Degradation of $^{14}$C-labeled Few Layer Graphene via Fenton Reaction: Reaction Rates, Characterization of Reaction Products, and Potential Ecological Effects

Yiping Feng,¹ Kun Lu,¹ Liang Mao,¹*, Xiangke Guo,¹ Shixiang Gao,¹ and Elijah J. Petersen²

¹ State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing, Jiangsu 210023, China;
² Material Measurement Laboratory, National Institute of Standards and Technology, Gaithersburg, MD 20899, USA

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Address correspondence to L. Mao, State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing 210093, P. R. China. Telephone: (86)25-89680393. Fax: (86)25-89680393. E-mail: lmao@nju.edu.cn.
ABSTRACT

Graphene has attracted considerable commercial interest due to its numerous potential applications. It is inevitable that graphene will be released into the environment during the production and usage of graphene-enabled consumer products, but the potential transformations of graphene in the environment are not well understood. In this study, $^{14}$C-labeled few layer graphene (FLG) enabled quantitative measurements of FLG degradation rates induced by the iron/hydrogen peroxide induced Fenton reaction. Quantification of $^{14}$CO$_2$ production from $^{14}$C-labeled FLG revealed significant degradation of FLG after 3 days with high H$_2$O$_2$ (200 mmol L$^{-1}$) and iron (100 µmol L$^{-1}$) concentrations but substantially lower rates under environmentally relevant conditions (0.2 to 20 mmol L$^{-1}$ H$_2$O$_2$ and 4 µmol L$^{-1}$ Fe$^{3+}$). Importantly, the carbon-14 labeling technique allowed for quantification of the FLG degradation rate at concentrations nearly four orders of magnitude lower than those typically used in other studies. These measurements revealed substantially faster degradation rates at lower FLG concentrations and thus studies with higher FLG concentrations may underestimate the degradation rates. Analysis of structural changes to FLG using multiple orthogonal methods revealed significant FLG oxidation and multiple reaction byproducts. Lastly, assessment of accumulation of the degraded FLG and intermediates using aquatic organism *Daphnia magna* revealed substantially decreased body burdens, which implied that the changes to FLG caused by the Fenton reaction may dramatically impact its potential ecological effects.
Keywords: $^{14}$C-labeled few layer graphene; Fenton reaction; degradation kinetics; $^{14}$CO$_2$ generation; quantification; characterizations

Abbreviations

$^{14}$C carbon-14
FLG few layer graphene
CNMs carbon nanomaterials
GO graphene oxide
TEM transmission electron microscopy
SEM scanning electron microscopy
XPS X-ray photoelectron spectroscopy
FTIR Fourier transform infrared
AFM atomic force microscopy
LSC liquid scintillation counting
HPLC high-performance liquid chromatography
LC-MS/MS liquid chromatography coupled with tandem mass spectrometry
GC-MS gas chromatography-mass spectrometry
1. Introduction

Graphene, a nanomaterial with a honeycomb lattice structure composed of planar $sp^2$ bound carbon atoms (Geim and Novoselov, 2007; Novoselov et al., 2004), has become one of the most intensively studied carbon nanomaterials (CNMs) for a number of potential applications (Geim, 2009; Stankovich et al., 2006; Sun et al., 2008). Since the application of graphene and its derivate has been greatly developed, it is inevitable that graphene will be released into the environment during the production and usage of graphene-enabled consumer products. It has been shown that graphene can induce cytotoxic effects such as cell membrane damage and bacterial toxicity and genotoxic effects on mammalian cells (Akhavan and Ghaderi, 2010; Bianco, 2013; Yang et al., 2013; Yang et al., 2010) and can accumulate in *Daphnia magna* (Guo et al., 2013).

