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Use of Flow Cytometry and Confocal Microscopy to Investigate the Infection of *Arthrospira fusiformis* by Cyanophages

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Maryamalsadat Zekri

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Contents

Abstract

Arthrospira fusiformis is a cyanobacterium occurring in harsh conditions of soda lakes. The characteristics of soda lakes are high temperature, high pH, and high turbidity. *A. fusiformis* is the main food source for certain species such as Lesser Flamingo (*Phoeniconaias minor*). Therefore, it is considered a key driver of several ecological cycles. Any fluctuations in population density of *A. fusiformis* affect flamingo communities and other food webs. The high protein content and non-toxicity of *A. fusiformis* makes it an eligible candidate for aquaculture to feed, for example, shrimps or the production of food supplements. Cyanophages are viruses infecting cyanobacteria thereby affecting their community composition. The transition from the lysogenic to the lytic cycle (cyanophage induction) is a possible cause of *A. fusiformis* sudden collapse.

In this study, we studied the potential of confocal laser scanning microscopy to detect cyanophage attacks in *Arthrospira*. We used flow cytometry to detect the cyanophage induction. We received samples collected from the lakes Nakuru and Oloidien, both located in Kenya and one cultivated sample called Deborah after the donator. We used 1.5 mg ml-1 mitomycin (MMC) on Deborah samples to induce prophages. Nakuru and Oloidien samples were treated with 24 hours' light and 24 hours' darkness. The samples were filtered, fixed and stored at – 80° C. Staining of nucleic acids of virus particles was done with SYBR green prior to flow cytometry. We scrutinized temporal alteration in thylakoids and the DNA of cyanobacterium by confocal laser scanning microscopy. For this, the samples were stored at room temperature in the darkness after fixation. Thylakoids are autofluorescent but, for DNA scrutiny, DAPI staining was used. The sampling was carried out at 8-time points (0, 3, 6, 20, 22, 25, 28, 32 hours after induction). The outcomes of flow cytometry histograms showed a significant increase in the number of viruses after induction with MMC in Deborah samples. Confocal images corresponded to the results of flow cytometry. By increasing the number of viruses, the structure of thylakoids was disturbed. The DNA was also denser and located on one side of the cell. Intensive light exposure is assumed as a trigger of cyanophage induction. In both Nakuru and Oloidien samples, the number of viruses increased after almost 24 hours. Meanwhile, the damage to the thylakoids was observed. However, we also observed the increased number of viruses and damage to the thylakoids in the samples stored under constant

darkness. The increased number of viruses can be as a result of the presence of heterotrophic bacteria which, can survive in the darkness. There was also another phenotype of a cyanobacterium which, can respond differently to the environmental factors. The damage to the thylakoids may also be due to the effect of glutaraldehyde as a fixative which may affect the ultrastructure of a cyanobacterium. The conclusion of our survey indicates the efficacy of confocal microscopy to investigate the ultrastructure of *A. fusiformis*. However, it calls for more detailed investigations on axenic cultures, the effect of environmental factors and, the effect of fixation.

Zusammenfassung

Arthrospira fusiformis ist ein Cyanobakterium, das unter extremen Bedingungen in Sodaseen vorkommt. Charakteristisch für Sodaseen sind hohe Temperatur, hoher pH-Wert und hohe Trübung. *A. fusiformis* ist die Haupt nahrungsquelle für viele Arten, wie zum Beispiel dem Zwergen Flamingo (Phoeniconaias minor). Daher wird in diesen System *A. fusiformis* als Motor für verschiedene ökologische Zyklen angesehen Schwankungen dieser Art wirken sich auf Flamingogemeinschaften und andere Nahrungsnetze aus. Der hohe Proteingehalt und die Ungiftigkeit von *A. fusiformis* machen es außerdem zu einem geeigneten Kandidaten für die Aquakultur, um beispielsweise Garnelen zu füttern Auch als Nahrungsergänzungsmittel findet sich A. fusiformis in der industriellen Verwertung wieder. Cyanophagen sind Viren, die Cyanobakterien infizieren und daher deren Population stark beeinflussen können. Vor allem der Übergang vom lysogenen zum lytischen Zyklus (Cyanophageninduktion) ist eine mögliche Ursache für den plötzlichen Kollaps von *A. fusiformis*.

Wir untersuchten, ob sich Vireninfektionen an Arthrospira mittels konfokaler Laserscanning Mikroskopie nachweisen lassen. In dieser Studie wurde die Cyanophageninduktion mittels Durchflusszytometrie getestet. Wir untersuchten Proben aus den Seen Nakuru und Oloidien, beide in Kenia gelegen, und eine stamm Deborah (Name der Spenderin). Wir verwendeten 1,5 mg ml-1 Mitomycin (MMC) bei Deborah-Proben, um Prophagen zu induzieren. Nakuru und Oloidien-Proben wurden mit 24-stunden Licht und 24- stunden Dunkelheit behandelt. Die Proben wurden durch einen Spritzenfilter filtriert, fixiert, und bei -80 ° C gelagert. Die Färbung der Virus-Nukleinsäuren für Durchflusszytometrie erfolgte mittels SYBR Green. Weiters untersuchten wir die Veränderung von Thylakoiden und der DNA im zeitlichen Verlauf mittels konfokaler Laserscanning Mikroskopie. Dafür wurden die Proben nach der Fixierung im Dunkeln bei Raumtemperatur gelagert. Thylakoide sind autofluoreszierend; für die DNA-Untersuchung wurde DAPI gefärbt. Die Probenahme wurde zu 8 Zeitpunkten (0, 3, 6, 20, 22, 25, 28, 32 Stunden nach Induktion) durchgeführt.

Die Ergebnisse der Durchflusszytometrie-Histogramme zeigten einen signifikanten Anstieg der Anzahl der Viren nach Induktion mit MMC in Deborah-Proben.Die konfokalen Bilder entsprachen den Ergebnissen der Durchflusszytometrie. Durch die Erhöhung der

Anzahl der Viren wurde die Struktur der Thylakoide gestört. Die DNA war auch dichter und befand sich auf einer Seite der Zelle. Intensive Belichtung wird auch als Auslöser der Cyanophageninduktion angenommen und sowohl in Nakuru als auch in Oloidien-Proben stieg die Anzahl der Viren nach fast 24 Stunden an. Außerdem wurde eine Schädigung der Thylakoide beobachtet. Wir beobachteten jedoch auch eine erhöhte Anzahl von Viren und Schädigungen der Thylakoide in den Proben, die bei konstanter Dunkelheit gelagert wurden. Dafür gibt es mehrere Erklärungen. Die erhöhte Anzahl von Viren kann auf das Vorhandensein heterotropher Bakterien zurückzuführen sein, die in der Dunkelheit überleben können. Es gab auch einen anderen Phänotyp eines Cyanobakteriums, der auf die Umweltfaktoren unterschiedlich reagieren kann. Die Schädigung der Thylakoide kann auch auf die Wirkung von Glutaraldehyd als Fixiermittel zurückzuführen sein, das die Ultrastruktur eines Cyanobakteriums beeinträchtigen kann. Unsere Studie zeigt die Wirksamkeit der konfokalen Mikroskopie zur Untersuchung der Ultrastruktur von *A. fusiformis*. Es sind jedoch weitere Versuche notwendig, um axenische Kulturen, sowie die Auswirkungen von Umweltfaktoren und der Fixierungen genauer zu analysieren.

1. INTRODUCTION

1.1. Soda lakes

1.1.1. Genesis of soda lakes

Geothermal activity and tectonic movements contribute to the evolution of many soda lakes so that soda lakes are common in regions with volcanic bed rock such as the eastern branch of East African Rift (Schagerl & Renaut, 2016). Although there are also soda lakes in nonvolcanic terrains, most East African soda lakes are located close to the volcanic rocks. For instance, the soil of the Nakuru area is volcanic origin with high porosity, permeability and loose structure which make this area highly susceptible to erosion, land subsidence and fractures during or after heavy rain (Odada et al, 2006). The importance of volcanism in soda lakes can be investigated in two aspects: chemically and physically. Regarding to the physical role of volcanism, central volcanoes and lava flows provide barriers that contain lakes bounded by rift faults (Schagerl & Renaut, 2016). Chemical weathering of volcanic rocks provides most of the ions and dissolved species. Ions such as Na^+, K^+, Ca^{2+}, Mg^{2+} and SiO_2 engage surface waters and ground waters through the weathering. Carbonic acid (H_2CO_3) which is consumed during weathering, results in more alkaline solutions because bicarbonate ions (HCO_3^-) are produced. Soda lakes formation is followed by high evaporation (Schagerl & Renaut, 2016).

1.1.2. Importance of soda lakes

Vareschi et al (1987) suggested that soda lakes are ideal systems for modeling because of their low degree of complexity. A big soda lake is an ideal environment to study microbial reactions and to determine the impact of these reactions on geochemical processes (Oremland et al, 1988). Primary production in lakes Nakuru and Elementaita (both in Kenya) have been recorded more than 10 g carbon m^{-2} per day (Melack & Kilham, 1974). However, in another study, the primary production of soda lakes of Kulunda steppe ranged between 0.01 to 1.32 g carbon m^{-2} per day (Kompantseva et al, 2009). The choice of a method, the frequency of measurement, and the length of incubations may change the result (Hammer, 1981). However, soda lakes are assumed one the most productive aquatic environment on earth (Jones & Grant, 1999).

Like any other ecosystems, soda lakes are also affected by anthropogenic activities, pollution and climate changes (Williams, 2002; Bettinetti et al, 2011; Wagawet al, 2019). The biodiversity of Lake Abijatai (Ethiopia) is threatened by anthropogenic activities. “Deforestation, expansion of agriculture, livestock, soda ash extraction and upstream irrigation led to a drop in water level and surface area, a change in physico-chemical and biological conditions, and a general deterioration in ecosystem condition. The phytoplankton community structure has switched from populations of *Arthrospira fusiformis* to non-*Arthrospira* ones, zooplankton communities have moved towards small-bodied rotifers, such as *Brachionus*, *Filinia* and *Lecane spp*., the fishery has totally collapsed and birds, such as Lesser Flamingoes (*Phoeniconaias minor* Geoffroy) and great white pelicans (*Pelecanus onocrotalus*), have migrated to nearby lakes” (Wagaw et al, 2019). Cyanobacteria such as *Arthrospira fusiformis, Anabaenopsis elenkinii* are prevailing and replacing coccoid *Chlorophyceae* in Lake Oloidien. It was suggested that a shift towards a eutrophic freshwater in Lake Naivasha and hypereutrophic alkaline-saline in Lake Oloidien is the reason (Ballot et al, 2009). To redress this situation, there is a need to raise the awareness of values of soda lakes, improved conservation measures prokaryote and effective management.

