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Investigating the impact of manufacturing process on the ecotoxicity of carbon nanofibers: a multi-aquatic species comparison

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ABSTRACT

Manufactured nanomaterial production is out pacing the ability to investigate environmental hazard using current regulatory paradigms, causing a backlog of materials requiring testing. To ameliorate this issue, regulatory bodies have proposed integrating safety into the production of novel nanomaterials, allowing for hazards to be identified early in development rather than aftermarket release. In addition, there is a growing interest in short-term ecotoxicity testing to rapidly identify environmental hazards. In this sense, the present study investigated three carbon nanofibers, created using different production methods, using short-term *in vitro* and *in vivo* exposures on fish cell lines, mussel hemocytes, crustacea and algae. The study investigated if differences in ecotoxicity hazard between the carbon nanofibers could be identified and, if so, which product could be considered less hazardous. A major challenge in assessing the potential hazards posed by manufactured nanomaterials is standardizing the preparation for testing. Standardized operating protocols have been proposed using protein to facilitate the preparation of stable stock suspension, which is not environmentally representative. As such, the study also assessed the potential impacts of these standardized protocols (with or without the use of protein) could have on the interpretation of environmental hazard. The results of the study demonstrated that there were clear differences between the three CNFs and that the dispersion protocol influenced the interpretation of hazard, demonstrating a need for caution when interpreting ecotoxicity in a regulatory context.

INTRODUCTION

The emergence of manufactured nanomaterials (MNMs) has the potential to completely revolutionize almost every facet of the global economy. Although achieving an accurate definition for MNMs is rather difficult, according to an EU recommendation, MNMs should have 50 % or more of the particles with between 1 nm-100 nm in at least one of its dimensions (EU, 2011).. It is estimated that the production of MNMs will be close to half a million tons by 2020 and will inevitably be released into the environment, with a final fate in aquatic ecosystems, making it an emerging concern for the environment (Canesi et al., 2015). MNMs are of unique concern as they display novel properties, mainly due to the reduction in size resulting in a consequent increase in the surface area to volume ratio, not observed in the source material of the nanoparticles, making it difficult to quantify potential hazards using conventional approaches. As a result, the ecotoxicity of MNMs cannot be estimated to be comparable to bulk materials and can lead to new unforeseen risks/hazards to human health and the environment (Auffan et al., 2009; Singh, 2016). This can be a challenge for regulatory programs in which novel paradigms may be necessary to address potential human and environmental impacts of MNMs.

One of the challenges associated with the production, marketing and use of MNMs is how to develop appropriate regulatory policies without hindering innovation. In this sense, it is essential to identify potential hazards of MNMs at an early stage in the production process to reduce the effort and resources required to demonstrate the product is safe and obtain approval by regulatory agencies for market use. From an environmental perspective, this can be achieved by investigating the potential ecotoxicity of the initial

MNMs prior to their use in products (Baun et al., 2008). There is however, a lack of knowledge for ecotoxicity data that limits the integration of this kind of information in the development of less environmentally hazardous MNMs (Schwarz-plaschg et al., 2017). This is most likely due to the fact that many authors and manufacturing companies focus research on the possible effects of MNMs towards human health with an emphasis on exposure risks towards factory workers or accidental release in confined spaces. When environmental hazards are considered, the possible exposure of biota in the environment is often perceived as a secondary concern. Nevertheless, the high production volume of MNMs will inevitably lead to their release (during the use and disposal of MNM based products) into the environment and their accumulation in aquatic media (Canessi et al., 2015). Therefore, it is essential to characterize their potential ecotoxicity.

Presently, ecotoxicity test guidelines (TGs) developed by the Organization for Economic Cooperation and Development (OECD) have been widely used in the assessment of potential hazards associated with novel materials. It has been recognized that OECD TGs are generally applicable to MNMs if particular issues derived from their peculiar physico-chemical properties are appropriately addressed (Kühnel and Nickel, 2014). These conclusions have been confirmed in a number of studies some of them referring to specific test guidelines. For instance Hund-Rinke et al., 2016 suggested preference be given to particular media preparation protocols and output measurement for OECD TG 201 and 202 that wouldn't require modifications to the initial TG. In this context, the OECD published a document giving guidance for sample preparation (OECD, 2012a) without specific changes to the TGs, considering that the main limitations were those related to the generation of appropriate test suspensions. These

TGs were also applied to single and multiple wall carbon nanotubes in the sponsorship program carried out at the OECD to study the applicability of OECD TGs to carbon based MNMs (OECD, 2015a, 2015b). Taking all this into account OECD TGs could be applied without major modifications as long as an appropriate test suspension is achieved.

As such, the preparation of test suspensions is a major issue in assessing the potential hazards posed by MNMs as it is well established that changes in the dispersion properties, such as the stability of the size of agglomerates/aggregates, can influence their toxicity (Hartmann et al., 2015; Kim et al., 2011; Langevin et al., 2018; Tantra et al., 2015). Special attention must also be paid to MNMs which have demonstrated difficulty in forming stable dispersions, such as nanocarbons, due to their intrinsic hydrophobicity. Previous research assessing nanocarbon ecotoxicity has also demonstrated a wide range of conflicting results, mainly associated with differences in the production process (Eckelman et al., 2012; Jackson et al., 2013). Considering the enormous variability in sizes, shapes and other physico-chemical properties of carbon-based MNMs on the market, it is necessary to establish consistent preparation techniques for this group of MNMs to avoid possible confounding factors when investigating their ecotoxicity.

A strict standardization is thus essential for the preparation of test suspensions to maintain comparability among MNMs when evaluating ecotoxicity (Laux et al., 2017). The development of standardized operating procedures (SOPs) for the preparation is also of interest in the context of regulation to guarantee intra and interlaboratory repeatability of results. Considering this, a dispersion SOP was defined in the European project NANOGENOTOX for the preparation of MNM suspensions and improved upon in the

project NANoREG (NANOGENOTOX, 2011). In a first step, a stable stock suspension is established by adding bovine serum albumin (BSA), which has been demonstrated to promote stable suspensions for hydrophobic MNMs (such as single and multi-walled carbon nanotubes) in a wide array of different test media (Vietti et al., 2013; Wang et al., 2010). This SOP was designed in the context of human toxicology however and its use in environmental studies is questionable.

