Are fluorescence-based chlorophyll quantification methods suitable for algae toxicity assessment of carbon nanomaterials?

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Using a multi-walled carbon nanotube (MWCNT) and graphene oxide (GO) as representative test materials, we evaluated the applicability of *in vivo* and *in vitro* chlorophyll-a (Chl-a) fluorescence quantification methods, which are used in standard algae ecotoxicity tests such as OECD 201 and ISO 8692. *In vivo* quantification of Chl-a from *Raphidocelis subcapitata* indicated a significant reduction in Chl-a fluorescence in the presence of MWCNTs due to shading, but a significant autofluorescence from GO that caused an overestimation of Chl-a concentration. *In vitro* Chl-a quantification methods employing a modified acetone and an ethanol extraction protocol reduced the influence of shading and autofluorescence, but both resulted in a significant loss of fluorescence signal in the presence of 100 mg L^{-1} MWCNTS (99-100%) and GO (21-52%). Chl-a reduction was dose dependent for both tested CNM materials, but effects were more pronounced for MWCNT, which caused a significant fluorescence reduction (16±0.3%) already at 1 mg L^{-1}. Further study of the CNM-algae-Chl-a interaction processes revealed that CNM can not only interact with live algae, but also efficiently adsorb extracted Chl-a. Our results showed that within 10 min, 95-100% of Chl-a extracted from two algae concentrations were adsorbed to MWCNT, while 35-60% of Chl-a was adsorbed to the GO. This study shows that Chl-a quantification by fluorescence determination is not a suitable method for ecotoxicity testing of CNM. However, a quick screening test for individual MNMs is recommended to determine if Chl-a adsorption is a significant process prior to selection of a quantification method.

Key words: Standard tests; adsorption; carbon nanotubes; graphene oxide;

Introduction

Many of the existing standard toxicity test guidelines (TGs) have been found to exhibit limitations when applied to assessment of manufactured nanomaterials (MNMs) (Sørensen *et al.*, 2015). As a result, there has been significant effort within the Organization for Economic Cooperation and Development (OECD), the International Standardization Organization (ISO) and large-scale research projects (e.g. EU FP7 projects Marina and NANoREG) to evaluate the applicability of existing TGs for MNMs. In 2009, the OECD Working Party on Manufactured Nanomaterials (WPMN) assessed the limitations of existing OECD TGs for MNMs (OECD, 2009), and published the results of a seven year testing programme as a series of dossiers in 2015. The general conclusion of OECD was that most TGs are considered suitable for the testing of MNMs, but common issues of harmonised dispersion preparation, sedimentation during exposure and dosimetry were highlighted. These issues have been the subject of individual studies (Hartmann *et al.*, 2010, Hund-Rinke *et al.*, 2010, Hartmann *et al.*, 2015) and a critical review of the dossiers has highlighted significant limitations with the conclusions drawn by OECD (Hansen *et al.*, 2017). In some cases, modifications were proposed for improving the relevancy of specific TGs towards MNMs.

In 2012, a guidance document addressed general issues such as sample preparation and dosimetry, but lacked specific focus on individual TGs (OECD, 2012). Despite further work, there is currently no official compilation of proposed nano-specific test modifications on OECD TGs for ecotoxicity, therefore the adaption of the test guidelines is hampered (Hund-Rinke *et al.*, 2016). Most recently, the EU FP7 project Marina has provided an overview of the progress on eight OECD ecotoxicity TGs, summarising the difficulties identified with individual TGs for MNMs and the proposed modifications.

OECD Guideline 201 (Freshwater Alga and Cyanobacteria, Growth Inhibition Test) (OECD, 2011) and ISO 8692:2004 (Water quality–freshwater algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*) (ISO, 2004) are two of the most commonly used standard aquatic ecotoxicity tests. The tests are quick and cost effective to perform, reliable and reproducible, employ organisms which are easy to maintain in permanent culture, and present the user with few ethical considerations compared to other species. The test is internationally recognised, and freshwater algae toxicity data are available for many chemicals and metals. However, significant variability in EC_{50} values for MNM effects on algal growth is reported, highlighting the importance of test reliability and reproducibility for interpretability (Menard *et al.*, 2011).

OECD 201 is considered to have limited issues with respect to sedimentation and dosimetry during exposure, due to samples being shaken during the incubation period (Hund-Rinke *et al.*, 2016). However, two key limitations regarding the applicability of OECD 201 to MNMs have been identified. The first is that the chelating agent EDTA, a component of the test medium, can interfere with metallic MNMs causing iron precipitation and resulting in unfavourable growth conditions (Kadar *et al.*, 2012, Hund-Rinke *et al.*, 2016, Mouchet *et al.*, 2016, Zhang *et al.*, 2016). It is now recommended that an EDTA-free algal medium should be considered when testing metal MNMs (Hund-Rinke *et al.*, 2016). The second is accuracy in determination of algal growth. Biomass determination through dry weight is not applicable in the presence of particles and interference was reported in both cell counting and *in vivo* measurement of chlorophyll-a (Chl-a) concentrations by fluorescence (Handy *et al.*, 2012a, Handy *et al.*, 2012b, Hartmann *et al.*, 2012, Hund-Rinke *et al.*, 2016). Cell counting in a haemocytometer is very laborious, has a large variance and may not truly reflect algal biomass if the mean cell size changes in response to a toxicant or other conditions (Hund-

Rinke *et al.*, 2016). In the case of *in vivo* fluorescence Chl-a measurements, MNMs can cause shading of the algae fluorescent signal, which reduces the response, underestimating the Chl-a concentration and overestimating the toxicity of the MNM being studied (Handy *et al.*, 2012a, Handy *et al.*, 2012b, Hartmann *et al.*, 2012, Sørensen *et al.*, 2015, Hund-Rinke *et al.*, 2016, Sørensen *et al.*, 2016). The term shading can refer to either (i) blocking of the light from the source to the algal cells, or (ii) blocking of the fluorescence signal from the algal cells to the detector. The main approach suggested for overcoming the issue of fluorescence signal shading is the extraction of the Chl-a from the algal cells and removal of the MNMs prior to *in vitro* fluorescence measurement of the algal extract (Hartmann *et al.*, 2012, Hund-Rinke *et al.*, 2016). Methods proposed include the use of acetone extraction (Mayer *et al.*, 1997, Sørensen *et al.*, 2016), ethanol extraction and filtration (ISO 10260) (ISO, 1992), and acetone extraction combined with MNM flocculation and sedimentation (no filtration) using locust bean gum (Cerrillo *et al.*, 2016, Hund-Rinke *et al.*, 2016).