1.1.3. Chemistry of soda lakes

When annual water loss by evaporation and evapotranspiration exceeds the annual inflow from precipitation or rivers, the lakes become saline. Therefore, the magnitude of evaporation and annual inflow affects lake levels. The level of salinity may differ over time in response to climate changes. Long-lasting evaporation leads to drying out of soda lakes; rainfall will result in rising water levels linked to a decrease of salinity levels (Schagerl, 2016). Some lakes, such as lake Nakuru in Kenya and Lake Abbe in Ethiopia respond to small climatic change so that they completely dry out from time to time (Schagerl & Renaut, 2016). Climate fluctuations cause large changes in the length and salinity of Nakuru; the changes can be seen daily, annually, decanally or longer time scales (Odada et al, 2006). Besides climatic conditions, anthropogenic activities such as intensive utilization (mainly as a result of population growth and soda ash production) affect lakes chemistry (Vincent. 2009; Schagerl, 2016).

According to Hammer (1986), the salinity of fresh waters is < 0.5% and waters with salinity more than 3.0% are assumed as salt lakes. Among those soda lakes are characterized by pH usually more than 9 (Schagerl & Renaut, 2016). Sodium (Na^+) is the most abundant cation and trona (Na_2CO_3/ $NaHCO_3^-$) is the most dominant salt in soda lakes (Sorokin et al, 2015) (Figure 1). CO_2 may contribute to trona and nahcolite (NaHCO3) crystallization and may explain why most economic soda deposits are present in volcanic regions (Schagerl, 2016). Carbonate species (HCO_3^- , CO_3^{2-}) are also dominant in soda lakes followed by Cl^-, fluoride (F^-), silica and variable concentration of SO_4^{2-}(Schagerl & Renaut, 2016).

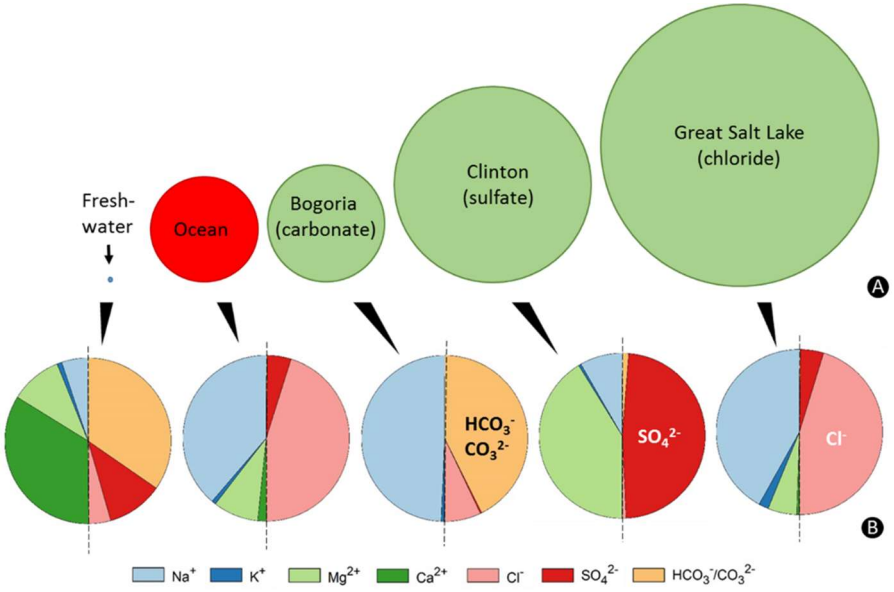


Figure 1- Lake comparisons. (A) Salinity ranges of fresh water, oceans and soda lakes; the circle areas correspond to salinity level. (B) the classification of lakes based on the chemical composition (meq L^{-1}) (Schagerl & Renaut, 2016).

Soda lakes are dominated by HCO_3^- (Boros & Kolpakova, 2018). Therefore, they provide a suitable environment for cyanobacteria growth. HCO_3^- and CO_3^{2-} are unlimited source of CO_2, which makes soda lakes the most productive environments in the world (Boros & Kolpakova, 2018).

1.1.4. Biology of soda lakes

Soda lakes are amongst the most productive ecosystems on earth due to high temperature, intense irradiance and good nutrient supply and a constant photoperiod over the year (Oduor and Schagerl, 2007). Many organisms such as cichlid fish, brine flies and large ciliates depend on soda lakes with extreme aquatic environment (Schagerl & Renaut, 2016) (Figure 2). Due to stable elevated pH as a result of high concentration of carbonate ions, soda lakes provide a rich diversity of haloalkaliphilic bacteria and archaea (Sorokin et al, 2014). High insolation and adequate supply of nutrients usually support abundant phytoplankton (Nurmi, 2010). The changes in water level due to rainfall and consequently conductivity affect the biodiversity of lakes such as lake Nakuru (Wambui et al, 2018). There are 70 mammal species, 400 bird species and over 200 plant species in lake Nakuru national park (Odada et al, 2006).

Figure 2- cichlid fish (left) (Maana & Sefc,2013); brine flies (middle) (Harris, 2011) and large ciliates (right) (Schagerl & Renaut, 2016): some organisms depend on environmental conditions of soda lakes

Despite the harsh conditions, soda lakes are inhabited by abundant microbial life and prokaryotes such as *A. fusiformis* and Archaea (Schagerl & Renaut, 2016) (Figure 3). These ecosystems are very different from other salty inland waters in respect to microbial richness and activity. The main reason is the different physico-chemical features (Sorokin, 2015). Buffer capacity of Soda lakes and soda solonchak soils is the reason of persistence high pH (> 8.5) and high salinity (> 35 g l−1). Aerotolerant microorganisms are more dominant in Soda solonchak soils (a pale or grey soil type found in arid to sub

humid regions) because of more air circulation (Sorokin et al, 2008) while, “soda lakes are the most suitable habitats to find anaerobic haloalkaliphilic microorganisms” (Sousa et al, 2015). High concentration of dissolved organic carbon also boost microbial life (Schagerl & Renaut, 2016).

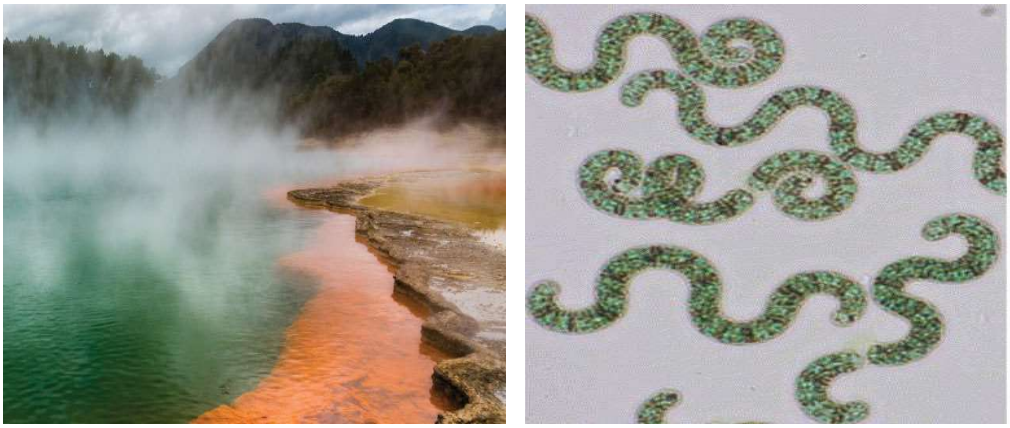


Figure 3- abundant prokaryotes in soda lakes such as *Arthrospira fusiformis* (right) (Mussagy, 2006) and Archaea (left) (Gaia et al, 2008)

Soda lakes of East Africa are spectacular environments, hosting some of the rarest species in the world and leading to the informally name “flamingo lakes”. There are two flamingo species occurring in the East African soda lakes: Greater Flamingo (*Phoenicopterus roseus*) and Lesser Flamingo (*Phoenicopterus minor*), the latter classified as “near threatened” (IUCN red list, 2012,2018). The Lesser Flamingo is the smallest but most numerous species of flamingo and lives in large colonies that can contain over 1 million birds. (http://www.theanimalfiles.com/birds/flamingos/lesser flamingo.html) (Figure 4).

“”The Lesser Flamingo, *Phoeniconaias minor*, is the dominating and characteristic bird species of alkaline–saline lakes and pans of East Africa” (Peduzzi et al, 2014). It is a primary consumer that prefers to ingest microphytes from the lake water surface (Schagerl & Renaut, 2016). The cyanobacterium *A. fusiformis* is one of the main primary producers, and is the preferred food of the Lesser Flamingo (Odada et al, 2006). As filter feeders (aquatic animals feeding on particles strained out of the water by using their

specialized filtering system), Lesser Flamingos consume photoautotrophic algal primary producers (Vareschi & Jacobs, 1985)

Figure 4- Lesser Flamingo (*Phoenicopterus minor*) communities contain over 1 million birds habituating in soda lakes of East Africa (www. cosmosmagazine.com/biology/africa-s-most-toxic-lakes-are-a-paradise-for-fearless-flamingos)

During the last two decades, the Lesser Flamingo colonies have faced a dramatic decline in their size. One reason can be anthropogenic disturbances such as attempts to extract sodium carbonate (a useful industrial material known as soda ash) from Lake Natron (Tanzania). Back in 1993, polluted water in Lake Bogoria and nearby Nakuru killed more than 20,000 Lesser Flamingos (Mlingwa & Baker, 2006). On the other hand, Lesser Flamingos are filter feeders and feed almost entirely on cyanobacteria. One investigation on the Lake Chitu (Ethiopia) showed only *A. fusiformis* as the preferred and mostly dominating food resource for flamingos (Krienitz & Kotut, 2010). It was mentioned that flamingos are specialized feeding on diatoms in the Lake Abijata and *Arthrospira* sp. in Lake Chitu; diatoms are only grazed, if the cyanobacteria are in low abundance. It was also concluded that the food availability affects distribution and abundance of flamingos (Krienitz & Kotut, 2010, Kumssa & Bekele, 2014). Another study on phytoplankton

composition, biomass, and flamingo population density in three alkaline-saline lakes of Kenya (Bogoria, Nakuru, and Oloiedien) in 2010 explored the link between sudden flamingo death and fluctuations in algal food quantity and quality (Krienitz & Kotut, 2010). In this study, unusual interruption in *A. fusiformis* dominance and the occurrence of *Anabaenopsis* or by *Picocystis salinarum* Lewin, prevailing *A. fusiformis* was reported. The large and slimy colonies of *Anabaenopsis* have the potential of blocking the flamingo's food filtration system; moreover, they might be able to produce cyanotoxins. It was concluded that food scarcity leads to either being susceptible to attacks of infective diseases, such as the ones caused by *Mycobacterium avium* and *Pseudomonas aeruginosa* or it predisposes birds to poisoning by cyanotoxins and pollutants, by reducing their capacity to handle toxic substances.