Overall the objective of the present study was to assess the applicability of short-term ecotoxicity testing (based on *in* vitro and *in* vivo approaches) for a poorly studied group of MNMs, carbon nanofibers (CNFs), in a regulatory context. Under the scope of the European Union's Horizon2020 project one of the industrial partners increased the production volume of CNFs using a scaled-up production process. The objectives of the study were to determine: i) if there were differences in ecotoxicological hazards between three industrial products from different production processes using short term testing based on *in vitro* and *in vivo* approaches, ii) which product might be considered less hazardous for the aquatic environment and iii) whether or not the use of dispersion agent such as BSA in the dispersion SOP influenced the interpretation of the results.

MATERIALS AND METHODS

Products

Chemicals for ecotoxicological testing on *in vitro* cell cultures were purchased from Sigma Aldrich unless otherwise stated. Eagle's Minimum Essential Medium (EMEM) with non-essential aminoacids, (NEAA) and Na Pyruvate without L-Glutamine, EMEM with Earle´s Balanced Salt Solution without L-Glutamine,

Penicillin/Streptomycin (P/S), 10,000 units penicillin and streptomycin per mL, L-Glutamine solution (200 mM), and 100x NEAA were obtained from Lonza (Barcelona, Spain). Serum-free/phenol red-free MEM was purchased from PAN Biotech (Aidenbach, Germany). Alamar Blue (AB) reagent and 5-Carboxyfluorescein diacetate-acetoxymethyl ester (CFDA-AM) were from Life Technologies. Bovine serum albumin (BSA) was from Merck (Darmstadt, Germany).

Materials necessary for preparing culture media for *M. edulis* hemocytes BSA, Leibovitz L-15 medium (L-15), sodium chloride (NaCl), potassium chloride (KCl), calcium chloride dihydrate ($CaCl_2$-$2H_2O$), magnesium sulphate heptahydrate ($MgSO_4$-$7H_2O$), magnesium chloride hexahydrate ($MgCl_2$-$6H_2O$), glucose, sodium citrate, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), fetal bovine serum (FBS), Penicillin g, streptomycin sulphate, gentamycin sulphate were purchased from Sigma Aldrich.

Products for testing on *Daphnia magna* immobilization and reproduction tests as well as algae growth inhibition test, were purchased from Fisher Scientific.

Nanomaterials

Grupo Antolin (Burgos, Spain), one of the industrial partners in the NanoReg2 project, provided three CNFs: GANF, GANFg and GATam. GANF is the initial product manufactured which is a CNF created through catalytic vapor deposition (CvD) using a natural gas and sulfur feed stock at temperatures greater than 1100°C in a floating catalyst reactor (Vera-Agullo et al., 2007; Weisenberger et al., 2009). Deposition of graphene layers is promoted by metallic Ni while catalytically inactive NiS allows for the

formation of helical-ribbons with a stacked cup structure. GANF could be considered as the base product design with the other two CNFs being derivatives of the initial one. GANFg CNFs are produced by super heating GANF at 2500°C, which decreases the interlayer spacing in the CNF and increases the purity, in terms of C content, by decreasing the concentration of O and H (Weisenberger et al., 2009). GATam is generated using a scaled-up version of the production process for GANF. Differences in materials provided by Grupo Antolin are summarized in table 1.

Preparation of stock suspensions

Suspensions were prepared following the SOP with and without BSA. For both dispersion methods, 15.36mg of the corresponding CNF were pre-wet with 30µL of absolute ethanol in glass vials. Stock suspensions were prepared using 0.05% BSA-water (w/v) to achieve a final concentration of 2.56 mg/mL of CNF. For the dispersion without BSA, Milli-Q water was used instead of the BSA solution. The stock suspensions were then placed in an ice-water bath solution and sonicated using a probe sonicator for 16 minutes. The sonicator used were, Vibra-Cell™ VCX 130 (Sonics, Newton, CT, USA) in the laboratory working with fish cells, Branson-S450 (FisherScientific) in the laboratory working with mussel cells, and Vibra-Cell™ VCX 750 (Sonics) in the laboratory performing the algae and Daphnia tests . To standardize the amount of energy provided to the suspension among laboratories, the NANoREG probe sonicator calibration SOP was used to identify the appropriate settings to achieve 7056 J of delivered acoustic energy.

Characterization of MNM suspensions

Dynamic light scattering (DLS) was used to determine the hydrodynamic size of CNFs in the stock and test suspensions at the start of the experiment and at the end. Transmission Electron Microscopy (TEM) was also used to observe the morphology of the CNFs in ultra-pure water as well as test media. For the preparation of TEM images of the CNF stock and test suspensions carbon-coated grids were hydrophilized using a glow discharge apparatus (K100X, Emitech, UK). The glow discharge was performed for 180 s at an air pressure of 10^{-1} mbar and an electric current of 40 mA. This treatment was applied to TEM grids prior to the suspension deposition as it prevents most of the artefactual agglomeration phenomena during the drying of the suspensions on the TEM grids (Dubochet et al., 1982). CNFs in stock suspensions and fish cell culture media were observed by means of a 1400 PLUS (JEOL Ltd., Japan) microscope, whereas, CNFs in *M. edulis*, were analyzed using a FEI Philips CM12® (OR, USA) microscope.

The CNFs suspensions were also analyzed through colorimetric analysis to characterize stability of the suspensions. Each suspension was measured across the 340-700nm light wavelength at 10nm intervals to determine the wavelength yielding the highest value, which resulted to be 340nm for all three CNFs. Samples were taken from the top of the media at 0, 2, 4, 6 and at every 24 h until the end of the experiment. The results were then normalized using a blank for each media suspension and values at each time point were adjusted relative to the starting absorbance.

