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Estuarine sediment hydrocarbon-degrading microbial communities demonstrate resilience to nanosilver

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ABSTRACT

Little is currently known about the potential impact of silver nanoparticles (AgNPs) on estuarine microbial communities. The Colne estuary, UK, is susceptible to oil pollution through boat traffic, and there is the potential for AgNP exposure via effluent discharged from a sewage treatment works located in close proximity. This study examined the effects of uncapped AgNPs (uAgNPs), capped AgNPs (cAgNPs) and dissolved Ag_2SO_4, on hydrocarbon-degrading microbial communities in estuarine sediments. The uAgNPs, cAgNPs and Ag_2SO_4 (up to 50 mg L^{-1}) had no significant impact on hydrocarbon biodegradation (80–92% hydrocarbons were biodegraded by day 7 in all samples). Although total and active cell counts in oil-amended sediments were unaffected by silver exposure; total cell counts in non-oiled sediments decreased from 1.66 to 0.84×10^7 g^{-1} dry weight sediment (dws) with 50 mg L^{-1} cAgNPs and from 1.66 to 0.66×10^7 g^{-1} dws with 0.5 mg L^{-1} Ag_2SO_4 by day 14. All silver-exposed sediments also underwent significant shifts in bacterial community structure, and one DGGE band corresponding to a member of Bacteroidetes was more prominent in non-oiled microcosms exposed to 50 mg L^{-1} Ag_2SO_4 compared to non-silver controls. In conclusion, AgNPs do not appear to affect microbial hydrocarbon-degradation but do impact on bacterial community diversity, which may have potential implications for other important microbial-mediated processes in estuaries.

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Introduction

Between 470 thousand and 8.3 million tonnes of petroleum hydrocarbons are released into aquatic environments globally each year (National Research Council, 2003). Although almost half of this is released naturally from oil seeps, the remainder comes from incidental deposition and represents a significant source of pollution (National Research Council, 2003). For example, between 2000 and 2013, 43 large marine oil spills (>700 tonnes) and 167 medium sized marine oil spills (7–700 tonnes) were reported (ITOPF, 2013). Estuarine systems are particularly susceptible to anthropogenic hydrocarbon contamination, with concentrations of polyaromatic hydrocarbons (PAHs) shown to exceed 100 mg kg^{-1} sediment in one UK location at Milford Haven (Woodhead et al., 1999). Of particular concern is the accumulation of low molecular weight PAHs such as naphthalenes, which have been found at concentrations of up to 2.4 mg kg^{-1} dry weight sediment in the Tyne estuary (Woodhead et al., 1999), and which are acutely toxic to aquatic invertebrates at concentrations as low as 8 μg L^{-1} (Sanborn and Malins, 1977). In addition, many high molecular weight PAHs such as chrysene, which has been found at concentrations of up to 6.94 mg kg^{-1} dry weight sediment at Milford Haven (Woodhead et al., 1999), are classed as carcinogens and can cause chronic toxic effects in fish and invertebrates (Barron et al., 2003). Fortunately, there are over 175 known bacterial genera that are able to utilize a range of different crude oil compounds as a source of carbon and energy, thus petroleum hydrocarbons that contaminate aquatic environments are commonly biodegraded by *in situ* microbial communities (Prince et al., 2010). For example, the stimulation of indigenous oil-degrading bacteria and their corresponding

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biodegradation genes were notably observed following the Deepwater Horizon event in 2010 (Hazen et al., 2010; Lu et al., 2011).

Recent advances in nanotechnology have led to an increase in the concentration of engineered nanoparticles reaching aquatic systems, where they pose a direct risk to *in situ* microbial communities and their processes (Moore, 2006; Nowack and Bucheli, 2007; Klaine et al., 2008; Baker et al., 2013). Ionic silver and silver nanoparticles (AgNPs) are used as a broad spectrum antimicrobial agent in consumer products including medical equipment, clothing, pharmaceuticals, food storage containers, children's toys, cosmetics, optical devices and household appliances (Kim et al., 2007; PEN, 2014). The release of AgNPs from many of these products through general wear and tear results in entry of AgNPs into aquatic systems where they have the potential to detrimentally impact microbial hydrocarbon-degraders and rates of petroleum biodegradation. Furthermore, exposure to multiple stressors such as crude oil and AgNPs may have a synergistic detrimental effect on communities. For example, Sargian and Mas (2007) observed a synergistic toxic effect of crude oil and UVB radiation on microplankton communities. The impact of AgNPs on aquatic microbial communities and specifically hydrocarbon-degraders is currently poorly understood (Oberdörster et al., 2005; Moore, 2006; Nowack and Bucheli, 2007; Klaine et al., 2008).

The present study focused on the Colne estuary, UK, as a model system from which to measure the effect of AgNPs on estuarine microbial communities, due to its susceptibility to oil pollution (Coulon et al., 2012) and the presence of a large wastewater treatment works which is a potential release site for AgNPs (Fig. 1). The overall aim of the study was to determine the effects of two types of AgNPs: uncapped (uAgNPs) and capped (cAgNPs) on the community structure and activity of hydrocarbon-degrading microbial communities in estuarine sediments. The cAgNP were coated with methoxypolyethylene-glycol (mPEG), a capping agent which is commonly used to stabilize AgNPs and aid dispersion (Christian et al., 2008; Crane et al., 2008). A silver salt (Ag_2SO_4) was also used as a source of silver ions for comparison.

Materials and methods

Silver nanoparticles

The uncapped silver nanoparticles (uAgNPs) were purchased as a dry powder (American Elements). A stock suspension (1 g L^{-1}) was prepared in sterile ultra-high purity (UHP) water and dispersed by sonication (75 Watts, 40 kHz) in an ultrasonic water bath (Decon F5 minor, Decon Ultrasonics Ltd) for 30 min prior to use. The capped silver nanoparticles (cAgNPs, capped with methoxypolyethylene glycol) were provided as a well-dispersed 0.9 g L^{-1} suspension of mPEG-coated AgNPs by Dr Paul Christian (University of Manchester, UK). Both nanoparticles have been thoroughly characterized previously (Beddow et al., 2014). The cAgNPs were found to be prominently present as single particles with an average diameter of 17–40 nm (depending on characterization technique) and a zeta potential of −37 mV when dispersed in UHP water, while the uAgNPs tended to form aggregates with an average diameter of 118–188 nm and a zeta potential of −32 mV (Beddow et al., 2014). A

