Journal of Hazardous Materials 343 (2018) 200–207

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Research paper

Chronic toxicity of graphene and graphene oxide in sequencing batch bioreactors: A comparative investigation

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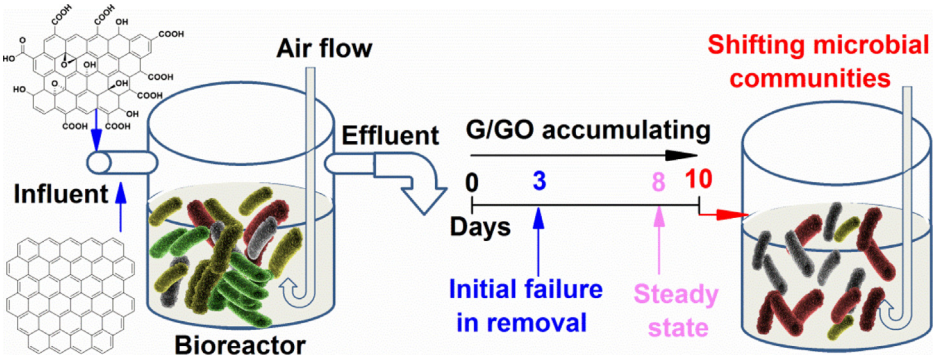
HIGHLIGHTS

- G and GO effects started at concentrations as low as 5.26 and 3.64 mg/L.
- The ammonia, phosphate and COD removals reached steady state after 8 days.
- AOB and PAO abundances started to recover after 8 days.
- GO and G impacted differently the microbial populations in the sludge.
- GO exhibited higher toxicity then G to microbial communities.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 1 June 2017
Received in revised form
17 September 2017
Accepted 19 September 2017
Available online 20 September 2017

Keywords:
Graphene oxide
Graphene
Phosphate accumulating bacteria
Ammonia oxidizing bacteria
Community structure

ABSTRACT

The present study investigates the chronic toxicity of graphene (G) and graphene oxide (GO) in activated sludge. Sequencing batch bioreactors were fed with influents containing 0, 1 and 5 mg L^{-1} of GO or G (12 h cycles) for ten days. Reduction in performance of the bioreactors in relation to chemical oxygen demand, ammonia and phosphate removals was observed after three days in the bioreactors fed with 5 mg L^{-1} of nanomaterials. After about eight days, these reactors reached a steady state nutrient removal, which corresponded to recovery of certain groups of ammonia oxidizing bacteria and phosphate accumulating bacteria despite the increasing accumulation of nanomaterials in the sludge. These results suggested that biological treatment can be affected transiently by initial exposure to the nanomaterials, but certain groups of microorganisms, less sensitive to these nanomaterials, can potentially strive in the presence of these nanomaterials. Results of 16S rRNA gene deep sequencing showed that G and GO affected differently the microbial communities in the activated sludge. Between the two nanomaterials investigated, GO presented the highest impact in nutrient removal, gene abundance and changes in microbial population structures.

1. Introduction

Recently, graphene-based nanomaterials have been intensively used due to the unique characteristics and broad applications of these materials in different fields, such as water and wastewater treatment, medical devices, electronic and aerospace [1–3]. The global market of these nanomaterials is expected to grow and

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http://dx.doi.org/10.1016/j.jhazmat.2017.09.032
0304-3894/

reach up to $986.7 million dollars over the next 5 years [4]. A study by Lazareva & Keller estimated, based on nanomaterial production data of 2010, that the presence of carbon nanotubes, a graphene-based nanomaterial, in the wastewater treatment plants of New York City would be around 1.5 mg kg^{-1} and 0.2 μg L^{-1} per year in biosolids and treated effluents, respectively [5]. These results showed that this carbon-based nanomaterial will tend to be retained in biosolids. In this study, Keller's group did not examine G or GO productions and releases, but if GO and G follow the same trends as carbon nanotubes, these nanomaterials will also end up accumulating in the sludge over time.

In previous studies, the impact of carbon-based nanomaterials in wastewater treatment has been investigated mostly in very high concentrations and for one or three days as acute toxicity assays [6–9]. These studies aimed to simulate worse case scenarios in case of industrial spills. These nanomaterials, however, will most likely be introduced in the wastewater treatment plants in much smaller concentrations than the ones previously investigated since the release of nanomaterials will be the result of direct consumption and disposal, as well as wear and tear of products containing CNTs, G or GO. Hence, in this study, we aim to simulate the entrance and accumulation over time of GO and G in the wastewater influent when introduced in smaller and more realistic amounts. We hypothesize that G, which is more hydrophobic and prone to aggregation in aqueous environment, will interact and accumulate more in the sludge than GO. We also hypothesize that accumulation of the GO and G in the sludge will eventually hinder the biological treatment process due to the antimicrobial properties of these nanomaterials. To investigate these hypotheses, bioreactors were set up, which were fed with low concentrations of G and GO in the influent for up to 10 days. Through these ten days, we monitored the performance of the reactors by investigating chemical oxygen demand, microbial metabolic activity, nitrogen and phosphate removals, as well as gene abundances for ammonia oxidizing bacteria (AOB), phosphate accumulating microorganisms (PAO) and ammonium monooxygenase (*amoA*) genes. The changes in microbial diversity structure and abundance were also determined using the 16S rRNA gene deep sequencing technique. We also monitored the release of these nanomaterials in the effluents using a previously published technique [10]. Through mass balance, we determined the accumulation of these nanomaterials in the sludge over time.

