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Ecotoxicological assessment of a graphene-based commercial ink toward freshwater ecosystems: from single-species tests to a microcosm approach

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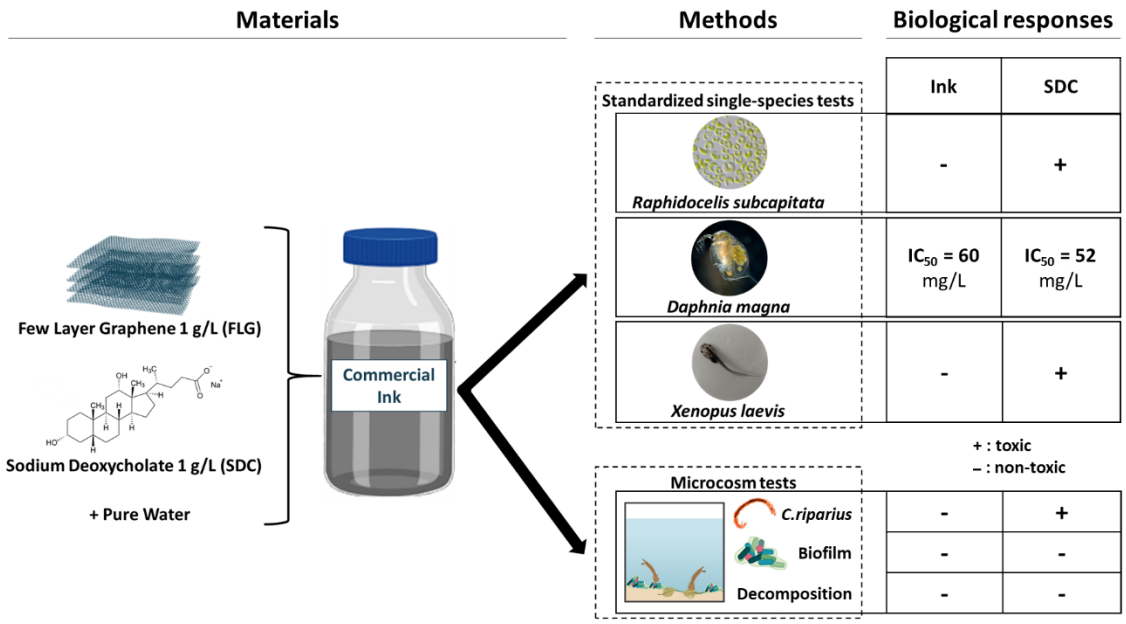
Abstract

The growing use of nanomaterials, including few-layer graphene (FLG) under various forms such as inks, raise concerns about their potential ecological impacts. This study evaluates the ecotoxicity on the aquatic compartment of a commercial ink composed of FLG stabilized with sodium deoxycholate (SDC) at a concentration of 1 g/L, through single-species bioassays and microcosm approaches on various aquatic organisms. The algae *Raphidocelis subcapitata*, the crustacean *Daphnia magna*, and the amphibian larvae *Xenopus laevis* were exposed to 0; 0.1; 10 and 50 mg/L of ink or SDC alone. The microcosm study was conducted with the ink or SDC at 0.1 mg/L, focusing on biofilm (diatoms and bacterial consortium) integrity and life-history traits of the dipteran *Chironomus riparius*. Results showed that although the ink exhibited weak toxicity in single-species bioassays or in microcosms, SDC alone caused significant toxic effects, depending on the biological model and concentration. Cellular alterations were observed in algae exposed to 0.1 mg/L of SDC, while acute toxicity occurred at concentrations over 50 mg/L in daphnia. Growth inhibition was observed in amphibian larvae at 0.1 and 1 mg/L without genotoxic effect. Microcosm study revealed slight changes in biofilm diversity and inhibition of chironomid growth by SDC (0.1 mg/L). This study highlights the importance of comprehensive ecological risk assessments for graphene-based materials and associated co-contaminants, emphasizing the environmental risks posed by SDC. The findings demonstrate the value of realistic approaches to evaluate the hazards of these chemicals and suggest that their release into the aquatic environments could pose significant ecological threats.

Keywords: nanomaterials; graphene; ecotoxicology; bioassays; microcosm; sodium deoxycholate.

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Graphical Abstract



Highlights

- Graphene-based ink showed no toxicity, unlike dispersant SDC alone.
- SDC altered chironomid's life traits under environmentally relevant conditions.
- In combination, FLG adsorbed SDC, reducing its bioavailability and toxicity.

I. Introduction

Carbon-based nanomaterials (CBNs), including graphene-based materials (GBMs), emerged at the beginning of the 21st century and quickly attracted industrial interest for the development of new applications due to their numerous optical, electrical, and mechanical properties [1–3]. CBNs are currently among the most extensively developed nanomaterials [4,5]. The markets for CBNs and GBMs are projected to reach respectively 16.3 and 1.5 billion US dollars by 2027, with an expected compound annual growth rate of 16.5% between 2022 and 2027 [6]. Such increasing production and widespread use of GBMs would increase the likelihood of their release into the environment. Indeed, they are likely to be released at all stages of their lifecycle from production to recycling, and reach aquatic ecosystems, which are receptors for most of pollutants [7]. This raises concerns about their potential toxicity towards aquatic organisms.

While most of the research conducted focused on potential effects towards humans and mammals using *in vitro* cell systems, environmental aspects are relatively underexplored [8–10]. Among the available ecotoxicological studies, most of them focused on raw graphene materials, such as graphene oxide (GO), reduced graphene oxide (rGO), or few-layer graphene (FLG), documenting various toxic effects including membrane impairment in algae and bacteria [11,12], tissues damages, oxidative stress or genotoxicity in vertebrates and invertebrates [13,14]. Overall, while these groups have been studied more extensively, the main adverse effects have predominantly been observed in organisms from lower trophic levels [10,15], which play a crucial role in biogeochemical and energy cycles at the ecosystemic scale. However, only few studies have examined the hazards associated with finished products containing GBMs, which are more likely to be released into the environment during their life cycle.

The originality of this study lies in its focus on a finished product, a commercial FLG-based ink as few studies have focused on its toxicity [16,17]. Specifically, FLG consists of multiple layers of graphene sheets. Carbon atoms in each graphene sheet are bounded by sp^2 covalent bonds in a hexagonal lattice. As non-oxidized graphene is polar and hydrophobic, dispersing agents are used to improve its dispersion stability in aqueous media [9,18]. This ink uses sodium deoxycholate (SDC), an ionic surfactant, as a dispersant to stabilize graphene in aqueous media. As such dispersants could further modify the environmental behaviour and toxicity of GBMs [19,20], and given the likelihood of co-release of FLG and dispersants into aquatic environments, it becomes essential to study their combined effects [21,22].

To address this concern, we used two approaches: (i) standardized single-species tests and (ii) a more environmentally realistic approach based on the use of microcosm systems. Single-species tests are well established in ecotoxicology, and are widely used to assess the effects of pollutants, and nanomaterials in particular, on key species from different trophic levels. In this study, three representative organisms were selected: the green alga *Raphidocelis subcapitata* which forms the basis of food webs and performs many essential functions: primary production and oxygen generation, the crustacean *Daphnia magna* which is a widely established pelagic invertebrate model organism for the regulatory assessment of substances, and the larvae of the amphibian *Xenopus laevis*, which is an established model for assessing the genotoxic potential of contaminants. These species are frequently used in regulatory frameworks due to their sensitivity to pollutants and their ecological relevance in freshwater ecosystems [23,24]. Although these single-species tests provide valuable information on the toxicological effects of

compounds, they fail to capture the complexity of natural ecosystems, where multiple species interact and indirect effects can occur [25]. Thus, a more environmentally relevant approach using microcosm systems, which mimic natural aquatic ecosystems [26], was integrated to the study. These model ecosystems were previously shown to be relevant for the assessment of environmental hazards associated to nanoparticles [25]. Microcosm study was conducted to expose a food chain composed of a mixed biofilm, ensuring primary production, interacting with the dipteran *Chironomus riparius* as a primary consumer and decomposers. This model organism was selected for this approach as it plays a key role in lentic aquatic ecosystems, performing various essential ecological functions including organic matter recycling, nitrogen cycling, sediment bioturbation, while also serving as an important food source for higher trophic level organisms [27,28]. The biofilm is composed of the benthic diatom *Nitzschia palea,* inoculated with a natural bacterial consortium. Complex biofilms play a major role in global primary productivity, carbon storage, nutrient biogeochemical cycles, and form the basis of many food chains [29,30].

In this context, the aim of this study was to evaluate the toxicity of a commercial FLG-based ink formulation in order to (i) better understand the impact of this GBM on aquatic organisms in line with the considerations of public authorities in the global risk assessment, and (ii) assess the ecological risks by contrasting single-species test results with those from microcosms, thereby better understanding how GBMs and dispersants interact and affect aquatic ecosystems. Our findings will contribute to a deeper understanding of the environmental impact of graphene-based products, especially when co-released with dispersants such as SDC, and will inform future ecological risk assessments.

II. Materials and Methods

II.1. Nanomaterial characteristics

The GBM is a commercial ink produced and supplied by the company BeDimensional® used as a conductive or coating material [31,32] and composed of two elements: few-layer graphene (FLG), obtained through the exfoliation of graphite and sodium deoxycholate (SDC, C_{24}-H_{39}-NaO_4, 414.58 g/mol, [302-95-4]), an ionic detergent also known as bile salt, used as dispersant. To prepare the ink, a dispersion of graphite (Merck, 10 g/L), sodium deoxycholate (Merck, 10 g/L), and Milli-Q water was stirred at 40 rpm (Digital Overhead Stirrer 100-240V, 50/60Hz, 50-2200RR). The suspension was then processed through a high-pressure homogenizer using a reaction chamber with a diameter of 87 µm under a pressure of 200 MPa, powered by a hydraulic piston. The dispersion was reprocessed 5 times using the same parameters. The ink was stored in a tinted glass bottle at 4°C to limit both photo-and bio-degradation. For all experiments, nanoparticles were dispersed by bath sonication for 10 minutes before introduction into the exposure medium. The physicochemical characteristics of the tested ink are detailed in Table 1 and Figure S1.