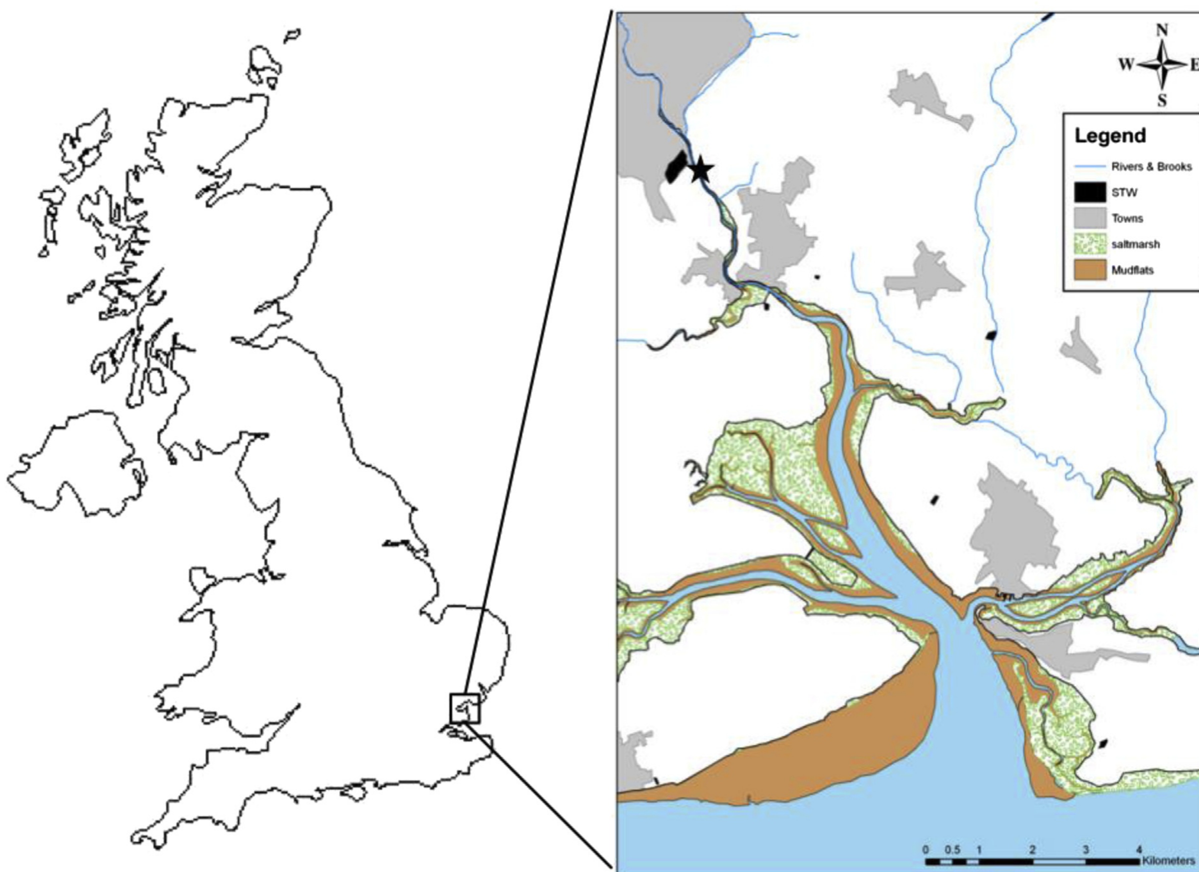

Fig. 1. Map of the UK with an enlarged box showing the Colne estuary. The sampling site (the Hythe, marked with a star) is located directly downstream of a large sewage treatment works (STW). Map provided by Dr Steve McMellor, University of Essex, UK.

1 g L^{-1} stock solution of Ag_2SO_4 (Sigma Aldrich) was used as a source of Ag^+.

Sample site and analysis

Surface sediments (top 2 cm) and nearby overlying water samples (top 50 cm) were collected at low tide in March 2011 from the Hythe, Colne estuary, UK (51°52′47.3″N; 0°55′43.1″E) (Fig. 1) and transferred back to the laboratory within 2 h. Water salinity was determined using a portable refractometer (CETI) and pH was measured using a pH electrode and meter (3310, Jenway). Sediment slurries were prepared from 1 part sediment and 2 parts overlying water. The total organic carbon (TOC) content of sediment slurries and the anion content of slurry pore waters were measured using a Shimadzu TOC-VCSH and a Dionex ICS-3000 (Thermo Scientific) respectively. Sediment dry weights were determined by drying 5 g wet weight slurry at 60 °C (Hybaid oven) to a constant weight.

Experimental setup

Glass serum bottles (120 mL) were cleaned to remove any traces of hydrocarbons before autoclaving, according to the method of Johnson et al. (2011). Triplicate aerobic sediment slurry microcosms containing 20 mL sediment slurry, 0.1% (v/v) weathered Forties crude oil (weathered by distillation at 230 °C to remove the lower molecular weight (C_1–C_{12}) alkanes and to sterilize) and uAgNPs, cAgNPs or Ag_2SO_4 at final concentrations of either 0.5 or 50 mg L^{-1}. The lower concentration of 0.5 mg/L was selected on the basis that toxicity effects of AgNPs have been observed for concentrations in the range of ~μg/L to 1 g/L (Handy et al., 2008; Yu et al., 2013). The higher concentration of 50 mg/L was selected on the basis of potential concentrations that may arise in the event of an incidental spill (Gottschalk et al., 2009). The AgNP concentrations used in this study were similar to those used in other studies (Sondi and Salopek-Sondi, 2004; Morones et al., 2005; Lowry et al., 2012).

Triplicate control microcosms without the addition of silver and crude oil were also prepared. Killed controls (killed by the addition of 10 g L^{-1} mercuric chloride (Sigma Aldrich) were prepared for every treatment type. The viability of killed controls was checked by plating 100 μL sediment slurry onto triplicate R2A agar plates (Reasoner and Geldrich, 1985) and incubating for two weeks at 20 °C. Following the addition of AgNPs, the pH of the microcosms remained unchanged and was between pH 7.5–7.7. Bottles were crimp sealed with PTFE-lined caps, incubated at 20 °C in the dark with shaking (100 rpm) and destructively sampled after days 0, 7 and 14.

Hydrocarbon extraction and GC-MS analysis

Total Petroleum Hydrocarbons (TPH) were extracted from whole sediment slurry microcosms at days 0, 7 and 14, and gas chromatography with mass spectrometry (GC-MS) analysis was conducted according to the method of McKew et al. (2007). Percent biotic degradation was determined by comparing hydrocarbon concentrations in live sediment slurries to those in killed sediment slurries at each time point (i.e. after 0, 7 and 14 days). The day 0 data were used to calculate for abiotic loss of hydrocarbons between days 0 and 7 days (data not shown). The percentage hydrocarbon remaining was calculated relative to killed controls. No abiotic losses were observed.

