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RESEARCH ARTICLE

Effects of fullerene (C60), multi-wall carbon nanotubes (MWCNT), single wall carbon nanotubes (SWCNT) and hydroxyl and carboxyl modified single wall carbon nanotubes on riverine microbial communities

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Abstract Commercial production of nanoparticles (NP) has created a need for research to support regulation of nanotechnology. In the current study, microbial biofilm communities were developed in rotating annular reactors during continuous exposure to 500 $\mu g\ L^{-1}$ of each nanomaterial and subjected to multimetric analyses. Scanning transmission X-ray spectromicroscopy (STXM) was used to detect and estimate the presence of the carbon nanomaterials in the biofilm communities. Microscopy observations indicated that the communities were visibly different in appearance with changes in abundance of filamentous cyanobacteria in particular. Microscale analyses indicated that fullerene (C60) did not significantly ($p < 0.05$) impact algal, cyanobacterial or bacterial biomass. In contrast, MWCNT exposure resulted in a significant decline in algal and bacteria biomass. Interestingly, the presence of SWCNT products increased algal biomass, significantly in the case of SWCNT-COOH ($p < 0.05$) but had no significant impact on cyanobacterial or bacterial biomass. Thymidine incorporation indicated that bacterial production was significantly reduced ($p < 0.05$) by all nanomaterials with the exception of fullerene. Biolog assessment of carbon utilization revealed few significant effects with the exception of the utilization of carboxylic acids. PCA and ANOSIM analyses of denaturing gradient gel electrophoresis (DGGE) results indicated that the bacterial communities exposed to fullerene were not different from the control, the MWCNT and SWNT-OH differed from the control but not each other, whereas the SWCNT and SWCNT-COOH both differed from all other treatments and were significantly different from the control ($p < 0.05$). Fluorescent lectin binding analyses also indicated significant ($p < 0.05$) changes in the nature and quantities of exopolymer consistent with changes

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in microbial community structure during exposure to all nanomaterials. Enumeration of protozoan grazers showed declines in communities exposed to fullerene or MWCNT but a trend for increases in all SWCNT exposures. Observations indicated that at 500 µg L^{-1}, carbon nanomaterials significantly alter aspects of microbial community structure and function supporting the need for further evaluation of their effects in aquatic habitats.

Keywords Carbon nanotubes · Fullerenes · Effects · Microbial activity · Diversity · Metabolism

Introduction

The development and application of nanotechnology have raised significant concerns about the adverse effects of nanomaterials on human health and the environment. Manufactured nanomaterials have been reported as potentially more toxic than larger particles of the same composition because of their large specific surface area and unique catalytic properties. The commercial production of nanomaterials and their incorporation into a variety of products have generated a need for research to support regulation of the nanotechnology sector (Handy et al. 2008). Worldwide CNT production values were estimated by Mueller and Nowak (2008) to range from 350 to 500 tons/year. Recent estimates of demand for CNTs are in the range of 3700–4100 tons increasing to 10,500–12,000 tons by 2020. Nanoparticles produced in large amounts include carbon black, fullerenes and a range of carbon nanotubes. Carbon nanomaterials have a wide variety of applications due to their: high tensile strength, electronic conductance, semi-conductor potential, high surface area and potential for sorption (Ajayan et al. 1999; Ajayan and Zhou 2001; Ball 2001). These applications include aerospace, fibre production, semiconductors, sorbents and remediation (Ajayan and Zhou 2001; Lee et al. 2005). Carbon-based nanomaterials are also finding applications in water treatment, waste water treatment, drug delivery, as well as food packaging preservation (Theron et al. 2008). Their use in cosmetics has been extensive and would likely contribute to aquatic environmental loading (Chae et al. 2010). Commercial scale production and use with growing demand raises clear concerns regarding the potential health and environmental effects of these materials (Dreher 2004).

The applications proposed for this technology suggest that nanoparticles could enter aquatic systems through direct discharges and from industrial as well as domestic wastewater effluents. In their review, Petersen et al. (2011) note that most CNTs are part of a variety of composites that may weather or even be incinerated. The modelling exercises of Gottchalk et al. (2009, 2010) and Mueller and Nowack (2008) assume that the bulk of CNTs manufactured is part of a variety of polymer-containing products assumed to enter the environment through landfills. The fraction reaching wastewater treatment plants, which is largely unknown, will be derived from clothing and fabric manufacturing and other textile applications (Kohler et al. 2008). Of greatest concern may be their proposed use in pollution control and in situ remediation where they may be released intentionally (Mauter and Elimelech 2008; Apul et al. 2012).

Environmental concentrations of nanomaterials are largely unknown, although modelling has permitted estimates, ranging from 620 kg $year^{-1}$ for nanosilver and 47,300 kg $year^{-1}$ for TiO_2 entering the surface waters of Switzerland (Mueller and Nowack 2008). Releases of this magnitude would result in nano to microgram levels in the receiving environment (Mueller and Nowack 2008). Modelling-based estimates of CNTs in the aquatic environment are generally in the low ng L^{-1} (Gottshalk et al. 2009). Of course, hot spots may arise as a result of manufacturing, transport and disposal. Although relatively little is known regarding actual environmental concentrations, concerns have been raised about the potential toxicity and environmental impacts of CNTs (Handy et al. 2008; Petersen et al. 2011). Indeed, a number of studies have shown that carbon nanomaterials have antimicrobial properties under pure culture conditions (e.g., Kang et al. 2007; Ghafari et al. 2008; Kang et al. 2009; Neal 2008). Carbon nanomaterials appear to require close contact and may disrupt membranes as a result of electrostatic, oxidative or physical puncturing interactions and most notably by production of reactive oxygen or nitrogen species (Jackson et al. 2013). Complex microbial community studies have concentrated on impacts in sewage effluents and sludges (Kang et al. 2009; Yin et al. 2009; Luongo and Zhang 2010; Goyal et al. 2010). While Tong et al. (2007) and Chung et al. (2011) examined effects of fullerene (C60) and multiwalled carbon nanotubes (MWCNT) respectively on a soil microbial community, they found no effect on diversity based on DGGE, biomass or enzyme activity. Velzeboer et al. (2011) examined the effects of high levels of MWCNTs in aquatic sediments with no effect on invertebrate diversity and increased numbers during 3-month exposures. In contrast, their long-term (15 months) study detected significant effects of 2000 µg L^{-1} MWCNT on sediment community structure (Velzeboer et al. 2013). However, conditions, such as high concentrations of organic matter found in complex environmental systems, including soil, anaerobic sludge and wastewater effluent, may mitigate carbon nanomaterial toxicity to varying degrees (Tong et al. 2007; Kang et al. 2009; Lawrence et al. 2016). In terms of environmental fate and effects, it is apparent that these nanomaterials may be modified upon entering the environment undergoing aggregation (Chen et al. 2004), coating with organic matter and cations, as well as potential modification by oxidants or microorganisms (Hyung et al. 2007). Lawrence et al. (2016) describe the nature of the coatings developing on MWCNTs and SWCNTs in

association with aquatic biofilms demonstrating that they are highly complex and result in a reduction in toxicity through suppression of ROS production. Toxicity of nanomaterials in the natural environment is intimately linked to environmental parameters, including natural organic matter and solution chemistry which may be far more important in dictating the toxicology of the nanomaterials than the as manufactured state (Petersen et al. 2011).