In vitro testing

Fish cell line preparation and exposure.

Two fish cell lines PLHC-1 (*Poecilipis lucida* liver cells*)* and CLC (*Cyprinus carpio* leucocytes) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). PLHC-1 was maintained in EMEM (with NEAA and Na Pyruvate without L-Glutamine) supplemented with 1 % L-glutamine, 1 % P/S and 5 % fetal bovine serum (FBS) at 30 °C, in an atmosphere of 5% CO_2. For cell treatments, medium was supplemented with 10 % FBS. CLC was cultured in EMEM (Earle´s Balanced Salt Solution without L-Glutamine) supplemented with 1% L-glutamine, 1% P/S, 1% NEAA and 10% FBS at 28°C, 5% CO_2. Both cell lines were sub-cultured twice a week using trypsin ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) in phosphate buffered saline (PBS).

PLHC-1 cells were seeded in 96-well plates (Greiner Bio-One GmbH, Germany) at a density of 5 x 10^4 cells/mL, whereas CLC cells were plated on poli-L-lysine coated 96-well plates (Greiner Bio-One GmbH, Germany) at a density of 7.5 x 10^4 cells/mL. Following a 24 h incubation, culture media was refreshed with media containing MNMs for 8 serial concentrations ranging from 0 to 256 mg/L in a 100 µL volume for 72 h. 256 mg/L was selected as it is highest possible concentration (1/10 of the media) able to be tested. Culture media for test concentrations below 256 mg/L were supplemented with stock media, BSA or ultra-pure water, prior to the addition of media containing MNMs to guarantee concentrations of stock media were consistent across test concentrations.

Fish cell line cytotoxicity measurements.

Cell viability was measured on the same set of cells according to a modified version (Lammel et al., 2013; Lammel and Navas, 2014) of a protocol described by Dayeh et al. (2013). After 72 hours of exposure to MNMs, medium was removed and cells were washed twice with PBS and incubated with 1.25% (v/v) AB and 4 µM CFDA-AM in serum-free/phenol red-free MEM (containing 1% NEAA). Fluorescence was measured on a microplate reader (Tecan GENios, Männendorf, Switzerland) at a wavelenght of 535/590 nm (excitation/emission) for AB and at 485/535 nm for CFDA-AM after 30 min of incubation in the dark. Cells were washed with PBS and incubated with 100 µL of neutral red (NR) solution (33 µg/mL in serum-free/phenol red-free MEM containing 1% NEAA) for 1 h in the dark. Following incubation, cells were rinsed with PBS and the retained dye was extracted with 100 µL of an acidified (1% glacial acetic acid) 50% ethanol/49% Milli-Q water solution.

Potential interference by the CNF dispersions with the cytotoxicity assays were tested in the presence of cells to simulate a more realistic assay scenario. Cells were seeded and exposed to CNFs in the same way as for the toxicity tests. Fluorescence readings of exposed cells were taken at the same wavelengths used for the three cyotoxicity assays before and after washing the cells twice with PBS. Measurements were repeated following the addition of serum-free/phenol red-free MEM (containing 1% NEAA) to the cells. Next, cells were incubated in the dark under exposure conditions with the conversion products of AB (0.1 and 1 µM of resorufin) and CFDA-AM (0.4 and 4 µM of 5-carboxyfluorescein) and fluorescence was read at time 0 and after 30 min.

After the washing step, NR (33 µg/mL) prepared in extraction solution was added and fluorescence was determined at time 0 and after 1 h at the corresponding wavelengths.

M. edulis cell culture preparation and exposure.

M. edulis individuals were collected from a relatively clean site, Saint-Cast-le-Guildo (48°37′48″N 2°15′24″W), previously identified as suitable for experimental research (Chevé et al., 2014). Mussels were placed in artificial sea water (30 psu, at 15°C with a 12 h light/dark cycle) for a 2-day acclimation period (unfed) prior to testing. A primary cell culture on *M. edulis* hemocytes was established following the methodology described in Barrick et al. (2018) with minor adjustments. Briefly, hemolymph was extracted from 50 mussels using a 23-gauge, 2 mL syringe containing 0.1 mL of Alseve (ALS) buffer (20.8 g/L glucose, 8 g/L sodium citrate, 3.36 g/L EDTA, 22.5 g/L NaCl, pH 7.0) (Cao et al., 2003). The total volume of the hemolymph was then stored and cell viability and cell concentration were determined through trypan blue exclusion method. Hemocyte was diluted to reach a concentration of $1x10^6$ cells/mL using the ALS solution. 200 µL of hemolymph per well was then seeded into a tissue culture treated 96-well microplate (Corning, VWR, France) to reach a concentration of $2x10^5$ cells/well. The plate was then placed into an incubator at 18°C (3.5% CO_2) for 30 minutes. After 30 minutes, hemolymph was aspirated and replaced with adjusted L-15 medium (20.2 g/L NaCl, 0.54 g/L KCl, 0.6 g/L CaCl2, 1g/L $MgSO_4$, 3.9 g/L $MgCl_2$) containing 100 units/mL penicillin G, 100µg/mL streptomycin, 40 µg/mL gentamycin, 10% glucose and 10% FBS, (pH 7.0). Cells were left to adhere overnight prior to exposure. After 24 hours, cell culture media was refreshed with cell culture media containing MNMs in suspension (0 - 256 mg/L). Culture media for the 10 exposure conditions were prepared in Eppendorf

tubes and vortexed for 10 seconds prior to refreshing media. Exposure conditions below 256 mg/L were supplemented with stock media, BSA or ultra-pure water depending on which SOP was being tested, prior to preparing the serial dilutions to ensure consistency across test conditions. Both dispersion techniques were tested in parallel and on the same microplate to reduce the risk of inter-plate variation influencing the interpretation of results. Wells containing no cells were also prepared for all test concentrations to account for potential interference as well as to limit the risk of false positive results (Drasler et al., 2017). The cell culture was then returned to the incubator for 24 hours.