Nucleic acid extraction

Nucleic acids were extracted from sediment slurry subsamples (1 mL) at days 0, 7 and 14, using a bead-beating method described by Stephen et al. (1996) with the following modifications: nucleic acids were resuspended in 100 μL sterile distilled water containing 0.1% (v/v) DEPC and stored at −80 °C. Prior to bead-beating, the zirconia/silica beadbeating beads (Bio Spec Products Inc.) were baked at 180 °C for 4 h. All glassware was cleaned with RNaseZAP™ (Sigma Aldrich), all solutions were prepared with DEPC-treated UHP water and RNase-free plasticware was used throughout.

Reverse transcription of total RNA

Subsamples (25 μL) of the nucleic acid extractions were treated with Turbo DNA-free™ DNase (Invitrogen) according to the manufacturer's instructions. A control PCR was performed to ensure complete removal of DNA using the primer pair F341-GC/R534 (Muyzer et al., 1993). All control PCRs confirmed the absence of DNA in DNase-treated RNA extracts. Reactions (total 50 μL) contained 1 μl RNA template, 1x PCR buffer containing 1.5 mM $MgCl_2$ (Qiagen), 0.4 μM of each primer, 200 μM of each dNTP and 1 U *Taq* DNA polymerase (TopTaq™, Qiagen). Thermocycling consisted of 95 °C for 5 min followed by 28 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with a final elongation step of 72 °C for 7 min. PCRs were performed using a Gene Amp® PCR system 9700 Thermocycler (Applied Biosystems). Reverse transcription of 1 μL of the RNA template was carried out using BioScript™ Reverse Transcriptase (Bioline) with 2 μM random hexamers (Invitrogen), according to the manufacturer's instructions. The resulting cDNA was stored at −20 °C.

PCR-DGGE analysis of bacterial 16S rRNA genes

PCR amplifications targeting the bacterial 16S rRNA gene were performed on sediment DNA and cDNA using the primer pair F341-GC/R534 (Muyzer et al., 1993) and the conditions described in Section 2.6. Resulting DNA fragments (8 μL) were analysed by Denaturing Gradient Gel Electrophoresis (DGGE) as per the method of Muyzer et al. (1993). Analysis was performed on a D-Code System (Bio-Rad) using 8% (w/v) polyacrylamide (acrylamide:bisacrylamide, 37:1) and a denaturing gradient from 40% to 60 %. Gels were run at 100 V for 16 h. Gels were silver stained as described by Nicol et al. (2005) and band presence/absence data was recorded. Selected DGGE bands were excised, purified using a QIAquick PCR purification kit (Qiagen) and sequenced unidirectionally by GATC Biotech using the reverse primer. Sequences were checked for ambiguous bases and identified to the nearest match (NCBI BLAST, http://blast.ncbi.nlm.nih.gov).

Enzyme activity

Microbial enzyme activity in sediment slurry microcosms was measured by a fluorescein diacetate (FDA) hydrolysis method as described by Adam and Duncan (2001). Spectrophotometric analysis (at 490 nm) was carried out using a Versamax Microplate reader (Cape Cod Inc.). Enzyme activity was calculated based on the concentration of hydrolysed FDA present in samples when plotted against a fluorescein sodium salt (Sigma Aldrich) standard curve (0–2.5 μg mL^{-1}).

Total and active cell counts

A cell staining method was developed herein that maximised cell detachment from particles and minimised background

fluorescence, whilst maintaining the integrity of microorganisms. Total and active microbial cell counts in sediment slurry microcosms were determined by 4′,6-diamidino-2-phenylindole (DAPI) and 5-cyano-2,3-di-(p-tolyl)tetrazolium chloride (CTC) cell staining. Briefly, 100 μL samples were diluted 10-fold in 10 mM tetrasodium pyrophosphate and sonicated in an ultrasonic water bath (75 Watts, 40 kHz; Decon F5 Minor, Decon Ultrasonics Ltd) containing crushed ice for 10 min. Large particles were left to settle for 1 min before 10 μL subsamples were further diluted (1000-fold final dilution) and stained with 5 mM CTC (Sigma–Aldrich) for 2 h in the dark at 30 °C with shaking (100 rpm). Samples were fixed with formaldehyde (2% (v/v) final concentration) for 1 h at room temperature and counterstained with DAPI (Sigma) (1 μg mL^{-1} final concentration) for 15 min.

Stained samples were filtered onto 0.2 μm black polycarbonate membrane filters (Whatman), transferred to microscope slides, coated with a drop of ProLong® Gold antifade reagent (Invitrogen) and left overnight in the dark at room temperature to dry. Microscopic analysis was performed using a Nikon Eclipse Ti Widefield fluorescent microscope at 600× magnification, with UV light passage through DAPI (342–380 nm excitation, 435–485 nm emission, 400 nm DM) and Cy3 (510–560 excitation, 572.5–647.5 nm emission, 565 nm DM) filters. Fifteen fields of view per filter (i.e. a total of 30 per sample) were imaged. Total (DAPI-stained) and active (CTC-stained) cells were counted and analysed using the software ImageJ (National Institutes of Health).

Statistical analyses

Two-way repeated measures analysis of variance (ANOVA) with pairwise comparisons was used to test for significant differences between samples in enzyme activity, cell counts and percent active cells. One way ANOVAs and *t*-tests were used to further test for significant differences between samples in enzyme activity, cell counts and percent active cells at each time point, and to test for significant differences in hydrocarbon-degradation (PASW Statistics v18, IBM). Similarity matrices (Jaccard S7 coefficient) were performed based on DGGE gel band profiles (binary data) in Primer 6 (Primer-E Ltd). Significant differences between communities were explored using analysis of similarities (ANOSIM), and were displayed as non-metric multidimensional scaling (NMDS) plots (Primer-E Ltd).

Results

Effect of AgNPs on microbial hydrocarbon biodegradation

Whilst Total Petroleum Hydrocarbon (TPH) biodegradation varied between silver treatments ($F_{6,20} = 4.97$, $P < 0.01$), exposure to all forms of silver at 0.5 and 50 mg L^{-1} had no significant impact on TPH biodegradation by day 7 compared to non-silver exposed controls ($P > 0.05$) (Fig. 2A). Although background hydrocarbon concentrations in the sediments were low (<0.5% of the concentration added experimentally), there was a significant reduction (between 76 and 89 %; $t_4 = 8.51$, $P = 0.001$) in TPH with the live control microcosms (i.e. not exposed to silver) by day 7 compared to killed controls which was attributed to biodegradation by *in situ* microbial communities (Fig. 2A).