One critical question regarding the fate of graphene in the environment is the extent and rate of degradation (Bai et al., 2014; Kotchey et al., 2012). If graphene can be degraded to CO$_2$, graphene may not accumulate in the environment with time, which would decrease the likelihood of potential adverse effects. A recent study showed that treatment of pristine multilayered graphene by H$_2$O$_2$ at physiologically
and environmentally relevant concentrations (1 to 10000 × 10^{-6} mol L^{-1}) was found to cause holes and defects in the surface of graphene (Xing et al., 2014). The hydroxyl radical (•OH) was considered to play an important role in the oxidative degradation of graphene (Xing et al., 2014; Zhou et al., 2012). In addition, the distinctly different reactivities have been observed for graphene-based materials with varying oxygen contents, defects and numbers of layers (Figure S1). For example, researchers have found that graphene oxide (GO), a precursor of graphene, can be degraded by photo-Fenton reaction (Bai et al., 2014; Zhou et al., 2012), or by enzymatic reaction with horseradish peroxidase and H_{2}O_{2} (Kotchey et al., 2011; Kotchey et al., 2012). However, reduced graphene oxide resisted degradation under the same experimental condition (Kotchey et al., 2011). Moreover, photoreaction of GO under sunlight or UV irradiation resulted in reduced graphene oxide nanosheets with many holes and defects (Hou et al., 2015; Matsumoto et al., 2011). It was also found that the intermediate photoproducts which have reduced sizes, decreased oxygen functionalities and low molecular-weight are more slowly degraded by photoreaction compared to parent GO (Hou et al., 2015). Overall, the conclusions regarding the degradation of graphene-based nanomaterial were mostly drawn from qualitative results and did not provide quantitative degradation rates (Bai et al., 2014; Hou et al., 2015; Kotchey et al., 2011; Kotchey et al., 2012; Matsumoto et al., 2011; Xing et al., 2014; Zhou et al., 2012).
Fenton chemistry ($\text{Fe}^{2+}/\text{Fe}^{3+}/\text{H}_2\text{O}_2$) is well known as a strong oxidizing agent with the production of $\cdot\text{OH}$. It has been developed and applied in wastewater treatment, especially in treating aromatic organic pollutants (Ensing et al., 2003; Neyens and Baeyens, 2003; Pignatello et al., 2006). Recently, the Fenton and photo-Fenton reactions were found to have significant implication on the treatment of carbon nanotubes (CNTs) (Allen et al., 2009; Fan et al., 2007) and GO (Bai et al., 2014; Zhou et al., 2012). Researchers have demonstrated that this simple and economical approach could effectively degrade CNTs and GO into CO$_2$ and some intermediate products which largely consist of adjacent aromatic rings with carboxylic acid groups (Allen et al., 2009; Bai et al., 2014; Zhou et al., 2012). In addition, Fenton chemistry can functionalize CNMs and was shown to cut GO into graphene quantum dots via photo-Fenton reaction (Zhou et al., 2012). Furthermore, the reaction of CNMs by Fenton chemistry may undertake similar oxidation processes as by enzymatic biodegradation systems. Therefore, Fenton chemistry was applied to understand the degradation mechanism of CNMs in biodegradation systems, especially for the enzymatic-catalyzed systems which with ferric heme iron ($\text{Fe}^{3+}$) in the catalytic active center (Allen et al., 2009; Andon et al., 2013; Bai et al., 2014; Kagan et al., 2010). Moreover, the naturally occurring Fenton chemistry plays a significant role in the environmental fate of the pollutants (Fukushima and Tatsumi, 2001; Sawyer et al., 1996). Thus, the reaction of CNMs by Fenton chemistry could reveal important information on the environmental fate of CNMs.
In previous studies, several techniques have been applied to characterize and quantify the degradation of CNMs (see Table S1 for a summary of studies on CNMs degradation). However, most of these techniques utilized (e.g., Raman spectroscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) and UV-vis-IR spectroscopy) may have a limited capacity for CNMs quantification in environmentally relevant matrices as a result of interferences or an insufficiently low detection limit (Fores-Cervantes et al., 2014; Petersen et al., 2011b). In contrast, the $^{14}$C isotope, a long-life radioactive isotope, is a sensitive tracer and $^{14}$C-isotopic quantification methods can overcome the limitations described above for other techniques. $^{14}$C-labeling has been applied to quantitatively tracking the biodistribution of CNMs (Georgin et al., 2009; Guo et al., 2013; Petersen et al., 2009; Petersen et al., 2008; Petersen et al., 2011a; Petersen et al., 2011b). Recently, $^{14}$C-labeling was also applied to study the microbial degradation of multiwall carbon nanotubes under complex reaction conditions (Zhang et al., 2013). Microbial degradation of multiwall carbon nanotubes resulted in $^{14}$CO$_2$ as the end product under some conditions (Zhang et al., 2013).

In this study, we treated $^{14}$C-labeled few layer graphene (FLG) by iron/H$_2$O$_2$-driven Fenton chemistry. We demonstrate that FLG can be completely degraded by the Fenton reaction by measuring the FLG degradation rates under a range of conditions using $^{14}$C-labeling to quantify FLG residues and $^{14}$CO$_2$ release. By measuring $^{14}$C, FLG degradation kinetics with various concentrations of iron,
hydrogen peroxide, and FLG were quantitatively evaluated. FLG degradation was tested using iron and hydrogen peroxide concentrations relevant for wastewater treatment of organic pollutants (Kusic et al., 2007; SafarzadehAmiri et al., 1997) and lower and more environmentally relevant conditions (Xing et al., 2014). In addition, degradation products were identified using multiple orthogonal techniques to fully understand the reaction and determine if the techniques were in agreement. Moreover, the accumulation of FLG before and after the Fenton reaction was tested using *D. magna*, a standard test aquatic organism (USEPA, 2002; OECD, 2004), to probe the potential ecological impacts of degraded FLG.

2. **Materials and methods**

2.1. **Materials**

Phenol (≥99.5% purity) and hydrogen peroxide (H$_2$O$_2$, 30% wt) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). $^{14}$C-ring-labeled phenol in an ethanol solution (≥ 97% purity, determined by HPLC radiochromatogram) was purchased from Moravek Biochemicals and Radiochemicals (CA, USA). Analytical-grade tetrahydrate (FeCl$_2$•4H$_2$O), ammonium dihydrogenphosphate (NH$_4$H$_2$PO$_4$), ferric chloride (FeCl$_3$•6H$_2$O), hydrochloric acid (HCl) and anhydrous sodium sulfate
NaSO_4 were purchased from Nanjing Chemical Reagent Co., Ltd (Nanjing, China).

Ultrapure water was used in this experiment (>18 MΩ). Dichloromethane and methanol of HPLC/SPECTRO grade were purchased from TEDIA (Fairfield, USA).

Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

2.2. Synthesis, purification, and characterization of 14C-labeled FLG

14C-labeled FLG was synthesized as described in our previous study (Guo et al., 2013). A full description of synthesis process and characterization of 14C-labeled FLG is provided in the Supplementary Information (SI) and summarized here. Briefly, FLG was successfully synthesized by graphitization and exfoliation of sandwich-like FePO_4/dodecylamine hybrid nanosheets and then purified using hydrochloric acid to remove the iron catalysts to below the detection limit (0.005 mg/L) by inductively coupled plasma optical emission spectroscopy (Optima 5300DV). TEM and SEM showed crumpled nanosheets of FLG and agglomeration (Figure S2). Atomic force microscopy (AFM) measurements showed the thickness of FLG is 1.05 to 4.05 nm.
and mostly was in the range of 1.35 to 1.95 nm, which would correspond to graphene that is four layers thick, and that the lateral size distribution ranged from 60 to 590 nm with two major peaks at 90 and 365 nm (Lu et al., 2015). High resolution TEM image indicates that the interlayer distance is about 0.344 nm, which is the interlayer distance of graphite. The strong D (1343 cm\(^{-1}\)), G (1579 cm\(^{-1}\)), and 2D (2686 cm\(^{-1}\)) bands in Raman spectra are common in graphene with multilayers structure. Elemental analysis by X-ray photoelectron spectroscopy (XPS) indicates that the atomic ratio of FLG is C: O: H: N is 89: 6: 1: 4. Also, XPS-peak-fitting analysis of the average carbon element content shown that most of carbon participate in C=C and C–C bonds. UV-vis spectra indicate no obvious UV-vis absorption region of this FLG. The specific radioactivity of the purified FLG was 16.12 ± 0.59 mCi g\(^{-1}\) (the uncertainty indicates the standard deviation of triplicate samples). To measure potential carbon-14 impurities in FLG, the dispersed FLG were extracted using dichloromethane and n-hexane mixtures, and the extracts were analyzed using liquid scintillation counting (LSC) and gas chromatography-mass spectrometry (GC-MS). Results did not show a significant increase in the radioactivity or obvious chemical peaks in GC-MS chromatograms of the extracted solutions, indicating that carbon-14 byproducts were not formed from the synthesis, purification, or dispersion processes of FLG (Guo et al., 2013).

2.3. Assessment of FLG degradation under varying reaction conditions.
The following experiments were conducted to determine the degradation behavior of FLG by Fenton chemistry at environmentally realistic concentrations of Fe$^{3+}$ and H$_2$O$_2$ (Xing et al., 2014). Briefly, 20 mL of $^{14}$C-FLG suspensions with initial concentrations of 20, 50, 100 or 500 μg L$^{-1}$ were prepared and 100 μmol L$^{-1}$ Fe$^{3+}$ and 2 mM H$_2$O$_2$ were added to initiate the reaction. These FLG concentrations are at least three orders of magnitude lower than that (100 mg FLG L$^{-1}$) used in previous studies (Bai et al., 2014; Zhou et al., 2012). To study this reaction using a broader range of conditions, treatment of 500 μg L$^{-1}$ of $^{14}$C-FLG with 4 μmol L$^{-1}$ Fe$^{3+}$ and 0.2 or 20 mM H$_2$O$_2$ was also evaluated. Experiments were also performed to assess FLG transformation through reactions mediated by Fenton reagents under different reaction conditions with higher concentrations of iron and hydrogen peroxide (Fe$^{3+}$ and H$_2$O$_2$ concentrations were varied from 25 to 200 μmol L$^{-1}$ or 0.2 to 200 mmol L$^{-1}$, respectively) potentially relevant for treatment of wastewater with FLG (Kusic et al., 2007; SafarzadehAmiri et al., 1997). Similar experiments were also performed with FLG and either H$_2$O$_2$ or Fe$^{3+}$. Lastly, an experiment was conducted testing 500 μg L$^{-1}$ $^{14}$C-FLG with 200 mmol L$^{-1}$ H$_2$O$_2$ and 50 μmol L$^{-1}$ Fe$^{3+}$ for 10 days to assess if the FLG would be completely converted to CO$_2$.

The reactions were carried out in 40-mL glass vials equipped with polytetrafluoroethylene (PTFE)-lined screw caps and incubated on a rotary shaker operating at 150 rpm at 25 °C. Each reactor contained a 20 mL reaction medium that was adjusted to pH 4; the reasons for using this pH value are that ferric ions can form
iron hydroxides, which will not react with hydrogen peroxide, at neutral and alkaline conditions (Lu et al., 2001) and that this pH was used in related previous studies (Bai et al., 2014; Zhou et al., 2012). H$_2$O$_2$ was added last to each reactor to initiate the reaction. To quantify the production of $^{14}$CO$_2$, gas in the reaction vial headspace was sampled after 0, 4, 8, 12, 24, 36, 48, 60, 72 h, 96, 120 and 144 h using a syringe with valve, and then added to 2-mL of a 5 M NaOH solution. The NaOH solution was transferred to 3 mL of alkaline scintillation cocktail (Oxysolve C-400; Zinsser Analytic, Frankfurt, Germany). At each of the times listed above, 100 μL of the reaction suspension from each vial was removed and mixed with 3 mL of alkaline scintillation cocktail. The corresponding radioactivity in the samples was analyzed by LSC (LS6500; Beckman Coulter, USA). The limit of detection of this experimental system was approximately 54 ng $^{14}$C L$^{-1}$. Three replicates were tested at each reaction condition. All experiments were performed in the dark to prevent photolysis reactions.