Although these studies used different reference MNMs (TiO_2, Ag and Au) (Hartmann *et al.*, 2012, Hund-Rinke *et al.*, 2016), no carbon-based MNMs (CNMs) were included. Carbon nanotubes (CNTs) and graphene family nanomaterials (GFNs) represent a large group of CNMs with increasing production volumes. CNTs and GFNs exhibit unique physicochemical properties that differ quite significantly from spherical metal and metal oxide MNMs and have implications for their environmental fate and effects (Jackson *et al.*, 2013, Zhao *et al.*, 2014, Hu *et al.*, 2016). One feature in particular, is the high adsorption capacity of CNMs for organic chemicals (Chen *et al.*, 2007, Kah *et al.*, 2014, Glomstad *et al.*, 2016, Zindler *et al.*, 2016). The propensity for CNMs to adsorb organic compounds raises the issue of Chl-a adsorption in algae ecotoxicity studies employing extraction methods.

In the current study, we evaluate the applicability of different fluorescence-based Chl-a quantification methods for the assessment of CNMs using a multi-walled CNT (MWCNT) and a graphene oxide (GO) as test materials. The suitability of *in vivo* fluorescence approaches are evaluated using OECD Guideline 201 (OECD, 2011). *In vitro* fluorescence approaches are evaluated using a Chl-a extraction method described in the ISO 10260 guideline (ISO, 1992), and also by applying a modified acetone extraction and flocculation method developed in the EU FP7 project Marina (Hund-Rinke *et al.*, 2016) and benchmarked in the EU FP7 project NANoREG (Cerrillo *et al.*, 2016).

Methods

Materials

The MWCNTs were supplied by the Joint Research Centre and is known widely as NM-400. The GO was supplied by Abalonyx AS (Oslo, Norway). Nanomaterial stock dispersions of 2560 mg L^{-1} were prepared by probe sonication (Branson Ultrasonics Sonifier S-450; Branson Ultrasonics, USA) at 15 % amplitude for 13 minutes and 45 seconds. This method delivers an acoustic delivered power of 7.35±0.05 Watt and an accumulated dose of 7056±103 J to the dispersion, which was determined using the calorimetric described by Taurozzi et al. as a basis (Taurozzi *et al.*, 2012). Stock dispersions of GO were prepared in MilliQ water. Due to the hydrophobicity of the MWCNT, the dispersion procedure was enhanced through the inclusion of a pre-wetting step with 96% ethanol and the addition of 20 mg L^{-1} natural organic matter (NOM, Suwannee river). Working dispersions were prepared by diluting the stock dispersions either in OECD algae growth media TG201 (for GO exposures) or TG201 containing 10 mg L^{-1} NOM (TG201+NOM; for MWCNT exposures). NOM was dissolved in MilliQ or TG201 by magnetic stirring for 24 h followed by repeated filtration (0.2 μm

Nalgene filter unit, Thermo Fisher Scientific Inc., USA) to remove undissolved material.

Nanomaterial Characterisation

SEM was performed with an Hitachi S-5500 ultra high resolution SEM equipped with bright field and dark field STEM detectors and a Bruker Quantax EDS system. The MWCNT samples were dispersed in isopropanol and prepared on lacey carbon TEM grids. GO was dispersed in high purity water and similarly prepared on a lacey carbon grid. Prior to imaging, all samples were cleaned of volatile organic contaminants using a UV ozone cleaner. An acceleration voltage of 30kV was used for images. The hydrodynamic diameter (HDD) and zeta potential of MWCNT and GO were determined with a Zetasizer ZS (Malvern Instruments, UK) in stock dispersions and exposure dispersions (TG201 and TG201+NOM) prepared at a concentration of 100 mg L^{-1}. It is acknowledged that the usefulness of dynamic light scattering is limited for MNMs with high aspect ratios and high polydispersity (e.g. CNTs).

Algae culture

The algae used for the experiments in this study derived from a continuous in-house *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*) culture. The algae culture is maintained at 60 – 120 µE/m^2/s at a constant temperature of 21±2 °C. Regular monitoring confirms that the culture fulfils the recommended quality criteria regarding growth rate and sensitivity for ecotoxicological testing as specified in ISO 8692:2004 (ISO 2004).

Chl-a determination

In this study, we determined Chl-a as surrogate for total Chl. All Chl-a measurements were performed using a fluorimeter (TD700, Turner design, USA) with an optical kit (10-037R; Turner design, USA) suited for *in vivo* and extracted Chl-a measurements (excitation 340-500 nm; emission >665 nm).

In vivo Chlorophyll-a determination

In order to investigate the feasibility of *in vivo* Chl-a determination in algae exposed to CNM, MWCNT and GO were added to algae dispersions and the fluorescence signal determined. Algae were diluted in either TG201 (for GO exposures) or TG201+ NOM (MWCNT exposures) to result in fluorescence values of approximately 25 AU (arbitrary units), which corresponds to approximately 25000 cells mL^{-1} (See Supplementary Information section for more details). Following dilution, MWCNT and GO were added to the algae to reach a final concentration of 100 mg L^{-1}. Algae-CNM dispersions were carefully shaken and left standing still in the dark for 10 minutes to enable algae-CNM interactions, but prevent any toxicological response, before measuring fluorescence as described above. Algae prepared at similarly concentrations in TG201 and TG201+NOM, respectively were used as controls. Control and CNM exposure groups were measured in triplicates (n=3).