As has been noted in a wide variety of studies (Haak and McFeters 1982a, b; Lawrence et al. 2004; Battin et al. 2009), microbial communities represent the base of the food web, driving most biogeochemical cycles and so-called ecosystem services. Therefore, it is essential to examine the fate and effects of these materials under controlled but representative conditions. Biofilms are already known as extensive natural sinks for metals, pesticides, antimicrobial agents and a variety of other environmental contaminants (Dynes et al. 2006a, b; Wolfaardt et al. 1994). Based on this and observations regarding apparent scavenging of nanoparticles by bacterial EPS (Liu et al. 2007), Battin et al. (2009) suggested that biofilms may be exposed to higher levels of nanoparticles than planktonic communities. It is suggested that exopolymeric substances (EPS) represent the most likely point of interaction for nanomaterials with the biological compartment of aquatic ecosystems and that both direct and indirect effects may occur as a result of bioaccumulation of carbon nanomaterials in the EPS pool and their subsequent introduction into the food web. We have used complex river biofilm communities and multimetric analyses to assess the fate and effects of a wide range of environmental contaminants, including municipal wastewater effluents, metals, pharmaceuticals and personal care products (Lawrence et al. 2004, 2005, 2009, 2012). Rotating annular reactors have been used to generate communities on defined substrata that are then subjected to analyses that are microscopic, molecular, genomic, functional and activity based. Here, we have applied this approach in conjunction with scanning transmission X-ray microscopy analyses to assess the exposure, fate and effects of a panel of carbon nanomaterials in these complex microbial communities.

Materials and methods

Microcosm operation

The experimental setup and reactor design for biofilm development has been described in detail previously (Lawrence et al. 2000, 2004). Natural river water (South Saskatchewan River, Saskatoon, SK, Canada) was used as inoculum and as a source of carbon and nutrients. The nutrients and nanoparticles were added directly to the individual reactors using a peristaltic pump. Nutrient levels were assessed as described

by Chenier et al. (2003). Typical water chemistry for the South Saskatchewan River, an oligotrophic, carbon-limited, alkaline pH system is shown in Table 1. The reactors were maintained at 21 ± 2 °C in keeping with environmental conditions during the experimental period (May–June). The water was pumped through the reactors at a rate of 500 ml per day (one reactor volume) by using a multichannel peristaltic pump (Watson Marlow, Wilmington, MA). Treatments included the addition of 500 $\mu g \, L^{-1}$ of the following carbon nanomaterials (CNM): fullerene (C60), multi-wall carbon nanotubes (MWCNT), single wall carbon nanotubes (SWCNT) and SWCNT that are hydroxyl and carboxyl modified SWCNT-OH and SWCNT-COOH. Table 2 provides detailed information on the specific characteristics of the nanomaterials used in this study. Additional characterization using transmission electron microscopy as well as scanning transmission X-ray microscopy is presented by Lawrence et al. (2016). The concentration selected considers levels predicted by modelling in most cases "low" ng L^{-1} (Gottschalk et al. 2009; Mueller and Nowack 2008), although Boxall et al. (2007) predicted a steady-state fullerene concentration of 0.31 $\mu g \, L^{-1}$ and those showing effects in testing with complex communities (2000 $\mu g \, L^{-1}$, Velzeboer et al. 2013) as well as lowest observed effect concentrations in single species testing (algae, 53 $\mu g \, L^{-1}$, Schwab et al. 2011). Therefore, the concentration selected is greater than modelled estimates and the lowest observed effects concentration reported for algae but intended to be "more realistic" than the high milligram and gram levels used in many other exposures as well as using a community based rather than single species exposure. CNM suspensions were created by sonication (three cycles of 10 s on/off at 65 w/20 khz) in sterile water using a Bransonic Ultrasonic processor 10 s on/off at 65 w·20 khz prior to addition to the reactors. In addition, control reactors were operated that received river water alone. Biofilms were grown under treatment and control conditions in bioreactors for a period of 50 days, at which time coupons were removed for immediate analysis.

Table 1 Typical chemical data for Saskatchewan River water (spring and summer)

Parameter	Spring	Summer
Conductivity (μmhos cm^{-1})	451	429
pH	8.13	8.46
Turbidity (NTU)	2.7	5
Ammonia (mg N l^{-1})	0.04	0.03
Nitrate-nitrite (mg N l^{-1})	0.75	0.31
Orthophosphate (mg P l^{-1})	0.01	0.01
Dissolved organic carbon (mg C l^{-1})	3.5	3.0
Total suspended solids (mg l^{-1})	1	1

Table 2 Characterization of carbon nanomaterials

CNM	Purity	Dimensions	SSA	BD	TD	Functionalization
MWCNT	>95 %	OD > 50 nm/ID 5-15 nm/lgth 10–20 μm	>40 m²/g	0.05 g/cm³	2.1 g/cm³	0
SWCNT (308068-56-6)	>90 %	OD 1-2 nm/lgth 5–30 μm	>490 m²/g	0.14 g/cm³	–	0
SWCNT-OH	>90 %	OD 1-2 nm/lgth 5–30 μm	>490 m²/g	0.14 g/cm³	–	4 wt% (308068-56-6)
SWCNT-COOH (308068-56-6)	>90 %	OD1-2 nm/lgth 5–30 μm	>490 m²/g	0.14 g/cm³	–	2.75 w%
Fullerene C60	>99 %					

(CAS #)

SSA specific surface area, *BD* bulk density, *TD* true density

Supplier: M K Impex Canada (MKnano); 6382 Lisgar Drive; Mississauga, Ontario, L5N 6X1

Confocal laser scanning microscopy and image analysis

All stained and control materials were analyzed by confocal laser microscopy (Nikon -C2, Confocal laser microscope) attached to a Nikon Eclipse 80i standard light microscope, equipped with 488/543/633 nm excitation as well as reflection and transmission imaging (Nikon, Chiyoda, Tokyo, Japan) using fluorescent staining, Syto9 (Life Technologies, Burlington, ON, Canada) and *Triticum vulgaris* lectin-TRITC (Sigma, St. Louis, MI) to visualize bacterial cells and exopolymer respectively as described in detail (Neu et al. 2001).