2.4. Characterizations of FLG changes and products identification.

Experiments were also conducted to characterize changes to FLG remaining in the aqueous phase after the Fenton reaction and to identify the possible formation of intermediate products. Samples at two initial FLG concentrations, 500 μg L$^{-1}$ and 100 mg L$^{-1}$, were prepared to characterize changes to FLG via the Fenton reaction using instruments with different sensitivities. The 500 μg L$^{-1}$ samples were prepared using
the procedure described above in 40-mL glass vials with unlabeled FLG and Fe$^{3+}$ and 
H$_2$O$_2$ concentrations of 50 μmol L$^{-1}$ and 200 mmol L$^{-1}$, respectively. These samples 
were analyzed using UV-vis spectroscopy, Raman spectroscopy, AFM, and TEM. For 
techniques that required higher FLG concentrations, the reactions were conducted in 
100-mL beakers and stirred with a magnetic stir bar at room temperature. Unlabeled 
FLG, Fe$^{3+}$, and H$_2$O$_2$ concentrations of 100 mg L$^{-1}$, 50 μmol L$^{-1}$, and 200 mmol L$^{-1}$, 
respectively, were used. At set reaction times, FLG was sampled and analyzed using 
FTIR Spectroscopy, XPS, HPLC, and LC-MS/MS (detailed information on the 
analysis method and samples preparation are presented in the SI). In experiments 
designed to measure intermediate products, 200 mmol L$^{-1}$ H$_2$O$_2$ was additionally 
added to the reaction suspensions at day 3 to increase the mass of degradation 
byproducts. After incubation for 3 days or 5 days, 10 mL of the aqueous phase from 
the reaction mixture was sampled and sequentially extracted three times using 10 mL 
dichloromethane each time. The extracted solutions were combined, dried by rotary 
evaporation, and then the residue was dissolved in methanol. The reconstituted 
samples were completely transferred into 1.8-mL vials and then each was reduced to 
0.5 mL using a gentle stream of nitrogen gas. It should be noted that this method 
could not detect any volatile products that were created. This sample was sequentially 
analyzed by HPLC to identify any peaks and LC-MS/MS to determine the molecular 
weight of the possible products. HPLC was performed on an Agilent 1200 instrument 
equipped with the variable wavelength detector (VWD) by a 4.6 × 250 mm Eclipse
XDB C18 reverse-phase column (Agilent, USA) at 30 °C. The detection wavelength of VWD was 230 nm. The isocratic mobile phase was made up of methanol (70%) and water (30%) with a flow rate of 1.0 mL min\(^{-1}\) and the injection volume was 20 µL. LC-MS/MS analysis was carried out on a Thermo liquid chromatograph connected to a Thermo LCQ Advantages (Quest LCQ Duo, USA) mass spectrometer through an ESI interface. 10 µL of concentrated sample was injected into a Eclipse XDB C18 reverse-phase column (Agilent, USA) via a split injector (split ratio 1:5). The composition and operation of mobile phase were kept identical to those mentioned previously for HPLC. The mass spectrometer was set in negative ionization mode over the range m/z = 50-1200. Capillary voltage and cone voltage were 4.5 kV and 25 V, respectively. Desolvation and source temperatures were 300 and 120 °C, respectively. Nitrogen was used as sheath gas at a flow rate of 35 arb units and as auxiliary gas at a flow rate of 5 arb units. In addition, to quantitatively track the possible intermediate resulting from FLG degradation at different incubation times, reactions were performed with \(^{14}\)C-FLG at 500 µg L\(^{-1}\). At predetermined intervals (0, 6, 12, 36, 60, 72, and 84 hours after the reaction was initiated), 1 mL samples of the reaction solution were removed, filtered using a 0.45-µm Millipore membrane (cellulose acetate), and the radioactivity in the filtrate was quantified by LSC. In addition, the filtrate obtained from the 3 days reaction sample was extracted as described above and the radioactivity of extracts was also analyzed by LSC.
2.5. Accumulation of the degraded FLG and intermediates by D. magna.

*D. magna* neonates (< 24 h old) were exposed in untreated or treated FLG suspensions to explore the potential ecological effects of FLG after treatment with the Fenton reaction. The exposure suspensions used in these experiments were prepared using the following method: 500 μg L\(^{-1}\) \(^{14}\)C-labeled FLG was first treated by the Fenton reagent (50 μmol L\(^{-1}\) Fe\(^{3+}\) and 200 mmol L\(^{-1}\) H\(_2\)O\(_2\)). After allowing the reaction to occur for 3 days, the reaction was stopped by adding NaOH solution at a final concentration of 150 μmol L\(^{-1}\) to ensure Fe\(^{3+}\) was fully precipitated as Fe(OH)\(_3\).

Residual H\(_2\)O\(_2\) was eliminated by addition of catalase (5.0 μg L\(^{-1}\), reaction time was 2 h) and by confirming the hydrogen peroxide removal by monitoring the concentration of residual H\(_2\)O\(_2\) using UV-vis spectrophotometry at 220 nm. Preliminary data suggested that the added catalase was sufficient to consume residual H\(_2\)O\(_2\) and had no adverse impact on the *Daphnia*. Then reaction solutions were passed through a 0.45-μm syringe filter (cellulose acetate, 25mm, Sterile, Fisher Scientific, Pittsburgh, PA) to remove larger FLG particles and precipitated iron. The residual Fe\(^{3+}\) content in the filtrate was analyzed by acidifying the filtrate with HCl and then quantified using the potassium thiocyanate chromogenic reaction and inductively coupled plasma-optical emission spectroscopy (details can be found in the SI). The results showed that Fe\(^{3+}\) could be decreased to below the limit of detection (0.005 mg/L) for both measurements.
The residual radioactivity in the filtrate was measured as (6878.5 ± 460.4) dpm mL\(^{-1}\) by LSC which corresponds to a FLG concentration of (192.2 ± 12.9) µg L\(^{-1}\). CaCl\(_2\)·2H\(_2\)O, MgSO\(_4\)·2H\(_2\)O, NaHCO\(_3\) and KCl were added into the filtrate as the artificial freshwater (AF) at the final concentration of 58.8 mg L\(^{-1}\), 24.7 mg L\(^{-1}\), 13.0 mg L\(^{-1}\) and 1.2 mg L\(^{-1}\), respectively (hardness [Ca\(^{2+}\)]+ [Mg\(^{2+}\)] = 0.5 mmol L\(^{-1}\)), pH= 6.5±0.2. In addition, a dispersion of 192.2 µg L\(^{-1}\) of unreacted FLG was prepared in AF for comparison.