M. edulis cytotoxicity measurements.

The previously described method was adapted to *M. edulis* hemocytes to assess cell viability. Briefly, cell culture media was removed and the cells were washed twice with PBS adjusted more marine organisms (Le Marrec-Croq et al., 1999) and incubated with 10% (v/v) of AB and 2µM of CFDA-AM prepared in PBS for 30 minutes at 18°C (3.5% CO_2). AB was measured using the colorimetric method described by the manufacturer using a spectrophotometer at the light wavelengths (570 and 600nm) for absorbance. Readouts were measured at the start and end of the assay (Rampersad, 2012). To account for coloration and physical obstruction of light by CNFs remaining in the wells, the rate of change in dye reduction was used to measure cellular metabolism. The rate of change in the wells containing only CNFs were also measured to determine if the presence of the CNFs interfered with dye reduction. CFDA-AM was measured as previously described with wells containing only CNFs at each respective concentration being used to account for interference.

In vivo Testing

Algal growth inhibition tests.

Growth inhibition testing was conducted following OECD TG 201(OECD, 2011). Prior to exposure, *P. subcapitata* was cultured for 3 days at 22°C and constant illumination to ensure the algae was in the exponential growth phase. For the exposure assay algal densities were prepared at $8x10^3$ cells/mL and exposed to by adding medium prepared with CNFs using 8 concentrations ranging from 0.78 to 50 mg/L for 72 hours. Uniform exposure was maintained through magnetic stirring using a previously defined protocol (Manier et al., 2016). Algal growth was measured using fluorescence microplate reader (TECAN SAFIRE 2, Switzerland) at a wavelength of 438nm/685 nm (excitation/emission). Negative controls containing only CNF suspensions were performed in parallel to identify potential interference in the measurement by CNFs. Growth rates were determined as relative to the control. The use of BSA in the dispersion SOP was determined to interfere with algal growth. As a result, the dispersion protocol without BSA was the only protocol tested.

D. magna testing.

Acute toxicity testing was conducted following the OECD TG 202 (OECD, 2011) and chronic toxicity was conducted following OECD TG 211 (OECD, 2012). For each test, CNF suspensions were prepared by a dilution of the stock media in test medium (ISO or M4 medium) and stirred continuously prior to conducting the assays. Young daphnids, aged less than 24 hours at the start of the test, were exposed to 6 different concentrations of CNFs (3.125-100 mg/L) for a period of 48 hours. After 24h and 48h,

immobilization of the young daphnids exposed to CNFs was recorded and compared with control values.

For chronic reproduction test, young female *Daphnia magna* (10 animals at each test conditions) were exposed to the CNFs suspensions to 8 concentrations (0.19-25 mg/L). The total number of living offspring produced per parent animal were recorded after a 21 days exposure period and compared with control values. To maintain a uniform exposure in the water column concentrations, a complete renewal of the test media with new CNF suspensions was performed after 24 hours during the acute test and every working day for the chronic reproduction test.

Statistical Analysis

Effective concentration values (ECs) of toxicity were calculated by fitting to dose-response curves using the Hill equation using the REGTOX_software v.7.0.4 macro from Microsoft excel (http://www.normalesup.org/~vindimian/en_index.html).

RESULTS

Physico-chemical characterization of the CNFs

Transmission electron microscopy results indicated aggregates/agglomerates approximately 1 µm in size formed during the preparation of the stock suspension (Figure 1). No differences among CNFs as well as test media could be easily identified. DLS results indicated that frequency size distribution of CNF agglomerations could be measured reliably for the dispersions generated with the SOP when BSA was used (Table 2). DLS is typically considered unsuitable for carbon-based products as it assumes

particles in suspension are spherical. However, CNF agglomerates can be approximated as spherical and be analyzed as long as the polydispersion index (PdI) is low (Reinert et al., 2015). In general, the Z-average (d-nm) was below 500 nm with a low PdI of around 0.1-0.3, suggesting a low variation in the size distribution of the agglomerates in the stock suspensions. The stock suspensions, as approximated by DLS, of all three CNFs were comparable in aggregate/agglomerate size for GANF (489.3 d-nm), GATam (414.3 d-nm) and GANFg (479.8 d-nm). In PLHC-1 media, aggregates for GANF (471 d-nm), GATam (462.1 d-nm) and GANFg (393.1 d-nm) exhibited similar z-average values. In CLC media GANF (460.1 d-nm), GATam (426.6 d-nm) and GANFg (344 d-nm) z-averages were also similar to those detected in PLCH-1 media. In *M. edulis* hemocytes culture media there was a decrease in the size of the agglomerates by approximately half for GANF (260.1 d-nm), GATam (197.8 d-nm) and GANFg (244.1 d-nm). DLS results could not be accurately analyzed in media for *D. magna* and *P. subcapitata* due to high polydispersion. In media where DLS could be measured, the approximated agglomerate sizes suggested a stable suspension throughout the duration of the experiment.

Most of the dispersions prepared without BSA could not be accurately analyzed through DLS, indicating large agglomerate sizes and poor colloidal stability. Only in the case of GANFg suspension obtained in M. edulis culture media the z-average (174.9 d-nm) could be reliably measured having associated a PDI of 0.22.

Colorimetric results were analyzed in relation to the initial concentration of CNF in suspension approximated through absorbance. The results demonstrated that the stability of the stock suspensions was improved using when BSA with more of the CNF remaining in suspension for the duration of the experiment as summarized in table 3.

When testing the stability of the preparation using BSA in the cell culture media for both fish cell lines and mussels appeared to promote stability all three CNFs. When using the suspensions prepared without BSA, GANF and GATam displayed rapid sedimentation where as GANFg displayed higher stability for the duration of the experiment. With respect to test media for *D. magna*, GANF and GATam quickly fell out suspensions when prepared without BSA. GANFg was still able to be measured in suspension at the end of the experiment in OECD 201, 202 and 211 test media and when not using BSA. Again, BSA notably improved stability for the CNFs in OECD 201 and OECD 211 media.