Table 1 : Physicochemical characteristics of the ink. TGA: thermogravimetry under argon; TEM: transmission electron microscope; BET: Brunauer-Emett-Teller; NP: nanoparticles.

	Data	Methods
Nominal FLG concentration (g/L)	1	TGA
Nominal SDC concentration (g/L)	1	TGA

Number of graphene layers	2-20	TEM
NP lateral size (µm)	1-4	TEM
NP thickness (nm)	0.7-7	TEM
Specific surface area (SSA) (m^2/g ink)	7.7 ± 0.2	BET
Graphite residues (%)	40 ± 2.2	Raman

II.2. Behaviour of ink and SDC in the exposure media

The stability of the ink suspension, prepared by dilution at 10 mg/L in reconstituted water (294 mg/L $CaCl_2 \cdot 2H_2O$; 123.25 mg/L $MgSO_4 \cdot 7H_2O$; 64.75 mg/L $NaHCO_3$; 5.75 mg/L KCl) [33], was evaluated using a Turbiscan LAB equipment (Formulaction, Scientific Instruments) immediately following 5 min of sonication. This concentration was chosen to make sure to reach the detection threshold of the Turbiscan device. Particle sedimentation was monitored at room temperature for 24 hours with one measurement per minute by assessing the light transmission through the suspension at different heights in the measurement cell. In case of sedimentation, an increase in the transmission is expected, indicating a decrease in the number of particles in the water column.

To monitor SDC concentrations in the water column and potential adsorption on FLG particles, liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) was carried out. For this purpose, SDC was quantified in samples diluted to a nominal concentration of 10 mg/L of SDC alone or in ink solution. To quantify free SDC, ink samples were vacuum filtered through a 0.22 µm membrane to remove FLG particles. The potential impact of the filtration process on SDC concentration was evaluated by comparing filtered and unfiltered SDC solution to account for any losses due to the filtration process (such as adsorption on the filtration membrane). SDC was quantified in the prepared samples and after 24 h of incubation in exposure media. Samples were 100-fold diluted in a water/acetonitrile mixture (50:50 v/v), except for filtered ink samples which were injected without dilution. The diluted sample were homogenised for 15 sec, and 5 µL were injected in the LC-HRMS system. LC-HRMS analyses were performed with an Ultimate 3000 RSLC chromatographic system (ThermoScientific®) coupled to an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific®) equipped with an electrospray source. The chromatographic separations were performed using a Kinetex EVO C18 column (2.1 x 150 mm; 1.7 µm-Phenomenex) operating at 30°C and an injection volume of 5 µl. The flow rate was fixed at 0.40 mL/min with 0.1% of formic acid in water (A) and 0.1% of formic acid in acetonitrile (B) for mobile phases at the following gradient: initial, 65% A; 0–2 min linear, 55 % A linear; 2–5.5 min, 53.7 % A; 5.5–6 min, 0 % A linear following by washing and reconditioning of the column. The mass spectrometer was operated in negative ion mode with capillary voltage at −3,0 kV and a capillary temperature set at 320 °C. Nitrogen gas was used for nebulizing. The detection was performed using negative electrospray ionizations in the SIM mode (at 391 m/z). The quantification was performed using Xcalibur™ 4.4 software (Thermo Scientific) by extracting the ion signal of SDC at 391.2855 m/z. Calibration curve were prepared with SDC analytical standard (Avanti Polar Lipid) with a calibration range from 0,5 ng/ml to 2500 ng/ml using internal standard (DCA-d4- Avanti Polar Lipid).

II.3. Toxicity evaluation using single-species based assays

II.3.1. Green algae

The green algae *Raphidocelis subcapitata* was used as biological model for toxicity evaluation. The *R. subcapitata* strain was obtained from the Culture Collection of Algae and Protozoa (CCAP, Scotland) and supplied by INERIS (France). Algal cultures were grown under axenic conditions in a modified CHU N°10 basic medium [34] (Table S1). Cultivations were carried out in 2-L bottles closed with a screw cap with connection system to provide 0.22 µm filtered air and placed in a growth room at 20 ± 2 °C on a rotary shaker at 40 rpm with a light/dark period of 14 h/10 h supplied by a LED projector (VEGELED Floodlight, 150 W, 90-305 VAC, 120°). Experiments were conducted under a class II laminar flow hood to maintain axenic conditions. 1 mL of algal culture was placed in 12-well plates (COSTAR®, Corning Incorporated, Corning, NY) at 1 x 10^5 cells/mL in SPE culture medium (Table S1) and incubated for 24 hours under culture conditions prior exposure to the ink and SDC. To evaluate the effects of the test compounds, 1 mL of a 2X dispersed ink suspension or SDC solution was added to each well of the plates to reach a final volume of 2 mL per well and final concentrations of ink (expressed in mg/L of FLG) or SDC of 0, 0.1, 1 and 10 mg/L. During the exposure, the plates were placed under culture conditions (20 ± 2 °C; on a rotary shaker at 40 rpm; 14 h/10 h of light/dark). Prior to the experiment, the sensitivity of the strain was evaluated by exposure to increasing concentrations of potassium dichromate, resulting in a 50% growth inhibition (IC_{50}) of 0.54 ± 0.4 mg/L after 72 h of exposure, which is in consistent with previous studies [35]. In the case of ink and SDC, endpoints related to cell abundances and physiological parameters were measured after 24, 48, and 72 h of exposure using flow cytometry (Beckman-Coulter Cytoflex cytometer). The relative chlorophyll *a* content of *R. subcapitata* was determined by measuring the natural fluorescence emitted by chlorophyll at 690/50 nm after excitation with a 488 nm laser. The relative neutral lipid content of *R. subcapitata* was assessed using the fluorescent reagent BODIPY® (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene; 505/515), which has a strong affinity for lipids. The algae were incubated with the reagent at a final concentration of 1 µg/L for 1.5 minutes before analysis by flow cytometry. The fluorescence emitted by the BODIPY® reagent was collected using a 530/30 nm filter after excitation by a 488 nm laser. Collected data were analysed with Cytexpert software version 2.2.0.97. Results are presented as the mean fluorescence intensity (arbitrary units) collected, normalized as a percentage of the negative control.

II.3.2. Crustaceans

The toxicity of the tested products was evaluated using tests on daphnia from a laboratory maintained parental population of *Daphnia magna*. The daphnids were raised according to the OECD standard protocol [24], under a 16h/8h light/dark cycle at 21 ± 1°C in 1.5 L glass crystallizing jars containing reconstituted ISO water [33] (Table S2), with continuous aeration. Water replacement and removal of the new generation (juveniles) was done twice a week. Feeding was done *ad libitum* 2 to 3 times a week with the green alga *R. subcapitata* supplemented with extracts of marine macrophytes to avoid any form of deficiencies.
The experiment consists of a 48-hour immobilization test on *D. magna* (acute toxicity test) conducted according to Test Guideline 202 [24] under static exposure conditions (without renewal of food and exposure medium). Organisms used were neonates less than 24 h old and from the same batch to minimize genetic variability. They were exposed in groups of 5 (n=4) in 50-mL crystallizing dishes to dilution ranges of ink or SDC prepared with M4 medium to reach the concentrations of 0, 0.1, 1, 10, 50, and 100 mg/L (expressed in mg/L of FLG for ink). In addition, as a comparative effect, the toxicity of FLG alone was evaluated by washing of the ink through repeated ultrasonic baths in order to desorb the SDC from the FLG. Therefore, this standardized daphnia test was performed at the same FLG concentrations. Organisms were

exposed in a temperature-controlled incubator in constant darkness at 21 ± 1°C. To meet the validity criteria, the model's sensitivity was assessed using a range of concentrations of potassium dichromate [7778-50-9] as a positive control. After 48 h of exposure, each organism was visually inspected to count immobilized daphnids (motionless for more than 15 seconds). The effective concentration leading to immobilization of 50% of the organisms (IC_{50} – 48 h) was determined by non-linear 4-parameter regressions.

II.3.3. Amphibians

The ink toxicity was also evaluated towards *Xenopus laevis larvae* as an amphibian model species used in ecotoxicology [36]. *X. laevis* rearing was performed in the certified breeding facilities of the laboratory (under the approval number A31113002). To obtain larvae, adult reproduction was stimulated by injecting males with 50 IU of pregnant mare serum gonadotropin (PMSG 500; Intervet, France), while females were injected with 750 IU of human chorionic gonadotropin (HCG; Organon, France). Collected eggs were placed in an aquarium containing filtered water. For the experiment, all larvae used were from the same spawn to minimize genetic variability. Groups of 15 larvae, homogenous in size (measured at t0) and at the same developmental stage of 50 [37] (corresponding to the appearance of the hindlimb bud), were exposed for 12 days in semi-static conditions at 21°C ± 1°C under a 16h/8h light/dark cycle with daily feeding (with ground TetraPhyll ®) and renewal of exposure medium according to the procedure described in the international standard ISO 21427-1 [33]. The exposure was performed in 2-L glass crystallizing dishes. Ink and SDC alone were tested within the following concentration range: 0, 0.1, 1, and 10 mg/L. A positive control for genotoxicity was performed by exposing organism to cyclophosphamide (CP) at 20 mg/L ([6055-19-2], Sigma-Aldrich). After 12 days of exposure, *Xenopus* larvae were euthanized by immersion in a buffered tricaine methanesulfonate solution (MS-222, SigmaAldrich). Acute toxicity was monitored daily by visual inspection (lack of movement, no food intake, barrel swimming). Chronic toxicity was assessed by measurement of each larvae (ImageJ analysis software v.1.53, 2022) on photographs acquired with a binocular microscope to calculate the normalized growth rate (NGR) as follows [38]:

$$NGR = \left(\frac{Lt_{12} - MLt_0}{MLt_0} \times 100\right) \times \left(\frac{100}{MLCt_{12}}\right)$$

With Lt_{12} corresponding to the length of a larva at 12 days, MLt_0 as the average length of larvae on day 0, and $MLCt_{12}$ for the average length of larvae in the negative control after 12 days of exposure.

