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Slime moulds response to carbon nanotubes exposure: from internalisation to behaviour

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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 down side, their increasing presence in the environment poses potential threats to living organisms and the 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 mould *Physarum polycephalum*. Despite its ecological significance, its simplicity of organisation and its behavioral complexity, exposure of such organisms to nanoparticles has been poorly investigated. Slime moulds were exposed to DWCNTs using three routes of exposure (topical, food, environment). We first demonstrated that DWCNTs were rapidly internalised by slime moulds especially when DWCNTs were mixed with the food or spread out in the environment. Secondly, we showed that a six-week exposure to DWCNTs did not lead to bioaccumulation nor did it lead to persistence in the slime moulds when they entered a resting stage. Thirdly, we revealed that, two days following exposure, DWCNTs were almost entirely excreted from the slime moulds. Lastly, we uncovered that DWCNTs exposure altered the migration speed, the pseudopods formation and the expansion rate of the slime moulds. Our results extend our current knowledge of CNTs cytotoxicity and introduce *P. polycephalum* as an ideal organism to nanotoxicology.

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 revolutionised 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 et al., 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 functionalised without degrading their intrinsic mechanical properties which makes them the perfect compromise for many industrial applications (Flahaut et al., 2003; Simon et al., 2019).They also offer higher stability under chemical, mechanical and thermal treatments and a longer lifetime than MWCNTs (Green & Hersam, 2011). Lastly, they enhance electron transfer and thus provide better electrochemical behaviour than SWCNTs (Pumera, 2007).

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 & 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 a 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 (T. Y. Sun et al., 2016). Soil and water are the main environmental reservoirs for CNTs (Liné et al., 2017). Using a dynamic probabilistic material flow model, predicted CNTs concentration was estimated to be *ca.* $3.6.10^{-4}$ mg•L^{-1} in surface waters and approximatively $3.5.10^{-2}$ mg•kg^{-1} in natural and urban soil (T. Y. 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 & Yang, 2009), disrupt wall and membrane (Kang et al., 2007; Liu et al., 2009), induce stress response (Kang, Herzberg, 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 stabilised 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). Similarly to protozoa, both

fresh water (*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 behavioural complexity and due to the ease with which it can be cultivated and manipulated, the acellular slime mould *P. polycephalum* presents itself as an ideal model system to determine the cytotoxicity of CNTs in single cell organisms. *P. 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. *P. 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. *P. 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 centimetres (Figure 1A) 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 1C).

Both *P. polycephalum*'s motion and behaviour rely on a network of interconnected veins (Figure 1B). These veins contract and relax periodically, causing the cytoplasm to flow back and forth, a phenomenon called "shuttle streaming" (Matsumoto et al., 2008). The veins are porous, allowing respiratory gases, molecules, nuclei and organelles to be exchanged with the surrounding cytoplasm (Oettmeier et al., 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 et al., 2008, Alim et al., 2013). These contractions produce a pressure gradient that pushes the cytoplasm towards the cell periphery where local cytoskeletal reorganisation 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 et al., 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 et al., 1986; Ridgway & Durham, 1976; Shimizu & Tada, 1994). It has been shown that slime moulds respond to a variety of cues such as chemicals (Aldrich, 2012), light (Hato et al., 1976; Marwan, 2001), temperature (Wolf et al., 1997), humidity (Rakoczy, 1973), etc. *P. polycephalum* exhibits a rich repertoire of complex behaviours which makes it a model organism to study problem-solving in unicellular systems (Adamatzky, 2017; Oettmeier et al., 2017; Reid et al., 2015; Smith-Ferguson & Beekman, 2020; Vallverdú et al., 2018).

Although the importance of slime moulds to the environment is well acknowledged, only a single study was found on exposure of such organisms to carbon nanotubes (CNTs) (Gizzie et al., 2016). The objective of Gizzie et al (2016) study was not to measure the impact of CNTs on *P. polycephalum* but to hybridise the organism with nanomaterials. In contrast, the main objective of our work was to measure the impact of CNTs exposure on *P. polycephalum* behaviour. First, we verified that CNTs could be internalised into the cell using various routes of exposure. CNTs were either mixed with food, spread in the environment or administrated by topical application. Being a phagotrophic organisms, 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 behaviour including movement initiation and migration speed.

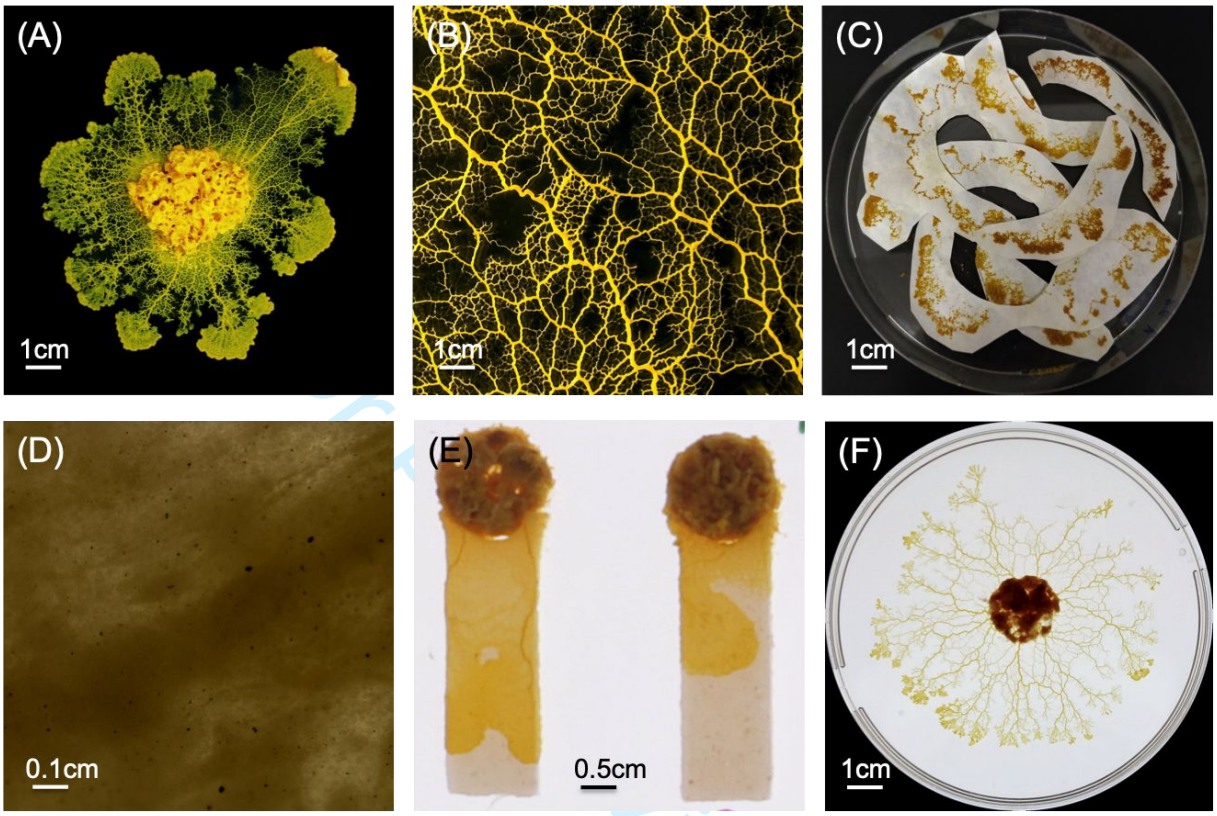

Figure 1: Pictures of *P. polycephalum*. These pictures illustrate (A) a macroplasmodium; the vegetative phase of *P. polycephalum*, (B) a magnified vein network, (C) a sclerotium, the dormant stage of *P. polycephalum*, (D) DWCNTs agglomerates internalised in a slime mould (E) two slime moulds migrating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

Materials and methods

1. *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 synthesise 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 a dihydrogen (H_2) and a methane (CH_4) 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 deionised water and 15 mL of 37% HCl for 1g 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, a 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 micrometres. Bundles typically had a maximum diameter of a few tens of nanometres. 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 fig 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 500W in a classical mixrowave oven) were used to facilitate the dissolution. Then, the CMC was transferred in a 1L bottle and tap water was added to obtain a 50 mg•L^{-1} CMC solution. Pristine DWCNT wet powder (equivalent of 50 mg in the dry form) was suspended in a 1L bottle with CMC solution (50 mg•L^{-1}) to obtain a 50 mg•L^{-1} DWCNT solution. The suspension was homogenised with a probe sonicator (Vibra Cell 75042, 20 kHz, 500W, 125 mm-diameter probe) cooled in an ice bath (programming of 3 sec ON and 3 sec OFF alternately and an amplitude of 30%, for 30 min).

