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ABSTRACT

Despite the growing interest for boron nitride nanotubes (BNNT) due to their unique properties, data on the evaluation of the environmental risk potential of this emerging engineered nanomaterial are currently lacking. Therefore, the ecotoxicity of a commercial form of BNNT (containing tubes, hexagonal-boron nitride, and boron) was assessed *in vivo* toward larvae of the amphibian *Xenopus laevis*. Following the exposure, multiple endpoints were measured in the tadpoles as well as in bacterial communities associated to the host gut. Exposure to BNNT led to boron accumulation in host tissues and was not associated to genotoxic effects. However, the growth of the tadpoles increased due to BNNT exposure. This parameter was associated to remodeling of gut microbiome, benefiting to taxa from the phylum Bacteroidetes. Changes in relative abundance of this phylum were positively correlated to larval growth. The obtained results support the finding that BNNT are biocompatible as indicated by the absence of toxic effect from the tested nanomaterials. In addition, byproducts, especially free boron present in the tested product, were overall beneficial for the metabolism of the tadpoles.

KEYWORDS

Boron nitride nanotubes; Xenopus; genotoxicity; gut microbiota; 16S

1. Introduction

Boron nitride nanotubes (BNNT) are nanoparticles structurally analogous to carbon nanotubes (CNTs) in which boron and nitrogen replace carbon atoms (Chopra et al. 1995). Increasing attention is being paid to these BNNT due to their unique properties such as high thermo-mechanical stability (Suryavanshi et al. 2004; Chen et al. 2004), electrical insulation (Radosavljević et al. 2003) and thermal conductivity (Chang et al. 2006). As high quality materials are increasingly available due to the improvement of synthesis techniques, BNNT are triggering great interest for the development of applications in a wide range of area such as composite improvement, medicine, hydrogen storage as well as aquatic pollution remediation (Kim et al. 2018; Kalay et al. 2015; Zhi et al. 2008; Merlo et al. 2018; Lale, Bernard, and Demirci 2018; Yu et al. 2018). Such keen interest for this material predicts a large-scale production in the near future. As any engineered nanomaterial, BNNT could be released into the environment during the whole material life cycle, from the production, the use and the waste disposal or recycling to reach significant levels in aquatic ecosystems (Yu et al. 2018; Mottier et al. 2017). For these reasons, this implies to evaluate its biocompatibility and its potential hazard for the environment before considering the mass use of this nanomaterial.

Studies related to BNNT toxicity remain scarce and inconsistent. Indeed, although many of the published works indicate biocompatibility of raw and functionalized BNNT *in vitro* as well as *in vivo* (Chen et al. 2009; Şen, Emanet, and Çulha 2016; Ciofani et al. 2010; Ciofani et al. 2008; Ciofani et al. 2014; Fernandez-Yague et al. 2015; Rocca et al. 2016; Salvetti et al. 2015), some studies highlighted cell type dependent cytotoxic and genotoxic effects (Augustine et al. 2019; Horváth et al. 2011; Çal and Bucurgat 2019). Ecotoxicological data are lacking

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while it is necessary for the sustainable development of BNNT-based products. Indeed, as for other nanoparticles, a large scale usage of BNNT would lead to releases into the environment at different stages of their life-cycle (Mottier et al. 2017; Sun et al. 2016; Keller and Lazareva 2014; Bundschuh et al. 2018). Thus, there is a need to fill knowledge gaps concerning the ecotoxicity of BNNT, particularly toward aquatic ecosystems as they constitute a receptacle for most contaminants. For this purpose, the amphibian *Xenopus laevis* was chosen as biological model to assess the ecotoxic potential of BNNT as larval stages were shown to be sensitive to contaminants, including nanoparticles (Evariste, Barret, et al. 2019; Mouchet et al. 2010; Bourdiol et al. 2013; Muzi et al. 2016; Saria et al. 2014; Colombo et al. 2017; Marín-Barba et al. 2018). Parameters related to the larval growth were shown to constitute endpoints of interests as they reflect the overall health status of the organisms. In a context of exposure to nanoparticles of carbon allotropes such as CNTs, toxicity leading to larval growth inhibition was described by the specific surface area of the tested nanoparticles (Mottier et al. 2016; Lagier et al. 2017). In addition, genotoxic effects could lead to non-negligible consequences as damaged DNA may cause cellular dysfunctions (Jackson and Bartek 2009) leading to the death of organisms and further impact the ability of populations to maintain (Sukumaran and Grant 2013). For this reasons, biomarkers related to genotoxicity are considered as a pertinent ecological endpoint at the population, community and ecosystem level (Anderson et al. 1994). Thus, alterations of genetic materials in amphibians would contribute to the decline of populations while this class contains many endangered species (Oertli et al. 2005).

It is now widely accepted that the growth of organisms is dependent from its capacity to acquire energy from trophic resources while this energy metabolism is can be strongly influenced by the metabolic capacities of the gut microbiota (Mithieux 2018; Musso, Gambino, and Cassader 2011; Cani 2014). In addition, the gut microbiome is emerging as a central target in environmental toxicology studies due to its contribution in the regulation of multiple physiological processes of the host but also in the metabolization of environmental pollutants (Claus, Guillou, and Ellero-Simatos 2016; Evariste, Barret, et al. 2019; Adamovsky et al. 2018). Alterations of the gut microbiome composition may dysregulate the normal physiological functioning of the host and lead to various diseases (Durack and Lynch 2019). Furthermore, in the amphibians, changes in the gut microbiota composition during early stages of life were shown to influence later life resistance to infections (Knutie et al. 2017). Environmental pollution constitutes a major factor influencing the composition of the gut flora and previous studies reported that a wide range of contaminants were able to induce gut dysbiosis in association to deleterious issues on host (Evariste, Barret, et al. 2019; Jin et al. 2017). Thus, the present work aims to investigate the consequences of an *in vivo* exposure of *X. laevis* tadpoles to BNNT combining monitoring of host physiology and gut bacterial community composition. The former endpoint constitutes an integrator of the overall host-metabolism while the latter one would allow to better understand the causes of potential metabolic changes induced by BNNT exposure. Overall, the results obtained in this study suggest that BNNT are rather biocompatible. Indeed, exposure to the nanoparticles did not induced irreversible genotoxic effects and stimulated the growth of the tadpoles. This increased growth was associated to remodeling of the host-associated gut microbiota benefiting to the phylum Bacteroidetes. Measurement of boron released in the exposure media and accumulated in the organisms suggest that the free boron associated to the nanoparticles are responsible for the beneficial effects observed.

2. Materials and methods

2.1. Synthesis and characterization of boron nitride nanotubes

Boron nitride nanotubes were obtained from BNNT LLC (USA). They correspond to the 'BNNT P1-Beta Products' range of materials as classified on the website of the company (http://www.bnnt.com/products). This material is described as containing hexagonal boron nitride (h-BN) and boron as well as being catalyst free (elemental B and $N > 99.9\%$) with ca. 25% of Boron in addition to BNNT. According to the manufacturer, the specific surface area is $>200\,m^2/g$. Sample was used as-received,

after simple grinding (agate mortar and pestle) in order to make it suitable for the experiments and characterization. The sample was characterized by X-ray diffraction (XRD), Brunauer-Emmet-Teller (BET), TGA, TEM, Raman and chemical elemental analysis.

X-ray diffraction analysis was performed using a Bruker D4 ENDEAVOR X-ray Diffractometer (Cu.Kα = 1.5406 Å). Specific surface area was measured with a Micrometrics Flow Sorb II 2300 by N_2 adsorption using the BET theory. Dry BNNT powder sample was first degassed at 100 °C under N_2 atmosphere for 2 h before being cooled down to 77 K (liquid nitrogen temperature) for adsorption of a monolayer of N_2. After heating the sample back to room temperature, the desorption peak was recorded. A calibration was done by injecting a known quantity of N_2 (in the same conditions as for the sample measurement). The uncertainty of the measurement was 3%. Thermogravimetric analysis was performed in air atmosphere at a heating rate of 1 °C/min using a SETARAM TAG 16. Transmission Electron Microscopy observations were performed using a TEM JEOL 1400 with an acceleration voltage of 120 kV after deposition of a few drops of a dilute suspension in ethanol on Copper TEM grids (Lacey carbon). Outer diameter of the BNNTs was measured from HRTEM images using ImageJ software. For chemical elemental analysis of Boron, the sample was digested in an open system with a mixture of sulfuric and nitric acids during 8 h. Elemental analysis was performed on an ICP-AES ICAP 6500 from Thermofisher Scientific, Bremen. Both the digestion and analysis were performed by CREALINS, 6Napse group (uncertainty of the measurement: 3%). Nitrogen and Oxygen elemental analyses were performed by ISA (CNRS, Lyon, France) by organic micro-analysis after total combustion at 1050 °C under helium/oxygen flux for nitrogen; total pyrolysis at 1080 °C under nitrogen flux for Oxygen. Raman signature was analyzed to get information on the structural quality of the nanotubes (Labram HR800 Horiba Yvon Jobin, λ = 532 nm). X-Ray photoelectron spectroscopy (XPS) was used to determine the quantitative atomic composition of the BNNT (XPS Kalpha ThermoScientific). IR analysis was performed using a Perkin Elmer Spectrum One FT-IR spectrometer and inclusion of the sample in a dry KBr pellet.

The stability of the BNNT suspension was evaluated using a Turbiscan LAB (Formulation) equipment, at room temperature. Suspensions were prepared at 10 mg L^{-1} in exposure media before performing analysis. The suspension was placed in a glass vial and both transmission and reflection were monitored vs time for 24 h (1 scan per minute).