In vitro Chlorophyll-a determination

In vitro Chl-a determination was evaluated using two different Chl-a extraction methods in order to investigate the effect of the extraction solvent on potential CNM interference. Experiments were conducted at algal concentrations resulting in an approximate fluorescence of 500 AU, which corresponds to approximately 500000 cells mL^{-1} (See Supplementary Information section for more details). These concentrations were based on typical values

found in previously conducted OECD 201 growth inhibition tests during the middle-end phase of the test. The CNMs were added to the algae to reach a concentration of 100 mg L^{-1}, selected based on the CLP hazard classification limit. The samples were carefully agitated, left standing for 10 min and extracted thereafter with the two methods described below. As controls, only algae in the respective growth media (TG201 or TG201+NOM) were extracted. Each condition was tested in triplicates (n=3). As first method we applied an acetone extraction of Chl-a as described in by Hund-Rinke et al., (Hund-Rinke *et al.*, 2016) and Cerrillo et al., (Cerrillo *et al.*, 2016). In brief, 2 mL of the exposure dispersions were transferred into 8.8 mL of acetone containing lotus bean gum (LBG), mixed by immersion and placed in the dark for 24 h before determining the fluorescence as described above. This method was adapted to testing MNMs using LBG to increase the flocculation and sedimentation of MNMs so that an MNM-free supernatant containing the extracted Chl-a can be isolated and analysed.

The second method investigated for extracting Chl-a for *in vitro* quantification was the ISO 10260 '*Measurement of biochemical Parameters - Spectrometric determination of the chlorophyll-a concentration*', using filtration (GF-F; Whatman, UK) and heated Chl-a extraction in 90% ethanol. The extract was allowed to cool to room temperature and filtered once more in order to remove particulate matter before measuring Chl-a fluorometrically as described above.

Dose dependency

Dose dependent impacts of CNMs on Chl-a quantification were studied using the modified acetone extraction method described above. MWCNT and GO were added to a high algae concentration (fluorescence approximately 600 AU) to reach the following exposure

concentrations: 100, 50, 10, 1, 0.1 and 0 mg L^{-1}. The exposures were carefully shaken and left standing still for approximately 10 minutes. To obtain homogeneous samples, the exposure dispersions were agitated and a 2 mL sample was transferred into 8.8 mL of acetone containing LBG, mixed by immersion and placed in the dark for 24 h. Chl-a fluorescence was determined fluorometrically as described above. Each condition was tested in triplicates (*n*=3).

Extraction point and time dependence

In this experiment we wanted to test if CNM interference derives from adsorption and following removal of algae through settling, or if CNMs can also interact with extracted Chl-a. Therefore, 100 mgL^{-1} MWCNT and GO were added to two algae concentrations: low (fluorescence approximately 15 AU) and high (fluorescence approximately 500 AU). The CNMs were added (i) to the algae 10 min before extraction, (ii) directly after the Chl-a extraction with acetone (as described above), and iii) after 24 h after Chl-a extraction, 10 minutes before fluorescence measurements. Due to potential interference of CNMs present in the dispersions, the later samples (iii) were filtered either through GF-F filters (Whatman, UK; MWCNT) or through 0.1 µm Omnipore PTFE filter (MerkMillipore Ltd, Ireland; GO). Following, the fluorescence was determined as described above. All conditions were tested as triplicates (*n*=3).

Microscopy

In order to visualise the interaction of CNM with live *R. subcapitata*, samples of high algae concentrations (600-700 AU) were mixed by agitation with CNMs to reach concentrations of 10 or 50 mg L^{-1} CNMs. Samples were inspected with a fluorescent microscope (Nikon

Eclipse 90i; Nikon, Japan) using a 10 and 20x objective and B-2A filter (optimised for blue excitation with a wide excitation bandpass). Further, the adsorption of Chl-a onto MWCNT was visualised. A highly concentrated Chl-a suspension was extracted from an enriched *R. subcapitata* culture using the previously described ISO method. The extracted Chl-a was subsequently added to 5 mL of 100 mg L^{-1} MWCNT and carefully shaken for 2 minutes and left standing in the dark for approximately 10 minutes. As background control dispersions containing only 100 mgL^{-1} MWCNT were used. The samples were filtered through 0.1 µm Omnipore PTFE filter (MerkMillipore Ltd, Ireland; GO), and the filters placed on microscopy slides. Images of MWCNT were taken with a Nikon Eclipse fluorescent microscope (Nikon, Japan) using a 40x objective and B-2A filter. Chl-a was visualised using fluorescence applying high exposure times (1.5 s) and high contrast mode.

Statistics

Data analyses were performed with GraphPad Prism 7 (GraphPad Software Inc., USA). Data sets were analysed for normality (Shapiro-Wilk normality test). Significant differences between treatments groups were analysed using either t-test (two groups) or one way ANOVA (multiple groups).

Results

CNM Characterisation

The physicochemical properties of the CNMs used in the study are summarised in Table 1. MWCNT NM-400 was supplied by the JRC repository and sub-samples of material from the same batch has been extensively characterised previously (Rasmussen *et al.*, 2014). Therefore, existing data are presented for size (thickness and length) and specific surface area

(SSA). The SSA of the MWCNT was reported as 189.3±8.1 m^2 g^{-1} using SAXS analysis and 254 m^2 g^{-1} using BET method. The MWCNT had a reported average length of 846±446 nm, and widths of 16.2±3.5 nm and 11±3 nm determined independently by two different laboratories (Rasmussen *et al.*, 2014). In house acquired STEM images of MWCNT NM-400 showed a similar size range (Figure 1A and B). The GO sheets were relatively heterogeneous, ranging from <100 nm to 1 µm in size (Figure 1C). The CNMs were also characterised in dispersion form, both as stock dispersions and in the standard algae toxicity media (TG201) used in the studies. Average particle size distribution (Z-ave) for MWCNTs was 3540±727 nm in the stock dispersion and 2480±1450 nm at 100 mg L^{-1} in TG201+NOM (Table 1). The large Z-ave value and high standard deviation is consistent with high aspect ratio MNMs analysed by dynamic light scattering. The zeta potential values were 0.8±1.6 mV and -20±2.8 mV, respectively, confirming unstable dispersions of MWCNT. Dispersions of GO were observed to be relatively stable and had a hydrodynamic diameter (HDD) of 245±14.6 nm in the stock dispersion and slightly smaller size of 214±7.22 nm in the algae growth medium TG201 at a concentration of 100 mg L^{-1}. The zeta potential values were -41±0.14 mV and -28±0.25 mV, respectively (Table 1).