Further analysis also revealed no significant effect of uAgNPs, cAgNPs or Ag_2SO_4 on alkane and PAH biodegradation ($P > 0.05$) (Fig. 2B and C). In general, all samples showed greater biodegradation of short chain alkanes (89–96% degradation of C_{11}–C_{20} alkanes) compared to long chain alkanes (62–79% degradation of C_{31-39} alkanes) and branched alkanes (59–77% degradation of pristane and phytane) by day 7 (Fig. 2D). There was also greater biodegradation of low ring-number PAHs (99–100 % degradation of naphthalene) compared to high ring-number PAHs (44–62% degradation of pyrenes and chrysenes) by day 7 (Fig. 2E).

Effect of AgNPs on bacterial community structure and composition

In non-oiled sediments, significant shifts in total bacterial community structure occurred in response to all silver treatments ($R = 0.61$, $P = 0.001$) (Fig. 3A). Specifically, by day 7, the total bacterial community composition of all non-silver exposed sediments were >80% similar to one another, but had <60% similarity to the communities in silver exposed sediment (at 0.5 and 50 mg L^{-1}). Specifically, DGGE band sequence analysis revealed that a member of the phylum Bacteroidetes (Band B5, Supplementary Fig. S1 and Supplementary Table 1) were more prominent in non-oiled microcosms exposed to 50 mg L^{-1} Ag_2SO_4 after both 7 and 14 days compared to non-silver exposed controls. In oil-amended sediments, the total bacterial community composition also underwent distinct shifts in response to all silver treatments ($R = 0.75$, $P = 0.001$) (Fig. 3B). By day 7, the total bacterial community composition of all non-silver exposed sediments demonstrated >80% similarity to one another, but had <60% similarity to communities in sediments exposed to silver (at 0.5 and 50 mg L^{-1}). Specifically, sequence analysis revealed that the intensity of a band closely related to a member of the genus *Aquabacterium* (Band B8, Supplementary Fig. S1 and Supplementary Table 1) was greater in oiled-sediments incubated with 0.5 and 50 mg L^{-1} cAgNP by day 7 compared to non-silver exposed controls. Furthermore, a member of the Phylum Firmicutes (Band B8, Supplementary Fig. S1 and Supplementary Table 1) was more prominent after 14 days in oil-amended sediments incubated with 50 mg L^{-1} Ag_2SO_4, compared to non-silver exposed controls.

Differences in the active members of sediment bacterial communities were also characterized (Fig. 3C and D, Supplementary Fig. S2). In non-oiled sediments, the active bacterial community composition underwent distinct shifts with all silver exposures ($R = 0.96$, $P = 0.001$) (Fig. 3C). Specifically, by day 14, the bacterial community composition of all non-silver exposed sediments demonstrated >80% similarity to one another and had <80% similarity to communities in sediments exposed to silver (at 0.5 and 50 mg L^{-1}). The active bacterial communities from oil-amended slurries also varied significantly between the microcosms exposed to silver and non-silver exposed controls ($R = 0.94$, $P = 0.001$) (Fig. 3D). Specifically, by day 7, the active bacterial community composition of all non-silver exposed sediments demonstrated >80% similarity to one another and had <80% similarity to communities in sediments exposed to silver (at 0.5 and 50 mg L^{-1}).

Time of measurement also had a significant impact on the total bacterial communities of both non-oiled ($R = 0.93$, $P = 0.001$) and oil-amended ($R = 0.98$, $P = 0.001$) sediments, with significant shifts in structure occurring after both 7 and 14 days ($P = 0.001$) (Fig. 3A and B). Bacterial taxa that shifted over time included a *Gaetbulibacter* spp. (Band 3, Supplementary Fig. S1, Supplementary Table S1) and two bands that were closely related to Flavobacteria (Bands 1 and 4, Supplementary Fig. S1, Supplementary Table S1), which were all present in non-oiled microcosms at day 0 but were not detected in samples by days 7 or 14. The time of measurement also had a significant impact on active bacterial community structure in both non-oiled ($R = 0.99$, $P = 0.001$) and oil-amended ($R = 0.85$, $P = 0.001$) sediments, with significant changes occurring after 14 days ($P = 0.001$) (Fig. 3C and D). Sequence analysis of selected bands from bacterial DGGE profiles revealed that *Hydrogenophaga* sp., *Pseudomonas* sp., *Aquabacterium citratiphilum,* and a Firmicutes clone (DGGE bands