2.2. *Xenopus laevis* rearing, breeding and exposure conditions

Xenopus laevis originated from our certified breeding facilities (under the approval number A31113002) and the experimental procedure was approved by an ethic committee (CEEA-073). Rearing and breeding conditions of *Xenopus* were performed as previously described (Mouchet et al. 2008; Mouchet et al. 2010). Pregnant mare's gonadotropin was injected in sexually mature individuals to induce spawning. Fecundated eggs obtained were bred in active charcoal filtered tap water at 22 ± 2 °C and fed with ground aquarium fish food (TetraPhyll®) until they reach stage 50 according to Nieuwkoop & Faber development table (Nieuwkoop and Faber 1958). Following the international standard ISO 21427-1 procedure (ISO/FDIS 21427-1.1, 2006), 20 larvae per experimental condition were exposed for 12 days under semi-static conditions with daily exposure media renewal. Larvae were fed daily *ad libitum* with ground aquarium fish food (TetraPhyll®, Tetra, Melle, Germany). Food was added twice a day, directly after media renewal and at the end of the day. Unconsumed food was removed daily during the media renewal process. Negative control (NC) condition was composed of reconstituted water devoid of contaminant (294 mg L^{-1} $CaCl_2.2H_2O$; 123.25 mg L^{-1} $MgSO_4.7H_2O$; 64.75 mg L^{-1} $NaHCO_3$; 5.75 mg L^{-1} KCl) (Evariste, Barret, et al. 2019; Mouchet et al. 2008) while positive control (PC) for genotoxicity assessment contained cyclophosphamide monohydrate ([6055-19-2], Sigma, France) at 40 mg L^{-1}. BNNT were dispersed by sonication before contamination of the exposure medium. Tested concentrations of BNNT were 0, 0.1, 1, and 10 mg L^{-1}.

2.3. Analysis of boron clearance in the water and accumulation in tadpoles

Boron concentration was measured in reconstituted water from the control and the 10 mg L^{-1} of BNNT conditions after 24 h of incubation in the presence and absence of tadpoles in the media. Water samples were centrifuged twice at 1500 × g during 20 min and filtered at 0.22 µm to remove nanotubes from the samples before boron analysis. After 12 days of exposure, boron concentration was measured in larvae from the control and the 10 mg L^{-1} of BNNT groups. Guts of tadpoles were removed before lyophilization of organisms, in order to avoid measurement of BNNT-related boron from the gut content. Boron concentration in the water and in the larvae was determined by ICP-AES at CREALINS laboratory (Lyon, France).

2.4. Micronucleus test and cell cycle analysis

The micronucleus assay was performed in accordance with the ISO 21427-1 guidelines (ISO/FDIS 21427-1.1, 2006). The micronucleus formation constitutes a good indicator of irreversible genotoxic effects, integrating aneugenic and clastogenic effects occurring after exposure to a genotoxic compound. For this purpose, after 12 days of exposure, *Xenopus* larvae were anesthetized by bathing in MS222 solution at 100 mg L^{-1} before collecting blood samples from cardiac puncture. Blood smears were prepared, fixed in methanol for 10 minutes before performing hematoxylin and eosin staining.

Micronucleated erythrocytes were accounted over a total of 1000 cells (MNE ‰) using optical microscopy (oil immersion lens, x1500). For cell cycle analysis, blood sub-samples were fixed using cold ethanol (70% v/v). Prior to the flow cytometric analysis, cells were rinsed using PBS and labeled with FxCycle™ PI/RNase Staining Solution (Life Technologies SAS) following manufacturer's recommendations. Propidium iodide fluorescence was measured using Cytoflex (Beckman Coulter, USA) equipped with a 488-nm excitation laser. For each sample, 10,000 events were acquired in a region corresponding to cells of interest after doublet discrimination using FSC-H versus FSC-A.

2.5. Larval growth measurement

The total length of larvae was measured at the beginning (d0) and at the end of the 12 days of exposure (d12) using ImageJ 1.49 software. Normalized growth rate was determined as previously described (Mottier et al. 2016; Lagier et al. 2017) using the following formula:

$$Normalized\ size\ (\%) = \left(\frac{Ld12 - MLd0}{MLd0} \times 100\right) \times \left(\frac{100}{MLCd12}\right)$$

Ld12 corresponds to the length of one larvae at 12 days, MLd0 is the mean length at day 0 of larvae from the exposure condition and MLCd12 is the mean length of larvae from the negative control at day 12.

2.6. Analysis of sequences from gut microbiota survey

Genomic DNA was extracted from the whole intestine of larvae using DNeasy PowerSoil kit (QIAGEN) according to manufacturer recommendations with the following adjustments: samples were incubated 10 min at 65 °C after adding the solution C1 and elution buffer C6 was incubated during 10 min before performing the last centrifugation. The V4-V5 region of the 16S rRNA gene was amplified using 515 F (5′-GTGYCAGCMGCCGCGGTA-3′)/928R (5′-CCCCGYCAATTCMTTTRAGT-3′) primer pair (Wang and Qian 2009) and the following PCR protocol: 94 °C for 120 s, 30 cycles of 94 °C for 60 s, 65 °C for 40 s, 72 °C 30 s and 72 °C for 10 min. Amplicon sequencing was performed using an Illumina MiSeq (2 × 250 pb) by the Get PlaGe platform (Genotoul, Toulouse, France).

Demultiplexed data were processed using FROGS (Find Rapidly OTU with Galaxy Solution) pipeline on Galaxy (Escudié et al. 2018). Contigs with a length between 380 and 500 pb were kept, clustered with Swarm (Mahé et al. 2014) with an aggregation distance of 3 and chimeras were removed. Filters were applied to remove singletons and keep for analysis OTUs with a minimum abundance of 0.005% of the sequences (Bokulich et al. 2013). The taxonomy affiliation was performed using Blastn against the Silva 132 database (pintail 80).

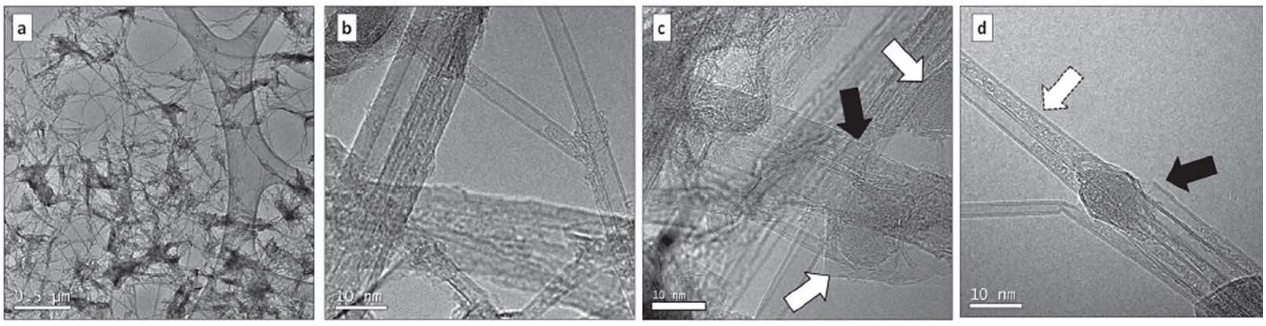

Figure 1. High-resolution TEM images of the BNNT sample. (a) overview at low magnification illustrating the fibrous structure. (b–d) High-resolution images showing on (b) the presence of multiwalled nanotubes (from 2 to 10 concentric walls), (c) damaged walls of some nanotubes (black arrow) and two isolated amorphous particles surrounded by some graphitic-like shells (white arrows), (d) some filling inside some nanotubes (dotted white arrow), a broken and open nanotube (black arrow) as well as an amorphous particle which seems to be at the end of a nanotube. The inset (same scale) of (d) also shows two isolated particles surrounded by some graphitic-like shells, one being amorphous while the other one (top) is crystallized.

2.7. Statistical analysis

Results from micronucleus assay were analyzed using McGill non-parametric test (Mcgill, Tukey, and Larsen 1978) on median values of each group of larvae. This test consists in comparing medians of samples of size n (where $n \geq 7$) and in determining their 95% confidence intervals (95% CI). 95% CI are expressed by $M \pm 1.57 \times \mathrm{IQR}/\sqrt{n}$, where M is the median and IQR is the inter-quartile range (Mcgill, Tukey, and Larsen 1978). The difference between the medians of the test groups and the median of the NC group is significant with 95% certainty if there is no overlap. For cell-cycle data, normality was assessed with Kolmogorov–Smirnov test and homogeneity of variances with Levene's test. One-way analysis of variance (ANOVA) followed by Tukey test were used to compare cell-cycle phase distribution among conditions.

For data manipulations of gut microbiota survey, OTUs counts, alpha diversity indexes and Weighted Unifrac Distances calculations as well as multidimentional scaling (MDS) plot were carried out using 'Phyloseq' R package (McMurdie and Holmes 2013). Graph visualization of OTUs relative abundances was performed using 'ggplot2' package (Wickham 2016) while differential abundance of bacterial genera between exposed conditions compared to the control group was performed using 'Deseq2' R package (Love, Huber, and Anders 2014). For multivariate analysis of variance between groups, PERMANOVA was performed using Adonis function from the 'vegan' R package (Oksanen et al. 2015).

3. Results and discussion

3.1. Bnnt characteristics

BET measurement indicated a specific surface area of 163 m^2/g, a little bit lower than the $> 200\ m^2/g$ claimed by the provider. TEM images (Figure 1(a)) shows that the sample is composed of nanotubes (2–10 walls, Figure 1(b)) and small nanoparticles encapsulated in graphitic-like h-BN shells (size *ca.* 10–15nm) (Figure 1(c,d)) as well as some more disorganized material. Part of the nanotubes are damaged or even broken (Figure 1(c,d), black arrows), which may be related to the grinding treatment that we have applied. On Figure 1(c,d), it is possible to see that while most nanoparticles look amorphous, a few are crystalized. All observed nanoparticles were coated with similar polyhedral graphitic-like h-BN shells. Outer diameter of the BNNT ranged from 2 to 14 nm with a mean outer diameter of 6 ± 2.6 nm (Figure 2).

We obtained through measurement of elemental analysis a total boron content between 38.6 and 42.5 wt. % (*ca.* 50.5 at. % using the highest weight value), 46.3 wt. % of Nitrogen (*ca.* 42.5 at. %) and 8.7 wt. % of Oxygen (*ca.* 7 at. %). If we make a first hypothesis that all the Nitrogen in the sample is in BN, then the excess of Boron is of *ca.* 8 at. %. Assuming that all the oxygen is involved in B_2O_3 (the most stable boron oxide), then the excess of Boron (corresponding to elemental boron) would be *ca.* 3.3 wt.%. This is rather far from the information from the provider, although this explains that variations are likely between different batches.