In vivo Chlorophyll-a determination

In vivo Chl-a fluorescence measurements revealed a high level of interference in the presence of both tested CNMs (Figure 2). Relative to the control samples, Chl-a fluorescence was significantly reduced for MWCNTs through shading of the algal cell fluorescent response, being measured as a significant (P<0.001) net loss of fluorescence in the system (Figure 2). Furthermore, the algae were observed to adsorb or attach significantly to the MWCNT aggregates (Figure 3A). In contrast, the measured fluorescence of the GO at both high and low concentrations was significantly (P<0.001) increased relative to the control samples

(Figure 2). This was due to autofluorescence of GO causing high fluorescent background levels that affected *in vivo* Chl-a quantification (Figure 2). Without algae present, GO autofluorescence at a concentration of 100 mg L^{-1} was >100 AU in the TG201 algae media, and was even higher >300 AU in MilliQ water (data not shown). In contrast to in MWCNT dispersions, algal cells remained dispersed in the presence of high GO concentrations (Figure 3B). Although adsorption of algal cells to GO might occur, this could not be determined with light microscopy due to the small size and stable dispersion of GO in the exposure media.

In vitro Chlorophyll-a determination

When using *in vitro* Chl-a determination, no direct fluorescence interference and autofluorescence was observed. However, a loss of fluorescence signal relative to control samples containing only algae was observed for both CNM types with both extraction methods applied (Figure 4). Fluorescence was completely reduced in the presence of 100 mg L^{-1} MWCNT using the modified acetone extraction (99.5±0.2%) and the EtOH extraction described in the ISO protocol (99.1±0.4%) (Figure 4A). Fluorescence was also decreased in the presence of 100 mg L^{-1} GO, however, the reduction was significantly less pronounced, being 21.5±4.7% when using the EtOH extraction method and 52±8 % (p=0.0049) when using the acetone extraction method (Figure 4B).

Dose dependence

When applying the acetone extraction method, a clear dose dependence of fluorescence reduction was found for both CNMs tested. However, the effects were more pronounced for MWCNT than for GO (Figure 5). A significant reduction in fluorescence of 16.5±4% was

observed for the MWCNT at a concentration of only 1 mg L^{-1} (P<0.0001). At the tested concentrations of 10, 50 and 100 mg L^{-1} the relative reduction in fluorescence was 65±1.6%, 95.5±0.3 and 98±0.1 %, respectively. In the GO exposures, a significant reduction in fluorescence was seen at concentrations of 50 and 100 mgL^{-1}, being 22.4±1.4% and 32±1.1%, respectively.

In vitro Chlorophyll-a determination – Extraction point and time dependence

In order to understand more about the adsorption process, the CNMs were added to both high and low algae concentrations at different time points in the extraction process: (i) added to the live algae immediately prior to extraction (TP1), (ii) added directly added after Chl-a extraction with acetone but prior to the 24 h settling period (TP2), and (iii) added after the 24 h settling period, 10 minutes before fluorescence measurements were made (TP3). Figure 6 summarises the degree of Chl-a adsorption of the two CNMs at both high and low algae concentrations at TP1-TP3. Chl-a fluorescence was significantly decreased (95 - >100 %) in the presence of 100 mg L^{-1} MWCNT for both low and high algae concentrations compared to controls at all TP. At all TPs, the decrease in fluorescence was more efficient (P<0.001) for the low algae concentration than for the high algae concentration (Figure 6A). Association of Chl-a with the MWCNTs was also observed microscopically (Figure 7). While no fluorescence signal was detected in the MWCNT only controls (Figure 7A and B), the presence of high Chl-a concentrations resulted in a fluorescence signal which was most intense and most stable in association with MWCNT aggregates (Figure 7D). Furthermore, the reduction in fluorescence was lower for the GO than for the MWCNTs at both test concentrations and at all TPs (Figure 6B). However, the results were generally consistent for this material at both concentrations and all TPs, with fluorescence reduction being 50-55% at the low concentration across all TPs and 35-60% at the high concentration. Again, there was a

slight trend towards greater fluorescence reduction when moving from TP3 to TP1.

Discussion

The current study assessed the applicability of Chl-a determination by fluorescence for algae toxicity assessment of CNMs. Our results show that the assessed *in vivo* and *in vitro* methods were not suitable for use with CNMs. *In vivo* Chl-a fluorescence measurements for CNMs highlighted two key interference processes; (i) shading of algal fluorescence, and (ii) autofluorescence. Shading of the fluorescent signal was the predominant interference process in the case of the MWCNTs. Moreover, MWCNTs were unstable, formed aggregates and settled out of the exposure dispersion removing any co-adsorbed algal cells (Table 1; Figure 3A). The co-adsorption of CNTs and other CNMs to algal cells in exposure studies is well documented (Schwab *et al.*, 2011, Long *et al.*, 2012, Sørensen *et al.*, 2015, Mouchet *et al.*, 2016, Nolte *et al.*, 2017). Critically, co-adsorption prevents separation of the two matrix elements, e.g. through CNM settling, which may have offered a solution for achieving accurate Chl-a measurements. Some studies have even detected individual CNTs in the cytoplasm of algal cells which may complicate quantification further (Rhiem *et al.*, 2015). Although shading of the algal cell fluorescence is also expected to be an issue in the case of GO, the results show that autofluorescence of this material is the dominant process, having an even greater impact on the ability to measure Chl-a fluorescence accurately. However, there was no clear evidence of co-adsorption between algal cells and GO (Figure 3B). The GO was relatively stable in the exposure dispersions, making it impossible to identify single GO sheets with light microscopy and therefore detect potential co-adsorption of GO and algal cells. The results of the current assessment support those from previous studies which concluded that *in vivo* measurement of Chl-a in tests employing MNMs is not feasible due to shading of algal fluorescence, algal cell adsorption processes and autofluorescence in similar wavelengths as

Chl-a (in the case of GO).