1.2. Arthrospira fusiformis

1.2.1. Characteristics of *Arthrospira fusiformis*

In 1967, *Arthrospira* was categorized as a "wonderful future food source" for the world because of its high protein content (Wan et al, 2016). The importance of *Arthrospira* sp. as a food supply has attracted a lot of attention (Belay & Gershwin, 2007). *Arthrospira* usually occurs under intense sunlight, high temperature and alkaline environment. *Arthrospira* is very common in most African soda lakes but it is also found in Europe (Serbia) (Fuzinato et al, 2010). The genus *Arthrospira* consist *of Arthrospira fusiformis, Arthrospira platensis, Arthrospira maxima* and *Arthrospira jenneri.* Flamingo *Phoeniconaias minor*, cichlid fish *Sarotherodon alcalicus* grahami, copepod *Lovenula africana*, dipteran larva *Leptochironomus deribae*, rotifer *Brachionus dimidiatus* (Vareschi & Jacobs, 1985) and soda tilapia fish *Alcolapia alcalicus grahami* Boulenger (Shagerl et al, 2015) feed on *Arthrospira* in Lake Nakuru. *Arthrospira* therefore can be treated as a "key species driving a majority of secondary production in these soda lakes".

Voronichin (1934) described *A. fusiformis* for the first time as *Spirulina fusiformis* from Lake Tanatar, Siberian steppe, Russia. In the soda lakes of East Africa, *Arthrospira fusiformis* forms an exceptionally high algal crop due to its high photosynthetic capacity (Schagerl et al, 2015); net photosynthetic rates in Lake Nakuru is very high at depths with optimal light conditions (230 kJ m^{-3} h^{-1}). Three strains of *A. fusiformis*, two strains isolated

from wastewater ponds in Mozambique and one from Lake Nakuru (Kenya) were studied to find out the toxin production.

Toxicity analysis of the three strains, following the growth cycle, detected neither microcystins nor anatoxin-a. The results indicated that the strains were not toxigenic under the experimental conditions applied (Mussagy et al, 2007). These findings point out the usability of *A. fusiformis* in seawater as food source for shrimps and could be considered a candidate for use in different applications such as in food supplements and in aquaculture (Mussagy et al, 2007).

1.2.2. Ultrastructure of *Arthrospira fusiformis*

A. fusiformis is a filamentous, unbranched cyanobacterium without heterocyst characterized by cylindrical shape, visibility of cross and helicoid appearance (Figure 5). The helical shape of *A. fusiformis* differs between and within species and is controlled by environmental conditions such as temperature. For example, *A. fusiformis* show great plasticity in morphology towards environmental factors like temperature and other physical and chemical factors (Gershwin & Belay, 2007; Kaggwa et al, 2013).

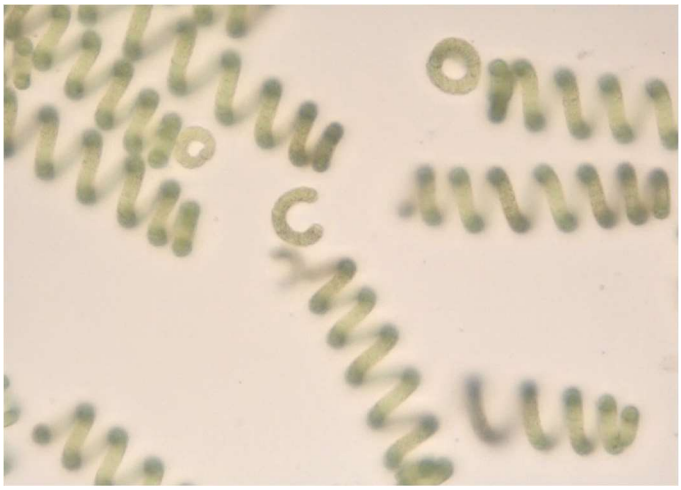


Figure 5- *Arthrospira fusiformis*; bar: 100 µm

Several studies have investigated the morphological, physiological and biochemical properties of this genus (Tomaselli, 1997; Cohen & Vonshak, 1991). Like all cyanobacteria, *A. fusiformis* consists of distinct and distinguishable compartments. These

compartments include a mucilage sheath whose thickness, pigmentation, consistency, and nature is greatly influenced by environmental factors.

The genetic material of cyanobacteria is made up of naked DNA dispersed in the central region of the cytoplasm. Like in other prokaryotes, the nucleus is not a bound organelle. Kazuyoshi and colleagues (2016) showed that DNA is homogeneously spread out across the cell, surrounded by thylakoids. However, after 12 hours' darkness/12 hours' light period, the DNA of the cyanobacterium *Synechococcus* sp elongated, became compacted at the end of the light period and subsequently divided to daughter cells, whereas it was diffused throughout the cytoplasm at other times (Kazuyoshi et al, 2016). Polyphosphate granules within the cyanobacterial cells are associated with compacted DNA, assuming that they provide phosphate during DNA synthesis (Kazuyoshi et al, 2016).

The photosynthetic apparatus in cyanobacteria is located in free thylakoids and contains photosynthetic pigments such as Chlorophyll a. Liberton and colleagues (2006) showed that the thylakoid membranes are in fact physically discontinuous from the plasma membrane in cyanobacterium *Synechocystis* sp (Liberton et al, 2006).

Phycobilisomes are additional structures to trap light energy of certain wavelengths helping cyanobacteria to exploit deeper waters where the quality and quantity of light is inappropriate for other photosynthetic organisms.

Environmental fluctuations, nutrients and cyanophages may affect the ultrastructure of cyanobacteria. By means of Transmission Electron Microscopy (TEM), it was shown that N limitation causes a drop in chlorophyll a and cellular nitrogen content after 14–15h and degradation of phycobilisomes and thylakoid membranes. However, nitrogen limitation did not affect polyphosphate bodies, carboxysomes, lipid granules, the cell envelope, or the extra-cellular glycocalyx (Stevens et al, 1981). UV light produced reactive oxygen species (ROS) and thereby caused lipid peroxidation and DNA strand breaks in cyanobacterium *Anabaena sp.* (He & Häder, 2002). Heavy metals and salt stress also inhibited the growth rate of cyanobacteria (Hardie et al, 1983; Surosz & Palinska, 2004; Sudhir & Murthy, 2004). On the other hand, some factors cause changes in one genus or

species but not in another one. For example, the lipid composition was affected by temperature in *Anacystis nidulans*, but not in *Anabaena variabilis* (Satoa et al, 1979).

1.3. Cyanophages

1.3.1. Introduction, structure and replication cycle

“Microorganisms constitute more than 90% of the living biomass in the sea and it is estimated that viruses kill approximately 20% of this biomass per day” (Suttle, 2007). It was shown that in every milliliter of ocean water, there are millions of virus-like particles (Bergh et al, 1989). “The abundance of viruses exceeds that of bacteria and archaea by approximately 15 fold. However, because of their extremely small size, viruses represent only approximately 5% of the prokaryotic biomass” (Suttle, 2007). Viruses are substantial agents of mortality in heterotrophic and autotrophic plankton (Proctor & Fuhrman, 1990). The host-specific, often strain-specific, nature of viral infection makes viruses powerful agents for controlling the community composition (Suttle, 2007). This has been incorporated into the “Kill the winner” model in which the diversity of the microbial community is controlled by viral infection. In aquaculture industry, viral diseases can cause enormous losses in production and revenue (Thingstad, 2000). Moreover, cellular components such as carbon and nutrients are released due to the lysis of heterotrophic and autotrophic microorganisms by viruses. It was stated that almost 25% of the total photosynthetically fixed carbon is channeled through the viral shunt, the virus-induced transformation of particulate matter to dissolved organic matter (Ostfeld et al, 2008)

Cyanophages are viruses that infect cyanobacteria (Jiang et al, 2019) and can be found in both, freshwater and marine environments, where they are a key regulator of cyanobacterial populations.

All cyanophages described thus far are Caudovirales (dsDNA-phages with a tail and a protein capsid surrounding genetic material) (Figure 6). Viruses attach to the host cell and transfers viral DNA to the host cell by tails. “Based on morphological characteristics, cyanophages are placed into the families Myoviridae, Siphoviridae and Podoviridae” (Singh et al, 2012).

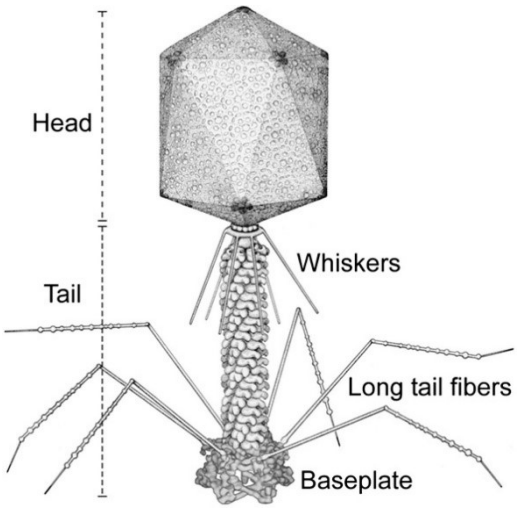


Figure 6- The structure of a cyanophage (Yap et al, 2016), representing the tail which helps the cyanopahes to attach to the host cell and head which contains DNA.