2. *Species and rearing conditions*

Slime moulds of *P. polycephalum* strain LU352 kindly provided by Professor Dr Wolfgang Marwan (Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany) was used for the experiments. The slime moulds were reared on a 1% agar medium with rolled oat flakes (Quaker Oats Company®) 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.

3. *Internalisation of pristine DWCNTs by slime moulds*

The aim of the first experiment was to find out how DWCNTs may be internalised within the slime mould. Circular slime moulds (14 mm Ø) were placed in the centre of a Petri dish (55 mm Ø) previously filled with 10 mL of 1 wt. % agar gel. 100 µL of DWCNT (50 mg•L^{-1}) were either dropped on rolled oats (Food treatment, n=20), directly on top of the slime mould (Topical treatment, n=20) or in the slime mould environment (Environment treatment, n=22). For the environment treatment, 2 drops of 50 µL were put on either side of the slime mould 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 hours. The slime moulds were then observed with a binocular microscope (Leica S9, 600 lp/mm, pi=0.4µm equipped with a MC170 HD camera) to quantify the DWCNTs internalisation. 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 mould were taken (depending on the size of the slime

mould) to count the number of static DWCNTs agglomerates (black spots, *Figure 1*). For each picture several zones of 0.5 mm² 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 mould, we also tracked circulating DWCNTs agglomerates within the organism. To this end, five veins per slime mould were video monitored for 100 seconds and the number of DWCNTs agglomerates circulating within the veins were counted (Movie S1).

4. Bioaccumulation of pristine DWCNTs into the slime mould

Beyond internalising DWCNTs, do the slime moulds accumulate the DWCNTs? The aim of the second experiment was to investigate if chronic exposure to DWCNTs led to bioaccumulation. 20 slime moulds were exposed daily to either a CMC solution (Control group N=20) or DWCNTs suspension (CNT group N=20), for 6 weeks. Slime moulds were reared in Petri dishes (55 mm Ø) and 1 mL of DWCNTs suspension (50 mg•L^{-1}) or CMC was spread on the food given daily to the slime moulds (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 internalisation. For 9 slime moulds chosen randomly, 12 pictures were taken the first week and the last week with a binocular microscope (Leica S9, 600 lp/mm, pi=0.4µm) and camera MC170 HD Leica. Static DWCNTs agglomerates were counted in four 0.5 mm² zone 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.

5. Persistence of pristine DWCNTs into the slime mould

When environmental conditions deteriorate, slime moulds enter a dormant state called sclerotium until conditions become favourable again. During the sclerotisation process, slime moulds lose 50% of their total protein content together with 40% of their DNA and 65% of their RNA (Aldrich, 2012). Slime mould can be easily re-activated from sclerotia if placed in favourable conditions. The aim of the third experiment was to examine if the DWCNTs internalized after a chronic exposure were excreted by the slime molds before entering the dormant stage. 240 samples of the slime moulds 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 moulds 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 moulds start to explore the agar gel, usually 24h 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 sec videos of 3 veins per slime mould were recorded with a binocular microscope and camera MC170 HD Leica to quantify circulating DWCNTs agglomerates. Ten pictures per slime mould were also taken. DWCNTs agglomerates were counted in 5 zones of 0.5 mm² chosen randomly on each picture leading to a total of 912 counts. As we used sample from the slime moulds tested in the bioaccumulation protocol, we were able to compare the number of static DWCNTs agglomerates in the slime mould before and after entering the dormant state.

6. Excretion of pristine DWCNTs from the slime mould

The aim of the fourth experiment was to evaluate the excretion rate of DWCNTs by the slime mould. Slime moulds were exposed once to DWCNTs using food as a route of exposure. Circular slime moulds (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 mould. We let the slime moulds feed and explore for 24h. Any food residues remaining after 24hours were discarded as they may provide a niche in which microorganisms can rapidly grow. As they explore, slime moulds leave behind a thick mat of non-living, translucent, extracellular slime. To prevents internalisation of DWCNTs that had been already excreted and left in the extracellular slime we replaced the agar gel after 48 hours so that the slime mould never explored the same area twice. Slime moulds were observed with a binocular microscope and camera MC170 HD Leica 24, 31, 48, 55, 72 and 79 h post exposure. 5 to 10 pictures were taken for each slime mould sample and DWCNTs agglomerates were counted using ImageJ software. 5 veins per slime mould were video monitored for 100 seconds to quantify circulating DWCNTs. We also measured the area of the static agglomerates using the software ImageJ to investigate a potential deagglomeration through time.

7. Migration speed following a single exposure to DWCNTs

The aim of the fifth experiment was to investigate how a single exposure to DWCNTs affects the slime mould behaviour. In this experiment a circular slime mould (10 mm Ø) had to migrate on a bridge (35 mm long, 100 mm wide) made of 10% w/v powdered oat-agar (Figure 1E). Slime mould were 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 mould (Topical treatment), on few oat flakes resting on the slime mould (Food treatment) or on the bridge (Environment treatment). Hence, 80 slime mould were tested for each treatment and each group, leading to total of 480 assays. After one night's incubation (*ca.* 15 h), the distance travelled (in mm) on each bridge was measured with a ruler.

8. First pseudopod and expansion rate following a chronic exposure to DWCNTs

The aim of the sixth experiment was to investigate how a chronic exposure to DWCNTs affects the slime mould performance. Twenty slime moulds were exposed chronically to DWCNTs or CMC control treatment (see bioaccumulation protocol) for 6 weeks. Each week, a circular sample of each slime mould (Ø 13 mm) was introduced in the centre of a petri dish (Ø 55 mm) containing a layer of agar (1% in tap water) (Figure 1F). The dishes were placed in a thermoregulated chamber and pictures were taken every 5 min for 36 hours with a digital Canon 70D camera. For each slime mould, we measured the time of appearance of the first pseudopod, which is an indicator of slime mould healing process, as well as the expansion rate. Expansion rate was computed as the difference between the final surface (the area of the slime moulds when it reached a distance of 15 mm from its original location) and the initial surface (the area of the slime mould when we started the experiment) divided by the time to reach the final surface. The pictures were analysed using the software Image J.

9. Statistical analysis

To assess the difference in the various parameters measured between the treatments, we used linear models, or linear mixed models, or generalised 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 week 1 to 6). When needed, a random effect: the slime mould 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 (Table S1-S11).

Results

Our main objective was to investigate the fate of DWCNTs in slime moulds and their potential effect on behaviour. *P. Polycephalum* crawls over the soil surface where it can encounter various materials, including DWCNTs.

1. *Internalisation of pristine DWCNTs by slime moulds*

Our first objective was to identify the most efficient route of exposure to DWCNTs to maximize their internalisation. The number of static DWCNTs agglomerates was considerably high when the slime mould encountered the DWCNTs while exploring their environment, five times higher than when DWCNTs were presented together with food ($p<0.001$, Figure 2A, Table S1) and fifty times higher than when DWCNTs were dropped directly on the slime mould ($p<0.001$, Figure 2A, 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 topical application ($p<0.001$, $p<0.001$ and $p=0.513$ for Food *vs* Topical, Environment *vs* Topical and Food *vs* Environment respectively, Figure 2B, Table S2). When the slime moulds 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 internalised. These observations were later confirmed by picture and video analyses revealing that there were almost no static or circulating DWCNTs agglomerates in the slime mould.

