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Manon Ternois, Maxence Mougou, Emmanuel Flahaut, Audrey Dussutour. Slime molds response to carbon nanotubes exposure: from internalization to behavior. *Nanotoxicology*, In press, 10.1080/17435390.2021.1894615 . hal-03425756v1

**HAL Id: hal-03425756**

**<https://hal.science/hal-03425756v1>**

Submitted on 19 Mar 2021 (v1), last revised 11 Nov 2021 (v2)

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Ternois, Manon  and Mougou, Maxence  and Flahaut, Emmanuel  and Dussutour, Audrey *Slime molds response to carbon nanotubes exposure: from internalization to behavior.* (2021) *Nanotoxicology*. 1-16. ISSN 1743-5390

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# Slime molds response to carbon nanotubes exposure: from internalization to behavior

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## ABSTRACT

Carbon nanotubes (CNTs) offer attractive opportunities due to their physical, electrical, mechanical, optical, and thermal properties. They are used in a wide range of applications and are found in numerous consumer products. On the downside, their increasing presence in the environment poses potential threats to living organisms and ecosystems. The aim of this study was to evaluate the toxicity of double-walled carbon nanotubes (DWCNTs) on a new model system: the acellular slime mold *Physarum polycephalum*. Despite its ecological significance, its simplicity of organization, and its behavioral complexity, exposure of such organisms to nanoparticles has been poorly investigated. Slime molds were exposed to DWCNTs using three routes of exposure (topical, food, environment). We first demonstrated that DWCNTs were rapidly internalized by slime molds especially when DWCNTs were mixed with the food or spread out in the environment. Secondly, we showed that a 6-week exposure to DWCNTs did not lead to bioaccumulation nor did it lead to persistence in the slime molds when they entered a resting stage. Thirdly, we revealed that 2 days following exposure, DWCNTs were almost entirely excreted from the slime molds. Lastly, we uncovered that DWCNTs exposure altered the migration speed, the pseudopods formation, and the expansion rate of the slime molds. Our results extend our current knowledge of CNTs cytotoxicity and introduce *P. polycephalum* as an ideal organism for nanotoxicology.

## KEYWORDS

Slime mold; carbon nanotubes; ecotoxicology; behavior

## Introduction

Recent years have witnessed a growing interest in nanomaterials due to their specific electrical, optical, thermal, and magnetic properties that enhance the performance of the final product (Guinée et al. 2017). Nanomaterials may be of different nature including metals, oxides, or carbon-based. Carbon nanotubes (CNTs) were brought to the forefront in 1991 by Sumio Iijima and since then they have revolutionized several fields of research with their extraordinary properties and applications (De Volder et al. 2013). They are incorporated in a wide variety of consumer products (Guinée et al. 2017) and used in many fields of applications such as biomedical engineering, energy production and storage, nanoelectronics, mechanical engineering, and so forth (Gabaudan et al.

2019; Simon, Flahaut, and Golzio 2019). CNTs can be single-walled (SWCNTs), multi-walled (MWCNTs), or at the interface such as the double-walled carbon nanotubes (DWCNTs). DWCNTs offer several advantages in comparison to both SWCNTs and MWCNTs. They show enhanced properties thanks to an outer wall protecting the inner tube. Hence, DWCNTs can be functionalized without degrading their intrinsic mechanical properties which makes them the perfect compromise for many industrial applications (Flahaut et al. 2003; Simon, Flahaut, and Golzio 2019). They also offer higher stability under chemical, mechanical and thermal treatments and a longer lifetime than MWCNTs (Green and Hersam 2011). Lastly, they enhance electron transfer and thus provide better electrochemical behavior than SWCNTs (Pumera 2007).

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 Supplemental data for this article can be accessed [here](#).

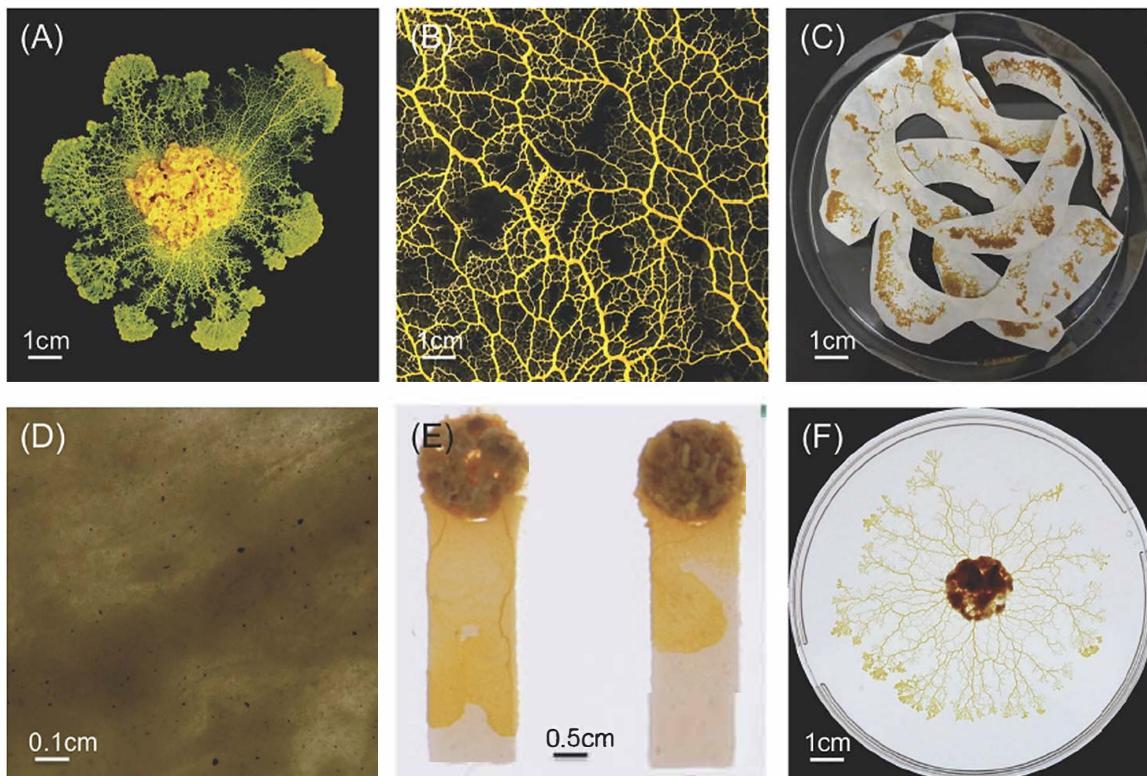
Despite the attractive properties of CNTs for numerous applications, some studies have raised concerns regarding the potential risks of these nanomaterials to the environment (Francis and Devasena 2018). CNTs are released in the environment throughout their life cycle by accidental release during production, transport, storage, uses of the final product (Mouchet et al. 2007), and as waste from sewage treatment or incineration plants (Petersen et al. 2011). The fate of CNTs and their impact depends on their transport, diffusion, degradation, bioaccumulation, transformation, and persistence. Hence, to evaluate the potential risks associated with CNTs, it is primordial to quantify their presence in each compartment of the environment. However, it is difficult to measure precisely the quantity of CNTs in environmental matrices, and models are often used to predict environmental concentrations (Sun et al. 2016). Soil and water are the main environmental reservoirs for CNTs (Liné, Larue, and Flahaut 2017). Using a dynamic probabilistic material flow model, predicted CNTs concentration was estimated to be *ca.*  $3.6 \times 10^{-4} \text{ mg} \cdot \text{L}^{-1}$  in surface waters and approximately  $3.5 \times 10^{-2} \text{ mg} \cdot \text{kg}^{-1}$  in natural and urban soil (Sun et al. 2016).

Single-cell organisms play an important role in nutrients cycling and a change in their activity could indicate a response to an environmental stressor (Chung et al. 2011). Several studies indicate that MWCNTs and SWCNTs present in soils repress enzymatic activities of bacteria, reduce microbial biomass (Chung et al. 2011; Ge et al. 2016; Jin et al. 2013), and alter soil microbial community composition (Jin et al. 2014; Kerfahi et al. 2015). It was also shown in soil bacteria that SWCNTs inhibit growth, reduce viability (Arias and Yang 2009), disrupt wall and membrane (Kang et al. 2007; Liu et al. 2009), induce stress response (Kang et al. 2008), and affect denitrification activity (Zheng et al. 2014). CNTs are hydrophobic, but stable CNTs suspension can persist for over one month in natural surface water when stabilized by natural organic matter (Hyung et al. 2007). Hence negative impacts of CNTs on aquatic single-cell organisms have also been observed. Among these single-cell organisms, ciliates and unicellular algae have been the most studied to assess CNTs' adverse effects (reviewed in Jackson et al. 2013). SWCNTs and MWCNTs can be

readily ingested by various ciliates (*Tetrahymena thermophila*, *Tetrahymena thermophila pyriformis*, *Pseudocohnilembus persalinus*, and *Stylonychia mytilus*) and can alter the motility, induce growth inhibition, cause cell aggregation, decrease viability, impair predation and alter the intracellular trafficking of vesicles (Chan et al. 2009; Ghafari et al. 2008; Guo et al. 2008; Mortimer et al. 2016; Weijie et al. 2020; Zhu et al. 2006). Similar to protozoa, both freshwater (*Chlorella vulgaris*, *Pseudokirchneriella subcapitata*) and marine algae (*Dunaliella tertiolecta*) are sensitive to CNTs and exhibit reduced growth and stress response when exposed (Long et al. 2012; Schwab et al. 2011; Wei et al. 2010).

Due to its comparably simple structure in relation to its behavioral complexity and due to the ease with which it can be cultivated and manipulated, the acellular slime mold *Physarum polycephalum* presents itself as an ideal model system to determine the cytotoxicity of CNTs in single cell organisms. *Physarum polycephalum* is a remarkable organism belonging to the Myxomycetes and the Amoebozoa. It is a phagotrophic organism commonly observed in association with decaying plant material in terrestrial ecosystems. *Physarum polycephalum*, as a major component of the detritus food chain, is essential in nutrient cycling. Bacteria and fungi are the primary natural food source of *P. polycephalum*, and these are phagocytized (Aldrich 2012). Food particles are first captured in flask-like invaginations of the membrane and then isolated in vacuoles which are released into the cytoplasm. *Physarum polycephalum* can also take up droplets of medium by pinocytosis (Aldrich 2012). The vegetative state of *P. polycephalum* is a large mobile polynucleated cell also called plasmodium. It can extend to up to hundreds of square centimeters (Figure 1(A)) and be severed into viable and structurally similar yet smaller plasmodia. Upon contact, these plasmodia can fuse with each other to form a unique plasmodium (Aldrich 2012). Lastly, a starving plasmodium can encapsulate and enter a dormant stage called sclerotium (Figure 1(C)).

Both *P. polycephalum*'s motion and behavior rely on a network of interconnected veins (Figure 1(B)). These veins contract and relax periodically, causing the cytoplasm to flow back and forth, a phenomenon called 'shuttle streaming' (Matsumoto, Takagi, and Nakagaki 2008). The veins are porous, allowing



**Figure 1.** Pictures of *P. polycephalum*. These pictures illustrate (A) a macroplasmidium; the vegetative phase of *P. polycephalum*, (B) a magnified vein network, (C) a sclerotium, the dormant stage of *P. polycephalum*, (D) DWNTs agglomerates internalized in a slime mold (E) two slime molds migrating on a bridge and (F) one slime mold exploring an agar gel in a petri dish.

respiratory gases, molecules, nuclei and organelles to be exchanged with the surrounding cytoplasm (Oettmeier, Lee, and Döbereiner 2018). This open network distributes the cytoplasm throughout the cell body and allows to maintain homeostasis. This network is also responsible for the plasmodium migration at a speed of up to 4 cm per hour, through the interplay of intracellular flow and rhythmic vein contractions (Alim et al. 2013; Matsumoto, Takagi, and Nakagaki 2008; Alim et al. 2013). These contractions produce a pressure gradient that pushes the cytoplasm toward the cell periphery where local cytoskeletal reorganization leads to the formation of fan-shaped leading fronts. These fronts extend and retract in synchrony with the shuttle streaming of the cytoplasm (Lewis et al. 2015; Matsumoto, Takagi, and Nakagaki 2008). The frequency and the amplitude of the contractions and by extension the shape, size, and motion of the plasmodium depend on the external cues encountered in the environment (Matsumoto, Ueda, and Kobatake 1986; Ridgway and Durham 1976; Miyake et al. 1994). It has been shown that slime molds respond to a variety of cues such as chemicals

(Aldrich 2012), light (Hato et al. 1976; Marwan 2001), temperature (Wolf, Niemuth, and Sauer 1997), humidity (Rakoczy 1973), etc. *P. polycephalum* exhibits a rich repertoire of complex behaviors which makes it a model organism to study problem-solving in unicellular systems (Adamatzky 2017; Oettmeier, Brix, and Döbereiner 2017; Reid et al. 2015; Smith-Ferguson and Beekman 2020; Vallverdú et al. 2018).