In general, cyanophages replicate through two dominant cycles: the lytic cycle and the lysogenic cycle. Degradation of host-DNA in concert with viral nucleic acid-replication followed by the synthesis of virus-encoded protein defines the lytic cycle. Lysogeny, or the lysogenic cycle (also temperate cycle), is characterized by the integration of the bacteriophage nucleic acid into the host bacterium's genome. In this cycle, the bacterium replicates normally. The genetic material of the bacteriophage transport to the daughter cells of the host. Later trigger-events (DNA-damage e.g. through UV-radiation or MMC or mostly unknown environmental factors) cause the INDUCTION of the prophage and proliferation of the phage via the lytic cycle. One of the few characterized lysogenic phages, AS-1 (Myoviridae family), was induced by elevated temperatures (Chu et al, 2011). The difference between these two cycles is that in the lysogenic cycle the spread of virus DNA occurs through the usual prokaryotic reproduction while virus DNA replication in the lytic cycle is quick and results in cell destruction and the release of viral progeny (virions) (Figure 7). Early studies investigating the impact of cyanophages on the ultrastructure of infected cells using electron microscopy showed that especially

thylakoids can show significant changes (Sherman & Haselkorn 1970). However, such changes have hardly been investigated by means of modern methods as CFLSM.

Cyanophages can alter the metabolism (Ginzburg et al, 1968; Thompson et al, 2011; Rosenwasser et al, 2016) and the replication of their hosts (Jiang et al, 2019) thereby influencing the structure of the cyanobacterial community (Mann, 2003; Roux et al, 2016). It has been reported that “UV, mitomycin C and heavy metals such as copper or cadmium can induce the lytic cycle” (Singh et al, 2012). Detrimental conditions such as nutritional status or any pathogenic condition to which the bacterium is susceptible represent a trigger of either lytic or lysogenic cycles (Singh et al, 2012). It was also shown that temperature is a crucial factor to induce the progression of cyanophage AS-1 (Myoviridae family) from lysogenic to lytic cycle (Chu et al, 2011).

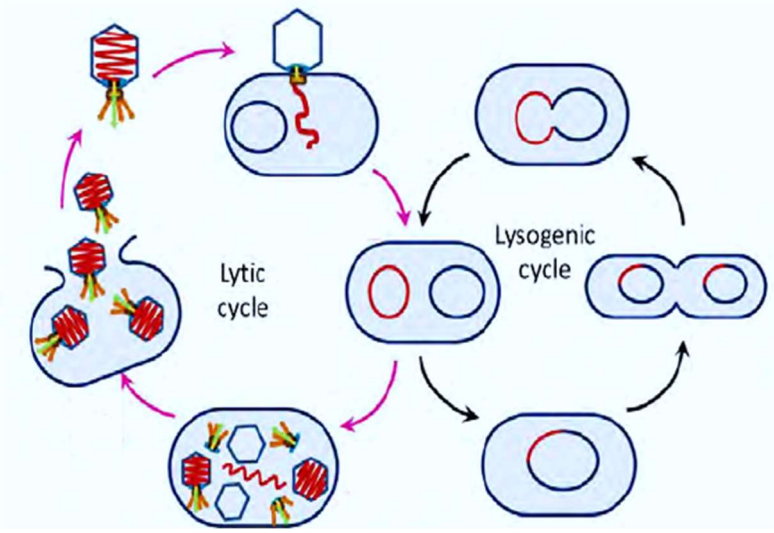


Figure 7- The comparison of lytic and lysogenic cycle (Orlova, 2012)

Cyanophages are resistence to different pH and therefore, this may not be a very important factor affecting the distribution of phages (Singh et al, 2012). Viral propagation is frequently constrained by phosphor-limitation but enhanced by elevated pCO2 (Cheng et al, 2019). Cyanophages show different activity in various depths which can be related to the fact that the temperature and CO2 accumulation vary with depth (Singh et al, 2012).

1.3.2. Cyanphages and cyanobacteria

Suttle and colleagues (1990) studied the effect of viral infection on primary production and observed a reduction in initial fluorescence as a sign of chlorophyll a after infection. They concluded that not only grazing and nutrient limitation, but also viruses are responsible for changes in phytoplankton structure and substantial reduction (78%) in primary production in the oceans (Figure 8). Later, Suttle (1992) showed that, accompanied by decreasing in vivo chlorophyll-autofluorescence, viruses infecting phytoplankton communities reduce CO2-fixation by almost 50%.

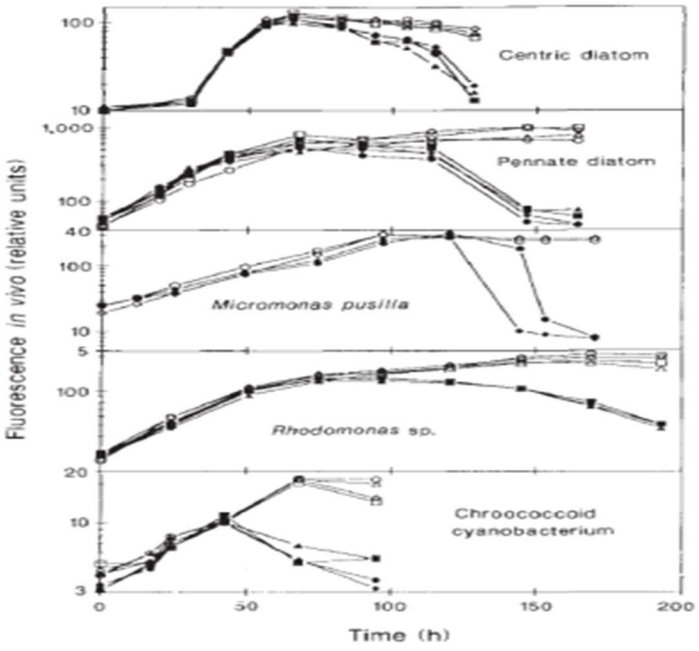


Figure 8- The effect of adding naturally occurring marine viruses to cultures of their phytoplankton hosts (Suttle et al, 1990)

1.4. Scrutiny of small particles by flow cytometry

Flow cytometry is a technique to study cells or particles, suspended in a fluid. Viruses range in size between 17-350 nm (Lippe, 2018), thus frequently fall below the discriminable size of conventional flow cytometers. Caudovirales (including cyanophages) typically have capsids 45 – 170nm in diameter. However, improvements as better hydrodynamic focusing, more-powerful lasers, and forward light scatter detectors with greater sensitivity (Zamora & Aguilar 2018) as well as the use of fluorescent dyes, like SYBR Green I for DNA-staining allow counting of VLPs (Brussaard 2004, Rowley, 2012).

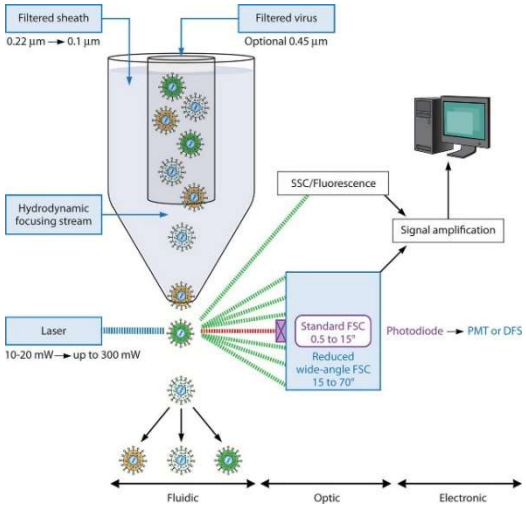


Figure 9- Flow Cytometry apparatus, including the fluidic, optic, and electronic components (Lippe, 2018).

The detection of viruses is done by either “labeling the surface of the viral particles with antibodies” or “indirectly via a secondary antibody or labeling the DNA or RNA of viruses with nucleic acid fluorescent dyes” (such as SYBR Green). In general, many different markers can be observed simultaneously which makes flow cytometry a remarkable technique. It enables us to probe many subpopulations of cells and analyze large numbers of cells with great statistical value (Lippe, 2018).

1.5. Ultrastructure studies by means of confocal microscopy

Confocal laser scanning microscopy is an optical imaging technique in which a pinhole excludes out-of-focus light to increase resolution and contrast. In confocal microscopy, opposite to the conventional microscopes, a small beam of light penetrates at one narrow depth. In addition, capturing multiple two-dimensional images at different depths in a sample enables the reconstruction of three-dimensional structures ("optical sectioning"). High resolution and labeling techniques with fluorescent dyes facilitate the visualization of photosynthetic pigments and/or DNA structure. Fluorescence techniques were used to investigate the distribution of photosynthetic pigments in cyanobacterial cells (*Synechocystis sp*.). Vermaas et al (2008) showed the heterogeneity between thylakoid rings as phycobilisomes mainly occur along the outer thylakoids where the photosystem II is prevalent while photosystem I occurs mainly on inner rings of thylakoids.

1.6. Aims of the study

Cyanophage- cyanobacteria interaction is of great interest due to ecological, biological and commercial importance of cyanobacteria. More details about this interaction empower us to, on one hand, prevent ecological disturbances due to the loss of cyanobacteria and, on the other hand, provide the infrastructure to use cyanobacteria, such as *A. fusiformis*, in food, pharmaceutical and sanitary industries.

Based on the results of several studies (Suttle et al,1990; Suttle & Chan, 1994; Jacquet et al, 2013; Peduzzi et al, 2014), we assume that cyanophages can cause sudden collapses of *A. fusiformis* blooms. In order to better understand the effects of cyanophage infection on *A. fusiformis* cells we wanted to investigate the temporal and spatial changes in (a) the distribution of DNA inside the cells and (b) the modifications of thylakoid ultrastructure during infection using confocal laser scanning microscopy. Beforehand, flow cytometry should be used to investigate cyanophage infections in cultures of three distinct *A. fusiformis* isolates.

