An amoeba phagocytosis model reveals a novel developmental switch in the insect pathogen *Bacillus thuringiensis*.

Beeton M. L.[1], Atkinson D. J.[2] and Waterfield N. R.[1]*

*Corresponding author. Tel. +44(0)1225 384292. Email. bssnw@bath.ac.uk

[1] *Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath, BA2 7AY, United Kingdom.* [2] *Health Protection Agency, Porton Down, Sailsbury, UK.*

Keywords.
Bacterial filaments, *Bacillus thuringiensis*, *B. cereus*, *B. anthracis, Acanthamoeba polyphaga*.

Abstract.

The *Bacillus cereus* group bacteria contain pathogens of economic and medical importance. From security and health perspectives, the lethal mammalian pathogen *B. anthracis* remains a serious threat. In addition the potent insect pathogen *Bacillus thuringiensis* is extensively used as a biological control agent for insect pests. This relies upon the industrial scale induction of bacterial spore formation with the associated production of orally toxic Cry-toxins. Understanding the ecology and potential alternative developmental fates of these bacteria is therefore important. Here we describe the use of an amoeba host model to investigate the influence of environmental bactivorous protists on both spores and vegetative cells of these pathogens. We demonstrate that the bacteria can respond to different densities of amoeba by adopting different behaviours and developmental fates. We show that spores will germinate in response to factors excreted by the amoeba, and that the bacteria can grow and reproduce on these factors. We show that in low densities of amoeba, that the bacteria will seek to colonise the surface of the amoeba as micro-colonies, resisting phagocytosis. At high amoeba densities, the bacteria change morphology into long filaments and macroscopic rope-like structures which cannot be ingested due to size

exclusion. We suggest these developmental fates are likely to be important both in the ecology of these bacteria and also during animal host colonisation and immune evasion.

1. Introduction.

Early observations by Joseph Leidy in 1849 first identified microscopic filaments in the gut lumen of termites, but at the time the genus of these organisms was unknown and he simply referred to them as *Arthromitus*. It was not until a study by Margulis *et al.*, looking at boiled intestines of insects that these *Arthromitus* were identified as *Bacillus cereus* group bacteria (Margulis et al., 1998). Feinberg also characterised a number of *Bacillus cereus* strains as commensal inhabitants of termites, millipedes, sow bugs and cockroaches (Feinberg et al., 1999). The *Bacillus cereus* group bacteria are also known as notorious infective agents of both invertebrates (*B. thuringiensis*) and mammals (*B. anthracis*). Recent observations of filamentous *Bacillus* in insects have focused on the association with the hindgut of *Blaberus giganteus*, the large tropical American cockroach (Feinberg et al., 1999). This study observed the effects of diet on the cockroach and the subsequent effect on filamentation of the associated *Bacillus* strains.

Filamentous morphology in bacteria has also been observed in numerous human pathogens as a proposed mechanism by which the bacteria might evade cell-mediated immunity (Justice et al., 2008). For example, uropathogentic *E. coli* (UPEC) form filaments on the rat bladder epithelium. Mutants lacking functional SulA, the protein responsible for preventing FtsZ ring formation and subsequent septation, were found to be attenuated in a mouse model. Crucially this was only in the presence of a functional host TLR-4 mediated response confirming a critical role for filamentation in cellular immune evasion (Justice et al., 2006). It has been suggested that this filamentation response first evolved to counter predation by protozoa in the soil, and has subsequently been redeployed for evading the cellular immunity of metazoan hosts (Molmeret et al., 2005) (Harb et al., 2000). It has also

previously been shown that *Salmonella* can form filaments *in vitro* (Stackhouse et al., 2012), in response to both amoeba and animal cells (Birmingham et al., 2005).

Interestingly the formation of filaments and larger multi-filament “ropes” has previously also been observed in another member of the *Bacillus* genus, *B. subtilis* (Mendelson et al., 2003). In this case the investigators explain the developmental switch as an alternative mode of motility. While both the *B. cereus* group bacteria and *B. subtilis* are abundant in the soil, genomic analysis has confirmed that the *B. cereus* group cannot efficiently utilise complex plant carbohydrates like *B. subtilis* (Ivanova et al., 2003). Rather they are adapted for the utilization of animal derived material. It has been proposed that they exist mainly as a dormant spore form in the soil until they can infect an animal host. Soil dwelling bacteria are under constant threat of predation by grazing amoeba. Indeed this selection pressure is likely to be far higher than that imposed by the less frequent likelihood of death resulting from phagocytosis by an animal professional immune cell. There has been a great deal of interest in the ecology of known animal pathogens when not in their recognised hosts. It is desirable to understand the environmental interactions of the important animal pathogens *B. thuringiensis* and *B. anthracis* for agricultural, ecological and health-security reasons.

In this study we examined how the entomopathogenic bacterium *B. thuringiensis* (and two other members of the *B. cereus* group) respond to the bactivorous amoebae *Acanthamoeba polyphaga* and *Dictyostelium discoideum*. We show that these bacteria can sense the presence of factors released by amoeba to trigger spore germination. In addition we show that vegetative cells respond by either (i) chemotactic homing, attachment and surface colonisation or (ii) by inducing a developmental switch into a filamentous form. These filaments may remain surface attached or swim freely, ultimately forming macroscopic rope like structures. This developmental stage correlates with previous observations of “Arthromitus” filaments seen in insect guts (Margulis et al., 1998).

2. Materials and Methods.

2.1 Strains and culture conditions.

Bacterial strains used in this study were as follows; *B. thuringiensis israelensis* 4Q7 (a strain cured of all plasmids), *B. thuringiensis israelensis* 4Q5 (contains only the pBtoxis plasmid), and *B. thuringiensis israelensis* 4Q7 *gfp* labelled, *B. thuringiensis israelensis* 4Q7 Δ*plcR* (Salamitou et al., 2000), *Bacillus cereus* (NCT 14579) and *B. anthracis* Sterne ASC 1. Bacterial cultures were routinely grown in LB broth at 28^{o}C with agitation. The *Acanthamoeba polyphaga* and *Dictyostelium discoideum* (AX2) were maintained as monolayers in 76 cm^{3} tissue culture flasks at 23^{o}C in axenic PYG and HL5 media respectively.

2.2 Co-culture assays.

Attached amoeba were scrapped off the bottom of a prior culture with a sterile cell scraper and harvested from the medium by centrifugation at 1 000 xG for 5 minutes. The cells were washed twice in sterile PBS (harvested using centrifugation) before finally re-suspending in a volume of PBS equivalent to the starter culture. They were then seeded into the desired medium in a 25 well plate using 5-fold dilutions *A. polyphaga* or *D. discoideum* were seeded and allowed to adhere for 1 hour. Bacteria from overnight cultures were pelleted at 13 000 xG for 2 minutes and washed twice in sterile PBS. Ten-fold dilutions of bacteria were then added on top of the adherent amoeba and co-incubated at 25^{o}C. Each well was observed after 4 hours and 24 hours. Light microscopy of samples was performed and visualised (including time-lapse filming) using the NIS-elements “Br” software and a Nikon inverted microscope.

2.3 Conditioned PBS.

A. polyphaga were washed, as described above, and then incubated as monolayers in 76 cm^{3} tissue culture flasks at 23^{o}C in sterile PBS overnight.

The following day the PBS was decanted and any detached amoeba were removed by gentle centrifugation at 1000 xG for 5 minutes. To ensure full removal of amoeba cells the conditioned PBS was then passed through a 0.22 µm filter. For the determination of growth factors in the conditioned PBS aliquots were either passed through a 5 kDa molecular weight cut-off column or heated to 70^{o}C for 15 minutes.

2.4 Preparation of samples for scanning electron microscopy SEM.

The co-culture assays were set up as previously described, but with a Thermanox cover slip (Nunc) on the base of the well as a surface for the amoeba to adhere to. At the required point the medium was aspirated and replace with the fixative solution of 2.5% glutaraldehyde and 1% potassium ferrocyanide, post-fixed in aqueous 1% osmium tetroxide and stained in2% aqueous uranyl acetate in the dark. The sample was then dehydrated through an acetone series and dried. Samples were then coated with gold and analysed under a JEOL JSM6480LV scanning electron microscope (JEOL Tokyo, Japan).

2.5 Preparation of samples for transmission electron microscopy (TEM).

Media was aspirated and replaced with 2.5% glutaraldehyde and PBS fixative solution and post fixed in aqueous 1% osmium tetroxide and 2% potassium ferrocyanide. Samples were then encapsulated in 3 % agarose and stained with 2 % aqueous uranyl acetate in the dark. The sample was then dehydrated through an acetone series and infiltrated and embedded in Spurrs epoxy resin (TAAB, premix). Ultra-thin sections were cut using an ultra-mirotome (Leica, Reichert Ultracut E). Sections were then analysed under a JEOL JEM1200 transmission electron microscope (JEOL Tokyo, Japan).