Ten *D. magna* neonates (<24 h) were exposed to 30 mL of the reacted and unreacted FLG samples. Triplicate containers were sampled after 0.5, 4, 10, and 24 h. There was no feeding during the exposure experiments. Assessment of *Daphnia* immobilization was conducted on the organism to evaluate the toxicity of degradation intermediates during the exposure period. After the exposure duration, *D. magna* were placed in a beaker containing 30 mL clean water and pipetted vigorously to remove FLG particles attached to their carapaces. This step was repeated three times. We demonstrated in our previous study that this procedure can effectively minimize the contributions from the attached FLG to the total mass of FLG associated with the *D. magna* (Guo et al., 2013). Then, the *D. magna* from each container were added to scintillation vials with 3 mL of Gold Star cocktail, ultrasonicated for 20 min (100 W, P = 7.52 J s\(^{-1}\)), allowed to sit for at least 24 h in the dark at room temperature, and then analyzed using LSC. The radioactivity from blank samples (i.e., *D. magna* not exposed to FLG) was subtracted from the neonate uptake results. After *D. magna*
removal, aqueous-phase radioactivity was also measured as described above to
determine the concentration of FLG remaining suspended.

2.6. Data analysis.

Data on $^{14}$CO$_2$ generation during the examined times was fitted to the zero-order
kinetics $C_t - C_0 = kt$, using Origin 8.5 software, where $C_0$ is the initial percentage of
$^{14}$CO$_2$ generated from Fenton reagent mediated FLG reaction, $C_t$ is $^{14}$CO$_2$ percentage at
time $t$, and $k$ is $^{14}$CO$_2$ generation rate constant. When determining the rate constants
for the degradation processes, all data points for each time point were used. Statistical
analyses (t-tests) were performed using SPSS 18.0 (PASW Statistics, IBM Company);
differences were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Assessment of FLG removal at varying reaction conditions.

We first explored the degradation of FLG by Fe$^{3+}$/H$_2$O$_2$-driven Fenton reaction at
environmentally relevant conditions by measuring $^{14}$CO$_2$ generation (Figure 1). FLG
can be slowly transformed into CO$_2$ by 4 µmol L$^{-1}$ Fe$^{3+}$ and 0.2 or 20 mmol L$^{-1}$ H$_2$O$_2$
after a 6 day reaction (Figure 1 (a)). The total production of $^{14}$CO$_2$ at day 6 was
around 0.1 % and 0.6 % of the initial \(^{14}\)C-labeled FLG for 0.2 or 20 mmol L\(^{-1}\) H\(_2\)O\(_2\) with 4 µmol L\(^{-1}\) Fe\(^{3+}\), respectively. In contrast, significantly faster degradation can be observed after increasing the Fe\(^{3+}\) concentration to 100 µmol L\(^{-1}\), in which case the total production of \(^{14}\)CO\(_2\) after day 3 was 1.5 % and 12 % of the initial \(^{14}\)C-labeled FLG for 0.2 or 20 mmol L\(^{-1}\) H\(_2\)O\(_2\), respectively. In experiments testing the impact of the initial FLG concentration on the degradation rate (Figure 1 b), the total amount of \(^{14}\)CO\(_2\) produced after 5 days was approximately 6.2, 3.8, 2.9 and 1.3 % of the initial 20, 50, 100 and 500 µg L\(^{-1}\) \(^{14}\)C-labeled FLG. This suggests that measurements made using higher FLG concentrations, as done in all previous studies (see Table S1) may significantly underestimate the rate of Fenton reactions at lower concentrations. This finding highlights the importance of highly sensitive quantitative measurements using carbon-14 labeled FLG.

Although we have demonstrated that Fe\(^{3+}/H_2O_2\)-driven Fenton reaction could cause FLG degradation as measuring the production of \(^{14}\)CO\(_2\) at environmentally relevant conditions, it is important to study the degradation kinetics of FLG via Fenton reaction across a broader range of conditions to quantitatively evaluate the degradation kinetics. These conditions could potentially be used, for example, to treat wastewater with graphene. As shown in Figure 2 and Figure 3, when testing different initial Fe\(^{3+}\) and H\(_2\)O\(_2\) concentrations, a significant fraction of FLG, ranging from 2.0% to 50%, was transformed into \(^{14}\)CO\(_2\), and \(^{14}\)CO\(_2\) generation increased with longer reaction times. Total \(^{14}\)CO\(_2\) generation increased from 1.5 % to 32.8 % of the total
initial radioactivity in a dose-dependent manner as the H$_2$O$_2$ dosages from 0.2 to 200 mmol L$^{-1}$ at a constant Fe$^{3+}$ dosage (100 µmol L$^{-1}$) (Figure 2a and Figure 3a). We also fitted the kinetics of relative generation of $^{14}$CO$_2$ using a zero-order model ($C_t-C_0=kt$) to obtain the reaction rate (k) for each reaction condition, as listed in Table 1. While the reaction rate increased with increasing H$_2$O$_2$ concentrations, a different trend was observed when testing various concentrations of Fe$^{3+}$ (25 to 200 µmol L$^{-1}$) with a fixed H$_2$O$_2$ concentration (200 mmol L$^{-1}$). In this case, the maximum mineralization rate was measured at a Fe$^{3+}$ dosage of 50 µmol L$^{-1}$ (Figure 2c, Figure 3b and Table 1). However, raising the Fe$^{3+}$ dosage (from 75 to 200 µmol L$^{-1}$) did not result in further increases in the degradation rate. The radioactivity remaining in the aqueous phase decreased at rates consistent with $^{14}$CO$_2$ generation (Figure 2b and 2d). When incubating FLG with only H$_2$O$_2$, approximately 1.8% of the initial FLG was transformed into $^{14}$CO$_2$ with 200 mmol L$^{-1}$ H$_2$O$_2$ after 72 h incubation (Figure S3a, b). This result was in agreement with a previous result that demonstrated FLG degradation by H$_2$O$_2$ (Xing et al., 2014). Conversely, degradation was not observed with any concentration of Fe$^{3+}$ tested in the absence of H$_2$O$_2$ (Figure S3c, d).