However, we need a better understanding of prophage induction and the possible role of cyanophages for this genus. Therefore, the investigation of ultrastructural alterations in A. fusiformis cells during infection is of central interest. We studied temporal and spatial

changes in the distribution of DNA inside the cells and modifications of thylakoid ultrastructure to obtain a more detailed perception of the effective mechanism of cyanophages infecting *A. fusiformis*. We used flow cytometry to detect cyanophage infections in three cultures. Confocal microscopy enabled us to follow the temporal changes in ultrastructure of *A. fusiformis* after cyanophage infection. By comparing healthy and infected cells, we were able to study alteration of thylakoids and DNA as two main components in cyanobacteria.

2. MATERIAL AND METHODS

2.1. Materials

2.1.1. Samples

We used three strains. Two strains originate from the two East African soda lakes Nakuru and Oloidien, one more was kindly provided by an algal culture farm from France. This strain is called Deborah after the donator. Lake Nakuru (Kenya, 0˚22'S, 36˚5'E) is an alkaline- saline lake located in the East African Rift Valley that stretches from Tanzania through Kenya to Ethiopia (Odada et al, 2006) (Figure 10). Air temperature varies between 10ºC and 29ºC. "The climate ranges from cold and humid to arid and semi-arid. Mean annual rainfall is approximately 1000mm with peaks in the months of November to December and April to May" (State of Environment Report 2009, 11). Lake Oloidien (Kenya, 00° 50´s, 36°17 ´E, altitude 1900 m) is situated about 20 km of Naivasha town in the Rift Valley (Lyngs, 1996). Lake Oloidien (Figure 11) is a former bay of Lake Naivasha (the largest freshwater lake in the Kenyan Rift Valley with a hydrologically closed basin (Ballot et al, 2009). In Lake Oloidien, the pH ranged from 9.3 to 9.9, conductivity values between 3.89 and 5.27 mS cm-1. The phytoplankton is dominated by cyanobacteria and Chlorophyceae (Ballot et al, 2009).

Figure 10- The location of Lake Nakuru in Kenya (http://underatree.overblog.com/lake-nakuru-national-park)

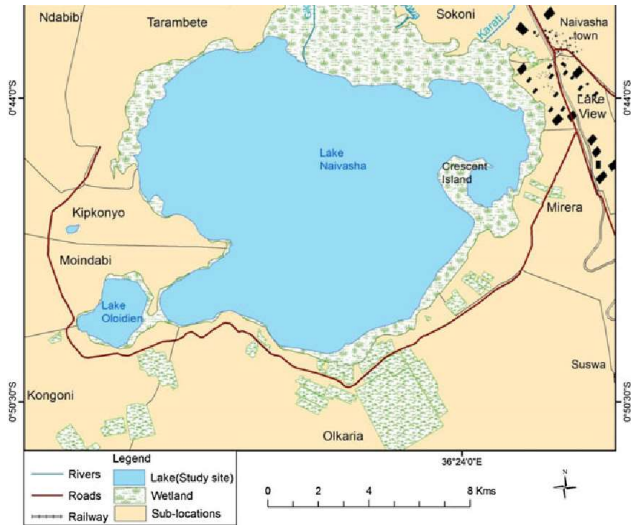


Figure 11- The location of Lake Oloidien in Kenya (Rindoria et al, 2015)

Samples for cyanophage screening within *A. fusiformis* cells by confocal microscope and flow cytometry were concentrated with a plankton net from unialgal cultures (mesh size 30 µm). In the following, the strains were called “Nakuru”, “Oloidien”, and “Deborah”. After sampling, *Arthrospira* sp was isolated and cultivated in Zarrouk medium (Table 1) at 32°C in 100ml Erlenmeyer flasks under constant light (40µE m-2 s-1) and 100rpm shaking.

Table 1- Zarrouk medium composition (Michele et al, 2015)

Reagents	Amount (g L^{-1})
$NaHCO_3$	16.8
K_2HPO_4	0.50
$NaNO_3$	2.5
K_2SO_4	1.00
NaCl	1.00
$MgSO_4.7H_2O$	0.20
$CaCl_2$	0.04
$FeSO_4.7H_2O$	0.01
EDTA	0.08
Solution A5	1 mL
Solution B6	1 mL

Solution A5: (g L^{-1}): H_3BO_3, 2.86; $MnCl_2.4H_2O$, 1.81; $ZnSO_4.7H_2O$, 0.222; $Na_2MoO_4.2H_2O$, 0.390; $CuSO_2.5H_2O$, 0.079. Solution B6: (mg L^{-1}): NH_4VO_3: 22.86; $KCr(SO_4)_2$. 12 H_2O: 192; $NiSO_4$. $6H_2O$: 44.8; Na_2WO_4. $2H_2O$: 17.94; $TiOSO_4$. $8H_2O$: 61.1; $CO(NO_3)_2$. $6H_2O$: 43.98.

2.1.2. Chemicals

Phosphate buffered saline (PBS) (Thermo Fisher Scientific) at pH 7.2 was used as sheeth fluid in flow cytometry. PBS is a buffer solution containing disodium hydrogen phosphate (726.0 $mgL^{-1)}$, sodium chloride (9000.0 mgL^{-1}) and, potassium dihydrogen phosphate (210.0 mgL^{-1}). For cleaning steps of flow cytometry (FCM), 70% (v/v) ethanol was used.

SYBR Green 1 (Thermo Fisher Scientific) is one of the most sensitive nucleic acid stains available for detecting double-stranded DNA (dsDNA). SYBR Green 1 is a suitable dye for use in laser scanning microscopy because of its low background and “spectral characteristics that closely match light sources and filters sets”. SYBR Green 1 turned out to be the most useful dye for staining aquatic bacteria better than SYBR Gold, Pico Green or RiboGreen (Griebler et al, 2001). The most concern about SYBR Green 1 is that it is highly mutagenous and should be handled very carefully. We applied SYBR Green to label DNA for flow cytometry investigation. To prepare a SYBR Green 1 working solution, 1 µl of 10.000x stock was mixed with 199 µl distilled water.

TE buffer is commonly used in molecular biology, especially in procedures involving DNA or RNA. The name "TE" is derived from its components: 10 mM of Tris base and 1mM ethylenediaminetetraacetic acid (EDTA), pH 8.0 EDTA a molecule that chelates cations like Mg^{2+}. 5 ml of 10xTE was mixed with 45 ml distilled water and used to dilute samples for flow cytometry.

Glutaraldehyde ($C_5H_8O_2$) is a water-soluble oily liquid, containing two aldehyde groups, used as a disinfectant and as a fixative for biological tissues. We applied 0.2% glutaraldehyde to fix samples for confocal microscopy.

DAPI (4, 6-diamidino-2-phenylindole) (Thermo Fisher Scientific) is a fluorescent dye that binds to adenine–thymine regions in double strand DNA (dsDNA). “DAPI can pass through an intact cell membrane, it can be used to stain both live and fixed cells” (https://www.revolvy.com/page/DAPI). We applied 0.1 µg/ml DAPI diluted in PBS to stain DNA for confocal microscopy.

Mitomycin C (MMC; $C_{15}H_{18}N_4O_5$) was used to induce prophages as shown in many studies (Humphrey et al, 1995; Ortmann et al, 2002; Stoddard et al, 2007). In this study, 1.5 $\mu g ml^{-1}$ mitomycin C was used to stimulate prophages induction.

2.2. Methods

2.2.1. Flow Cytometry

In this study, we applied mitomycin C to detect the occurrence of lysogenic phages. Nakuru and Deborah were exposed to pH > 12 for 72 hours to eliminate other cyanobacteria. The pH was adjusted by using 1 M NaOH solution. It was added gradually to increase 1 pH every 30 minutes. The applied media was without bicarbonate because of the buffering effect. The Oloidien culture was not treated with high pH (>12) as it could not survive at this pH. The pH of Oloidien culture was 8.

The results of FCM of Nakuru and Oloidien were not adequate to detect lysogenic phages but, we observed a diurnal cycle which led us to find out if the cyanophage in Nakuru and Oloidien is light-dependent. Therefore, we applied light/dark incubation to answer this question.

We took 300 ml of Nakuru and Oloidien cultures and divided them evenly into two sterile Erlenmeye flasks. One flask of each culture was fully covered with foil paper to prevent light and the another one was placed under the constant light. One flask of Deborah was induced with 1.5 µg/ml mitomycin C at the early exponential growth phase. After treatment, all samples were taken every three hours for confocal microscopy and flow cytometry experiments (3, 6, 20, 22, 25, 28, 31 hours, respectively).

We used a flow cytometer of the type BD FACSAria™ IIIu. SYBR Green 1 was excited using a blue laser (488 nm). To compute flow rates for further calculations, 1 ml distilled water was weighted on the analytic balance to four digits then placed in the flow cytometers for 5 minutes. Then, remaining distilled water was weighted again. The weighting procedure was repeated 3 times. By following formula, the final flow rates were calculated:

V= (before (g) – After (g)/5) * 1000

We used the flow rates settings 2, 5, 9 with the range of 33.48, 52.14 and 84.68, µ/minutes, respectively to stay in the statistically reasonable range of 200-1200 detected events per second. Table 2 shows various flow rates used for treatments and samples.

Table 2- the flow rates vary between the treatments and time points

Time points (hours after the treatment)	Oloidien		Nakuru		Deborah	
	Light	Dark	Light	Dark	-MMC	+MMC
0	2	5	9	9	9	5
3	2	2	5	5	5	5
6	2	2	2	2	5	5
20	2	2	2	9	5	5
22	2	2	2	5	5	2
25	2	2	5	9	2	2
28	5	9	9	9	5	2
31	9	9	5	5	2	2

For the flow rate check the thresholds for forward scatter (FSC), side-scattered light (SSC) and SYBR Green 1 were set to 300, 300 and 530, respectively. FSC is a measure of “the amount of the laser beam that passes around the cell” and “gives a relative size for the cell. If one uses a known control or standard beads with a known size”, it is possible to determine the relative size of the cells based on the size of the control or standard. SSC is a “measurement provides information about the internal complexity (i.e. granularity) of a cell. The interface between the laser and intracellular structures causes the light” which reflects from the structures. Laser light of the similar wavelength of the laser (488nm) reflected by organelles in the cell is considered as SSC.