2.6 Inhibitors of phagocytosis.

A. polyphaga were exposed to commercially obtained cytochalasin D (1µg/ml), cycloheximide (100 µg/ml) or bafilomycin (5µM) for 2 hours prior to

the addition of either bacterial spores or vegetative cells. A further two hours of incubation was required to allow for phagocytosis to occur. The numbers of spores or vegetative cells within the amoeba were then counted by light microscopy. In each assay 20 amoeba per well were examined in triplicate giving a total number of 60 amoeba per treatment. This was repeated three times and the data was collated and analysed. To determine statistical significance the Mann-Whitney statistical test was employed to compare the number of phagocytosed cells between the treated and untreated.

3. Results.

3.1 Behavioural and morphological developmental switches in *Bacillus* can be triggered by amoeba.

We examined the interactions between the closely related *B. thuringiensis*, *B. cereus* and *B. anthracis* with the bactivorous amoebae *A. polyphaga* and *D. discoideum* by static co-incubation of vegetative bacterial cells in PBS with the amoeba at a range of cell densities and ratios. Certain repeatable outcomes of these interactions were observed (Figure 1). The density of the amoeba dictated the behaviour of the bacteria. At a high density of amoeba (Figure 1, top row) the bacteria ceased to septate and grew as long filaments. Conversely, at a low density of amoeba (Figure 1, bottom row), the bacteria would show chemotactic swimming toward the amoeba (see movie nw1). They would show localised attachment to specific areas upon the amoeba surface (Figure 2). This region was found at the trailing edge of the motile amoeba. Heavy colonisation of the cell surface was seen to lead to the eventual death of the amoeba. It is not known if this was due to direct pathogenic activity of the bacteria, a competition for resources or a physical disruption of the amoeba cell surface properties. When an “intermediate” density of amoeba was used (Figure 1, middle row) we saw that the phenotype of the bacteria became dependent upon the ratio of bacteria/amoeba. At a high bacteria/amoeba ratio, the bacteria again completely colonised the surface of amoeba, ultimately killing them. At a low

ratio, the bacteria once again adopted the filamentous morphology. An intermediate phenotype of the bacteria is illustrated on the middle panel of Figure 1. This represents loose chains of elongated cells in addition to amoeba colonisation. It should be noted that in sporadic cases we observed apparent bacterial persistence in the amoeba cytoplasm. It was not clear what variables were triggering this and whether the bacteria were actively invading the amoeba or simply being taken up by phagocytosis. Nevertheless in these cases the bacteria were seen to persist for at least 6 hours and often overnight (data not shown). Incubation in amoeba free PBS resulted in the sporulation of the bacteria as expected (data not shown).

When spores were added to the amoeba, we saw germination followed by filament formation (see movie nw2). Significant phagocytosis of many spores was seen to occur showing them to be a target for amoeba predation (see movie nw3). It was also possible to observe germinated spores inside the food vacuoles of the amoeba (Figure 3). However it should be noted that we also observed significant germination of spores in the surrounding medium. It is therefore difficult to determine the precise sequence of events and confirm whether spores were able to germinate once taken up by the amoeba. In the absence of amoeba as expected no germination in the PBS was seen. These experiments confirmed that the bacteria were able to sense and respond to the presence of the amoeba, either by germination (when in spore form) or chemotaxis and attachment and/or filamentation of vegetative cells.

To examine the impact of PapR-PlcR mediated bacterial quorum sensing (QS) upon these phenotypes we repeated these experiments using a *B. thuringiensis* 4Q7 Δ*plcR* strain. The response of this strain was again identical to that of the wild-type demonstrating that this QS system is not involved. This was further confirmed by the exogenous application of high concentrations of a biologically active synthetic PapR based on the 9 amino acid terminal residues of both the *B. cereus* strain and *B. thuringiensis* strains used (data not shown) (Slamti and Lereclus, 2002). Again these experiments confirmed no involvement of the PapR QS system. In should be noted that these phenotypes were also exhibited by *B. anthracis* strain Sterne ASC1

(performed in class III containment - data not shown), and in this species the *papR* QS pheromone gene is frame-shifted rendering the QS system inactive. Furthermore, *B. thuringiensis* strain 4Q7 has been cured of all plasmids indicating that chromosomal factors alone are responsible for this effect. This is supported by the observations that the same phenotypes are also seen with *B. cereus* and *B. anthracis* in addition to experiments using *B. thuringiensis* strain 4Q5 (data not shown) which has lost all but the pBtoxis plasmid (Berry et al., 2002).

To determine the influence of culture "substrate" on this effect, we also challenged bacteria with amoeba on nutrient free solid agar medium made with amoeba growth salts solution alone. In this environment the filament formation was even more extensive with all bacteria adopting a filamentous form. Again in this form the amoebas were unable to phagocytose them (see movie nw4).

Finally we also tested the effect of co-incubation of a second model amoeba, *Dictyostelium discoideum*, with *B. thuringiensis* 4Q7. Figure 4 illustrates that again the bacteria ceased to septate and grew as long surface attached filaments in a manner similar to that for the *Acanthamoeba* experiments.

3.2 Description of the *Bacillus* filaments.

Filaments ranged in size from 20 µm up to several mm and were motile. Under light microscopy at 40x no obvious signs of septation could be seen suggesting a continuous hyphae like structure. To examine this further we performed scanning electron microscopy (SEM) on the early stages of bacterial-amoeba interactions. The filamentous bacteria were seen associated with amoeba often at several times their normal length 15 µm (Figure 5A). Elongating cells were smooth in appearance and had no surface signs of septation. The SEM micrographs also suggested that the amoebas were actively attempting to phagocytose the adherent bacilli as evidence by the formation of phagocytic cup like structures (Figure 5B). Additionally the SEM data gave a magnified view of the localisation of bacilli to regions upon the

surface of the amoeba (Figure 2 and 5B). Despite many attempts at visualization of the internal structure of the filaments to observe septa directly using Transmission Electron Microscopy (TEM) failed due to an inability to find suitable longitudinal sections.

It was noted that over time, free swimming filaments would entwine and weave together to form complex rope like structures (Figure 6A) which became visible by eye. Although it was possible to watch the formation of ropes from filamentous cells using time-lapse microscopy, we nevertheless used a Gfp-labelled *B. thuringiensis* to demonstrate the rope formation and rule out the possibility of fungal contamination (Figure 6B). Furthermore the "quality" of the ropes depended upon the levels of nutrients available. For example, addition of 1% rich PYG amoeba medium to the PBS gave a more open rope structure (Figure 6C) while the addition of 10% PYG gave thicker and denser structures (Figure 6D). Furthermore, when ropes which had formed in amoeba conditioned PBS were transplanted into rich growth media (LB or PYG), the filaments and ropes were observed to disintegrate apparently by septation, and release single motile bacilli once more. This demonstrates that this developmental state is reversible and dependent upon environmental factors.

3.3 Internal localisation of *Bacillus* using TEM.

The condition and location of internalised vegetative cells and spores were examined using TEM. As discussed above, we often observed amoeba which were seen to contain a large number of persisting bacilli, moving around with a similar vitality to uninfected cells. While it was possible to clearly see phagocytosed spores inside large food vacuoles using light microscopy (movie nw3), the location of internalised vegetative cells could not be determined. We therefore used TEM to determine if these vegetative cells were membrane bound or free in the cytoplasm. As can be seen in Figure 7AB, the majority of the bacilli were contained within small tight fitting membrane bound vesicles. TEM also revealed germinated spores within the food vacuoles of the amoeba (Figure 7C). Closer magnification revealed an

unknown residue surrounding the internalised spores (Figure 7D) which we speculate represents the remains of the shed spore coat.

3.4 Bacterial uptake mechanisms.

In order to further investigate the uptake mechanism of the internalised bacilli, we used various drugs to inhibit different aspects of phagocytosis. When compared with untreated amoeba, only the eukaryotic translation inhibitor, cycloheximide significantly reduced the number of internalised spores (median of 3 spores and 1 spore per amoeba, respectively; p value of <0.001) (Figure 8). When compared with untreated amoeba those treated with bafilomycin contained more spores (median of 3 spores and 4 spores per amoeba, respectively; p value of <0.001). The comparison of amoeba with those treated by the potent inhibitor of actin polymerization, cytochalasin D showed no significant difference. There was greater uptake of vegetative cells (1 cell per amoeba) in the untreated amoeba compared with cytochalasin D and cycloheximide in which no bacterial uptake was seen. Bafilomycin treated amoeba contained significantly more vegetative cells than the untreated cells (p value of <0.05). There was a significant difference when comparing the uptake of spores versus vegetative cells in all treatments with more spores being taken up relative to the vegetative cells (p value <0.001). This suggests that the vegetative cells are activity resisting phagocytosis in these assays, consistent with surface colonisation and micro-colony formation described above.

3.5 Effects of *A. polyphaga* conditioned PBS on *B. thuringiensis* development.

To determine if the germination and filamentous phenotypes were a result of soluble secreted/excreted substances from the amoeba or contact dependent signalling we exposed *B. thuringiensis* to filtered PBS which had been conditioned overnight by incubation with amoeba. Vegetative cells incubated in this conditioned PBS again showed the filamentous developmental switch. This medium also induced the germination and outgrowth of spores.