Exopolymer analyses

Lectins labeled with either fluorescein isothiocyanate or TRITC (Sigma, St. Louis, MO) or Cy5 (Research Organics, Cleveland, OH) were applied for exopolymer analyses. The lectins *T. vulgaris* (β(1,4) *N*-acetyl glucosamine, *N*-acetyl neuraminic acid), *Arachis hypogaea* (terminal β-galactose, *N*-acetyl galactosamine (associated with algal-cyanobacterial polymers)), *Canavalia ensiformis* (α-linked mannose or glucose residues), *Glycine* max (terminal α- or β-linked *N*-acetylgalactosamine; associated with algal-cyanobacterial polymers) and *Ulex europaeus* (α-L-fucose) were used alone or in combination for in situ analyses of polymer composition. Staining, imaging, image analyses and calculations of lectin binding volumes were carried out by using the equations of Neu et al. (2001).

Protozoan and micrometazoan enumeration

Protozoa and micrometazoa were enumerated using phase contrast microscopy. Samples were removed from the reactors on a weekly basis and the numbers of protozoa and micrometazoa manually counted on replicate 2 cm² subsamples using phase contrast microscopy.

Carbon utilization spectra

Carbon utilization spectra were determined for biofilm samples using commercial Eco-plates (Biolog, Hayward, CA) (Lawrence et al. 2004).

Chemical analyses

All effluents from the reactors and carbon nanomaterials working solutions were assessed for the presence of metal contaminants by subjecting waters and materials to ICP-MS analyses. Samples were submitted to the Saskatchewan Research Council Analytical Facility in Saskatoon SK for extraction and determination of metal levels by ICP-MS.

Scanning transmission X-ray microscopy and data analysis

STXM data was measured on beamline 10ID1 at the Canadian Light Source (CLS, Saskatoon, SK, Canada) (Kaznatcheev et al. 2007). Further details of the X-ray fluorescence detector and its operation are presented elsewhere (Hitchcock 2012).

All STXM samples were prepared by deposition of 1–5 μL of the biofilm solution material onto Si_3N_4 windows (1 × 1 mm, thickness 100 nm on a 200-μm thick chip, 5 mm × 5 mm, Norcada Inc., Edmonton, Canada) and analysed as described in detail (Dynes et al. 2006a, b; Lawrence et al. 2016). STXM was used analytically by measuring image sequences at specific energies (Jacobsen et al. 2000) or from image difference maps which are the difference of on- and off-resonance images (Dynes et al. 2006a). Data analysis was performed using aXis2000 (Hitchcock 2014).

Molecular analyses

Total community DNA extraction For each treatment bioreactor, a frozen (−80 °C) polycarbonate strip was aseptically cut (2 cm²) and transferred to a 50-ml polypropylene tube

(Falcon, Becton Dickinson, Franklin Lanes, NJ). Bacterial cells from the frozen biofilm samples were removed from the polycarbonate strip with a sterile metal scraper and total DNA was extracted by using the FastDNA spin kit for soil (Bio101 systems Qbiogene, Carlsbad, CA) according to the manufacturer's instructions.

PCR amplification The bacterial 16S rRNA gene was amplified using "universal" primers to perform DGGE. The primer consensus sequence forward was 5′- CCT ACG GGA GGC AGC AG -3′ (preceded by a GC clamp for DGGE (not for sequencing) = CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC G (40 nt)) and reverse, 5′- CCG TCA ATT CMT TTG AGT TT-3′ position (length) 341–357 (17 nt) and 907–926 (20 nt), respectively, and the amplified PCR fragment size was 586 base pairs (Muyzer et al. 1993; Muyzer and Ramsing 1995). PCR amplification was conducted in a 25-µl reaction volume containing 1 µl of DNA template, 10 pmol of each appropriate primer as described by Muyzer et al. (1993, Muyzer and Ramsing 1995), 1.25 U Taq DNA polymerase (New England Biolabs Ipswich, MA.), 1× PCR buffer, 2.5 mM $MgCl_2$ and 200 µM dNTPs. A touchdown PCR program using the PTC-200 thermocycler (MJ Research, Inc. Waltham, MA) consisted of an initial denaturation step of 94 °C for 5 min, followed by 10 cycles of denaturation at 94 °C for 1 min, annealing at 66 °C (decreasing in each cycle by 1 °C) for 1 min and an elongation step of 72 °C for 1 min. Following these steps, another 20 cycles of 95 °C for 1 min, annealing at 56 °C for 1 min and elongation at 72 °C for 1 min, with a final elongation step of 72 °C for 7 min, were performed. The correctly sized PCR product was verified by electrophoresis on a 1.5 % w/v agarose gel in 1.0× Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA) for 1.0 h at 100 V. Gels were stained using ethidium bromide and documented using the AlphaImager 3300 gel documentation and image analysis system (Alpha Innotech Corporation, San Leandro, CA).

Denaturing gradient gel electrophoresis analysis

After the specificity and size of the amplified products were checked on agarose gels, the PCR product was separated by denaturing gradient gel electrophoresis analysis (DGGE) (Muyzer et al. 1993; Muyzer and Ramsing 1995) using an Ingeny phorU2 system (Ingeny, Leiden, The Netherlands). Aliquots (20 ul) of PCR product were mixed with 4 µL of loading dye buffer and resolved on a 6 % (w/v) polyacrylamide gel in 1.0× TAE buffer using denaturing gradients from 45 to 65 % (100 % denaturant contains 7 M urea and 40 % deionized formamide). DGGE was carried out at 40 V for 10 min and then 100 V for 18 h at 60 °C. After electrophoresis, the gel was stained with SYBR Green I (1:10,000 dilution; Molecular Probes, Eugene, OR) for 15 min, with gentle agitation and photographed using the AlphaImager 3300 gel documentation and image analysis system (Alpha Innotech Corporation, San Leandro, CA).

Experimental design and statistical analyses

The experimental design consisted of an untreated control and exposure to carbon-based nanoparticles at 500 µg L^{-1}. River biofilm communities were allowed to develop in the absence of the nanomaterials or in their presence for 50 days. Each treatment had 3 identical replicate reactors randomly assigned to it on the reactor bench (replications). Each analysis was done on subsamples from randomly selected biofilm coupons from among the 12 identical coupons in each replicate reactor. The confocal laser scanning microscopy (CLSM) imaging was done at 5 random locations across a transect on a 1 cm^2 piece of the biofilm coupon from each reactor $n = 3$. Subsampling for other analyses, protozoan counts, chlorophyll-a, thymidine incorporation, carbon utilization analyses as well as molecular DGGE analyses was also carried out using three randomly selected subsamples from among the 12 identical coupons in each replicate reactor ($n = 3$). Analysis of variance was used to detect significant differences among sample means at $p < 0.05$. Analyses were carried out using the commercial package, MiniTab (State College, PA, USA).

