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

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Keywords:	Slime mould, Carbon nanotubes, Ecotoxicology, Behaviour
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 <i>Physarum polycephalum</i> . 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

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	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 <i>P. polycephalum</i> as an ideal organism to nanotoxicology.
Note: The following files were submitted by the author for peer review, but cannot be converted to PDF. You must view these files (e.g. movies) online.	
MovieS1_MS_Ternois et_al.mp4	



# 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.

## 31 Introduction

32 Recent years have witnessed a growing interest in nanomaterials due to their specific electrical,  
33 optical, thermal and magnetic properties that enhance the performance of the final product (Guinée  
34 et al., 2017). Nanomaterials may be of different nature including metals, oxides, or carbon-based.  
35 Carbon nanotubes (CNTs) were brought to the forefront in 1991 by Sumio Iijima and since then they  
36 have revolutionised several fields of research with their extraordinary properties and applications (De  
37 Volder et al., 2013). They are incorporated in a wide variety of consumer products (Guinée et al., 2017)  
38 and used in many fields of applications such as biomedical engineering, energy production and storage,  
39 nanoelectronics, mechanical engineering, and so forth (Gabaudan et al., 2019, Simon et al., 2019).  
40 CNTs can be single-walled (SWCNTs), multi-walled (MWCNTs) or, at the interface such as the double-  
41 walled carbon nanotubes (DWCNTs). DWCNTs offer several advantages in comparison to both SWCNTs  
42 and MWCNTs. They show enhanced properties thanks to an outer wall protecting the inner tube.  
43 Hence, DWCNTs can be functionalised without degrading their intrinsic mechanical properties which  
44 makes them the perfect compromise for many industrial applications (Flahaut et al., 2003; Simon et  
45 al., 2019). They also offer higher stability under chemical, mechanical and thermal treatments and a  
46 longer lifetime than MWCNTs (Green & Hersam, 2011). Lastly, they enhance electron transfer and thus  
47 provide better electrochemical behaviour than SWCNTs (Pumera, 2007).

48 Despite the attractive properties of CNTs for numerous applications, some studies have raised  
49 concerns regarding the potential risks of these nanomaterials to the environment (Francis & Devasena,  
50 2018). CNTs are released in the environment throughout their life cycle by accidental release during  
51 production, transport, storage, uses of the final product (Mouchet et al., 2007), and as a waste from  
52 sewage treatment or incineration plants (Petersen et al., 2011). The fate of CNTs and their impact  
53 depends on their transport, diffusion, degradation, bioaccumulation, transformation and persistence.  
54 Hence, to evaluate the potential risks associated with CNTs, it is primordial to quantify their presence  
55 in each compartment of the environment. However, it is difficult to measure precisely the quantity of  
56 CNTs in environmental matrices, and models are often used to predict environmental concentrations  
57 (T. Y. Sun et al., 2016). Soil and water are the main environmental reservoirs for CNTs (Liné et al., 2017).  
58 Using a dynamic probabilistic material flow model, predicted CNTs concentration was estimated to be  
59 *ca.*  $3.6 \cdot 10^{-4} \text{ mg} \cdot \text{L}^{-1}$  in surface waters and approximately  $3.5 \cdot 10^{-2} \text{ mg} \cdot \text{kg}^{-1}$  in natural and urban soil (T.  
60 Y. Sun et al., 2016).

61 Single cell organisms play an important role in nutrients cycling and a change in their activity could  
62 indicate a response to an environmental stressor (Chung et al., 2011). Several studies indicate that  
63 MWCNTs and SWCNTs present in soils repress enzymatic activities of bacteria, reduce microbial  
64 biomass (Chung et al., 2011; Ge et al., 2016; Jin et al., 2013) and alter soil microbial community  
65 composition (Jin et al., 2014; Kerfahi et al., 2015). It was also shown in soil bacteria that SWCNTs inhibit  
66 growth, reduce viability (Arias & Yang, 2009), disrupt wall and membrane (Kang et al., 2007; Liu et al.,  
67 2009), induce stress response (Kang, Herzberg, et al., 2008) and affect denitrification activity (Zheng et  
68 al., 2014). CNTs are hydrophobic, but stable CNTs suspension can persist for over one month in natural  
69 surface water when stabilised by natural organic matter (Hyung et al., 2007). Hence negative impacts  
70 of CNTs on aquatic single cell organisms have also been observed. Among these single cell organisms,  
71 ciliates and unicellular algae have been the most studied to assess CNTs adverse effects (reviewed in  
72 Jackson et al., 2013). SWCNTs and MWCNTs can be readily ingested by various ciliates (*Tetrahymena*  
73 *thermophila*, *Tetrahymena thermophila pyriformis*, *Pseudocohnilembus persalinus* and *Stylonychia*  
74 *mytilus*) and can alter the motility, induce growth inhibition, cause cell aggregation, decrease viability,  
75 impair predation and alter the intracellular trafficking of vesicles (Chan et al., 2009; Ghafari et al., 2008;  
76 Guo et al., 2008; Mortimer et al., 2016; Weijie et al., 2020; Zhu et al., 2006). Similarly to protozoa, both

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3 77 fresh water (*Chlorella vulgaris*, *Pseudokirchneriella subcapitata*) and marine algae (*Dunaliella*  
4 78 *tertiolecta*) are sensitive to CNTs and exhibit reduced growth and stress response when exposed (Long  
5 79 et al., 2012; Schwab et al., 2011; Wei et al., 2010).

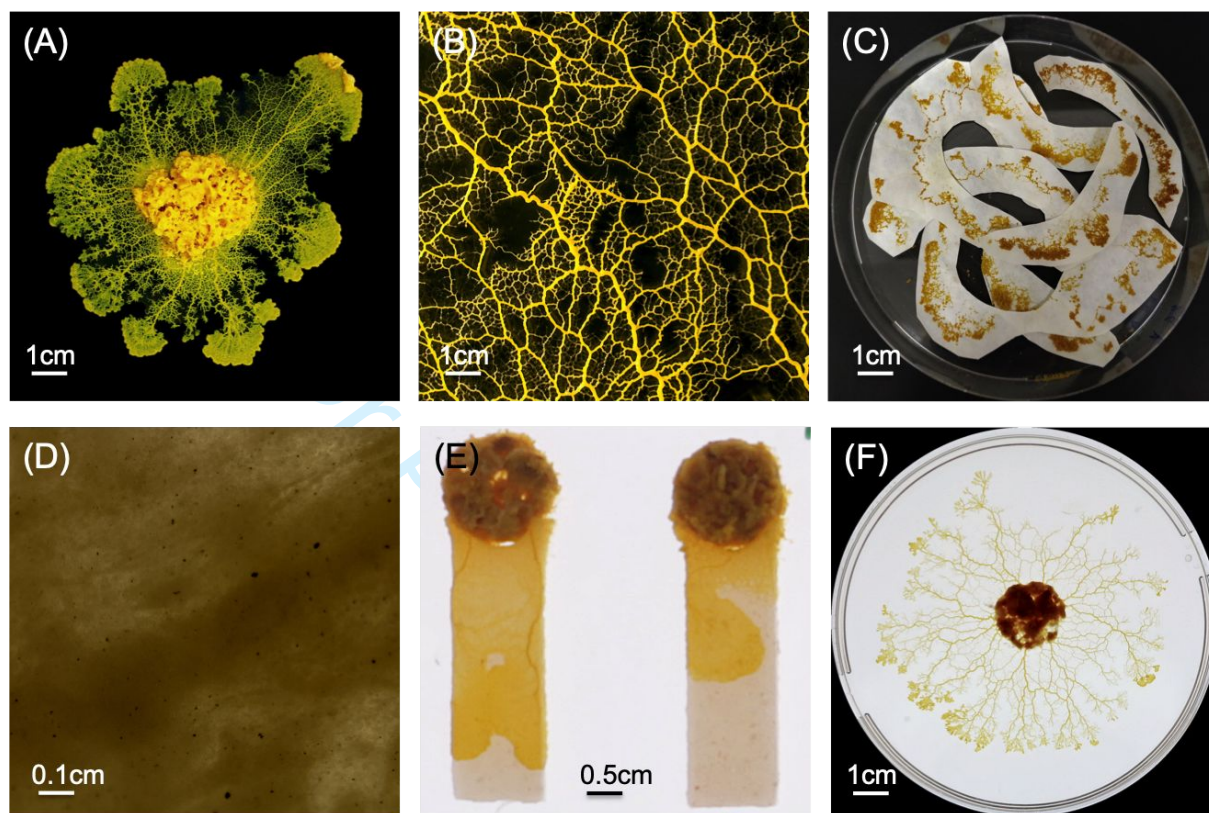
7 80 Due to its comparably simple structure in relation to its behavioural complexity and due to the ease  
8 81 with which it can be cultivated and manipulated, the acellular slime mould *P. polycephalum* presents  
9 82 itself as an ideal model system to determine the cytotoxicity of CNTs in single cell organisms. *P.*  
10 83 *polycephalum* is a remarkable organism belonging to the Myxomycetes and the Amoebozoa. It is a  
11 84 phagotrophic organism commonly observed in association with decaying plant material in terrestrial  
12 85 ecosystems. *P. polycephalum*, as a major component of the detritus food chain is essential in nutrient  
13 86 cycling. Bacteria and fungi are the primary natural food source of *P. polycephalum*, and these are  
14 87 phagocytized (Aldrich, 2012). Food particles are first captured in flask-like invaginations of the  
15 88 membrane and then isolated in vacuoles which are released into the cytoplasm. *P. polycephalum* can  
16 89 also take up droplets of medium by pinocytosis (Aldrich, 2012). The vegetative state of *P. polycephalum*  
17 90 is a large mobile polynucleated cell also called plasmodium. It can extend to up to hundreds of square  
18 91 centimetres (Figure 1A) and be severed into viable and structurally similar yet smaller plasmodia. Upon  
19 92 contact, these plasmodia can fuse with each other to form a unique plasmodium (Aldrich, 2012). Lastly,  
20 93 a starving plasmodium can encapsulate and enter a dormant stage called sclerotium (Figure 1C).