Owing to the significant limitations in achieving accurate determination of Chl-a *in vivo*, recent studies have attempted to develop *in vitro* approaches for Chl-a determination (Hartmann *et al.*, 2012, Cerrillo *et al.*, 2016, Hund-Rinke *et al.*, 2016). In the current study, the issues concerning shading of algal fluorescence and autofluorescence during the Chl-a fluorescence measurements were removed when the *in vitro* approaches were applied. However, the concentration-dependent loss of Chl-a fluorescence signal observed in the *in vitro* studies indicates that the Chl-a is being removed from the system through adsorption to the CNMs (Figure 5). The results from the studies assessing the two different extraction methods for *in vitro* Chl-a determination from algal cells showed that the presence of 100 mgL^{-1} MWCNT caused a total loss of Chl-a fluorescence signal in the extracted samples relative to controls. Similarly, GO exhibited a reduction in Chl-a fluorescence relative to the control samples, however, the reduction was less than that observed for the MWCNTs. Furthermore, the reduction in fluorescence was less when using the EtOH extraction and filtration procedure compared to the acetone extraction method reported by Hunde-Rinke et al. [8]. This suggests that the partition coefficient for Chl-a between the CNMs and the two organic solvents is different, with the EtOH retaining a greater proportion of Chl-a than the acetone. However, reduction was still around 20% and is therefore sufficiently high that an accurate determination of Chl-a values is not possible. The differences in Chl-a concentration reduction observed between the MWCNT and GO indicate that the presence of significant amounts of oxygen in the GO material reduces the hydrophobicity of the material and therefore its adsorption affinity for algae cells and Chl-a compared to the MWCNTs. This was supported by the increased level of dispersion stability observed for the GO compared to the

MWCNT (Table 1).

A higher adsorption affinity of MWCNTs was also seen in dose-dependence experiments using acetone extraction. Here, the MWCNTs caused a significant reduction of Chl-a signal at a concentration of only 1 mgL^{-1}. A comparable level of reduction was only reached in GO exposure at a concentration of 50 mg L^{-1}. The dose dependent interactions show that the level of Chl-a adsorption will be a function of the algae to CNM ratio, although the current study does not go as far as determining adsorption isotherms similar to those previously reported for specific persistent organic pollutants and CNMs (Chen *et al.*, 2007, Kah *et al.*, 2014, Glomstad *et al.*, 2016, Zindler *et al.*, 2016). Critically, this dose dependent signal reduction of Chl-a means that a CNM concentration-dependent quantity of the extracted Chl-a will be removed from the sample matrix during the removal of the CNMs. This in turn, might lead to a false interpretation of dose dependent CNM toxicity. Despite the lower degree of Chl-a fluorescence signal reduction observed for GO, it is clear that accurate total Chl-a concentrations cannot be determined for either material, highlighting severe limitations with the proposed modifications to OECD 201 for CNMs.

A more detailed assessment of the adsorption process was conducted in an attempt to identify possible modifications to the *in vitro* method of Hund-Rinke et al., (Hund-Rinke *et al.*, 2016) that would reduce or even remove the impact of adsorption processes on the fluorescence measurements. To achieve this, the CNMs were added to both high and low algae concentrations (reflecting algae concentrations at the start and end of the growth period), and at different time points in the extraction process. Irrespective of when the CNMs were added to the test system, significant adsorption was observed for both CNMs at both algae concentrations and at all TPs studied (Figure 6). This shows not only that CNM-algae

399 co-adsorption occurs, but that CNMs readily adsorb the extracted Chl-a. This was confirmed
400 by microscopic observations, in which high Chl-a fluorescence was shown associated with
401 MWCNT aggregates (Figure 7D). The shortest contact time for CNMs and Chl-a used in the
402 current study was 10 min (TP3). However, even within this very short timeframe, Chl-a
403 adsorption at TP3 was observed to be >100% for the MWCNTs at the low algae concentration
404 and approximately 95% for the high algae concentration. The level of Chl-a adsorption was
405 even higher at TP1 and TP2. Although the degree of Chl-a adsorption to GO was significantly
406 lower than that observed for the MWCNTs at all TPs, it was still determined as ~50% for the
407 low algae concentration and ~35% for the high algae concentration at TP3. Again, Chl-a
408 adsorption was typically higher at TP1 and TP2 for GO. The results show that the Chl-a
409 adsorption process occurs extremely rapidly (< 10 min) and therefore further modification of
410 the OECD 201 method proposed by Hunde-Rinke et al., (Hund-Rinke et al., 2016) does not
411 appear feasible.
412
413 Consistent with most adsorption models (e.g. Langmuir, Freundlich, and the Dubinin-
414 Ashtakhov model based on Polanyi theory) (Yang *et al.*, 2006, Kah *et al.*, 2011, Glomstad *et*
415 *al.*, 2016), the decrease in fluorescence was more efficient (P<0.001) for the low algae
416 concentration than for the high algae concentration at all TPs (Figure 6A) for MWCNT.
417 Although the reduction in fluorescence due to adsorption was lower for the GO than for the
418 MWCNTs at both test concentrations and at all TPs (Figure 6), the level was still greater than
419 30% indicating that reliable Chl-a measurement reflecting accurate algal growth during the
420 test cannot be achieved. Interestingly, the measured reduction in fluorescence for MWCNTs
421 in low algae concentrations was greater than 100% at all TPs (Figure 6A). It is suggested that
422 the MWCNTs also adsorb the NOM present in the exposure media. The adsorption of NOM
423 to CNTs is well known, and the stabilising effect this has on CNT dispersions is the main

reason why NOM is increasingly utilised in aquatic ecotoxicity studies with such materials (Cupi *et al.*, 2015, Linard *et al.*, 2015, Cerrillo *et al.*, 2016, Glomstad *et al.*, 2016).