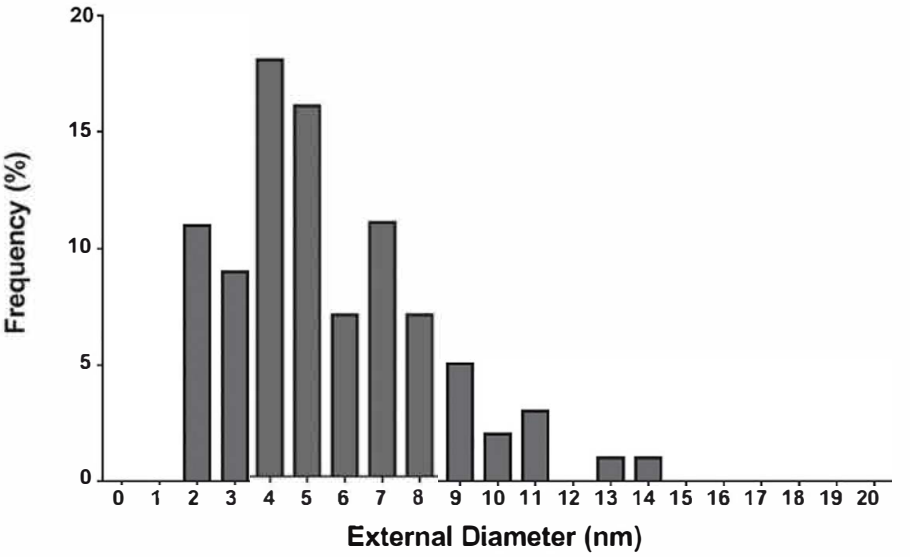

Figure 2. BNNT outer diameter distributions evaluated from the HRTEM images ($n = 91$).

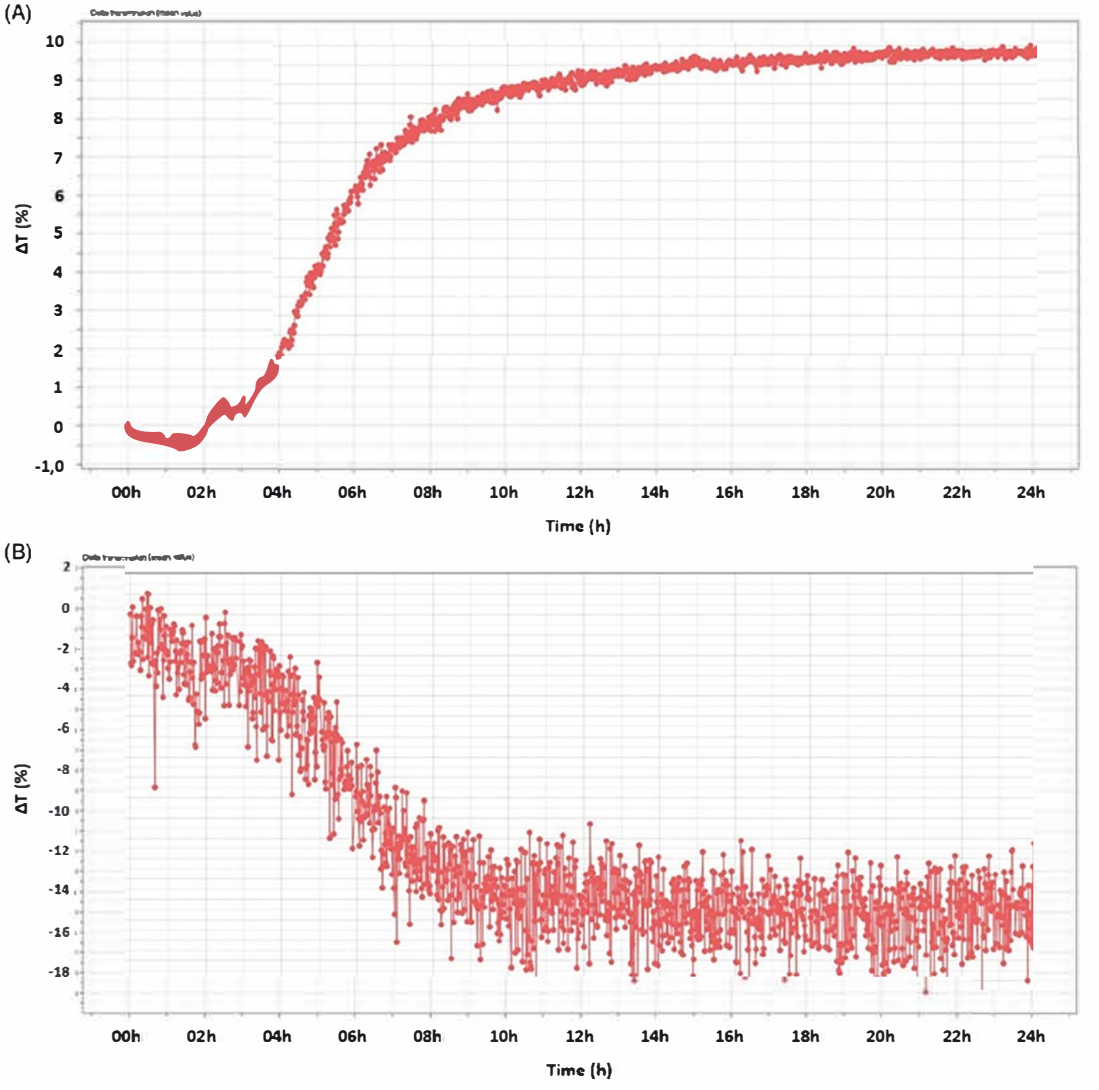

Figure 3. Variation of the transmission vs time for suspensions of BNNT at 10 mg L^{-1} in exposure media measured at the top of the vial (A) and at the bottom of the vial (B).

Details results from the XRD, XPS, TGA, Raman and IR analysis are detailed in the Supplementary information.

The variation of the transmission data (compared to t0) vs time at the top and at the bottom of the vial is presented in Figure 3(A,B) respectively. At the top of the vial, transmission progressively increased as the BNNTs were settling down. On the opposite, the transmission progressively decreased at the bottom of the vial as the sediment was forming. In both cases, it is obvious that most of the clarification/sedimentation took place in 8-10 h, at a rather constant speed (before slowing down) (Supplementary Figure S5).

3.2. Boron concentration in the exposure media and larvae

Analysis of boron concentration in the water indicated that reconstituted water from the control condition was devoid of boron. In the 10 mg L^{-1} of BNNT condition, 0.32 mg L^{-1} of boron were measured after 24 h of incubation in the media in absence of Xenopus tadpoles while after 24 h in presence of larvae, the concentration of boron decreased below 0.1 mg L^{-1} (quantification limit). During the exposure, larvae produced BNNT-containing feces according to the color differences with the control group. At the end of the 12 days of exposure, 2 ± 4 µg g^{-1} of boron were measured in the whole tissues of the larvae from the control group. Boron concentration in larvae exposed to 10 mg L^{-1} of BNNT reached 183 ± 83 µg g^{-1} of tissue.

BNNT were shown to be structurally very stable (Chen et al. 2017; Song et al. 2014) and there is few chance for the digestive tract of tadpoles to constitute drastic enough conditions allowing BNNT degradation as strong acidic conditions are needed to dissolve this material (Kleinerman et al. 2017). In addition, carbon-based structural homologues of BNNT were shown to accumulate in the lumen of *Xenopus* tadpoles during the exposure while a transfer across the intestinal wall was not evidenced (Mouchet et al. 2011). Thus, crossing of intestinal barrier is not likely to occur in the case of BNNT exposure and it is highly probable that the boron concentration measured in the water as well as in the tissues of the larvae originated from free boron as byproduct found in the commercial form of the tested BNNT. However to confirm this hypothesis, the use of imaging technics would be needed to determine if BNNT are present or not in the biological tissues. Indeed, amphibians were previously shown to accumulate free boron in polluted sites and are able to tolerate boron concentration in the water up to 10 mg L^{-1} for extended period without adverse effects (Eisler 1990; Emiroğlu et al. 2010). Measurement performed under the experimental conditions indicated a full clearance of the water column by the larvae over 24 hours, leading to rejection of BNNT in the animal feces.

3.3. Micronucleus assay and cell cycle analysis

Xenopus larvae exposed to cyclophosphamide at 40 mg L^{-1} (positive control = PC) exhibited significantly higher MNE compared to the control group (negative control = NC), validating results obtained from the micronucleus assay. Among experimental conditions containing BNNT, although a trend to an increasing number of micronucleated cells was observed after exposure to 10 mg L^{-1} of the nanoparticle, no statistically significant increase of MNE ‰ was accounted (Figure 4(A)). Analysis of erythrocyte cell cycle highlighted a significant decrease in S-phase cells in conditions containing BNNT compared to the negative control (ANOVA, S-phase: $p < 0.001$), while the percentage of G0/G1 cells increased and G2/M decreased only after exposure to 1 mg L^{-1} of BNNT (ANOVA, G0/G1: $p < 0.001$; G2/M: $p < 0.001$) (Figure 4(B)).