21 94 Both *P. polycephalum*'s motion and behaviour rely on a network of interconnected veins (Figure 1B).  
22 95 These veins contract and relax periodically, causing the cytoplasm to flow back and forth, a  
23 96 phenomenon called "shuttle streaming" (Matsumoto et al., 2008). The veins are porous, allowing  
24 97 respiratory gases, molecules, nuclei and organelles to be exchanged with the surrounding cytoplasm  
25 98 (Oettmeier et al., 2018). This open network distributes the cytoplasm throughout the cell body and  
26 99 allows to maintain homeostasis. This network is also responsible for the plasmodium migration at a  
27 100 speed of up to 4 cm per hour, through the interplay of intracellular flow and rhythmic vein contractions  
28 101 (Alim et al., 2013; Matsumoto et al., 2008, Alim et al., 2013). These contractions produce a pressure  
29 102 gradient that pushes the cytoplasm towards the cell periphery where local cytoskeletal reorganisation  
30 103 leads to the formation of fan shaped leading fronts. These fronts extend and retract in synchrony with  
31 104 the shuttle streaming of the cytoplasm (Lewis et al., 2015; Matsumoto et al., 2008). The frequency and  
32 105 the amplitude of the contractions and by extension the shape, size and motion of the plasmodium  
33 106 depend on the external cues encountered in the environment (Matsumoto et al., 1986; Ridgway &  
34 107 Durham, 1976; Shimizu & Tada, 1994). It has been shown that slime moulds respond to a variety of  
35 108 cues such as chemicals (Aldrich, 2012), light (Hato et al., 1976; Marwan, 2001), temperature (Wolf et  
36 109 al., 1997), humidity (Rakoczy, 1973), etc. *P. polycephalum* exhibits a rich repertoire of complex  
37 110 behaviours which makes it a model organism to study problem-solving in unicellular systems  
38 111 (Adamatzky, 2017; Oettmeier et al., 2017; Reid et al., 2015; Smith-Ferguson & Beekman, 2020;  
39 112 Vallverdú et al., 2018).

40 113 Although the importance of slime moulds to the environment is well acknowledged, only a single study  
41 114 was found on exposure of such organisms to carbon nanotubes (CNTs) (Gizzie et al., 2016). The  
42 115 objective of Gizzie et al (2016) study was not to measure the impact of CNTs on *P. polycephalum* but  
43 116 to hybridise the organism with nanomaterials. In contrast, the main objective of our work was to  
44 117 measure the impact of CNTs exposure on *P. polycephalum* behaviour. First, we verified that CNTs could  
45 118 be internalised into the cell using various routes of exposure. CNTs were either mixed with food, spread  
46 119 in the environment or administrated by topical application. Being a phagotrophic organisms, we  
47 120 expected the slime molds to readily internalize CNTs. Second, we evaluated if CNTs accumulated over  
48 121 time when cells were continuously exposed to CNTs for 6 weeks. Bioaccumulation of CNTs is a major  
49 122 issue, as it means that CNTs could move up along the food chain. Third, we measured the persistence



123 of CNTs within the cell when cells were exposed to CNTs for 6 weeks. If the CNTs remain into the cell,  
 124 they might be transferred to the next generation. Fourth, we estimated the **excretion** rate of CNTs  
 125 when cells were exposed only once to CNTs. Last, we measured the effect of CNTs exposure on  
 126 behaviour including movement initiation and migration speed.



127  
 128 **Figure 1: Pictures of *P. polycephalum*.** These pictures illustrate (A) a macroplasmodium; the vegetative  
 129 phase of *P. polycephalum*, (B) a magnified vein network, (C) a sclerotium, the dormant stage of *P.*  
 130 *polycephalum*, (D) DWCNTs agglomerates internalised in a slime mould (E) two slime moulds migrating  
 131 on a bridge and (F) one slime mould exploring an agar gel in a petri dish.

## 132 133 **Materials and methods**

### 134 **1. CNT synthesis and dispersion**

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

137 We used the CCVD (catalytic chemical vapor deposition) technique to synthesise the DWCNTs.  
 138 Magnesia (MgO) was used as support material for *in situ* generated catalytic nanoparticles of cobalt  
 139 and molybdenum (Flahaut et al., 2003). CCVD is a widely used method due to its capacity to produce  
 140 CNTs with a high yield and possibly also a high purity with easily controllable reaction conditions to  
 141 tune the desired type of carbon nanotubes (Yeoh et al., 2009). First, the catalytic powder was placed  
 142 in a ceramic container which was positioned in a tube furnace. The synthesis was performed with a  
 143 dihydrogen (H<sub>2</sub>) and a methane (CH<sub>4</sub>) mixture flow. Methane served as the source of carbon by catalytic  
 144 decomposition on the cobalt and molybdenum nanoparticles, leading to the formation of carbon  
 145 nanotubes. The obtained black nanocomposite powder was composed of DWCNTs and the catalytic  
 146 support, which was dissolved by addition of a concentrated aqueous solution of hydrochloric acid (3.5

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3 147 mL of deionised water and 15 mL of 37% HCl for 1g of powder) and left overnight. Then, a vacuum  
4 148 filtration was carried out using a nitrocellulose membrane (0.45  $\mu\text{m}$  pore size). Successive filtrations  
5 149 were performed to remove the excess of HCl and the solution of Mg, Co and Mo salts. Finally, a last  
6 150 washing was carried out using tap water.

8  
9 151 As described in our earlier work (Flahaut et al. 2003), the DWCNTs samples contained 80% of DWCNTs,  
10 152 10% SWCNTs and 5% MWCNTs (triple-walled CNTs). The outer-diameter distribution of the CNTs was  
11 153 between 1 and 3 nm. The length was difficult, if not impossible, to assess because the nanotubes  
12 154 formed entangled bundles. Considering that the length of bundles may reach 100  $\mu\text{m}$ , we estimated  
13 155 the length of individual CNTs to be between a few  $\mu\text{m}$  to tens of micrometres. Bundles typically had a  
14 156 maximum diameter of a few tens of nanometres. The elemental analysis indicated that our DWCNTs  
15 157 samples contained residual cobalt (*ca.* 3.5 - 4 wt.%) and molybdenum (*ca.* 1.0 wt.%) mainly in the form  
16 158 of nanoparticles tightly encapsulated in concentric carbon shells. The chemical analysis of carboxylic  
17 159 groups usually led to 0-0.5 mM/g (Bortolamiol et al. 2014), and the oxygen content was typically 3-  
18 160 4 wt.% (see fig S1 for more details).

21 161 Due to their low water solubility, DWCNTs were suspended in a solution of carboxymethyl cellulose  
22 162 (CMC). CMC (carboxymethyl cellulose) is a cellulose derivative used as an additive in human food and  
23 163 is harmless for living organisms (E466). It is commonly used to provide stability to pristine CNTs  
24 164 suspensions (Bourdiol et al., 2013). First, 50 mg of CMC were weighed and placed in a vial with a few  
25 165 mL of tap water. Ultrasonic bath (*ca.* 1 minute) and microwave heating (a few seconds at 500W in a  
26 166 classical mixrowave oven) were used to facilitate the dissolution. Then, the CMC was transferred in a  
27 167 1L bottle and tap water was added to obtain a 50  $\text{mg}\cdot\text{L}^{-1}$  CMC solution. Pristine DWCNT wet powder  
28 168 (equivalent of 50 mg in the dry form) was suspended in a 1L bottle with CMC solution (50  $\text{mg}\cdot\text{L}^{-1}$ ) to  
29 169 obtain a 50  $\text{mg}\cdot\text{L}^{-1}$  DWCNT solution. The suspension was homogenised with a probe sonicator (Vibra  
30 170 Cell 75042, 20 kHz, 500W, 125 mm-diameter probe) cooled in an ice bath (programming of 3 sec ON  
31 171 and 3 sec OFF alternately and an amplitude of 30%, for 30 min).

## 35 172 2. Species and rearing conditions

37 173 Slime moulds of *P. polycephalum* strain LU352 kindly provided by Professor Dr Wolfgang Marwan (Max  
38 174 Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany) was used for the  
39 175 experiments. The slime moulds were reared on a 1% agar medium with rolled oat flakes (Quaker Oats  
40 176 Company®) in Petri dishes (140 mm  $\varnothing$ ). They were kept in the dark in a thermoregulated chamber at a  
41 177 temperature of 25 degrees Celsius and a humidity of 80%. Slime mold were transferred every day on  
42 178 a new agar medium on which was spread a single layer of rolled oat flakes. All experiments were also  
43 179 carried out in a thermoregulated chamber and pictures were taken with a Canon 70D digital camera.