Although the importance of slime molds to the environment is well acknowledged, only a single study was found on the exposure of such organisms to carbon nanotubes (CNTs) (Gizzie, Mayne, and Adamatzky 2016). The objective of Gizzie, Mayne, and Adamatzky (2016) study was not to measure the impact of CNTs on *P. polycephalum* but to hybridize the organism with nanomaterials. In contrast, the main objective of our work was to measure the impact of CNTs exposure on *P. polycephalum* behavior. First, we verified that CNTs could be internalized into the cell using various routes of exposure. CNTs were either mixed with food, spread in the environment, or administered by topical application. Being a phagotrophic organism, we

expected the slime molds to readily internalize CNTs. Second, we evaluated if CNTs accumulated over time when cells were continuously exposed to CNTs for 6 weeks. Bioaccumulation of CNTs is a major issue, as it means that CNTs could move up along the food chain. Third, we measured the persistence of CNTs within the cell when cells were exposed to CNTs for 6 weeks. If the CNTs remain into the cell, they might be transferred to the next generation. Fourth, we estimated the excretion rate of CNTs when cells were exposed only once to CNTs. Last, we measured the effect of CNTs exposure on behavior including movement initiation and migration speed.

## Materials and methods

### *CNT synthesis and dispersion*

In this study, we used DWCNTs as they can be considered as a general model for carbon nanotubes, representing both thin and flexible SWCNTs and multiwall CNTs.

We used the CCVD (catalytic chemical vapor deposition) technique to synthesize the DWCNTs. Magnesia (MgO) was used as support material for *in situ* generated catalytic nanoparticles of cobalt and molybdenum (Flahaut et al. 2003). CCVD is a widely used method due to its capacity to produce CNTs with a high yield and possibly also a high purity with easily controllable reaction conditions to tune the desired type of carbon nanotubes (Yeoh et al. 2009). First, the catalytic powder was placed in a ceramic container which was positioned in a tube furnace. The synthesis was performed with dihydrogen (H<sub>2</sub>) and a methane (CH<sub>4</sub>) mixture flow. Methane served as the source of carbon by catalytic decomposition on the cobalt and molybdenum nanoparticles, leading to the formation of carbon nanotubes. The obtained black nanocomposite powder was composed of DWCNTs and the catalytic support, which was dissolved by addition of a concentrated aqueous solution of hydrochloric acid (3.5 mL of deionized water and 15 mL of 37% HCl for 1 g of powder) and left overnight. Then, a vacuum filtration was carried out using a nitrocellulose membrane (0.45 μm pore size). Successive filtrations were performed to remove the excess of HCl and

the solution of Mg, Co and Mo salts. Finally, the last washing was carried out using tap water.

As described in our earlier work (Flahaut et al. 2003), the DWCNTs samples contained 80% of DWCNTs, 10% SWCNTs, and 5% MWCNTs (triple-walled CNTs). The outer-diameter distribution of the CNTs was between 1 and 3 nm. The length was difficult, if not impossible, to assess because the nanotubes formed entangled bundles. Considering that the length of bundles may reach 100 μm, we estimated the length of individual CNTs to be between a few μm to tens of micrometers. Bundles typically had a maximum diameter of a few tens of nanometers. The elemental analysis indicated that our DWCNTs samples contained residual cobalt (*ca.* 3.5–4 wt.%) and molybdenum (*ca.* 1.0 wt.%) mainly in the form of nanoparticles tightly encapsulated in concentric carbon shells. The chemical analysis of carboxylic groups usually led to 0–0.5 mM/g (Bortolamiol et al. 2014), and the oxygen content was typically 3–4 wt.% (see Figure S1 for more details).

Due to their low water solubility, DWCNTs were suspended in a solution of carboxymethyl cellulose (CMC). CMC (carboxymethyl cellulose) is a cellulose derivative used as an additive in human food and is harmless for living organisms (E466). It is commonly used to provide stability to pristine CNTs suspensions (Bourdiol et al. 2013). First, 50 mg of CMC were weighed and placed in a vial with a few mL of tap water. Ultrasonic bath (*ca.* 1 minute) and microwave heating (a few seconds at 500 W in a classical microwave oven) were used to facilitate the dissolution. Then, the CMC was transferred in a 1 L bottle and tap water was added to obtain a 50 mg·L<sup>-1</sup> CMC solution. Pristine DWCNT wet powder (equivalent of 50 mg in the dry form) was suspended in a 1 L bottle with CMC solution (50 mg·L<sup>-1</sup>) to obtain a 50 mg·L<sup>-1</sup> DWCNT solution. The suspension was homogenized with a probe sonicator (Vibra Cell 75042, 20 kHz, 500 W, 125 mm-diameter probe) cooled in an ice bath (programming of 3 s ON and 3 s OFF alternately and an amplitude of 30%, for 30 min).

### *Species and rearing conditions*

Slime molds of *P. polycephalum* strain LU352 kindly provided by Professor Dr. Wolfgang Marwan (Max

Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany) were used for the experiments. The slime molds were reared on a 1% agar medium with rolled oat flakes (Quaker Oats Company<sup>®</sup>) in Petri dishes (140 mm Ø). They were kept in the dark in a thermoregulated chamber at a temperature of 25 degrees Celsius and a humidity of 80%. Slime mold were transferred every day on a new agar medium on which was spread a single layer of rolled oat flakes. All experiments were also carried out in a thermoregulated chamber and pictures were taken with a Canon 70D digital camera.

### ***Internalization of pristine DWCNTs by slime molds***

The aim of the first experiment was to find out how DWCNTs may be internalized within the slime mold. Circular slime molds (14 mm Ø) were placed in the center of a Petri dish (55 mm Ø) previously filled with 10 mL of 1 wt. % agar gel. 100 µL of DWCNT (50 mg·L<sup>-1</sup>) were either dropped on rolled oats (Food treatment,  $n=20$ ), directly on top of the slime mold (Topical treatment,  $n=20$ ), or in the slime mold environment (Environment treatment,  $n=22$ ). For the environment treatment, 2 drops of 50 µL were put on either side of the slime mold and spread out with a brush to cover the entire gel as evenly as possible. All the petri dishes were stored in a thermoregulated chamber (25 °C) for 16 h. The slime molds were then observed with a binocular microscope (Leica S9, 600 lp/mm,  $\pi = 0.4 \mu\text{m}$  equipped with a MC170HD camera) to quantify the DWCNTs internalization. The DWCNTs that we observed using the binocular microscope were much larger than bundles and correspond to agglomerates. 8 to 10 pictures of each slime mold were taken (depending on the size of the slime mold) to count the number of static DWCNTs agglomerates (black spots, [Figure 1](#)). For each picture, several zones of 0.5 mm<sup>2</sup> were delimited randomly and DWCNTs were counted in each zone. Data acquisition was performed using the software ImageJ. As we could not preclude that some static agglomerates were trapped in the extracellular slime layer surrounding the cell and not actually inside the slime mold, we also tracked circulating DWCNTs agglomerates within the organism. To this end, five veins per slime mold were video-monitored for 100s and the number of DWCNTs

agglomerates circulating within the veins were counted (Movie S1).

### ***Bioaccumulation of pristine DWCNTs into the slime mold***

Beyond internalizing DWCNTs, do the slime molds accumulate the DWCNTs? The aim of the second experiment was to investigate if chronic exposure to DWCNTs led to bioaccumulation. 20 slime molds were exposed daily to either a CMC solution (Control group  $n=20$ ) or DWCNTs suspension (CNT group  $n=20$ ), for 6 weeks. Slime molds were reared in Petri dishes (55 mm Ø) and 1 mL of DWCNTs suspension (50 mg·L<sup>-1</sup>) or CMC was spread on the food given daily to the slime molds (1 g of rolled oats). We used food as a route of exposure as we demonstrated in the first experiment that it was the most efficient method for DWCNTs internalization. For 9 slime molds chosen randomly, 12 pictures were taken the first week and the last week with a binocular microscope (Leica S9, 600 lp/mm,  $\pi = 0.4 \mu\text{m}$ ) and camera MC170 HD Leica. Static DWCNTs agglomerates were counted in four 0.5 mm<sup>2</sup> zones chosen randomly on each picture leading to a total of 432 counts for each treatment, using the software ImageJ, to find out if there was an increase in the number of CNT agglomerates over time (bioaccumulation). We also measured the area of the agglomerates using the software ImageJ to investigate a potential deagglomeration through time.

### ***Persistence of pristine DWCNTs into the slime mold***

When environmental conditions deteriorate, slime molds enter a dormant state called sclerotium until conditions become favorable again. During the sclerotization process, slime molds lose 50% of their total protein content together with 40% of their DNA and 65% of their RNA (Aldrich 2012). Slime mold can be easily re-activated from sclerotia if placed in favorable conditions. The aim of the third experiment was to examine if the DWCNTs internalized after a chronic exposure was excreted by the slime molds before entering the dormant stage. 240 samples of the slime molds used in the second experiment were successfully turned into a dormant state ( $n=20$  for each treatment every week for

6 weeks). The transition from plasmodia to sclerotia was initiated by placing the slime molds on a moist filter paper for 4 days to dry. Six months after entering the dormant state, 60 samples were chosen randomly and were reactivated to test the viability of the sclerotia (5 samples per treatment and per week). To this end, the sclerotia were soaked in water and placed in petri dishes (55 mm Ø) on agar gel (1%). Once revived, slime molds start to explore the agar gel, usually 24 h after the reactivation of the sclerotia.

Then, 20 sclerotia were re-activated one year later for each treatment (10 samples from week 1 and 10 samples from week 6). One sclerotium out of 20 could not be re-activated in each treatment. The day following the reactivation, 120 s videos of 3 veins per slime mold were recorded with a binocular microscope and camera MC170HD Leica to quantify circulating DWCNTs agglomerates. Ten pictures per slime mold were also taken. DWCNTs agglomerates were counted in 5 zones of 0.5 mm<sup>2</sup> chosen randomly on each picture leading to a total of 912 counts. As we used a sample from the slime molds tested in the bioaccumulation protocol, we were able to compare the number of static DWCNTs agglomerates in the slime mold before and after entering the dormant state.

#### ***Excretion of pristine DWCNTs from the slime mold***

The aim of the fourth experiment was to evaluate the excretion rate of DWCNTs by the slime mold. Slime molds were exposed once to DWCNTs using food as a route of exposure. Circular slime molds (10 mm Ø) were placed in a square Petri dish (120 mm wide) previously filled with 10 mL of 1 wt. % agar gel. Few oat flakes soaked in a DWCNTs suspension (volume 40 µL) were placed 5 mm away from the slime mold. We let the slime molds feed and explore for 24 h. Any food residues remaining after 24 h were discarded as they may provide a niche in which microorganisms can rapidly grow. As they explore, slime molds leave behind a thick mat of non-living, translucent, extracellular slime. To prevent internalization of DWCNTs that had been already excreted and left in the extracellular slime we replaced the agar gel after 48 h so that the slime mold never explored the same area twice. Slime molds were observed with a binocular

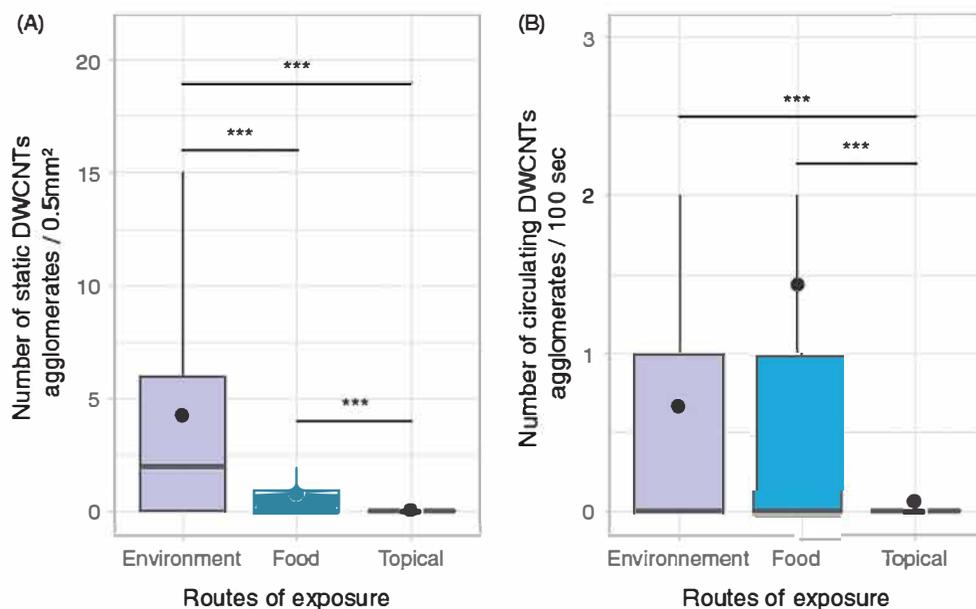
microscope and camera MC170HD Leica 24, 31, 48, 55, 72 and 79 h post-exposure. 5–10 pictures were taken for each slime mold sample and DWCNTs agglomerates were counted using ImageJ software. Five veins per slime mold were video-monitored for 100 s to quantify circulating DWCNTs. We also measured the area of the static agglomerates using the software ImageJ to investigate a potential deagglomeration through time.