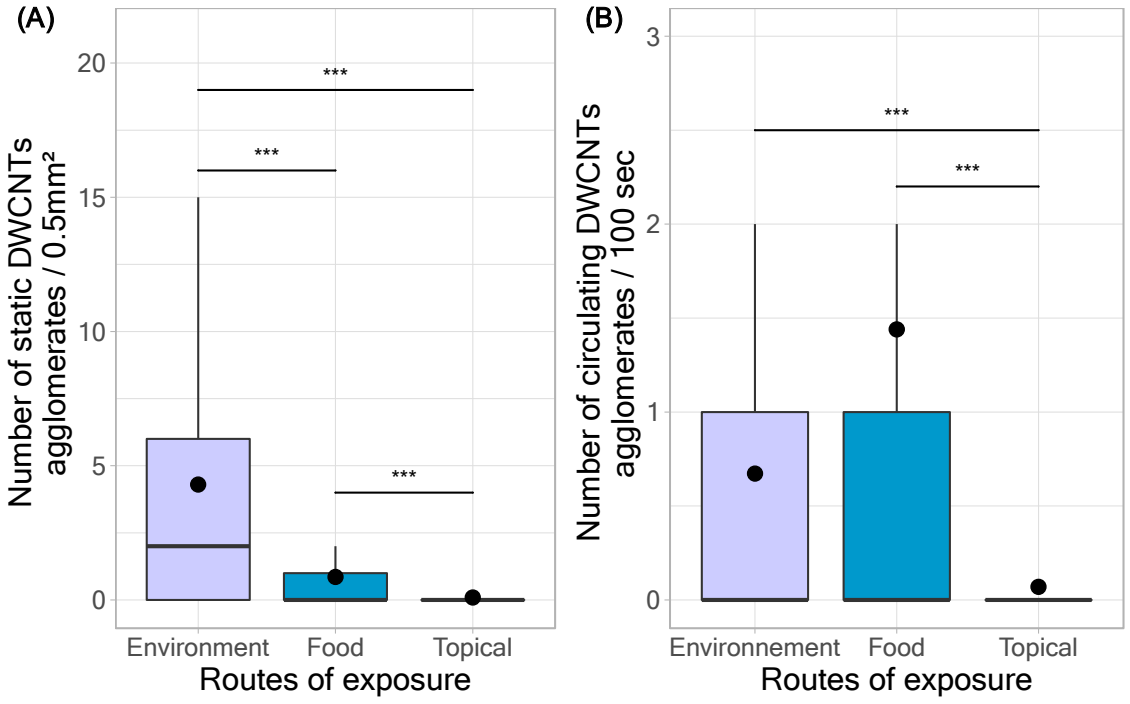

Figure 2. Internalisation of pristine DWCNTs by slime moulds. Number of static (A) and circulating (B) DWCNTs agglomerates as a function of the route of exposure: environment, food or topical. A total of 2342 pictures and 310 videos were analysed. The boxes extend from lower to upper quartile values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

2. Bioaccumulation of pristine DWCNTs into the slime mould

Here, the aim was to evaluate if DWCNTs accumulated within the slime mould when DWCNTs were added daily to the food for an extended period of time (6 weeks). Surprisingly despite daily exposure to DWCNTs, slime mould 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, Table S3 and Table S4, Figure 3A and Figure 3B).

3. Persistence of pristine DWCNTs into the slime mould

Even though DWCNTs did not accumulate over time, a certain quantity of DWCNTs were retained in the slime moulds. Thus, we next examined if DWCNTs altered the induction of the dormant state and the reactivation of the slime mould and if they persisted throughout the dormant state. All the slime moulds could enter a dormant state and be re-activated after 6 months regardless of the treatment. After one year, only 2 out of 40 slime moulds 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 3C) regardless of the extent of exposure to DWCNTs (p=0.272, Table S5, Figure 3C). 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 an evidence that the DWCNTs agglomerates internalised by slime moulds were later excreted before entering the dormant state.

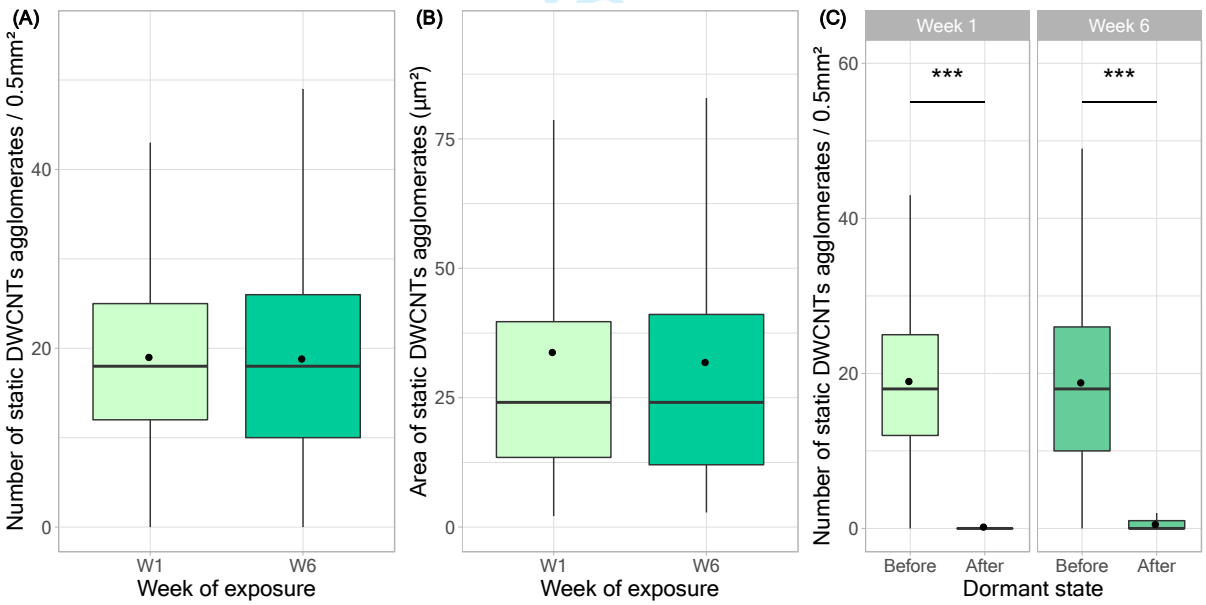

Figure 3. Bioaccumulation and persistence of pristine DWCNTs into the slime mould. (A) Number of static DWCNTs agglomerates as a function of the number of weeks of exposure. (N= 416 and N=368 pictures analysed for week 1 and week 6 respectively); (B) Area of the static agglomerates as a function of the number of weeks of exposure N= 317 and N=297 pictures analysed 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 analysed before and after entering the formant 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

4. *Excretion of pristine DWCNTs from the slime mould*

Knowing that slime moulds did not accumulate DWCNTs and could excrete them in the environment, we examined the duration and dynamics of excretion of DWCNTs for slime moulds exposed only once to DWCNTs. Both the number of static and circulating DWCNTs agglomerates dropped drastically after 24h (Time effect p<0.001, Table S6 and S7, Figure 4A and 4B). 24 hours after a single exposure to DWCNTS static and circulating agglomerates were spotted in half of the pictures and the videos, respectively. 79 hours after the exposure only few static DWCNTs agglomerates were spotted on 2 pictures out of 420 and a single occurrence of 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 4C). The area of the static agglomerates did not change through time (time effect p=0.714, Table S8)