## 46 180 3. Internalisation of pristine DWCNTs by slime moulds

48 181 The aim of the first experiment was to find out how DWCNTs may be internalised within the slime  
49 182 mould. Circular slime moulds (14 mm  $\varnothing$ ) were placed in the centre of a Petri dish (55 mm  $\varnothing$ ) previously  
50 183 filled with 10 mL of 1 wt. % agar gel. 100  $\mu\text{L}$  of DWCNT (50  $\text{mg}\cdot\text{L}^{-1}$ ) were either dropped on rolled oats  
51 184 (Food treatment, n=20), directly on top of the slime mould (Topical treatment, n=20) or in the slime  
52 185 mould environment (Environment treatment, n=22). For the environment treatment, 2 drops of 50  $\mu\text{L}$   
53 186 were put on either side of the slime mould and spread out with a brush to cover the entire gel as evenly  
54 187 as possible. All the petri dishes were stored in a thermoregulated chamber (25°C) for 16 hours. The  
55 188 slime moulds were then observed with a binocular microscope (Leica S9, 600 lp/mm,  $\text{pi}=0.4\mu\text{m}$   
56 189 equipped with a MC170 HD camera) to quantify the DWCNTs internalisation. The DWCNTs that we  
57 190 observed using the binocular microscope were much larger than bundles and correspond to  
58 191 agglomerates. 8 to 10 pictures of each slime mould were taken (depending on the size of the slime



1  
2  
3 192 mould) to count the number of static DWCNTs agglomerates (black spots, *Figure 1*). For each picture  
4 193 several zones of 0.5 mm<sup>2</sup> were delimited randomly and DWCNTs were counted in each zone. Data  
5 194 acquisition was performed using the software ImageJ. As we could not preclude that some static  
6 195 agglomerates were trapped in the extracellular slime layer surrounding the cell and not actually inside  
7 196 the slime mould, we also tracked circulating DWCNTs agglomerates within the organism. To this end,  
8 197 five veins per slime mould were video monitored for 100 seconds and the number of DWCNTs  
9 198 agglomerates circulating within the veins were counted (Movie S1).

#### 199 4. *Bioaccumulation of pristine DWCNTs into the slime mould*

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

#### 213 5. *Persistence of pristine DWCNTs into the slime mould*

214 When environmental conditions deteriorate, slime moulds enter a dormant state called sclerotium  
215 until conditions become favourable again. During the sclerotisation process, slime moulds lose 50% of  
216 their total protein content together with 40% of their DNA and 65% of their RNA (Aldrich, 2012). Slime  
217 mould can be easily re-activated from sclerotia if placed in favourable conditions. The aim of the third  
218 experiment was to examine if the DWCNTs internalized after a chronic exposure were excreted by the  
219 slime molds before entering the dormant stage. 240 samples of the slime moulds used in the second  
220 experiment were successfully turned into a dormant state (N=20 for each treatment every week for 6  
221 weeks). The transition from plasmodia to sclerotia was initiated by placing the slime moulds on a moist  
222 filter paper for 4 days to dry. Six months after entering the dormant state, 60 samples were chosen  
223 randomly and were reactivated to test the viability of the sclerotia (5 samples per treatment and per  
224 week). To this end, the sclerotia were soaked in water and placed in petri dishes (55 mm Ø) on agar  
225 gel (1%). Once revived, slime moulds start to explore the agar gel, usually 24h after the reactivation of  
226 the sclerotia.

227 Then, 20 sclerotia were re-activated one year later for each treatment (10 samples from week 1 and  
228 10 samples from week 6). One sclerotium out of 20 could not be re-activated in each treatment. The  
229 day following the reactivation, 120 sec videos of 3 veins per slime mould were recorded with a  
230 binocular microscope and camera MC170 HD Leica to quantify circulating DWCNTs agglomerates. Ten  
231 pictures per slime mould were also taken. DWCNTs agglomerates were counted in 5 zones of 0.5 mm<sup>2</sup>  
232 chosen randomly on each picture leading to a total of 912 counts. As we used sample from the slime  
233 moulds tested in the bioaccumulation protocol, we were able to compare the number of static  
234 DWCNTs agglomerates in the slime mould before and after entering the dormant state.

#### 235 6. *Excretion of pristine DWCNTs from the slime mould*

1  
2  
3 236 The aim of the fourth experiment was to evaluate the **excretion** rate of DWCNTs by the slime mould.  
4 237 Slime moulds were exposed once to DWCNTs using food as a route of exposure. Circular slime moulds  
5 238 (10 mm  $\varnothing$ ) were placed in a square Petri dish (120 mm wide) previously filled with 10 mL of 1 wt. %  
6 239 agar gel. Few oat flakes soaked in a DWCNTs suspension (volume 40  $\mu$ L) were placed 5 mm away from  
7 240 the slime mould. We let the slime moulds feed and explore for 24h. Any food residues remaining after  
8 241 24hours were discarded as they may provide a niche in which microorganisms can rapidly grow. As  
9 242 they explore, slime moulds leave behind a thick mat of non-living, translucent, extracellular slime. To  
10 243 prevents internalisation of DWCNTs that had been already **excreted** and left in the extracellular slime  
11 244 we replaced the agar gel after 48 hours so that the slime mould never explored the same area twice.  
12 245 Slime moulds were observed with a binocular microscope and camera MC170 HD Leica 24, 31, 48, 55,  
13 246 72 and 79 h post exposure. 5 to 10 pictures were taken for each slime mould sample and DWCNTs  
14 247 agglomerates were counted using ImageJ software. 5 veins per slime mould were video monitored for  
15 248 100 seconds to quantify circulating DWCNTs. **We also measured the area of the static agglomerates**  
16 249 **using the software ImageJ to investigate a potential deagglomeration through time.**

#### 20 250 7. *Migration speed following a single exposure to DWCNTs*

21 251 The aim of the fifth experiment was to investigate how a single exposure to DWCNTs affects the slime  
22 252 mould behaviour. In this experiment a circular slime mould (10 mm  $\varnothing$ ) had to migrate on a bridge (35  
23 253 mm long, 100 mm wide) made of 10% w/v powdered oat-agar (Figure 1E). Slime mould were first  
24 254 gently placed in contact with the bridge and then exposed to DWCNTs suspension (CNT group) or CMC  
25 255 only (Control group). Three routes of exposure were tested: a drop of DWCNTs suspension (20  $\mu$ L) was  
26 256 either placed directly on the slime mould (Topical treatment), on few oat flakes resting on the slime  
27 257 mould (Food treatment) or on the bridge (Environment treatment). Hence, 80 slime mould were tested  
28 258 for each treatment and each group, leading to total of 480 assays. After one night's incubation (*ca.* 15  
29 259 h), the distance travelled (in mm) on each bridge was measured with a ruler.

#### 30 260 8. *First pseudopod and expansion rate following a chronic exposure to DWCNTs*

31 261 The aim of the sixth experiment was to investigate how a chronic exposure to DWCNTs affects the  
32 262 slime mould performance. Twenty slime moulds were exposed chronically to DWCNTs or CMC control  
33 263 treatment (see bioaccumulation protocol) for 6 weeks. Each week, a circular sample of each slime  
34 264 mould ( $\varnothing$  13 mm) was introduced in the centre of a petri dish ( $\varnothing$  55 mm) containing a layer of agar (1%  
35 265 in tap water) (Figure 1F). The dishes were placed in a thermoregulated chamber and pictures were  
36 266 taken every 5 min for 36 hours with a digital Canon 70D camera. For each slime mould, we measured  
37 267 the time of appearance of the first pseudopod, which is an indicator of slime mould healing process,  
38 268 as well as the expansion rate. Expansion rate was computed as the difference between the final surface  
39 269 (the area of the slime moulds when it reached a distance of 15 mm from its original location) and the  
40 270 initial surface (the area of the slime mould when we started the experiment) divided by the time to  
41 271 reach the final surface. The pictures were analysed using the software Image J.

#### 42 272 9. *Statistical analysis*

43 273 To assess the difference in the various parameters measured between the treatments, we used linear  
44 274 models, or linear mixed models, or generalised mixed model (function `lm` or `lmer` or `glmer`, Package  
45 275 `lme4`) in R (RStudio Version 1.2.1335). The models were fitted by specifying the fixed effects  
46 276 (explanatory variables) depending on the experiment: treatment (categorical predictor with two  
47 277 modalities: Control and CNT), the route of exposure (categorical predictor with three modalities: Food,  
48 278 Environment and Topical) and /or the exposure duration (continuous predictor, from week 1 to 6).  
49 279 When needed, a random effect: the slime mould identity was also added to the model. The dependent  
50 280 variables that did not fit linear model requirements were transformed using the "bestNormalize"