Fish cell lines

Results of cytotoxicity in fish cell lines appear in Table 4 (initial dispersion with BSA) and in Table 5 (initial dispersion without BSA). The use of BSA did not appear to have a deep influence in the cytotoxicity of CNFs when tested on fish cell lines. In PLHC-1 cells CNFs cytotoxicity was not detectable for some of the used assays at the concentrations tested (EC25 or EC50 > 256 mg/L). In a number of cases, CNFs provoked some kind of interference with the fluorescent readouts at the higher concentrations tested preventing the calculation of accurate EC25 or EC50. However, for all the three CNFs the EC25 for CFDA-AM assay could be calculated (ranging from 10.5 to 58.0 mg/L). In addition, for some of the CNFs and assays used the calculated EC25 and EC50 values were close to the maximal concentrations applied (Tables 4 and 5).

In the CLC cells, as in the PLHC-1, no strong differences in cytotoxicity were observed depending between the two dispersion SOPs. GANF provoked a decrease in

cell viability detected by means of the AB and CFDA-AM assays allowing the calculation of EC25 (ranging from 3.9 to 12.8 mg/L) and of EC50 (ranging from 18.9 to 51.4 mg/L, respectively). However, the NR assay did not allow the calculation of the EC50 (>256 mg/L). In the case of the GANFg all the EC25 and EC50values were higher than 32 mg/L and in some cases it could only be determined that they were above the highest concentration used (>256 mg/L). The cytotoxicity caused by GATam could be detected through the AB (EC50 of 46.7 mg/L in the dispersion with BSA and of 26.1 mg/L in the dispersion without BSA) and the CFDA-AM (EC50 of 89.9 mg/L in the dispersion with BSA and of 49.7 mg/L in the dispersion without BSA) assays. The NR assay led to EC50 values higher or close to the highest concentration used, depending on the use of BSA or not in the initial dispersion.

M. edulis hemocytes

Results indicated that the effects (EC50 values) on cellular metabolism (Alamar Blue) were observed at lower concentrations for GANF (36.9 mg/L) and GATam (120.3 mg/L) when the dispersion was without BSA as compared to dispersions with BSA for GANF (219.8 mg/L) and GATam (178.6 mg/L). The inverse was observed for GANFg, which showed higher EC50 values in the AB assay when no BSA was applied (83.4 mg/L) compared to the assays with the dispersion with BSA (34.4 mg/L). Higher concentrations were necessary to observe effects on cell membrane integrity (CFDA-AM) but a similar pattern for GANF could be seen in that effects were observed at lower concentrations without BSA (159.5 mg/L) than in tests with BSA (219.8 mg/L). For GATam (>256 mg/L) and GANFg (191.1 mg/L) EC50 values were similar in the CFDA-

AM assay with or without BSA being used (ranging from 191.1mg/L to >256mg/L for both CNFs).

OECD TG 201

Interferences with the fluorescence analyses occurred, due to a shading effect of the CNFs potentially limiting algal growth, at concentrations higher than 12.5 mg/L in the algal growth inhibition test and consequently these conditions were removed from the analysis as a result. When comparing EC50 values with those generated when no BSA was used, the lowest values were found with GATam (2.12 mg/L) with similar values for GANF (3.09 mg/L) and higher values for GANFg (8.48 mg/L).

OECD 202 & 211

A clear effect of the three CNFs on the mobility of *D. magna* was observed when the test suspension was prepared without BSA. EC50 values for GANFg (5.83 mg/L) were lower than those for GANF (9.99 mg/L) and GATam (8.88 mg/L). No effects were observed for GANF (>100 mg/L) and GANFg (>100 mg/L) when BSA was used. The EC50 value for GATam (58.81 mg/L) was much higher than this observed when the stock suspension was prepared without BSA.

The use of BSA in the dispersion SOP established a stable stock suspension that could easily be reestablished through vortex, allowing for chronic toxicity testing to be conducted. As a result, chronic testing was only conducted when BSA was added in stock suspension. A clear inhibition of *D. magna* reproduction was observed whatever the CNF

tested. In addition, the GAtam CNFs showed a lower EC50 (0,.32mg/L) than those of GANFg CNF (EC50 = 6.18mg/L) and GANF CNF (EC50 = 1.58mg/L).

DISCUSSION

In the assessment of MNM hazards, it is essential to use multiple endpoints, generated *in vitro* and *in vivo*, with several species as there may be different potential mechanisms of toxicity (Oleszczuk et al., 2015). The present study followed a tiered approach applying several *in vitro* assays on fish and mussel hemocyte cells to elucidate different mechanisms of toxicity to help in the design of higher tier *in vivo* ecotoxicity testing. Thereafter, two *in vivo* assays, that are used in several regulatory paradigms (e.g. CLH, REACH), were used to gain essential information about the possible environmental hazards of the tested substances. In addition, these short-term assays were accompanied by the reproduction toxicity test in *daphnia* to potential determine if long term ecotoxicity could discriminate between the three CNFs.

Unexpectedly, these minute changes in the production process do lead to differences in the ecotoxicological responses among endpoints investigated. One potential explanation could be the ease in which the CNFs dispersed, with GANFg displaying more stability compared to GANF and GATam regardless of dispersion SOP or medium. This could be linked to the fact that GANFg was reported to have a higher purity (structural and chemical) than GANF and GATam, which could limit its ability to interact with the test media, effectively reducing the probability of aggregate formation. Differences in CNF stability due to changes in production method could have potential implications on the assessment of ecosystem hazards as a prolongation in the water

column could promote environmental transportation and impact organisms with life cycles within the water column more so than benthic organisms (Jackson et al., 2013). Consequently, a less stable CNF would also sediment rapidly and could be sequestered in an ecosystem, posing a long-term risk to the environment and a strong impact on sediment dwelling organisms. The present study highlights this challenge in assessing MNM hazards as three nearly identical CNFs, produced by the same industrial partner, can have divergent, species-specific impacts highlighting the need for multispecies assessment of MNM ecotoxicity.