To prepare the samples, 3 ml of each cultured strain was taken and filtered through a syringe filter with 0.45 µm pore size. Then, 588 µl of filtered sample was fixed with 12 µl 0.2% glutaraldehyde for 10 minutes. The fixed samples were then placed in liquid N for 30 minutes and stored at -80°^{C} until analysis.

Exactly before running the flow cytometry, the samples need to be prepared by dilution with TE buffer at different ratios. Table 3 shows the dilution rates of the samples. The total

amount of the sample and diluent was 500 µl. again the dilution was based on achieving an event-rate of 200-1200 events per second. For further calculations in FCM, the threshold for the number of events was assumed 200-1000/minutes. The number of events more than the threshold was considered as a too condense sample.

Table 3- the dilution rates varying between cultures and treatments

Time points (hours after the treatment)	Oloidien		Nakuru		Deborah	
	Light	Dark	Light	Dark	-MMC	+MMC
0	0.25	0.25	0.25	0.25	0.1	0.1
3	0.25	0.25	0.25	0.25	0.1	0.1
6	0.25	0.25	0.25	0.25	0.1	0.02
20	0.25	0.02	0.25	0.02	0.1	0.1
22	0.1	0.02	0.1	0.02	0.1	0.1
25	0.02	0.02	0.02	0.02	0.1	0.1
28	0.1	0.1	0.1	0.1	0.1	0.1
31	0.1	0.1	0.1	0.1	0.1	0.1

After dilution, 5 µl of SYBR Green 1 was added to each sample and then heated to 80° C for exactly 10 minutes. Then, the samples were immediately placed in cold water to cool down. The thresholds of FSC, SSC and SYBR Green were set to 470, 500 and 570, respectively. The blank sample contained 1000 µl TE and 10 µl SYBR Green.

2.2.2. Confocal Microscopy

We used the confocal laser scanning microscope CLSM Leica TCS SP5x/Leica DM 6000Cs) for dsDNA and thylakoid observation. It is an upright microscope stand, and we used its UV diode and Aragon laser. All images were taken with the 63x oil immersion objective.

2.2.2.1. Sample preparation

980 µl of the samples incubated in either light/dark or treated with mitomycin C and were fixed with 0.2% v/v (final) glutaraldehyde and kept at the room temperature in the dark

until analysis. Shortly before observation in the microscope, a drop of the liquid sample was placed on the glass slide and covered with a coverslip.

2.2.2.2. DNA investigation

For DNA scrutiny with DAPI, the UV diode (405 nm, 15% laser power) and a 420-480 nm long-pass filter were used. The scan speed was 400 ms-1 per pixel, the pinhole size was 102.86. The resolution was 1024×1024 and the z step size was between 0.25 - 0.46 according to the number of individual images. Stack size was set to 20-30 images to reduce the bleaching during imaging.

2.2.2.3. Thylakoids investigation

We used the chlorophyll autofluorescence to study the thylakoid structure. The argon laser line of 496 nm was used at the intensity of 15% in combination with 650-700 nm long-pass filter for chlorophyll examination of thylakoids. The pinhole size was adjusted to 102.86 for the optical image acquisition and data processing. The scan speed was 400 ms per pixel. 20-30 optical serial sections were taken with adjusted intervals. The time settings for all series were below 1:30 minute. The gain was adjusted to obtain the optimal chlorophyll and DNA fluorescence image.

2.2.2.4. 3D rendering

For 3D reconstruction and volume rendering, we used Amira software 6.2.0. Three stacks of 20 images taken by confocal microscopy were used to acquire 3D images.

3. RESULTS

3.1. Flow cytometry showed explicit oscillations in the number of viruses

3.1.1. Deborah treated with and without MMC

The results of the flow cytometry experiments are presented in the following graphs. Figure 12 shows the number of viruses in Deborah, treated with and without MMC over the period of 31h. MMC was added at 0h. In comparison to the untreated sample 20h after addition of MMC the concentration of viruses significantly increased, with a peak 3 times above starting levels. In comparison, virus counts in the untreated sample slightly decreased over time.

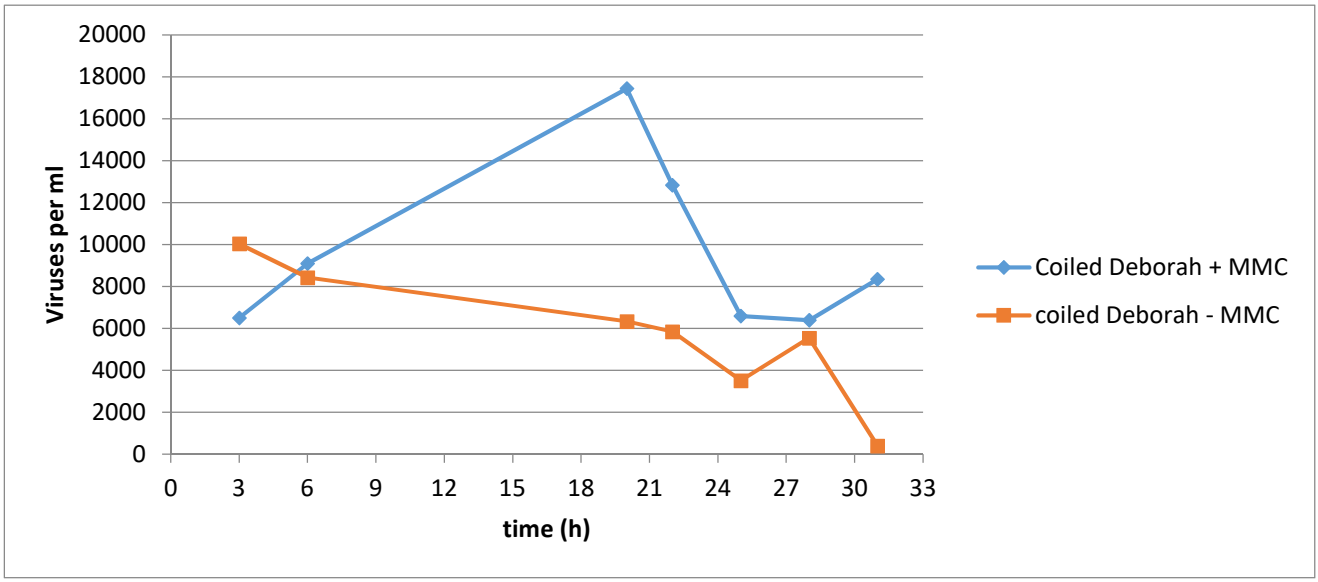


Figure 12- the number of viruses detected in Deborah by flow cytometry treated with and without MMC at 8 time points

3.1.2. Nakuru and Oloidien treated with and without MMC

Figures 13 and 14 show the number of viruses in Nakuru and Oloidien, which were treated with and without MMC over the period of 31h. MMC was added at 0h. The concentration of viruses significantly increased at time point 22h, in both treated and untreated samples. Any differences between the two treatments were observed, therefore we could not show prophage induction in Nakuru and Oloidien. However, there is an oscillation every almost 20 hours. The oscillation was assumed to be related to the diurnal cycle of cyanophages.

This brought up the question of whether the cyanophages in Nakuru and Oloidien are light-dependent. To answer this question, we conducted light and dark incubation.

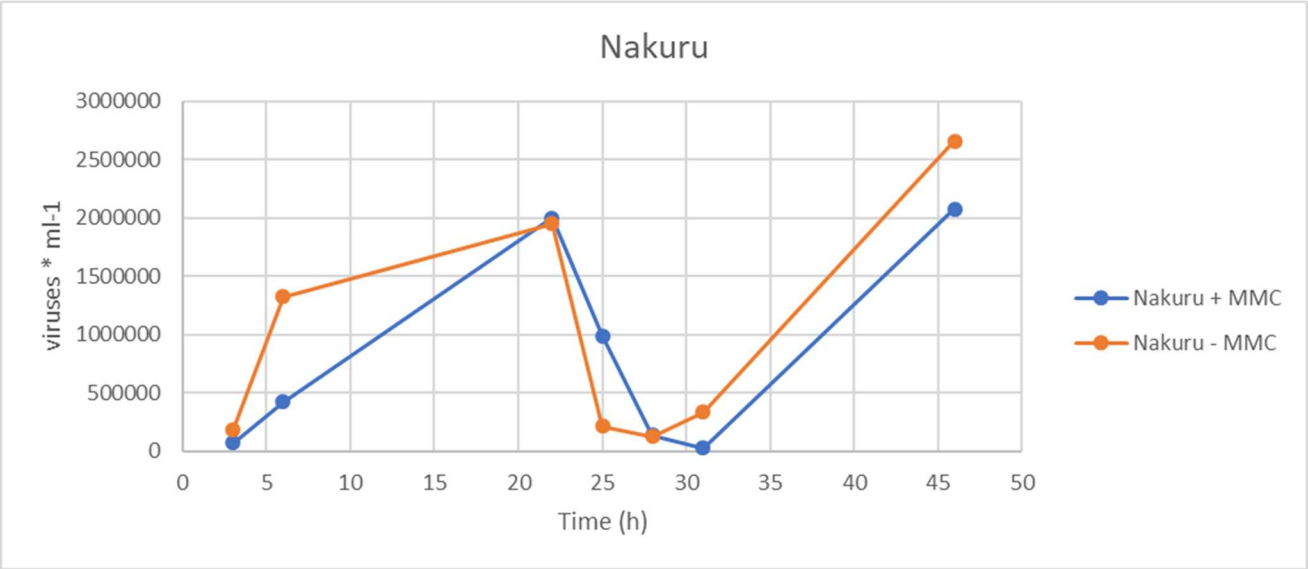


Figure 13- the number of viruses detected in Nakuru by flow cytometry treated with and without MMC at 8 time points