Band detecting, matching and processing of DGGE gels were completed with the GelCompare II software 4.6 (Applied Maths, Kotrijk, Belgium). Fingerprint data was processed by generating a band-matching table (Boon et al. 2002). The binary data was exported and compared by principal component analysis (PCA) with PRIMER v6 software (PrimerE-Ltd Lutton, UK). Statistical analyses of PCA scores generated from the first two axes were run using an analysis of similarity (ANOSIM) with PRIMERr v6 software (Clarke 1993). The inclusion of DGGE ladders allowed GelCompareII to normalize the position of bands in all of the lanes under examination. PRIMER v6 was also used to perform PCA and cluster analyses on other data sets obtained from the analyses, i.e. carbon utilization, biomass, lectin binding, etc.

Results and discussion

Algae

Impacts on photosynthetic organisms may arise through a variety of direct and in direct mechanisms; however, it is generally agreed that effects are largely driven by direct interactions with the nanomaterials (Jackson et al. 2013). In the case of algae direct shading of the cells particularly as a result of agglomeration of CNTs and algae cells appears to a possible

source of inhibition. Schwab et al. (2011) exposed *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* to either pristine or oxidized CNTs. They reported that for *C. vulgaris* the lowest observed effects concentration (LOEC) was 0.53 mg L^{-1} for each type of CNT, whereas *P. subcapitata* was much less sensitive with reduced growth only when exposed to dispersed pristine CNT at LOEC 5.5 mg L^{-1}. They determined that irreversible binding of CNTs to the cell surfaces and formation of aggregates resulting in shading was the mechanism (Schwab et al. 2011). In contrast, Bennet et al. (2013) examined interactions of five CNTs with *P. subcapitata* concluding that shading was not a factor in toxicity which they ascribed to photoactivity of the CNTs which reduced growth by up to 200 %. At the concentration used in the current study and based on STXM imaging of the biofilms (Fig. 3), it appears that shading did not play a role in these complex communities (see also Lawrence et al. 2016). Long et al. (2012) carried out studies investigating the effects of a panel of MWCNTs with diameters 10, 20–40, and 60–100 nm on *C. vulgaris*. These authors reported that growth inhibition of *C. vulgaris* occurred at EC50 values of 41.0, 12.7 and 12.4 mg L^{-1}, corresponding to increasing diameter of the CNTs. Our observations suggest no effect of diameter given that both MWCNTs and SWCNT had effects on algal biomass (Fig. 1). These authors also reported that placing the cultures in the dark markedly reduced toxicity (EC50 values of 62.2, 36.8 and 46.3 mg L^{-1} respectively), concentrations far in excess of 500 μg L^{-1}. Thus, as is frequently the case in the literature, light mediated toxicity is indicated for CNTs; however, this was not addressed in the current study. Exposure of the algae *Dunaliella tertiolecta* to carboxylated MWCNT indicated that the LOEC for oxidative stress and photosynthesis occurred at 10 mg L^{-1} (Wei et al. 2010), an LOEC well above the exposure concentrations achieved here. Our observations on river biofilm communities indicated that only two of the CNTs had a significant effect on algal biomass and no product

had an effect on the cyanobacterial biomass (Fig. 1). Velzeboer et al. (2008) similarly reported no effects of nominal concentrations of up to 100 mg/L fullerene (C60) and SWCNTs. MWCNT exposures resulted in a significant ($p < 0.05$) reduction in algae biomass, while SWCNT-COOH in the reactor and biofilm community resulted in a decline in algal biomass ($p < 0.05$) (Fig. 1). STXM observations would suggest that CNTs were integrated into the biofilm community and in intimate association with cells in the biofilm. The distribution and quantities did not appear consistent with coating and shading of the algae; therefore, other mechanisms maybe acting in this case. In general, high values for no observed effects have been reported with the exception of *C. vulgaris* were the LOEC was 53 μg L^{-1} for each type of CNT (Schwab et al. 2011) and our observation of effects for MWCNT and SWCNT-COOH treatments at 500 μg L^{-1}.

Bacteria

As outlined by Jackson et al. (2013) and others, there are a number of recognized mechanisms for the antimicrobial properties of CNTs, they: (i) disrupt membranes due to strong electrostatic interactions, by oxidation of the membrane or physically puncturing the cell envelop particularly during cell-CNT agglomeration, (ii) production of reactive oxygen species has been observed for CNTs and suggested as a significant source of damage to the cell, DNA, proteins, etc. It has also been noted that: (iii) numerous impurities coming from manufacturing catalysts and suspension solutions may be major sources of toxicity found in CNTs (Kang et al. 2007, 2008; Luongo and Zhang 2010, Arias and Yang 2009, Yang et al. 2010; Nel et al. 2006; Musee et al. 2011). Fullerenes have been reported to exhibit antimicrobial properties by a number of authors (Fortner et al. 2005; Lyon et al. 2006, 2008; Fang et al. 2007); however, there remains a lack of clarity with regard to the mechanisms involved. It has been suggested that fullerene (C60) has a high affinity for electrons and thereby disrupts the electron transport chain of bacteria precipitating cell death (Lyon et al. 2008). Our observations suggest that with the exception of observable effects on protozoa, other metrics were not impacted by the presence of fullerene in the river biofilm communities.

In this regard, Kang et al. showed that the main CNT mechanism contributing to the inactivation of *Escherichia coli* was direct contact with the highly purified CNTs (Kang et al. 2007, 2008). It was also apparent that size and length were critical factors in this interaction with the much smaller size of SWCNTs apparently contributing to their increased toxicity relative to MWCNTs (Kang et al. 2008). A number of investigations have noted the relative toxicity SWCNT > MWCNT (Jia et al. 2005; Kang et al. 2007, 2008, 2009). Jia et al. (2005) examined a panel of SWNTs and MWNTs (with a diameter of 10–20 nm, MWNT10), and fullerenes (C60) which were: all
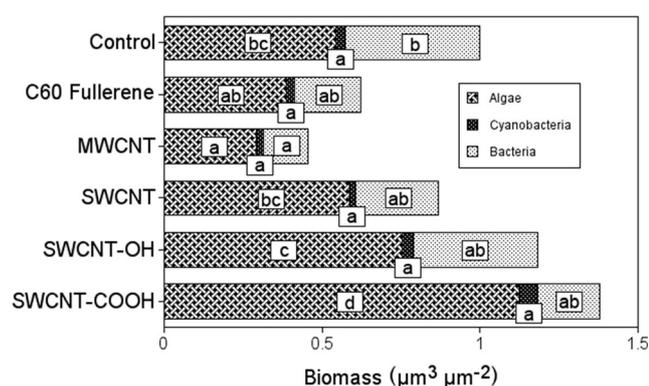

Fig. 1 Results of ANOVA ($n = 3$, $p < 0.05$) using the results of bacterial, cyanobacterial and algae biomass determined by digital image analyses of confocal image stacks (representative 3D projections in Fig. 8). Parameters indicated by *different letters* are significantly different from the control at $p < 0.05$

made of carbon, covered a range of surface areas, sizes and chemical properties. Using mammalian cells (guinea pig macrophages) and a phagocytosis assay they found that in terms of toxicity SWNTs > MWNT10 > C60. The lowest observed effects concentrations reported were 0.38 μg cm^{-2} for SWCNTs and 3.06 μg cm^{-2} MWCNT and C60. Similarly, other authors have found that for interactions with pure cultures, river water and effluents SWCNTs have exhibited the highest toxicity relative to other CNTs (Kang et al. 2009). It has also been claimed that MWCNT may be sites where bacteria proliferate (Akhavan et al. 2009). The difference in toxicity between these two CNTs has been ascribed the nanometric size of SWCNTs which allows greater interaction with the bacterial cell (Kang et al. 2008).