Importantly, the bacilli were able to utilise nutrients in the amoeba conditioned PBS to divide and grow, showing a typical sigmoidal growth curve when cultured at 37°C with shaking aeration. In order to gain a better understanding of the molecules which were responsible for the germination signal we heated the conditioned PBS to 70^{o}C for 15 minutes in order to denature any protein structures. Nevertheless the spores were still able to germinate confirming that the signal is not heat-labile. We used molecular weight cut-off columns to size fractionate the conditioned medium, which confirmed that the germination signal was less than 5 kDa. Mass-spec analysis of the amoeba conditioned PBS indicated a heterogeneous mix of molecular species which varied between bioactive replicates (data not shown).

4. Discussion.

It has been proposed that single celled eukaryotyes in the environment can act as "training grounds" for the evolution of novel mechanisms of cellular immune evasion (Molmeret et al., 2005) (Waterfield et al., 2004). Indeed bacteria have been combating environmental bactivorous eukaryotes such as protists and nematodes for a significantly longer period of evolutionary time than they have had to resist immune killing by higher animals such as insects or mammals. The sheer number of "interactions" between protists and bacteria in the soil and the continuing selection pressure of predation from protists will continue to drive an intense arms race of genetic novelty. Evidence supporting this hypothesis has previously come from studies examining the interactions between *Legionella pneumophila* and *Acanthamoeba*. *L. pneumophila* has been shown to multiply and kill both human macrophages and free-living amoebae and that the same genes are expressed for replication in *Acanthamoeba* and macrophage (Segal and Shuman, 1999) (Rowbotham, 1980) (Horwitz and Silverstein, 1980). These observations have led to suggestions that bacteria evolved to become intracellular pathogens after surviving phagocytosis and adapting to the intracellular environment of protists. Here we have shown that the *Bacillus cereus* group bacteria can modify their behaviour and developmental fate

depending upon the presence and the relative cell numbers of a predatory amoeba.

The surface colonisation response. When exposed to a low density of amoeba, the bacteria used flagella-mediated motility to swim to the amoeba, showing chemotactic homing. They then attached via their pole to the surface of the amoeba, and localized to specific regions of the cell. Time-lapse microscopic analysis revealed that this area usually represented the trailing edge of motile amoeba typically near regions of contractile vacuole discharge. Micro-colonies of bacteria formed in these regions as a result of multiple attachment events, although the role of cell replication in expansion of these micro-colonies remains unclear. This phenotype resembled “pack swarming” described for *Pseudomonas aeruginosa* (Dacheux et al., 2001). Dacheux *et al.* demonstrated that when *P. aeruginosa* were co-cultured with neutrophils and macrophages, the bacteria rapidly accumulated on the surface of the immune cell. Chemotactic mutants of *Pseudomonas* lacked this phenotype. We speculate that the bacilli are being attracted by a “food signal” released by the amoeba into the PBS medium.

While surface attached bacilli on the whole appeared to be resistant to phagocytosis, some cells were seen to be internalised sporadically. It is not clear if this represented a failure of the bacteria to prevent phagocytosis, deliberate invasion or a more cooperative farming behaviour. It is formally possible that this situation represents bacterial “farming” by the amoeba, feeding the bacteria with excretions and on occasion harvesting them.

The filament and rope formation response. When the bacteria were challenged with a higher density of amoeba the bacterial phenotype switched to the formation of long filaments. This was seen to occur by bacilli both free in the medium and also by surface attached cells, which sometimes detached and swam away once they became long filaments. Interestingly, when an “intermediate” density of amoeba was used, the developmental fate of the bacteria became dependant up the ratio of bacteria/amoeba. The observations are consistent with the bacteria reacting to a density dependant

"signal" from the amoeba. High density of amoeba leads to a high concentration of "signal" and *vice versa*. In the intermediate concentration of "signal", bacterial density may influence the outcome through "titration" or "signal metabolism" effects.

Light microscopy and SEM showed the surface of the bacterial filaments to be smooth, suggesting the bacteria simply cease septation and grow as hyphae. The high swimming motility and spiralling movement of these filaments was seen to be the driving force behind the entwining of multiple neighbouring filaments to form macroscopic multicellular rope like structures. Continued elongation then served to lengthen these "ropes". The use of a Gfp-labelled *B. thuringiensis* strain confirmed the identity of these hyphae and ruled out the possibility of fungal contamination. The molecular basis for this phenotype is still unknown, but by using a *B. thuringiensis* 4Q7 Δ*plcR* strain or by exogenously applying a synthetic PapR quorum sensing peptide to both *B. thuringiensis* and *B. cereus* we were able to rule out any involvement with the PlcR regulon which is known to regulate transcription of many secreted factors and autolysins. The observation of this phenotype in *B. anthracis* cultures (which lacks this QS system) also supported this finding. Interestingly white "flecks" are often seen in the large scale cultures used to produce vaccine strains for anthrax (unpublished observations), suggesting this developmental state is also being triggered under those conditions. It is possible that this could have an influence of vaccine production and quality.

The growth of *Bacillus* in a filamentous form has previously been noted. In 1849 Joseph Leidy first described the growth of what was termed *Arthromitus* cells attached to the guts of termites. At the time it was not known that these cells belong to the genus *Bacillus* until the work of Margulis *et al.*, who examined the boiled intestines of a number of different soil associated insects (Margulis et al., 1998). This developmental state is possibly more representative of how the *Bacillus cereus* group bacteria live in the environment, when not in their resistant spore dispersal form. The short, motile single cells maintained in laboratory culture scenarios may in fact be a rather transient developmental form in nature. Previous studies with *B.*

anthracis also showed that cells were able to take on a filamentous phenotype when grown in an artificial rhizosphere system (Saile and Koehler, 2006).

We speculate that filament formation in *Bacillus cereus* group represents a primitive, yet effective mechanism of evasion from phagocytic predation from either protists or professional immunity cells such as insect hemocytes or mammalian macrophages.

The spore germination response. Germination and filament formation of spores and vegetative cells in *A. polyphaga* conditioned PBS confirmed that the developmental signal was not contact dependant. In addition, the ability of the bacteria to grow in this conditioned PBS suggests that simple stress-response is not a contributing factor. Indeed incubation in normal PBS led to the starvation-stress triggered production of bacterial spores as expected. We therefore conclude that the *A. polyphaga* excrete factors/nutrients which the *Bacillus* can utilize for replication. An alternative source of nutrients would be amoeba cell death and lysis, although in our experiments we saw no obvious evidence of this. Attempts at using mass-spectrometry to identify the relevant nutrients, were inconclusive, indicating a heterogeneous mix of small molecules, which was variable between different replicates of conditioned PBS. Nevertheless we were able to determine that the compounds used by the bacilli were heat stable up to 70^{o}C and less than 5 kDa, which would include small organic molecules and ions. This correlates with our finding which showed conditioned PBS could also promote germination of spores, a process which requires the presence of monovalent cations (Foerster and Foster, 1966). Light microscopy revealed the germination of spores in response to the presence of amoeba and TEM revealed germinating spores within the food vacuoles. Nevertheless it was not possible to determine if germination was occurring inside the vacuoles or had begun just before ingestion. Nevertheless this correlates with previous reports which show that *B. anthracis* spores in fact require phagocytic uptake by lung macrophages before they can germinate in the aetiology of inhalational anthrax (Sanz et al., 2008).

We propose two compatible hypotheses to explain the germination response. Firstly, the spores may simply be germinating in response to increased nutrients excreted/secreted by the amoeba. Secondly, the spores may germinate in order to avoid phagocytic destruction by the predatory amoeba.

Uptake mechanisms. The use of various drug inhibitors suggested that the uptake mechanism for both spores and vegetative cells was dependent upon active phagocytosis by the amoeba. For example the translation inhibitor cycloheximide abolished all bacterial uptake. It was also apparent that the amoeba were digesting many of the phagocytosed bacteria as evidenced by the increased number of vegetative cells and spores present when the phagosome acidification inhibitor bafilomycin, was applied. This also suggests that phagocytosed bacteria are not very efficient at preventing the phagosome maturation process. The reduced number of vegetative cells internalised in the presence of the actin polymerisation inhibitor cytochalasin D also argues that active cellular processes are important in internalisation.

Overall, however fewer vegetative cells were taken up by amoeba compared with spores. This indicates that the active surface colonisation in micro-colonies and/or filament formation, can contribute toward resilience to phagocytic destruction. Indeed the process of filament formation engenders a “size exclusion” principle which has been previously suggested as a major driving force in driving bacterial population composition in aquatic environments (Jurgens and Matz, 2002).

5. Conclusion

In conclusion we investigated how both invertebrate and mammalian pathogens belonging to the *Bacillus cereus* group respond to the presence of bactivorous amoeba. They have evolved several specific mechanisms to respond to the threat of predation. These include; (1) germination of spores when they sense the presence of amoeba; (2) surface colonisation and inhibition of phagocytosis and (3) the formation of filaments and “ropes” too large to be ingested. The ability of the bacteria to germinate in the presence of

amoeba and to colonise the cell surface and apparently grow and reproduce on their excretions has implications for the survival of these pathogens in the environment outside their recognised hosts. In addition the discovery of the alternative rope-like developmental stage also has significant implications for understanding their ecology. For example, how does this affect the fate of *B. thuringiensis* in the soil when used in widespread spraying for insect pest control strategies? Also does this process occur during colonisation of the insect gut or invasion of the bacteria into the insect hemocoel as a means to combat cellular immunity *in vivo*?