The reaction kinetics studies showed that Fenton chemistry driven by H$_2$O$_2$ and Fe$^{3+}$ can cause FLG degradation into CO$_2$. As shown in Scheme S1, Fenton-like reactions can generate reactive oxygen species (ROS) such as •OH and •OOH. These reactive oxygen species are considered the critical factor in the transformation of the FLG (Pignatello et al., 2006). These generated reactive oxygen species could attack...
and destroy FLG to form defect sites, and finally decompose FLG into CO₂ (Fan et al., 2007; Zhou et al., 2012). The H₂O₂ concentration is directly proportional to the generation rate of ROS in Fenton-like reaction (Ensing et al., 2003; Neyens and Baeyens, 2003). With increasing Fe³⁺ dosage, ROS can be formed at higher rates by the increased reaction rates with H₂O₂. However, excess iron ions may consume the active oxygen species (Equations 3 and 5 in Scheme S1) and thus reduce the Fenton reaction efficiency (Lin and Gurol, 1998).

3.2. Characterization of FLG after Fenton reaction: intermediate products.

When treating 500 µg L⁻¹¹⁴C-FLG with 200 mmol L⁻¹ H₂O₂ and 50 µmol L⁻¹ Fe³⁺ for 10 days, we determined that the residual radioactivity in reaction solutions was below the detection limit (< 54 ng ¹⁴C L⁻¹), thus showing that FLG was completely transformed into CO₂. While the ultimate end product of FLG degradation is CO₂, degradation products other than, or as precursors of CO₂, are also likely to be produced prior to complete conversion to CO₂. As such, the residual radioactivity in the aqueous phase as shown in Figure 2 may contain oxidized FLG and degradation intermediates. As shown in Figure S4, approximately 15% of the remaining radioactivity was measured in the filtrate from the 3 day reaction sample while the radioactivity in the filtrate from the unreacted FLG was not detectable. In addition, the filtrate obtained from the 3 day reaction sample (containing 15% radioactivity)
was extracted, and the remaining radioactivity in the reconstituted solution was determined to be 3.6% of the initial radioactivity using LSC revealing that. FLG degradation generated low molecular weight products. The extraction solutions from the degradation of a high concentration of FLG (100 mg L\(^{-1}\)) were analyzed by HPLC and LC-MS/MS to identify the possible products. HPLC and LC-MS/MS chromatograms and LC-MS base peak chromatograms, which usually monitors only the most intense peaks in each spectrum, for FLG degradation products after 3 or 5 days of reaction showed a series of new peaks compared to unreacted FLG (Figure S5, S6, and S7). Larger magnitude signals were present for the reaction samples for the LC-MS base peak chromatograms after a 3 day treatment compared to the 5 day treatment. The majority of peaks are large molecular weight components in the solution of 3 day of reaction, such as m/z 497.15, 610.36, 723.38, 836.43, 949.49 and 1062.45 (Figure S7). In the extract from the 5 day reaction sample, the low molecular weight components, such as m/z 118.06, 215.07, 282.15, 222.17, 301.03 and 425.25 appeared as the most intense peaks (Figure S7). Based on the analysis of related studies (Allen et al., 2009; Bai et al., 2014; Hou et al., 2015; Zhang et al., 2013; Zhou et al., 2012), and according to the MS/MS spectra (Figure S7) of the detected intermediate product, possible molecular formulas and structures of these low molecular weight intermediates were deduced, as shown in Figure S6.

3.3. Characterization of FLG after Fenton reaction: changes to the FLG particles.
Figure 4a provides visual evidence of FLG degradation after 3 and 5 days of incubation by showing how the color of the vial decreases after incubation with Fe$^{3+}$ and H$_2$O$_2$. UV-Vis spectrometry analysis between 280 and 800 nm of FLG samples incubated for 0, 12, 24, 36, 48, 60, and 72 h showed a similar decrease (Figure S8).

Progress of the Fenton reaction of FLG was also assessed by TEM and AFM (Figure S9 and Figure 4d, e). At 0 days, the FLG was observed to be integrated on the basal plane and many of them were agglomerated. After 1 day reaction, the agglomerated FLG became more dispersed, a phenomenon that could be explained by an increased concentration of hydrophilic functional groups on the surface of FLG.

After reaction for 3 days, holes in the FLG were observed. The results in Figure 4d show that the height of the flat FLG sheet is $\approx 1.4$ nm, and after the Fenton reaction for 3 days, FLG was oxidized to contain holes but the height of the FLG was still $\approx 1.4$ nm (Figure 4e). By day 5, the FLG plane was barely visible using TEM, indicating that the majority of FLG has been completely oxidized. A similar degradation process was also observed in the enzymatic oxidation of GO with increased incubation time (Kotchey et al., 2011).