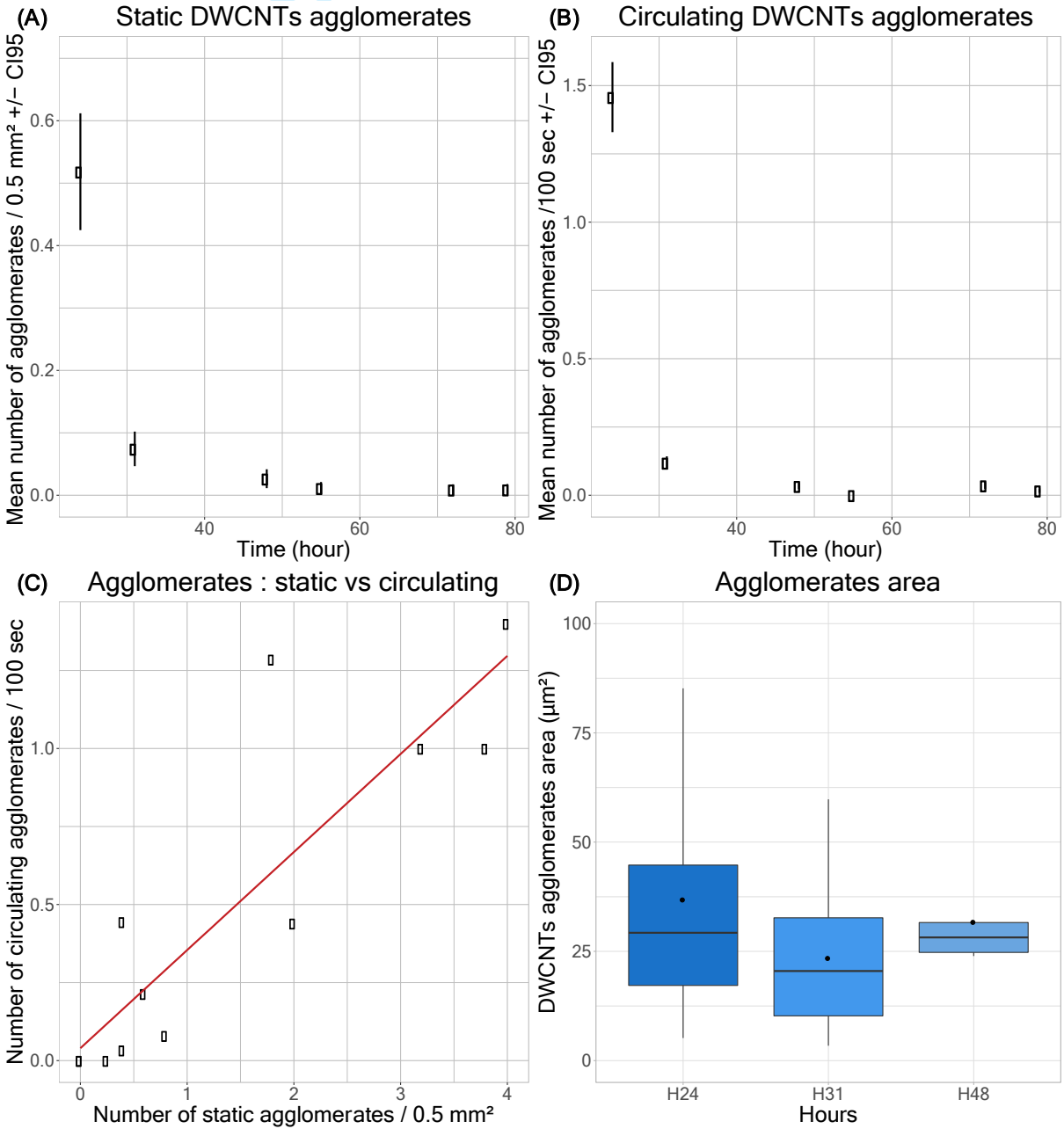

Figure 4: Excretion of pristine DWCNTs from the slime mould. Number of static (per 0.5 mm2) (A) and circulating (per 100sec) (B) DWCNTs agglomerates observed for slime moulds exposed once to DWNCTs. N= 1688 pictures and N=352 videos analysed. Error bars are Confidence intervals. (C) Correlation between 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.

5. Migration speed following a single exposure to DWCNTs

Here, the objective was to investigate if a single exposure to DWCNTs altered the slime moulds migration speed depending on the route of exposure. When the DWCNTs were associated to the food, the slime moulds were significantly slower than the controls (p=0.001, Table S9, Figure 5A). In contrast, we observed no difference in speed between the slime moulds 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 5B and 5C).

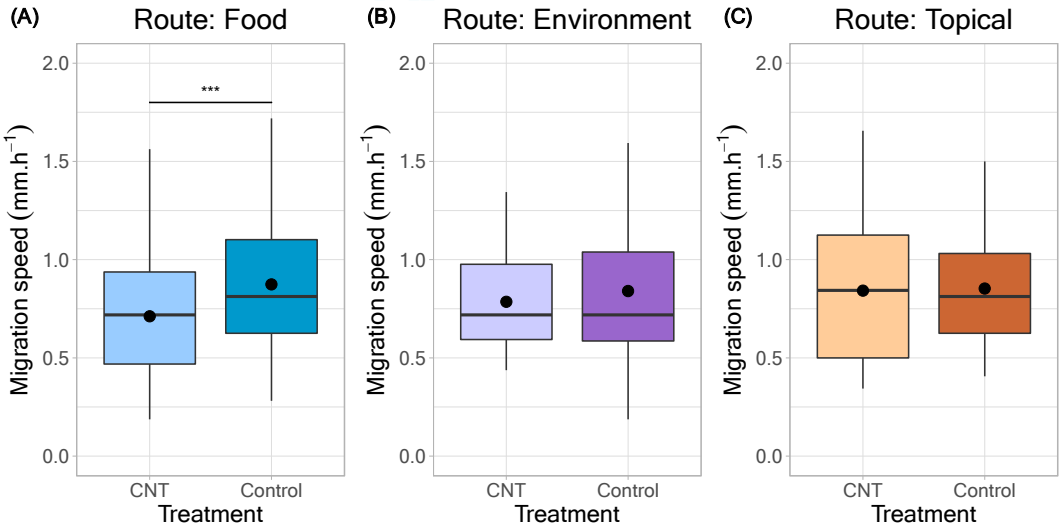

Figure 5: Migration speed following a single exposure to DWCNTs as a function of the route of exposure: environment, food or topical. 80 slime moulds per treatment and per route of exposure were observed. The boxes extend from lower to upper quartile values and vertical black lines extend to most extreme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

6. First pseudopod and expansion rate following a 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 6A, Figure S2). Similarly, the expansion rate was slower for the slime mould exposed to the DWCNTs when compared to the controls (treatment effect p=0.026, Table S13, Figure 6B, Figure S3).

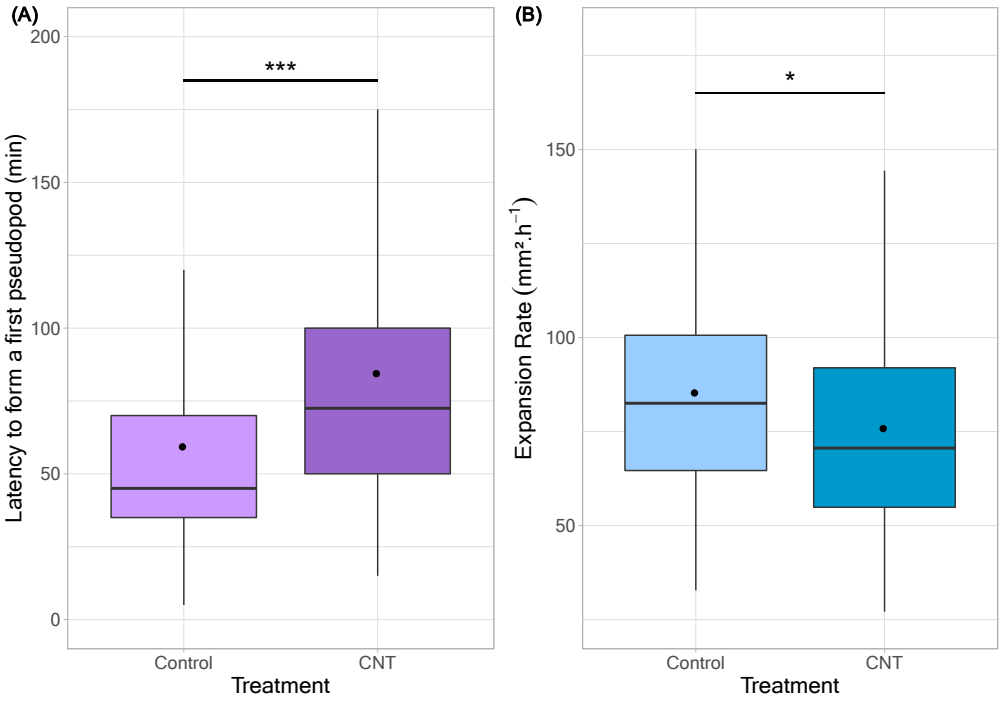

Figure 6: Behaviour analyses. (A) Latency to form a first pseudopod following a chronic exposure to DWCNTs. (B) Expansion rate following a chronic exposure to DWCNTs. 40 slime moulds were observed per treatment and per week for a total of 480 slime moulds. The boxes extend from lower to upper quartile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

Discussion

Taken together, our results indicate that slime moulds can internalise DWCNTs and that DWCNTS exposure affects behavioural performances.

Deciphering the mechanisms responsible for CNTs internalisation 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 moulds, 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 for food, it phagocytes microorganisms and continuously secretes a thick extracellular slime (Reid et al., 2012). Hence slime moulds can either internalise CNTs through phagocytosis while feeding or trapped CNTs in their own mucus while crawling on the substrate. Topical application of CNTs was the least effective route of exposure. The presence of black streaks where the CNTs were deposited indicate that the CNTs were not all passively internalised through membrane pores. In slime moulds, objects smaller than 1 µm can be endocytosed within vesicles (Gizzie et al., 2016; Mayne et al., 2011; Mayne & Adamatzky, 2015). For instance, Mayne *et al.* (2011) showed that magnetite nanoparticles can be internalised by endocytosis in slime moulds.