When conducting a literature review, one finds that there is a lack of information regarding the ecotoxicity of CNFs. There are however a number of studies conducted on multiwalled and single walled carbon nanotubes (MWCNTs and SWCNTs). While CNTs are not directly comparable to CNFs they do provide a framework for understanding the toxicity of other nanocarbons of fibril nature. CNTs have been previously shown to have EC50 values for *P. subcapitata* ranging from 1.8 to 24 mg/L and cause immobilization in *D. magna* at concentrations ranging from 9 to 25 mg/L (Jackson et al., 2013; Schwab et al., 2011; Zhu et al., 2009). In the context of ecotoxicological hazards and thresholds of toxicity (ECHA, 2017), these concentrations of CNTs would be considered to result in low (>100mg/L) to high (<10mg/L) toxicity towards *D. magna*. As a result, it is difficult to conclude where or not carbon-based nanomaterials are inherently a risk towards the environment.

This highlights an essential issue in hazard assessment of MNMs in that the generation of stable suspensions, and the respective influence of the protocol used, alters the final determination of ecotoxicity. In the present work, a SOP previously established

and applied in several large EU research projects, involving a number of laboratories, was used to investigate the hazards of the CNFs. This SOP uses BSA as a dispersant to increase the stability of the stock suspension which facilitates an appropriate exposure of the MNMs within biological systems, ensuring a constant concentration throughout the exposure period (limiting the sedimentation due to the formation of big aggregates or agglomerates), which is a prerequisite to calculate an accurate assessment of ecotoxicity. When colloidal suspension stability is poor, it is difficult to maintain uniform exposure conditions throughout the experiment and makes the detected toxicity unclear as to whether it is due to the MNMs or a decrease in the probability of physical contact with big aggregates or agglomerates. At the same time, the use of a dispersant allows a better comparability of results between MNMs. One of the key questions that the present work investigated was the influence of BSA as a dispersant on the interpretation of CNF ecotoxicity. One potential explanation for the results in the present study is that the use of BSA coats the CNFs and effectively reduces the toxicity as evidenced by testing with *D. magna*. This could potentially be due to the reduction of the release of impurities (Nickel, Sulfur, hydrocarbons) from the CNFs (GANF and GATam) or the a decreased of physical interaction between *D. magna* and the CNFs (Oleszczuk et al., 2015). Previous research also suggested that proteins in test media may alter the organisms ability to interaction with carbon based MNMs (Lukhele et al., 2015). The present study appears to support this idea as BSA visually reduced the interaction between CNFs and *D. magna* (adsorption onto shell, antenna and other parts of the organisms) (Figure 2).

In vitro testing also elucidated unexpected information regarding the three CNFs. Results for the fish cell lines demonstrated a notable difference between the CLC and

PLCH-1 cells. Leucocytes from *C. carpio* were affected by the exposure to the CNFs with some clear adverse effects. Leucocytes play a role in the clearance of pollutants and are most likely one of the first cell types to respond to CNF exposure. Based on the EC50 values GANFg displayed the lowest toxicity towards this cell line. This however, was not in agreement with the results for *M. edulis* hemocytes which suggested GANFg may be more hazardous. One potential explanation for this is that the culture media for *M. edulis* has a higher ionic concentration which can lead to alterations in the CNFs' behavior. In the case of the PLHC-1 cells, the differences in toxicity among CNFs were smaller but, again, GANFg shows a slightly lower toxicity than the other CNFs.

Previous work on carbon-based MNMs has demonstrated a need for a surfactant to establish a stable suspension. Many of the common surfactants (Tween 20 and isopropanol) however have been demonstrated to be toxic towards sensitive aquatic organisms including *D. magna* (Olasagasti et al., 2009). The use of BSA has been proposed as a suitable dispersant for toxicity testing of MNMs as it has been demonstrated to have little to no adverse effects on test organisms. In the present study the obtained results demonstrate that the use of BSA can influence the interpretation of ecotoxicity in some of the assays used, which can influence its efficacy in identifying environmental hazards. Although, proposing a solution or an appropriate protocol for the dispersion of nanocarbons (with or without BSA) is out of the scope of this work, it is evident that this must be seriously considered when proposing dispersion protocols to be applied in internationally accepted regulatory testing.

CONCLUSION

In order to apply environmental hazard assessments in an industrial setting, ecotoxicity testing needs to be able to discriminate between products that are very similar in design. In this sense, Grupo Antolin provided three CNFs produced using different production methods with the aim of determine if “safety” could be integrated in the development of their product. Unexpectedly, the three CNFs did demonstrate some differences with their ecotoxicity hazards towards the test organisms, which suggests that differences in purity (chemical or structural) could determine ecotoxicity. Representative organisms from multiple levels trophic levels and ecosystems were selected in the present study to better establish a holistic environmental hazard assessment that CNFs pose. In this sense, it becomes possible to make a prediction as to whether or not these MNMs pose a significant environmental risk. The current study highlights key challenges associated with environmental risk assessment as there is variation in interspecies responses when exposed to the CNFs. Despite this, there does seem to be evidence suggesting that GANFg may be less hazardous for the environment (by comparison with the other CNFs) due to the observation of improved stability, which may alter which environmental compartment is impacted, and that chemical purity may play a role in the hazards associated with the CNFs, as evidenced by the OECD TGs. In order to verify this however, an analysis of environmental fate needs to be conducted to determine whether or not these CNFs have different potentials for bioaccumulation.