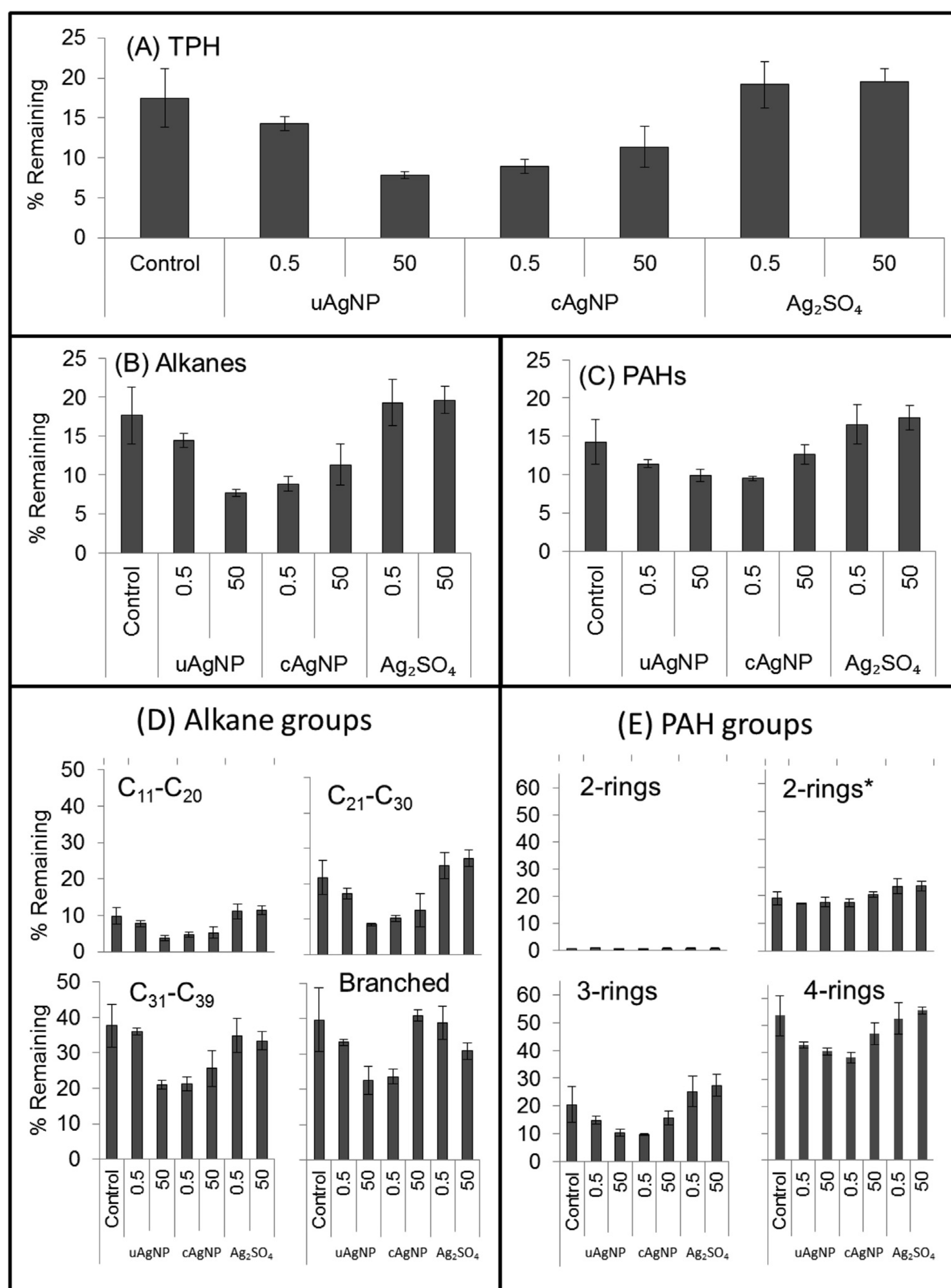

Fig. 2. Biodegradation of (A) total petroleum hydrocarbons (TPH), in sediment slurry microcosms after 7 days incubation with 0.5 and 50 mg L^{-1} uAgNPs, cAgNPs or Ag_2SO_4. Controls were not exposed to any form of silver. Results show % hydrocarbons remaining where 0.1% (v/v) crude oil was added at day 0, and were calculated relative to killed controls. Results are further broken down into (B) alkanes, (C) PAHs, (D) alkane groups (including C_{11}–C_{20}, C_{21}–C_{30}, C_{31}–C_{49} and branched alkanes pristane and phytane), and (E) PAH groups (including naphthalenes (2-rings), fluorenes and dibenzothiophenes (2-rings*), phenanthrenes and anthracenes (3-rings) and chrysenes (4-rings)). (Error bars represent the standard error of the mean, $n = 3$). Rapid initial biodegradation (days 0–7) was followed by negligible biodegradation between days 7–14, therefore hydrocarbon biodegradation results are presented at day 7 only.

B6-B9, Supplementary Fig. S1, Supplementary Table S1) were detected in oil-amended microcosms but not in non-oiled microcosms.

Effect of AgNPs on microbial enzyme activity

Microbial enzyme activity in sediments subjected to crude oil and silver exposure was measured over the whole study (Supplementary Fig. S3). Microbial enzyme activities in both non-oiled and oil-amended sediments were not significantly affected by the addition of uAgNPs, cAgNPs or Ag_2SO_4 up to 50 mg L^{-1}, over 14 days ($F_{6,20} = 0.945$, $P > 0.05$ and $F_{6,20} = 4.01$, $P > 0.05$, respectively). However, enzyme activity was significantly greater in oil-amended controls (i.e. no addition of silver) compared to non-oiled control microcosms by days 7 (291 compared to 110 μg fluorescein hydrolysed g^{-1} dws; $t_4 = -11.07$, $P < 0.001$) and 14 (565 compared to 230 μg fluorescein hydrolysed g^{-1} dws; $t_4 = -9.74$, $P = 0.001$) days. Enzyme activity also varied significantly over time in both non-oiled ($F_{2,8} = 662.14$, $P < 0.001$) and oil-amended ($F_{2,8} = 145.95$, $P < 0.001$) microcosms. Specifically, in non-oiled