#### ***Migration speed following a single exposure to DWCNTs***

The aim of the fifth experiment was to investigate how a single exposure to DWCNTs affects slime mold behavior. In this experiment, a circular slime mold (10 mm Ø) had to migrate on a bridge (35 mm long, 100 mm wide) made of 10% w/v powdered oat-agar (Figure 1(E)). Slime mold was first gently placed in contact with the bridge and then exposed to DWCNTs suspension (CNT group) or CMC only (Control group). Three routes of exposure were tested: a drop of DWCNTs suspension (20 µL) was either placed directly on the slime mold (Topical treatment), on few oat flakes resting on the slime mold (Food treatment), or on the bridge (Environment treatment). Hence, 80 slime mold were tested for each treatment and each group, leading to a total of 480 assays. After one night's incubation (ca. 15 h), the distance traveled (in mm) on each bridge was measured with a ruler.

#### ***First pseudopod and expansion rate following a chronic exposure to DWCNTs***

The aim of the sixth experiment was to investigate how chronic exposure to DWCNTs affects slime mold performance. Twenty slime molds were exposed chronically to DWCNTs or CMC control treatment (see bioaccumulation protocol) for 6 weeks. Each week, a circular sample of each slime mold (Ø 13 mm) was introduced in the center of a petri dish (Ø 55 mm) containing a layer of agar (1% in tap water) (Figure 1(F)). The dishes were placed in a thermoregulated chamber and pictures were taken every 5 min for 36 h with a digital Canon 70D camera. For each slime mold, we measured the time of appearance of the first pseudopod, which is an indicator of the slime mold healing process, as



**Figure 2.** Internalization of pristine DWCNTs by slime molds. Number of static (A) and circulating (B) DWCNTs agglomerates as a function of the route of exposure: environment, food or topical. A total of 2342 pictures and 310 videos were analyzed. The boxes extend from lower to upper quartile values and vertical black lines extend to most extreme data point. The horizontal black lines indicate median values and the dots represent the mean. \*\*\* $p < 0.001$ .

well as the expansion rate. Expansion rate was computed as the difference between the final surface (the area of the slime molds when it reached a distance of 15 mm from its original location) and the initial surface (the area of the slime mold when we started the experiment) divided by the time to reach the final surface. The pictures were analyzed using the software Image J.

### Statistical analysis

To assess the difference in the various parameters measured between the treatments, we used linear models, or linear mixed models, or generalized mixed model (function `lm` or `lmer` or `glmer`, Package `lme4`) in R (RStudio Version 1.2.1335). The models were fitted by specifying the fixed effects (explanatory variables) depending on the experiment: treatment (categorical predictor with two modalities: Control and CNT), the route of exposure (categorical predictor with three modalities: Food, Environment, and Topical), and/or the exposure duration (continuous predictor, from weeks 1 to 6). When needed, a random effect: the slime mold identity was also added to the model. The dependent variables that did not fit linear model requirements were transformed using the 'bestNormalize' function ('bestNormalize' package). The outcomes

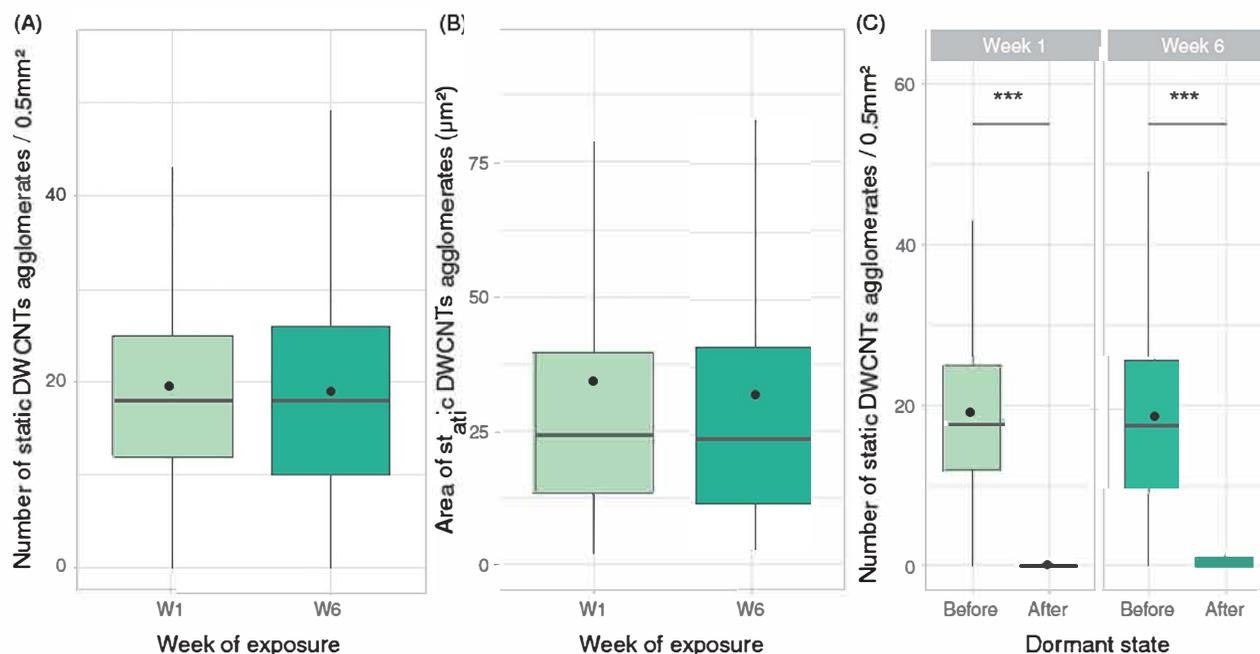
of all the models are presented in the supplementary information (Tables S1–S11).

### Results

Our main objective was to investigate the fate of DWCNTs in slime molds and their potential effect on behavior. *Physarum polycephalum* crawls over the soil surface where it can encounter various materials, including DWCNTs.

#### Internalization of pristine DWCNTs by slime molds

Our first objective was to identify the most efficient route of exposure to DWCNTs to maximize their internalization. The number of static DWCNTs agglomerates was considerably high when the slime mold encountered the DWCNTs while exploring their environment, five times higher than when DWCNTs were presented together with food ( $p < 0.001$ , Figure 2(A), Table S1) and fifty times higher than when DWCNTs were dropped directly on the slime mold ( $p < 0.001$ , Figure 2(A), Table S1). The number of circulating DWCNTs agglomerates was also significantly higher when food or the environment were the routes of exposure when compared to the topical application ( $p < 0.001$ ,  $p < 0.001$ , and  $p = 0.513$  for Food vs Topical,



**Figure 3.** Bioaccumulation and persistence of pristine DWCNTs into the slime mold. (A) Number of static DWCNTs agglomerates as a function of the number of weeks of exposure. ( $n = 416$  and  $n = 368$  pictures analyzed for week 1 and week 6 respectively); (B) Area of the static agglomerates as a function of the number of weeks of exposure  $n = 317$  and  $n = 297$  pictures analyzed for week 1 and week 6 respectively); (C) Number of static DWCNTs agglomerates before and after entering a dormant state ( $n = 864$  and  $n = 1687$  pictures analyzed before and after entering the dormant state respectively). The boxes extend from lower to upper quartile values and vertical black lines extend to most extreme data point. The horizontal black lines indicate median values and the dots represent the mean.  $***p < 0.001$ .

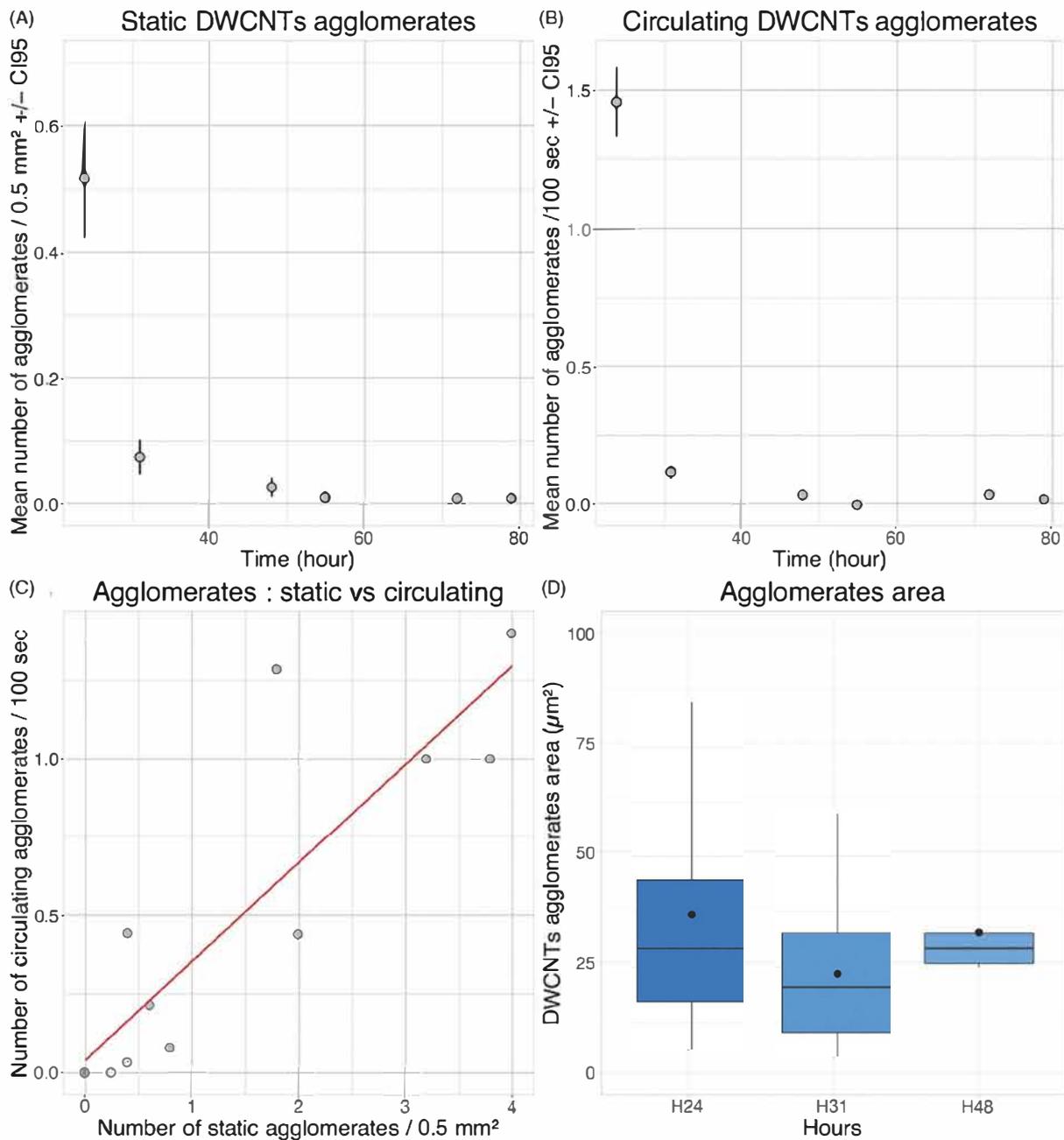
Environment vs Topical and Food vs Environment respectively, Figure 2(B), Table S2). When the slime molds were exposed to DWCNTs by topical application, black streaks were observed on the surface of the organism where the DWCNTs suspension was dropped. This clearly indicates that a significant proportion of DWCNTs was not internalized. These observations were later confirmed by picture and video analyses revealing that there were almost no static or circulating DWCNTs agglomerates in the slime mold.

#### **Bioaccumulation of pristine DWCNTs into the slime mold**

Here, the aim was to evaluate if DWCNTs accumulated within the slime mold when DWCNTs were added daily to the food for an extended period of time (6 weeks). Surprisingly despite daily exposure to DWCNTs, slime mold did not accumulate DWCNTs agglomerates over time. Both the number and the area of DWCNTs agglomerates remained constant between the first and the last week of exposure ( $p = 0.213$  and  $p = 0.639$ , Tables S3 and S4, Figure 3(A,B)).

#### **Persistence of pristine DWCNTs into the slime mold**

Even though DWCNTs did not accumulate over time, a certain quantity of DWCNTs were retained in the slime molds. Thus, we next examined if DWCNTs altered the induction of the dormant state and the reactivation of the slime mold and if they persisted throughout the dormant state. All the slime molds could enter a dormant state and be reactivated after 6 months regardless of the treatment. After one year, only 2 out of 40 slime molds could not be re-activated, one for each treatment. Hence, the DWCNTs did not alter the dormancy process. The number of static DWCNTs agglomerates dropped drastically after the dormant state ( $p < 0.001$ , Table S5, Figure 3(C)) regardless of the extent of exposure to DWCNTs ( $p = 0.272$ , Table S5, Figure 3(C)). We noticed that the filter papers holding the sclerotia were covered with black streaks which appeared to be DWCNTs agglomerates when observed with a binocular microscope. These observations provide evidence that the DWCNTs agglomerates internalized by slime molds were later excreted before entering the dormant state.