Recommendations

Based on the evidence presented in the current study, it is recommended that all MNMs to be subjected to algal growth tests should first undergo a rapid screening test to determine their affinity for Chl-a adsorption. A detailed approach presented in the Supplementary Information is summarised briefly here. A stock dispersion of the test MNM in freshwater algae media should be prepared and added to a solution of extracted Chl-a in algae test media. The resulting sample should be shaken for 10 mins and the MNMs removed by filtration or flocculation and settling. A control sample, containing only Chl-a, should also be prepared and treated in the same way. The fluorescence of the resulting Chl-a should then be measured and the percentage loss of Chl-a to the test MNM determined. It is suggested that Chl-a adsorption >10% renders the method unsuitable for the selected MNM and an alternative method must be used. Where Chl-a adsorption is <10%, the method is considered suitable and can be used as part of the toxicity test.

Based on the recommendations by Hund-Rinke et al. (Hund-Rinke *et al.*, 2016) and the results of the current study, it is suggested that both *in vivo* and *in vitro* fluorescence determination of Chl-a have limitations and do not represent viable standard methods, which need to be applicable to all MNM types. Cell counting using automated systems can also be limited as agglomerates can be in the size range of the algae and systems are not able differentiate between the two under these circumstances. Although the method is very time consuming and therefore costly, it is recommended that manual cell counting remains the only reliable approach currently available for accurately quantifying algal cell growth in acute

toxicity methods. Owing to the importance of standardised algae acute toxicity tests from a regulatory perspective, it is also recommended that alternative approaches for the quantification of algal growth is prioritised in the near future.

Conclusion

The currently available algae toxicity test methods are not fully suited to the hazard assessment of the MNMs. A number of issues arise during both the exposure period and in the algal growth quantification methods available, including *in vivo* and *in vitro* fluorescence measurements. *In vivo* quantification is limited due to the issue of MNMs shading algal fluorescence and is unable to provide reliable Chl-a concentrations. *In vitro* fluorescence quantification, based on extraction of the Chl-a followed by removal of the test MNMs, has recently been recommended as the best alternative to *in vivo* quantification. Whilst this remains the case for MNMs which do not exhibit a significant level of algae or Chl-a adsorption, the approach is not suitable CNMs and other MNMs which have a strong adsorptive properties. At present, algal growth quantification by cell counting seems to remain the only method which has potential applicability for all MNM types, but this method is very time consuming and not necessarily suitable for standard toxicity tests which need to be time and cost effective.

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Declaration of Interest

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Tables

Table 1. Properties of MWCNT (NM-400) and GO used in this study. The hydrodynamic diameter (HDD) is given as z-average. For HDD, polydispersity index (PDI) and zeta potential values are presented as mean±SD; *n*=3.

Material	Identifier	Surface area (m^2 g^{-1})	Size (nm)	HDD (nm)		PDI		Zeta potential (mV)	
				Stock[e]	Exposure[f]	Stock[e]	Exposure[f]	Stock[e]	Exposure[f]
MWCNT	NM-400	254*[a] 189.3±8.1*[b]	11±3/16.2±3.5*[c] 846 ± 446*[d]	3540±727	2480±1450	0.99±0.013	0.92±0.92	0.8±1.6	-20±2.8
GO	GO	n.a.	<100 nm – 1 µm	245±14.6	214±7.22	0.40±0.018	0.37±0.023	-41±0.14	-28±0.25

*Data reproduced from Rasmussen et al. (Rasmussen *et al.*, 2014)
a: Derived using BET method
b: Derived using SAXS
c: MWCNT thickness (the two values shown represent independent measurements conducted in two different laboratories)
d: MWCNT length
e: Stock concentration of CNMs were 2560 mg L^{-1}, in MilliQ (GO) or MilliQ+20 mgL^{-1} NOM (MWCNT)
f: The exposure concentration of the CNMs was 100 mg L^{-1} in TG201 (GO) or TG201+10 mgL^{-1} NOM (MWCNT)