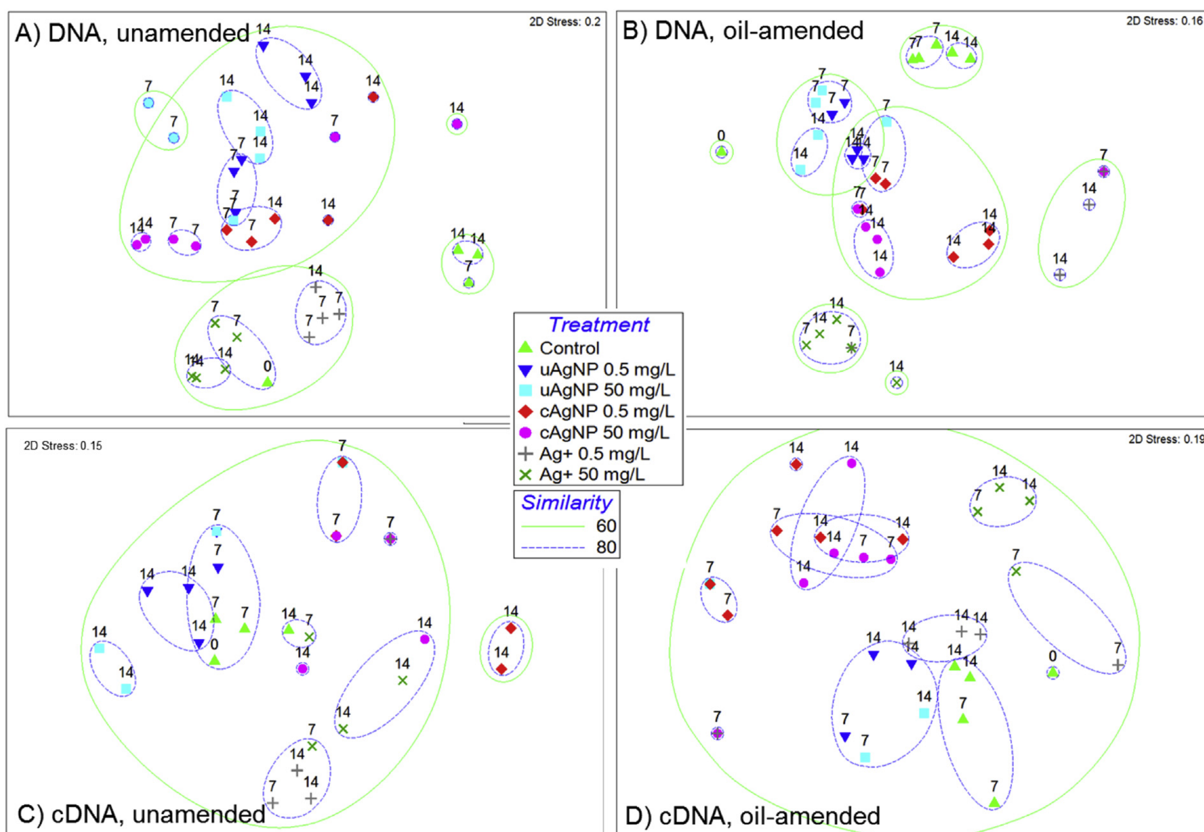

Fig. 3. Non-metric multidimensional scaling (NMDS) biplots of bacterial community structure based on DGGE band analysis of PCR-amplified 16S rRNA genes (A–B) and gene transcripts (C–D) (Jaccard coefficient). Biplots show non-oiled microcosms (A and C), and oil-amended microcosms (B and D). Communities are labelled by time (0, 7 or 14 d), and treatment (control, uAgNPs, cAgNPs or Ag_2SO_4 at 0.5 or 50 mg L^{-1}). Dotted lines represent communities with >60% and 80% similarity. 3D stress were lower for A (0.12), B (0.09), C (0.08) and D (0.11).

control microcosms, enzyme activity decreased from 606 to 110 μg fluorescein hydrolysed g^{-1} dws between days 0 and 7 ($P = 0.005$). After day 7, there was an increase in enzyme activity from 110 to 230 μg fluorescein hydrolysed g^{-1} dws by day 14 ($P = 0.01$). In oil-amended control microcosms, enzyme activity reduced from 605 to 291 μg fluorescein hydrolysed g^{-1} dws between days 0 and 7 ($P = 0.01$), then increased to 565 μg fluorescein hydrolysed g^{-1} dws between days 7 and 14 ($P = 0.001$).

Effect of AgNPs on total and active microbial cells

Total microbial cell counts are shown in Fig. 4A and B and the proportion of active cells are shown in Fig. 4C & D. Significant reductions in the total cell numbers after 14 days in non-oiled microcosms were measured ($F_{6,19} = 5.69$, $P < 0.005$) in sediments exposed to 50 mg L^{-1} cAgNPs (from 1.66 to 0.84 × 10^7 cells g^{-1} dws; $P = 0.05$), 0.5 mg L^{-1} Ag_2SO_4 (from 1.66 to 0.66 × 10^7 cells g^{-1} dws; $P < 0.01$), and 50 mg L^{-1} Ag_2SO_4 (from 1.66 to 0.90 × 10^7 cells g^{-1} dws; $P < 0.05$) (Fig. 4A). There was also significant inhibition to the proportion of active cells by day 7 in non-oiled microcosms exposed to 50 mg L^{-1} cAgNPs (with a reduction from 30 to 23%; $P < 0.05$), and 0.5 mg L^{-1} Ag_2SO_4 (with a reduction from 30 to 18%; $P < 0.01$) (Fig. 4C).

Total cell counts also varied significantly over time in both non-oil-amended ($F_{2,8} = 111.45$, $P < 0.001$) and oil-amended microcosms ($F_{2,8} = 91.89$, $P < 0.001$). Specifically, cell counts in non-oiled controls (i.e. no added silver) increased between days 7 and 14 from 7.0 × 10^6 to 1.7 × 10^7 cells g^{-1} dws ($P = 0.001$) (Fig. 4A). In oil-amended controls, total cell counts decreased between days 0 and 7 from 7.8 × 10^6 to 6.1 × 10^6 cells g^{-1} dws ($P < 0.01$), then increased significantly between days 7 and 14 from 6.1 × 10^6 to 9.1 × 10^6 cells g^{-1} dws ($P < 0.05$) (Fig. 4B). Time of measurement also had a significant effect on the proportion of active cells in oil-amended microcosms ($F_{2,8} = 77.36$, $P = 0.001$). Specifically, there was a significant decrease in active cells from 27% to 8% between days 0 and 14 in oil-amended control microcosms ($P = 0.01$) (Fig. 4D). The addition of crude oil had a significant effect on the proportion of active cells in sediments. By day 7, oil-amended controls (i.e. non-silver-exposed) contained a significantly lower proportion of active cells (14%) compared to non-oiled controls (30%) ($t_4 = 6.48$, $P < 0.005$).

Discussion

This study is one of the first to investigate the impact of engineered AgNPs on microbial hydrocarbon biodegradation. We demonstrated that whilst AgNPs do not appear to effect the ability of microbial hydrocarbon-degraders to biodegrade crude oil, they do affect the structure of estuarine microbial communities, which may have potential implications for other important microbial-mediated processes in estuaries. Specifically, exposure to uAgNPs, cAgNPs and Ag_2SO_4 (up to 50 mg L^{-1}) had no significant impact on total petroleum hydrocarbon (TPH) biodegradation by sediment microbial communities over 7 days, suggesting that the activity of microbial hydrocarbon-degraders was highly resistant to the antimicrobial effects of silver. Indeed, resilience and even stimulation of microbial communities in response to nanosilver have been observed previously.