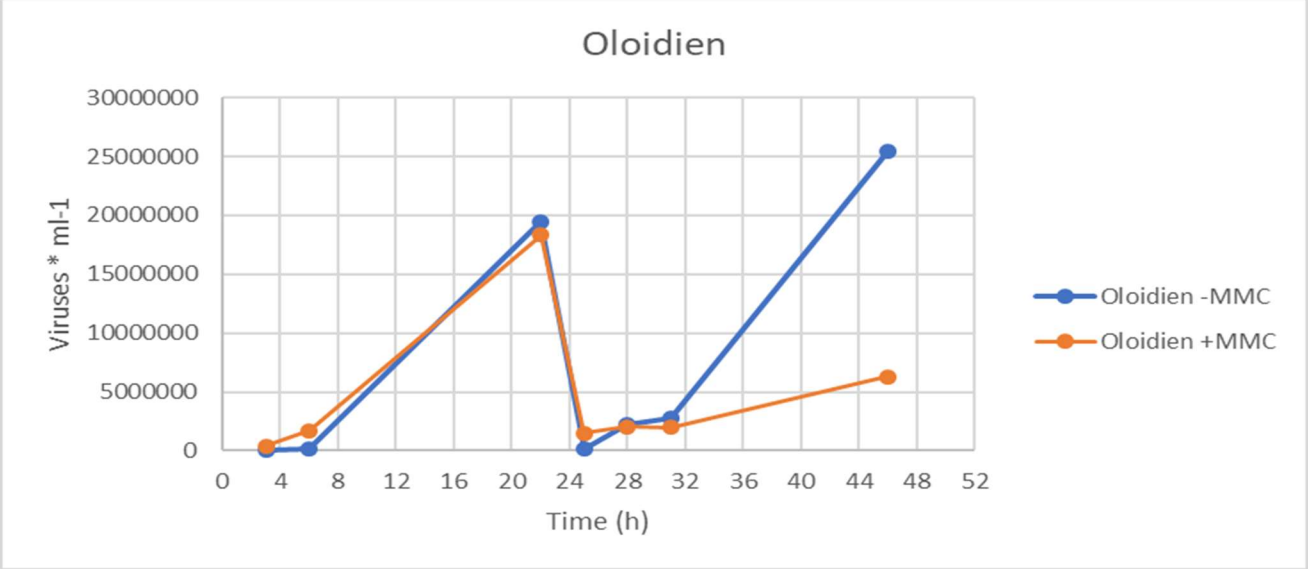


Figure 14- the number of viruses detected in Oloidien by flow cytometry treated with and without MMC at 8 time points

3.1.3. Light and dark incubation of Nakuru

Variations in the number of viruses between samples of Nakuru grown under constant light and samples incubated in the darkness is presented in the figure 15. In the sample incubated under constant light, the number of viruses did not change for 20 hours. There is a notable increase in the number of viruses in the next 5 hours before it decreased strongly between 25-28 hours. At time point 25 hours, there is an increase in the number of viruses in both cultures.

In the sample incubated in the dark for 31 hours a similar pattern was observed. There are no fluctuations in the number of viruses for 6 hours followed by a significant increase. The number of viruses increased significantly at time point 25 hours. There is a strong drop in the number of viruses at time point 28 hours. Between 28 to 31 hours, the number of viruses increased slightly.

Although the pattern between light and dark incubation is similar, the increase in the number of viruses in dark incubation is much higher than in the light incubation.

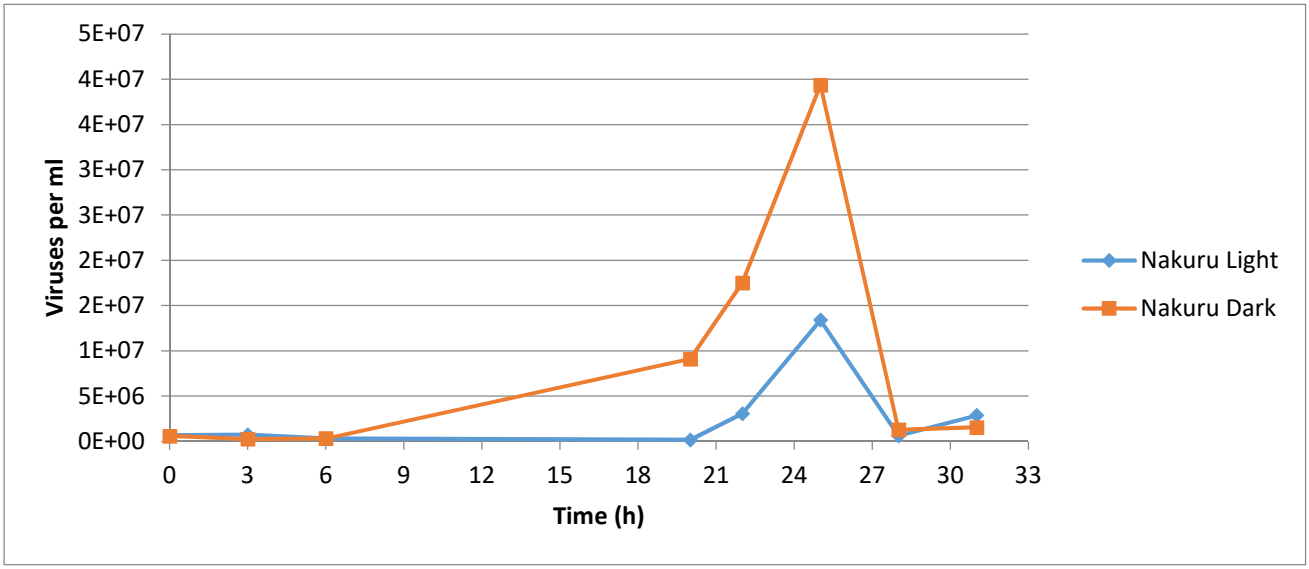


Figure 15- the number of viruses detected in Nakuru by flow cytometry under constant light and dark incubation at 8 time points

3.1.4. Light and dark incubation of Oloidien

Figure 16 shows the number of viruses in Oloidien under permanent light and dark incubation for 31 hours. In both cultures there is a slight increase in the number of viruses until 25h (dark) and 28h (light) both showed a 6-7fold increase in viral particles, followed by a step decrease 3h later. There is a slight increase in the number of viruses until 20 hours in both incubations, then a short drop for two hours in light incubation and another significant rise at time point 22 hours. The number of viruses decreased at 25h (dark) and 28 (light). Although there is a similar pattern between light and dark incubations, the peak in the number of viruses in light incubation occurred 3 hours later (Figure 16).

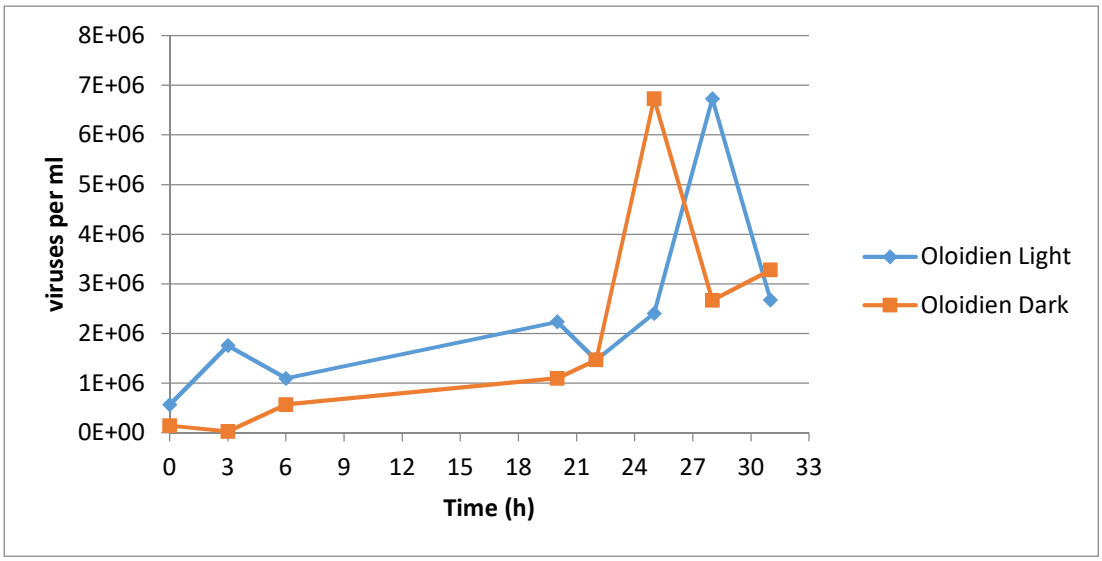


Figure 16- the number of viruses detected in Oloidien by flow cytometry incubated under constant light and darkness at 8 time points

3.2. Confocal Microscopy provided 3D visualization of ultrastructure of *A. fusiformis*

In our confocal microscopy studies, DAPI showed an acceptable level of affinity to DNA and labeling (Figure 17). The labeled DNA was completely detectable in the central parts of the cells although it is surrounded with thylakoids (Figure 18). DAPI also showed proper affinity to ssDNA. So, it is possible to see RNA or DNA of viruses (in the case of infection) along with DNA of cyanobacteria.

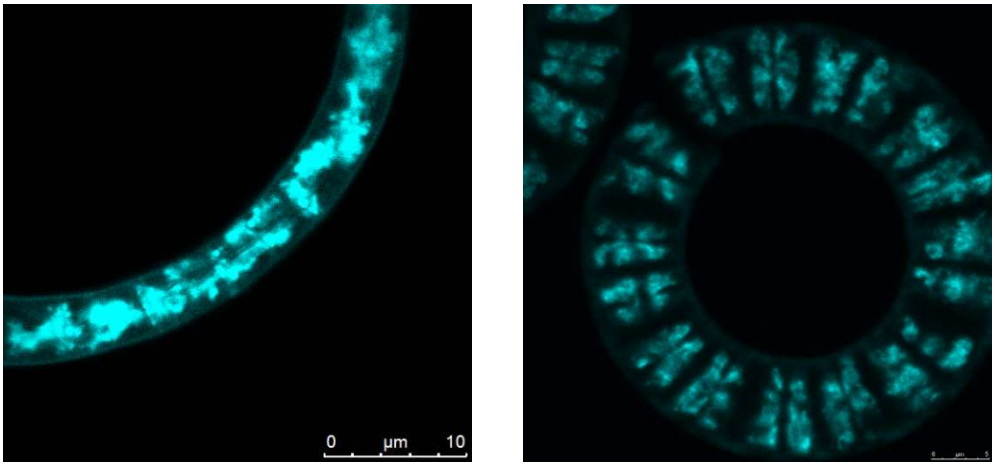


Figure 17- The DNA of the strain Nakuru labeled with DAPI

Thylakoids are auto fluorescent and detectable in at excitation wavelengths of 650-700 nm. Thylakoids are organized at the periphery of cell, embracing the DNA.