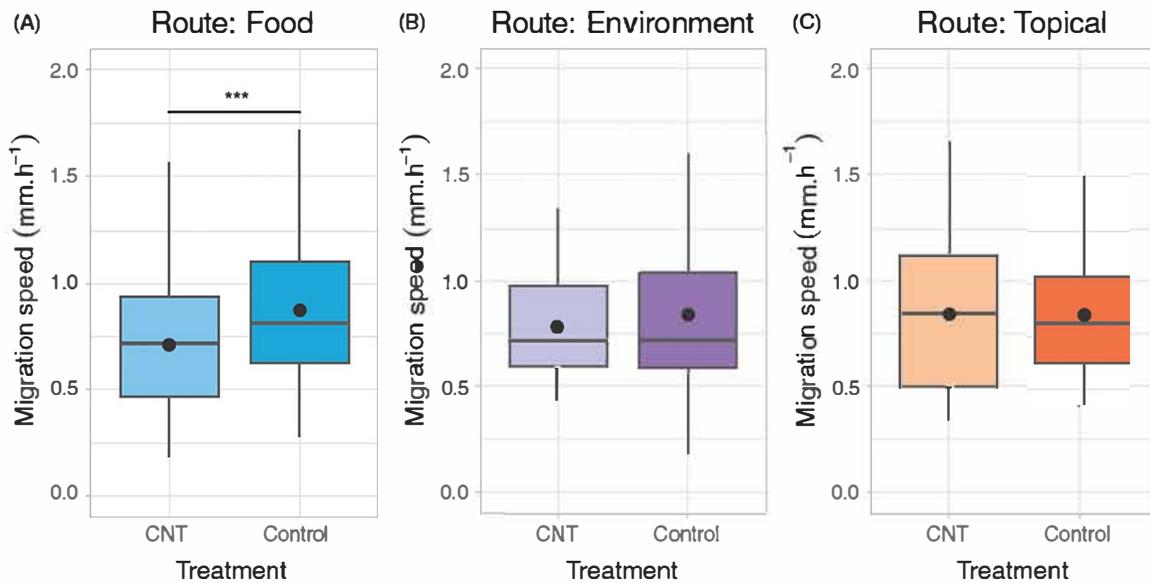


**Figure 4.** Excretion of pristine DWCNTs from the slime mold. Number of static (per 0.5 mm<sup>2</sup>) (A) and circulating (per 100s) (B) DWCNTs agglomerates observed for slime molds exposed once to DWCNTs.  $n = 1688$  pictures and  $n = 352$  videos analyzed. Error bars are Confidence intervals. (C) Correlation between the number of static aggregates and the number of circulating ones ( $n = 12$  plasmodia). The red line corresponds to the regression line ( $p < 0.001$ ,  $R^2 = 0.77$ ). (D) Area of the static agglomerates as a function of time ( $n = 280$  agglomerates in total). The boxes extend from lower to upper quartile values and vertical black lines extend to most extreme data point. The horizontal black lines indicate median values and the dots represent the mean.

#### Excretion of pristine DWCNTs from the slime mold

Knowing that slime molds did not accumulate DWCNTs and could excrete them in the environment, we examined the duration and dynamics of excretion of DWCNTs for slime molds exposed only once to DWCNTs. Both the number of static and circulating DWCNTs agglomerates dropped drastically

after 24 h (Time effect  $p < 0.001$ , Table S6 and S7, Figure 4(A,B)). 24 h after a single exposure to DWCNTs static and circulating agglomerates were spotted in half of the pictures and the videos, respectively. 79 h after the exposure only a few static DWCNTs agglomerates were spotted on 2 pictures out of 420 and a single occurrence of



**Figure 5.** Migration speed following a single exposure to DWCNTs as a function of the route of exposure: environment, food or topical. 80 slime molds per treatment and per route of exposure were observed. The boxes extend from lower to upper quartile values and vertical black lines extend to most extreme data point. The horizontal black lines indicate median values and the dots represent the mean. \*\*\* $p < 0.001$ .

circulating DWCNTs agglomerates was recorded out of 60 videos. As expected, the number of static agglomerates is highly correlated to the number of circulating ones ( $p < 0.001$ , Figure 4(C)). The area of the static agglomerates did not change through time (time effect  $p = 0.714$ , Table S8)

#### Migration speed following a single exposure to DWCNTs

Here, the objective was to investigate if a single exposure to DWCNTs altered the slime mold migration speed depending on the route of exposure. When the DWCNTs were associated with the food, the slime molds were significantly slower than the controls ( $p = 0.001$ , Table S9, Figure 5(A)). In contrast, we observed no difference in speed between the slime molds exposed to DWCNTs and the controls when they were exposed via the environment or via a topical application ( $p = 0.626$  and  $p = 0.685$ , respectively, Table S10 and S11, Figure 5(B,C)).

#### First pseudopod and expansion rate following chronic exposure to DWCNTs

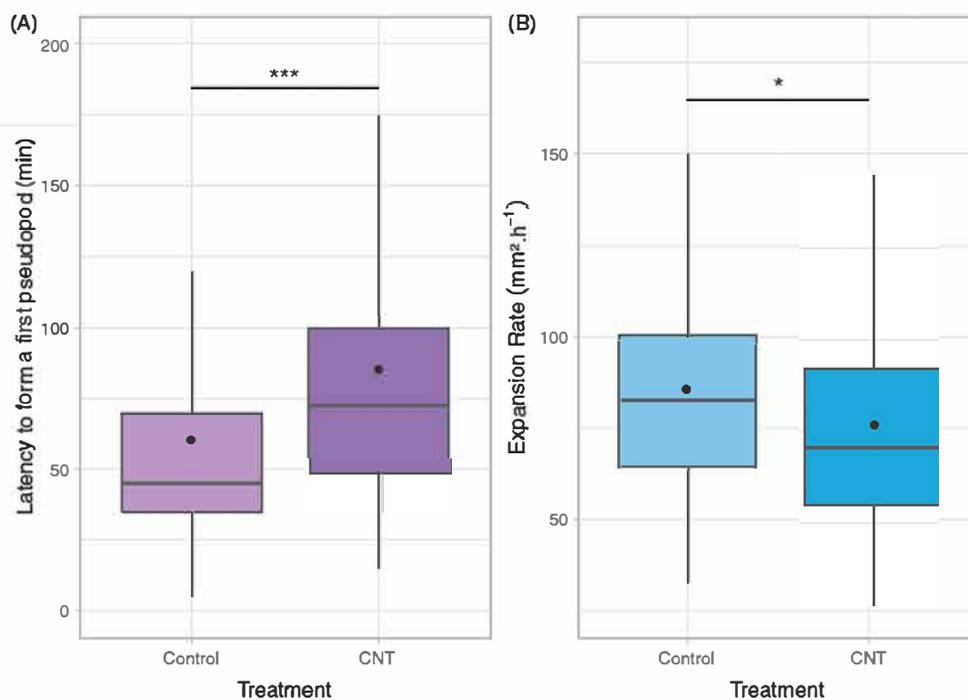
In the last experiment, the aim was to examine the effect of DWCNTs' daily exposure on performance. We used two proxies of performance: the appearance of the first pseudopod and the expansion rate.

The delay in the appearance of the first pseudopod was longer when the organisms were exposed to the DWCNTs when compared to the controls (treatment effect  $p < 0.001$ , Table S12, Figure 6(A), Figure S2). Similarly, the expansion rate was slower for the slime mold exposed to the DWCNTs when compared to the controls (treatment effect  $p = 0.026$ , Table S13, Figure 6(B), Figure S3).

#### Discussion

Taken together, our results indicate that slime molds can internalize DWCNTs and that DWCNTS exposure affects behavioral performances.

Deciphering the mechanisms responsible for CNTs' internalization into live cells is essential both from an ecological point of view but also to design CNT-based delivery systems. CNTs' toxicity or efficacy depends on their ability to cross cell boundaries and membranes. In slime molds, we demonstrated that as in other grazing protists (Ghafari et al. 2008) spreading out the CNTs in the environment or presenting them together with food were the most effective routes of exposure. As *P. polycephalum* continuously explores its environment in search of food, it phagocytoses microorganisms and continuously secretes a thick extracellular slime (Reid et al. 2012). Hence slime molds can either internalize CNTs through phagocytosis while



**Figure 6.** Behavior analyses. (A) Latency to form a first pseudopod following a chronic exposure to DWCNTs. (B) Expansion rate following a chronic exposure to DWCNTs. 40 slime molds were observed per treatment and per week for a total of 480 slime molds. The boxes extend from lower to upper quartile values and vertical black lines extend to most extreme data point. The horizontal black lines indicate median values and the dots represent the mean. \* $p < 0.05$ ; \*\*\* $p < 0.001$ .

feeding or trapped CNTs in their own mucus while crawling on the substrate. The topical application of CNTs was the least effective route of exposure. The presence of black streaks where the CNTs were deposited indicates that the CNTs were not all passively internalized through membrane pores. In slime molds, objects smaller than  $1\mu\text{m}$  can be endocytosed within vesicles (Gizzie, Mayne, and Adamatzky 2016; Mayne et al. 2011; Mayne and Adamatzky 2015). For instance, Mayne et al. (2011) showed that magnetite nanoparticles can be internalized by endocytosis in slime molds.

CNTs are persistent in the environment due to their low biodegradation, so the assessment of their potential for bioaccumulation is of major concern (Bjorkl, Tobias, and Petersen 2017). Despite a daily exposure to CNTs via food for 6 weeks, no bioaccumulation was observed within the cell of *P. polycephalum*. Hence, slime molds were internalizing and eliminating CNTs continuously for 6 weeks. Most living organisms, when exposed to CNTs, eliminate these nanoparticles quickly. For instance, mussels, zebrafish, oligochaete, lugworms, and earthworms exposed to CNTs for more than 2 weeks eliminate the nanoparticles within a couple of days (Cheng et al. 2009; Galloway et al. 2010;

Hanna, Miller, and Lenihan 2014; Li et al. 2013; Petersen, Huang, and Weber 2008). In the excretion experiment, we showed that there was a drop in the numbers of static and circulating CNTs agglomerates after 24 h, and the majority of CNTs were excreted from the cell at 48 h post-exposure. Therefore, the egestion of CNTs from the cell occurred rapidly which explains why only a few CNTs agglomerates persisted throughout the sclerotization process which lasts at least 72 h in average. We noticed significant black streaks on the filter paper where the slime had migrated before going into dormancy. These observations corroborate that slime molds egested most of the CNTs back in the environment before entering a dormant state. The egestion of CNTs raises the possibility that these CNTs might have been ingested again when the slime explored the filter paper upon awakening. We attempted to quantify the CNTs agglomerates excreted by the slime molds in the environment. We noticed that the number of agglomerates detected in the environment was very low (Figure S4). However, our counting methods were rather rough, as agglomerates smaller than  $5\mu\text{m}$  could not be detected with our binocular microscope.

CNTs have been shown to impact many microorganisms (Chen et al. 2019) and slime molds are no exception (Gizzie, Mayne, and Adamatzky 2016). In our experiment, we observed that both a single and chronic exposure to CNTs affected the slime mold behavior. We noticed that slime moved more slowly when exposed to CNTs while foraging (Figure 5(A)) or exploring (Figure 6). These differences could result from a defect in the motor or growing systems as movement and growth are intimately connected in slime molds. We can propose various processes that might explain our results based on *in vitro* and *in vivo* studies performed on various eukaryotic cell lines. First, direct contact with CNTs might have damaged the cell membrane and altered its function (Kang, Mauter, and Elimelech 2008). This could explain the delay in forming the first pseudopod (Figure 6(A)). Second, internalization of CNTs might have led to the production of reactive oxygen species which in turn might have altered the structure and function of the cells (Andersen et al. 2009; Chen et al. 2019; Müller et al. 2010; Pacurari et al. 2012; Singh et al. 2014; Srivastava et al. 2011). Last, once inside the cell, CNTs might have interacted with intracellular filaments: actin (Holt et al. 2010, 2012; Shams et al. 2014), DNA (Li, Peng, and Qu 2006; Sargent et al. 2012; Singh et al. 2014) and tubulin polymers (microtubules) (Li, Peng, and Qu 2006; Li et al. 2006; Pampaloni and Florin 2008) leading to migratory defects, DNA breakage and mitotic blockage. Interestingly, the deleterious effects produced by SWCNTs and MWCNT reported in the literature differ. SWCNTs interact mostly with DNA (Dong et al. 2015; García-Hevia et al. 2014; Li, Peng, and Qu 2006; Rodríguez-Fernandez et al. 2012; Sargent et al. 2012; Singh et al. 2014), while MWCNTs interfere with actin and microtubules (Dong et al. 2015; García-Hevia et al. 2014; Rodríguez-Fernandez et al. 2012). However, the reason for such differences is difficult to explain because authors have worked with CNTs exhibiting different morphologies (bundling, length) and may simply have focused on different endpoints. Movement in slime molds, as mentioned earlier, relies on a driving force generated by the actin and myosin filaments surrounding the veins. Any change in actin filaments induced by CNTs might have induced a reorganization of the cell cytoskeleton at a larger scale and altered cell

migration. Slime mold growth is associated with synchronous mitosis occurring every 8–10 h. Knowing that microtubules are responsible for DNA segregation during mitosis, any alteration of the microtubule cytoskeleton might have compromised cell growth.

## Conclusions

The interaction between living organisms and CNTs is a critical issue. Here we showed that CNTs are taken up by slime molds and could therefore move up the food chain. In addition, we demonstrated that the internalization of CNTs altered the behavior of slime molds and by extend could impede their adaptability to the environment. Our results extend our current knowledge on CNTs and their effects on cellular systems and introduce the slime mold *P. polycephalum* as a new model system to study CNTs toxicity. Slime molds offer multifaceted advantages. First, being giant cells, they allowed us to observe internalized CNTs agglomerates *in vivo* with little equipment. Second, being single-cell organisms, they enable us to measure the impact of CNTs at the cell level on multiple parameters: morphology, motility, and differentiation. Third, slime molds exhibit primitive forms of cognition (such as learning, memory, anticipation, and decision making) and thus provide an ideal model to measure the impact of CNTs on behavior (Vallverdú et al. 2018). Lastly, slime molds display many features of animal cells such as high molecular complexity and a large diversity of signaling molecules (Schaap et al. 2016). As such, they could constitute an interesting model to test CNTs application in drug delivery and medical imaging in future studies.