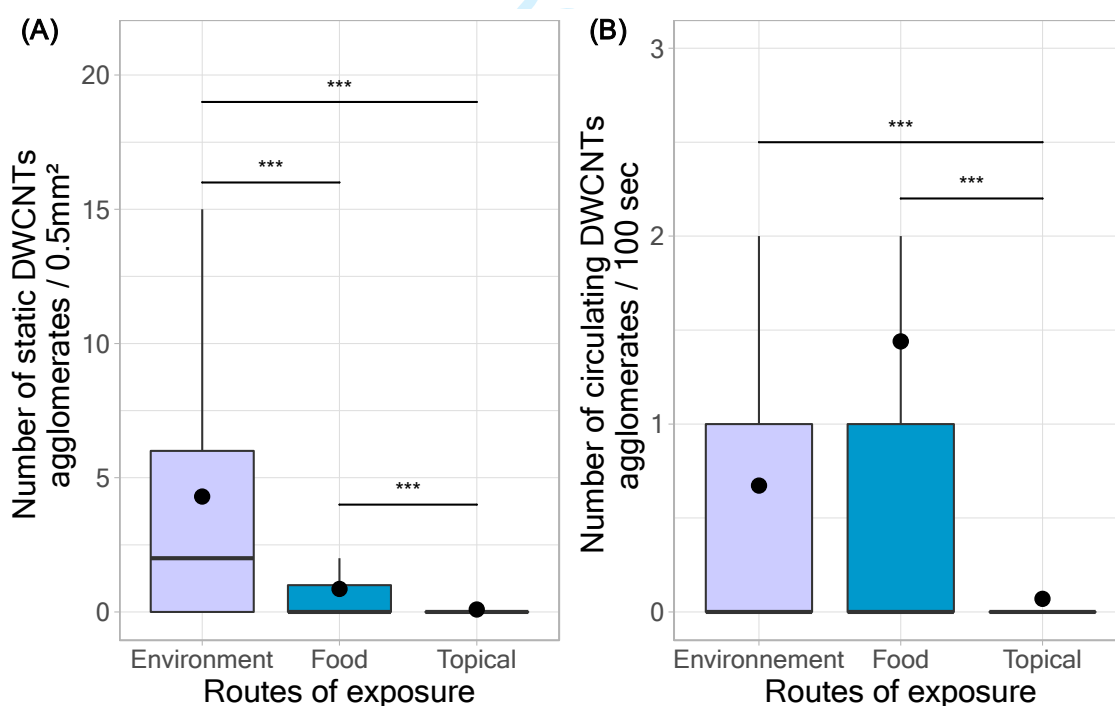
function (“bestNormalize” package). The outcomes of all the models are presented in the supplementary information (Table S1-S11).

## 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.



301  
302 **Figure 2. Internalisation of pristine DWCNTs by slime moulds.** Number of static (A) and circulating (B)  
303 DWCNTs agglomerates as a function of the route of exposure: environment, food or topical. A total of  
304 2342 pictures and 310 videos were analysed. The boxes extend from lower to upper quartile values  
305 and vertical black lines extend to most extreme data point. The horizontal black lines indicate median  
306 values and the dots represent the mean. \*\*\* $p < 0.001$

307

## 2. Bioaccumulation of pristine DWCNTs into the slime mould

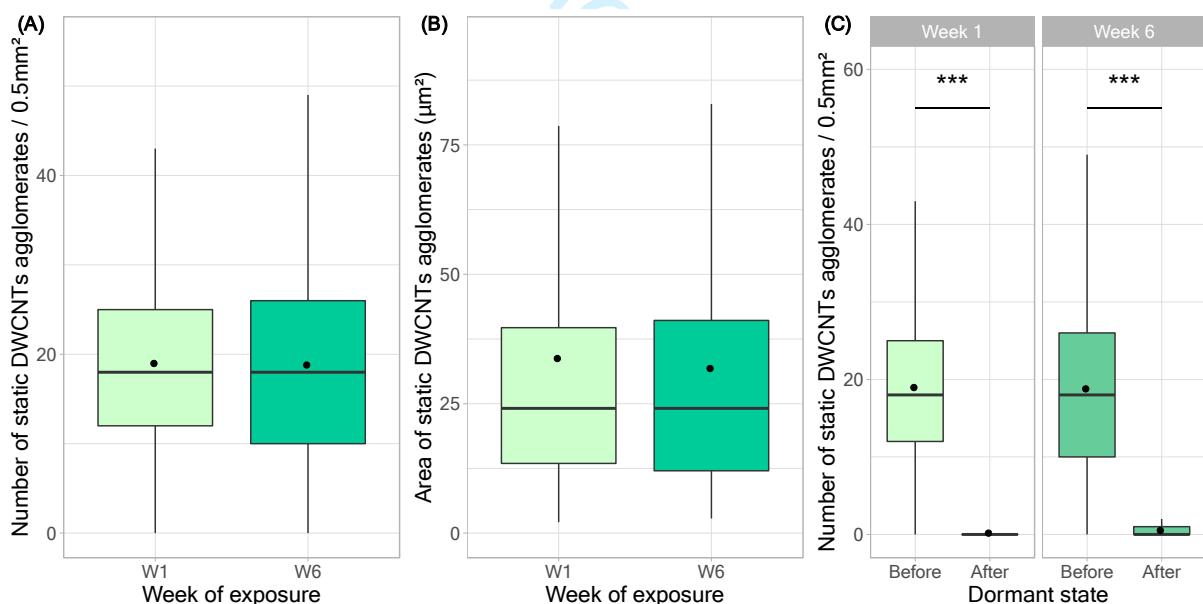
Here, the aim was to evaluate if DWCNTs accumulated within the slime mould when DWCNTs were added daily to the food for an extended period of time (6 weeks). Surprisingly despite daily exposure to DWCNTs, slime mould did not accumulate DWCNTs agglomerates over time. Both the number and the area of DWCNTs 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).

314

## 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.

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328

**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. \*\*\* $p<0.001$

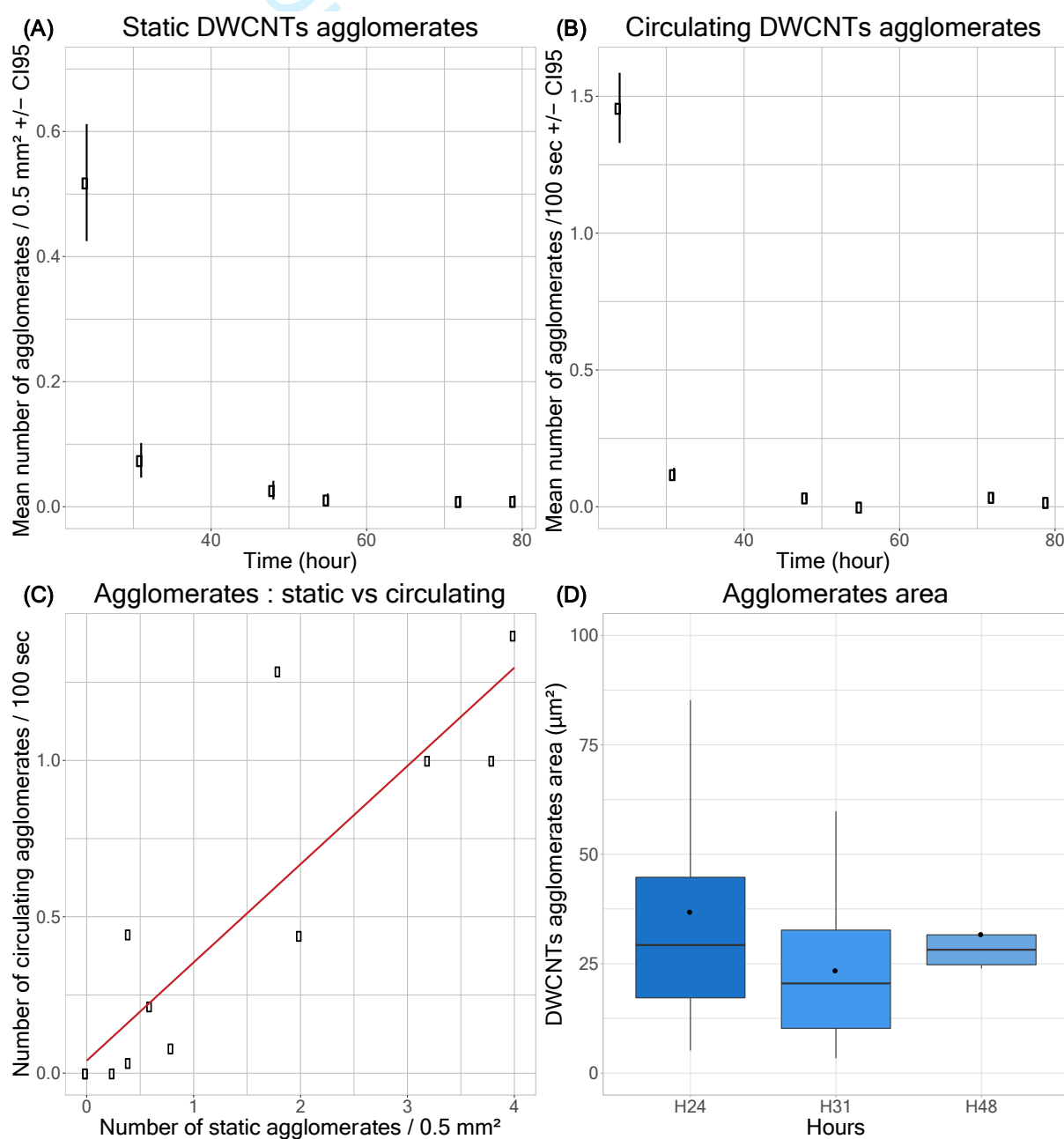
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#### 4. *Excretion of pristine DWCNTs from the slime mould*

