time PCR	
	Taqman probe: (TM1389F)	FAM -CTTGTACACA CCGCCGTC- BHQ1	Taqman real-time PCR	

^a Boldface indicates the functionalization of DNA nucleotide probes used for both MLNPs-based hybridization and Taqman real-time PCR.

Plasmid dsDNA standards

PCR products of *tetQ* gene (170 bp) were obtained from the amplification of gDNA, which was extracted from activated sludge mixed liquor, with the gene-specific primer set as shown in Table 1. The PCR-amplified and purified gene was ligated to a pCR2.1-TOPO vector, and the plasmid was transformed into competent *E. coli* cell using Invitrogen TOPO TA cloning kit. A plasmid DNA was extracted from *E. coli* clones using Qiagen Mini-prep kit. Clones were screened by colony PCR with M13 primers as well as restriction enzyme digestion using EcoRI (New England Biolabs, MA). The copy numbers of the plasmid was calculated from the DNA concentration determined by measuring absorbance at 260 nm and the molecular weight of *tetQ* gene, and that of the TOPO vector plasmid. Each plasmid dsDNA was 10-fold serially diluted to be used for the construction of standard curves for the MLNP-DNA assay.

MLNPs based hybridization-in-solution assay

The design of DNA oligonucleotide probes (Table 1) for antibiotic resistance genes was based on *tetQ* gene sequences available in GenBank Z21523¹³ and M19652,^{30,31} respectively. A 96-well high-throughput platform equipped with a 96-well plate and a magnet (Dyna-Invitrogen, Carlsbad, CA) was used for DNA hybridization and washing. The overall procedure of the MLNPs-based hybridization-in-solution assay is outlined in Fig. 1. Neutravidin-encapsulated MLNPs were mixed with 100 μ L of DIG easy hybridization buffer (Roche Diagnostic, Basel, Switzerland) and biotinylated probe DNA, and incubated for 2 h at 37 °C in a hybridization oven (Techne, Burlington, NJ). Post washing biotinylated MLNPs-DNA probes were hybridized with both target DNA (*i.e.*, dsDNA plasmid standards, gDNA extracted from microcosms, or cDNA derived from RNA extracted from microcosms) and signaling probe DNA labeled

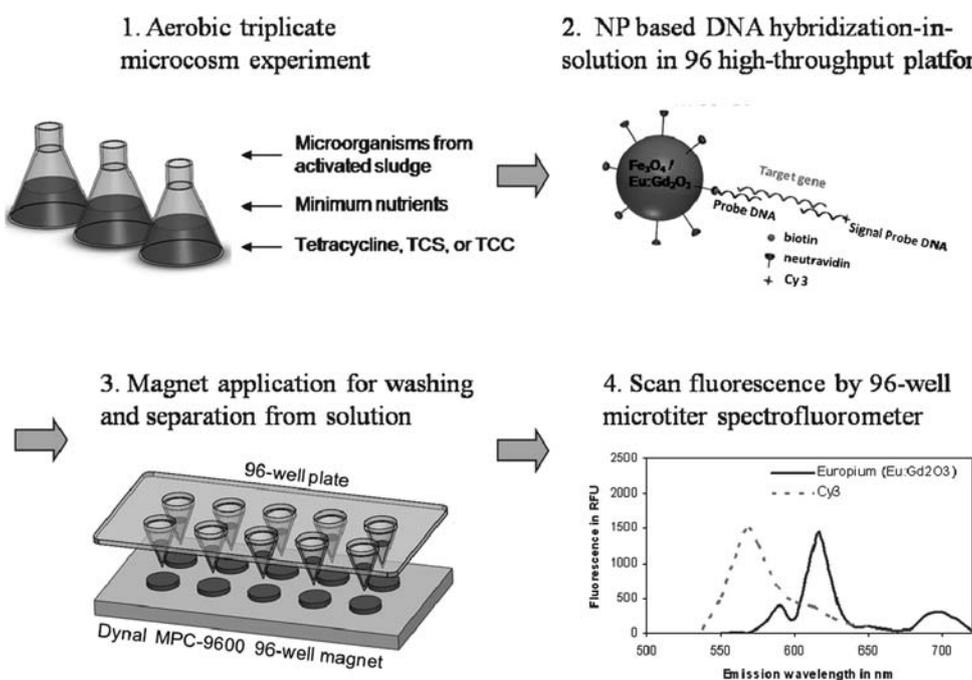


Fig. 1 Schematic diagram of a 96-well high-throughput MLNPs-based DNA hybridization-in-solution assay.