CNTs are persistent in the environment due to their low biodegradation, so the assessment of their potential for bioaccumulation is of major concern (Bjorkl et al., 2017). Despite a daily exposure to CNTs

via food for 6 weeks, no bioaccumulation was observed within the cell of *P. polycephalum*. Hence, slime moulds were internalising 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 two weeks eliminate the nanoparticles within a couple of days (Cheng et al., 2009; Galloway et al., 2010; Hanna et al., 2014; S. Li et al., 2013; Petersen et al., 2008). In the excretion experiment, we showed that there was a drop in the number of static and circulating CNTs agglomerates after 24 hours, and the majority of CNTs were excreted from the cell at 48 hours post exposure. Therefore, the egestion of CNTs from the cell occurred rapidly which explains why only few CNTs agglomerates persisted throughout the sclerotisation process which last at least 72h 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 moulds 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 moulds in the environment. We noticed that the number of agglomerates detected in the environment were very low (Figure S4). However, our counting methods was rather rough, as agglomerates smaller than 5 µm could not be detected with our binocular microscope.

CNTs have been shown to impact many microorganism (Chen et al., 2019) and slime moulds are no exception (Gizzie et al., 2016). In our experiment, we observed that both a single and a chronic exposure to CNTs affected the slime moulds behaviour. We noticed that slime moved more slowly when exposed to CNTs while foraging (Figure 5A) 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 moulds. 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, et al., 2008). This could explain the delay in forming the first pseudopod (Figure 6A). Second, internalisation 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 (X. Li, Peng, & Qu, 2006; Sargent et al., 2012; Singh et al., 2014) and tubulin polymers (microtubules) (X. Li, Peng, & Qu, 2006; X. Li, Peng, Ren, et al., 2006; Pampaloni & 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; X. Li, Peng, & Qu, 2006; Rodriguez-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; Rodriguez-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 moulds, 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 reorganisation of the cell cytoskeleton at larger scale and altered cell migration. Slime mould growth is associated to synchronous mitosis occurring every 8 to 10 hours. 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 moulds and could therefore move up the food chain. In addition, we demonstrated that the internalisation of CNTs altered the behaviour of slime moulds 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 mould *P. polycephalum* as a new model system to study CNTs toxicity. Slime moulds offer multifaceted advantages. First, being giant cells, they allowed us to observe internalised 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 moulds 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 behaviour (Vallverdú et al., 2018). Lastly, slime moulds display many features of animal cells such as a high molecular complexity and a large diversity of signalling 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.

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Authors' contributions. M.T., A.D. and E.F. conceived and designed the experiments. E.F. carried out 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.

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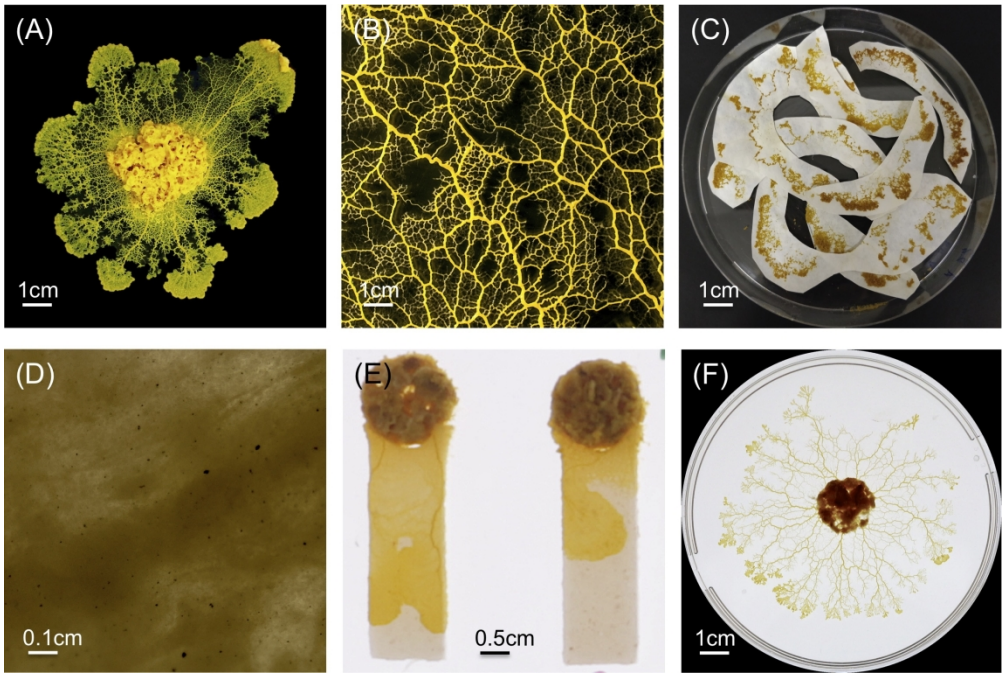

Figure 1: Pictures of P. polycephalum. These pictures illustrate (A) a macroplasmodium; the vegetative phase of P. polycephalum, (B) a magnified vein network, (C) a sclerotium, the dormant stage of P. polycephalum, (D) DWCNTs agglomerates internalised in a slime mould (E) two slime moulds migrating on a bridge and (F) one slime mould exploring an agar gel in a petri dish.

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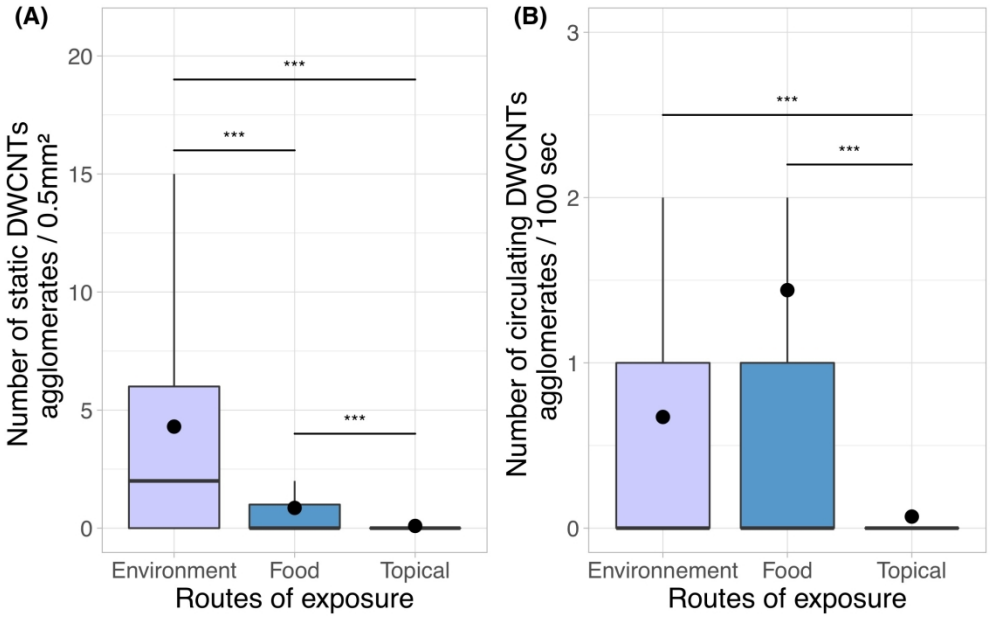

Figure 2. Internalisation of pristine DWCNTs by slime moulds. Number of static (A) and circulating (B) DWCNTs agglomerates as a function of the route of exposure: environment, food or topical. A total of 2342 pictures and 310 videos were analysed. The boxes extend from lower to upper quartile values 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

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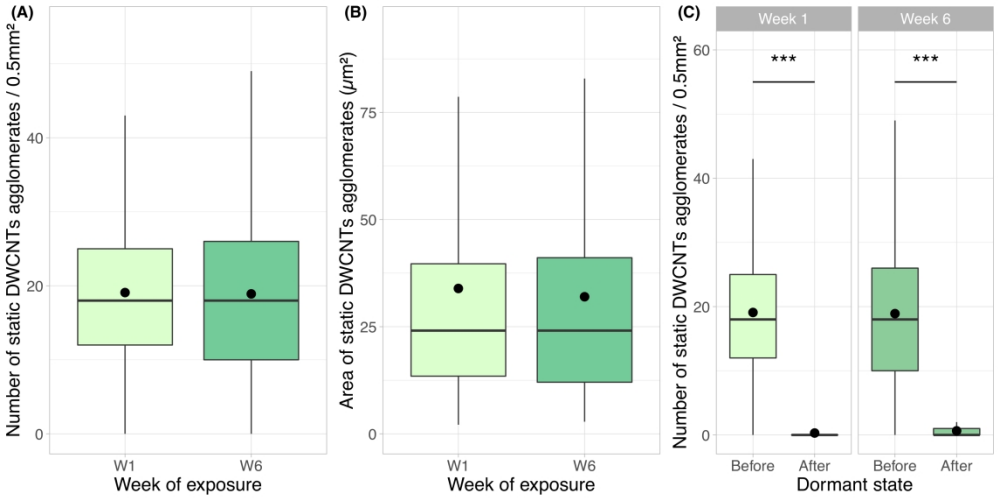