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)**

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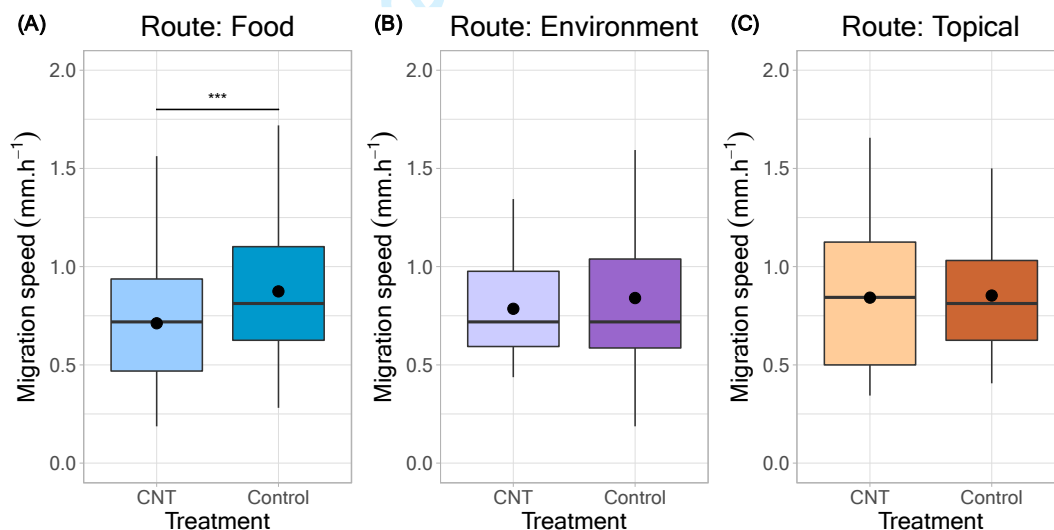
350

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

359

### 360 5. Migration speed following a single exposure to DWCNTs

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



367

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

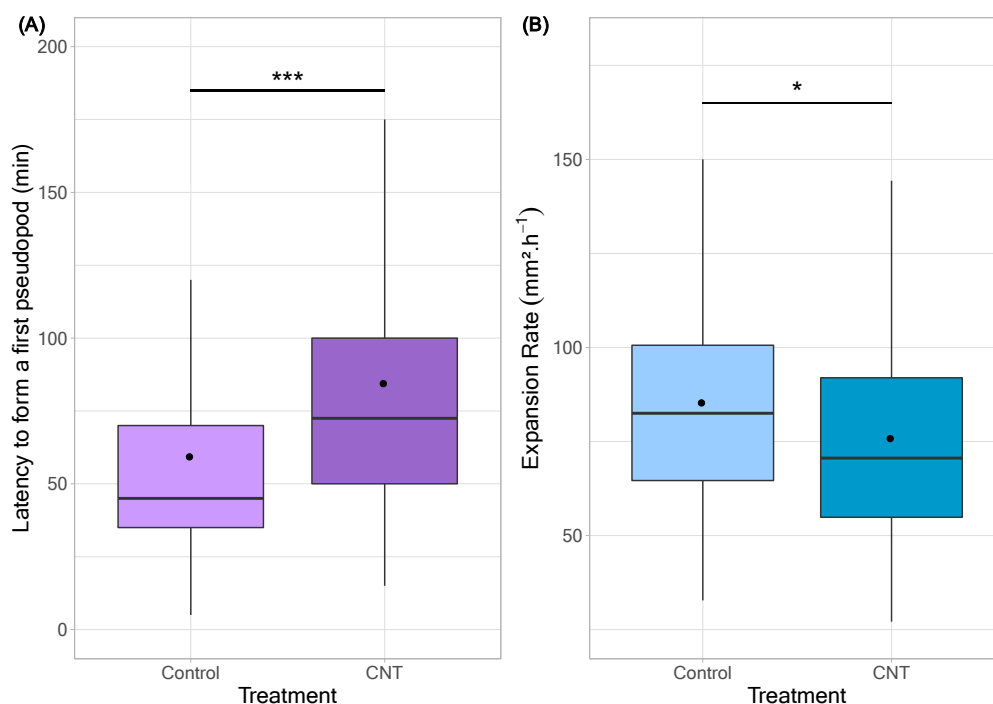
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### 373 6. First pseudopod and expansion rate following a chronic exposure to DWCNTs

374 In the last experiment, the aim was to examine the effect of DWCNTs daily exposure on performance.  
 375 We used two proxies of performance: the appearance of the first pseudopod and the expansion rate.  
 376 The delay in the appearance of the first pseudopod was longer when the organisms were exposed to  
 377 the DWCNTs when compared to the controls (treatment effect  $p < 0.001$ , Table S12, Figure 6A, Figure  
 378 S2). Similarly, the expansion rate was slower for the slime mould exposed to the DWCNTs when  
 379 compared to the controls (treatment effect  $p = 0.026$ , Table S13, Figure 6B, Figure S3).

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

387

## 388 Discussion

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

391 Deciphering the mechanisms responsible for CNTs internalisation into live cells is essential both from  
 392 an ecological point of view but also to design CNT-based delivery systems. CNTs toxicity or efficacy  
 393 depends on their ability to cross cell boundaries and membranes. In slime moulds, we demonstrated  
 394 that, as in other grazing protists (Ghafari et al., 2008) spreading out the CNTs in the environment or  
 395 presenting them together with food were the most effective routes of exposure. As *P. polycephalum*  
 396 continuously explores its environment in search for food, it phagocytes microorganisms and  
 397 continuously secretes a thick extracellular slime (Reid et al., 2012). Hence slime moulds can either  
 398 internalise CNTs through phagocytosis while feeding or trapped CNTs in their own mucus while  
 399 crawling on the substrate. Topical application of CNTs was the least effective route of exposure. The  
 400 presence of black streaks where the CNTs were deposited indicate that the CNTs were not all passively  
 401 internalised through membrane pores. In slime moulds, objects smaller than 1  $\mu\text{m}$  can be endocytosed  
 402 within vesicles (Gizzie et al., 2016; Mayne et al., 2011; Mayne & Adamatzky, 2015). For instance, Mayne  
 403 et al. (2011) showed that magnetite nanoparticles can be internalised by endocytosis in slime moulds.

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

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3 406 via food for 6 weeks, no bioaccumulation was observed within the cell of *P. polycephalum*. Hence,  
4 407 slime moulds were internalising and eliminating CNTs continuously for 6 weeks. Most living organisms,  
5 408 when exposed to CNTs, eliminate these nanoparticles quickly. For instance, mussels, zebrafish,  
6 409 oligochaete, lugworms and earthworms exposed to CNTs for more than two weeks eliminate the  
7 410 nanoparticles within a couple of days (Cheng et al., 2009; Galloway et al., 2010; Hanna et al., 2014; S.  
8 411 Li et al., 2013; Petersen et al., 2008). In the **excretion** experiment, we showed that there was a drop in  
9 412 the number of static and circulating CNTs agglomerates after 24 hours, and the majority of CNTs were  
10 413 **excreted** from the cell at 48 hours post exposure. Therefore, the egestion of CNTs from the cell  
11 414 occurred rapidly which explains why only few CNTs agglomerates persisted throughout the  
12 415 sclerotisation process which last at least 72h in average. We noticed significant black streaks on the  
13 416 filter paper where the slime had migrated before going into dormancy. These observations corroborate  
14 417 that slime moulds egested most of the CNTs back in the environment before entering a dormant state.  
15 418 The egestion of CNTs raises the possibility that these CNTs might have been ingested again when the  
16 419 slime explored the filter paper upon awakening. We attempted to quantify the CNTs agglomerates  
17 420 excreted by the slime moulds in the environment. We noticed that the number of agglomerates  
18 421 detected in the environment were very low (Figure S4). However, our counting methods was rather  
19 422 rough, as agglomerates smaller than 5  $\mu\text{m}$  could not be detected with our binocular microscope.

24 423 CNTs have been shown to impact many microorganism (Chen et al., 2019) and slime moulds are no  
25 424 exception (Gizzie et al., 2016). In our experiment, we observed that both a single and a chronic  
26 425 exposure to CNTs affected the slime moulds behaviour. We noticed that slime moved more slowly  
27 426 when exposed to CNTs while foraging (Figure 5A) or exploring (Figure 6). These differences could result  
28 427 from a defect in the motor or growing systems as movement and growth are intimately connected in  
29 428 slime moulds. We can propose various processes that might explain our results based on *in vitro* and  
30 429 *in vivo* studies performed on various eukaryotic cell lines. First, direct contact with CNTs might have  
31 430 damaged the cell membrane and altered its function (Kang, Mauter, et al., 2008). This could explain  
32 431 the delay in forming the first pseudopod (Figure 6A). Second, internalisation of CNTs might have led to  
33 432 the production of reactive oxygen species which in turn might have altered the structure and function  
34 433 of the cells (Andersen et al., 2009; Chen et al., 2019; Müller et al., 2010; Pacurari et al., 2012; Singh et  
35 434 al., 2014; Srivastava et al., 2011). Last, once inside the cell, CNTs might have interacted with  
36 435 intracellular filaments: actin (Holt et al., 2010, 2012; Shams et al., 2014), DNA (X. Li, Peng, & Qu, 2006;  
37 436 Sargent et al., 2012; Singh et al., 2014) and tubulin polymers (microtubules) (X. Li, Peng, & Qu, 2006;  
38 437 X. Li, Peng, Ren, et al., 2006; Pampaloni & Florin, 2008) leading to migratory defects, DNA breakage  
39 438 and mitotic blockage. Interestingly, the deleterious effects produced by SWCNTs and MWCNT reported  
40 439 in the literature differ. SWCNTs interact mostly with DNA (Dong et al., 2015; García-Hevia et al., 2014;  
41 440 X. Li, Peng, & Qu, 2006; Rodriguez-Fernandez et al., 2012; Sargent et al., 2012; Singh et al., 2014), while  
42 441 MWCNTs interfere with actin and microtubules (Dong et al., 2015; García-Hevia et al., 2014; Rodriguez-  
43 442 Fernandez et al., 2012). However, the reason for such differences is difficult to explain because authors  
44 443 have worked with CNTs exhibiting different morphologies (bundling, length) and may simply have  
45 444 focused on different endpoints. Movement in slime moulds, as mentioned earlier, relies on a driving  
46 445 force generated by the actin and myosin filaments surrounding the veins. Any change in actin filaments  
47 446 induced by CNTs might have induced a reorganisation of the cell cytoskeleton at larger scale and  
48 447 altered cell migration. Slime mould growth is associated to synchronous mitosis occurring every 8 to  
49 448 10 hours. Knowing that microtubules are responsible for DNA segregation during mitosis, any  
50 449 alteration of the microtubule cytoskeleton might have compromised cell growth.