Genetic toxicity was assessed by the Micronucleus Assay, based on the determination of the number of micronucleated erythrocytes (MNE) for 1000 observed erythrocytes of the circulating blood. For this purpose, blood samples were collected *via* cardiac puncture from each larva using heparinized micropipettes (20% solution $C_{12}H_{19}NO_{20}S_3$, Sigma Aldrich). Micronuclei were counted from blood smears made on histological slides before fixation in methanol for 10 minutes and then staining with Groat's hematoxylin (ISO, 2006). Micronucleated erythrocytes were blindly counted on a total of 1000 cells (MNE ‰) under light microscopy (oil immersion lens, x1500) by a single operator.

II.3. Toxicity evaluation using microcosm systems

II.3.1. Composition and experimental design

Nine microcosms (glass tanks measuring 20 × 20 × 20 cm, L × W × H; effective volume: 7 L) were used for this experiment. The substrate of each microcosm consisted of 700 mL of ISO sand (EN 1961) and 300 mL of Fontainebleau sand (99% silica). A volume of 4 L of commercial natural spring water Volvic® (Ca^{2+}: 12 mg/L, Na^{+}: 12 mg/L, Mg^{2+}: 8 mg/L, K^{+}: 6 mg/L, Cl^{-}: 1 mg/L, and Si: 32 mg/L) was used to establish a water column of 15 cm in the experimental systems [39]. To maintain adequate oxygenation, each tank was equipped with a bubbling aeration system. Lighting was provided by fluorescent tubes (JBL solar ultra, Natur 9000°K LT 24 WT5-HQ) with a 12h/12h light/dark cycle (Fig. S2). Temperature (22.2 ± 0.2 °C), conductivity (231.2 ± 28.4 µS/cm at 25°C), pH (8.63 ± 0.36), redox potential (95.9 ± 14 mV at 25°C), and dissolved oxygen (8.5 ± 0.7 mg/L at 25°C) were monitored at day 0 (D0), day 8 (D8), and day 28 (D28, final time). Nitrogenous products (NO_3^{-}, NO_2^{-}, and NH_4^{+}), dissolved organic carbon (2.5 ± 0.9 mg/L), and suspended matter (4.5 ± 2.7 mg/L) were also monitored without significant differences between the groups. Three different exposure conditions were tested: 1) negative control (Ctrl), 2) ink at a final concentration of 0.1 mg/L of FLG (INK), and 3) sodium deoxycholate alone, at a final concentration of 0.1 mg/L (SDC). Each condition was replicated three times with a random allocation of microcosms. Pollutants were added in microcosm systems once, after one month of microcosm operating, allowing time for the development of the lowest trophic chain link, *i.e.* a biofilm formed from an axenic culture of the benthic diatom *Nitzschia palea* (CPCC-160, Canadian Phycological Culture Centre, University of Waterloo,) and a microbial consortium collected from the Garonne River (Muret (31), France; x: 43.46053, y: 1.33208; January 2023). Each microcosm was inoculated with 1.5 x 10^7 bacterial cells and 5 x 10^6 cells of *N. palea*. The resulting biofilm allowed for primary production and the stabilization of nitrogenous products. At the beginning of the operation, 15 slices of senescent alder (*Alnus glutinosa*) leaves of the same diameter (2.5 cm) were added as a food source for the primary consumers and to allow monitoring of the organic matter decomposition activity. The end of the tank cycle marked the beginning of the experiment (D0). At D0, 40 *Chironomus riparius* larvae of stage 1 (24 h old) were added to each tank. After 24 h, allowing larval burial, contaminations with test products were conducted. Exposure lasted 28 days, the time required for the complete emergence of chironomids under control conditions.

II.3.2. Biofilm sampling, DNA extraction, PCR, sequencing and bioinformatic analysis

To monitor the dynamics of biofilm composition, surface sediment was sampled at 3 different timepoints during the experiment, including day 0 (D0) prior contamination, after 8 days of exposure to the test products (D8) and at the end of the exposure (D28). Total DNA was extracted from sediment samples (561.7 ± 48.4 mg) using the Qiagen® power soil DNA isolation kit, according to the manufacturer's recommendations. The quantity and the quality of DNA extracts were monitored using a NanoDrop 2000 UV spectrophotometer (Thermo Scientific).

The V4-V5 region of 16S rRNA gene was targeted using PCR1_515F (5'-GTGYCAGCMGCCGCGGTA-3') and PCR1_928R (5'-CCCCGYCAATTCMTTTRAGT-3') primers set [40]. PCR was performed in a final volume of 30 µL (22.9 µL of DNAse free PCR water; 3 µL of 10X PCR Buffer; 1 µL of DNA extract; 2.2 µL of primers set; 0.6 µL of dNTP (2.5 mM) and 0.3 µL of Taq DNA polymerase (5 U/mL - Sigma Aldrich) and using the following PCR protocol: 94°C – 120 s; (94°C – 30 s; 65°C – 40 s; 72°C – 30 s) x 30 cycles; 72°C for 10 minutes.

Sequencing of 16S rRNA genes amplicons was performed by the Get PlaGe platform (Genotoul, Toulouse, France) using Illumina MiSeq technology (2x250 pb). Bioinformatics was performed using QIIME 2 219.4 [41]. After importing data into QIIME2, forward and reverse Illumina demultiplexed sequences were joined and trimmed using DADA2 [42]. A total of 1,076,070 reads were obtained (minimum = 12,266; maximum = 47,834; mean = 32,608). All amplicon sequence variants (ASVs) were aligned with Multiple Alignment using Fast Fourier Transform (MAFFT) program and a phylogenetic tree was built using FastTree [43,44]. Taxonomy was assigned using naive Bayes taxonomy classifier with the Silva 138 reference database [45].

II.3.3. Benthic biofilm metabolic capability

Sediment samples from day 28 were used to characterize the metabolic profile of microbial communities thanks to BIOLOG® EcoPlates system consisting of 96-well plates, each with triplets of 31 different carbon sources and a control without a carbon source. For this purpose, 1.5 mL of sediments from each microcosm supplemented with 1 mL of Volvic® were vortexed to detach the biofilm from the substrate and suspend the microorganisms. The collected supernatant was diluted 1/100 with Volvic® water before use. 100 µL of diluted samples were transferred into each well of the plate. After inoculation, the EcoPlates were incubated in the dark under aerobic conditions at 22 ± 1°C for 144 h. Optical density (OD) at 590 nm was measured immediately after microbial seeding (h0) and monitored daily for 144 h using a CLARIOStar spectrophotometer (BMG Labtech). During these 144 h, the OD increased linearly ($R^2 = 0.97$) and did not reach a plateau phase. For each substrate, the absorbance was corrected by subtracting the absorbance of the control well containing only the medium. Negative values of corrected readings were set to zero. The average well color development (AWCD), indicative of substrate utilization, was calculated for all wells per plate as follows:

$$AWCD = (\sum D0_i) / 31$$

With DOi representing the corrected optical density of the i^{th} well.

The AWCD was also calculated for each guild of carbon sources, grouped into (1) carbohydrates, (2) carboxylic acids, (3) amino acids, (4) amines and amides, and (5) polymers as previously defined [46]. The AWCD reveals the activity rate of organisms and thus the degree of utilization of carbon source. To compensate for the effect of microorganism density on the AWCD measurement, OD values were corrected for the bacterial density in each well at t0, as determined by flow cytometry. All reported results correspond to data collected during the exponential growth phase at 96 h.

II.3.4. Chironomids

After 8 days of exposure, the time required for larvae to reach the 4th development stage under optimal conditions, 8 larvae were randomly sampled from each microcosm. Larval growth was determined based on normalized growth rate of the larvae calculated according to the method previously described. Moreover, developmental stage delay was determined based on the measurement of cephalic capsule width. Teratogenicity was assessed with a binocular by counting the number of mental deformities according to established classifications, including Köhn gaps, missing and additional teeth [47] of the retrieved and decolorized capsules in potassium hydroxide (15%, 95°C, 15 min). Chironomids emergence was monitored over time, with specimens collected daily for sex determination and measurement. The experiment ended

when all chironomids in the control group had emerged. Subsequently, the sediment was carefully sorted to collect any remaining larvae and determine mortality or emergence delay.

II.3.5. Leaf litter degradation

Leaf degradation was estimated based on the measurement of the total surface area of non-consumed leaf at the end of the experiment. Leaf slices were scanned at the beginning and at the end of the experiment at a resolution of 600 DPI. The images were processed using ImageJ software applying an 8-bit transformation and thresholding to quantify the degraded areas and calculate the degradation rate by chironomid larvae, which constitute the main decomposers of organic matter in such time-duration experiment and system [48].

II.4. Statistical analysis

Statistical analyses were performed using GraphPad Prism® (v.9.0.2) and Rstudio® (v.1.3.1073). Univariate ANOVAs followed by Tukey post-hoc test (multiple comparison test) were employed for all data. When the assumptions of normality and variance homogeneity were not met, data were transformed to meet these assumptions. The results of the micronucleus test were statistically analyzed using the non-parametric McGill test [49] on the median values of each larval group, as recommended in the standardized ISO 21427-1 procedure [33]. For chironomids sex ratio data over time, areas under the curve (AUC) were measured for each replica. These AUCs were then analyzed via univariate ANOVA. Concentrations leading to 50% immobilization of daphnids (IC_{50}) were calculated using non-linear 4-parameter regressions. An exploratory approach by principal component analysis (PCA) (ade4 package, Rstudio®) was employed on Biolog data to identify potential metabolic and/or functional impacts on microbial communities.