It is generally accepted that alterations of MNMs through stabilizing agents is undesirable but in some cases natural or dissolved organic matter, at the lowest possible concentration, may be used on a case-by-case basis (OECD, 2019). The results of the present study demonstrated that the use of BSA in preparation of the stock suspensions

significantly reduced the identification of toxicity, but it is unclear if this is due to functional differences between the materials or due to an alteration of stability profiles of the colloidal suspensions. In general, the dispersion SOP without BSA resulted in test suspensions with poor stability and rapid sedimentation of the CNFs, making it difficult to draw conclusions on CNF hazards and indicates a need for a stabilizing agent to investigate CNF ecotoxicity. This makes the use of BSA in the dispersion SOP a more suitable technique in establishing a stable stock suspension as it allows for the testing of hydrophobic materials. In the context of ecotoxicology however, the use of BSA is not recommended due to the difficulties in establishing environmental representation and additional research is required define a standardized dispersant for ecotoxicity testing. Presently, work is being conducted to identify a more suitable dispersant for environmental testing with some notable examples being carboxymethylcellulose sodium salt and gum arabic (Bourdiol et al., 2012). In the context of hazard/risk assessment the current study investigated safety in the context of the initial MNM. To fully determine the hazards associated with the CNFs, a link needs to be made with the final product and associated with risks across the life cycle of the product.

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Figures

Figure 1: TEM images of GANF, GATam and GANFg stock suspensions and of suspensions in the different media. Scale bars in stock suspension images, PLHC-1 and CLC medium suspension images indicate 0.5µm. Scale bars in *M. edulis* medium suspension images indicats 1µm.

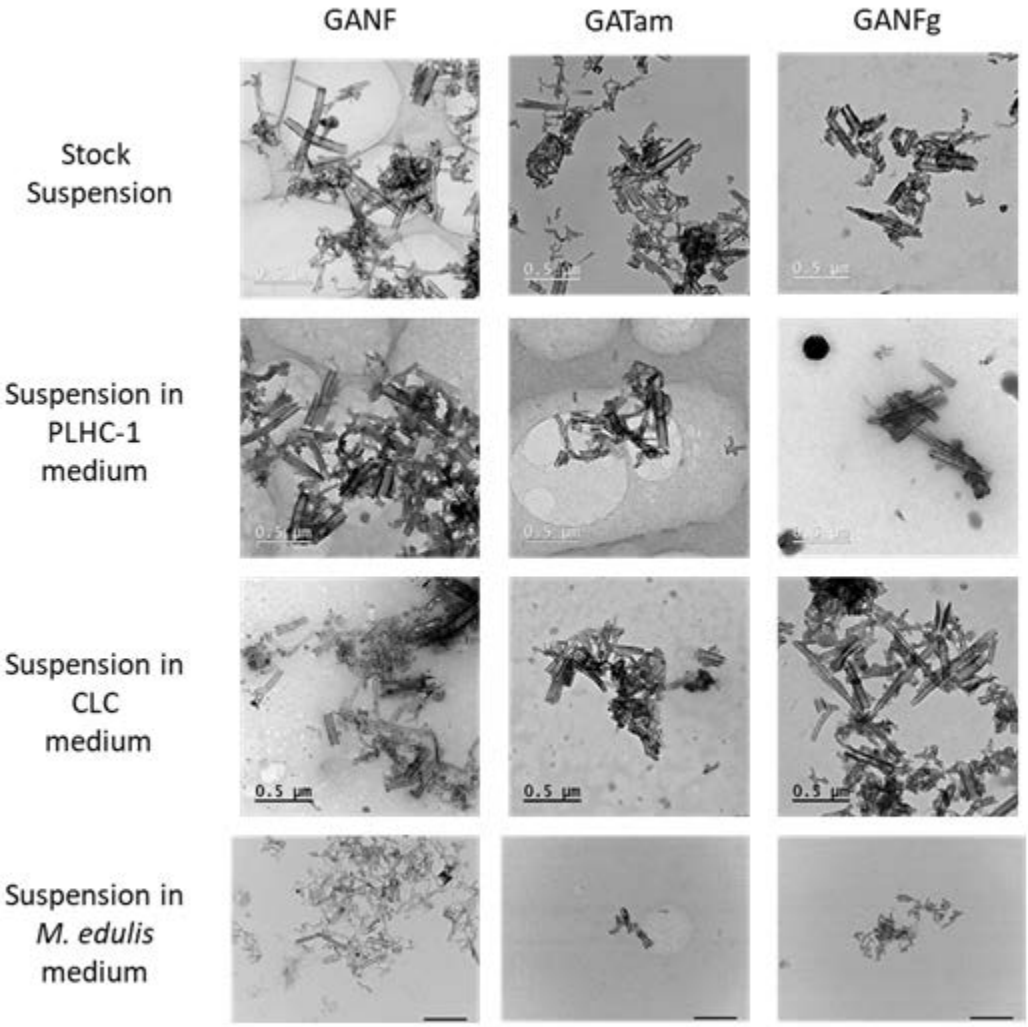

Figure 2: Images of *D. magna* exposed to GANFg (25mg/L) when testing was performed with BSA (A) or without BSA (B) in the preparation of the stock dispersion.

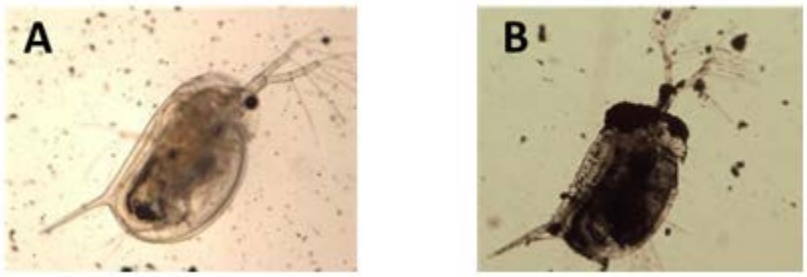

Table 1. Key properties of CNF provided by Grupo Antolin

Measured property	**Unit**	**GANF**	**GANFg**	**GATam**
Fiber diameter (TEM)	nm	20-80	20-80	20-80
Carbon purity (TGA)	%	>85	>99	>80
Apparent density	g/cc	~0.06	~0.08	~0.08
Specific surface area (BET N_2)	m^2/g	100-170	70-90	70-140
Graphitization degree (XRD)	%	≈70	≈90	≈60
Electrical resistivity	Ω-*m	$1*10^{-3}$	$1*10^{-4}$	$1*10^{-3}$

Table 2. CNF suspensions prepared using BSA in the dispersion SOP measured through DLS. N/A indicates results were not suitable for reporting. Size distribution graphs are provided in the supplemental materials.