## Authors' contributions

M.T., A.D., and E.F. conceived and designed the experiments. E.F. carried out the chemical synthesis of the CNTs sample. M.T and M.M performed the experiments and collected the data. M.T and A.D. performed the statistical analyses. M.T and A.D. produced the figures. M.T., A.D., and E.F. wrote the manuscript. A.D. and E.F. secured funding. All authors gave final approval for publication.

## Disclosure statement

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

## Funding

M.T. was financially supported by a grant from the 'Agence Nationale de la recherche' (reference no. ANR-17-CE02-0019-01-SMARTCELL, Principal investigator: A.D.) and the University of Toulouse and the Région Occitanie/Université Paul Sabatier (reference no. ALDOCT001112, Principal investigator: A.D.). A.D. and E.F. were supported by the MITI 'Mission pour les initiatives transverses et interdisciplinaires' funded by the CNRS. (reference: NTCCELL – Principal investigator: A.D.).

## References

- Adamatzky, A. 2017. "Thirty Seven Things to Do with Live Slime Mould." In *Advances in Unconventional Computing*, 709–738. Amsterdam, The Netherlands: Springer.
- Aldrich, H. 2012. *Cell Biology of Physarum and Didymium V1: Organisms, Nucleus, and Cell Cycle*. Amsterdam, The Netherlands: Elsevier.
- Alim, K., G. Amselem, F. Peaudecerf, M. P. Brenner, and A. Pringle. 2013. "Random Network Peristalsis in *Physarum polycephalum* Organizes Fluid Flows across an Individual." *Proceedings of the National Academy of Sciences of the United States of America* 110 (33): 13306–13311. doi:10.1073/pnas.1305049110.
- Andersen, A. J., S. H. Hashemi, T. L. Andresen, A. C. Hunter, and S. M. Moghimi. 2009. "Complement: Alive and Kicking Nanomedicines." *Journal of Biomedical Nanotechnology* 5 (4): 364–372. doi:10.1166/jbn.2009.1045.
- Bortolamiol, T., P. Lukanov, A. M. Galibert, B. Soula, P. Lonchambon, L. Datas, and E. Flahaut. 2014. "Double-Walled Carbon Nanotubes: Quantitative Purification Assessment, Balance Between Purification and Degradation and Solution Filling as an Evidence of Opening." *Carbon* 78: 79–90. doi:10.1016/j.carbon.2014.06.051.
- Arias, L. R., and L. Yang. 2009. "Inactivation of Bacterial Pathogens by Carbon Nanotubes in Suspensions." *Langmuir: The ACS Journal of Surfaces and Colloids* 25 (5): 3003–3012. doi:10.1021/la802769m.
- Bjorkl, R., D. A. Tobias, and E. J. Petersen. 2017. "Increasing Evidence Indicates Low Bioaccumulation of Carbon Nanotubes." *Environmental Science. Nano* 4 (3): 747–766. doi:10.1039/C6EN00389C.
- Bourdiol, F., F. Mouchet, A. Perrault, I. Fourquaux, L. Datas, C. Gancet, J.-C. Boutonnet, E. Pinelli, L. Gauthier, and E. Flahaut. 2013. "Biocompatible Polymer-Assisted Dispersion of Multi Walled Carbon Nanotubes in Water, Application to the Investigation of Their Ecotoxicity Using *Xenopus laevis* Amphibian Larvae." *Carbon* 54: 175–191. doi:10.1016/j.carbon.2012.11.024.
- Chan, T. S. Y., F. Nasser, C. H. St-Denis, N. C. Bols, and S. Tang. 2009. "Interactions between Single-Walled Carbon Nanotubes (SWNT) and Ciliates: SWNT Interfere with Ciliate Ecological Functions and Ciliates Transport/Transform SWNT." *MRS Online Proceedings Library Archives* 3 (6): 347–351.
- Chen, M., Y. Sun, J. Liang, G. Zeng, Z. Li, L. Tang, Y. Zhu, D. Jiang, and B. Song. 2019. "Understanding the Influence of Carbon Nanomaterials on Microbial Communities." *Environment International* 126: 690–698. doi:10.1016/j.envint.2019.02.005.
- Cheng, J., C. M. Chan, L. M. Veca, W. L. Poon, P. K. Chan, L. Qu, Y. P. Sun, and S. H. Cheng. 2009. "Acute and Long-Term Effects after Single Loading of Functionalized Multi-Walled Carbon Nanotubes into Zebrafish (*Danio rerio*)." *Toxicology and Applied Pharmacology* 235 (2): 216–225. doi:10.1016/j.taap.2008.12.006.
- Chung, H., Y. Son, T. K. Yoon, S. Kim, and W. Kim. 2011. "The Effect of Multi-Walled Carbon Nanotubes on Soil Microbial Activity." *Ecotoxicology and Environmental Safety* 74 (4): 569–575. doi:10.1016/j.ecoenv.2011.01.004.
- De Volder, M. F. L., S. H. Tawfick, R. H. Baughman, and A. J. Hart. 2013. "Carbon Nanotubes: Present and Future Commercial Applications." *Science* 339 (6119): 535–539. doi:10.1126/science.1222453.
- Dong, C., R. Eldawud, L. M. Sargent, M. L. Kashon, D. Lowry, Y. Rojanasakul, and C. Z. Dinu. 2015. "Carbon Nanotube Uptake Changes the Biomechanical Properties of Human Lung Epithelial Cells in a Time-Dependent Manner." *Journal of Materials Chemistry B* 3 (19): 3983–3992. doi:10.1039/C5TB00179J.
- Flahaut, E., R. Bacsa, A. Peigney, and C. Laurent. 2003. "Gram-Scale CCVD Synthesis of Double-Walled Carbon Nanotubes." *Chemical Communications* 12: 1442–1443.
- Francis, A. P., and T. Devasena. 2018. "Toxicity of Carbon Nanotubes: A Review." In *Toxicology and Industrial Health* 34 (3): 200–210. doi:10.1177/0748233717747472.
- Gabaudan, V., J. Touja, D. Cot, E. Flahaut, L. Stievano, and L. Monconduit. 2019. "Double-Walled Carbon Nanotubes, a Performing Additive to Enhance Capacity Retention of Antimony Anode in Potassium-Ion Batteries." *Electrochemistry Communications* 105: 106493. doi:10.1016/j.elecom.2019.106493.
- Galloway, T., C. Lewis, I. Dolciotti, B. D. Johnston, J. Moger, and F. Regoli. 2010. "Sublethal Toxicity of Nano-Titanium Dioxide and Carbon Nanotubes in a Sediment Dwelling Marine Polychaete." *Environmental Pollution* 158 (5): 1748–1755. doi:10.1016/j.envpol.2009.11.013.
- García-Hevia, L., F. Fernández, C. Grávalos, A. García, J. C. Villegas, and M. L. Fanarraga. 2014. "Nanotube Interactions with Microtubules: Implications for Cancer Medicine." *Nanomedicine* 9 (10): 1581–1588. doi:10.2217/nnm.14.92.
- Ge, Y., J. H. Priester, M. Mortimer, C. H. Chang, Z. Ji, J. P. Schimmel, and P. A. Holden. 2016. "Long-Term Effects of

- Multiwalled Carbon Nanotubes and Graphene on Microbial Communities in Dry Soil." *Environmental Science & Technology* 50 (7): 3965–3974. doi:10.1021/acs.est.5b05620.
- Ghafari, P., C. H. St-Denis, M. E. Power, X. Jin, V. Tsou, H. S. Mandal, N. C. Bols, and X. Tang. 2008. "Impact of Carbon Nanotubes on the Ingestion and Digestion of Bacteria by Ciliated Protozoa." *Nature Nanotechnology* 3 (6): 347–351. doi:10.1038/nnano.2008.109.
- Gizzie, N., R. Mayne, and A. Adamatzky. 2016. "On Modulating the *Physarum polycephalum* Plasmodium's Electrical Resistance, Resting Membrane Potential and Capacitance by Application of Nanoparticles and Nanostructures." *Organic Electronics* 32: 267–273. doi:10.1016/j.orgel.2016.02.033.
- Green, A. A., and M. C. Hersam. 2011. "Properties and Application of Double-Walled Carbon Nanotubes Sorted by Outer-Wall Electronic Type." *ACS Nano* 5 (2): 1459–1467. doi:10.1021/nn103263b.
- Guinée, J. B., R. Heijungs, M. G. Vijver, and W. J. G. M. Peijnenburg. 2017. "Setting the stage for debating the roles of risk assessment and life-cycle assessment of engineered nanomaterials." *Nature Nanotechnology* 12 (8): 727–733. doi:10.1038/nnano.2017.135.
- Guo, J., X. Zhang, S. Zhang, Y. Zhu, and W. Li. 2008. "The Different Bio-Effects of Functionalized Multi-Walled Carbon Nanotubes on *Tetrahymena pyriformis*." *Current Nanoscience* 4 (3): 240–245. doi:10.2174/157341308785161145.
- Hanna, S. K., R. J. Miller, and H. S. Lenihan. 2014. "Deposition of Carbon Nanotubes by a Marine Suspension Feeder Revealed by Chemical and Isotopic Tracers." *Journal of Hazardous Materials* 279: 32–37. doi:10.1016/j.jhazmat.2014.06.052.
- Hato, M., T. Ueda, K. Kurihara, and Y. Kobatake. 1976. "Phototaxis in True Slime Mold *Physarum polycephalum*." *Cell Structure and Function* 1 (3): 269–278. doi:10.1247/csf.1.269.
- Holt, B. D., H. Shams, T. A. Horst, S. Basu, A. D. Rape, Y. Wang, G. K. Rohde, M. R. K. Mofrad, M. F. Islam, and K. N. Dahl. 2012. "Altered Cell Mechanics from the inside: Dispersed Single Wall Carbon Nanotubes Integrate with and Restructure Actin." *Journal of Functional Biomaterials* 3 (2): 398–417. doi:10.3390/jfb3020398.
- Holt, B. D., P. A. Short, A. D. Rape, Y. Wang, M. F. Islam, and K. N. Dahl. 2010. "Carbon Nanotubes Reorganize Actin Structures in Cells and Ex Vivo." *ACS Nano* 4 (8): 4872–4878. doi:10.1021/nn101151x.
- Hyung, H., J. D. Fortner, J. B. Hughes, and J. H. Kim. 2007. "Natural Organic Matter Stabilizes Carbon Nanotubes in the Aqueous Phase." *Environmental Science & Technology* 41 (1): 179–184. doi:10.1021/es061817g.
- Jackson, P., N. R. Jacobsen, A. Baun, R. Birkedal, D. Kühnel, K. A. Jensen, U. Vogel, and H. Wallin. 2013. "Bioaccumulation and Ecotoxicity of Carbon Nanotubes." *Chemistry Central Journal* 7 (1): 154. doi:10.1186/1752-153X-7-154.
- Jin, L., Y. Son, J. L. DeForest, Y. J. Kang, W. Kim, and H. Chung. 2014. "Single-Walled Carbon Nanotubes Alter Soil Microbial Community Composition." *Science of the Total Environment* 466: 533–538.
- Jin, L., Y. Son, T. K. Yoon, Y. J. Kang, W. Kim, and H. Chung. 2013. "High Concentrations of Single-Walled Carbon Nanotubes Lower Soil Enzyme Activity and Microbial Biomass." *Ecotoxicology and Environmental Safety* 88: 9–15. doi:10.1016/j.ecoenv.2012.10.031.
- Kang, S., M. Herzberg, D. F. Rodrigues, and M. Elimelech. 2008. "Antibacterial Effects of Carbon Nanotubes: Size Does Matter!." *Langmuir: The ACS Journal of Surfaces and Colloids* 24 (13): 6409–6413. doi:10.1021/la800951v.
- Kang, S., M. S. Mauter, and M. Elimelech. 2008. "Physicochemical Determinants of Multiwalled Carbon Nanotube Bacterial Cytotoxicity." *Environmental Science & Technology* 42 (19): 7528–7534. doi:10.1021/es8010173.
- Kang, S., M. Pinault, L. D. Pfefferle, and M. Elimelech. 2007. "Single-Walled Carbon Nanotubes Exhibit Strong Antimicrobial Activity." *Langmuir: The ACS Journal of Surfaces and Colloids* 23 (17): 8670–8673. doi:10.1021/la701067r.
- Kerfahi, D., B. M. Tripathi, D. Singh, H. Kim, and S. Lee. 2015. "Effects of Functionalized and Raw Multi-Walled Carbon Nanotubes on Soil Effects of Functionalized and Raw Multi-Walled Carbon Nanotubes on Soil Bacterial Community Composition." *PLoS One* 10 (3): e0123042.
- Lewis, O. L., S. Zhang, R. D. Guy, and J. C. Del Álamo. 2015. "Coordination of Contractility, Adhesion and Flow in Migrating *Physarum Amoebae*." *Journal of the Royal Society Interface* 12 (106): 20141359. doi:10.1098/rsif.2014.1359.
- Li, S., F. Irin, F. O. Atore, M. J. Green, and J. E. Cañas-Carrell. 2013. "Determination of Multi-Walled Carbon Nanotube Bioaccumulation in Earthworms Measured by a Microwave-Based Detection Technique." *Science of the Total Environment* 445–446: 9–13. doi:10.1016/j.scitotenv.2012.12.037.
- Li, X., Y. Peng, and X. Qu. 2006. "Carbon Nanotubes Selective Destabilization of Duplex and Triplex DNA and Inducing B-A transition in solution." *Nucleic Acids Research* 34 (13): 3670–3676. doi:10.1093/nar/gkl513.
- Li, X., Y. Peng, J. Ren, and X. Qu. 2006. "Carboxyl-Modified Single-Walled Carbon Nanotubes Selectively Induce Human Telomeric i-Motif Formation." *Proceedings of the National Academy of Sciences of the United States of America* 103 (52): 19658–19663. doi:10.1073/pnas.0607245103.
- Liné, C., C. Larue, and E. Flahaut. 2017. "Carbon Nanotubes: Impacts and Behaviour in the Terrestrial ecosystem - A Review. In *Carbon*." *Carbon* 123: 767–785. doi:10.1016/j.carbon.2017.07.089.
- Liu, S., L. Wei, L. Hao, N. Fang, M. W. Chang, R. Xu, Y. Yang, and Y. Chen. 2009. "Sharper and Faster "nano darts" kill more bacteria: A Study of Antibacterial Activity of Individually Dispersed Pristine Single-Walled Carbon