For sequencing data, principal coordinates analysis (PCoA) plots were used to assess the variation between experimental conditions based on Bray-Curtis distances. Differences in beta diversity were assessed using permutational multivariate analysis of variance (PERMANOVA) with the package ‘vegan’ using the function ‘adonis’. Differences in the alpha diversity indexes and phyla relative abundances were analysed for each sampling time by one-way ANOVA followed by Tukey test. Differences in taxa abundances were analysed by the linear discriminant analysis (LDA) effect size (LEfSe) method [50].

III. Results & Discussion

III.1. Behaviour of compounds

Measurements of SDC concentration in the water column after ink or SDC addition were analysed by LC-HRMS. The measured concentrations of the free fractions of SDC after contamination with the nominal concentration of 10 mg/L were 10.2 ± 0.7 mg/L and 7.2 ± 0.4 mg/L in the SDC and ink solutions respectively, immediately after addition (Fig. 1A). This indicated that the target concentrations of SDC were reached in the different assays performed while in the ink, the presence of FLG reduced the free fraction of SDC by about 29.4 ± 11 %, probably due to SDC adsorption, as graphene family materials are known for their ability to adsorb various compounds, ranging from metal ions to aromatic compounds and biomolecules

[51,52]. After 24 hours, the concentration of free SDC did not change significantly (ANOVA & Tukey's test, p = 0,9119, Fig. 1A) suggesting the good stability of the SDC in the water column and a maximum adsorption capacity of the SDC on the FLG surface of about 300 mg/g. The adsorption capacity of SDC on the surface of multilayer graphene has already been studied, showing a maximum adsorption between 100 and 150 mg/g when the concentration of SDC in the medium is greater than 90 mg/L which is consistent [53]. The difference observed between the literature and our result could be due to differences in material characteristics (number of layers, surface…), but also to the dispersion method and the time before measurement [53].

Monitoring the sedimentation of FLG in ink within ISO exposure media using Turbiscan measurements revealed a significant increase in transmission in the water column over time (Fig. 1 B-C). This result indicates that approximately 40 % of the FLG contained in the ink sedimented within 24 hours and over 80 % after 72 hours. Pristine graphene is known for its poor stability in aqueous media. Surface modification, through the increase in oxygen-containing functions, or the use of surfactants, including SDC, can increase graphene stability and decrease agglomeration in aqueous media [54–57]. The sedimentation behavior of the tested material is in agreement with a previous study performed using a similar ink in freshwater [16]. Material concentration is known to influence FLG stability and the agglomeration state, with better stability in deionized water at around 0.1 mg/L [18]. However, the sedimentation behavior of the ink measured at 10 mg/L is consistent, although SDC slightly improves FLG stability and reduces agglomeration [16]. These interactions between FLG and SDC in the commercial ink alter the compounds bioavailability, which is an important parameter in toxicity studies. As a consequence, such behavior of the ink in the environment would decrease the bioavailability of graphene and the fraction of adsorbed SDC in the water column, while increasing the exposure of benthic organisms or those organisms feeding at the sediment-water interface [58–61].

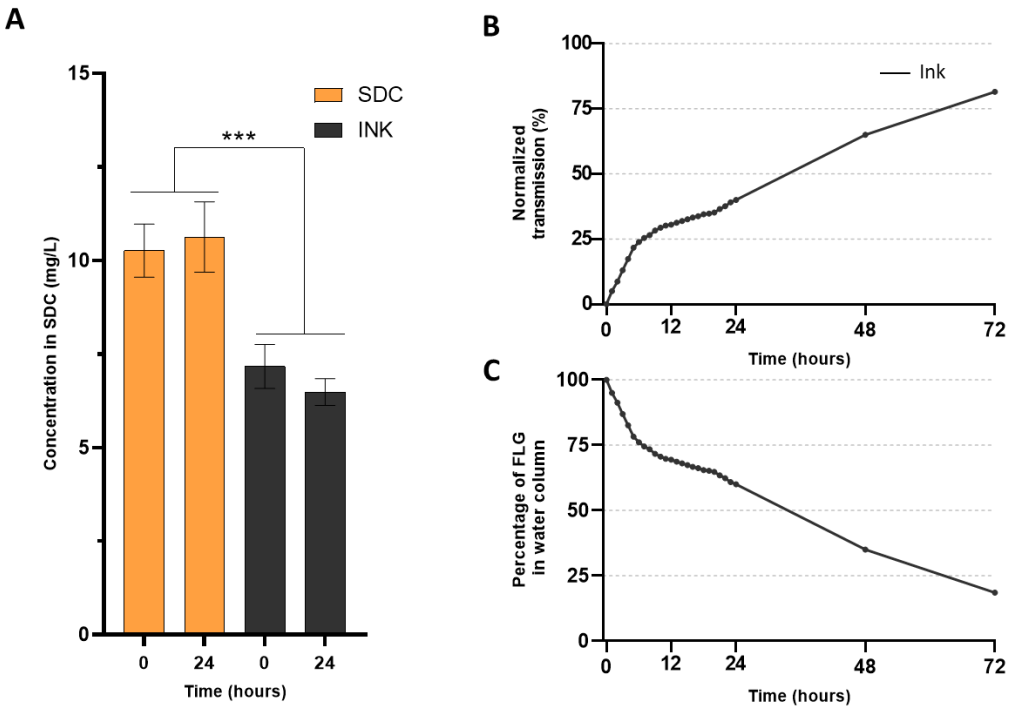


Figure 1: Characterization of ink and SDC behaviour in standardized exposure media (ISO, 2006). A) Effective concentrations of SDC (mg/L) in the water column measured in samples diluted to a nominal concentration of 10 mg/L of SDC solution alone and mixed with FLG to produce the ink at 0 and 24 h. B) Normalized transmission (%) in the water column over time in the presence of ink. C) Percentage of FLG from the ink in the water column over time. Data are presented as mean ± SD (n = 3 per condition). (ANOVA followed by Tukey. *: $p < 0.05$; **: $p < 0.01$ and ***: $p < 0.001$).

III.2. Effects on algae *Raphidocelis subcapitata*

The assessment of substance toxicity on the primary producer *R. subcapitata* enables the understanding of potential toxic effects on lower trophic levels within aquatic ecosystems. This test reveals significant effects, compared to the control group, in growth kinetics leading to inhibition of algal growth following 72 hours of exposure to 0.1 and 10 mg/L of SDC (ANOVA & Tukey's test, $p = <0.009$, Fig. 2A, B). The concentration leading to 50% of growth inhibition after 72h of exposure (IC_{50}) to SDC was calculated to be 104.4 mg/L. Cellular neutral lipid content, as determined by BODIPY® fluorescence, increased significantly by 69.5 ± 9.5% and 67.5 ± 9.5%, respectively, compared to the control group after exposure to 1 and 10 mg/L of SDC, respectively (ANOVA & Tukey's test, $p = <0.0001$, Fig. 2C). The relative chlorophyll content monitored from fluorescence intensity is significantly higher following exposure to SDC at 10 mg/L with a 23.36 ± 7.32% increase compared to the control group (ANOVA & Tukey's test, $p = 0.0245$, Fig. 2D).

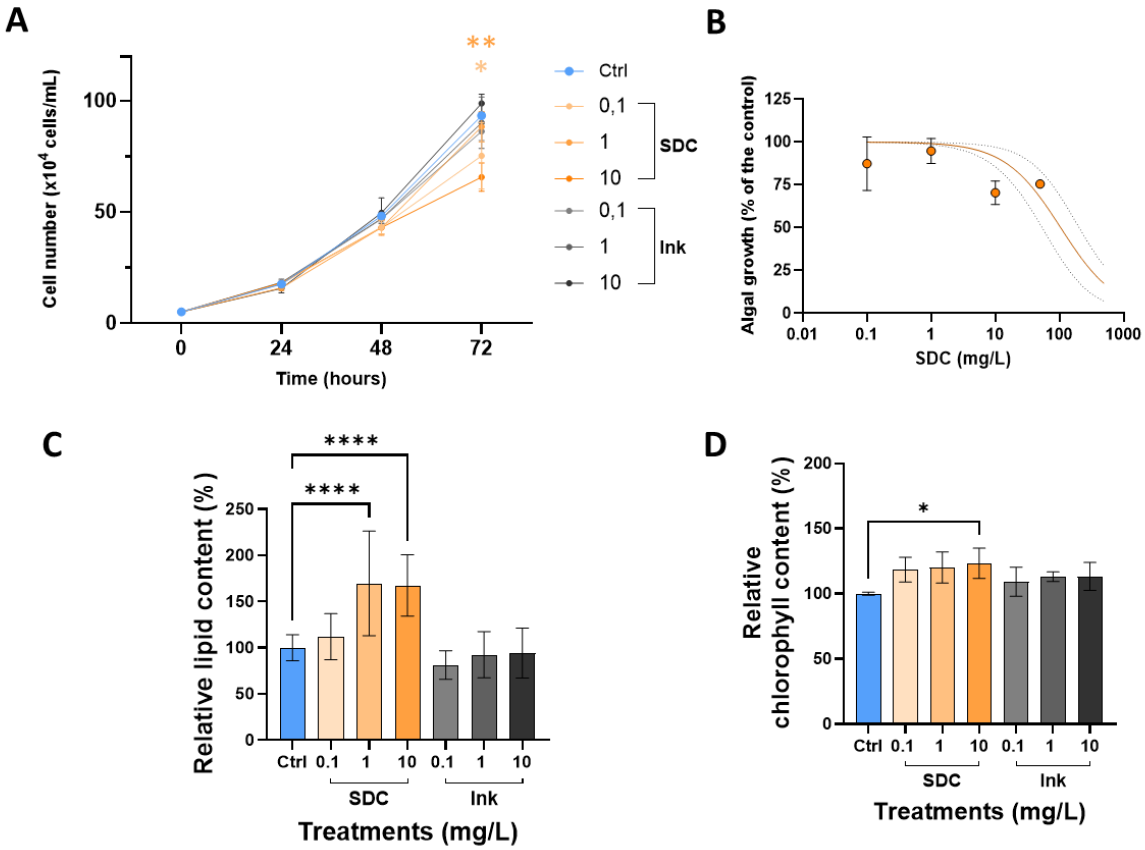


Figure 2: Effects of ink or SDC exposure towards the algae *Raphidocelis subcapitata*. A) Effects on the number of algal cells in culture over time when exposed to increasing concentrations of ink or SDC. B) Effects of 72h of an exposure to increasing concentrations of SDC on algal growth. C) Relative cellular content of neutral lipids expressed as percentage of the control group after 72h of exposure to different ink or SDC concentrations. D) Relative chlorophyll content expressed as percentage of the control in algae measured following 72h of exposure to different ink or SDC concentrations. Data are presented as mean ± SD (n = 3 per condition). (ANOVA followed by Tukey. *: $p < 0.05$; **: $p < 0.01$ and ***: $p < 0.001$).