The results obtained indicated that the commercial form of BNNT tested was not leading to irreversible genotoxic effects at the tested concentrations as no significant induction of micronucleated erythrocytes was observed. Thus, even if we cannot exclude that reversible DNA damages could occur through single strand break DNA, the obtained results indicated that BNNT exposure do not lead to permanent DNA alteration. This observation is contrary to *in vitro* studies indicating disturbances of cell proliferation and genotoxicity of BNNT (Fernandez-Yague et al. 2015; Horváth et al. 2011; Çal and Bucurgat 2019; Emanet et al. 2015), but consistent with the few available works performed *in vivo*. Indeed, no genotoxicity was observed in the planaria (Salvetti et al. 2015) as well as in drosophila (Demir and Marcos 2018) after BNNT exposure. In the latter study, BNNT showed antioxidant and antigenotoxic properties against a

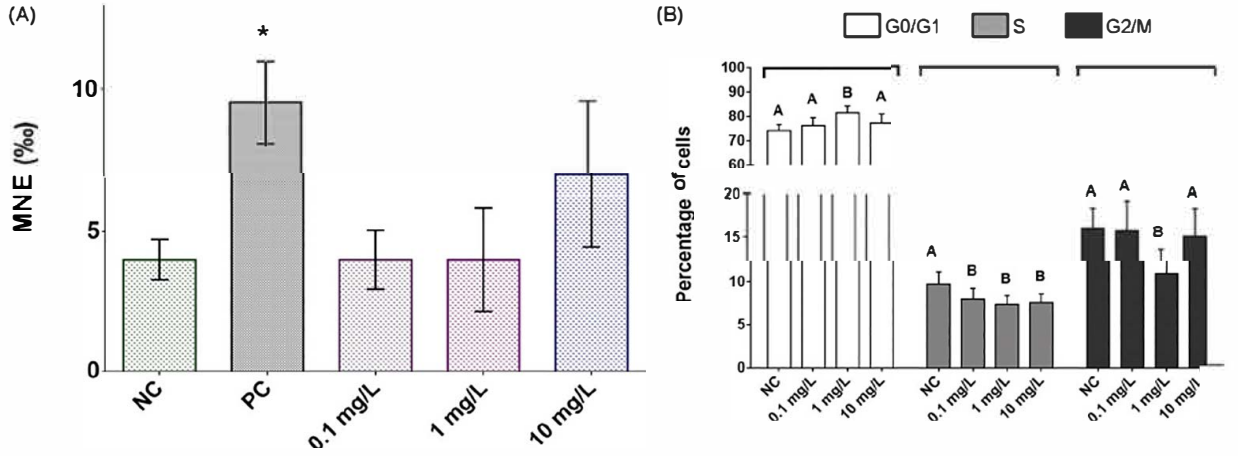

Figure 4. Micronucleus induction (A) and cell cycle (B) measured in erythrocytes of Xenopus tadpoles exposed for 12 days to increasing concentrations of BNNT. MNE: micronucleated erythrocytes; NC: negative control; PC: positive control; *significant difference compared to the NC (McGill test). For cell cycle analysis, $N = 12$, ANOVA $p < 0.001$ followed by Tukey test. Letters indicate significant differences between concentrations tested for each phase of the cell cycle.

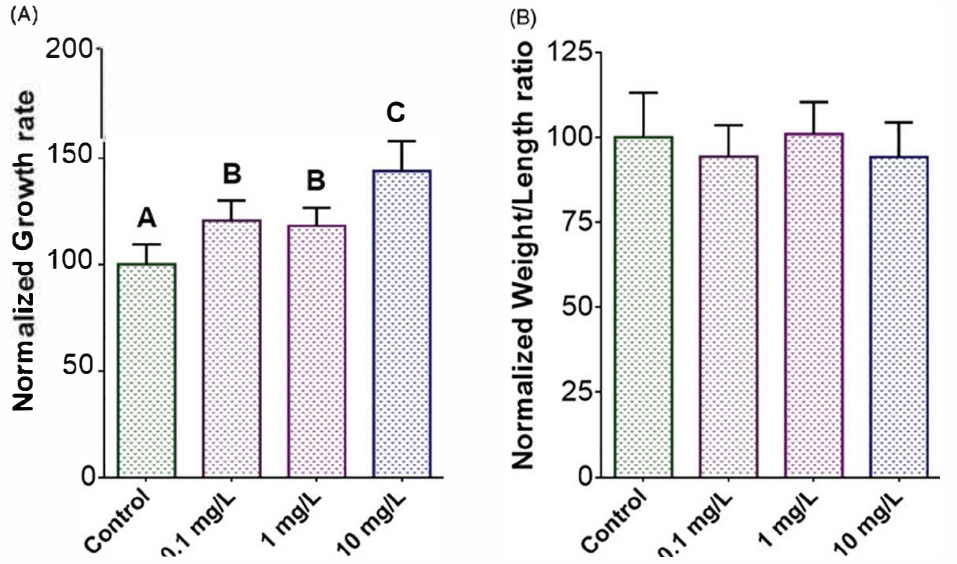

Figure 5. Normalized growth of *X. laevis* larvae measured after 12 days of exposure to increasing concentrations of BNNT. ANOVA $p < 0.001$ followed by Tukey test. Letters indicate significant differences between concentrations.

known genotoxic compound. In addition, similar effects were observed in *Xenopus laevis* exposed to other non-oxidized 1D carbon counterparts of BNNT (Mouchet et al. 2008; Mouchet et al. 2010). It was previously indicated that boron exert protective effects against genotoxic compounds in other biological models (Turkez 2008; Ince et al. 2014; Sarıkaya et al. 2016; Tepedelen, Soya, and Korkmaz 2016; Alak et al. 2019). For this reason, it is not possible yet to determine in these conditions whether free boron associated to the commercial mixture is protective against the genotoxic potential of the different types of BN nanoparticles or if BN are devoid of genotoxic potential.

3.4. Larval growth rate

Exposure to BNNT led to a significant increase of *Xenopus* tadpole growth in a dose-dependent manner (ANOVA, $p < 0.001$) (Figure 5(A)). Growth rate increased from $20.54 \pm 9.53\%$ at $0.1\ mg\ L^{-1}$ to $44 \pm 13.97\%$ at the highest tested concentration compared to negative control. However, mean weight/length ratio remained unchanged in larvae exposed to the tested conditions (ANOVA, $p = 0.143$) (Figure 5(B)).

Exposure to BNNT led to a dose-dependent increase of larval growth that is counterintuitive regarding to the literature indicating that no matter the number of dimension of the carbon-based

nanomaterial (CBN) considered, growth inhibition of *Xenopus* tadpoles was described by the surface area of exposure (Mottier et al. 2016; Lagier et al. 2017). As the surface area of the tested BNNT is comparable to those of the CBNs used in these studies and the observed effects are opposite, we can emphasize that this growth inhibition model is specific to CBNs and is thus not applicable for BNNT. The main hypothesis for mechanisms underlying growth inhibition following CBNs exposure was associated to nutrient depletion and reduction of nutrient intake due to the presence of agglomerated CBNs in the gut (Lagier et al. 2017). The accumulation of BNNT in the tadpole gut was not as clear as previously observed with carbon-based nanomaterials (CBNs) that is consistent with the absence of growth alteration measured. Thus, despite the capacity of BNNT to adsorb nutrients (Farmanzadeh and Ghazanfary 2014), the hypothesis of nutrient intake limitation is not likely to occur in the case of BNNT exposure.

Previous studies demonstrated that a deficiency ($< 0.003\ mg\ L^{-1}$) as well as too high concentrations of boron ($<50\ mg\ L^{-1}$) were detrimental for the development of amphibian embryo and impaired the reproduction of adults (Fort et al. 1998; Fort et al. 1999; Laposata and Dunson 1998; Fort et al. 2002). According to these studies, the boron concentrations measured in the exposure media of this work ($0.32\ mg\ L^{-1}$) do not represent a critical concentration impairing tadpole's physiology that is consistent. Despite the lack of literature regarding the enhanced growth of amphibian larvae exposed to boron, these effects were observed in fishes with no explanation of the mechanism involved in such growth increase (Eckhert 1998; Rowe et al. 1998; Öz, Inanan, and Dikel 2018). However, it was suggested that boron was playing a role in the thyroid axis of *X. laevis*, probably in the synthesis of T3 (Fort et al. 2002). Thus, among the possible pathways involved in this observed growth stimulation, it may in part occur from a stimulation of the metamorphosis by the boron.

3.5. Gut microbiota survey

Among the different tested conditions, richness was not affected (Observed species: ANOVA $p = 0.505$; Chao1: ANOVA $p = 0.826$). However, evenness calculated with Shannon index was shown to be significantly decreased after 12 days of exposure to $10\ mg\ L^{-1}$ of BNNT (ANOVA $p < 0.001$) (Figure 6(A)). Bacterial communities were shown to be significantly affected by the BNNT concentration as revealed by MDS performed with Weighted-Unifrac distances and PERMANOVA analysis ($F = 14.146$; $r^2 = 0.726$, $p = 0.001$) (Figure 6(B)). Pairwise comparisons indicated that gut bacterial communities were similar between the control group and the lowest tested concentration $0.1\ mg\ L^{-1}$, while the two other tested concentrations 1 and $10\ mg\ L^{-1}$ were different from each other and from any other conditions.

Three major phyla composed the gut microbiota of *X. laevis* tadpoles: Bacteroidetes, Proteobacteria and Firmicutes with relative abundance in the control group representing $62 \pm 8.5\%$, $24.7 \pm 8.3\%$ and $12.2 \pm 5.5\%$ of the whole microbial communities respectively (Figure 7). Exposure to BNNT led to a significant decrease of Proteobacteria relative abundance at 1 and $10\ mg\ L^{-1}$ of BNNT (ANOVA $p < 0.001$). On the opposite, a significant increase of Bacteroidetes is noticed at these concentrations (ANOVA $p < 0.001$), leading to a phylum relative abundance of $74 \pm 1.17\%$ at the highest tested concentration.

At the family level of the phylum Bacteroidetes, *Bacteroidaceae* were shown to significantly increase (ANOVA $p < 0.001$) at 1 and $10\ mg\ L^{-1}$ reaching $97.36 \pm 1.16\%$ of the overall Bacteroidetes in the higher BNNT concentration compared to the $82.24 \pm 8\%$ observed in the control group (Supplementary Figure S6A). On the contrary, other families such as *Spirosomaceae*, *Flavobacteriaceae*, *Crocinitomicaceae* or *Weeksellaceae* were shown to significantly decrease (ANOVA $p < 0.001$; $p < 0.001$; $p = 0.0356$; $p = 0.0023$ respectively). Similarly, inside the phylum Proteobacteria, taxa from the family *Magnetospirillaceae* were shown to significantly increase after exposure to $10\ mg\ L^{-1}$ of BNNT (ANOVA $p = 0.00116$) (Supplementary Figure S6B), while *Moraxellaceae* and *Burkholderiaceae* were shown to significantly decrease at this concentration (ANOVA $p < 0.001$ and $p = 0.00811$ respectively).