- Nanotube." *ACS Nano* 3 (12): 3891–3902. doi:10.1021/nn901252r.
- Long, Z., J. Ji, K. Yang, D. Lin, and F. Wu. 2012. "Systematic and Quantitative Investigation of the Mechanism of Carbon nanotubes' toxicity toward algae." *Environmental Science & Technology* 46 (15): 8458–8466. doi:10.1021/es301802g.
- Marwan, W. 2001. "Photomovement." In *Comprehensive Series in Photosciences*, Vol. 1, 561–587. Amsterdam, The Netherlands: Elsevier.
- Matsumoto, K., S. Takagi, and T. Nakagaki. 2008. "Locomotive Mechanism of Physarum Plasmodia Based on Spatiotemporal Analysis of Protoplasmic Streaming." *Biophysical Journal* 94 (7): 2492–2504. doi:10.1529/biophysj.107.113050.
- Matsumoto, K., T. Ueda, and Y. Kobatake. 1986. "Propagation of Phase Wave in Relation to Tactic Responses by the Plasmodium of *Physarum polycephalum*." *Journal of Theoretical Biology* 122 (3): 339–345. doi:10.1016/S0022-5193(86)80125-4.
- Mayne, R., and A. Adamatzky. 2015. "Toward Hybrid Nanostructure-Slime Mould Devices." *Nano Life* 05 (01): 1450007. doi:10.1142/S179398441450007X.
- Mayne, R., D. Patton, B. de Lacy Costello, A. Adamatzky, and R. C. Patton. 2011. "On the Internalisation, Intraplasmoidal Carriage and Excretion of Metallic Nanoparticles in the Slime Mould, *Physarum polycephalum*." *International Journal of Nanotechnology and Molecular Computation* 3 (3): 1–14.
- Mortimer, M., E. J. Petersen, B. A. Buchholz, E. Orias, and P. A. Holden. 2016. "Bioaccumulation of Multiwall Carbon Nanotubes in *Tetrahymena thermophila* by Direct Feeding or Trophic Transfer." *Environmental Science & Technology* 50 (16): 8876–8885. doi:10.1021/acs.est.6b01916.
- Mouchet, F., P. Landois, E. Flahaut, E. Pinelli, and L. Gauthier. 2007. "Assessment of the Potential in Vivo Ecotoxicity of Double-Walled Carbon Nanotubes (DWNTs) in Water, Using the Amphibian *Ambystoma Mexicanum*." *Nanotoxicology* 1 (2): 149–156. doi:10.1080/17435390701556080.
- Müller, L., M. Riediker, P. Wick, M. Mohr, P. Gehr, and B. Rothen-Rutishauser. 2010. "Oxidative Stress and Inflammation Response after Nanoparticle Exposure: Differences between Human Lung Cell Monocultures and an Advanced Three-Dimensional Model of the Human Epithelial Airways." *Journal of the Royal Society Interface* 7 (1): 27–40. doi:10.1098/rsif.2009.0161.focus.
- Oettmeier, C., K. Brix, and H. G. Döbereiner. 2017. "*Physarum polycephalum* - A New Take on a Classic Model System." *Journal of Physics D: Applied Physics* 50 (41): 413001. doi:10.1088/1361-6463/aa8699.
- Oettmeier, C., J. Lee, and H. G. Döbereiner. 2018. "Form Follows Function: Ultrastructure of Different Morphotypes of *Physarum polycephalum*." *Journal of Physics D: Applied Physics* 51 (13): 134006. doi:10.1088/1361-6463/aaab147.
- Pacurari, M., Y. Qian, W. Fu, D. Schwegler-Berry, M. Ding, V. Castranova, and N. L. Guo. 2012. "Cell Permeability, Migration, and Reactive Oxygen Species Induced by Multiwalled Carbon Nanotubes in Human Microvascular Endothelial Cells." *Journal of Toxicology and Environmental Health, Part A* 75 (2): 112–128. doi:10.1080/15287394.2011.615110.
- Pampaloni, F., and E.-L. Florin. 2008. "Microtubule Architecture: Inspiration for Novel Carbon Nanotube-Based Biomimetic Materials." *Trends in Biotechnology* 26 (6): 302–310. doi:10.1016/j.tibtech.2008.03.002.
- Petersen, E. J., Q. Huang, and W. J. Weber. 2008. "Ecological Uptake and Depuration of Carbon Nanotubes by *Lumbriculus Variegatus*." *Environmental Health Perspectives* 116 (4): 496–500. doi:10.1289/ehp.10883.
- Petersen, E. J., L. Zhang, N. T. Mattison, D. M. O'Carroll, A. J. Whelton, N. Uddin, T. Nguyen, et al. 2011. "Potential Release Pathways, Environmental Fate, and Ecological Risks of Carbon Nanotubes." *Environmental Science & Technology* 45 (23): 9837–9856. doi:10.1021/es201579y.
- Pumera, M. 2007. "Electrochemical Properties of Double Wall Carbon Nanotube Electrodes." *Nanoscale Research Letters* 2 (2): 87–93. doi:10.1007/s11671-006-9035-3.
- Rakoczy, L. 1973. "The Myxomycete *Physarum Nudum* as a Model Organism for Photobiological Studies." *Berichte Der Deutschen Botanischen Gesellschaft* 86 (1–4): 141–164.
- Reid, C. R., S. Garnier, M. Beekman, and T. Latty. 2015. "Information Integration and Multiattribute Decision Making in Non-Neuronal Organisms." *Animal Behaviour* 100: 44–50. doi:10.1016/j.anbehav.2014.11.010.
- Reid, C. R., T. Latty, A. Dussutour, and M. Beekman. 2012. "Slime Mold Uses an Externalized Spatial "memory" to navigate in complex environments." *Proceedings of the National Academy of Sciences of the United States of America* 109 (43): 17490–17494. doi:10.1073/pnas.1215037109.
- Ridgway, E. B., and A. C. H. Durham. 1976. "Oscillations of Calcium Ion Concentrations in *Physarum polycephalum*." *The Journal of Cell Biology* 69 (1): 223–226. doi:10.1083/jcb.69.1.223.
- Rodriguez-Fernandez, L., R. Valiente, J. Gonzalez, J. C. Villegas, and M. L. Fanarraga. 2012. "Multiwalled Carbon Nanotubes Display Microtubule Biomimetic Properties in Vivo, Enhancing Microtubule Assembly and Stabilization." *ACS Nano* 6 (8): 6614–6625. doi:10.1021/nn302222m.
- Sargent, L. M., A. F. Hubbs, S.-H. Young, M. L. Kashon, C. Z. Dinu, J. L. Salisbury, S. A. Benkovic, et al. 2012. "Single-Walled Carbon Nanotube-Induced Mitotic Disruption." *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 745 (1–2): 28–37. doi:10.1016/j.mrgentox.2011.11.017.
- Schaap, P., I. Barrantes, P. Minx, N. Sasaki, R. W. Anderson, M. Bénard, K. K. Biggar, et al. 2016. "The *Physarum polycephalum* Genome Reveals Extensive Use of Prokaryotic Two-Component and Metazoan-Type Tyrosine Kinase Signaling." *Genome Biology and Evolution* 8 (1): 109–125. doi:10.1093/gbe/ew237.

- Schwab, F., T. D. Bucheli, L. P. Lukhele, A. Magrez, B. Nowack, L. Sigg, and K. Knauer. 2011. "Are Carbon Nanotube Effects on Green Algae Caused by Shading and Agglomeration?" *Environmental Science & Technology* 45 (14): 6136–6144. doi:10.1021/es200506b.
- Shams, H., B. D. Holt, S. H. Mahboobi, Z. Jahed, M. F. Islam, K. N. Dahl, and M. R. K. Mofrad. 2014. "Actin Reorganization through Dynamic Interactions with Single-Wall Carbon Nanotubes." *ACS Nano* 8 (1): 188–197. doi:10.1021/nn402865e.
- Miyake, Y., H. Tada, M. Yano, and H. Shimizu. 1994. "Relationship between Intracellular Period Modulation and External Environment Change in Physarum Plasmodium." *Cell Structure and Function* 19 (6): 363–370. doi:10.1247/csf.19.363.
- Simon, J., E. Flahaut, and M. Golzio. 2019. "Overview of Carbon Nanotubes for Biomedical Applications." *Materials* 12 (4): 624. doi:10.3390/ma12040624.
- Singh, A. V., K. K. Mehta, K. Worley, J. S. Dordick, R. S. Kane, and L. Q. Wan. 2014. "Carbon Nanotube-Induced Loss of Multicellular Chirality on Micropatterned Substrate is Mediated by Oxidative Stress." *ACS Nano* 8 (3): 2196–2205. doi:10.1021/nn405253d.
- Smith-Ferguson, J., and M. Beekman. 2020. "Who Needs a Brain? Slime Moulds, Behavioural Ecology and Minimal Cognition." *Adaptive Behavior* 28 (6): 465–478. doi:10.1177/1059712319826537.
- Srivastava, R. K., A. B. Pant, M. P. Kashyap, V. Kumar, M. Lohani, L. Jonas, and Q. Rahman. 2011. "Multi-Walled Carbon Nanotubes Induce Oxidative Stress and Apoptosis in Human Lung Cancer Cell line-A549." *Nanotoxicology* 5 (2): 195–207. doi:10.3109/17435390.2010.503944.
- Sun, T. Y., N. A. Bornhöft, K. Hungerbühler, and B. Nowack. 2016. "Dynamic Probabilistic Modeling of Environmental Emissions of Engineered Nanomaterials." *Environmental Science & Technology* 50 (9): 4701–4711. doi:10.1021/acs.est.5b05828.
- Vallverdú, J., O. Castro, R. Mayne, M. Talanov, M. Levin, F. Baluška, Y. Gunji, A. Dussutour, H. Zenil, and A. Adamatzky. 2018. "Slime Mould: The Fundamental Mechanisms of Biological Cognition." *Bio Systems* 165: 57–70. doi:10.1016/j.biosystems.2017.12.011.
- Wei, L., M. Thakkar, Y. Chen, S. A. Ntim, S. Mitra, and X. Zhang. 2010. "Cytotoxicity Effects of Water Dispersible Oxidized Multiwalled Carbon Nanotubes on Marine Alga, *Dunaliella tertiolecta*." *Aquatic Toxicology* 100 (2): 194–201. doi:10.1016/j.aquatox.2010.07.001.
- Weijie, M., W. Chongnv, P. Xuming, J. Weixin, W. Yuhang, and S. Benhui. 2020. "TiO<sub>2</sub> Nanoparticles and Multi-Walled Carbon Nanotubes Monitoring and Bioremediation Potential Using Ciliates *Pseudocohnilembus persalinus*." *Ecotoxicology and Environmental Safety* 187: 109825. doi:10.1016/j.ecoenv.2019.109825.
- Wolf, R., J. Niemuth, and H. Sauer. 1997. "Thermotaxis and Protoplasmic Oscillations in Physarum Plasmodia Analysed in a Novel Device Generating Stable Linear Temperature Gradients." *Protoplasma* 197 (1–2): 121–131. doi:10.1007/BF01279890.
- Yeoh, W., K. Lee, S. Chai, K. Lee, and A. R. Mohamed. 2009. "Synthesis of High Purity Multi-Walled Carbon Nanotubes over Co-Mo/MgO Catalyst by the Catalytic Chemical Vapor Deposition of Methane." *New Carbon Materials* 24 (2): 119–123. doi:10.1016/S1872-5805(08)60041-4.
- Zheng, X., Y. Su, Y. Chen, R. Wan, M. Li, Y. Wei, and H. Huang. 2014. "Carboxyl-Modified Single-Walled Carbon Nanotubes Negatively Affect Bacterial Growth and Denitrification Activity." *Scientific Reports* 4: 5653. doi:10.1038/srep05653.
- Zhu, Y., Q. Zhao, Y. Li, X. Cai, and W. Li. 2006. "The Interaction and Toxicity of Multi-Walled Carbon Nanotubes With *Stylonychia mytilus*." *Journal of Nanoscience and Nanotechnology* 6 (5): 1357–1364. doi:10.1166/jnn.2006.194.