Analysis of the treated FLG samples with Raman spectroscopy also showed substantial degradation (Figure 4b). The observed D and G bands are distinctive of graphitic materials: the D band represents the disorder present in $sp^2$-hybridized carbon systems, while the G band represents the stretching of C-C bonds (Malard et
al., 2009; Wang et al., 1990). The ratio of the D band to G band can be used to evaluate the defects in graphitic materials (Pimenta et al., 2007). For day 0 and 2, the D:G ratio increased from (0.91±0.07):1.0 to (1.37±0.22):1.0 (p =0.021, t test), and at day 5, both the D and G bands disappeared. This result revealed that Fenton oxidation of the graphitic lattice would result in an increased frequency of defect sites and that the carbon atoms were transformed from \( sp^2 \) to \( sp^3 \) hybridization (Dresselhaus et al., 2010). At day 5, the disappearance of the D and G was attributed to the complete oxidation of the graphitic lattice.

FT-IR analysis revealed several peaks at 3400 cm\(^{-1}\) (O-H), 1710 and 1678 cm\(^{-1}\) (C=O), 1590 cm\(^{-1}\) (C=C), 1370 cm\(^{-1}\) (C=C or C-O-H), 1220 and 1060 cm\(^{-1}\) (C-O) on the surface of FLG after 3 d reaction (Figure S10) (Hwang and Li, 2010; Sun et al., 2012; Zielke et al., 1996). These results indicate that the oxygen-containing groups (hydroxyl, carboxyl, carbonyl, and epoxy groups) were introduced on the surface of FLG via the Fenton reaction. The XPS spectra in Figure S11 show the increase of O and decrease of C element on the surface of FLG with increasing reaction time up to 3 days; analyzing samples after 5 days was infeasible as a result of an inability to obtain a sufficient mass of FLG particles by centrifugation. Figure 4c and Table S2 summarize the surface elemental composition and oxygen species distribution that was determined by XPS (corresponding O1s spectra (Yu et al., 2011; Zielke et al., 1996) are provided in Figure S12). After 3 day reaction, the O:C ratio of FLG increased markedly from 0.04 to 0.17, indicating that FLG was oxidized and
oxygen-containing groups were introduced to the surface of FLG. Moreover, the oxygen species and carbon species distribution was significantly changed, the oxygen in OH, C-O and O-C=O increased with the reaction time. No significant increase of the O: C ratio was observed after reaction for 1 day.

3.4. Daphnia magna results.

Previous research on the degradation products from CNMs has suggested that the structures of the byproducts are polycyclic aromatic hydrocarbons (PAHs) (Allen et al., 2009; Bai et al., 2014; Hou et al., 2015; Zhang et al., 2013; Zhou et al., 2012), a result similar to the findings of this study on FLG degradation. However, these intermediate PAHs may have important toxicological implications (Bai et al., 2014). For example, it has been demonstrated that the oxidation products (e.g., MW < 3000 Da) of horseradish peroxidase-catalyzed degradation of single-wall carbon nanotubes will induce DNA damage (Pan et al., 2013). It was suggested that the products from photoreaction of GO are likely to exhibit different accumulation and toxic properties compared to parent GO (Hou et al., 2015), but the potential toxicological impacts of FLG degraded by the Fenton reaction and its byproducts are not yet well understood.

Our previous study revealed FLG accumulation in the gut tract of D. magna (Guo et al., 2013), a result similar to studies with other CNMs (Pakarinen et al., 2013; Petersen et al., 2009; Petersen et al., 2011a). However, the smaller size of degraded
FLG and low molecular weight compounds generated via the Fenton reaction showed different potential ecological effects (Figure 5). Substantial accumulation (17 μg mg\(^{-1}\)) of dry tissue) of FLG was measured in the *D. magna* exposed to unreacted FLG, while accumulation of the degradation intermediates after exposure for 24 h was nearly two orders of magnitude smaller (<0.02 μg mg\(^{-1}\)) (Figure 5a). No *D. magna* immobilization was observed in the exposure of the unreacted FLG or after exposure to the reacted FLG during the 24 h exposure period. Settling measurements of unreacted and reacted FLG suspensions after *Daphnia* removal showed significantly different behaviors (Figure 5b). After subtracting the fraction of unreacted and reacted FLG accumulated by the *Daphnia*, around 57% of the untreated FLG agglomerated and settled out of suspension over the 24 h period, while the settling percentage was less than 2% in the suspensions from the FLG treated by the Fenton reaction.

4. Conclusion

This study provides unambiguous experimental evidence that the Fe\(^{2+}/\) Fe\(^{3+}/\) H\(_2\)O\(_2\) -driven Fenton reaction can effectively degrade FLG. FLG can be completely transformed into CO\(_2\) by high concentrations of Fenton reagents (e.g., 200 mM H\(_2\)O\(_2\) and 50 μM Fe\(^{3+}\)), which implies that Fenton reaction can be potentially used for the complete degradation of FLG. In addition, by measuring the \(^{14}\)CO\(_2\) release, FLG
degradation kinetics under various reaction conditions were quantitatively evaluated. This study highlights a highly sensitive approach to quantitatively measure CNMs degradation for environmentally realistic conditions with low CNMs concentrations. Moreover, identification of degradation intermediates and characterization of the FLG changes using multiple orthogonal techniques provided critical information to fully understand the degradation process of FLG; results from each of these techniques were also in agreement. Importantly, we found that FLG degradation products, became more stable in water compared to the unreacted FLG, and were less easily accumulated in *D. magna* even though the organisms were exposed to higher average concentrations over the exposure period. Therefore, an important implication of this study is that FLG may be transformed via naturally occurring Fenton-like reactions, and the resulting change of FLG on the morphology, properties and the degradation intermediates should be taken into account when assessing its potential ecological risks.