At the genus scale, 12, 27, and 39 genera were differentially observed between the control group and the BNNT concentration of 0.1, 1, and $10\ mg\ L^{-1}$ respectively (excluding multi-affiliated and

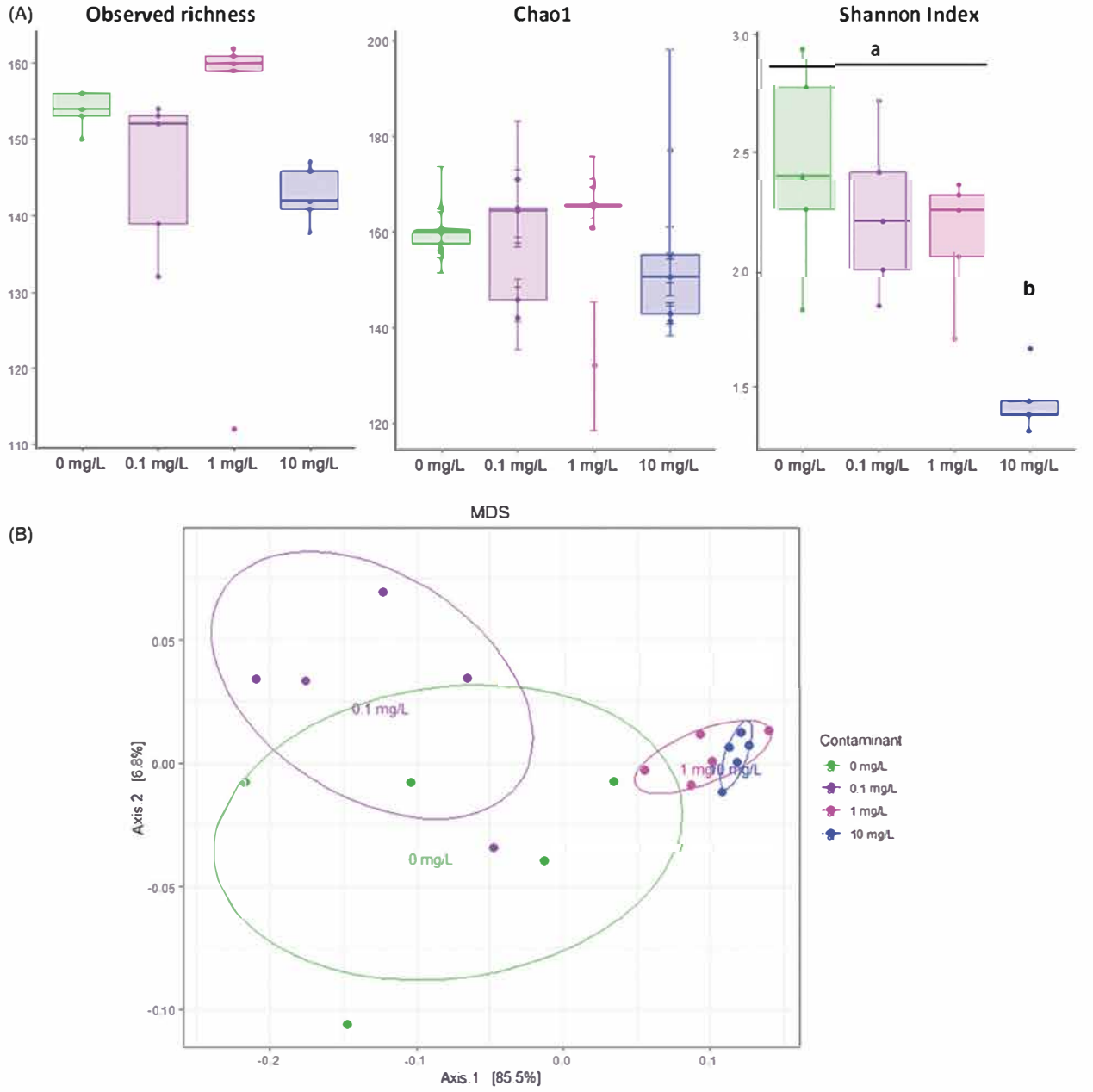

Figure 6. Effects of 12 days of exposure to BNNT on the structure of gut bacterial communities of X. laevis tadpoles. Observed richness, estimated richness (Chao1 index) and evenness (Shannon index) are compared between the exposure conditions (0, 0.1, 1 and 10 mg L^{-1}) (A). MDS plot of bacterial communities based on Unweighted unifrac distances (B).

unknown genera). Thus, even if the Firmicutes phylum was not globally affected, some genera were shown to be differentially observed between the control and the 1 and 10 mg L ^{1} of BNNT conditions (Supplementary Figure S7). Genera which relative abundance was shown to be unaffected, decreased or increased were classified as resistant, sensitive or opportunistic respectively. Thus, each category represented about 58.2% of the overall taxa for the resistant, 21.5 and 20.3% for the sensitive and opportunistic respectively.

Even if it is difficult to differentiate the part of the effects associated to indirect or direct effects of BNNT and/or to byproducts exposure, several possibilities can be considered concerning the effects observed on gut bacterial communities after the exposure of tadpoles to this commercial BNNT.

Considering the potential indirect effects, as previously mentioned, boron could play a role in the

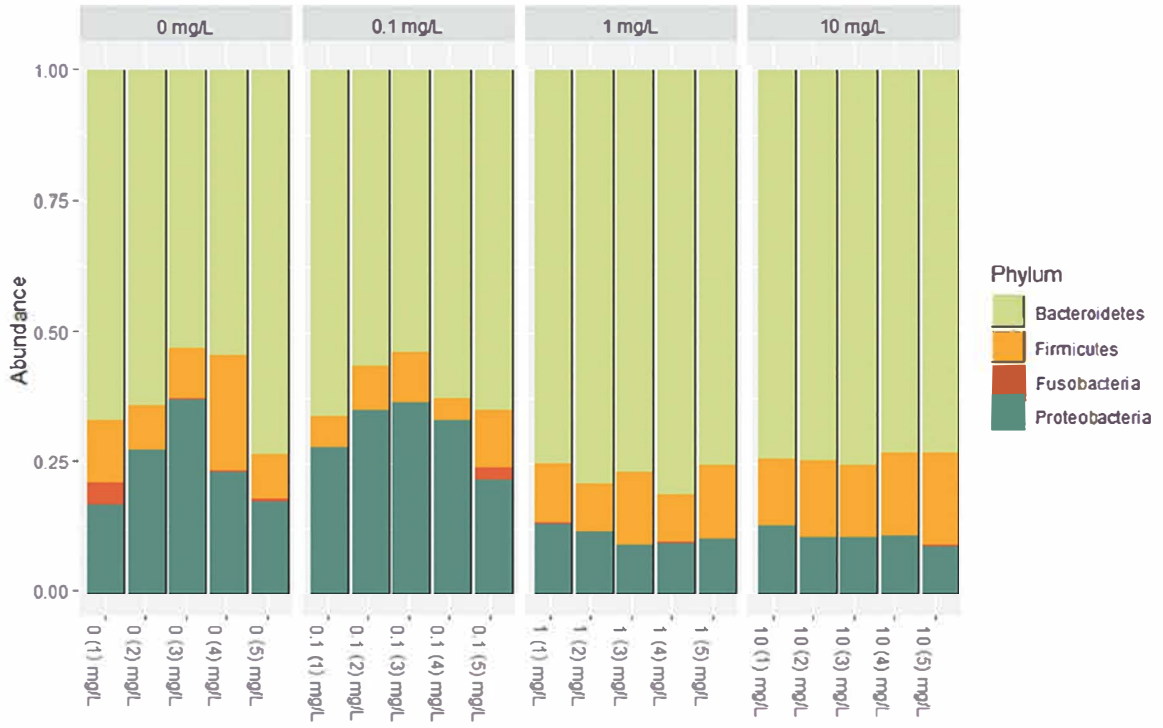

Figure 7. Phylum composing the gut microbiota after 12 days of exposure to BNNT concentrations ranging from 0 to 10 mg L^{-1}.

thyroid axis that is involved in the development and maturation of the gut (Chalmers and Slack 1998; Ishizuya-Oka 2011). It was also demonstrated that gut bacterial communities were changing during the metamorphosis of amphibians, notably through an increase of the phyla Bacteroidetes, a decrease of Proteobacteria and decrease of diversity (Kohl et al. 2013; Chai et al. 2018) which is consistent with our data. Thus, we cannot exclude that the changes occurring in the gut microbial communities could be in part associated to the stimulation of thyroid axis leading to the acceleration of gut maturation.

These changes in bacterial consortium could also be associated to direct effects of BNNT. Surprisingly, antibacterial properties of BN nanoparticles have not been thoroughly investigated yet (Merlo et al. 2018) and results available from the literature regarding the antibacterial activity are contradictory. Indeed, despite its potential to interact with biological membranes and to form heteroaggregates with bacteria (Thomas, Enciso, and Hilder 2015; Wang et al. 2015), it was indicated that raw BNNT at concentrations reaching 1 mg L ^{1} were not inducing *E.coli* and *S.aureus* growth inhibition (Nithya and Pandurangan 2014). In addition, exposure of *P. aeruginosa* strain to boron nitride nanosheets at a concentration of 10 mg L ^{1} was associated to a weak transcriptomic response compared to other tested nanoparticles and was not leading to growth inhibition (Mortimer et al. 2018) while another study observed bacteriostatic activities at 0.4 mg L ^{1} in multiple gram positive bacterial strains (Kıvanç et al. 2018). On the opposite, coating of copper surfaces with similar hexagonal boron nitride led to protective effects against bactericidal Cu (Parra et al., 2015). Moreover, exposure to 2D BN at high concentration (100 mg L ^{1}) induced damages to bacterial membranes in *E.coli* (Zhang et al. 2019). These studies were performed through exposure of single bacterial species in suspension in a liquid medium that is very different from the exposure conditions found in the gut. However, few data emphasizing the effects of BN nanoparticles on complex bacterial communities are available. Strong antibiofilm activities of 2D BN were observed in established biofilm composed of single bacterial species (Kıvanç et al. 2018).

As boron compounds were shown to exert weak toxicity against multiple bacterial strains (Minimum inhibitory concentration over 1 g L ^{1}) that possess a good tolerance to this element (Ahmed and Fujiwara 2010; Yilmaz 2012; Sayin, Ucan, and Sakmanoglu 2016), few direct effects from free

boron could be expected on gut microbial communities. Boron was shown to constitute an essential trace element for bacteria, contributing to physiological and metabolic activities (Kabu and Akosman 2013; Uluisik, Karakaya, and Koc 2018). Furthermore, boron was shown to be implied in bacterial communication through the activation of an extracellular signaling molecule (autoinducer AI-2), involved in quorum sensing (Chen et al. 2002). This process allows the regulation of gene expression and diverse physiological activities in response to fluctuations in bacterial density (Miller and Bassler 2001; Federle and Bassler 2003; Waters and Bassler 2005; Papenfort and Bassler 2016). Exposure to nanoparticles were previously shown to influence quorum sensing process (Singh et al. 2017; Xiao et al. 2016; Hayat et al. 2019). This could also be possible for the BN nanoparticle exposure. Thus, disturbances of quorum sensing from the gut bacteria could lead to changes in microbial communities (Thompson et al. 2015).