Acknowledgements

We would like to thank for their kind gifts, Didier Lecrus for the *B. thuringiensis* 4Q7 Δ*plcR* strain and Catherine Pears for the *Dictyostelium discoideum*.

References:

Berry, C., et al., 2002. Complete sequence and organization of pBtoxis, the toxin-coding plasmid of Bacillus thuringiensis subsp. israelensis. Applied Environmental Microbiology. 68**,** 5082-95.

Birmingham, C. L., et al., 2005. Salmonella-induced filament formation is a dynamic phenotype induced by rapidly replicating Salmonella enterica serovar typhimurium in epithelial cells. Infection and Immunity. 73**,** 1204-8.

Dacheux, D., et al., 2001. Pore-forming activity of type III system-secreted proteins leads to oncosis of Pseudomonas aeruginosa-infected macrophages. Molecular Microbiology. 40**,** 76-85.

Feinberg, L., et al., 1999. Arthromitus (Bacillus cereus) symbionts in the cockroach Blaberus giganteus: dietary influences on bacterial development and population density. Symbiosis. 27**,** 109-23.

Foerster, H. F., Foster, J. W., 1966. Endotrophic calcium, strontium, and barium spores of Bacillus megaterium and Bacillus cereus. Journal of Bacteriology. 91**,** 1333-45.

Harb, O. S., et al., 2000. From protozoa to mammalian cells: a new paradigm in the life cycle of intracellular bacterial pathogens. Environmental Microbiology. 2**,** 251-65.

Horwitz, M. A., Silverstein, S. C., 1980. Legionnaires' disease bacterium (Legionella pneumophila) multiples intracellularly in human monocytes. Journal of Clinical Investigation. 66**,** 441-50.

Ivanova, N., et al., 2003. Genome sequence of Bacillus cereus and comparative analysis with Bacillus anthracis. Nature. 423**,** 87-91.

Jurgens, K., Matz, C., 2002. Predation as a shaping force for the phenotypic and genotypic composition of planktonic bacteria. Antonie Van Leeuwenhoek. 81**,** 413-34.

Justice, S. S., et al., 2008. Morphological plasticity as a bacterial survival strategy. Nature Review Microbiology. 6**,** 162-8.

Justice, S. S., et al., 2006. Filamentation by Escherichia coli subverts innate defenses during urinary tract infection. Proceedings of the National Academy of Science U S A. 103**,** 19884-9.

Margulis, L., et al., 1998. The Arthromitus stage of Bacillus cereus: intestinal symbionts of animals. Proceedings of the National Academy of Science U S A. 95**,** 1236-41.

Mendelson, N. H., et al., 2003. The dynamic behavior of bacterial macrofibers growing with one end prevented from rotating: variation in shaft rotation along the fiber's length, and supercoil movement on a solid surface toward the constrained end. BMC Microbiol. 3**,** 18.

Molmeret, M., et al., 2005. Amoebae as training grounds for intracellular bacterial pathogens. Applied Environmental Microbiology. 71**,** 20-8.

Rowbotham, T. J., 1980. Preliminary report on the pathogenicity of Legionella pneumophila for freshwater and soil amoebae. Journal of Clinical Pathology. 33**,** 1179-83.

Saile, E., Koehler, T. M., 2006. Bacillus anthracis multiplication, persistence, and genetic exchange in the rhizosphere of grass plants. Applied Environmental Microbiology. 72**,** 3168-74.

Salamitou, S., et al., 2000. The plcR regulon is involved in the opportunistic properties of Bacillus thuringiensis and Bacillus cereus in mice and insects. Microbiology. 146 (Pt 11)**,** 2825-32.

Sanz, P., et al., 2008. Detection of Bacillus anthracis spore germination in vivo by bioluminescence imaging. Infect Immun. 76**,** 1036-47.

Segal, G., Shuman, H. A., 1999. Legionella pneumophila utilizes the same genes to multiply within Acanthamoeba castellanii and human macrophages. Infection and Immunology. 67**,** 2117-24.

Slamti, L., Lereclus, D., 2002. A cell-cell signaling peptide activates the PlcR virulence regulon in bacteria of the Bacillus cereus group. EMBO J. 21**,** 4550-9.

Stackhouse, R. R., et al., 2012. Survival and virulence of Salmonella enterica serovar enteritidis filaments induced by reduced water activity. Applied Environmental Microbiology. 78**,** 2213-20.

Waterfield, N. R., et al., 2004. Invertebrates as a source of emerging human pathogens. Nature Review Microbiology. 2**,** 833-41.

Figure legends

Figure 1. Light microscopy images of statically incubated co-cultures of *B. thuringiensis* 4Q7 and *A. polyphaga* in PBS at different cell densities and ratios. The inlaid numbers indicate the ratio of bacteria/amoeba. Black text in a white box show cases where the bacteria adopt a filamentous phenotype, and cannot be easily ingested. White text in a black box are cases where the bacteria remain as single cells and colonise the amoeba surface as micro-colonies. The ratio in the grey box shows an intermediate phenotype.

Figure 2. Micro-colony formation of *B. thuringiensis* 4Q7 on the surface of *A. polyphaga* observed using light microscopy (A) and scanning electron microscopy (B).

Figure 3. The presence of amoeba triggers *Bacillus cereus* spore germination. Following 22 hours incubation of *B. cereus* spores in PBS germination is absent (A), whereas incubation in PBS and *A. polyphaga* triggers spore germination (B). The presence of germinating spores (arrows) within vacuoles after two hours of exposure at either 37°C (C) or 28°C (D).

Figure 4. *B. thuringiensis* 4Q7 filament formation in response to *Dictyostelium*. Co-incubation between *B. thuringiensis* 4Q7 and *D. discoideum* in PBS results in filament formation similar to that seen with *A. polyphaga*.

Figure 5. Scanning electron micrographs of co-incubations between *B. thuringiensis* 4Q7 and *A. polyphaga*. (A) Long smooth filaments are seen associated with the surface of the amoeba as indicated by the solid arrows. (B) Amoebas appear to be actively trying to phagocytose the bacteria as evidenced by the presence of phagocytic cups (dashed-arrow).

Figure 6. Light microscopy of Gfp-labelled *B. thuringiensis* 4Q7 showing both individual filaments as well as multi-cellular rope-like structures resulting from the association of multiple bacterial filaments (A) and under Gfp-fluorescence illumination (B). The quality of the rope density varied depending of the level of available nutrients. The addition of 1% PYG medium (C) and 10% PYG (D).

Figure 7. TEM images of *A. polyphaga* and *B. thuringiensis* 4Q7. Bacteria can be seen in tightly fitting membrane bound vesicles (arrows) (A and B). The co-incubation of spores with the amoeba resulted in spore uptake and subsequent germination as indicated by the arrow (C). The residue (arrow) surrounding the germinating spores (D).

Figure 8. A box-plot comparison of the effects of cytochalasin D, cycloheximide and bafilomycin upon spore and vegetative cell uptake. Filled boxes represent vegetative cells and the open boxes, spores. Horizontal lines show the mean of each data set.