57 450 *Conclusions*

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3 451 The interaction between living organisms and CNTs is a critical issue. Here we showed that CNTs are  
4 452 taken up by slime moulds and could therefore move up the food chain. In addition, we demonstrated  
5 453 that the internalisation of CNTs altered the behaviour of slime moulds and by extend could impede  
6 454 their adaptability to the environment. Our results extend our current knowledge on CNTs and their  
7 455 effects on cellular systems and introduce the slime mould *P. polycephalum* as a new model system to  
8 456 study CNTs toxicity. Slime moulds offer multifaceted advantages. First, being giant cells, they allowed  
9 457 us to observe internalised CNTs agglomerates *in vivo* with little equipment. Second, being single cell  
10 458 organisms, they enable us to measure the impact of CNTs at the cell level on multiple parameters:  
11 459 morphology, motility and differentiation. Third, slime moulds exhibit primitive forms of cognition (such  
12 460 as learning, memory, anticipation, and decision making) and thus provide an ideal model to measure  
13 461 the impact of CNTs on behaviour (Vallverdú et al., 2018). Lastly, slime moulds display many features of  
14 462 animal cells such as a high molecular complexity and a large diversity of signalling molecules (Schaap  
15 463 et al., 2016). As such, they could constitute an interesting model to test CNTs application in drug  
16 464 delivery and medical imaging in future studies.  
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31 472 chemical synthesis of the CNTs sample. M.T and M.M performed the experiments and collected the  
32 473 data. M.T and A.D. performed the statistical analyses. M.T and A.D. produced the figures. M.T., A.D.  
33 474 and E.F. wrote the manuscript. A.D. and E.F. secured funding. All authors gave final approval for  
34 475 publication.  
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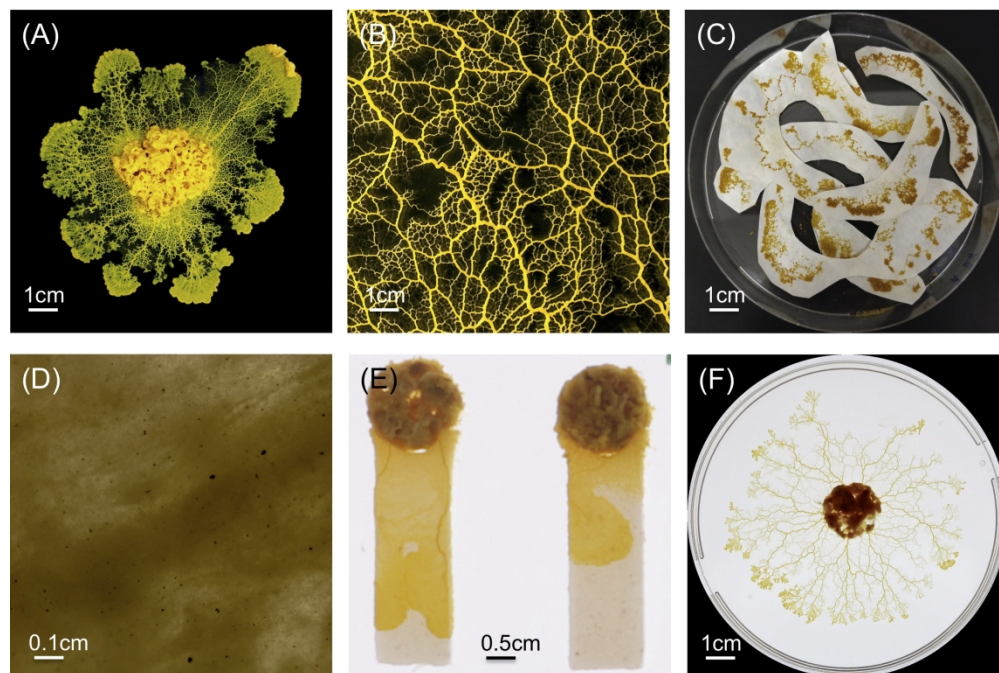


Figure 1: Pictures of *P. polycephalum*. These pictures illustrate (A) a macroplasmodium; the vegetative phase of *P. polycephalum*, (B) a magnified vein network, (C) a sclerotium, the dormant stage of *P. polycephalum*, (D) DWCNTs agglomerates internalised in a slime mould (E) two slime moulds migrating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

222x148mm (300 x 300 DPI)

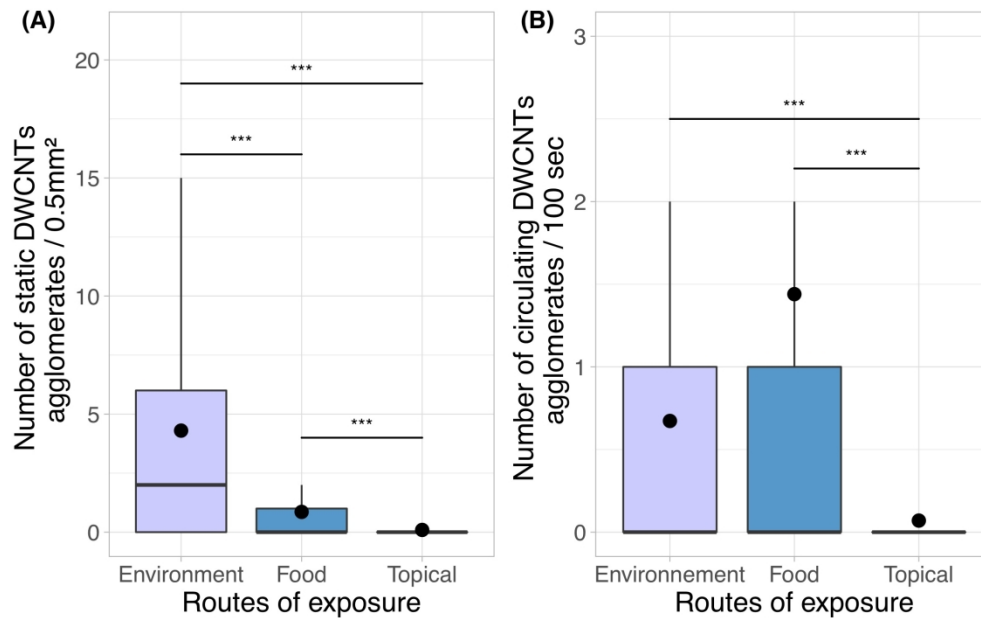


Figure 2. Internalisation of pristine DWCNTs by slime moulds. Number of static (A) and circulating (B) DWCNTs agglomerates as a function of the route of exposure: environment, food or topical. A total of 2342 pictures and 310 videos were analysed. The boxes extend from lower to upper quartile values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

203x127mm (300 x 300 DPI)

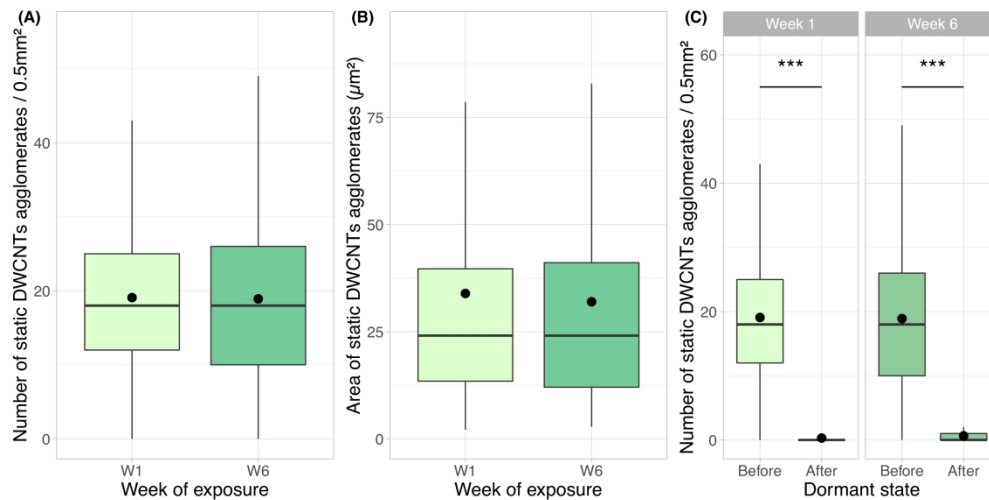


Figure 3. Bioaccumulation and persistence of pristine DWCNTs into the slime mould. (A) Number of static DWCNTs agglomerates as a function of the number of weeks of exposure. (N= 416 and N=368 pictures analysed for week 1 and week 6 respectively); (B) Area of the static agglomerates as a function of the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

304x152mm (300 x 300 DPI)