In contrast, we found that when bacterial biomass as determined by confocal laser microscopy and digital image analyses was considered, only the MWCNT treatment resulted in a significant ($p < 0.05$) reduction (Fig. 1). It has been noted that biofilms respond differently relative to planktonic bacteria to exposure to CNTs. Rodrigues and Elimelech (2010) found that to have the same impact on a biofilm required 10× the concentration of SWCNT relative to cells not protected by an EPS matrix. Similarly, Luongo and Zhang (2010) attributed a dose-dependent relationship between MWCNT concentration and respiration inhibition, where sheared mixed liquor demonstrated a greater degree of inhibition compared to unsheared mixed liquor. These authors suggested that the extracellular polymeric substances (EPS) associated with biological flocs which are intact in unsheared materials offer protection from the CNTs. It may be speculated that the EPS matrix modulates exposure to nanomaterials in biofilm systems contributing to variable outcomes depending upon CNT access to the cell envelop. The role of organic matter (Tong et al. 2012) in mitigating toxicity of CNTs in soils and aquatic environments parallels the effects of EPS in biofilm situations. These factors are particularly relevant given that most effects of CNTs appear to be extremely short range phenomena. As noted by Kang et al. (2007), direct close contact between the CNT and bacteria is proposed to cause bacterial cell death. Thus, aggregation and interactions with EPS may both mitigate in situ toxicity of these nanomaterials.

Protozoa

Protozoa and micrometazoans have been described as ecosystem engineers (Jones et al. 1994; Weerman et al. 2011) playing a critical role selective grazing impacting community composition (Glucksman et al. 2010; Lawrence et al. 2002) as well as regulation of carbon cycling and nutrient availability to higher trophic levels (Parry 2004). Thus, any impacts of CNTs on protozoa would have significant effects in natural and engineered aquatic systems. We observed that the presence of C60 fullerene and MWCNT had a negative impact on protozoan numbers over the time course of community development. In contrast, the three SWCNTs all appeared to stimulate protistan grazer populations (Fig. 2). Other authors have described negative impacts of oxidized SWCNTs and MWCNTs (Ghafari et al. 2008; Chan et al. 2013; Zhu et al. 2006) on ingestion and digestion of bacteria by protozoa. These processes may play a role in the current studies. During exposures to an oxidized SWCNT, Ghafari et al. (2008) documented the apparent loss of mobility and cell death at a LOEC of 1.6 mg L^{-1}, impaired bacterial feeding at LOEC 3.6 mg L^{-1} and loss of viability occurred at LOEC 6.8 mg L^{-1}. Similarly, Zhu et al. (2006) found that *Stylonychia mytilus* growth was inhibited by a LOEC of 1 mg L^{-1} of a functionalized MWCNT. However, these concentrations are significantly higher than those predicted for aquatic environments and substantially higher than those used in the current study (500 μg L^{-1}). Interestingly, and in contrast to our observations, Velzeboer et al. (2008, 2011) reported that lower doses of a MWCNT were in fact stimulatory to growth of *S. mytilus* in laboratory and field observations, whereas Velzeboer et al. (2013) in a longer term (15 months) study reported significant impacts of 2000 μg L^{-1} MWCNTs deemed a more realistic concentration, on sediment invertebrate communities. It may also be as observed for *Daphnia magna* that carbon nanomaterials (fullerenes) impede swimming behavior (Lovern et al. 2007). Thus, there are a number
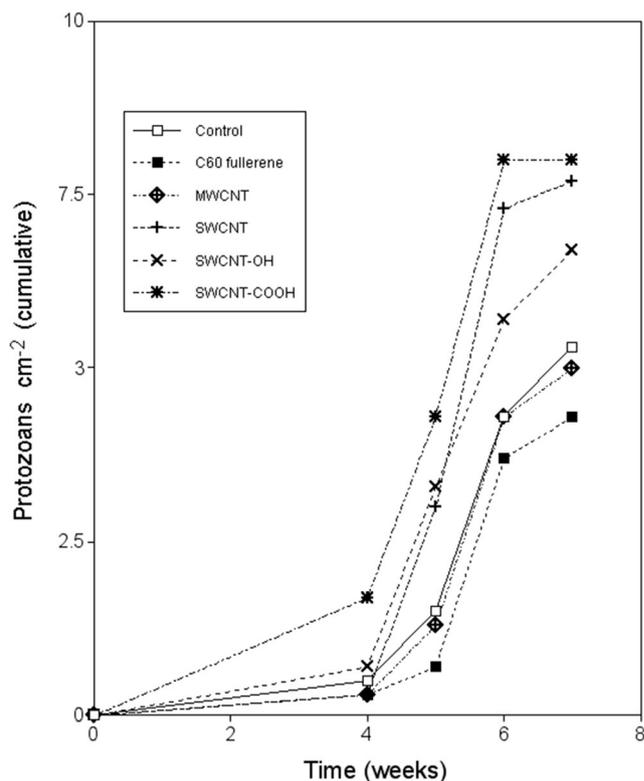

Fig. 2 Results of protozoan analyses by treatment show the cumulative counts for protozoa on a weekly basis ($n = 3$)

of potential mechanisms that may contribute to in situ effects;
it is also important to note that the diversity of protozoa active
in natural biofilm communities is not reflected in those used
for toxicological studies.