Figures

Figure 1

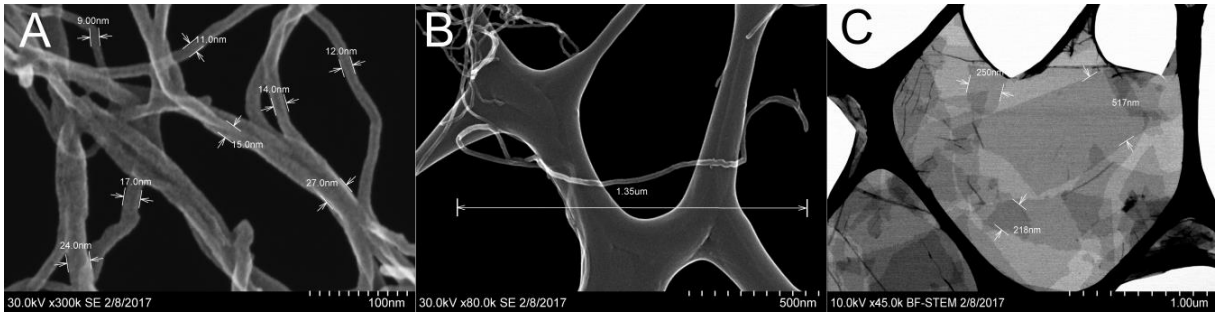


Figure 2

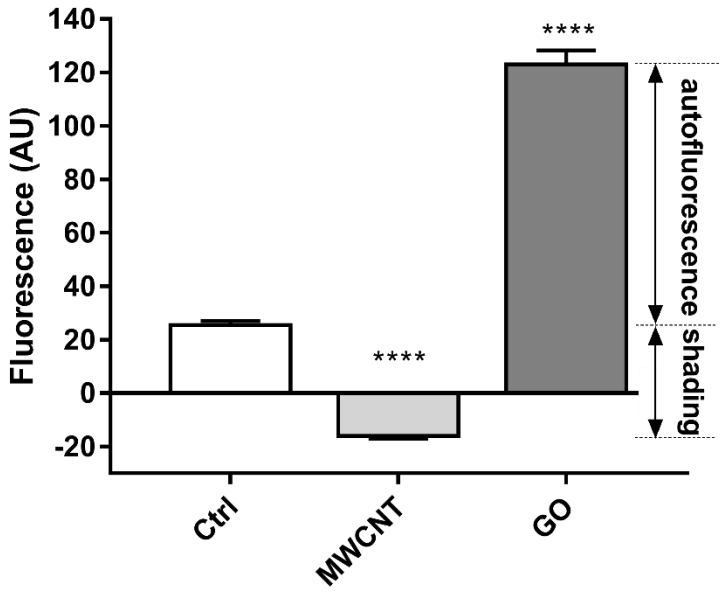


Figure 3

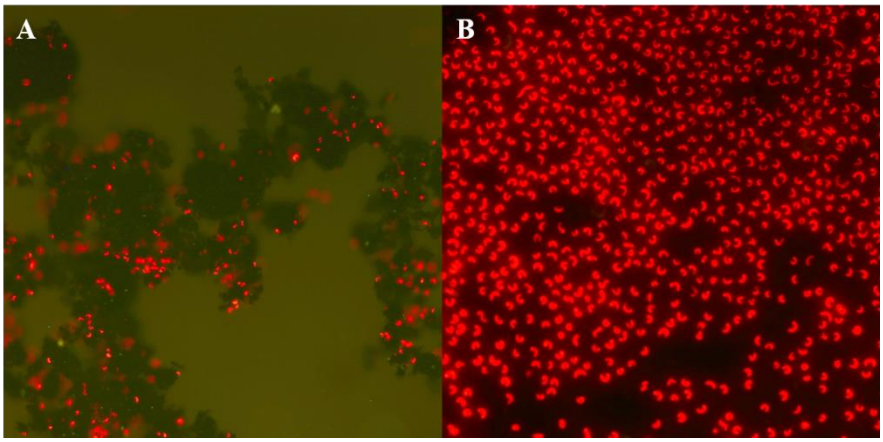
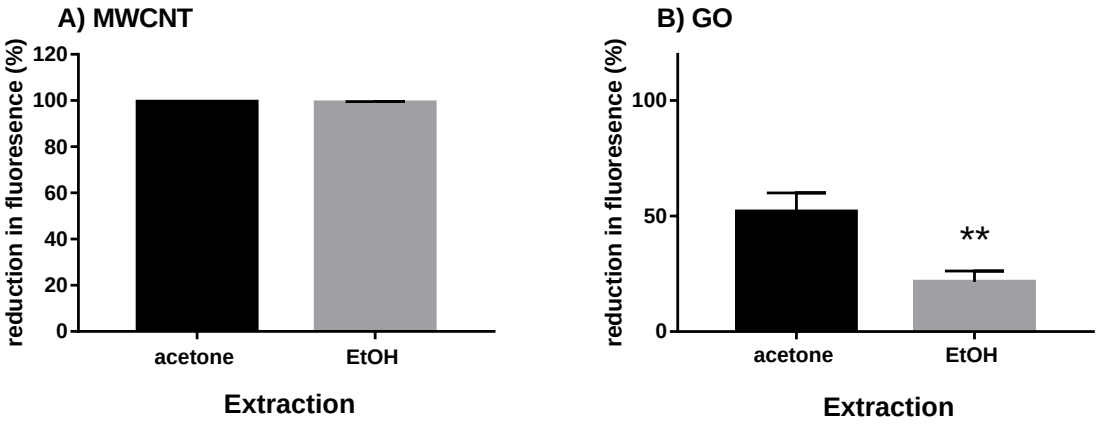


Figure 4



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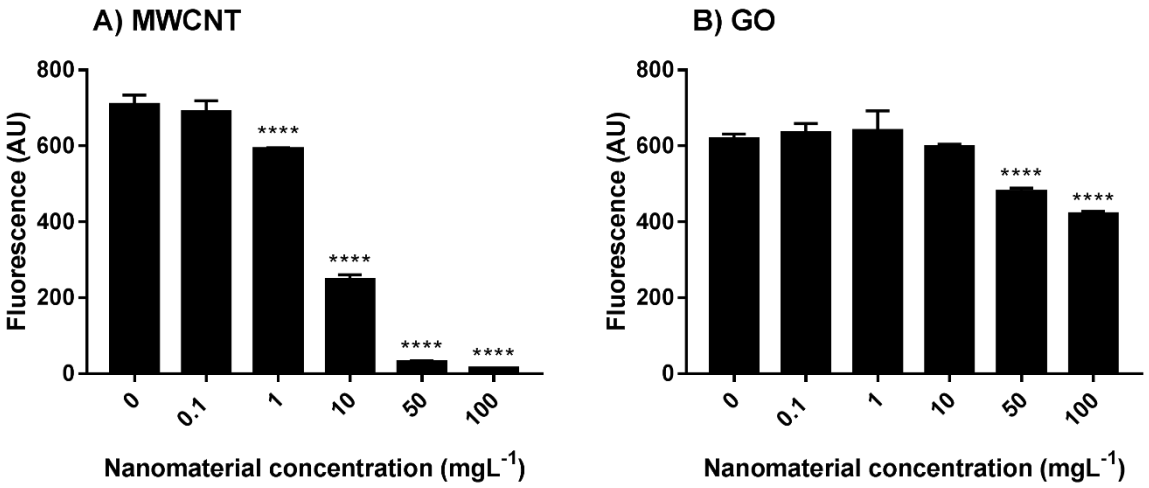