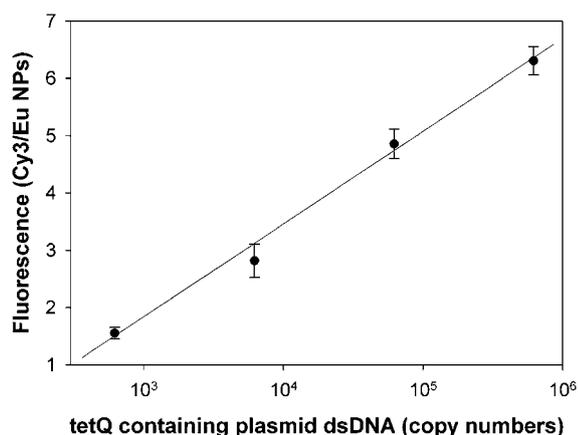


Fig. 2 A standard curve plotting the gene copy numbers and corresponding fluorescence in MLNPs-based hybridization using *tetQ* containing plasmid dsDNA. The linear relationship is $y = 1.631 \log x - 3.114$ (y : fluorescence, x : gene copy numbers), $R^2 = 0.99$. The signal and error bars represent average and standard deviations based on triplicate reactions.

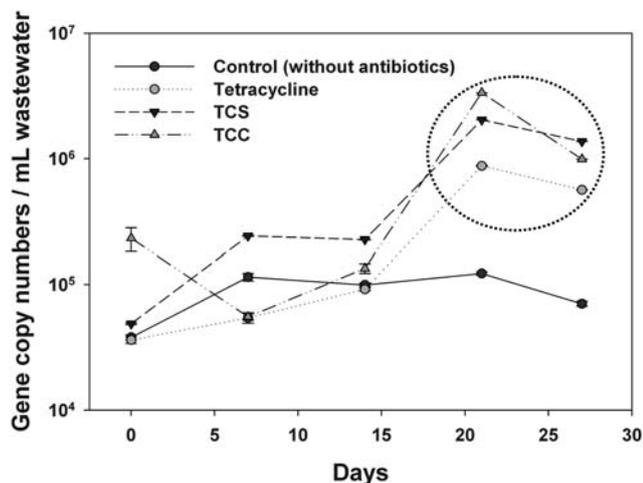


Fig. 3 *TetQ* gene quantification in microcosm reactors treated with antibiotics: Control (without antibiotic), tetracycline ($500 \mu\text{g L}^{-1}$), TCS ($300 \mu\text{g L}^{-1}$), or TCC ($100 \mu\text{g L}^{-1}$) by MLNPs-hybridization assay. Community genomic DNA was extracted from microcosms and used for hybridization. Microcosm batch reactors were operated for 4 weeks and samples were taken for DNA extraction every week. The signal and error bars represent average and standard deviations based on triplicate microcosm reactions.

with Cy3 at 37°C for 8 h with gentle mixing. Hybridized DNA complexes were separated from solution by a 96-well magnet, while non-hybridized DNA remained in solution and were subjected to the wash. The Cy3 ($\lambda_{\text{ex}} = 550 \text{ nm}$, $\lambda_{\text{em}} = 570 \text{ nm}$) and europium ($\lambda_{\text{ex}} = 260 \text{ nm}$, $\lambda_{\text{em}} = 616 \text{ nm}$) fluorescence were measured by a SpectraMax M2 spectrofluorometric microplate reader (Molecular Devices, Eugene, OR). Normalized fluorescence (Cy3/europium) of MLNP-based hybridization reactions was converted to gene copy numbers using the calibration curve shown in Fig. 2. Since the microcosm represents a synthetic wastewater environment, gene copy numbers were normalized by

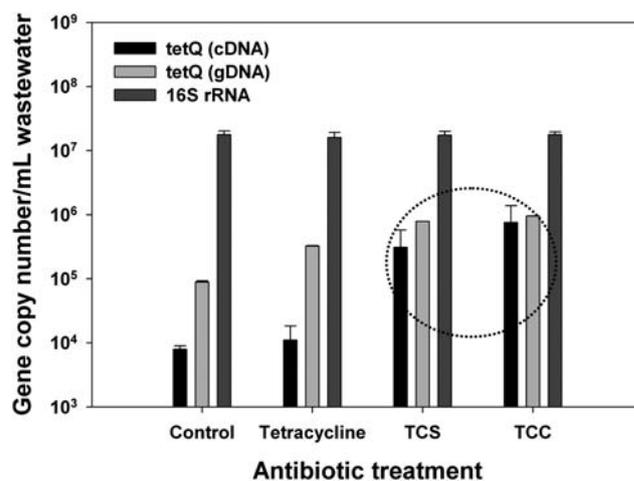


Fig. 4 Comparison between cDNA gene expression and gDNA quantification using MLNPs-DNA assay. cDNA was generated from RNAs extracted from pooled microcosm samples from weeks 0 through 4. gDNA copies depicted on the graph were obtained by combining and averaging from the gene copy numbers of each week's sample. 16S rRNA gene copies represent total bacterial abundance. The dotted circle shows the impact of TCS and TCC on the increased antibiotic resistant gene abundance and expression in comparison to the control. The signal and error bars represent average and standard deviations based on triplicate reactions.

the volume of wastewater, which was used for DNA or RNA extraction. Thus experimental results were reported as gene copies/mL wastewater as shown on y -axis of Fig. 3 and 4.