Community-level effects

The microbial community may be the most relevant level of
organization for assessing impacts of nanomaterials in aquatic
environments, particularly given that biofilms are thought to
be major sinks for nanomaterials (Battin et al. 2009) and are
major drivers of ecosystem function. A range of diversity and
functional assessments may be used to determine effects of
CNTs on the microbial community. To date, community-
focused studies in soils and water have not used more realistic
concentrations, i.e. reflecting modelled estimates (Gottschalk
et al. 2009, 2010; Mueller and Nowack 2008) which suggest
CNT levels would be from high ng L^{-1} to low µg L^{-1} at most.
Most frequently, exposures have been in the mg/L to low g
L^{-1} or g/kg range; in the current study, we have attempted
achieve an exposure of 500 µg L^{-1} in a constantly mixed
system (Lawrence et al. 2000). We have also applied a range
of approaches to control and estimate exposure in the biofilm
system, including ICP-MS analyses of water (Table 3) as well
as scanning transmission X-ray microscopy and
spectromicroscopy (STXM) of biofilms (Fig. 3). Our STXM
analyses would indicate that the "in biofilm" exposures are

Table 3 Results of ICP-MS analyses of reactor water

Metal Source	Cr	Co	Cu	Mn	Mo	Ni	Ag
Control	bld	bld	0.9	bld	1.6	1.2	bld
Fullerene C60	bld	bld	1	bld	1.6	1.2	bld
MWCNT	bld	bld	1	bld	1.6	1.5	bld
SWCNT	bld	bld	0.7	bld	1.6	1.2	bld
SWCNT-OH	bld	bld	0.6	bld	2.0	1.3	bld
SWCNT-COOH	bld	bld	0.8	bld	1.6	1.2	bld

Units = µg L^{-1}

Bld below limit of detection

very "patchy" and may be essentially zero at many locations
but may be locally very high in the biofilm SWCNTs. This
phenomenon is evident in Fig. 3 which shows an example of
mapping of the aggregated SWCNT carbon nanomaterials in
in the context of biofilm showing diatoms, bacteria and poly-
meric substances using STXM. We have also analysed the
nanomaterials for the presence of metals using STXM analysis
at the Ni 2p edge on pure compounds and in the biofilm.
Further, ICP-MS analyses were performed which did not re-
veal elevated levels of any metals in the reactor waters during
the experiment (Table 3). Exposure to CNTs in our experi-
ments did not result in significant changes in broad measures
such as biofilm thickness or chlorophyll-a content of the com-
munity (Fig. 4). Although digital imaging indicated signifi-
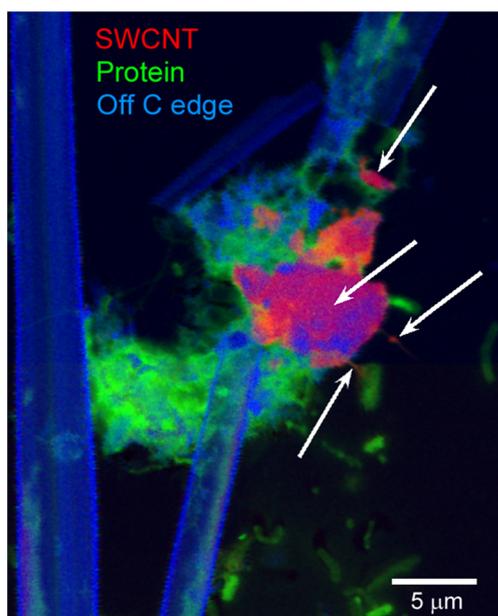cant changes ($p < 0.05$), a reduction in algal biomass in

Fig. 3 STXM C1s images of biofilm exposed to SWCNT illustrating
clumping, aggregation and localized distribution in the biofilm. *Red*
indicates SWCNT materials, *green* indicates protein, bacterial cells and
polymeric materials, *blue* indicates non-specific optical density showing
diatoms, *arrows* indicate large and small aggregates in the context of
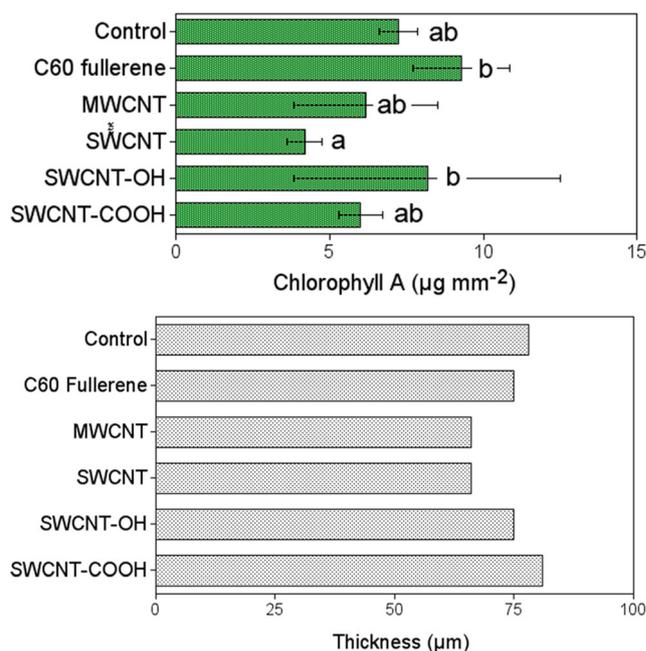diatoms, bacteria and polymeric substances

Fig. 4 Graphs of chlorophyll-a concentrations (*top*) and thickness in
biofilms exposed to the panel of carbon-based nanomaterials.
Parameters indicated by *different letters* are significantly different from
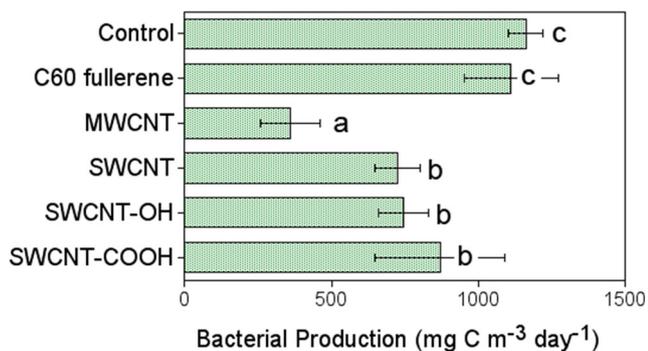the control at ($p < 0.05$, $n = 3$)

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Fig. 5 Graph of bacterial production based on thymidine incorporation. Parameters indicated by *different letters* are significantly different $p < 0.05$ ($n = 3$, ANOVA)

MWCNT treatment and an increase in algal biomass in SWCNT-COOH exposures (Fig. 1). Thymidine incorporation, a measure of bacterial production was not significantly affected ($p < 0.05$) by fullerene exposure but was reduced by MWCNT and all SWCNT exposures (Fig. 5). Soil and sediment-based studies have applied MWCNTs significantly reducing microbial biomass carbon and nitrogen as well as enzymatic activity when applied at 5 g kg^{-1}, but not at 0.5 g kg^{-1} (Chung et al. 2011). While Velzeboer et al. (2011) found that when MWCNTs were added at 2 g kg^{-1}, no impacts on invertebrate diversity were observed and macroinvertebrate numbers increased. Authors working with wastewaters have similarly described CNT induced toxicity on the microbial community. For example, Kang et al. (2009) reported toxic effects of MWCNT and SWCNT correlated with increasing bacterial cell inactivation in wastewaters. When Yin et al. (2009) examined SWCNT containing effluents, there was a reduction in chemical oxygen demand

removal by the microorganisms, which was confirmed by Goyal et al. (2010). Similarly, Luongo and Zhang (2010) found a decrease in microbial respiration correlated with increasing MWCNT concentration in a mixed liquor. Thus, our observations of negative impacts on bacterial production are in keeping with the literature.