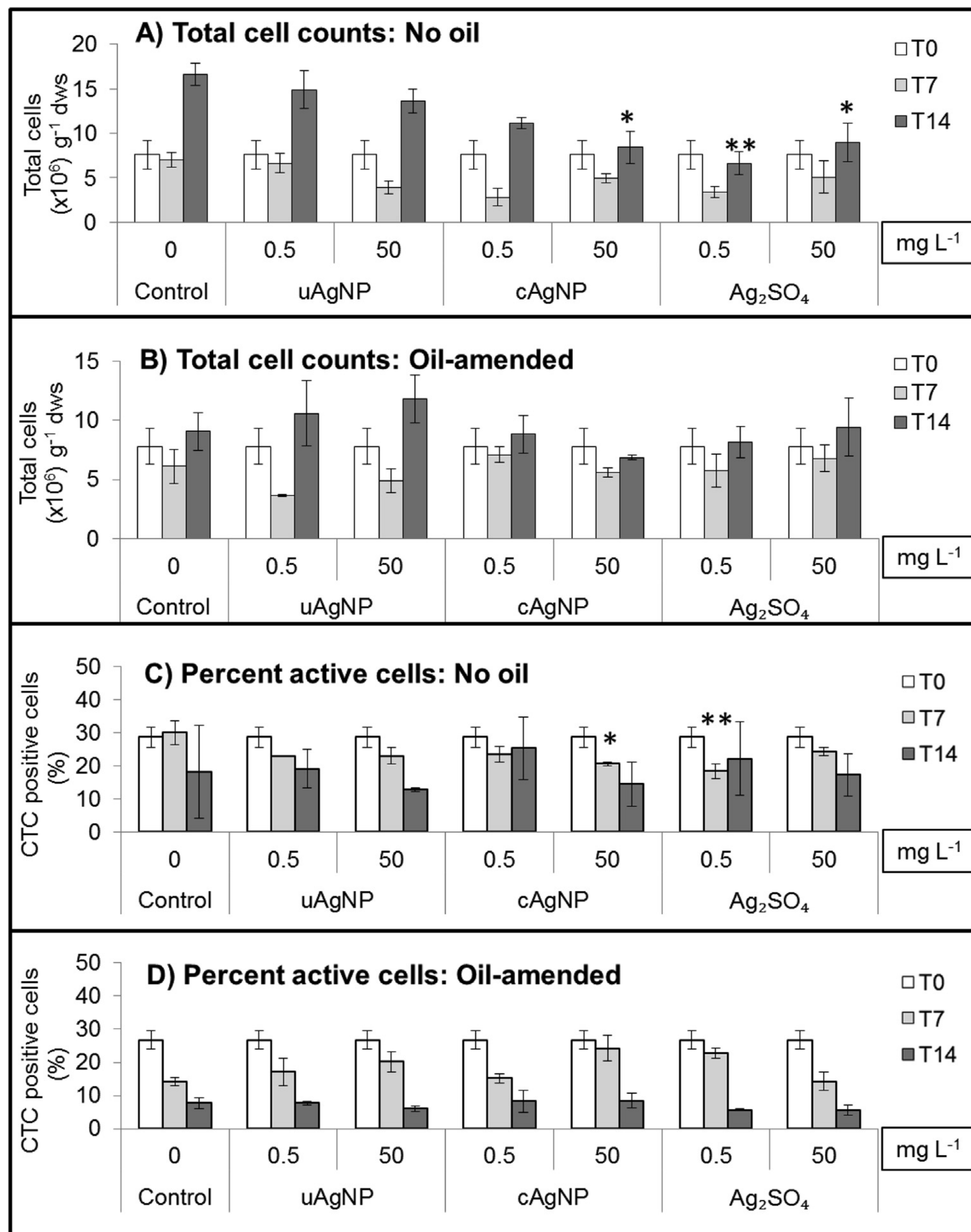

Fig. 4. Total microbial cells in (A) non-oiled microcosms, and (B) microcosms, and the proportion of active cells in (C) non-oiled microcosms, and (D) microcosms containing 0.1% (v/v) crude oil after 0 (T0), 7 (T7) and 14 (T14) days when exposed to 0.5 or 50 mg L^{-1} uAgNPs, cAgNPs or Ag_2SO_4. Controls were not exposed to any form of silver. Results that are significantly different to no-nanoparticle controls are shown by * ($p \leq 0.05$) and ** ($p \leq 0.01$). (Error bars represent the standard error of the mean, $n = 3$).

For example, Xiu et al. (2012) demonstrated a hormetic response (a favourable biological response to low toxin exposure) by *Escherichia coli* exposed to up to 16.4 mg L^{-1} AgNPs. Das et al. (2012a) recorded a 4–10% increase in activity by freshwater bacteria exposed to ≤ 0.02 mg L^{-1} AgNPs for 5 days (which was a lower concentration of nanosilver than those used in the present study). Das et al. (2012b) also demonstrated increased bacterial production and improved aminopeptidase hydrolysis affinity by communities exposed to up to 0.5 mg L^{-1} AgNPs over 48 h, and proposed that rare bacterial phylotypes containing silver resistance genes may have been stimulated by low concentrations of AgNPs (Das et al., 2012a,b). It is possible that hydrocarbon-degraders in estuarine sediments have acquired silver-resistance genes due to prolonged exposure to metal contaminants (Silver, 2003). Indeed, heavy metal contamination has been demonstrated previously in nearby estuaries along the Essex coastline (Emmerson et al., 1997; Matthiessen et al., 1999).

It is also possible that other factors contributed to the unperturbed activity of sediment hydrocarbon degraders in the Colne estuary in the presence of silver. For example, it is possible that the silver may have reacted with the low concentrations of sulphur-containing compounds (0.8%) present in the light Forties crude oil, muting any potential antimicrobial activity and contributing to the unperturbed rates of hydrocarbon degradation observed. It is also possible that the oil itself could have changed the dissolution properties of AgNPs, but detailed analyses of such effects were not part of this study. In addition, changes in silver speciation can occur depending on redox conditions and for ionic silver speciation is a major factor determining ecotoxicity.

Like Coulon et al. (2012), we generally observed high overall rates of crude oil biodegradation in Colne estuary sediments. Furthermore, hydrocarbon biodegradation followed a classical pathway whereby the breakdown of short chain alkanes exceeded that of long chain alkanes and branched alkanes by day 7, and the biodegradation of low ring-number PAHs was greater than that of high ring-number PAHs (Atlas, 1981). It was notable that fast initial rates of biodegradation slowed after day 7 and may have been caused by either nutrient limitation or the production of toxic metabolites (such as the ethanoic acid equivalents of the parent compounds (Johnson et al., 2011)) which inhibited *in situ* hydrocarbon-degraders, leading to reduced hydrocarbon biodegradation rates (Prince et al., 2003).

Despite the functional resilience of hydrocarbon-degraders in the Colne estuary, microbial communities from both non-oiled and oil-amended sediments underwent distinct shifts in response to all silver treatments. Microbial communities often demonstrate high sensitivity to disturbance events in terms of community structure (Allison and Martiny, 2008; Ager et al., 2010). However, the functions of microbial communities are not always lost as a result. For example, Wertz et al. (2007) demonstrated the resilience of microbes involved in denitrification and nitrite oxidation following a disturbance event, despite induced reduction in microbial diversity prior to the event. Doiron et al. (2012) also observed significant shifts in community structure after 3 days exposure to 5 and 50 $\mu g\ L^{-1}$ AgNPs. In the present study, a few rare DGGE bands increased in abundance in silver-exposed sediments. This phenomenon was also observed by Das et al. (2012a) in the presence of AgNPs at 0.01–1 mg L^{-1}, and may be related to the stimulation of a few rare phylotypes that are resistant to silver exposure.