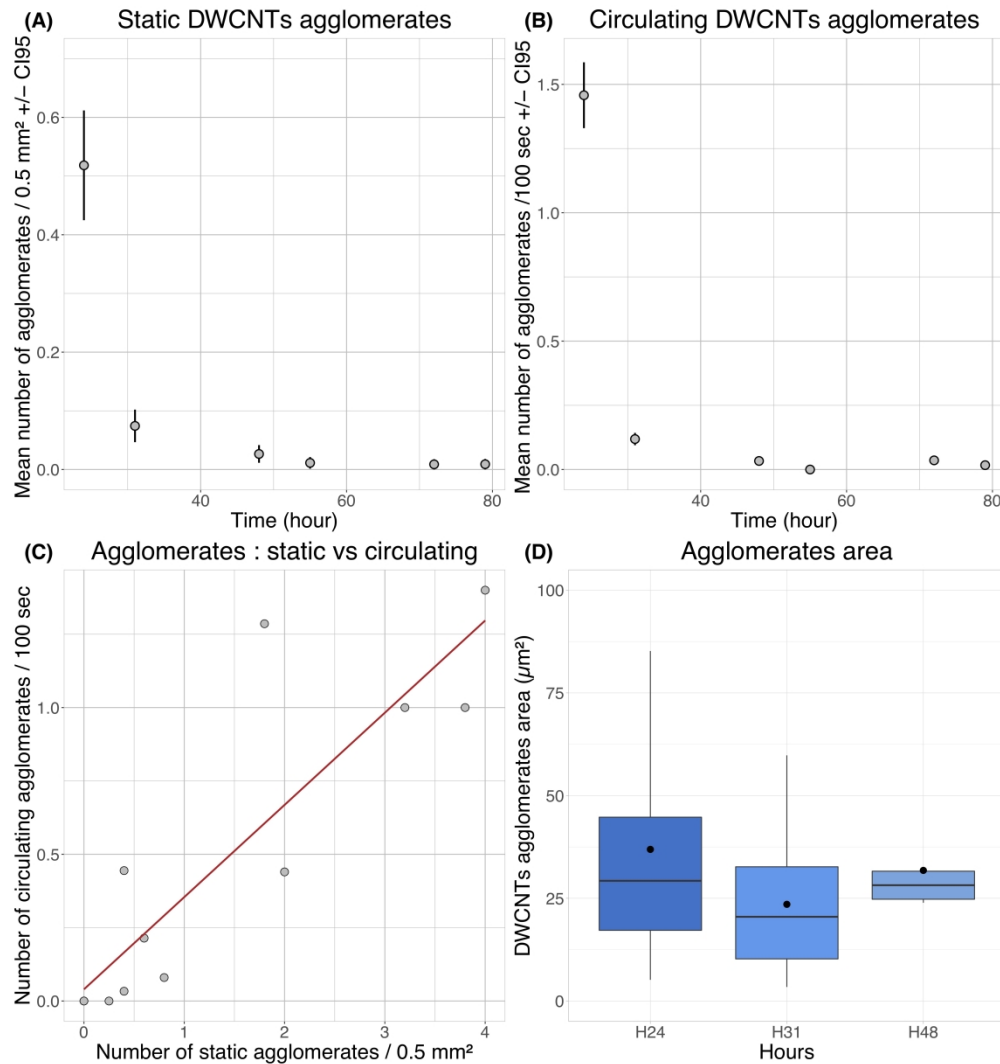


Figure 4: Excretion of pristine DWCNTs from the slime mould. Number of static (per 0.5 mm<sup>2</sup>) (A) and circulating (per 100sec) (B) DWCNTs agglomerates observed for slime moulds exposed once to DWNCTs. N= 1688 pictures and N=352 videos analysed. Error bars are Confidence intervals. (C) Correlation 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<sup>2</sup>=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.

406x431mm (300 x 300 DPI)

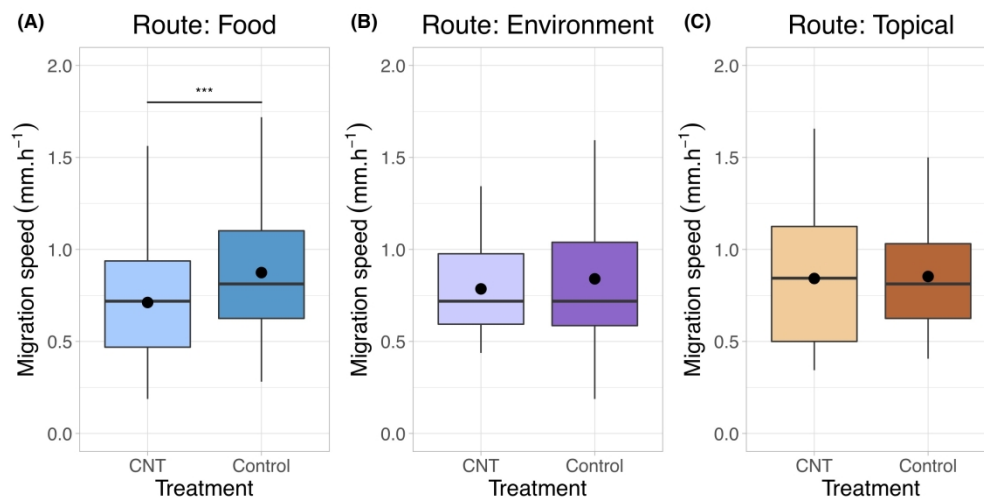


Figure 5: Migration speed following a single exposure to DWCNTs as a function of the route of exposure: environment, food or topical. 80 slime moulds per treatment and per route of exposure were observed. The boxes extend from lower to upper quartile values and vertical black lines extend to most extreme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

254x127mm (300 x 300 DPI)



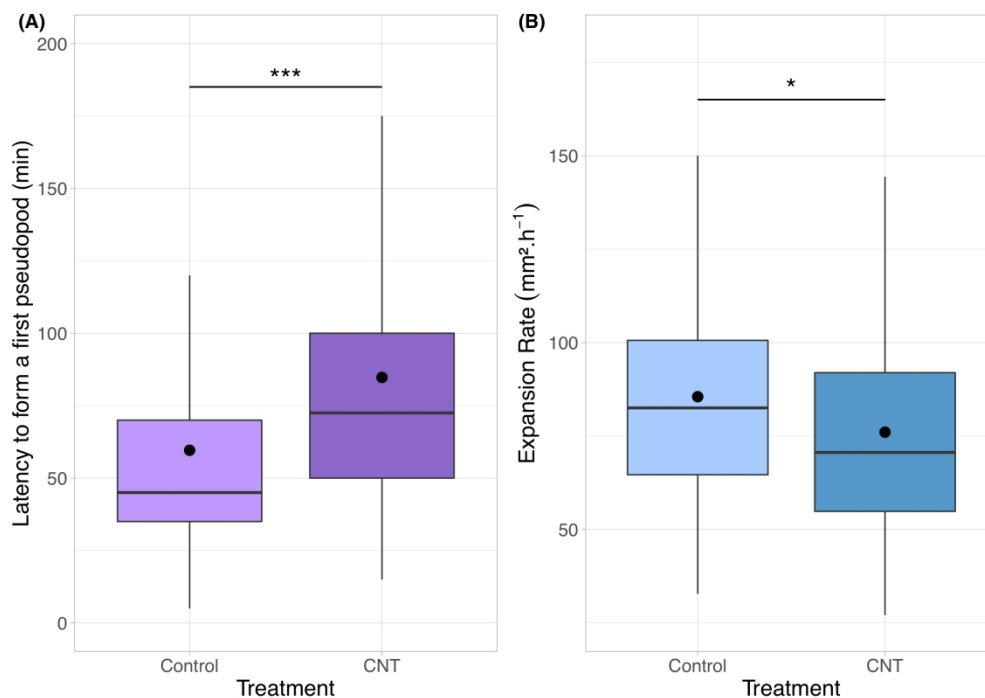


Figure 6: Behaviour analyses. (A) Latency to form a first pseudopod following a chronic exposure to DWCNTs. (B) Expansion rate following a chronic exposure to DWCNTs. 40 slime moulds were observed per treatment and per week for a total of 480 slime moulds. The boxes extend from lower to upper quartile 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)

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4 **Slime moulds response to carbon nanotubes exposure:**  
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6 **from internalisation to behaviour (supplementary materials)**  
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8

9 **Manon Ternois<sup>1,2</sup>, Maxence Mougou<sup>1,2</sup>, Emmanuel Flahaut<sup>2\*</sup> and Audrey Dussutour<sup>1\*†</sup>**  
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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).