Carbon utilization spectra generated using the Biolog Ecoplate system has proven to be a sensitive indicator of effects of various stresses on microbial communities and has been applied in a range of studies (Lawrence et al. 2004, 2009). Here, we applied the system to assess the impact of a panel of CNTs on carbon utilization in river biofilm communities. Curiously, of the 30 carbon substrates present in the assay, SWCNT-COOH did not suppress or increase utilization in any case, SWCNT suppressed utilization of 4-hydroxbenzoic acid and L-phenylalanine ($p < 0.05$) while fullerene increased utilization of glycogen and hydroxybutyric acid, MWCNT increased use of 4-hydroxybenzoic acid and hydroxybutryic acid utilization and SWCNT-OH treatments significantly increased utilization of L-phenylalanine and hydroxybutyric acid (Fig. 6). Although there are no other directly comparable studies in the literature, the narrow range of impacted carbon substrata appears unusual when compared to studies of impacts of metals (Lawrence et al. 2004) or pharmaceuticals (Lawrence et al. 2005) where much broader effects were detected.

To investigate the diversity and nature of the community, we applied fluorescent lectin binding analyses (Neu et al. 2001) to determine the amount and nature of the exopolymeric substances produced by the CNT exposed and reference communities. Figure 7 shows the results of these analyses, a shift in total EPS can be seen with significant reductions apparent
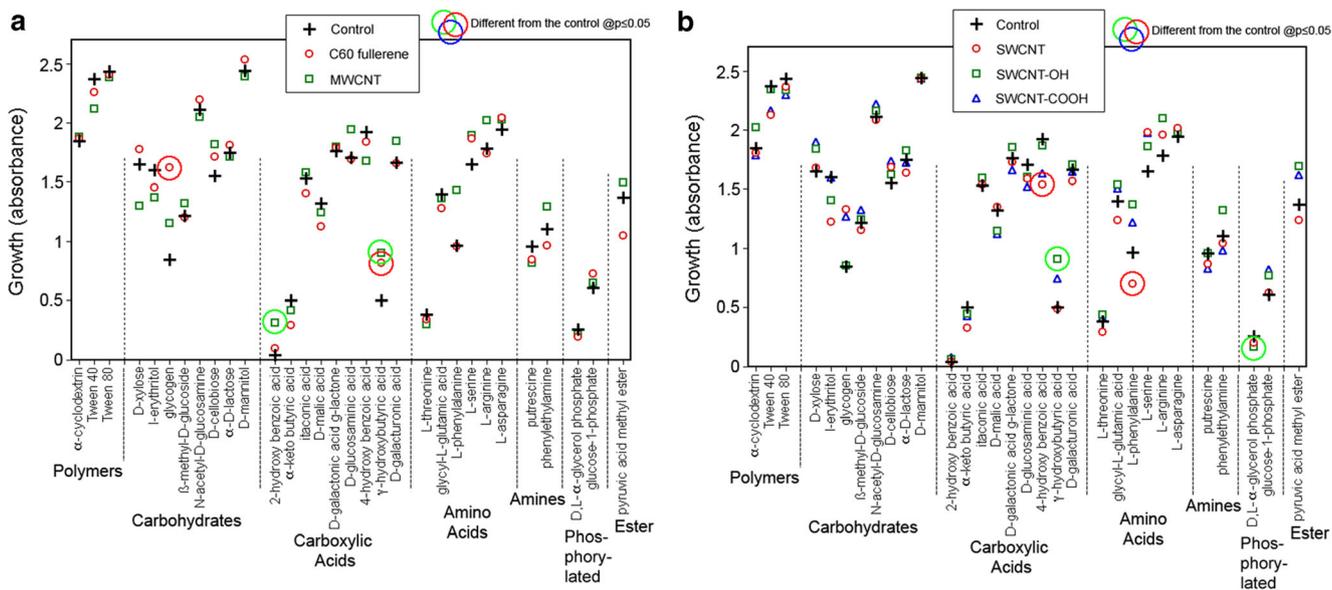

Fig. 6 Results of carbon utilization assays using the Biolog Ecoplate system showing shifts in utilization of specific substrates classes relative to the reference community ($n = 3$, ANOVA, $p < 0.05$)
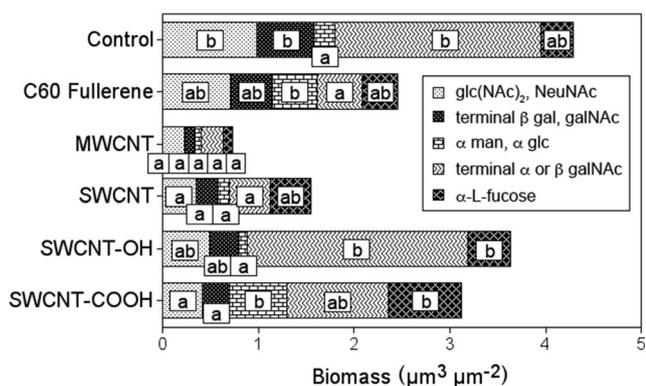
Fig. 7 Results of fluorescent lectin binding analyses of major sugar residues in the exopolymers of the treatment communities. *Bars with different letters* are significantly different from each other, the reference community and other treatments ($n = 3$, $p < 0.05$, ANOVA)

in the MWCNT and SWCNT treatments in particular. It is also evident that each treatment results in a unique EPS fingerprint based on the results from binding of the five lectin panel (Fig. 7). These patterns are reflective of the nature of the organisms producing the polymeric substances and thus the biodiversity of the community. Relative to reference conditions (Fig. 7), each community shows shifts in the amounts of terminal β-galactose, *N*-acetyl galactosamine residues (associated with algal-cyanobacterial polymers), but no significant change in α-L-fucose residues, which are more in keeping