Start of Experiment (Dispersion SOP with BSA)						
	GANF		GANFg		GATam	
Test media	Z-average (d-nm)	PdI	Z-average (d-nm)	PdI	Z-average (d-nm)	PdI
Stock suspension	489.3	0.13	479.8	0.18	414.3	0.23
Culture media (PLHC-1)	471	0.4	393.1	0.4	462.1	0.4
Culture media (CLC)	460.1	0.5	344.0	0.4	426.6	0.4
Culture Media (*M. edulis*)	260.1	0.36	479.8	0.18	414.3	0.23
D. magna (OECD 202)	N/A	N/A	N/A	N/A	N/A	N/A
D. magna (OECD 211)	N/A	N/A	N/A	N/A	N/A	N/A
P. subcapitata (OECD 201)	N/A	N/A	N/A	N/A	N/A	N/A
End of Experiment (Dispersion SOP with BSA)						

	GANF		GANFg		GATam	
Test media	Z-average (d-nm)	PdI	Z-average (d-nm)	PdI	Z-average (d-nm)	PdI
Stock suspension	527.5	0.12	441.2	0.29	389.4	0.16
Culture media (PLHC-1)	403.4	0.4	370.6	0.4	356.6	0.4
Culture media (CLC)	526.8	0.5	340.8	0.4	327.5	0.4
Culture Media (*M. edulis*)	527.5	0.12	441.2	0.29	389.4	0.16
D. magna (OECD 202)	N/A	N/A	N/A	N/A	N/A	N/A
D. magna (OECD 211)	N/A	N/A	N/A	N/A	N/A	N/A
P. subcapitata (OECD 201)	N/A	N/A	N/A	N/A	N/A	N/A

Table 3. Stability, measured through colorimetry, of CNF suspensions prepared with and without using BSA in the dispersion SOP. Values a presented as percentage relative to the start of the experiment (C/C_0*100).

Colorimetric Stability of Test Suspensions at End of the Experiment						
	GANF		GANFg		GATam	
Test Media	BSA	No BSA	BSA	No BSA	BSA	No BSA
Stock suspension	11.5	0	47.72	48.26	17.62	0
Culture media (PLHC-1)	72	27	95	71	97	30
Culture media (CLC)	69	28	92	95	103	37
Culture media (M. edulis)	47.88	0	75.19	89.8	86.34	0
D. magna (OECD 202)	20.6	0	8.79	16.73	37.28	0
D. magna (OECD 211)	81.55	0	87.35	6.39	66.86	0
P. subcapitata (OECD 201)	91.67	0	87.98	94.31	68.11	0

Table 4: EC_{25} and EC_{50} values calculated for the different assays when BSA was used in the stock suspension for the CNFs. > indicates EC values exceeded concentrations where results could reliably be analyzed.

EC25 and EC50 Values (Dispersion SOP with BSA)									
	GANF			GANFg			GATam		
	in vitro Testing								
	Alamar	CFDA-	Neutral	Alamar	CFDA-	Neutral	Alamar	CFDA-	Neutral

Cell Type	Blue		AM		Red		Blue		AM		Red		Blue		AM		Red	
	EC25	EC50	EC25	EC50	EC25	EC50	EC25	EC50	EC25	EC50	EC25	EC50	EC25	EC50	EC25	EC50	EC25	EC50
PLHC-1	>64	>64	10.5	>256	>256	>256	>32	>32	28.2	>256	>256	>256	>128	>128	32.2	>256	147	234.4
CLC	3.9	18.9	4.3	37.6	116	>256	>32	>32	114.7	252	>256	>256	15	46.7	14.7	89.9	113.4	>256
M. edulis Hemocytes	112.8	219.8	160.8	219.8	-	-	10.5	34.4	56.9	191.1	-	-	120.8	178.6	53.9	191.1	-	-

in vivo Testing

OECD Test	EC25	EC50	EC25	EC50	EC25	EC50
D. Magna OECD 202	>100	>100	>100	>100	58.81	62.8
D. Magna OECD 211	1.24	1.58	1.61	6.18	0.28	0.32
P. subcapitata OECD 201	1.87	3.09	5.12	8.48	1.04	2.12

Table 5: EC_{25} and EC_{50} values calculated for the different assays when BSA was not used in the preparation of the CNF stock suspensions. > indicates EC values exceeded concentrations where results could reliably be analyzed. – indicates that analysis was not conducted.

EC25 and EC50 Values (Dispersion without BSA)																		
	GANF						GANFg						GATam					
in vitro Testing																		
Cell Type	Alamar Blue		CFDA-AM		Neutral Red		Alamar Blue		CFDA-AM		Neutral Red		Alamar Blue		CFDA-AM		Neutral Red	
	EC25	EC50	EC25	EC50	EC25	EC50	EC25	EC50	EC25	EC50	EC25	EC50	EC25	EC50	EC25	EC50	EC25	EC50
PLHC-1	61.8	175.8	34.7	>256	>256	>256	201.7	>256	58.0	>256	>256	>256	69.3	160.0	50.8	>256	>256	>256
CLC	12.8	36.9	10.2	51.4	184.8	>256	35.3	101.2	32.2	220.9	>256	>256	8.1	26.1	11.2	49.7	65.8	215.6
M. edulis Hemocytes	8.93	36.9	27.8	159.5	-	-	42.8	83.4	44.4	>256	-	-	67.2	120.3	60.8	>256	-	-
in vivo Testing																		
OECD Test	EC25		EC50		EC25		EC50		EC25		EC50							
D. Magna OECD 202	6.81		9.99		3.94		5.83		5.5		8.88							