Green microalgae, such as *Raphidocelis subcapitata*, are commonly used in aquatic ecotoxicology [23,62] and in standardized tests, as the OECD Test Guideline 201 [63]. The importance of studying algae's sensitivity to various substances is essential as they are key organisms for ecosystem functioning [64]. As primary producers, green algae play a crucial role on the planet by performing multiple functions, such as energy production at the base of trophic networks [65], oxygen synthesis [66], and regulation of biogeochemical cycles, including the carbon cycle [67]. Based on the results obtained, treatments with SDC at 0.1 mg/L inhibited algal proliferation. Such effects have been observed with various ionic detergents such as sodium dodecyl sulfate and sodium alkyltrioxyethylene sulfate, with IC_{50} values ranging from 2.18 to 36.58 mg/L, depending on their molecular structures and weights [68]. In the case of SDC, IC_{50} has previously been shown to range from 100 to 500 mg/L which is consistent with our results, although we did not follow the full OECD 201 guidelines in our test [69]. The toxic mechanisms might involve the detergent's ability to alter the structure of membrane lipids [68,70], potentially leading to the disruption of the lipid bilayer, to which dividing cells are especially vulnerable [71]. Additionally, other impacts may be expected from these detergents,

which can bind to various bioactive macromolecules, such as starch, proteins, and peptides, or insert into different cellular fragments, causing metabolic dysfunctions [70].

The lack of effect induced by the FLG-based ink is consistent with the literature examining FLG alone. Indeed, growth inhibition in the diatom *N. palea* was previously observed only at concentrations above 50 mg/L of FLG alone [34]. However, as the sensitivity of algae to nanomaterials has previously been shown to vary depending on the species and the membrane properties of the cells [72,73], the observed results cannot be generalized to all algal species. In addition, the properties of the same type of graphene may vary from one synthesis process to another, thus potentially influencing toxicity. Shading effects leading to reduced growth rate, due to the presence of high concentrations of carbon-based nanomaterials, including FLG, have been described previously [34,72]. However, these effects were reported to occur at high concentrations which is consistent with the lack of growth inhibition observed in our study by direct contact at a concentration of 10 mg/L of ink.

Growth inhibition by SDC alone is associated with an increase in cellular neutral lipid content (Fig. 2C). This result has been frequently reported in algae under abiotic stress conditions, especially chemical stress [74,75]. However, studies on the impact of detergents on algae are scarce and do not directly address lipid reserves. Neutral lipids, primarily triacylglycerides (TAGs), play a major role in stress responses in algae [74,76]. TAGs serve as energy reserves, replacing starch under stress conditions [77]. They also function in protective and detoxification mechanisms by binding to free radicals and accumulating in photosynthetic centers to prevent phototoxicity under stress conditions. They are often associated with oxidative stress [77]. This result should be linked to the monitoring of chlorophyll content. While an increase in chlorophyll levels in algae exposed to the highest SDC concentration is observed (Fig. 2D), a decrease in relation with reduced photosynthetic efficiency under chemical stress situations is reported [78–80]. However, similar response patterns would suggest the occurrence of compensation mechanisms to maintain cellular energy under oxidative stress [74,76,80–82]. In association with increased cellular lipid content, an increase in chlorophyll would lead to enhanced photosynthetic efficiency, allowing for better CO_2 fixation and improved carbon allocation to mechanisms involving sugars or lipids [81,82].

Overall, based on the results collected, the toxic effects of the commercial ink tested appear to be primarily in the sodium deoxycholate fraction.

III.3. Effects on *Daphnia magna*

To study the toxicity of the test products on *Daphnia magna*, immobilization was examined in presence of the ink, SDC and FLG alone. Daphnia immobilization increases in a dose-dependent manner when exposed to the ink and to the SDC (Fig. 3A-B). After 48 hours, no impairment of mobility is measured at ink or SDC concentrations less than 10 mg/L. The 48-hour EC_{50} values determined by the 4-parameter nonlinear regression were 52.10 mg/L and 59.98 mg/L for ink and SDC exposure, respectively (Fig. 3A-B). No immobilization was observed below 150 mg/L of FLG alone. At this concentration, only 30 ± 10 % immobilization of daphnia was observed.

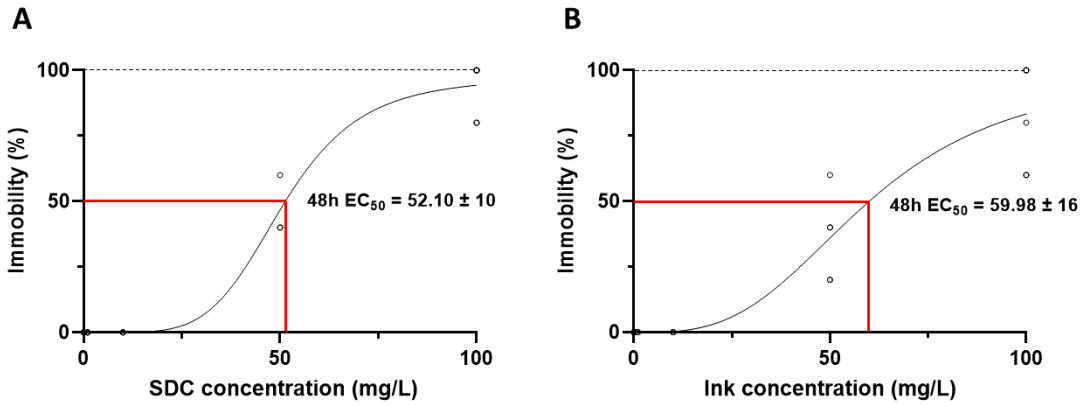


Figure 3: Percentage of *Daphnia magna* immobilization as a function of SDC concentration (A) and ink (B). Concentration response curves with 48h EC_{50} calculated and presented as mean ± CI95% (n = 4 per condition).

Daphnia magna is one of the gold standard model organisms in ecotoxicology and has been widely used to evaluate the toxic potential of nanomaterials, including graphene family materials [63,83–85]. Based on the available literature, FLG and GO were previously reported to induce no severe acute toxicity [26,86], which is consistent with the results obtained in this work, indicating an acute toxicity of the tested pristine material above 150 mg/L. However, while weak toxicity was observed, previous studies indicated that exposure to GBM was associated with the induction of sublethal effects including oxidative stress and long-term alteration of organism traits [87–90], accumulation in adult organisms with possible vertical transfer to the neonates [91].

It has been pointed out that while the OECD daphnia-based guidelines are appropriate for assessing the ecotoxic potential of soluble chemicals such as SDC, the standard procedure might lead to biased conclusions in the case of nanomaterials, including GBM, as their behaviour differs from that of chemicals in solution [63,85,92,93]. Based on the dispersion stability performed, daphnids in the water column are exposed to the initial target dose of the graphitic components of the ink only during the first few hours, resulting in a non-uniform exposure scenario over the 48-hour test duration. As suggested previously [63], the inclusion of a renewal of the exposure medium after 24 h of exposure would have increased the sensitivity of the test. However, the effects observed after exposure to SDC alone suggest that the mixture toxicity is mainly due to the freely bioavailable SDC. In addition, the higher 48h EC_{50} value measured in the case of ink compared to the SDC solution may be due to the lower amount of freely bioavailable SDC due to FLG adsorption which supports the results observed in algae.

III.4. Effects on *Xenopus laevis* tadpoles

The study conducted on *Xenopus* tadpoles enables the monitoring of the effects on amphibian development as well as evaluating the genotoxic potential of the compounds. During the exposure, no sign of acute toxicity (lack of movement, no food intake, barrel swimming) was observed. At the end of the exposure, results showed a significant decrease in growth rates of 29 ± 8% and 30 ± 8% in larvae exposed to 0.1 and 1 mg/L of SDC, respectively, compared to unexposed larvae (ANOVA & Tukey's test, p = 0.003 and 0.002, Fig. 4A). Ink does not affect this parameter. Concerning the assessment of genotoxic effects, larvae exposed to the positive

control exhibit a significantly higher number of micronucleated cells compared to larvae from the negative control group (6 ± 0.44 *vs* 2 ± 1.15), validating the test results. No genotoxicity was observed in larvae exposed at any concentration of ink and SDC, compared to the control group (McGill Test, Fig. 4B).