Figure 3. Bioaccumulation and persistence of pristine DWCNTs into the slime mould. (A) Number of static DWCNTs agglomerates as a function of the number of weeks of exposure. (N= 416 and N=368 pictures analysed for week 1 and week 6 respectively); (B) Area of the static agglomerates as a function of the number of weeks of exposure N= 317 and N=297 pictures analysed 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 analysed before and after entering the formant 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

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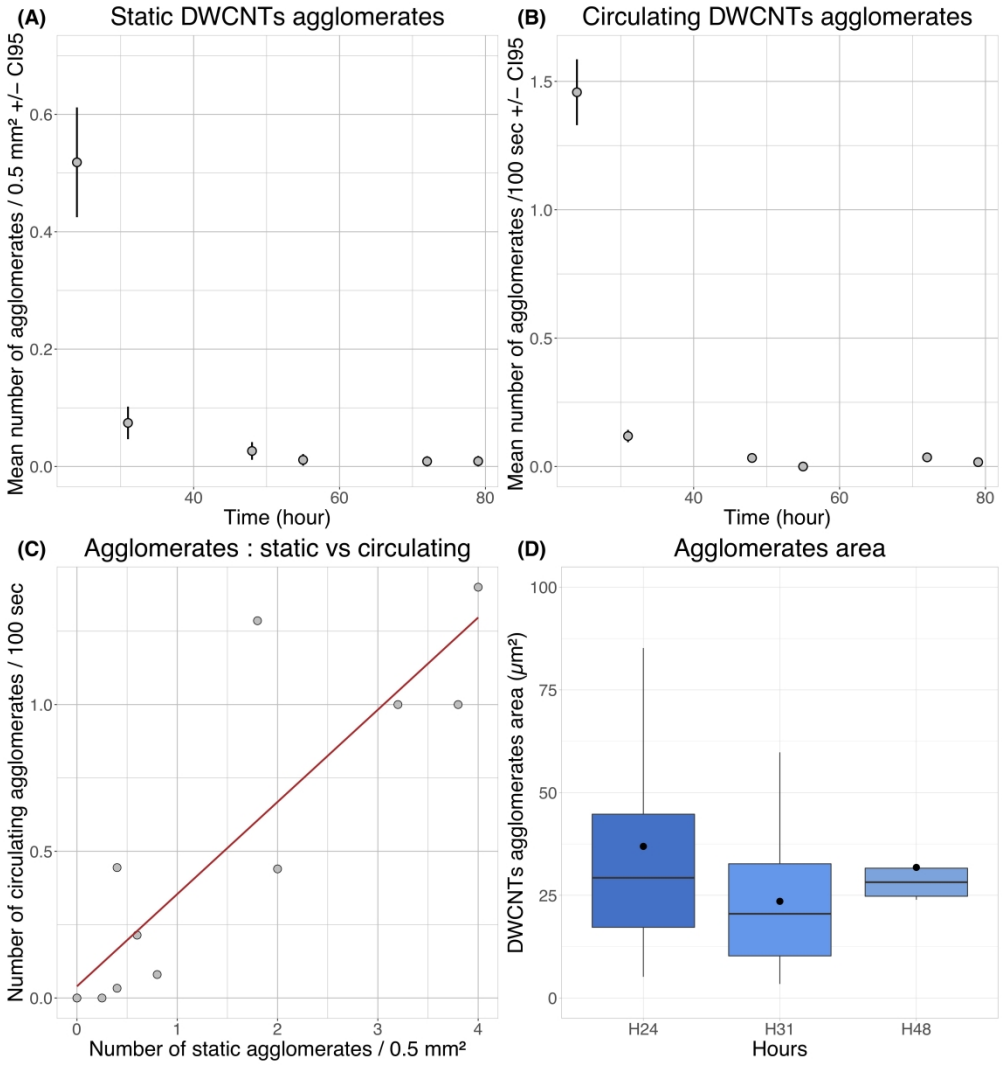

Figure 4: Excretion of pristine DWCNTs from the slime mould. Number of static (per 0.5 mm2) (A) and circulating (per 100sec) (B) DWCNTs agglomerates observed for slime moulds exposed once to DWNCTs. N= 1688 pictures and N=352 videos analysed. Error bars are Confidence intervals. (C) Correlation between the number of static aggregates and the number of circulating ones (N=12 plasmodia). The red line corresponds to the regression line ($p < 0.001$, $R2=0.77$). (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.

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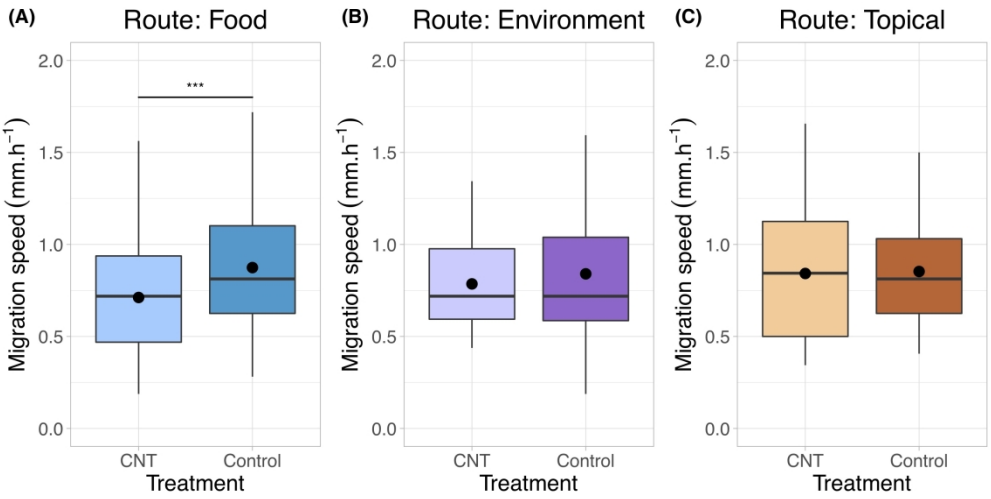

Figure 5: Migration speed following a single exposure to DWCNTs as a function of the route of exposure: environment, food or topical. 80 slime moulds per treatment and per route of exposure were observed. The boxes extend from lower to upper quartile values and vertical black lines extend to most extreme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

254x127mm (300 x 300 DPI)

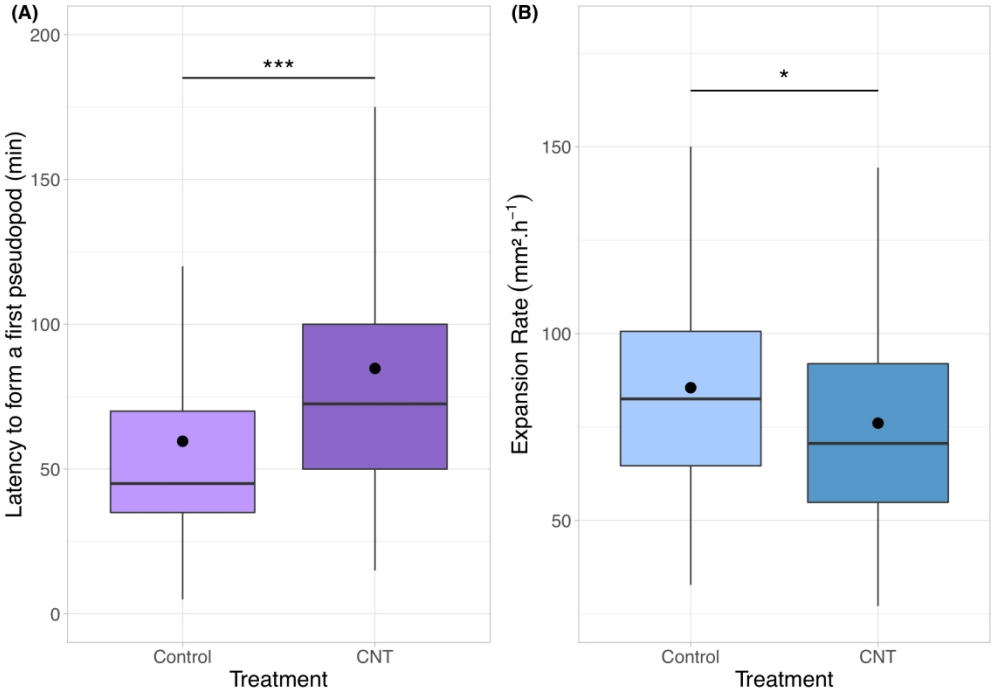

Figure 6: Behaviour analyses. (A) Latency to form a first pseudopod following a chronic exposure to DWCNTs. (B) Expansion rate following a chronic exposure to DWCNTs. 40 slime moulds were observed per treatment and per week for a total of 480 slime moulds. The boxes extend from lower to upper quartile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

254x177mm (300 x 300 DPI)

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

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

	Internalisation: Number of static agglomerates Pairwise comparisons				
Predictors	*Estimate*	*SE*	*z.ratio*	*p*	*df*
[Environment] vs [Food]	1.76	0.397	4.42	**<0.001**	2338.00
[Environment] vs [Topical]	4.36	0.430	10.12	**<0.001**	2338.00
[Food] vs [Topical]	2.60	0.395	-6.58	**<0.001**	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).