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630 Figure 6

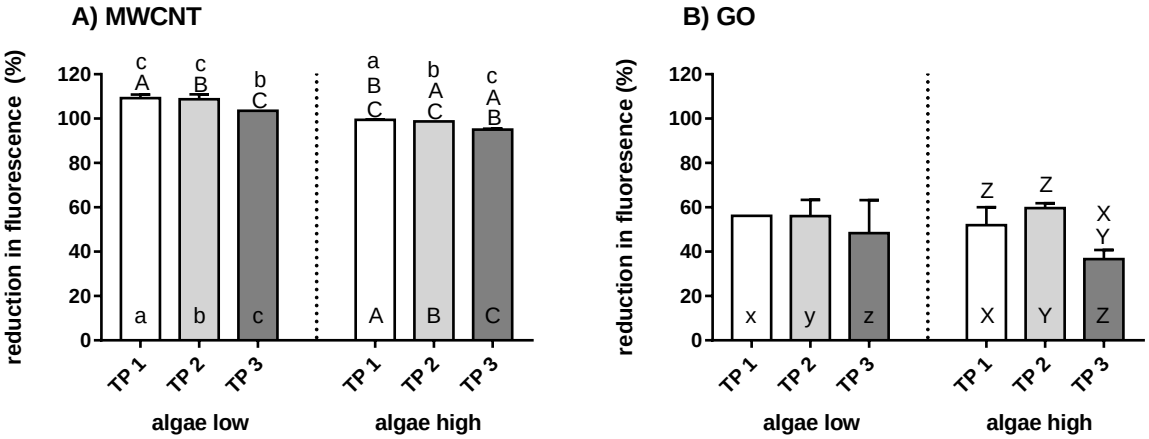

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Figure 7

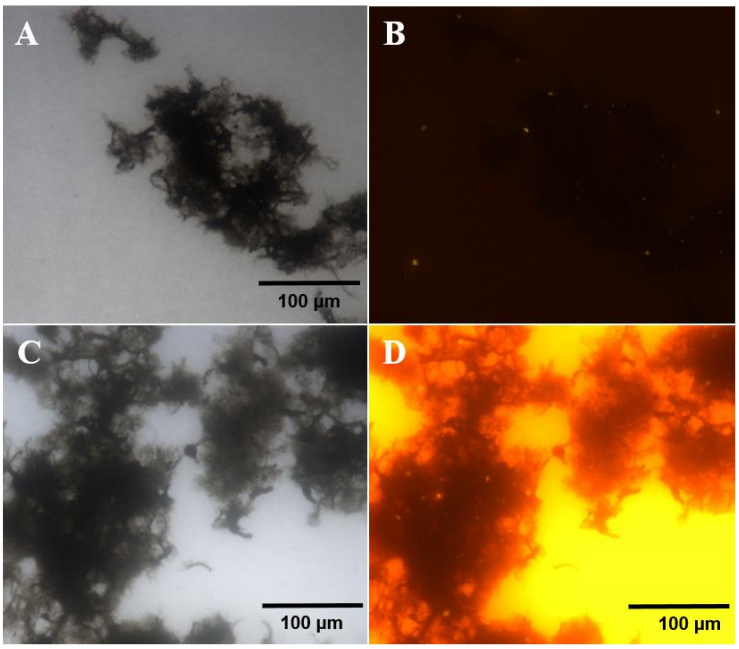

Figure captions

Figure 1. Electron microscopic images of A, B) MWCNT in SEM mode and C) GO in STEM mode showing the size of the CNMs used in this study.

Figure 2. *In vivo* fluorescence measurements of algae in control groups (Ctrl), and in the presence of 100 mg L^{-1} MWCNT and GO, respectively. The data is shown as mean±SD, n=3. Significant differences are indicated (P<0.05).

Figure 3. A) Algae adsorbed to agglomerates of MWCNT (10 mgL^{-1} in TG201+NOM), and B) Homogenous dispersion of algae in the presence of 50 mgL^{-1} GO (in TG201).

650 Figure 4. Reduction in algal Chl-a fluorescence, relative to control samples containing only
651 algae, after extraction with two different methods: acetone extraction with enhanced
652 flocculation (LBG) and EtOH extraction followed by filtration in the presence of A) 100 mg L^{-1}
653 MWCNT and B) 100 mg L^{-1} GO added. Data are presented as mean±SD, *n*=3. Significant
654 differences in fluorescence reduction between acetone and EtOH extraction are shown
655 ($P<0.05$).
656
657 Figure 5. Reduction in fluorescence (AU) of Chl-a extracts in the presence of different
658 concentrations of A) MWCNT and B) GO. Chl-a was extracted with acetone and subjected to
659 flocculation and settling in the presence of LBG. Data is shown as mean±SD, *n*=3. Significant
660 differences compared to control groups (0 mgL^{-1}) are indicated ($P<0.0001$).
661
662 Figure 6. Relative reduction of Chl-a fluorescence compared to control using a modified OECD
663 extraction protocol. Fluorescence was determined at two algae concentrations (low and high)
664 in the presence 100 mg L^{-1} MWCNT (A) and GO (B). Data bars represent samples with addition
665 of CNMs at different time points during the Chl-a extraction: TP1: CNMs added to the live
666 algae; TP2: CNMs added directly added after Chl-a extraction; TP3: CNMs added 10 minutes
667 before fluorescence measurements. The data is shown as mean±SD, *n*=3. Significant
668 differences between groups are indicated by different letters ($P<0.05$).
669
670 Figure 7. Microscope images of MWCNT. A, B) without Chl; C, D) MWCNT mixed with
671 extracted Chl. Fluorescence images (B, D) were taken at the same illumination and microscope
672 settings. Chlorophyll is seen in bright red surrounding the MWCNT. Background Chl is seen
673 as yellow after rapid bleaching during illumination.