Internalisation: Number of circulating agglomerates					
<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				

Internalisation: Number of circulating agglomerates					
Pairwise comparisons					
<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	<0.001	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).**

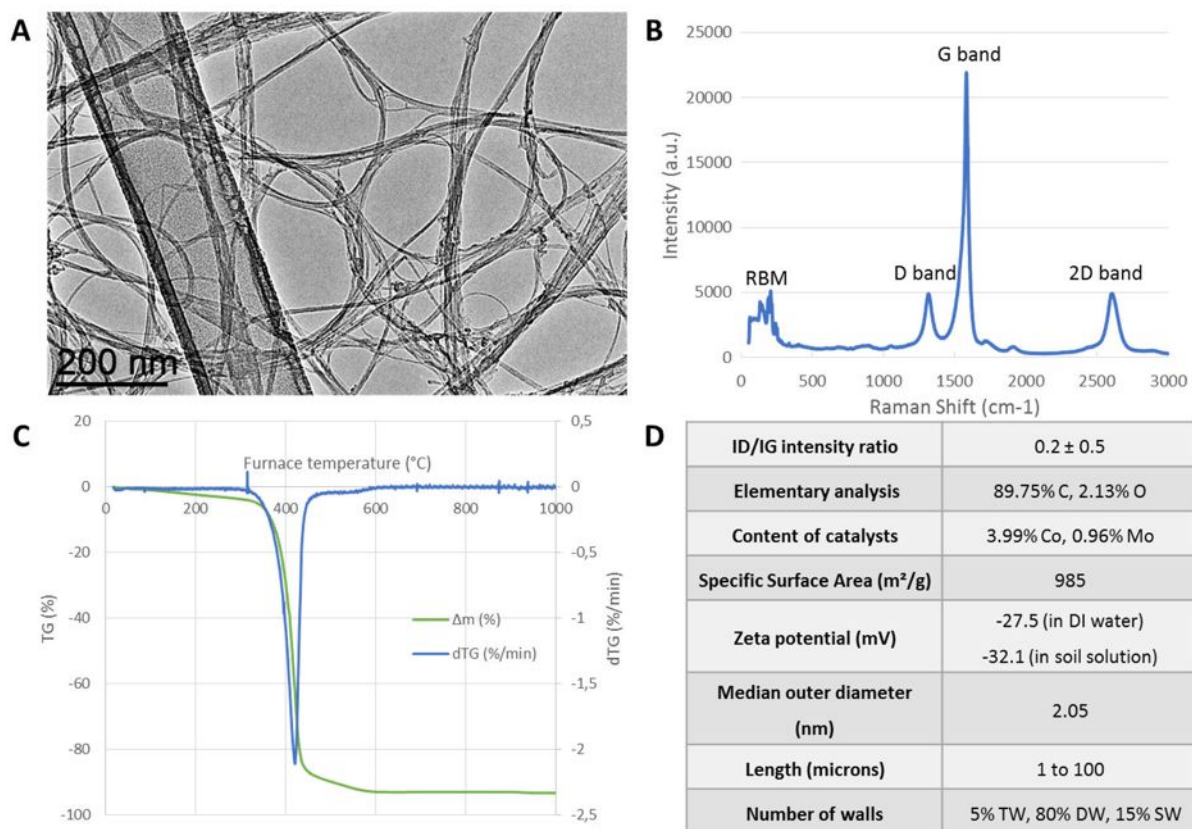
To assess the difference in latency to the first pseudopod between the two treatments; we used a linear mixed model (function `lmer`, error family: Gaussian, Package `lme4`). The model was fitted by specifying the fixed effects: treatment (categorical predictor, NTC vs Control), week (continuous predictor, 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>	<b>Expansion rate</b>				
	<i>Estimates</i>	<i>CI</i>	<i>Statistic</i>	<i>p</i>	<i>df</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				
$N_{\text{Replicate}}$	40				
Observations	480				
Marginal $R^2$ / Conditional $R^2$	0.121 / 0.137				

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

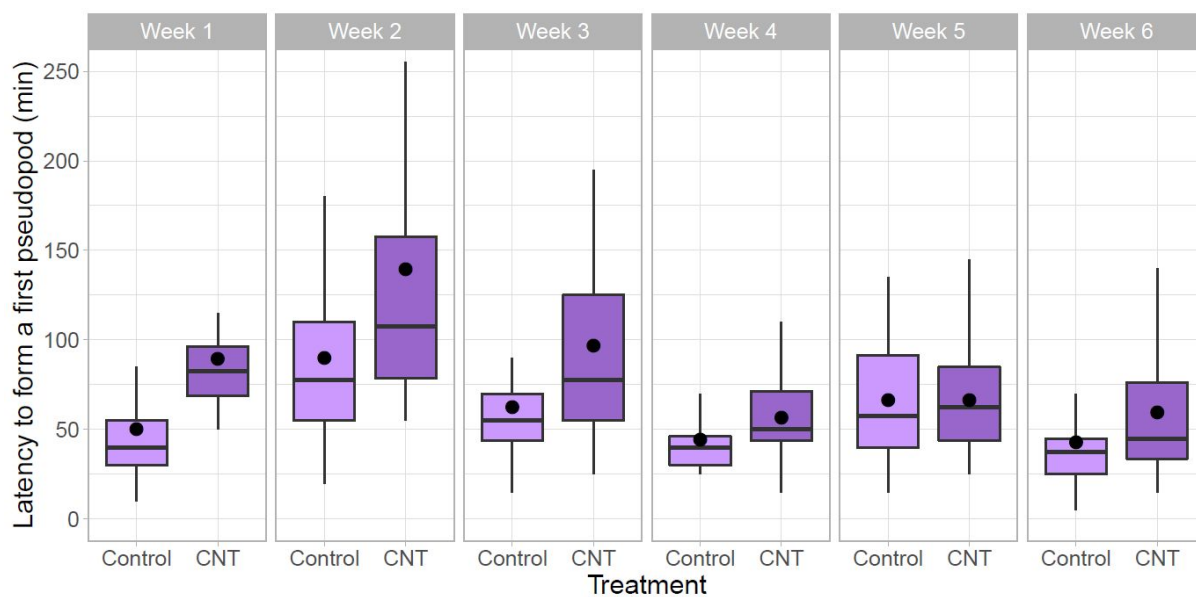
To assess the difference in expansion rate between the two treatments; we used linear mixed model (function lmer, error family: Gaussian, Package lme4). The model was fitted by specifying the fixed effects: treatment (categorical predictor, NTC vs Control), week (continuous predictor, week 1 to week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





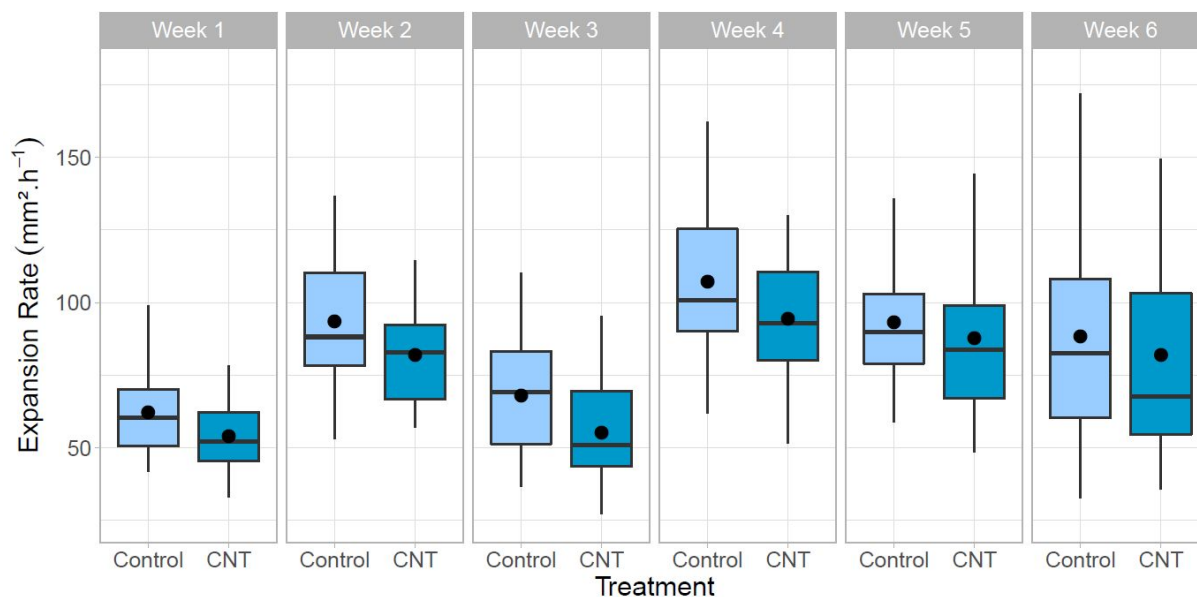
**Figure S1: Figure associated to the methods section.**

**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).



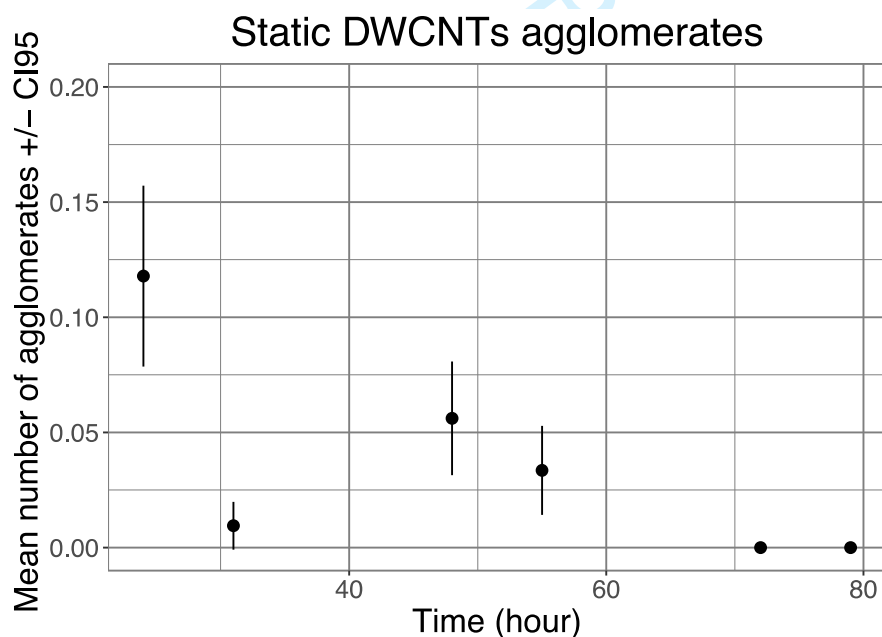
**Figure S2: Figure associated to Figure 6 (A).**

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

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

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