Figure_1
Click here to download high resolution image

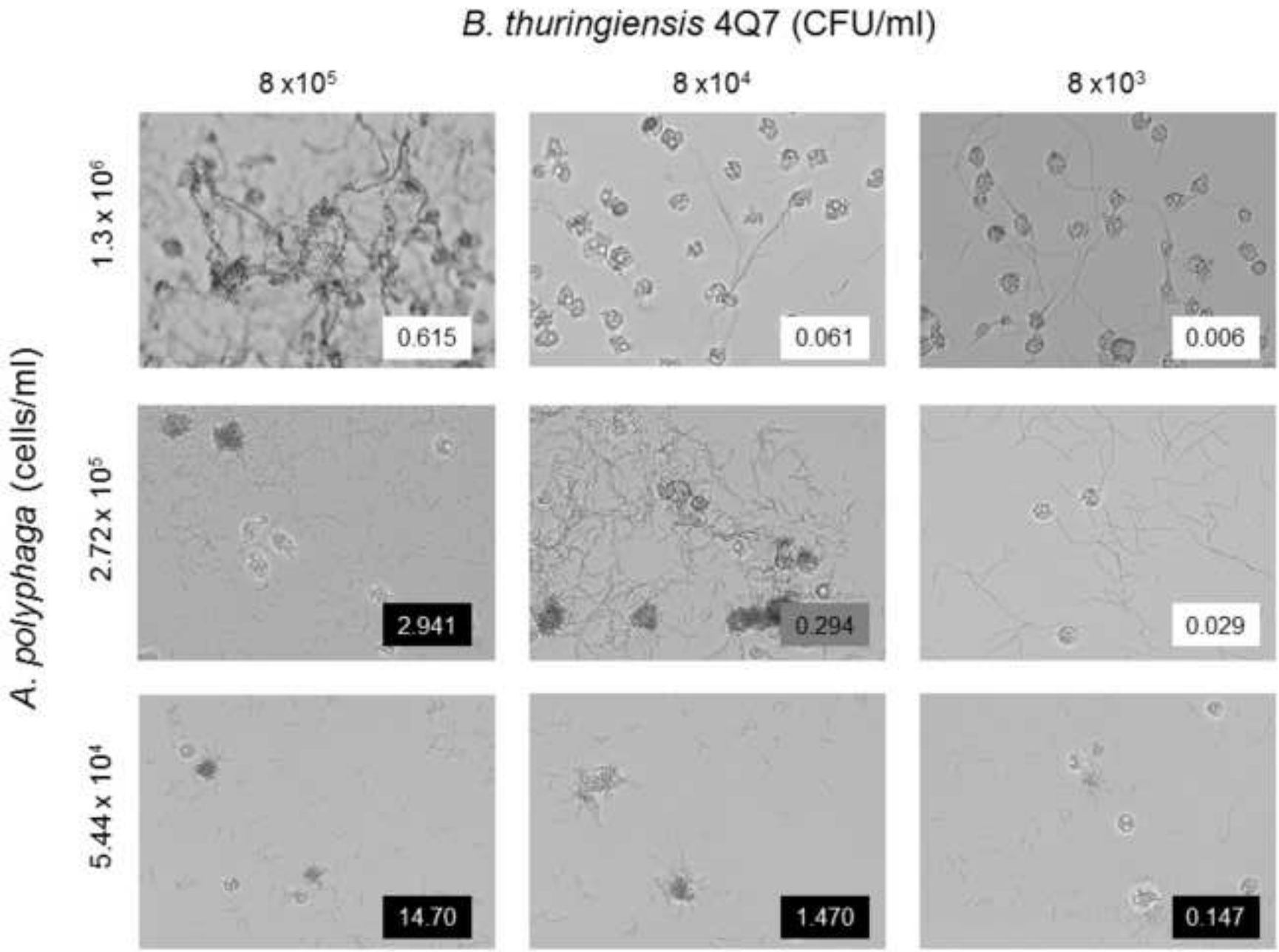


Figure_2
Click here to download high resolution image

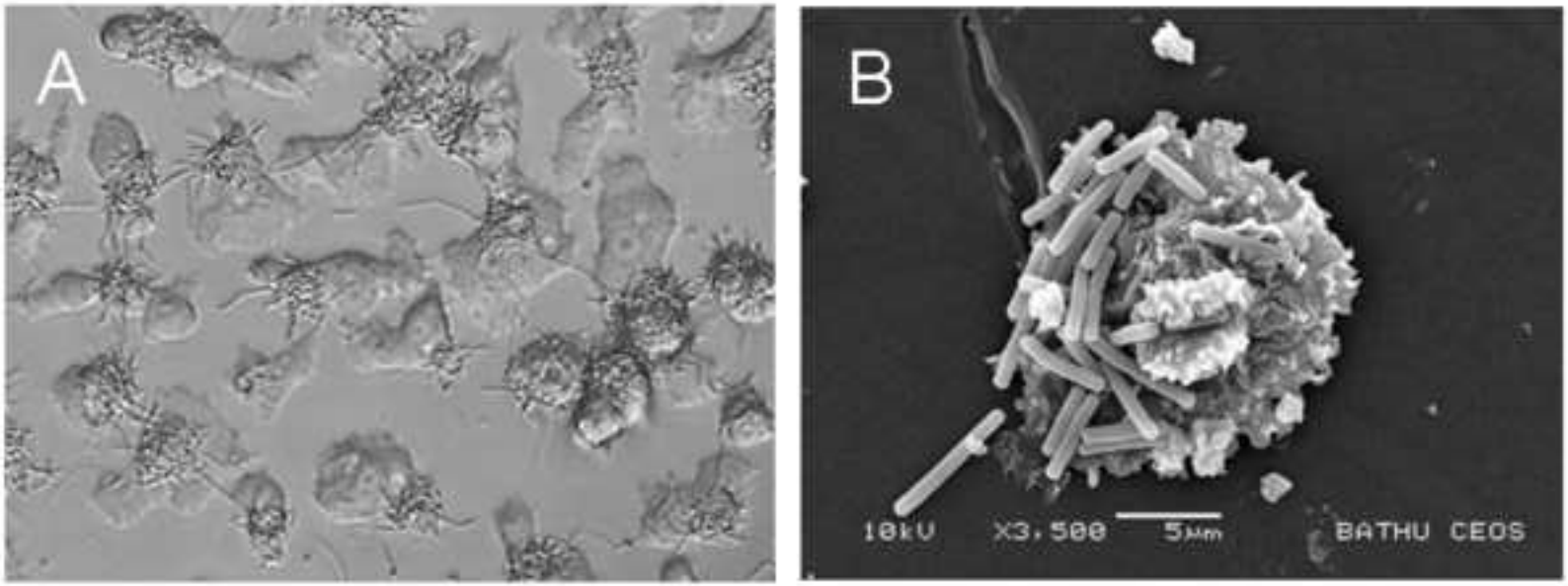


Figure_3

Click here to download high resolution image