3.6. Statistical correlations

Correlation analysis indicated strong positive correlations among tadpole biometric parameters. Significant negative correlation between the phylum Proteobacteria and Bacteroidetes are noticed (Pearson, $r = -0.91$ $p < 0.001$). Firmicutes/Bacteroidetes ratio was shown not to be correlated with any of the larvae biometric parameters or growth rate. However, phylum Bacteroidetes was shown to be significantly and positively correlated with larval length ($r = 0.62$, $p = 0.0034$), growth rate ($r = 0.51$, $p = 0.0211$). On the contrary, phylum Proteobacteria was shown to be significantly negatively correlated with larval length ($r = -0.69$, $p = 0.0008$) and growth ($r = -0.55$, $p = 0.012$).

Changes in the Firmicutes/Bacteroidetes ratio of gut microbiota was shown to be associated to metabolic disorders in several species (Ley et al. 2005; Ley, Peterson, and Gordon 2006; Haiser and Turnbaugh 2013; Li et al. 2013). In this study, the F/B ratio was not significantly affected (ANOVA, $p = 0.2$) and was not correlated to growth parameters following exposure to BN. Thus, we can suggest that the growth stimulation observed is not associated to the potential induction of a metabolic disorder in host. On the contrary, phylum Bacteroidetes was positively correlated with growth parameters of the tadpoles. Nevertheless, the most known biological function of members of the phylum Bacteroidetes from the gut is the degradation of biopolymers such as polysaccharides to produce carbohydrates (Thomas et al. 2011; Johnson et al. 2017). Thus, this increase of Bacteroidetes relative abundance can be associated to improved production of carbohydrates and production of energy, leading to an improved fitness for the host. The use of other omic techniques such as transcriptomic would be needed in order to determine the links between the changes in gut flora and modifications of functional capacities.

4. Conclusion

The aim of this study is to fill lacking data concerning the ecotoxicity of a commercial form of boron nitride nanotubes toward the aquatic compartment. We used an original approach based on the measurement of toxicological endpoints including genotoxicity and growth parameters of an amphibian species as well as bacterial communities associated to the host gut. The obtained results indicate an overall biocompatibility of the tested BN mixture toward *X. laevis* tadpoles. Significant induction of larval growth was shown to be correlated with changes in the gut microbial communities of the host. These changes in host physiology are most probably due to indirect effects of byproducts, especially free boron that could stimulate the maturation of the gut, benefiting to bacteria favoring the host metabolism. Thus, BNNT alone represent a minor threat for amphibians in aquatic environments. However, due to its sorption capacities, interactive effects with other common contaminants such as PAHs, heavy metals or pesticides are remaining to be assessed to fully characterize its ecotoxic potential.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Ecotoxicological assessment of commercial Boron Nitride Nanotubes towards *Xenopus laevis* tadpoles and host-associated gut microbiota

Supplementary information

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Detailed BNNT characteristics

XRD pattern showed 2 main peaks at ca. 2θ = 25.9° and 42.4°. The first one may correspond to the (002) line of h-BN (although the interlayer spacing is larger than expected at 3.44 Å instead of 3.36 Å), while the second one may be a superimposition of the (100) and (101) lines.

XPS analysis (Figure S1 and Table S1) revealed 40.2 at.% of Boron involved in B-N bond, and 39.9 at.% of Nitrogen involved in the same bond, thus BN represents at least 80 at. % of the total. It is however impossible from the XPS analysis to make the difference between, BN involved in nanotubes and BN particles. The only exogenous element evidenced by XPS analysis was Carbon, which is likely to come from contamination during sample handling and preparation. 4.4 at.% of Boron would be involved in O-B-N bonds and 1.6 at. % would be involved in direct B-O bonds. This confirms that the sample contains a large majority of BN but that some boron oxide is also likely to be present, while pure elemental boron seems excluded (no B-B bond identified).

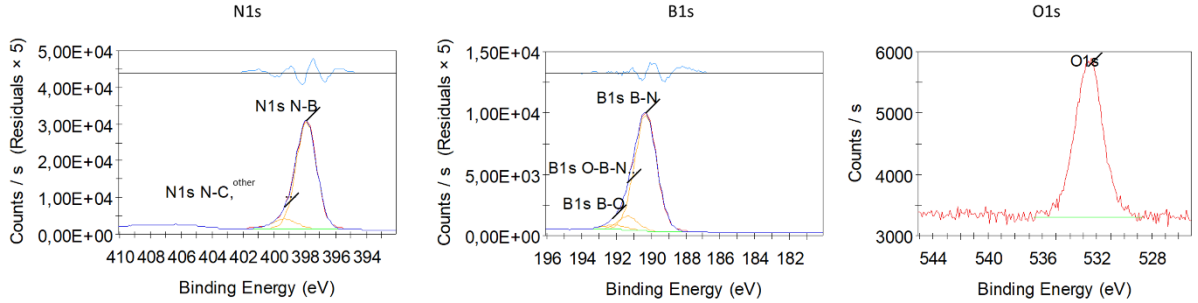

Figure S1: N1s, B1s and O1s deconvoluted X-ray photoelectron spectroscopy (XPS) spectra of BNNT.

Table S1: Assignments of C1s, B1s and C1s XPS peaks for BNNT

Name	**Peak BE**	**FWHM eV**	**Atomic %**
B1s B-N	190.31	1.48	40.24
B1s O-B-N...	191.35	1.35	4.36
B1s B-O	192.20	1.52	1.63
C1s	284.57	1.66	5.82
N1s N-B	397.91	1.53	39.94
N1s N-C, others	399.32	1.83	4.40
O1s	532.57	2.19	3.61

TGA analysis (Figure S2) indicated a good thermal stability of the BNNTs in air until 425°C, temperature at which some oxidation started to occur in 2 steps. The first step was observed between 425°C and 825°C, while the second one, much more pronounced, began at 825°C and stopped only close to 990°C. The first oxidation step was earlier attributed to the oxidation of elemental boron [1] but should have a different origin here as elemental boron was ruled out from the XPS analysis. The comparison with purified BNNTs (prepared by air oxidation treatment followed by washing in boiling

water, for characterization purpose only) revealed that the first oxidation step was absent in the purified material, indicating that it may be related either to BN not included in BNNTs, or to boron compounds (oxide, boric acid) present in the raw material.

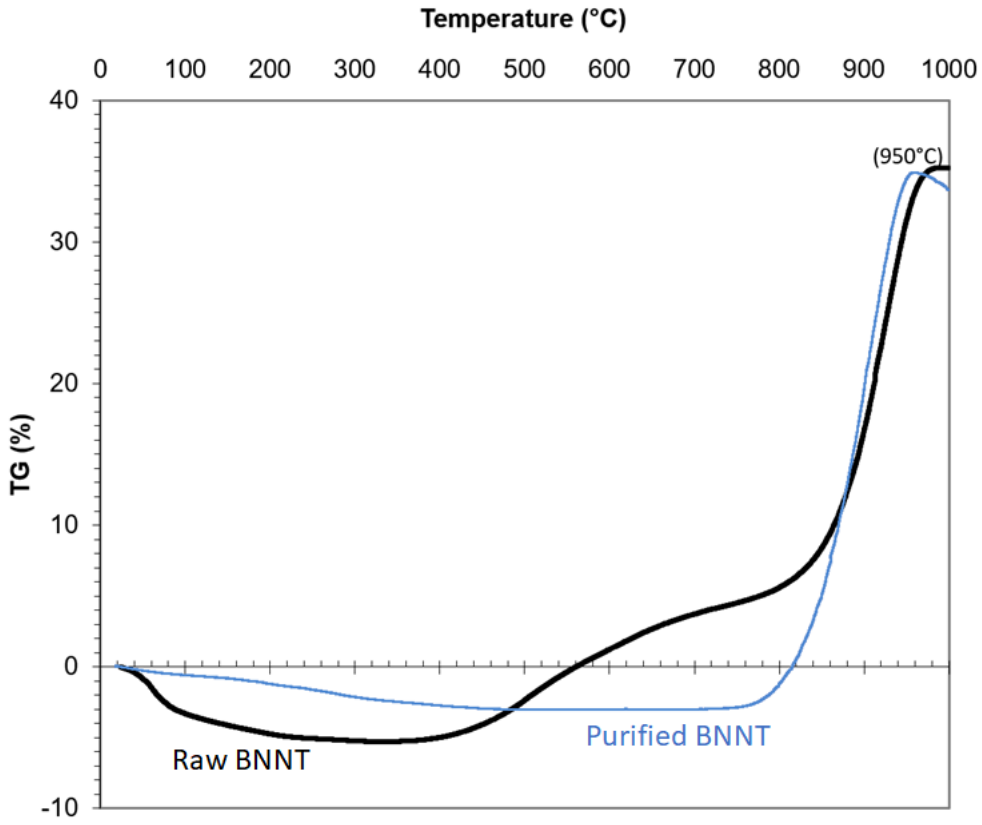

Figure S2: TGA in air: Comparison between raw (black) and purified BNNT (blue).

Raman analysis at 532 nm (Figure S3) revealed mainly a single peak at *ca*. 1370 cm^{-1} attributed to the E_{2g} mode related to in-plane vibration of the B-N bond. However, it seems that the same peak may be observed both for BNNTs and h-BN [1]. XRD analysis revealed mostly a main broad peak just below 26° corresponding to the (002) line in BNNTs [1,2], while the characteristic peak of h-BN for the same line was obviously absent from the BNNT sample. This analysis also ruled out the presence of detectable amounts of crystallized boric acid or boron oxide. The other peaks cannot be used to make a clear difference between h-BN and BNNTs.

Figure S3: Raman analysis of BNNTs (532 nm).