	Internalisation: Number of circulating agglomerates				
Predictors	*Incidence Rate Ratios*	*CI*	*Z value*	*p*	*df*
Intercept	0.43	0.25 – 0.75	-2.98	**0.003**	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	**<0.001**	306.00
Random Effects					
σ^2	1.56				
$\tau_{00\ Replicate}$	1.14				
ICC	0.42				
$N_{\ Replicate}$	62				
Observations	310				
Marginal R^2 / Conditional R^2	0.345 / 0.622				

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

	Accumulation: Number of static agglomerates				
Predictors	*Incidence Rate Ratios*	*CI*	*Z value*	*p*	*df*
Intercept	19.01	18.01 – 20.07	106.82	**<0.001**	781.00
[Time] (Week)	0.98	0.95 – 1.01	-1.25	0.213	781.00
Random Effects					
σ^2	0.05				
$\tau_{00\ Replicate}$	0.01				
ICC	0.10				
$N_{Replicate}$	9				
Observations	784				
Marginal R^2 / Conditional R^2	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).

	Area				
Predictors	*Estimates*	*CI*	*Statistic*	*p*	*df*
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
Random Effects					
σ^2	0.95				
$\tau_{00\ Replicate}$	0.06				
ICC	0.06				
$N_{Replicate}$	9				
Observations	610				
Marginal R^2 / Conditional R^2	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).

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

	Excretion: Number of static agglomerates				
Predictors	*Incidence Rate Ratios*	*CI*	*Z value*	*p*	*df*
Intercept	0.32	0.05 – 1.99	-1.22	0.223	1625.00
[Time] (Hours)	0.93	0.89 – 0.96	-3.92	**<0.001**	1625.00
Random Effects					
σ^2	5.62				
$\tau_{00\ Replicate}$	4.52				
ICC	0.45				
$N_{\ Replicate}$	72				
Observations	1628				
Marginal R^2 / Conditional R^2	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).

	Excretion: Number of circulating agglomerates				
Predictors	*Incidence Rate Ratios*	*CI*	*Z value*	*p*	*df*
Intercept	2.76	0.54 – 14.23	1.21	0.225	349.00
[Time] (Hours)	0.91	0.88 – 0.95	-4.74	**<0.001**	349.00
Random Effects					
σ^2	4.72				
$\tau_{00\ Replicate}$	2.40				
ICC	0.34				
$N_{\ Replicate}$	72				
Observations	352				
Marginal R^2 / Conditional R^2	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).

	Area				
Predictors	*Estimates*	*CI*	*Statistic*	*p*	*df*
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
Random Effects					
σ^2	0.94				
$\tau_{00\ Replicate}$	0.07				
ICC	0.07				
$N_{\ Replicate}$	17				
Observations	279				
Marginal R^2 / Conditional R^2	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).

	Migration speed - Route of Exposure: Food				
Predictors	*Estimates*	*CI*	*Statistic*	*p*	*df*
Intercept	0.25	0.03 – 0.46	2.29	**0.024**	158.00
[Treatment]	-0.50	-0.80 – -0.19	-3.23	**0.001**	158.00
Observations	160				
R^2 / R^2 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).

	Migration speed - Route of Exposure: Environment				
Predictors	*Estimates*	*CI*	*Statistic*	*p*	*df*
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^2 / R^2 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).

	Migration speed - Route of Exposure: Topical				
Predictors	*Estimates*	*CI*	*Statistic*	*p*	*df*
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^2 / R^2 adjusted	0.001 / -0.005				

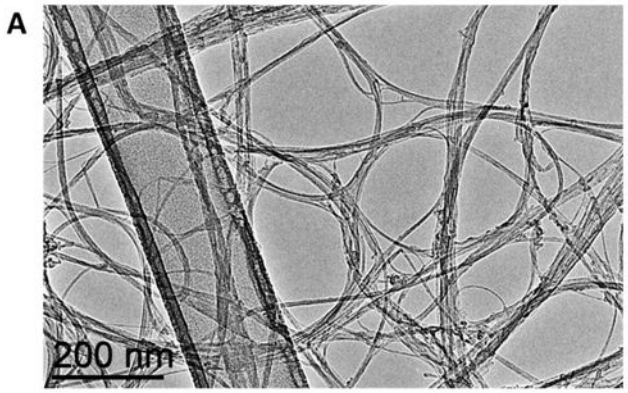
Table S11: Statistics associated to Figure 5 (C).
To assess the difference in migration speed between the two treatments when the route of exposure was Topical; we used a linear model (function lm, Package lme4). The model was fitted by specifying the fixed effect: treatment (categorical predictor, NTC *vs* Control). The dependent variable (migration speed) was normalized using the function bestNormalize (Package bestNormalize).

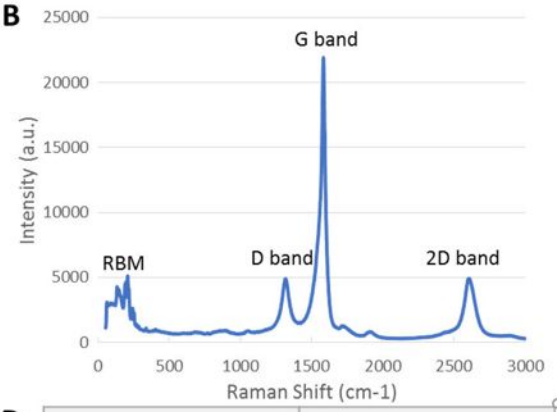
	Latency first pseudopod				
Predictors	*Estimates*	*CI*	*Statistic*	*p*	*df*
Intercept	0.04	-0.22 – 0.31	0.32	0.747	474.00
[Treatment: NTC vs Control]	1.08	0.70 – 1.46	5.58	**<0.001**	474.00
[Week]	-0.10	-0.16 – -0.03	-2.82	**0.005**	474.00
[Treatment*Week]	-0.14	-0.24 – -0.05	-2.98	**0.003**	474.00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

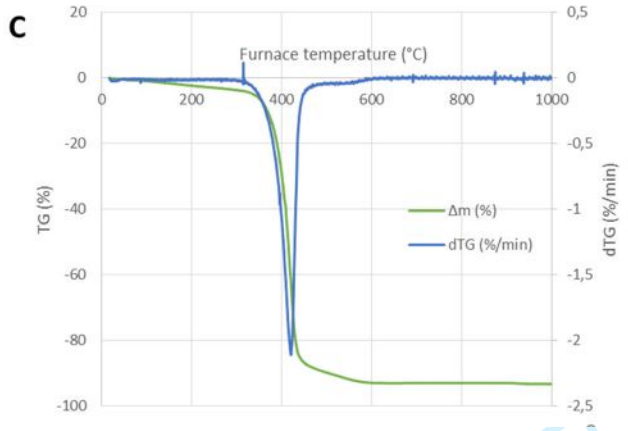
Table S12: Statistics associated to Figure 6 (A).
To assess the difference in latency to the first pseudopod between the two treatments; we used a linear mixed model (function lmer, error family: Gaussian, Package lme4). The model was fitted by specifying the fixed effects: treatment (categorical predictor, 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).

	Expansion rate				
Predictors	*Estimates*	*CI*	*Statistic*	*p*	*df*
Intercept	-0.40	-0.67 – -0.12	-2.83	**0.005**	474.00
[Treatment: NTC vs Control]	-0.44	-0.83 – -0.05	-2.22	**0.026**	474.00
[Week]	0.16	0.09 – 0.23	4.63	**<0.001**	474.00
[Treatment*Week]	0.03	-0.07 – 0.12	0.52	0.601	474.00
Random Effects					
σ^2	0.87				
$\tau_{00\ Replicate}$	0.02				
ICC	0.02				
$N_{\ Replicate}$	40				
Observations	480				
Marginal R^2 / Conditional R^2	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).