Real-time PCR assay

Five μL of gDNA dilutions were added to a real-time PCR reagent mix ($15 \mu\text{L}$ as a final reaction volume), which contains $1 \times$ AB power SYBR green master mix (Applied Biosystems, Foster City, CA) and primers ($0.2 \mu\text{M}$). The reaction for *tetQ* gene quantification was performed with a 7300 real-time PCR system (Applied Biosystems) at the following conditions: 95°C for 15 s; 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C 31 s; dissolution analysis of 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s.¹⁵ Standard curve was constructed between the gene copy numbers in reaction mixtures and the corresponding real-time PCR threshold cycles (C_T) by using 10-fold serial dilutions of the *tetQ* plasmid. TaqMan real-time PCR assay was performed to quantify the universal bacterial 16S rRNA gene in order to estimate the total bacteria numbers in the microcosms. The reaction mixture ($20 \mu\text{L}$) for TaqMan assay contained $2 \times$ TaqMan Universal PCR Master Mix (Applied Biosystems), $0.8 \mu\text{mol L}^{-1}$ of forward and reverse primers, $0.2 \mu\text{mol L}^{-1}$ of TaqMan probe³² shown in Table 1, and $5 \mu\text{L}$ of extracted gDNA. Thermal cycling program was 40 cycles of 95°C for 15 s and 56°C for 1 min with an initial cycle of 95°C for 10 min.

Results and discussion

DNA quantification assay using nanoparticles

We developed a quantitative high-throughput MLNPs-based DNA assay to quantify one of the tetracycline resistance

determinants, which is the *tetQ* gene. A strong linear relationship ($R^2 = 0.99$) between \log *tetQ* gene copies and fluorescence signals (Cy3/Europium) was achieved (Fig. 2) by the hybridization of different concentrations of plasmid dsDNA standards with cloned *tetQ* gene. The linear portion of the standard curve for \log gene copy numbers *versus* fluorescence (Cy3/Europium) extended over three orders of magnitude for *tetQ* gene. Detection limit was approximately 620 gene copies per 100 μL reaction of MLNPs-based hybridizations. In comparison to other non-PCR based DNA assays, our MLNPs based method is 2–4 orders of magnitude more sensitive than a quantum-dots based hybridization assay which had a detection limit of 10^4 – 10^6 gene copies in a gene expression study.¹

Antibiotic resistance monitoring in microcosms

To demonstrate the applicability of the newly developed MLNPs-hybridization assay for quantitative gene monitoring in environmental samples, the abundance of *tetQ* gene in wastewater microcosm incubations was quantified over 28 days period (Fig. 3). Community genomic DNA extracted from the microcosms, with and without the addition of tetracycline, TCS, or TCC, was used for the gene abundance quantification. The ratio of optical density (OD) at 260 nm and 280 nm (*i.e.*, $\text{OD}_{260}/\text{OD}_{280}$), which represents the purity of gDNA against protein (or phenol) contamination, was ranged from 1.8 to 1.9 for all gDNA extracted. As the optimum range of $\text{OD}_{260}/\text{OD}_{280}$ for pure DNA extract is within 1.8 through 2.0, our results indicate that the extracted genomic DNA from the reactors does not contain the contaminants. The level of *tetQ* gene abundance measured in a control microcosm (*i.e.*, no antibiotic addition) represents the background level ($\sim 10^{4.5}$ gene copies) of *tetQ* gene in activated sludge mixed liquor in the wastewater treatment plant. We observed that *tetQ* gene copies were increased by the addition of tetracycline, TCS, or TCC after 3–4 week of incubation. This increase was depicted by the dotted circle in Fig. 3. In the treatment with $500 \mu\text{g L}^{-1}$ of tetracycline *tetQ* gene copies increased from $10^{4.5}$ to $10^{5.5}$ gene copies per mL wastewater; in $300 \mu\text{g L}^{-1}$ of TCS and $100 \mu\text{g L}^{-1}$ of TCC treatments, *tetQ* gene copies per mL sample increased from $10^{4.5}$ to 10^6 , respectively. There was no significant change of gene abundance in the control microcosms (without tetracycline, TCS or TCC). An interesting observation was that TCS and TCC elicited a higher increase of tetracycline resistance based on *tetQ* gene copies than tetracycline would have elicited alone. The observed significant increase of *tetQ* gene copies with the addition of TCS or TCC implies that TCS or TCC may play a role in triggering tetracycline resistance by the mechanism of co-selection in complex microbial communities.