Finally, from IR analysis (Figure S4), it appears that this is not easy to clearly differentiate between h-BN and BNNTs (although H. Harrison et al have recently proposed a method based on FTIR spectroscopy to do so [1]. For BNNTs, a longitudinal mode is expected at 1369 cm-1, while a tangential circumferential one is expected at 1545 cm^{-1} [3]. These 2 peaks are observed but are very broad and strongly overlap in our sample. In h-BN, a transverse optical mode and a longitudinal mode both resonate near 1350 cm^{-1}, which are not clearly resolved in our sample. However, the peak at 1540 cm^{-1} was also experimentally reported for h-BN [4]. We observe a narrow peak at 811 cm^{-1}, attributed to an out-of-plane vibration (Radial) vibration of B-N bonds. Peaks in the 970 – 1170 cm^{-1} range are often attributed to oxidized forms such as boron oxide, boric acid or metaboric acid [5] but are observed at different places in our sample (925, 1032, 1102 cm-1) and exhibit a very low intensity.

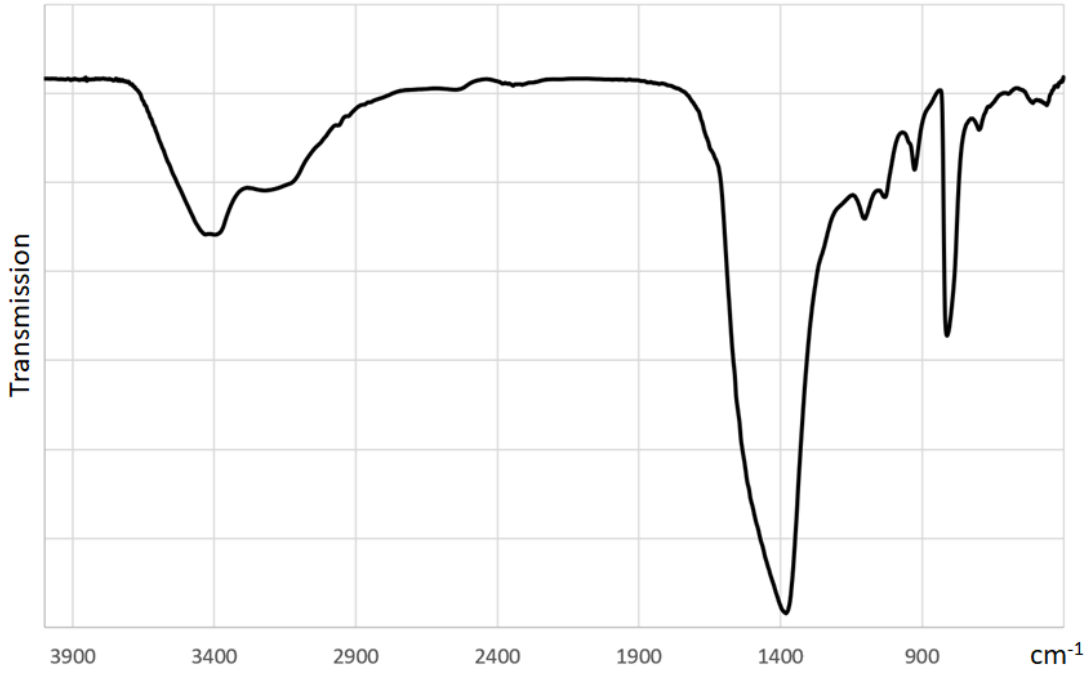

Figure S4: FTIR analysis of BNNTs.

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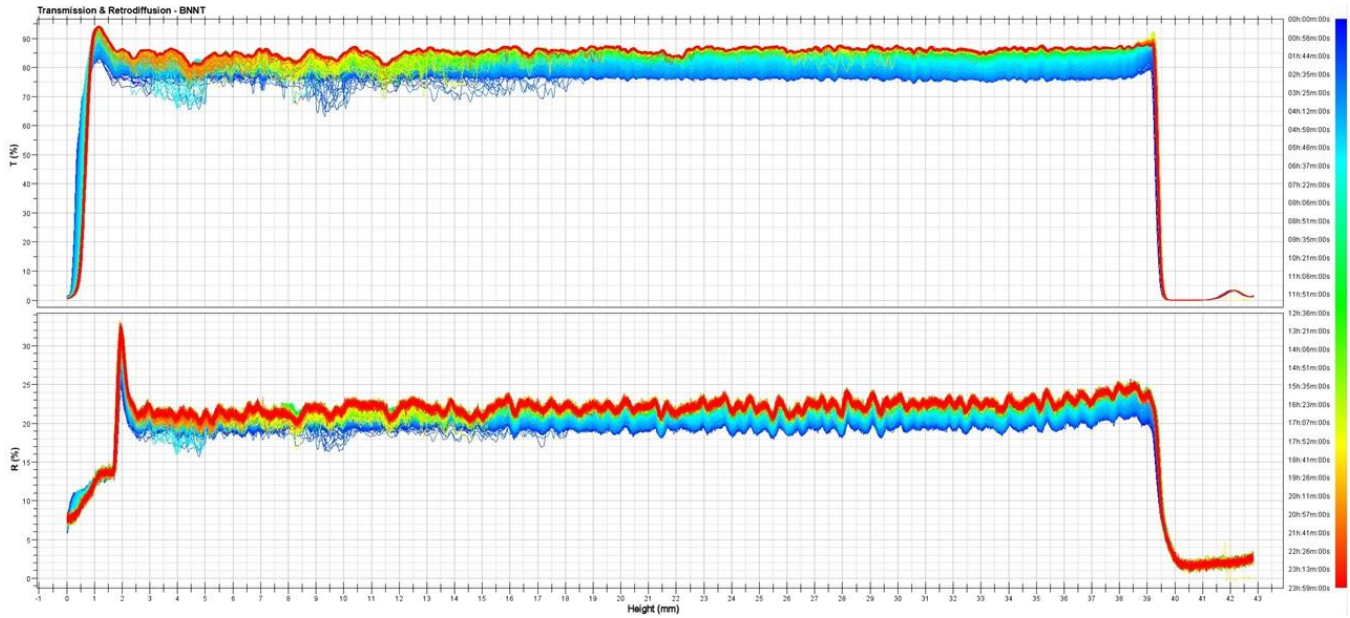
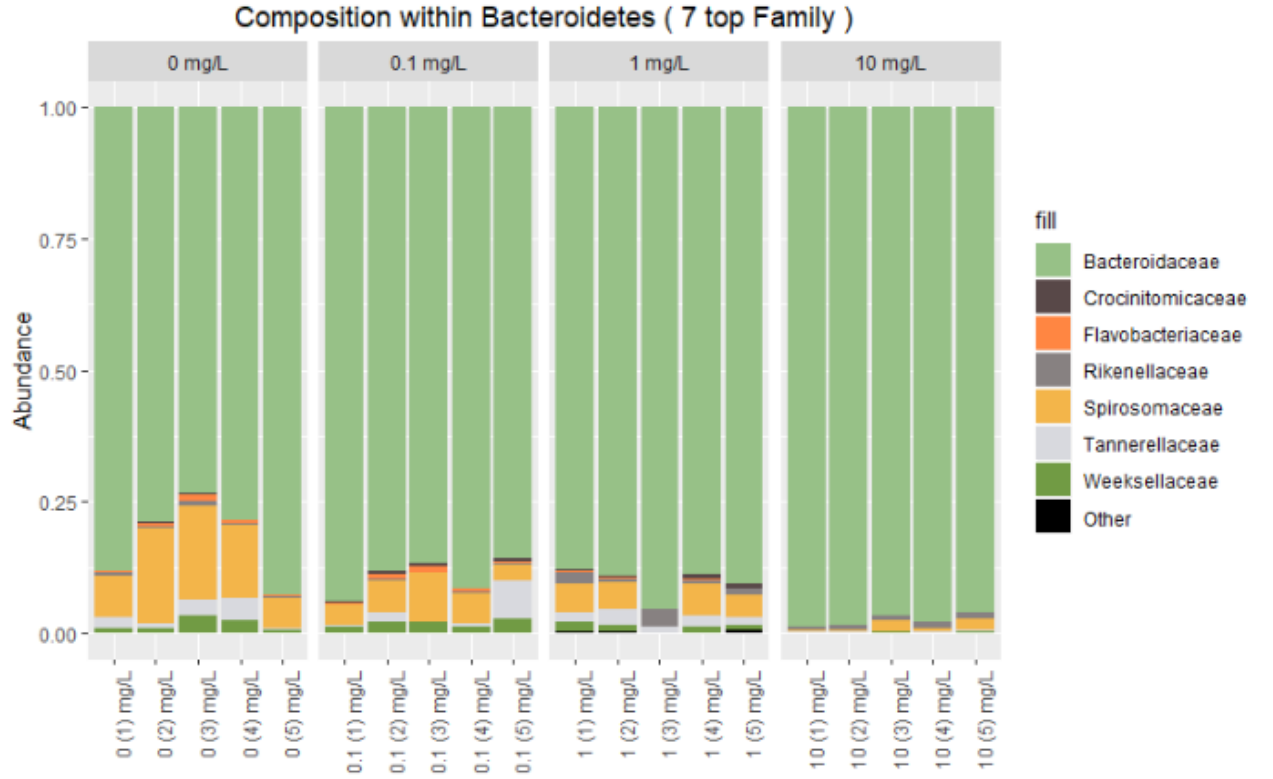


Figure S5: Variation of the transmission and reflexion *vs* time and height of the sample (compared to t0) of the suspensions of BNNT at 10 mg.L^{-1} in exposure media.