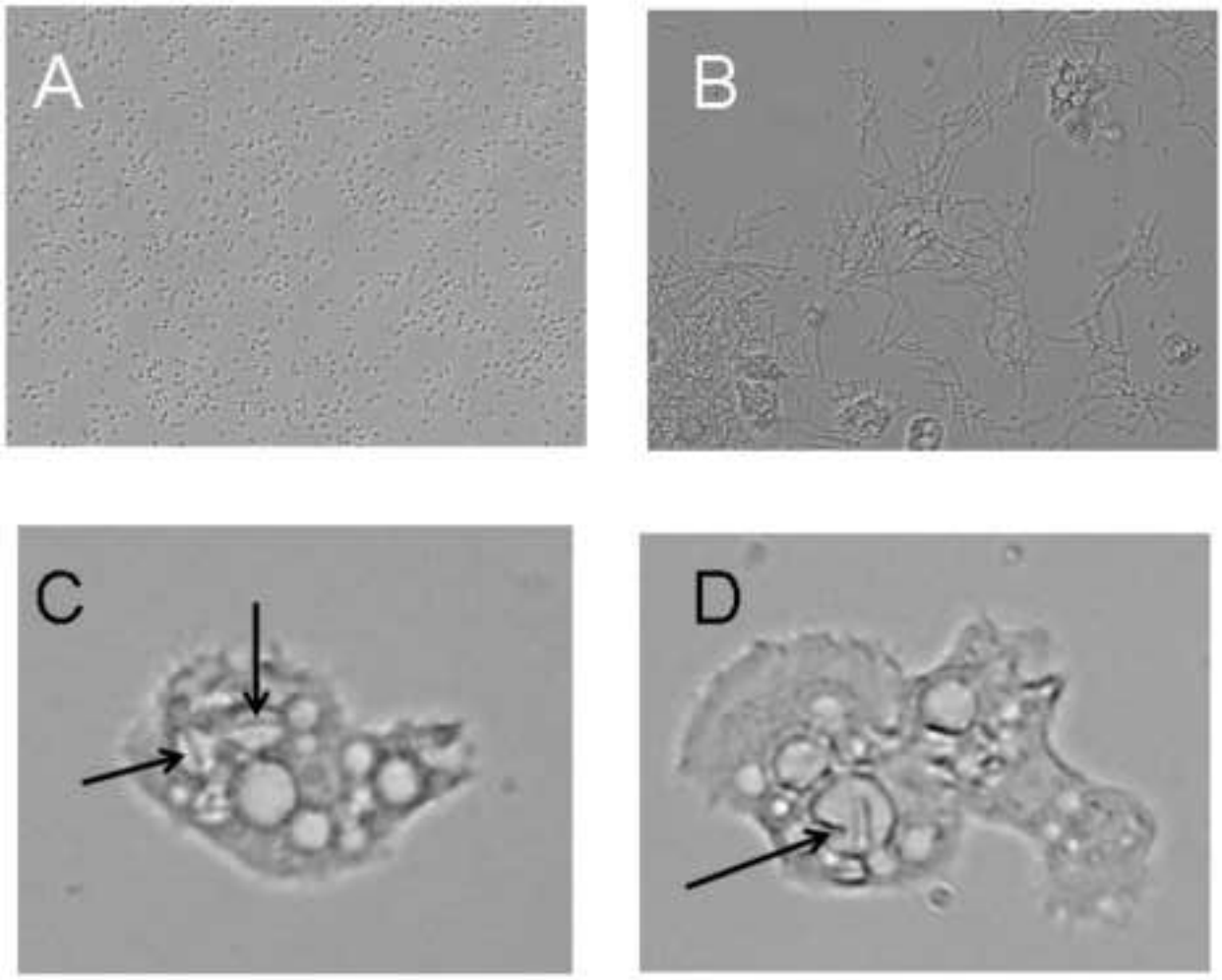


Figure_4
Click here to download high resolution image

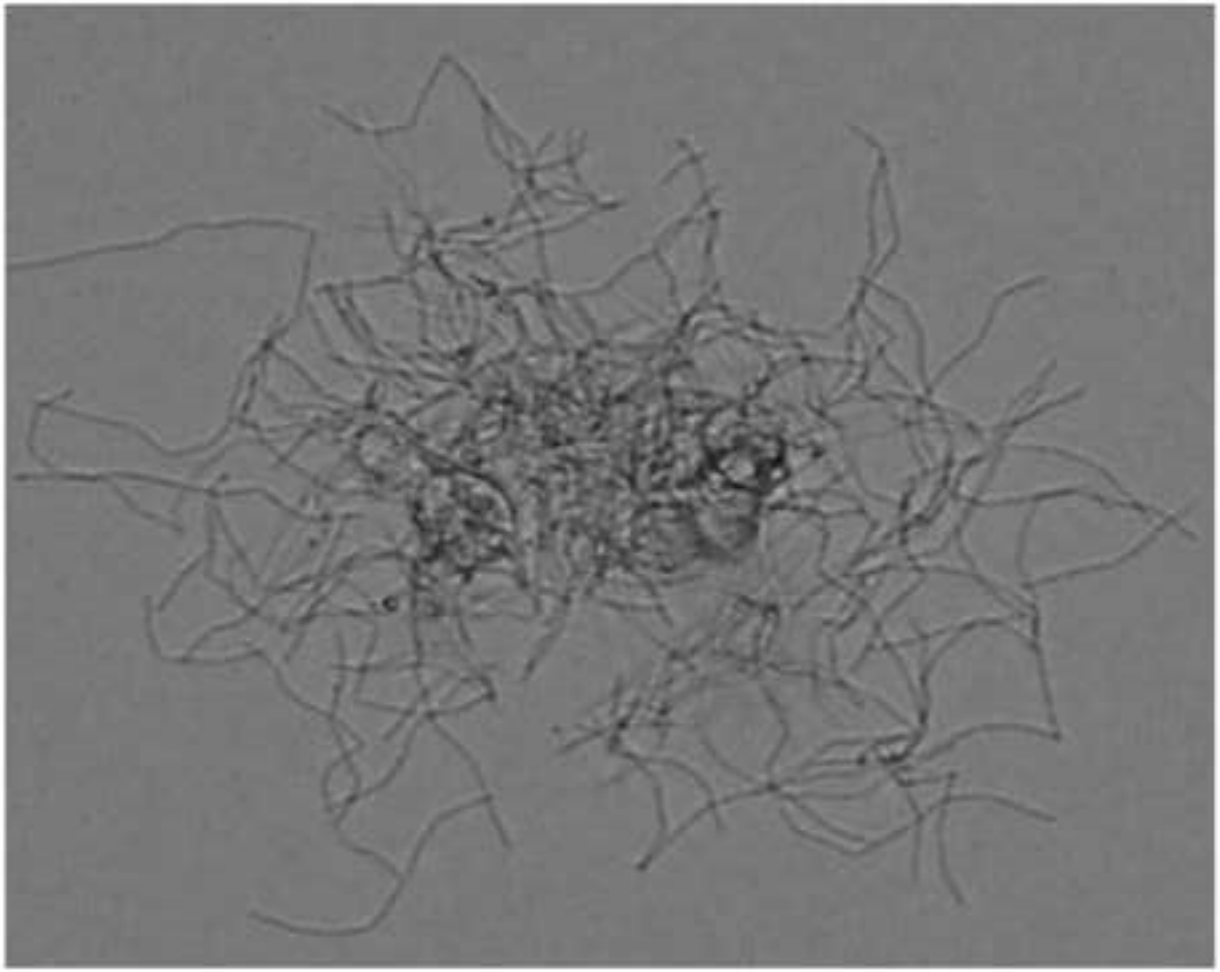


Figure_5

Click here to download high resolution image

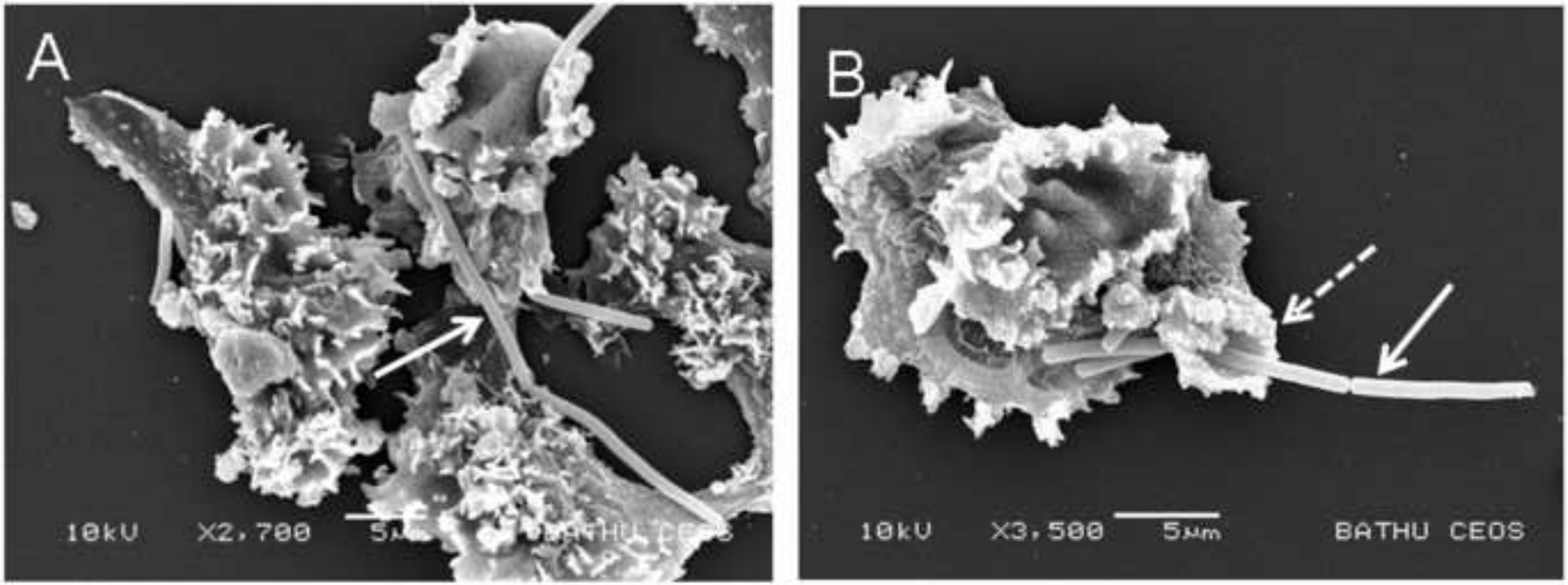