Antibiotic resistance gene expression

To investigate treatment effects and measure changes in metabolic activity, antibiotic resistance gene expression was monitored by cDNA quantification with the MLNPs assay. Similar to the observed difference in gene abundance measured with gDNA, the addition of TCS or TCC significantly increased the *tetQ* gene expression (*i.e.*, cDNA copies) above control levels (95% confidence level, two-sided t-test). This increase was greater

than the increase in *tetQ* gene expression achieved by tetracycline addition (in Fig. 4, it is depicted by the dotted circle). *TetQ* cDNA level in the control and tetracycline treatment was similar (p-value = 0.628: greater than 0.1). However the *tetQ* gene cDNA copy numbers between the control and TCS/TCC were significantly different (p-values = 0.04 (TCS), 0.009 (TCC): less than 0.1). A combined perspective of antibiotic/pharmaceuticals effects on antibiotic resistance gene expression, gene abundance, and bacterial population size, was shown by the quantification of cDNA, gDNA, and 16S rRNA gene copies, respectively (Fig. 4). Total bacterial population represented by 16S rRNA gene copies was in the range of 10^7 to 10^8 gene copies/mL throughout the incubation period. Results are indicative that the gene expression of antibiotic resistance could be induced by the exposure to common antibacterial products such as TCS and TCC as well as tetracycline.

Assay validation by comparison to real-time PCR

A real-time PCR assay was used for the verification of MLNPs-DNA assay sensitivity and range of quantification. Genomic DNA extracted from microcosm reactors was also used as a template in the real-time PCR assay and the results were compared to the MLNPs-DNA assay data. *TetQ* gene copies quantified by both real-time PCR and MLNPs-DNA assay (Fig. 5) were positively correlated ($R^2 = 0.93$) in the microcosm samples. Real-time PCR technology is widely used for detecting gene abundance and expression and is frequently employed in the studies of antibiotic resistance. Our MLNP-based DNA assay has a capability comparable to real-time PCR in terms of linearity, throughput, and assay sensitivity. Our unique assay configuration employing MLNPs as a carrier and internal calibration enables accurate and sensitive quantification. In addition, the assay itself has greater flexibility than real-time PCR and can be adapted for use in different platforms, such as microchannel (study in progress) and microplate systems. Therefore it has a potential to be fabricated into a portable miniaturized device. Furthermore, our method can potentially be used to quantify multi-analytes simultaneously by adopting nanoparticles doped with multiple colors of lanthanides (*e.g.*, Terbium, Samarium, Ithrium).

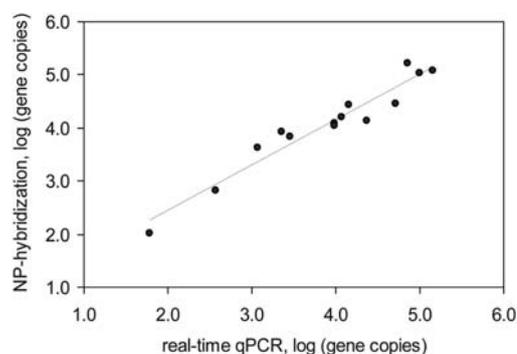


Fig. 5 Correlation between *tetQ* gene copy numbers in microcosm batch reactors, determined by real-time PCR *versus* MLNPs-based hybridization assay. Each point represents one microcosm reactor and the linear relationship is expressed by the equation $y = 0.859x + 0.718$ ($R^2 = 0.93$).

To further apply this assay to complex microbial communities in the environment, additional study will be required to understand the limits of our technology. For example, co-existing compounds such as humic acids in soils could potentially influence the sensitivity of hybridization assay. Although our hybridization based technology overcomes the inhibition that is related to the amplification process in real-time PCR, further elucidation of the other environmental factors (*i.e.* potential inhibitors to the nanoparticle based hybridization) is required in order to apply our monitoring technology in the complicated environment.

Conclusion

Magnetic/luminescent nanoparticles based DNA hybridization assay has successfully demonstrated its capability of gene quantification for the microbial antibiotic resistance monitoring in environmental samples. Our assay utilizes the advantages of efficient magnetic separation of MLNPs-DNA hybrids and precise quantification by the internal calibration *via* the fluorescence of nanoparticles. Measurement of *tetQ* gene abundance and expression indicated that antibiotic resistances can be significantly induced by the co-selection of antibacterial reagents (*i.e.*, TCS, TCC) as well as tetracycline. This rapid, accurate, and non-PCR based MLNPs-DNA assay described here, is potentially applicable for *in situ* monitoring of antibiotic resistance as well as the elucidation of microbial dynamics in the environment.

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