# Slime moulds response to carbon nanotubes exposure: from internalisation to behaviour (supplementary materials)

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<b>Internalisation: Number of static agglomerates</b>					
<i>Predictors</i>	<i>Incidence Rate Ratios</i>	<i>CI</i>	<i>Z value</i>	<i>p</i>	<i>df</i>
Intercept	3.06	1.69 – 5.56	3.68	<b>&lt;0.001</b>	2338.00
[Environment] vs [Food]	0.17	0.08 – 0.38	-4.42	<b>&lt;0.001</b>	2338.00
[Environment] vs [Topical]	0.01	0.01 – 0.03	-10.12	<b>&lt;0.001</b>	2338.00
<b>Random Effects</b>					
$\sigma^2$	1.46				
$\tau_{00}$ Replicate	1.10				
ICC	0.43				
N <sub>Replicate</sub>	48				
Observations	2342				
Marginal R <sup>2</sup> / Conditional R <sup>2</sup>	0.543 / 0.740				

<b>Internalisation: Number of static agglomerates</b>					
<b>Pairwise comparisons</b>					
<i>Predictors</i>	<i>Estimate</i>	<i>SE</i>	<i>z.ratio</i>	<i>p</i>	<i>df</i>
[Environment] vs [Food]	1.76	0.397	4.42	<b>&lt;0.001</b>	2338.00
[Environment] vs [Topical]	4.36	0.430	10.12	<b>&lt;0.001</b>	2338.00
[Food] vs [Topical]	2.60	0.395	-6.58	<b>&lt;0.001</b>	2338.00

**Table S1: Statistics associated to Figure 2 (A).**

To assess the difference in internalization of DWCNTs between the three routes of exposure; we used a generalized linear mixed model (function glmer, error family: Poisson, Package lme4). The model was fitted by specifying the fixed effect: treatment (categorical predictor, routes of exposure) and the random effect: Replicate (slime mould identity). Pairwise comparisons were conducted using the package emmeans (method= pairwise).

<b>Internalisation: Number of circulating agglomerates</b>					
<i>Predictors</i>	<i>Incidence Rate Ratios</i>	<i>CI</i>	<i>Z value</i>	<i>p</i>	<i>df</i>
Intercept	0.43	0.25 – 0.75	-2.98	<b>0.003</b>	306.00
[Environment] vs [Food]	1.53	0.72 – 3.27	1.10	0.271	306.00
[Environment] vs [Topical]	0.10	0.03 – 0.29	-4.25	<b>&lt;0.001</b>	306.00
<b>Random Effects</b>					
$\sigma^2$	1.56				
$\tau_{00}$ Replicate	1.14				
ICC	0.42				
$N_{\text{Replicate}}$	62				
Observations	310				
Marginal $R^2$ / Conditional $R^2$	0.345 / 0.622				

<b>Internalisation: Number of circulating agglomerates</b>					
<b>Pairwise comparisons</b>					
<i>Predictors</i>	<i>Estimate</i>	<i>SE</i>	<i>Z.ratio</i>	<i>p</i>	<i>df</i>
[Environment] vs [Food]	-0.43	0.387	-1.10	0.513	2338.00
[Environment] vs [Topical]	2.32	0.546	4.24	<b>&lt;0.001</b>	2338.00
[Food] vs [Topical]	2.75	0.547	5.02	<b>&lt;0.001</b>	2338.00

**Table S2: Statistics associated to Figure 2 (B).**

To assess the difference in internalization of DWCNTs between the three routes of exposure; we used a generalized linear mixed model (function glmer, error family: Poisson, Package lme4). The model was fitted by specifying the fixed effect: treatment (categorical predictor, routes of exposure) and the random effect: Replicate (slime mould). Pairwise comparisons were conducted using the package emmeans (method= pairwise).

<b>Accumulation: Number of static agglomerates</b>					
<i>Predictors</i>	<i>Incidence Rate Ratios</i>	<i>CI</i>	<i>Z value</i>	<i>p</i>	<i>df</i>
Intercept	19.01	18.01 – 20.07	106.82	<b>&lt;0.001</b>	781.00
[Time] (Week)	0.98	0.95 – 1.01	-1.25	0.213	781.00
<b>Random Effects</b>					
$\sigma^2$	0.05				
$\tau_{00}$ Replicate	0.01				
ICC	0.10				
N <sub>Replicate</sub>	9				
Observations	784				
Marginal R <sup>2</sup> / Conditional R <sup>2</sup>	0.002 / 0.101				

**Table S3: Statistics associated to Figure 3 (A).**

To assess accumulation of DWCNTs throughout the weeks; we used a generalized linear mixed model (function `glmer`, error family: Poisson, Package `lme4`). The model was fitted by specifying the fixed effect: Time (categorical predictor: Week1 vs Week 6) and the random effect: Replicate (slime mould identity).

<b>Area</b>					
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>Statistic</i>	<i>p</i>	<i>df</i>
Intercept	-0.01	-0.21 – 0.19	-0.10	0.920	606.00
[Week]	-0.04	-0.20 – 0.12	-0.47	0.639	606.00
<b>Random Effects</b>					
$\sigma^2$	0.95				
$\tau_{00}$ Replicate	0.06				
ICC	0.06				
N <sub>Replicate</sub>	9				
Observations	610				
Marginal R <sup>2</sup> / Conditional R <sup>2</sup>	0.000 / 0.063				

**Table S4: Statistics associated to Figure 3 (B).**

To assess the difference in agglomerates area throughout the weeks; we used a linear model (function `lmer`, Package `lme4`). We used linear mixed model (function `lmer`, error family: Gaussian, Package `lme4`). The model was fitted by specifying the fixed effects: week (continuous predictor, week 1 to week 6) and the random effect: Replicate (slime mould identity). The dependent variable (area) was normalized using the function `bestNormalize` (Package `bestNormalize`).

<b>Persistence: Number of static agglomerates</b>					
<i>Predictors</i>	<i>Incidence Rate Ratios</i>	<i>CI</i>	<i>Statistic</i>	<i>p</i>	<i>df</i>
Intercept	0.40	0.31 – 0.50	-7.92	<b>&lt;0.001</b>	2507.00
[Time] (after/before dormancy)	47.83	42.85 – 53.39	68.95	<b>&lt;0.001</b>	2507.00
[Week]	1.02	0.99 – 1.05	1.10	0.272	2507.00
<b>Random Effects</b>					
$\sigma^2$	0.48				
$\tau_{00}$ Replicate	0.24				
ICC	0.33				
N <sub>Replicate</sub>	20				
Observations	2511				
Marginal R <sup>2</sup> / Conditional R <sup>2</sup>	0.817 / 0.878				

**Table S5: Statistics associated to Figure 3 (C).**

To assess persistence of DWCNTs after the dormancy period; we used a generalized linear mixed model (function `glmer`, error family: Poisson, Package `lme4`). The model was fitted by specifying the fixed effects: Time (categorical predictor: After vs Before dormancy), Week (categorical factor: Week 1 vs Week 6) and the random effect: Replicate (slime mould identity).

<b>Excretion: Number of static agglomerates</b>					
<i>Predictors</i>	<i>Incidence Rate Ratios</i>	<i>CI</i>	<i>Z value</i>	<i>p</i>	<i>df</i>
Intercept	0.32	0.05 – 1.99	-1.22	0.223	1625.00
[Time] (Hours)	0.93	0.89 – 0.96	-3.92	<b>&lt;0.001</b>	1625.00
<b>Random Effects</b>					
$\sigma^2$	5.62				
$\tau_{00}$ Replicate	4.52				
ICC	0.45				
N <sub>Replicate</sub>	72				
Observations	1628				
Marginal R <sup>2</sup> / Conditional R <sup>2</sup>	0.177 / 0.544				

**Table S6: Statistics associated to Figure 4 (A).**

To assess excretion of static DWCNTs agglomerates throughout time; we used a generalized linear mixed model (function `glmer`, error family: Poisson, Package `lme4`). The model was fitted by specifying the fixed effect: Time (continuous predictor) and the random effect: Replicate (slime mould identity).

<b>Excretion: Number of circulating agglomerates</b>					
<i>Predictors</i>	<i>Incidence Rate Ratios</i>	<i>CI</i>	<i>Z value</i>	<i>p</i>	<i>df</i>
Intercept	2.76	0.54 – 14.23	1.21	0.225	349.00
[Time] (Hours)	0.91	0.88 – 0.95	-4.74	<b>&lt;0.001</b>	349.00
<b>Random Effects</b>					
$\sigma^2$	4.72				
$\tau_{00}$ Replicate	2.40				
ICC	0.34				
N <sub>Replicate</sub>	72				
Observations	352				
Marginal R <sup>2</sup> / Conditional R <sup>2</sup>	0.329 / 0.555				

**Table S7: Statistics associated to Figure 4 (B).**

To assess excretion of circulating DWCNTs agglomerates throughout time; we used a generalized linear mixed model (function `glmer`, error family: Poisson, Package `lme4`). The model was fitted by specifying the fixed effect: Time (continuous predictor) and the random effect: Replicate (slime mould identity).

<b>Area</b>					
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>Statistic</i>	<i>p</i>	<i>df</i>
Intercept	0.13	-0.72 – 0.99	0.30	0.760	275.00
[Week]	-0.01	-0.04 – 0.02	-0.37	0.714	275.00
<b>Random Effects</b>					
$\sigma^2$	0.94				
$\tau_{00}$ Replicate	0.07				
ICC	0.07				
N <sub>Replicate</sub>	17				
Observations	279				
Marginal R <sup>2</sup> / Conditional R <sup>2</sup>	0.001 / 0.071				

**Table S8: Statistics associated to Figure 4 (D).**

To assess the difference in agglomerates area through time; we used a linear model (function `lmer`, Package `lme4`). We used linear mixed model (function `lmer`, error family: Gaussian, Package `lme4`). The model was fitted by specifying the fixed effects: week (continuous predictor, week 1 to week 6) and the random effect: Replicate (slime mould identity). The dependent variable (area) was normalized using the function `bestNormalize` (Package `bestNormalize`).

<b>Migration speed - Route of Exposure: Food</b>					
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>Statistic</i>	<i>p</i>	<i>df</i>
Intercept	0.25	0.03 – 0.46	2.29	<b>0.024</b>	158.00
[Treatment]	-0.50	-0.80 – -0.19	-3.23	<b>0.001</b>	158.00
Observations	160				
R <sup>2</sup> / R <sup>2</sup> adjusted	0.062 / 0.056				

**Table S9: Statistics associated to Figure 5 (A).**

To assess the difference in migration speed between the two treatments when the route of exposure was food; we used a linear model (function `lm`, Package `lme4`). The model was fitted by specifying the fixed effect: treatment (categorical predictor, NTC vs Control). The dependent variable (migration speed) was normalized using the function `bestNormalize` (Package `bestNormalize`).

<b>Migration speed - Route of Exposure: Environment</b>					
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>Statistic</i>	<i>p</i>	<i>df</i>
Intercept	0.04	-0.18 – 0.26	0.35	0.726	158.00
[NTC vs Control]	-0.08	-0.39 – 0.23	-0.49	0.626	158.00
Observations	160				
R <sup>2</sup> / R <sup>2</sup> adjusted	0.002 / -0.005				

**Table S10: Statistics associated to Figure 5 (B).**

To assess the difference in migration speed between the two treatments when the route of exposure was Environment; we used a linear model (function `lm`, Package `lme4`). The model was fitted by specifying the fixed effect: treatment (categorical predictor, NTC vs Control). The dependent variable (migration speed) was normalized using the function `bestNormalize` (Package `bestNormalize`).

<b>Migration speed - Route of Exposure: Topical</b>					
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>Statistic</i>	<i>p</i>	<i>df</i>
Intercept	0.03	-0.19 – 0.25	0.29	0.774	158.00
[NTC vs Control]	-0.06	-0.38 – 0.25	-0.41	0.685	158.00
Observations	160				
R <sup>2</sup> / R <sup>2</sup> adjusted	0.001 / -0.005				

**Table S11: Statistics associated to Figure 5 (C).**

To assess the difference in migration speed between the two treatments when the route of exposure was Topical; we used a linear model (function `lm`, Package `lme4`). The model was fitted by specifying the fixed effect: treatment (categorical predictor, NTC vs Control). The dependent variable (migration speed) was normalized using the function `bestNormalize` (Package `bestNormalize`).