Figure_6
Click here to download high resolution image

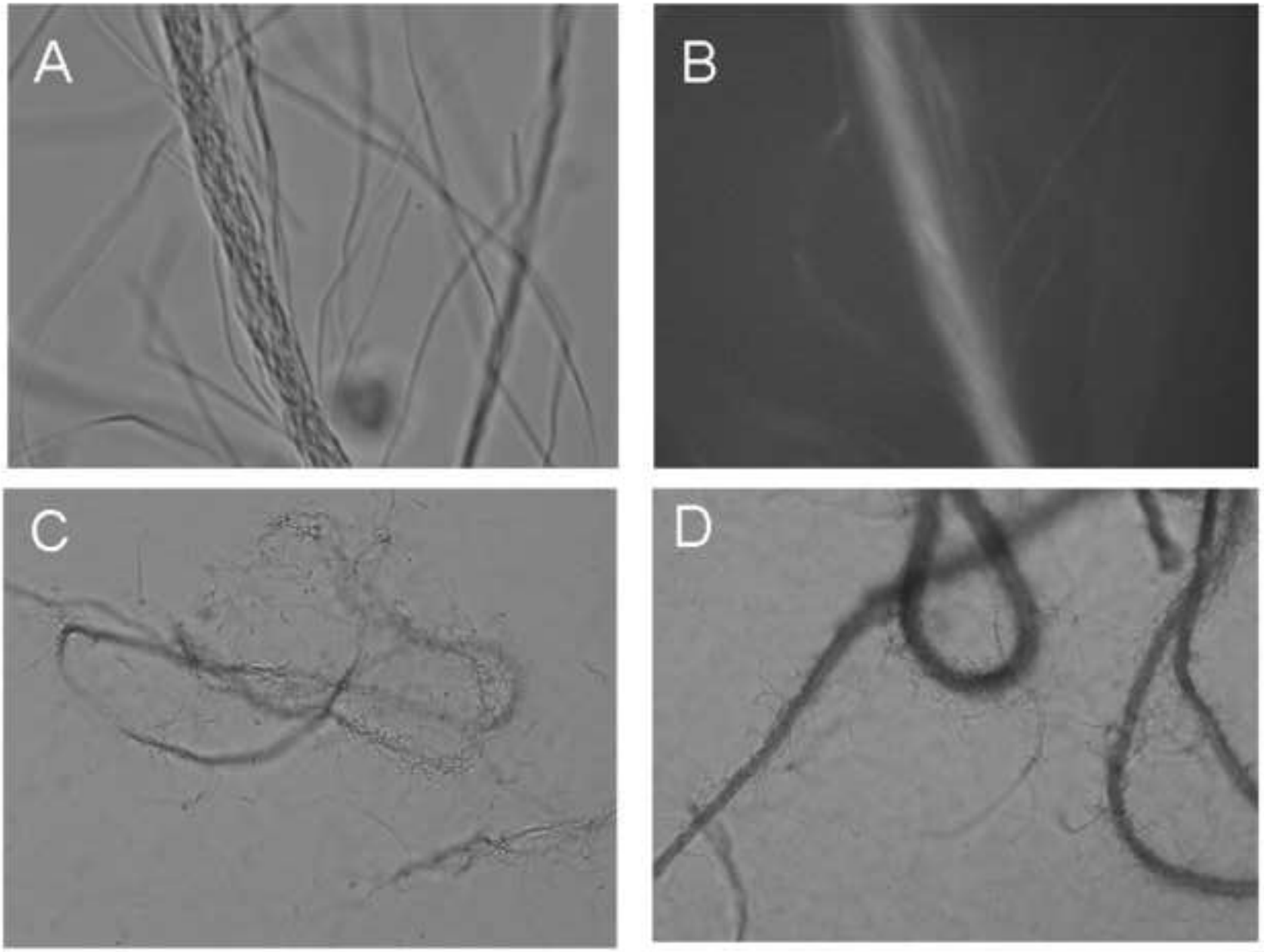


Figure_7

Click here to download high resolution image

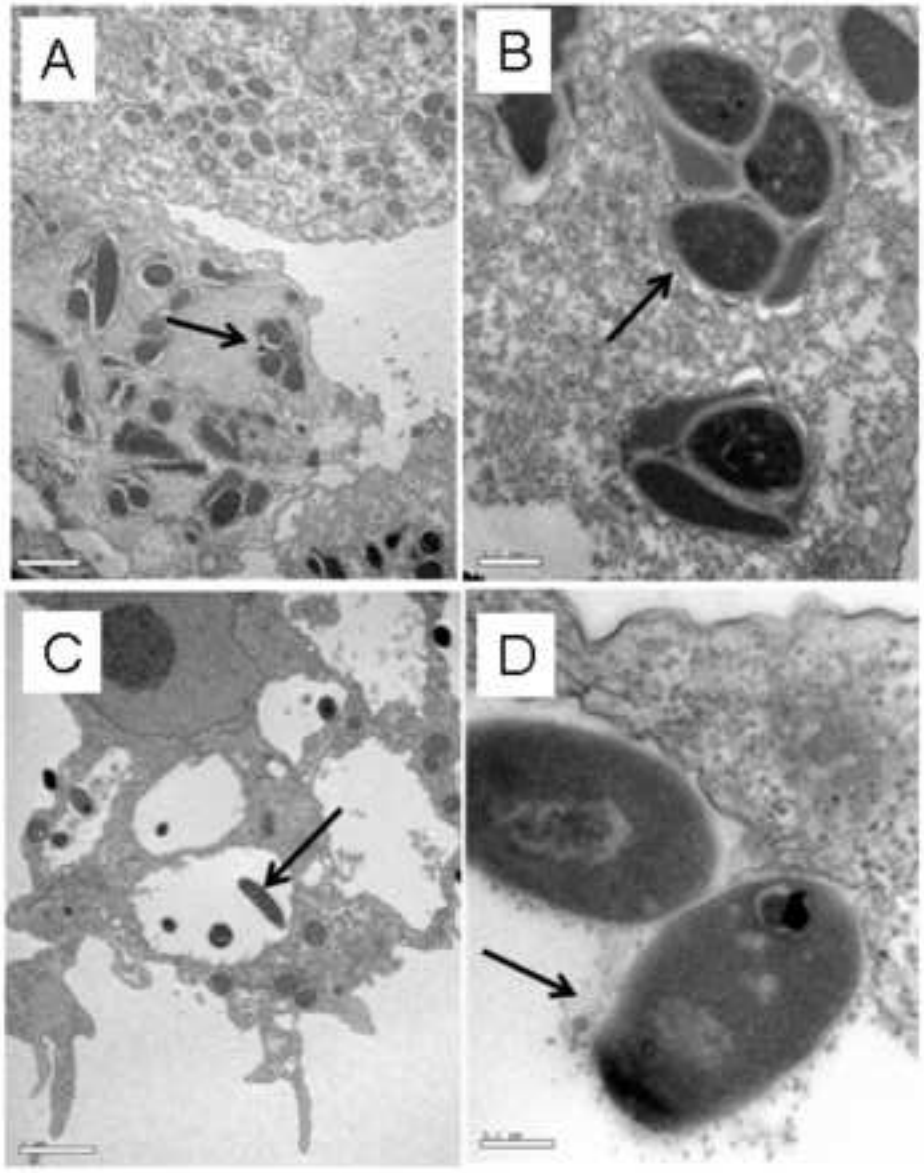
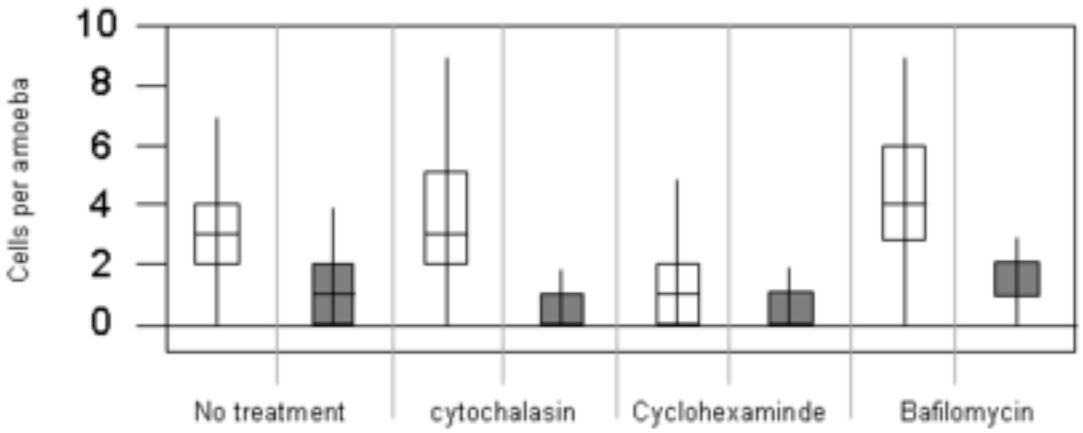