A



B

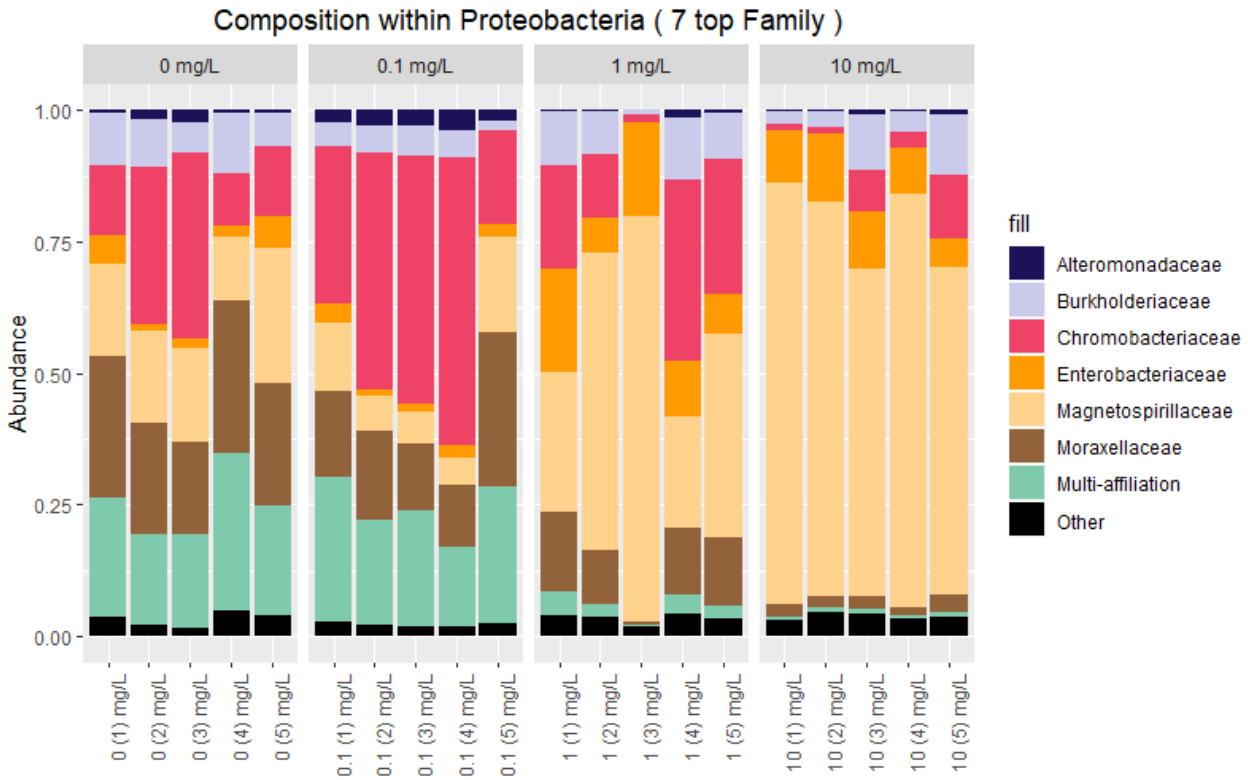


Figure S6: Families from Bacteroidetes (A) and Proteobacteria (B) phylum composing the gut microbiota after 12 days of exposure to BNNT concentrations ranging from 0 to 10 mg/L.

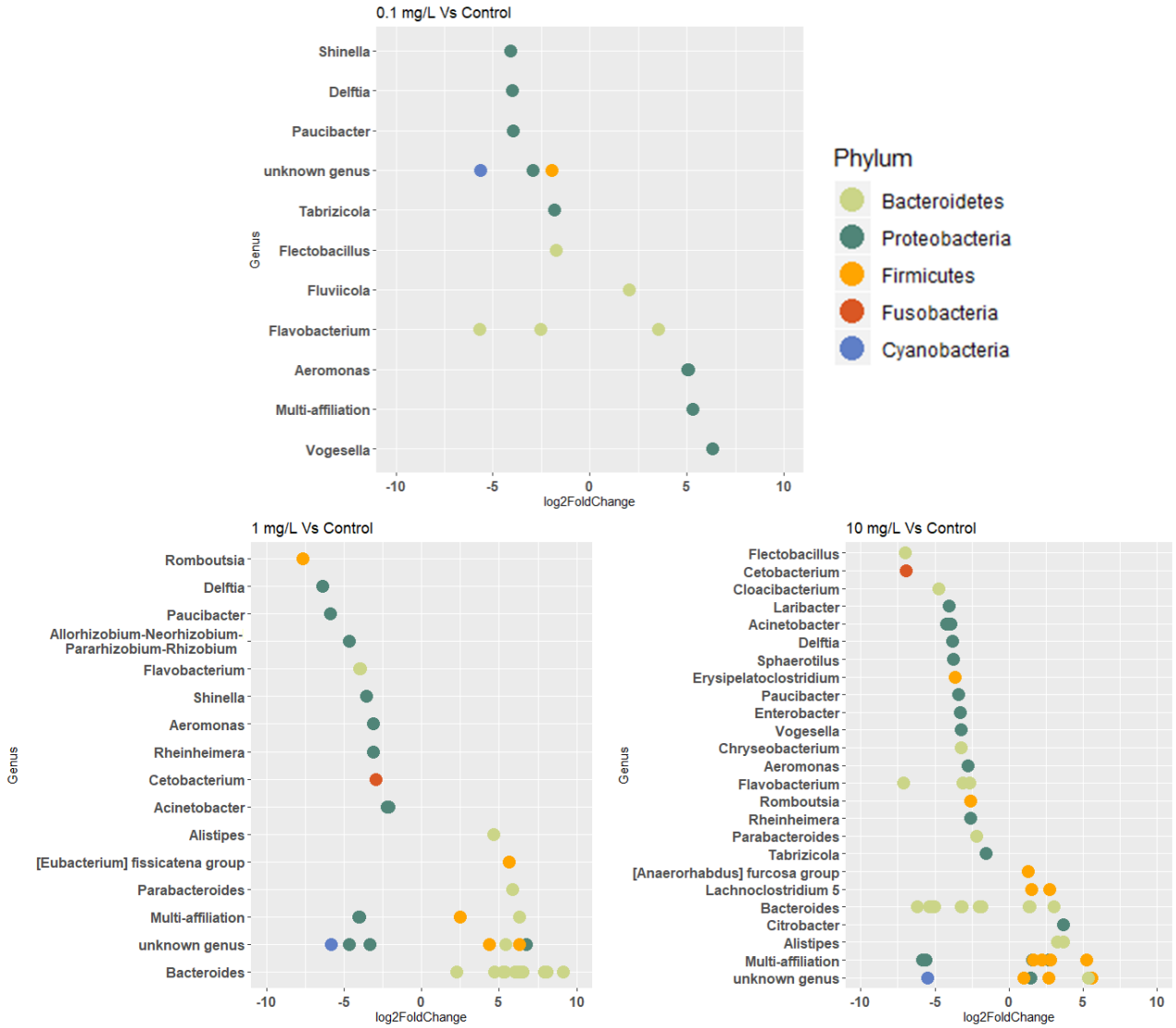


Figure S7: Bacterial genera differentially observed (p < 0.01) between the exposure conditions compared to the control group after 12 days of exposure. Positive or negative log2-fold change values indicate enriched or decreased OTUs in the exposure conditions respectively.

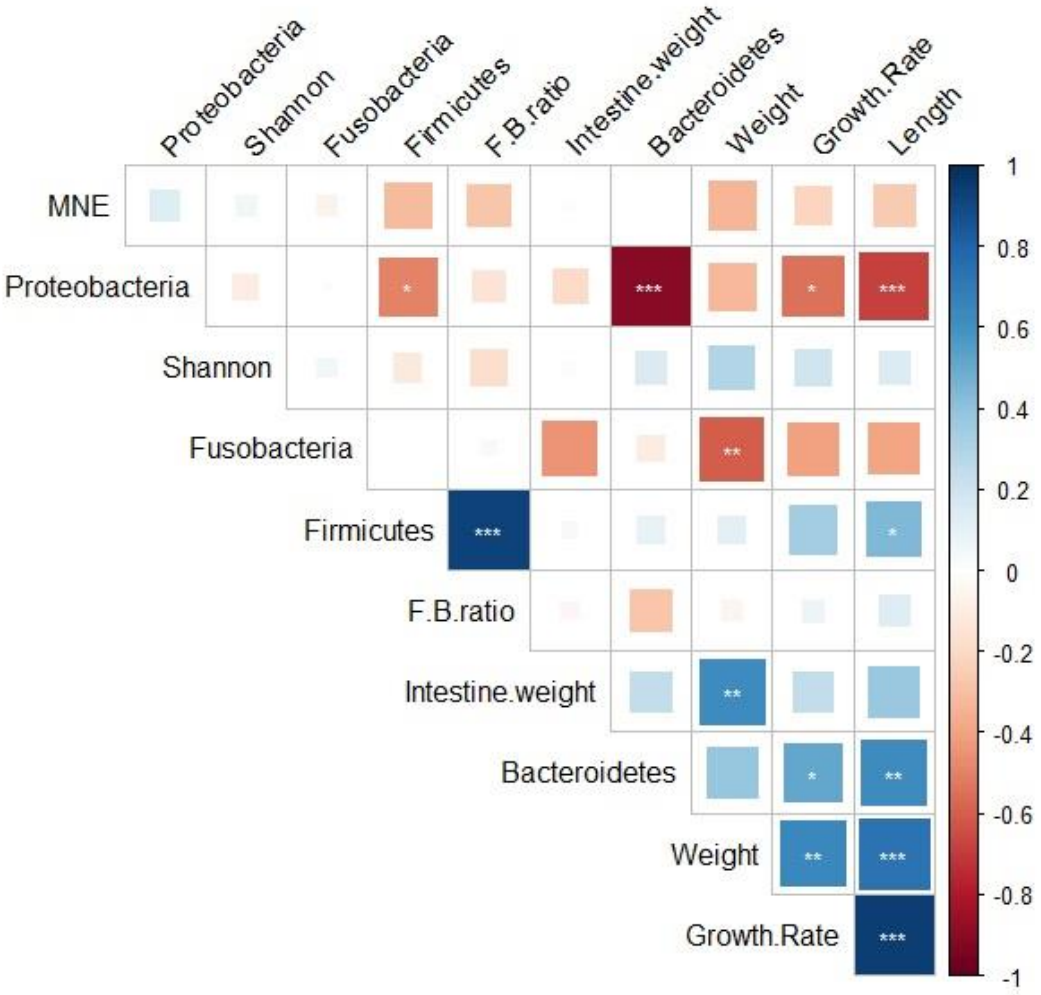


Figure S7: Correlogram showing pairwise Pearson correlations between measured variables. Positive correlations are displayed in blue and negative correlations in red color. Color intensity and the size of the square are proportional to the correlation coefficients as displayed in the legend. Significant p-value significant (p-value < 0.05) are indicated as follow: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. MNE = Micronucleated erythrocytes, Shannon = Shannon index; F.B.ratio = Firmicutes/Bacteroidetes ratio.