with bacterial EPS. Visual comparison of the confocal microscopy images representative of those used for digital image analyses (Figs. 1, 4 and 7) demonstrate the unique appearance of the communities (Fig. 8). That the bacterial diversity in these communities has changed was confirmed by the PCA analyses of the results of DGGE analyses (Fig. 9). Other authors have addressed the impact of CNTs on microbial diversity using approaches such as DGGE (Muyzer and Ramsing 1995; Muyzer et al. 1993). The application of denaturing gradient gel electrophoresis (DGGE) did not identify any changes in the microbial community structure in soil (Tong et al. 2007) or anaerobic biosolids (Nyberg et al. 2008) when exposed to C60 fullerenes. These results parallel those obtained in the current study where the C60 fullerene community is not significantly different (ANOSIM $p < 0.05$) from the control community based on its DGGE fingerprint. Tong et al. (2012) demonstrated that repeated applications of "as manufactured" SWCNTs affected microbial community structure as measured by DGGE and altered metabolic activity in a low organic matter soil. However, the authors concluded that the SWCNT treatments did not produce "significantly altered" microbial communities. They did, however, detect changes in specific organisms detected by sequencing DGGE bands but concluded that only minor changes were observed in community structure when SWNTs were applied at 1000 μg g^{-1} soil weekly, accumulating to 6000 μg g^{-1} over
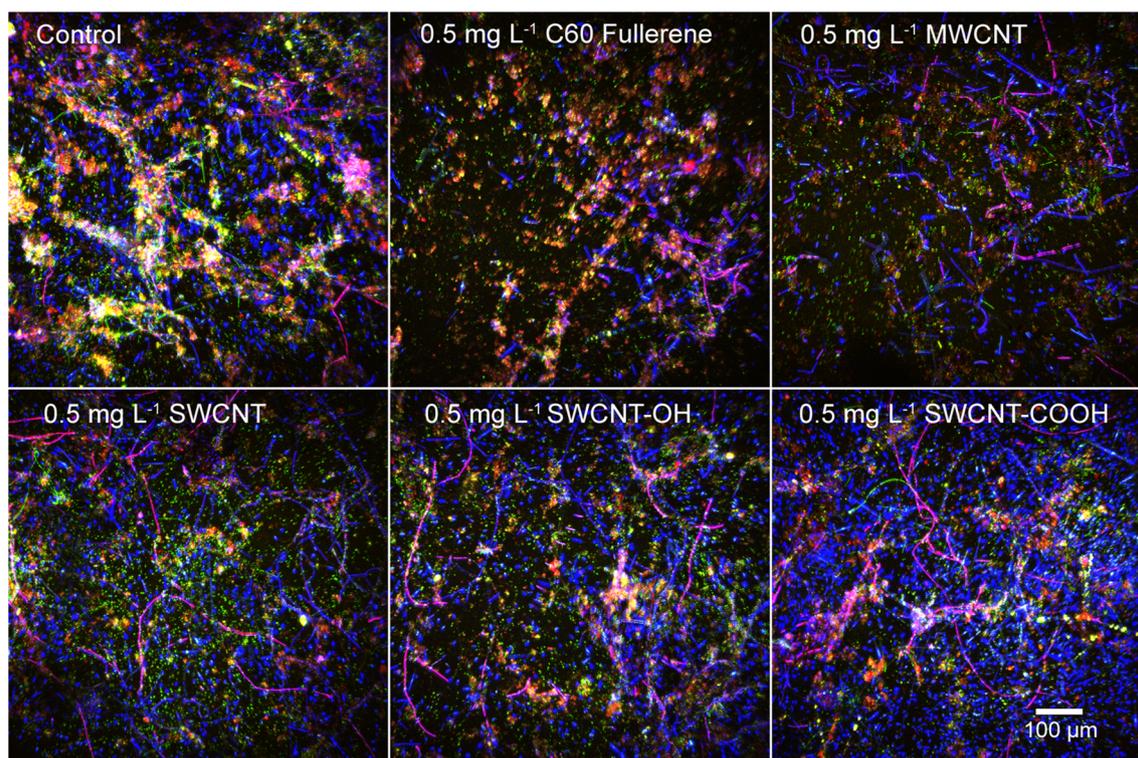

Fig. 8 Representative CLSM photomicrographs of control, exposed to C60 fullerene, MWCNT, SWCNT, SWCNT-OH and SWCNT-COOH continuously for an 8-week experimental period. Bacteria (*green*), *Triticum vulgaris*-TRITC lectin binding polymer (*red*), photosynthetic biomass (*blue/magenta*)

Bacterial DNA DGGE
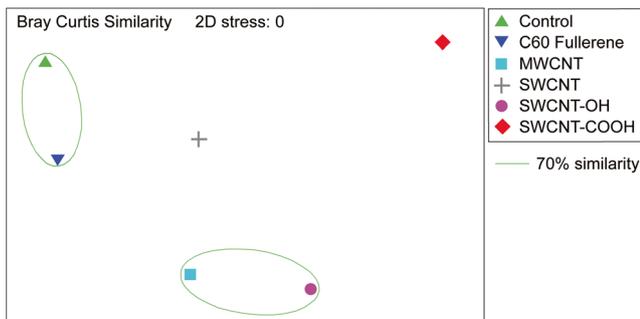### Carbon Nanotubes and C60 Fullerene

Fig. 9 Analyses of DGGE analyses using ANOSIM where $r = 0.78$ and $p < 0.001$ confirmed a significant difference between communities exposed to the various CNTs and C60 fullerene. Each point represents the average of 3 replicate gels, $n = 3$

6 weeks (Tong et al. 2012). It is apparent in the current study (Fig. 9) that exposure to both MWCNT and SWCNT variants can result in significant shifts in the nature of the eubacterial community. ANOSIM analyses of the DGGE fingerprints indicated that the MWCNT community was significantly different from the control and other CNT exposures. Similarly, the SWCNT exposed community was unique ($p < 0.05$) relative to other communities, while the modified SWCNTs resulted in communities similar to each other but significantly different from the control as well as fullerene and SWCNT-exposed biofilm communities. These differences may occur as a result of direct impacts or toxicity such as selection for or against specific community members. However, the observed impacts on protozoan grazing (Fig. 2) may also cause shifts in the bacterial community (see discussion above). CNTs and fullerene may also interfere with the tight linkages in terms of carbon and energy flow in the community through impacts on particular types of organisms or by shifting flows between bacteria and photosynthetic organisms that are vital to community structure and function (Haack and McFeters 1982a, b). It is also possible that their sorptive capacity may create limiting conditions by scavenging, metals, micronutrients or nutrients. However, these effects require further investigation.

Conclusions

A multimetric approach allowed assessment of the impacts of a panel of carbon-based nanomaterials on structure and function of river biofilm communities. Although the tested level was lower than in many earlier studies, significant effects of all materials could be detected on some aspect of community structure or function. It is useful to note that this occurred despite the aggregation, transformations and coatings that occurred, altering CNT properties and reducing toxicity (Lawrence et al. 2016) Fullerene C60 appeared to have minimal effects with the exception of protozoan grazing and

carbon utilization. Biomass measures such as thickness, chl-a, bacteria and cyanobacterial biomass were relatively insensitive to any changes induced by the CNT exposures. In contrast, metabolic and molecular measures were more indicative of effects in the community. STXM observations confirmed that the nanomaterials integrated into the developing communities with a patchy distribution, although estimates would suggest that the biofilm may concentrate the CNTs to an equivalent local concentration of 200 ppm. Given that close contact appears to be required for toxicity to occur, this implies that in situ exposures to the short range effects of CNTs and fullerene in the community are highly variable. Biofilm EPS have been viewed as a significant sink for nanomaterials as well as a regulator of exposure, perhaps shielding cells from direct interaction with nanomaterials in the environment. Based on community level screening, it may be suggested that SWCNT-OH>SWCNT-COOH>SWCNT>MWCNT>C60 Fullerene.

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