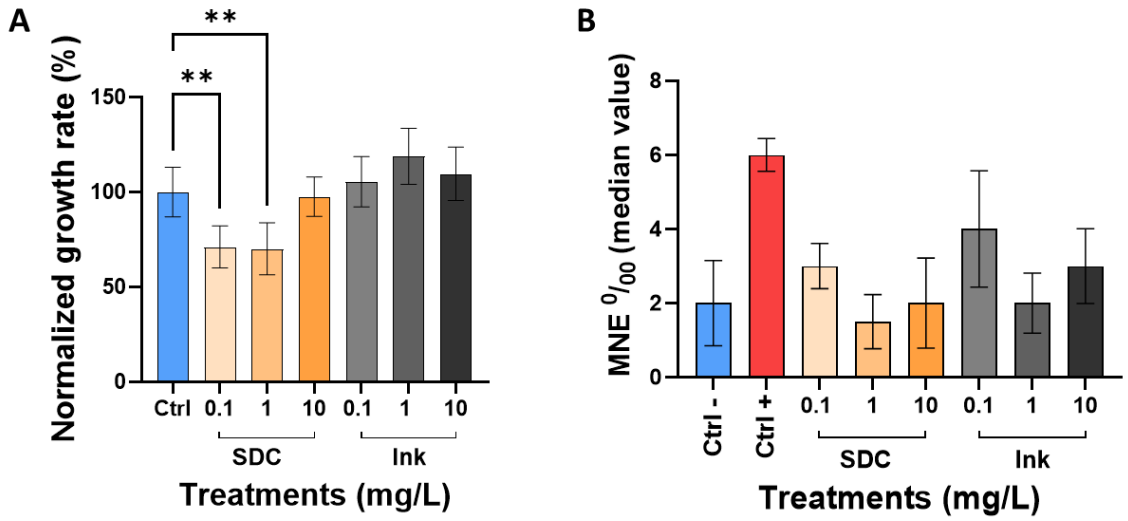


Figure 4: Effects of exposure to the commercial FLG-based ink or dispersant on *X. laevis* tadpoles. A) Effects on larvae normalized growth rate. Data are presented as mean ± SD (n = 15 per condition). (ANOVA followed by Tukey. *: p<.05; **: p < 0.01 and ***: p < 0.001). B) Micronucleus induction measured in erythrocytes (MNE) in ‰. Data are presented as median ± CI95% (n = 15 per condition). (McGill non-parametric test).

GBMs including FLG were previously shown to induce growth inhibitions towards *Xenopus laevis* tadpoles [38,94,95]. Such effects were associated to metabolic alterations [96], gut microbiota remodeling [97] without inducing specific endocrine disruptive effects [98]. Moreover, the growth inhibitory effects following any carbon allotrope exposure could be predicted based on the specific surface of the materials, leading to a 50% growth inhibition (IC_{50}) after exposure to a SSA of 7.42 m²/L [38,94]. Based on the characteristics of the ink, exhibiting a specific surface area of 7.7 m²/g of ink, IC_{50} would be at a mass metric equivalent of ~963 mg/L (equivalent to a surface 7.42 m²/L), which is consistent with the absence of growth inhibition observed after exposure to 10 mg/L of ink, corresponding to 0.077 m²/L.

Bile salts such as SDC used as an ionic surfactant have been previously described for their potential as dispersants for colloidal processing of carbon-based nanomaterials [57,99]. From a physiological perspective, bile salts are involved in the cholesterol catabolism, are important in lipid and protein digestion and shape the gut microbiota through antimicrobial activities or act as tumor promoters by interfering with cellular signaling, including Ca^{2+} trafficking [100–102]. To our knowledge, the effects of SDC on *X. laevis* have not been previously investigated on the endpoints monitored. In amphibian cells, deoxycholate has been shown to modulate cellular permeability [103], which could potentiate the effects of co-contaminants [104]. In the case of the ink exposure, no toxicity was observed, in contrast to single exposure to SDC, indicating that the presence of FLG in the ink reduces the dispersant toxicity on the endpoints analysed. Exposure to the ink and especially to SDC has previously been shown to induce detoxification activities in the juvenile rainbow trout, through activation of ethoxyresorufin-O-deethylase (EROD) activity and Cyp1A1 enzyme activity in the liver [16]. While the induction of these

mechanisms remains to be confirmed in our biological model, similar pathways cannot be excluded in amphibians and could be associated to the detected growth inhibition. While surfactant toxicity is influenced by its nature or environmental conditions [105], similar hypothesis were made in other amphibian species exposed to non-ionic surfactants such as poly-ethoxylated tallow amine, alcohol ethoxylate or alcohol ethoxy sulfate that were previously shown to induce development alterations in anurans [106,107]. In *X. laevis*, these effects were associated to mitochondrial dysfunction through collapse of the electrochemical gradient which is a marker of oxidative stress [106,108]. However, the non-linear dose-response of growth inhibition following exposure to SDC alone cannot be explained yet.

In amphibians, the genotoxic potential of GBMs would be associated to the oxidation state of GBMs, materials with high oxidation degree such as GO inducing oxidative stress leading to an increased occurrence of circulating micronucleated erythrocytes [59,109]. Thus, the lack of effect is consistent with a previous study [95], since the oxygen content of the FLG is low and the SDC alone, used as a dispersant, does not induce genotoxicity. In agreement with the previous data in algae and daphnids, it appears that the toxicity of the SDC towards *X. laevis* is higher than that of the final product containing FLG.

III.5. Microcosm exposure

III.5.1. Effects on microbial communities

The metabolic profile of the biofilm at the community level was monitored using Biolog® Ecoplate during exposure. The study of these profiles under different exposure conditions revealed no significant difference in the biofilm capacity to use of various carbon sources, neither at the global scale or at the guild level (Fig. S3).

The 16S rRNA sequencing analysis allowed to monitor the microbial dynamics in the experimental systems over time, depending on the treatment applied. Based on Bray-Curtis dissimilarities, PCoA indicates that the main factor contributing to the overall shift in microbial communities is the time over the treatment, while no interaction between the two factors was evidenced (PERMANOVA, $p = 0.001$ for the factor time of exposure; $p = 0.008$ for treatment and $p = 0.970$ for interaction) (Fig. 5 A). The treatment factor was considered for further analysis of the effects of the test products. Considering the treatment effects and based on Bray-Curtis distances, the results indicate that the microbial community structure of the biofilm exposed to SDC is dissimilar to the control group, while the biofilm exposed to ink is not, due to higher variability in the response (PERMANOVA, $p = 0.047$) (Fig. 5B). According to the alpha diversity indexes, the presence of ink or SDC does not affect the observed diversity while the presence of SDC is associated with a significant increase in the Pielou evenness index (ANOVA, $p = 0.217$ and $p = 0.012$ respectively) (Fig. 5C-D).

The relative abundance of the algal compartment of the biofilm (taxa associated with the chloroplast) was not affected by any treatment during the experiment (ANOVA, $p = 0.385$). Regarding prokaryotes, the bacterial compartment of the biofilm in the control group is composed of 4 major phyla including *Proteobacteria* (69.3 ± 5.9%), Planctomycetota (13.9 ± 4.8%), Bacteroidota (8.5 ± 1.8%) and Patescibacteria (1.6 ± 1.5%) (Fig. 5E). Exposure to SDC or ink resulted in a slight 10 % decrease in the relative abundance of taxa from the Proteobacteria phylum (ANOVA, $p = 0.049$). Analysis of differential taxa abundances using linear discriminant analysis (LDA) effect size (LEfSe) between the control group and the SDC-exposed biofilm revealed differential abundances of minor taxa (with a relative abundance

ranging from 0.05 to 1.5% of the reads) between SDC and FLG exposures. Thus, exposure to SDC was associated with a decrease in the relative abundance of taxa belonging to the genera *NS11-12* marine group (phylum: *Bacteroidota*), *Blastomonas* (phylum: *Bacteroidota*) or *Arsenibacter* (phylum: *Bacteroidota*), in favour of bacteria belonging to the genera *Pirellula* (phylum: *Planctomycetota*) and *Edaphobaculum* (phylum: *Bacteroidota*), whose relative abundance increased (Fig. 5F).