Time of measurement also effected the total and active microbial communities. It is possible that a bottle effect (whereby environmental communities undergo changes when subjected to laboratory conditions) may have also played a role in the observed changes in community structure (Ferguson et al., 1984; Massana et al., 2001; Gattuso et al., 2002; Agis et al., 2007). This could be overcome in future by scaling-up the size of experimental microcosms or by taking measurements *in situ*.

It has been shown previously that AgNPs are capable of binding to bacterial surface proteins inhibiting enzyme activity (Wigginton et al., 2010). In the present study, microbial enzyme activity in both non-oiled and oil-amended sediments were unaffected by all silver treatments. Therefore, the activities of *in situ* sediment microbial communities from the Colne estuary are highly resistant to AgNP exposure. In general, greater microbial enzyme activity was observed in oil-amended microcosms compared to non-oiled microcosms, which is likely to have resulted from the provision of crude oil as a carbon and energy source, leading to the stimulation of specific hydrocarbon-degrading microbial communities and an increase in the production of associated enzymes involved in hydrocarbon biodegradation (Fleeger et al., 2003; Sargian et al., 2007).

The effect of silver on actively respiring sediment microorganisms and total sediment microorganisms was determined herein, as not all microbial cells within environmental samples are live or active (Proctor and Souza, 2001). Sediment bacteria are notoriously difficult to visualize under a microscope as they adhere to sediment particles (Epstein and Rossel, 1995; Proctor and Souza, 2001) which obscure cells and create autofluorescence (Weinbauer et al., 1998). Although a method was developed in this study that maximised cell detachment from sediment particles and minimized background fluorescence, complete elimination of non-specific fluorescence was unavoidable and may have contributed to variability in the results.

Despite their lack of impact on hydrocarbon-degradation, exposure to 0.5–50 mg L^{-1} Ag_2SO_4 and 50 mg L^{-1} cAgNPs had a detrimental impact on total cell counts in non-oiled sediment slurry microcosms after 14 days. In addition, the proportion of active cells in non-oiled microcosms was significantly reduced by day 7 in response to 0.5 mg L^{-1} Ag_2SO_4 and 50 mg L^{-1} cAgNPs. Although one previous study found no effect of AgNPs up to 1 mg L^{-1} on estuarine sediment microbial cell abundance (Bradford et al., 2009), others have also demonstrated a detrimental effect of AgNPs on total microbial cell counts after shorter exposure periods (from 1 h) and lower AgNPs concentrations (from 5 to 1000 $\mu g\ L^{-1}$) than those used herein (Doiron et al., 2012; Das et al., 2012a).

Oil-amended microcosms were unaffected by silver exposure in terms of total cell abundance and the proportion of active cells, which was surprising as exposure to multiple stressors has previously been shown to have a synergistic detrimental effect on microbial communities (Sargian and Mas, 2007). In agreement with our findings, Mühling et al. (2009) found no additional effect of up to 2 mg L^{-1} AgNPs on estuarine bacterial cell counts when exposed to a range of antibiotics over 50 days. It is possible that the stimulation of specific microorganisms following the provision of crude oil as a carbon and energy source may have counteracted any reductions in the number of silver-susceptible species observed herein (Fleeger et al., 2003; Sargian et al., 2007).

The physicochemical conditions of an environment can also have a profound effect on AgNP size and solubility. AgNPs entering surface water are likely to become associated with natural colloids and so settle out and become incorporated into the sediment. Furthermore, in some aquatic systems (particularly marine), nanoparticles tend to aggregate, the rate of which is dependent on surface charge, particle shape and size, and pH of the medium (Whiteley et al., 2013). As a result of aggregation, surface area and dissolution potential are reduced. Capped nanoparticles have enhanced colloidal stability and so capped and uncapped particles may behave differently from each other (Badawy et al. 2010; Fabrega et al. 2011). In addition, the capping agent can affect the size and surface charge of particles which may affect particle toxicity which in turn affect the diversity and activity of microbial communities.

In the present study, water salinity was low and total organic carbon (TOC) content was high (Supplementary Table S2), suggesting that the AgNPs were unlikely to aggregate but may have become coated with natural organic matter which can reduce rates of Ag^+ dissolution (Baalousha et al., 2008; Handy et al., 2008; Christian et al., 2008; Liu and Hurt, 2010; Unrine et al., 2012). Indeed, we have previously demonstrated that both the uAgNPs and cAgNPs do not aggregate, and undergo reduced rates of Ag^+ dissolution when dispersed in freshwater from the Colne estuary (Poole, 2013). Unlike the cAgNPs, the uAgNPs had no impact on sediment microbial cell counts or the proportion of active cells in the present study, suggesting that the uAgNPs were less toxic than the cAgNPs. Differences in size are likely to have led to differential toxic effects between the cAgNPs and uAgNPs (Beddow et al., 2014). Specifically, the smaller cAgNPs would have increased contact with cell membranes and increased rates of toxic Ag^+ dissolution (Sondi and Salopek-Sondi, 2004; Sotiriou et al., 2011; Ma et al., 2012). Such differences in toxicity between the cAgNPs and uAgNPs will in turn differentially affect the microbial community diversity and activity.

Conclusion

AgNPs are likely to enter estuarine systems following increasing production and use of engineered nanomaterials in consumer products (Gottschalk et al., 2009). Despite this, little is currently

known about the potential impact of AgNPs on hydrocarbon-degrading microbial communities in estuarine sediments. The Colne estuary provided a model system from which to measure the effect of AgNPs on hydrocarbon-degraders, due to its susceptibility to oil pollution and the close proximity to a large wastewater treatment works which is a potential release site for AgNPs. Overall, whilst AgNPs have the potential to shape estuarine sediment bacterial community structure and reduce total cell numbers, communities were resilient in terms of hydrocarbon biodegradation and enzyme activity, suggesting that microbial hydrocarbon biodegradation in estuarine systems may be resistant to the antimicrobial effects of AgNPs. However, other key microbial-driven processes (such as nitrification) may be more sensitive to the antimicrobial effects of AgNPs and at a greater risk. It is thus important to continue to measure the effects of AgNPs on microbial communities.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2014.09.004.

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