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Appendix A. Supplementary Data

Supplementary data related to this article can be found at the Water Research website.

References


Table 1. Kinetics results obtained by fitting \(^{14}\text{CO}_2\) generation rate to zero-order plots under different concentrations of \(\text{H}_2\text{O}_2\), \(\text{Fe}^{3+}\) and FLG.

<table>
<thead>
<tr>
<th>(\text{H}_2\text{O}_2) (\mu\text{M} \text{Fe}^{3+})</th>
<th>(\text{Fe}^{3+}) (\mu\text{M} \text{H}_2\text{O}_2)</th>
<th>FLG (mM)</th>
<th>k (% h(^{-1}))</th>
<th>(mM)</th>
<th>k (% h(^{-1}))</th>
<th>(µM)</th>
<th>k (% h(^{-1}))</th>
<th>(µg L(^{-1}))</th>
<th>k (% h(^{-1}))</th>
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<tr>
<td>500 µg L(^{-1}) FLG, 100 µM Fe(^{3+})</td>
<td>500 µg L(^{-1}) FLG, 4 µM Fe(^{3+})</td>
<td>500 µg L(^{-1}) FLG, 200 mM H(_2)O(_2)</td>
<td>2 mM H(_2)O(_2), 100 µM Fe(^{3+})</td>
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<td></td>
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<td>0.2</td>
<td>0.0007±0.0001</td>
<td>25</td>
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<tr>
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<td>20</td>
<td>0.0043±0.0005</td>
<td>50</td>
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</table>

The uncertainty value is the standard error for the calculated k; the \(R^2\) values were \(\geq 0.92\) for all condition.
Figure 1. Relative yield of $^{14}$CO$_2$ over time resulting from Fenton reagent mediated FLG reaction under various environmentally relevant conditions. (a), [FLG] = 500 µg L$^{-1}$, [H$_2$O$_2$]= 0.2 or 20 mmol L$^{-1}$, [Fe$^{3+}$] = 4 µmol L$^{-1}$ for open symbols and 100 µmol L$^{-1}$ for solid symbols, 25 °C, pH= 4.0; (b), [FLG] = 20, 50, 100 or 500 µg L$^{-1}$, [H$_2$O$_2$]= 2 mmol L$^{-1}$, [Fe$^{3+}$] = 100 µmol L$^{-1}$, 25 °C, pH= 4.0. Error bars represent standard deviations (n= 3).
Figure 2. Relative yield of $^{14}$CO$_2$ and removal of FLG over time resulting from Fenton reagent mediated FLG reaction at different H$_2$O$_2$ (a) and Fe$^{3+}$ (c) concentrations. The residual radioactivity over time in the reaction samples with varied concentrations of H$_2$O$_2$ (b) or Fe$^{3+}$ (d). Experimental conditions: (a) and (b) [FLG] = 500 µg L$^{-1}$, [Fe$^{3+}$] = 100 µmol L$^{-1}$, 25 °C, pH= 4.0; (c) and (d): [FLG] = 500 µg L$^{-1}$, [H$_2$O$_2$]= 200 mmol L$^{-1}$, 25 °C, pH=4.0. Error bars represent standard deviations (n= 3).
Figure 3. Mass recovery of $^{14}$C-FLG after 3 days Fenton reaction at different initial FeCl$_3$ and H$_2$O$_2$ dosages by combining the aqueous phase concentration and the mass of $^{14}$CO$_2$.

Experimental conditions: (a) [FLG] = 10 µg, [Fe$^{3+}$] = 100 µmol L$^{-1}$, 25 °C, pH= 4.0; (b): [FLG] = 10 µg, [H$_2$O$_2$]= 200 mmol L$^{-1}$, 25 °C, pH=4.0. Error bars represent standard deviations (n= 3).
Figure 4. Characterization of FLG during the Fenton-like reaction. (a) Photograph of FLG in systems variously containing Fe$^{3+}$ and/or H$_2$O$_2$, including a system containing Fe$^{3+}$ at 50
µmol/L (G+Fe); a system containing 100 mmol/L H₂O₂ (G+H); and a system containing both 50 µmol/L Fe³⁺ and 100 mmol/L H₂O₂ for Day 3 or 5. (b) and (c) displays the Raman spectra and XPS analysis of the FLG reacted with Fenton reagent at different times. (d) and (e) show AFM images of FLG and FLG reacted with Fenton reagent at Day 3. The inserted dotted line in Figures (d) and (e) shows the height profile of FLG film. Experimental conditions: (a), (b), (d) and (e), [FLG]= 500 µg L⁻¹, [Fe³⁺] = 50 µmol L⁻¹, [H₂O₂] = 200 mmol L⁻¹; (c), [FLG]= 100 mg L⁻¹, [Fe³⁺] = 50 µmol L⁻¹, [H₂O₂]= 200 mmol L⁻¹; 25 °C, pH = 4.0.
Figure 5. The uptake of *D. magna* after exposure of unreacted FLG and its degradation intermediates up to 24 h (a). Measured residual concentrations and settling percentage of unreacted FLG and its degradation intermediates in uptake experiment suspensions after *D. magna* removal (b). Reaction conditions: \([\text{FLG}] = 500 \, \mu\text{g L}^{-1}, [\text{Fe}^{3+}] = 50 \, \mu\text{mol L}^{-1}, [\text{H}_2\text{O}_2] = 200 \, \text{mmol L}^{-1}, 25 ^\circ\text{C}, \text{pH} = 4.0, 3\, \text{days}.\)