<b>Latency first pseudopod</b>					
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>Statistic</i>	<i>p</i>	<i>df</i>
Intercept	0.04	-0.22 – 0.31	0.32	0.747	474.00
[Treatment: NTC vs Control]	1.08	0.70 – 1.46	5.58	<b>&lt;0.001</b>	474.00
[Week]	-0.10	-0.16 – -0.03	-2.82	<b>0.005</b>	474.00
[Treatment*Week]	-0.14	-0.24 – -0.05	-2.98	<b>0.003</b>	474.00
<b>Random Effects</b>					
$\sigma^2$	0.80				
$\tau_{00}$ Replicate	0.03				
ICC	0.03				
N <sub>Replicate</sub>	40				
Observations	480				
Marginal R <sup>2</sup> / Conditional R <sup>2</sup>	0.179 / 0.206				

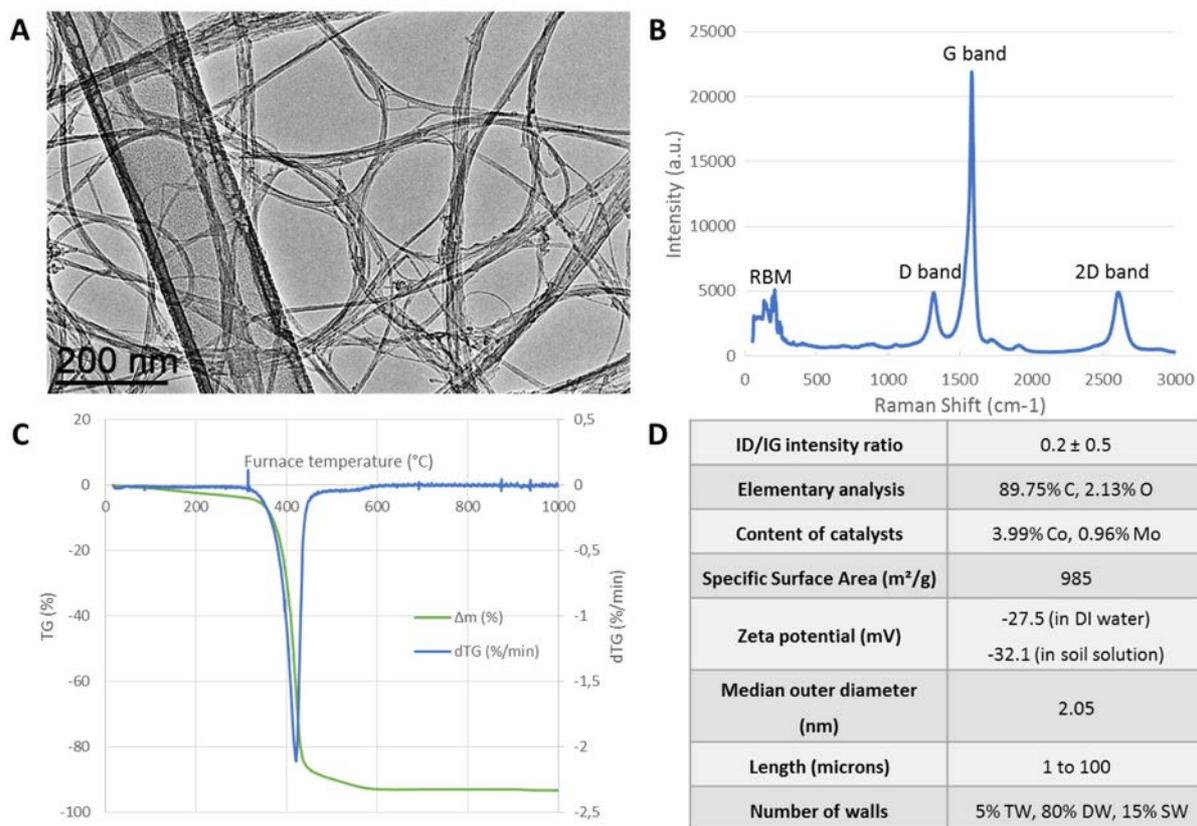
**Table S12: Statistics associated to Figure 6 (A).**

To assess the difference in latency to the first pseudopod between the two treatments; we used a linear mixed model (function `lmer`, error family: Gaussian, Package `lme4`). The model was fitted by specifying the fixed effects: treatment (categorical predictor, NTC vs Control), week (continuous predictor, week 1 to week 6) and the random effect: Replicate (slime mould identity). The dependent variable (migration speed) was normalized using the function `bestNormalize` (Package `bestNormalize`).

<i>Predictors</i>	<i>Estimates</i>	<b>Expansion rate</b>			<i>df</i>
		<i>CI</i>	<i>Statistic</i>	<i>p</i>	
Intercept	-0.40	-0.67 – -0.12	-2.83	<b>0.005</b>	474.00
[Treatment: NTC vs Control]	-0.44	-0.83 – -0.05	-2.22	<b>0.026</b>	474.00
[Week]	0.16	0.09 – 0.23	4.63	<b>&lt;0.001</b>	474.00
[Treatment*Week]	0.03	-0.07 – 0.12	0.52	0.601	474.00
<b>Random Effects</b>					
$\sigma^2$	0.87				
$\tau_{00}$ Replicate	0.02				
ICC	0.02				
N <sub>Replicate</sub>	40				
Observations	480				
Marginal R <sup>2</sup> / Conditional R <sup>2</sup>	0.121 / 0.137				

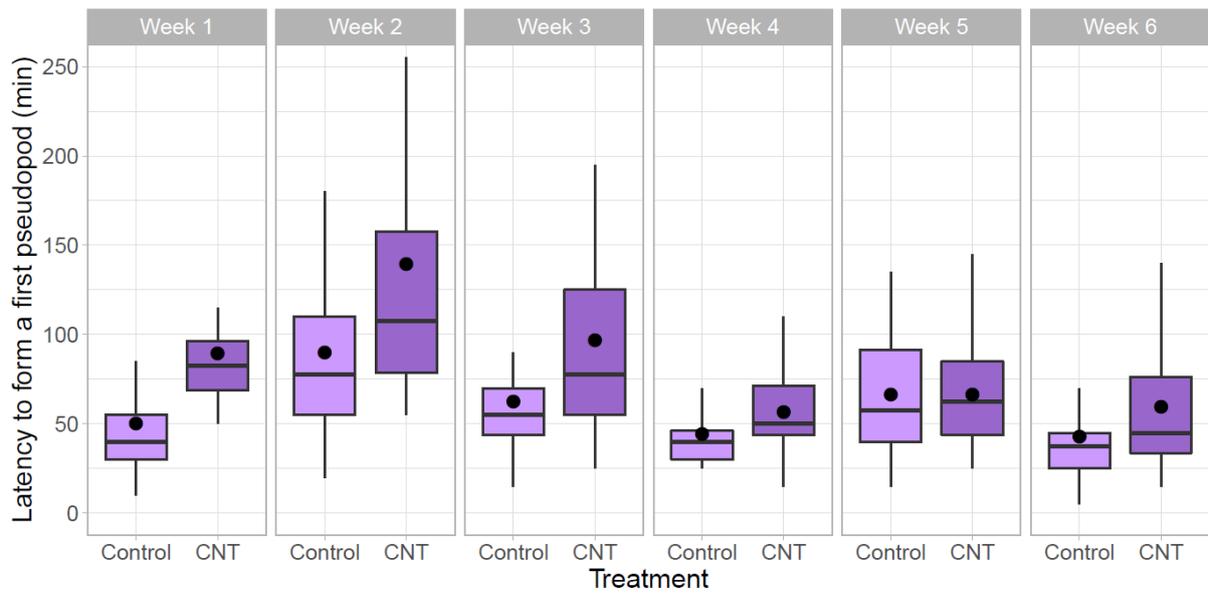
**Table S13: Statistics associated to Figure 6 (B).**

To assess the difference in expansion rate between the two treatments; we used linear mixed model (function `lmer`, error family: Gaussian, Package `lme4`). The model was fitted by specifying the fixed effects: treatment (categorical predictor, NTC vs Control), week (continuous predictor, week 1 to week 6) and the random effect: Replicate (slime mould identity). The dependent variable (expansion rate) was normalized using the function `bestNormalize` (Package `bestNormalize`).



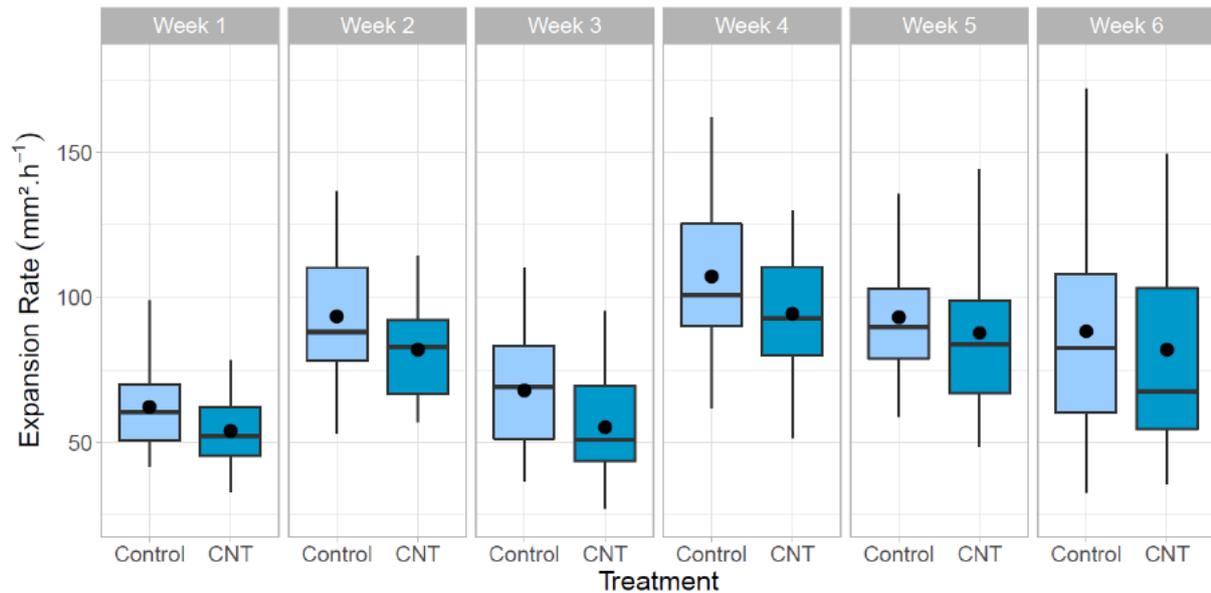
**Figure S1: Figure associated to the methods section.**

**CNT characterization** (A) TEM image of the DWCNTs. (B) CNT powder Raman scattering spectrum obtained using a 633 nm wavelength laser. (C) The weight loss profile obtained from TGA analysis in air atmosphere (1°/min). (D) Table summarizing the physicochemical characteristics (TW = triple walled, DW = double walled, SW = single walled).



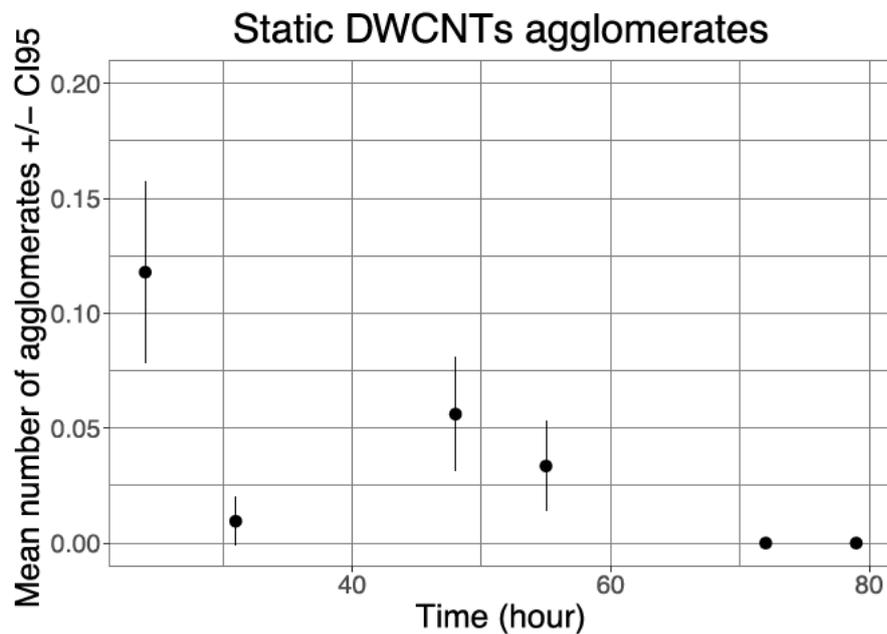
**Figure S2: Figure associated to Figure 6 (A).**

Latency to form a first pseudopod following a chronic exposure to DWCNTs. 40 slime molds were observed per treatment and per week for a total of 480 slime moulds. The boxes extend from lower to upper quartile values and vertical black lines extend to most extreme data point. The horizontal black lines indicate median values and the dots represent the mean.



**Figure S3: Figure associated to Figure 6 (B).**

Expansion rate following a chronic exposure to DWCNTs. 40 slime molds were observed per treatment and per week for a total of 480 slime moulds. The boxes extend from lower to upper quartile values and vertical black lines extend to most extreme data point. The horizontal black lines indicate median values and the dots represent the mean



**Figure S4: DWCNTs excreted in the environment from the slime mould.** Number of static DWCNTs agglomerates observed in the mucus (in 0.5mm<sup>2</sup> zones) for slime moulds exposed once to DWNCTs. N=1184 pictures analysed. Error bars are Confidence intervals

**Video S1: Video of a *P. polycephalum* vein.**

Circulation of DWCNTs in a vein of *P. polycephalum* exposed to DWCNTs via food.