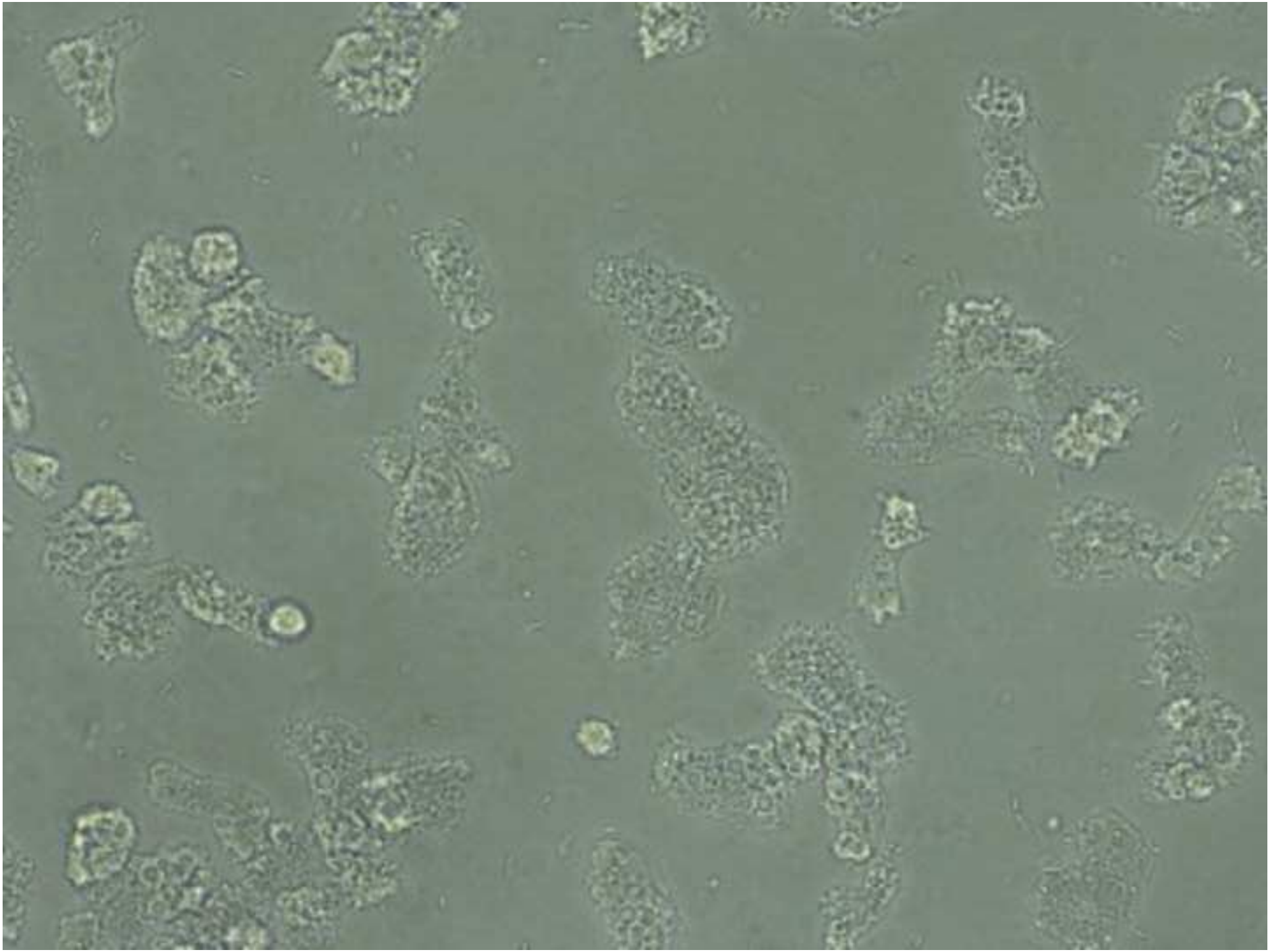
Figure_8

Click here to download high resolution image



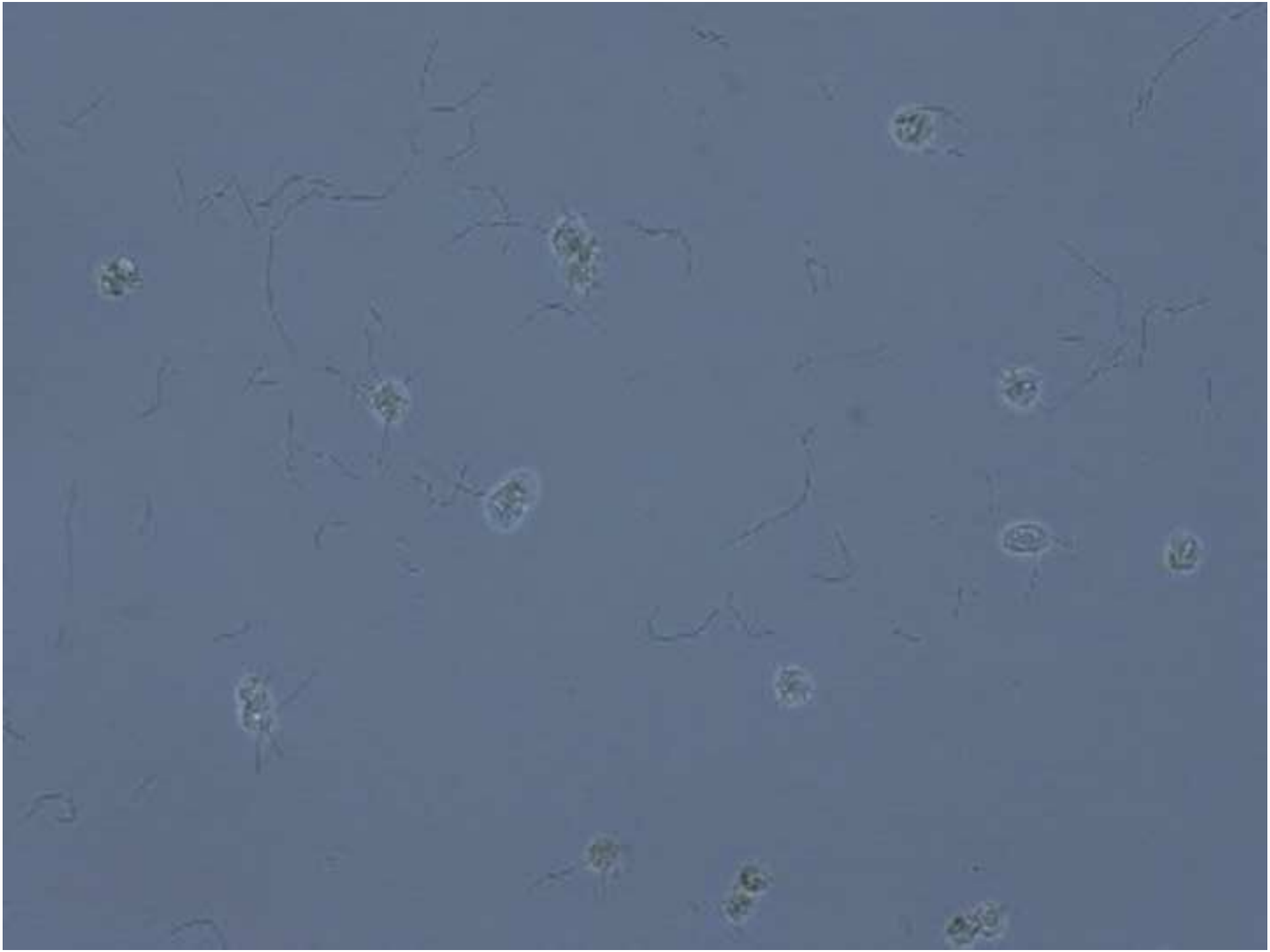
Video Still

Click here to download high resolution image



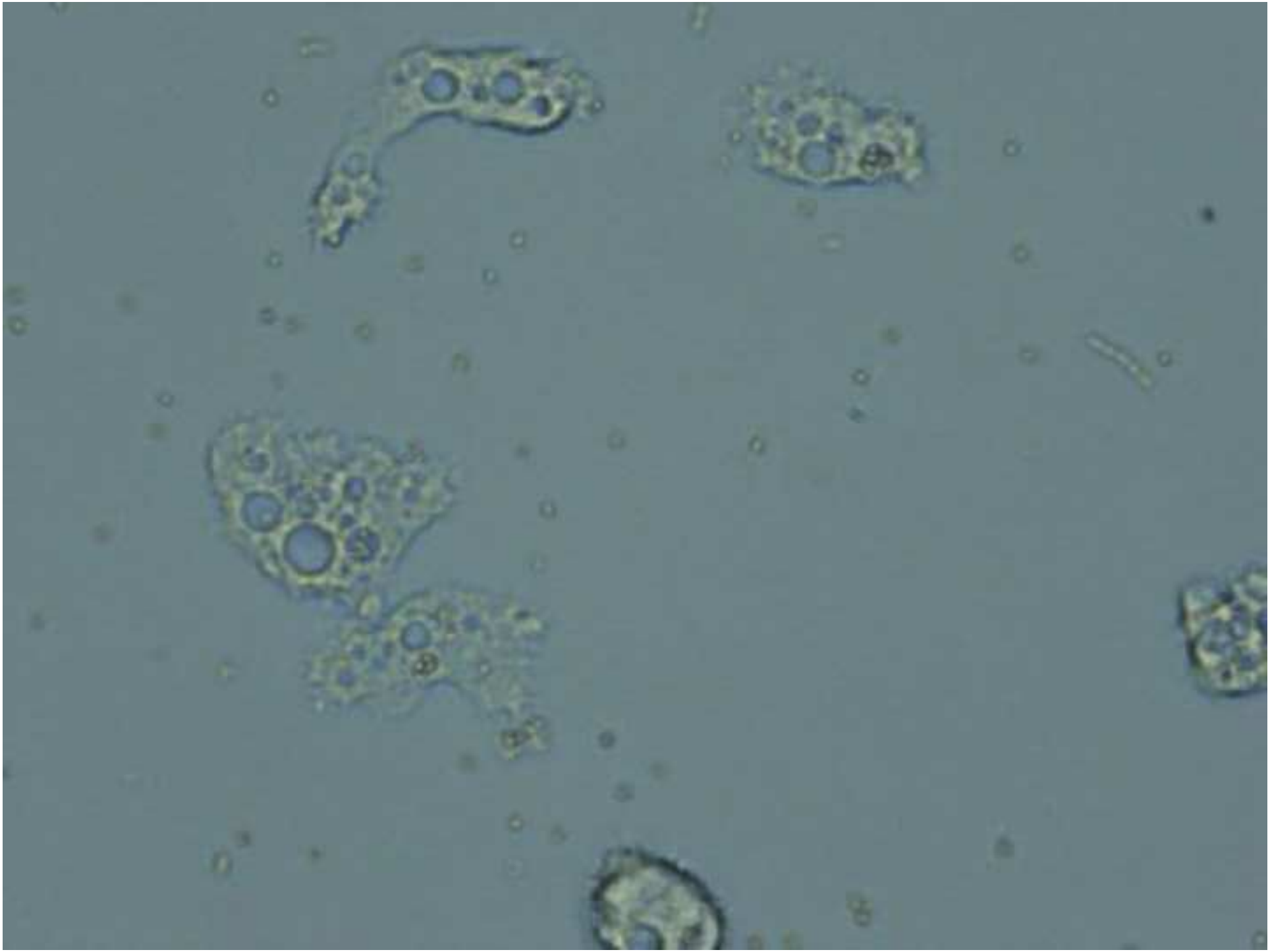
Video Still

Click here to download high resolution image



Video Still

Click here to download high resolution image



Video Still

Click here to download high resolution image