2. Materials and methods

2.1. Preparation of G, GO and activated sludge batch reactors with continuous feeding of nanomaterials

The G was purchased from XG Science (U.S.A.) and GO was synthesized following the modified Hummer's method [11]. The characterizations of G and GO can be found in supporting information (Fig. S1) and in our previous studies [12,13]. Suspension stocks for G and GO were prepared at 1000 mg L^{-1} using probe sonication for 5 min prior to use (30 kHz, Tekmar sonic disruptor, U.S.A). The stock solutions were diluted at 1 and 5 mg L^{-1} using sterile synthetic wastewater (SWW) prior to use.

All the reactors were set up with activated sludge freshly collected from the aeration basin from the Sims South Bayou Wastewater Treatment Plant (Houston, TX. USA). The reactors were fed with SWW (Table S1) with or without the nanomaterials every 12 h. Detailed information about the components of the reactor, reactor set-up and the SWW preparation procedure can be found in the supporting information. Briefly, a total of five reactors were set up in triplicate with the acclimated sludge. The experimental design included control reactors (without nanomaterials) and reactors continuously fed with 1 or 5 mg L^{-1} of G or 1 or 5 mg L^{-1} of GO. All reactors followed the same 12 h cycle as the control reactors. For analysis in each cycle, a volume of 500 mL of effluent was taken and a volume of 500 mL SWW, with or without nanomaterials, was added as influent to the reactor. Each reactor received in each cycle the same influent concentration of nanomaterial that was used at the initial reactor set up. The results of the triplicate reactors were averaged and their standard deviations were calculated.

2.2. Analysis of chemical parameters

At each cycle, the effluent of each reactor was analyzed for the following nutrient removals: ammonia (NH_3-N), nitrate (NO_3 -N), phosphate (PO_4^{3-}) and chemical oxygen demand (COD). Effluent and influent were collected and filtered through 0.2 μm sterile syringe filters (Corning, U.S.A). Ammonia and nitrate were analyzed using Orion Dual Star benchtop equipped with Orion™ High-Performance Ammonia Electrode and Orion™ Nitrate Electrodes (Thermo Scientific). Hach kits were used to measure phosphate and COD with a spectrophotometer DR3900 (Hach, U.S.A). All the results were expressed as days running the reactor versus concentrations measured (mg L^{-1}). Metabolic activity of the microbes in activated sludge was also investigated using Vibrant Cell Metabolic Assay kit (Invitrogen) (see supporting information).

2.3. DNA extraction and real time PCR (RT-PCR)

Activated sludge samples were collected at 0, 2, 4, 6, 8 and 10 days. Amounts of 0.5 g of settled activated sludge were collected for DNA extraction with the PowerWater DNA isolation kit (Mobio, U.S.A.). All DNA samples were investigated with RT-PCR for three genes, namely: AOB, *amoA* and PAO. The analyses of the abundances of the genes were determined using standard curves with serial dilutions of known concentrations of the genes cloned into a plasmid provided by the TOPO TA cloning kit (Invitrogen). Details related to the standard curves and RT-PCR conditions were described in our previous study [9]. All samples were run in triplicate. The triplicate DNA extracts from each triplicate reactors were averaged out. The results were expressed as concentrations of gene copy number, which were normalized to 1 ng of DNA template, versus concentrations of nanomaterials.

2.4. 16S rRNA metagenomics sequencing

Metagenomics of sludge samples from reactors with and without G and GO were investigated using Illumina Miseq (Genome Sciences, Bioscience Division, Los Alamos National Laboratory, New Mexico). The 16S rRNA gene libraries for each sample were prepared as described by Illumina with only modifications in the PCR amplification procedure. The conserved region targeted for analysis was the V4 region of the 16S rRNA gene using the primers F515 and R806 with Illumina adapters and barcodes [14]. More details on the library preparation and analysis can be found in the supporting information. Pair-end sequencing was employed using 600 – cycle MiSeq® Reagent Kit V3 (Illumina, U.S.A.). The output results from sequencing were analyzed using Ilumina basespace 16S Metagenomics v1.0.1. The data of metagenomics were deposited on the NCBI database under accession number SRP082429.

3. Results and discussions

3.1. Impact of G and GO on chemical oxygen demand (COD)

In a conventional wastewater treatment, COD removal is used as a mean to determine the treatment quality of biological pro-