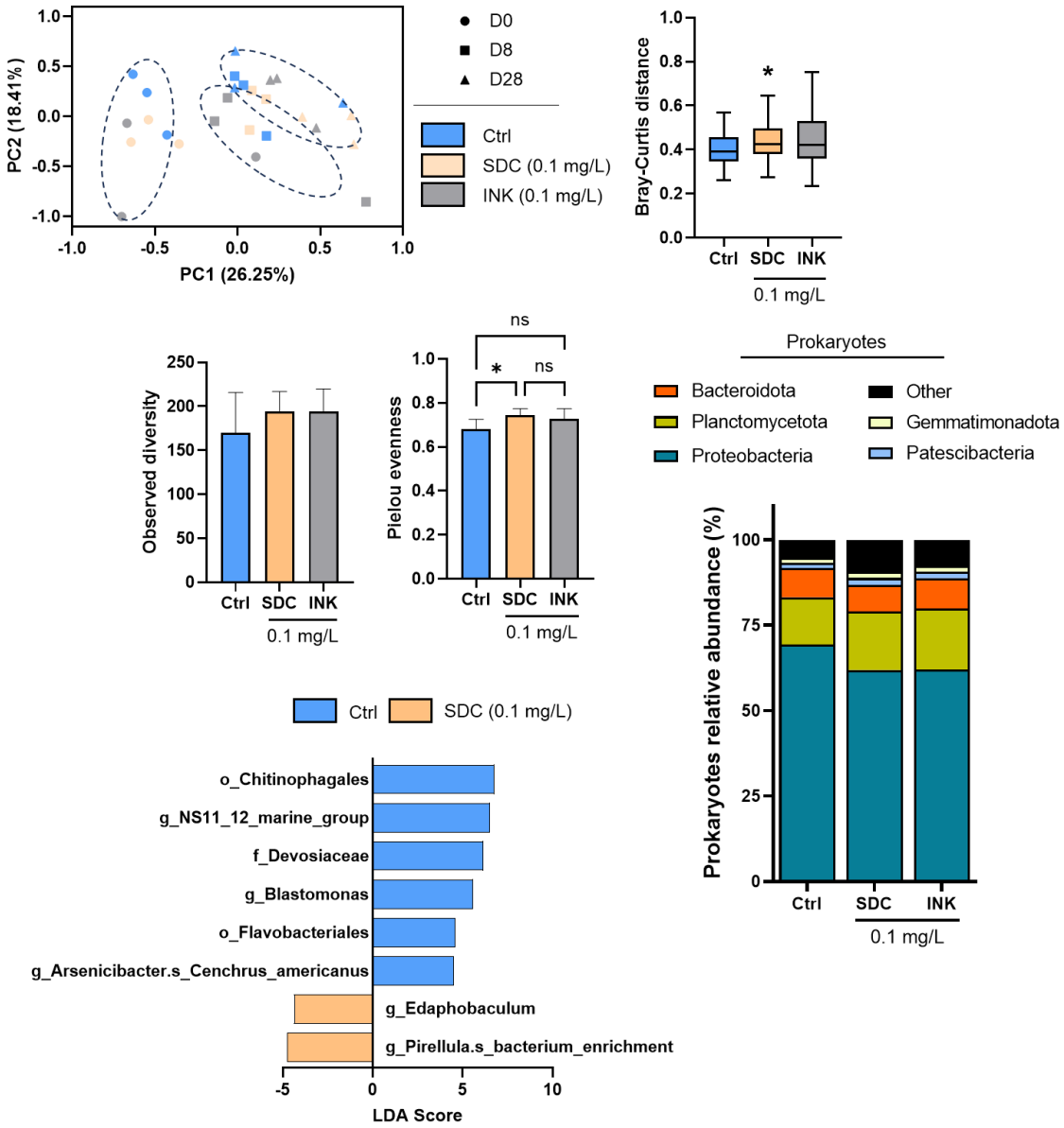


Figure 5: Effects of exposure to SDC and INK towards biofilm prokaryote communities. A) PCoA based on Bray-Curtis distances. B) Bray-Curtis distance. C) Observed diversity. D) Pielou evenness index. E) Relative abundances of the main phyla recovered in the biofilm. F) Taxa identified as differentially abundant between the control and the SDC-exposed group through LEfSe analysis. N = 9 samples per exposure condition (3 for each treatment duration). For alpha-diversity metrics and relative abundances of phyla, differences across groups were tested using ANOVA followed by Tukey's post-hoc test when $p < 0.05$. Differences in Bray-

Curtis distances were tested using PERMANOVA followed by pairwise comparison test using the control group as reference. * indicates $p < 0.05$.

Monitoring the effects of GBM-containing products on microbial communities is of importance due to their crucial role in aquatic ecosystems as they constitute the first level of the food chain and are involved in the regulation of biogeochemical cycles. While the main body of literature available is based on studies focusing on the effects of pristine materials towards single species models of bacteria or algae, the literature focusing on complex microbial assemblies (*i.e.* biofilm) remains limited [110], especially when considering the evaluation of end products containing GBMs. In mesocosm systems, the biofilm compartment or organisms living at the sediment-water interface are supposed to be especially at risk given the sedimentation behaviour of the tested Ink. Antibacterial activity or deleterious effects towards algae were previously reported to be associated to the production of reactive oxygen species (ROS) due to physical interactions with the cell membranes, lipids, proteins and nucleic acids, leading to the disruption of biochemical reactions [111–113]. In addition, the toxicity of GBMs has also been suggested to depend on the nature of the surface functions, [11,114], with the material with the highest oxygen content resulting in more pronounced antimicrobial activities against mixed aquatic biofilms [115]. Thus, the weak effects on bacterial community structure dynamics measured in the current study are consistent with the type and dose of the FLG used to produce the ink, as well as the low dose tested. The toxicity of GBMs could be mitigated depending on the maturity of the biofilm, due to the presence of EPS protecting bacteria as demonstrated by Guo and coworkers [116]. As in our study, the biofilm was allowed to develop for a long period prior to ink contamination (corresponding to a mature biofilm), and due to the presence of exopolymeric substances (EPS)-secreting algae that stabilize GBMs [34,59], the EPS-embedded biofilm would be protected from direct contact, limiting the expression of FLG toxicity. Thus, chronology appears as a critical factor in the experiments, as it is possible that ink contamination at the same time as inoculation with the microbial compartment would have led to the development of divergent biofilms dominated by more tolerant bacteria, in the microcosm system.

However, even if the measured toxic effects on the microbial compartment are low, EPS may have limited the penetration of FLG in the biofilm while the hydrophobicity of the SDC may have led to its diffusion, exerting specific antimicrobial activities and leading to more deleterious effects compared to FLG [117]. Indeed, SDC has previously been shown to act as an antimicrobial agent and modulate the bacterial biofilm production or virulence factor [118,119]. Thus, its presence in the microcosm could influence the dynamics between sensitive and tolerant taxa of the biofilm, leading to changes in the diversity indices measured. However, these changes are not associated with shifts in the biofilm metabolic profile probably due to functional redundancy between taxa. Overall, and in line with the previous data, the lack of effects associated with ink exposure compared to SDC suggests that FLG mitigates the SDC toxicity in ink formulations, limiting the toxicity of the final product toward this biological compartment. Although not evaluated in the present work, accumulation of SDC or FLG from the ink in the biofilm could contribute to the trophic transfer of these contaminants and potentially impact higher trophic levels [59,120].

III.5.2. Effects towards *C. riparius* development and functional traits

At the end of the experiment, no mouthpart deformations were observed regardless of the exposure, indicating the absence of teratogenic effects. A significant difference in larval growth rates was measured at D8 between larvae from the control group and those exposed to SDC or ink, resulting in a 14.87 ± 4.7% and 13.60 ± 4.7% reduction in larval size, respectively (ANOVA & Tukey's test, p = 0.0073 and 0.0154, Fig. 6A). In emerged male adults, the difference between the size of Chironomus from the control condition and SDC persisted, with exposed individuals being on average 8.5 ± 2.04% smaller (ANOVA Tukey's test, p = 0.0002, Fig. 6B). Only a non-significant trend in size reduction was observed in adult females exposed to the ink and SDC (ANOVA, p = 0.0629, Fig. 6C).

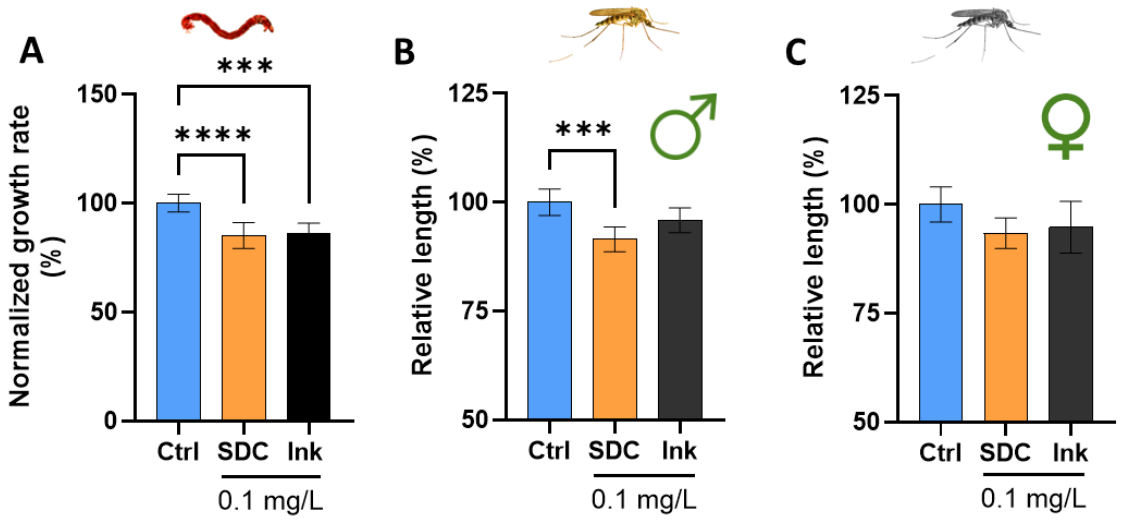


Figure 6: Effects of ink and SDC exposure in *C. riparius* with the normalized growth rate of 4-instar larvae after 8 days of exposure in % (**A**), the relative length of male adults in % (**B**) and the relative length of female adults in % (**C**). Data are presented as mean ± SD (n > 8 per condition). (ANOVA followed by Tukey. *: p < 0.05; **: p < 0.01 and ***: p < 0.001).

No significant difference in emergence dynamics was observed between the groups for either sex based on the median emergence day parameter (ANOVA & Tukey's test, p = 0.07 and 0.15, Fig. 7A-B). Monitoring of the sex ratio during exposure shows a lower female/male ratio in Chironomus exposed to SDC compared to the control (ANOVA & Tukey's test, p = 0.0073, Fig. 7C-D). At the end of the exposure, no significant difference in leaf litter decomposition was observed compared to the negative control, with an average decomposition rate of 51 ± 5.23% for each group (ANOVA, p = 0.1280, Fig. S4).