D

ID/IG intensity ratio	0.2 ± 0.5
Elementary analysis	89.75% C, 2.13% O
Content of catalysts	3.99% Co, 0.96% Mo
Specific Surface Area (m^2/g)	985
Zeta potential (mV)	-27.5 (in DI water) -32.1 (in soil solution)
Median outer diameter (nm)	2.05
Length (microns)	1 to 100
Number of walls	5% TW, 80% DW, 15% SW

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).

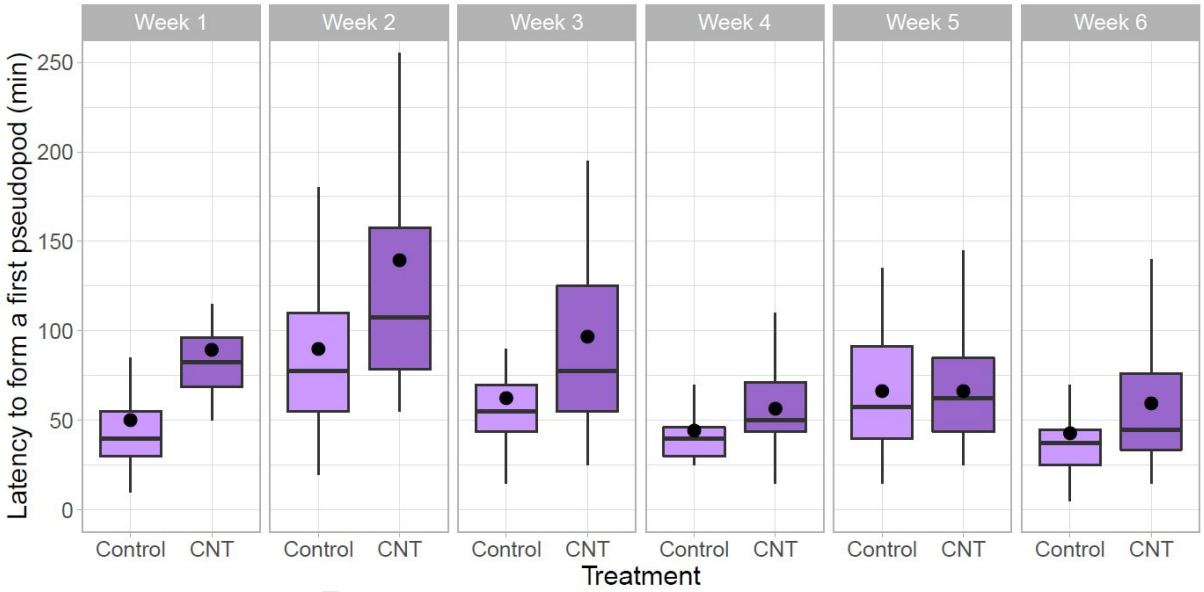


Figure S2: Figure associated to Figure 6 (A).
Latency to form a first pseudopod following a chronic exposure to DWCNTs. 40 slime molds were observed per treatment and per week for a total of 480 slime moulds. The boxes extend from lower to upper quartile values and vertical black lines extend to most extreme data point. The horizontal black lines indicate median values and the dots represent the mean.

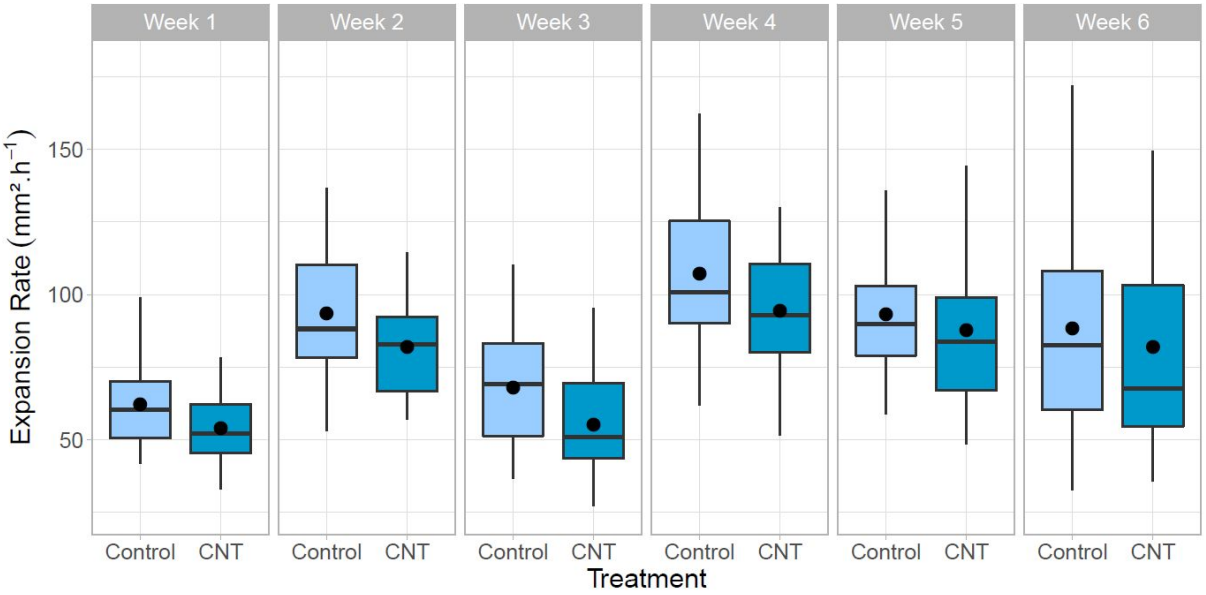


Figure S3: Figure associated to Figure 6 (B).
Expansion rate following a chronic exposure to DWCNTs. 40 slime molds were observed per treatment and per week for a total of 480 slime moulds. The boxes extend from lower to upper quartile values and vertical black lines extend to most extreme data point. The horizontal black lines indicate median values and the dots represent the mean

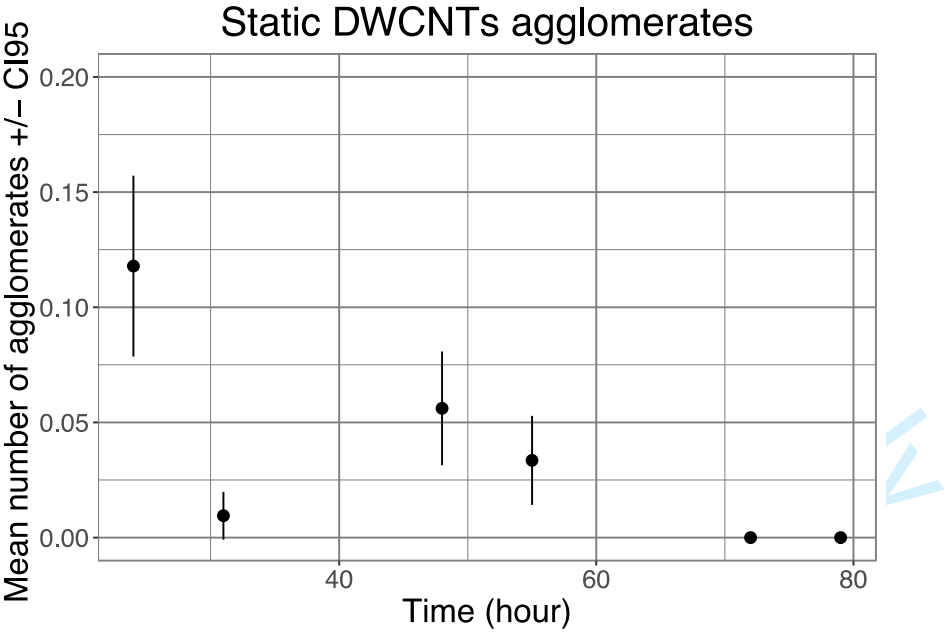


Figure S4: DWCNTs excreted in the environment from the slime mould. Number of static DWCNTs agglomerates observed in the mucus (in 0.5mm2 zones) for slime moulds exposed once to DWNCTs. N=1184 pictures analysed. Error bars are Confidence intervals

Video S1: Video of a *P. polycephalum* vein.
Circulation of DWCNTs in a vein of *P. polycephalum* exposed to DWCNTs via food.