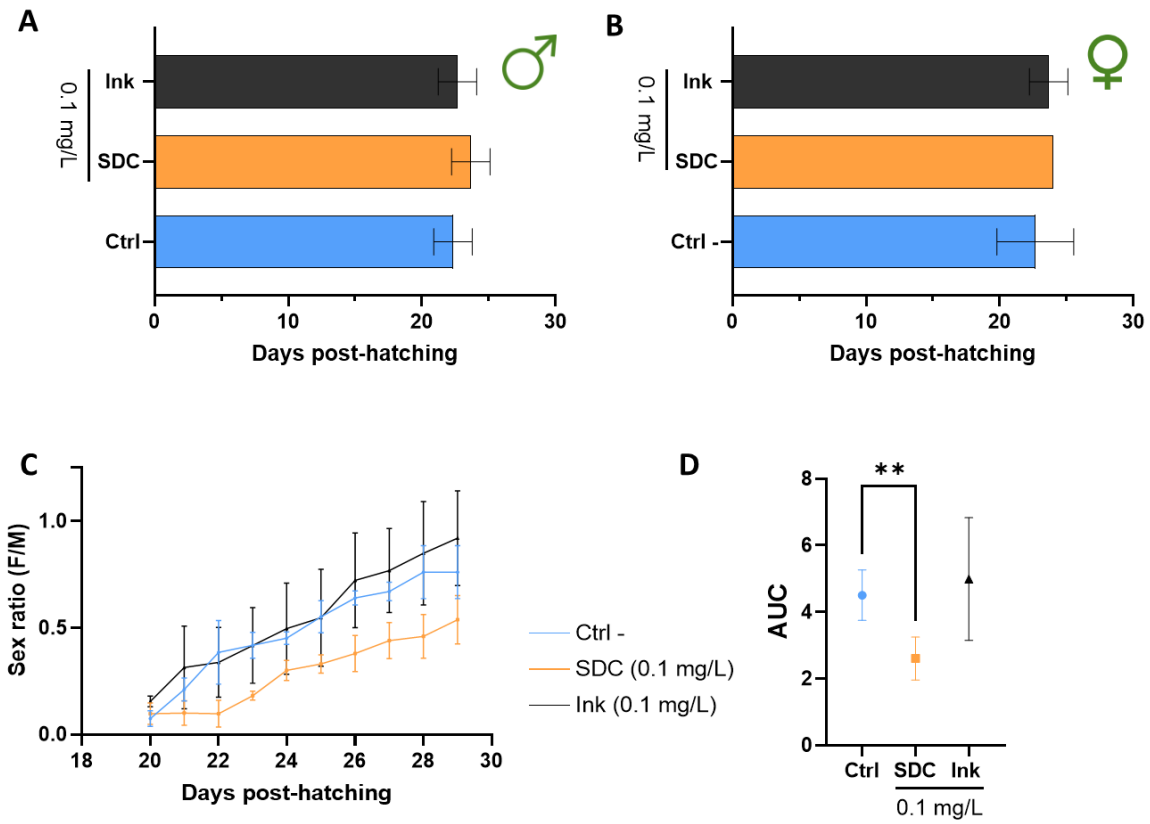


Figure 7: Effects of exposure to ink and SDC in *C. riparius*. A) Median day of emergence of adult males. B) Median day of emergence of adult females. C) Chironomid sex ratio over time (number of females / number of males). D) Area under the curve (AUC) calculated for the sex ratio over time. Data are presented as mean ± SD (n = 3 per condition) (ANOVA followed by the Tukey test. *: $p < 0.05$; **: $p < 0.01$ and ***: $p<0.001$).

Chironomids are widely used model species in ecotoxicology through laboratory-based assays or field studies and have been suggested to constitute a suitable model for the assessment of nanomaterial toxicity [121,122]. Several studies have previously evaluated the effects of pristine CBMs such as GO, carbon nanofibers or fullerenes on chironomids [123–127]. In the case of direct exposure to GO with different physicochemical properties, larval growth inhibitory effects were associated with the upregulation of genes involved in the chironomid endocrine system [126]. The possible involvement of endocrine disruption, probably mainly due to the presence of SDC, cannot be excluded in our study, because even if pupation blockade or teratogenic effects were observed at the end of the experiment, the persistence of the growth alteration and changes in the sex ratio with a more pronounced impact in females, suggest such a pathway [125,126]. The literature focusing on the effects of bile salts or non-ionic surfactants towards *C. riparius* is scarce. However, while the available data suggests that the species is tolerant to non-ionic detergents such as nonylphenol [128], bile acids such as SDC are signalling molecules that can bind to chitin components [129], interact with conserved receptors across species, and influence development through the modulation of metabolic pathways and affect growth [130–132].

GBMs including GO have also been shown to accumulate in the gut of chironomids and to induce oxidative stress [125,126]. Direct contact between GO and the gut microbiota of organisms has been shown to induce dysbiosis and intestinal inflammation in several other

biological models including fish, amphibians, mice or humans [97,133–136]. Collectively, oxidative stress, gut microbiota dysbiosis and endocrine disruption following exposure to environmental contaminants have been described as pathways that may contribute to impaired energetic balance and lead to growth inhibition, developmental defects, and altered traits in chironomids [137–139]. As FLG has been shown to exert effects closely related to those of GO in various biological models [61,140], the observed effects of growth inhibition without developmental delay after 8 days of exposure to the ink components in microcosm systems are consistent with the data from the literature, although the detailed mechanisms involved remain to be elucidated. Overall, changes in life-history traits alterations at the larval stage appear to be persistent at the adult stage following exposure to SDC but not to the ink, suggesting that different pathways may be involved and that in the latter case, adaptive responses allow the initial stress to be overcome.

In the microcosm system, chironomids, as shredders and deposit feeders, preferentially feed on biofilm at early developmental stages, whereas they are the main decomposers of alder leaves at later larval stages [48,141]. Litter decomposition rate, used as a marker of functional traits integrative of chironomid activities, was shown to be negatively correlated with the occurrence of mouthpart deformities, indicative of teratogenic effects in contaminated sites [142]. In the case of GBMs, a previous study using microcosm system showed no direct toxicity to GO to chironomids [59]. However, the environmental relevance of this test system allowed to unveil indirect effects of GO occurring through changes in the feeding behaviour of chironomids associated with the complex shift in the biofilm composition due to GO contamination, resulting in an increased litter consumption in contaminated systems [59]. Taken together, the lack of teratogenic effects, the weak changes in the biofilm composition, and the unaltered degradation rate measured in experimental units contaminated with ink components are consistent with the absence of indirect effects and suggest that direct toxicity to *C. riparius*, resulting in changes in life-history traits, occurs primarily.

Reduced larval and adult growth has previously been shown to predict population decline and reproductive success, which is consistent with the data obtained [143–145]. From an ecological perspective, and consistent with the data obtained following exposure to SDC, the induction of persistent growth inhibition at the adult stage, modification of the emergence dynamics and sex ratio behaviour to a lower number of available females, decreasing mating opportunities and reducing the probability of population maintenance. Reduced chironomid biomass in ecosystems could have deleterious effects on ecosystem functioning on a larger scale, but also because they constitute an important food source for higher trophic level organisms that feed on them such as fish, amphibians or birds [146–148].

IV. Conclusion

This study provides a comprehensive ecotoxicological assessment of a commercial ink containing FLG and SDC as a dispersant. The research aimed to elucidate the potential hazard posed by this product to aquatic ecosystems, with a particular focus on its effects towards organisms across multiple trophic levels using both single-species tests, covering concentrations ranging from 0.1 to 10 mg/L, and a more environmentally relevant system using microcosm approaches to evaluate the potential adverse effects at low doses (0.1 mg/L).

The results demonstrate that the raw ink does not exhibit significant direct toxicity to the organisms tested. However, it is evident that SDC, a component of this ink, shows direct effects

when in solution alone. Toxicity levels vary depending on the biological model and experimental conditions. The alga *Raphidocelis subcapitata* showed significant sensitivity to the ink, with observable disruptions in chlorophyll and neutral lipid content after exposure at 1 and 10 mg/L, respectively, indicating cellular stress. The crustacean *Daphnia magna* also showed acute toxic responses, but only at high concentrations (>50 mg/L). In addition, the larvae of the amphibian *Xenopus laevis* showed sublethal developmental effects even at the lowest dose (0.1 mg/L), which could translate into more severe impacts under chronic exposure or in more complex ecological settings such as natural environments. The microcosm study, which included interactions between primary producers and consumers, highlighted effects within the trophic network. The presence of SDC at 0.1 mg/L in the test systems affected the life cycle of the dipteran *Chironomus riparius*, a key species in sediment bioturbation and organic matter decomposition.

Overall, this study highlights the importance of considering the ecological implications of end products containing graphene-based materials, such as FLG which are increasingly being used in industrial applications. The results suggest that the release of such products into the aquatic environment could pose significant risks, particularly due to the co-release of dispersants that may affect organisms at all trophic levels. Future research should focus on long-term environmental monitoring and the development of experimental systems that more closely mimic environmental realities to assess functional and indirect effects. In addition, regulatory frameworks need be adapted to account for the emergence of new co-contaminants associated to the development of graphene-based composites, and to ensure that their environmental impact is properly assessed and mitigated.

Ethical standards

The experimental procedures were performed under the authorization of the vertebrate animal experimentation facility n° B31113002 and were approved by the Ethics Committee n° 073 (authorization n° #34208-2021120214298638 v3), in accordance with French and European legislation on animal experimentation (European Directive 2010/63/EU).

Conflicts of interest

The authors declare no conflict of interest.

CRediT

Florian Chapeau: Conceptualization, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Van Xuan Nguyen:** Investigation, Writing – review & editing. **Maialen Barret:** Writing – review & editing. **Boris Eyheraguibel:** Methodology, Resources, Writing – review & editing. **Maialen Barret:** Writing – review & editing. **Eric Pinelli:** Conceptualization, Supervision, Writing – review & editing. **Emmanuel Flahaut:** Data curation, Methodology, Resources, Writing – review & editing. **Laury Gauthier:** Funding acquisition, Writing – review & editing. **Florence Mouchet:** Funding acquisition, Project administration, Conceptualisation, Supervision, Witing – review & editing. **Lauris Evariste:** Conceptualization, Supervision, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing.

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