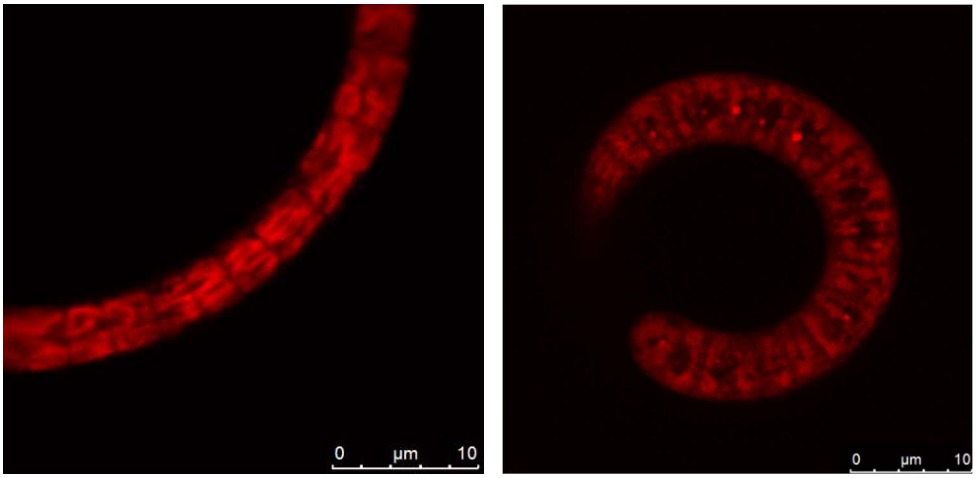


Figure 18- The thylakoids formed a network at the periphery of the thylakoids

The 3D visualization of DNA and DNA-thylakoid overlays done with Amira software is shown in figure 19. In figure 19a, we can see the overlay of DNA and thylakoids and in figure 19b the DNA structure (labeled by DAPI) is shown. It becomes clear by 3D images that the thylakoids are distinctively organized in the periphery and the DNA is mainly detectable at the center of a cell.

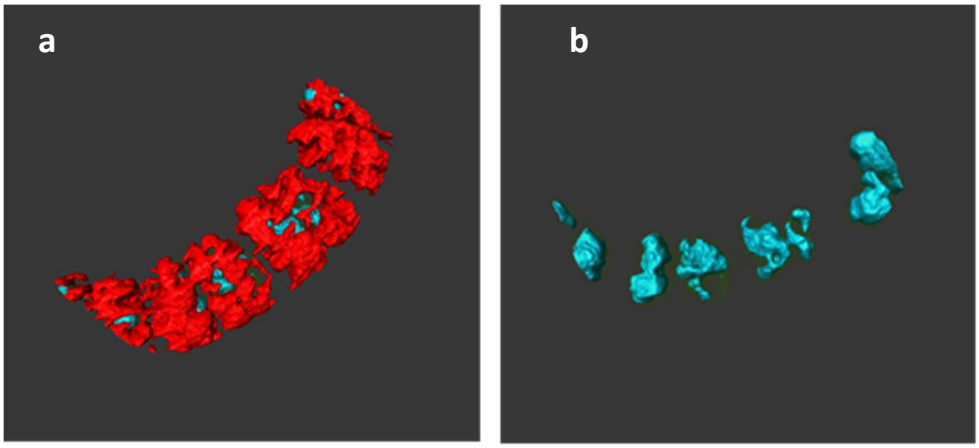


Figure 19- 3D visualization of DNA and thylakoids embracing the DNA by AMIRA software; a) overlay of thylakoids and DNA-fluorescence, b) DNA only

3.2.1. Deborah not-induced

As an example for staining, figure 20 shows “raw images” with DNA and thylakoids at time point “25 hours” taken by confocal microscopy. These two images show no visible change in the structure of thylakoids and the location of the DNA. The thylakoids are still well structured and surround the DNA which is almost homogenously spread out through the cells.

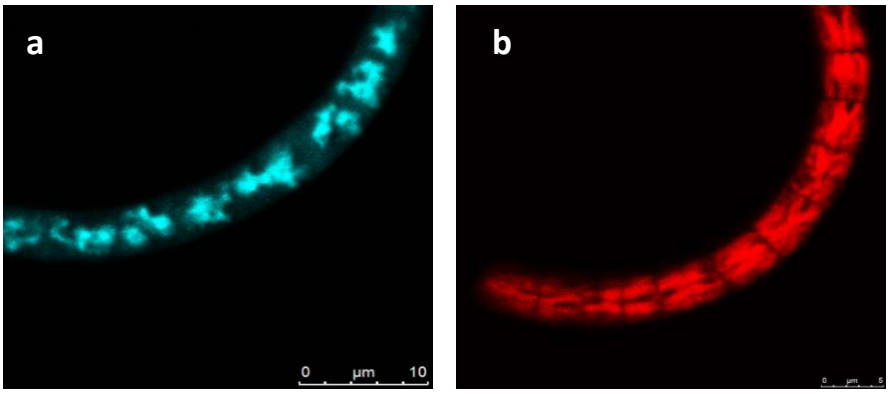
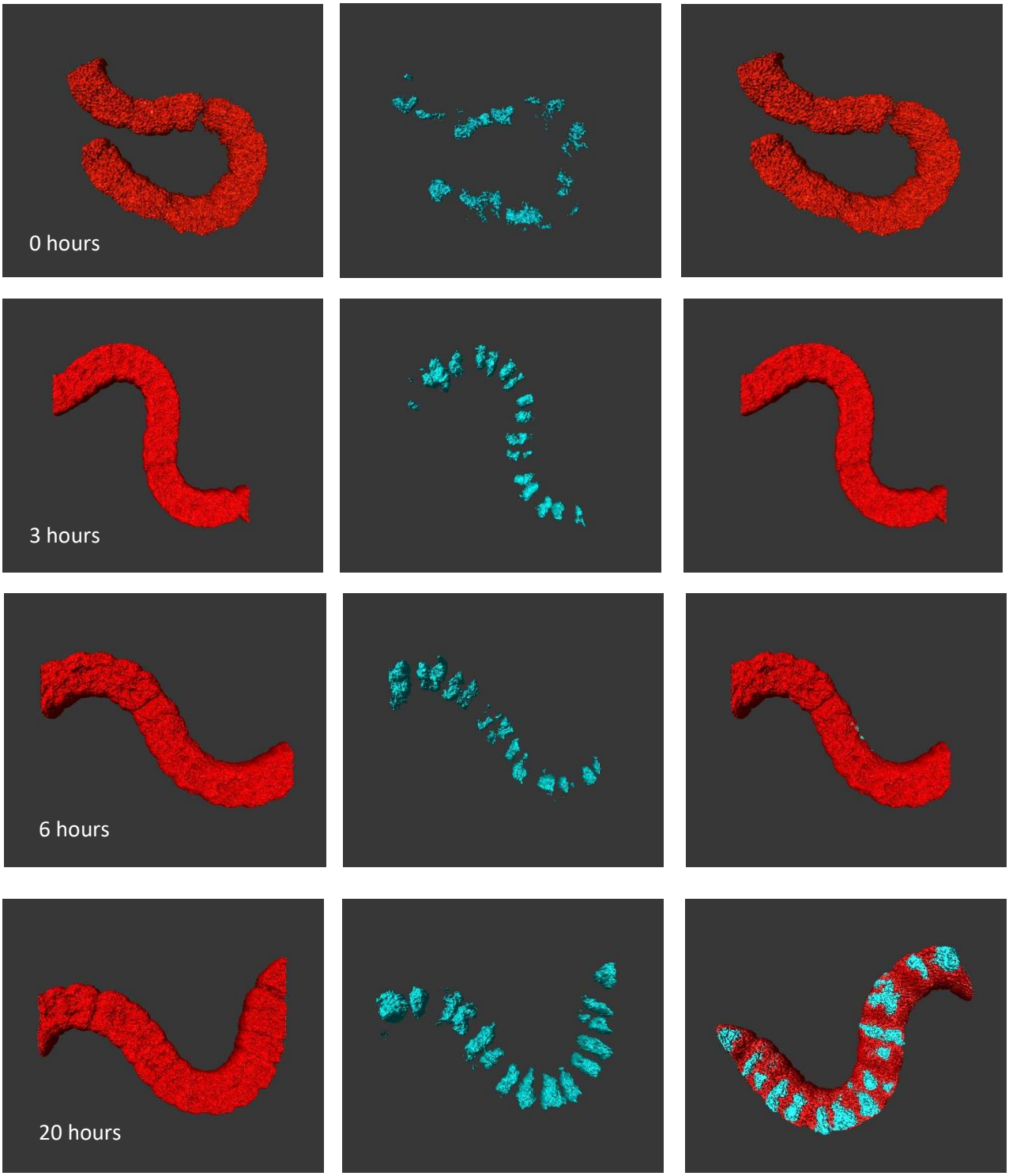


Figure 20- The confocal images of DNA (a) and thylakoids (b) of not-induced Deborah 25 hours after the beginning of the experiment

The 3D images of DNA and thylakoids are represented in figures 20 and 21 to show temporal changes in these two organelles at 8 consecutive time points. The 3D images of not-induced Deborah in figure 20 show that the thylakoids form a dense network at the periphery of the cell, properly wrapping the DNA. There is no visible disturbance or alteration in the structure of thylakoids. The DNA clearly expands throughout the cells. The right images show the overlay images of DNA and thylakoids. The overlay images represent that the thylakoids completely surround the DNA. On overlay images of time points 20, 22, 25, 28 and 31 hours, we removed some of the stacks by AMIRA software to show the location of DNA within the thylakoids. There are no significant changes in the DNA and thylakoids through the time points.


0 hours
3 hours
6 hours
20 hours

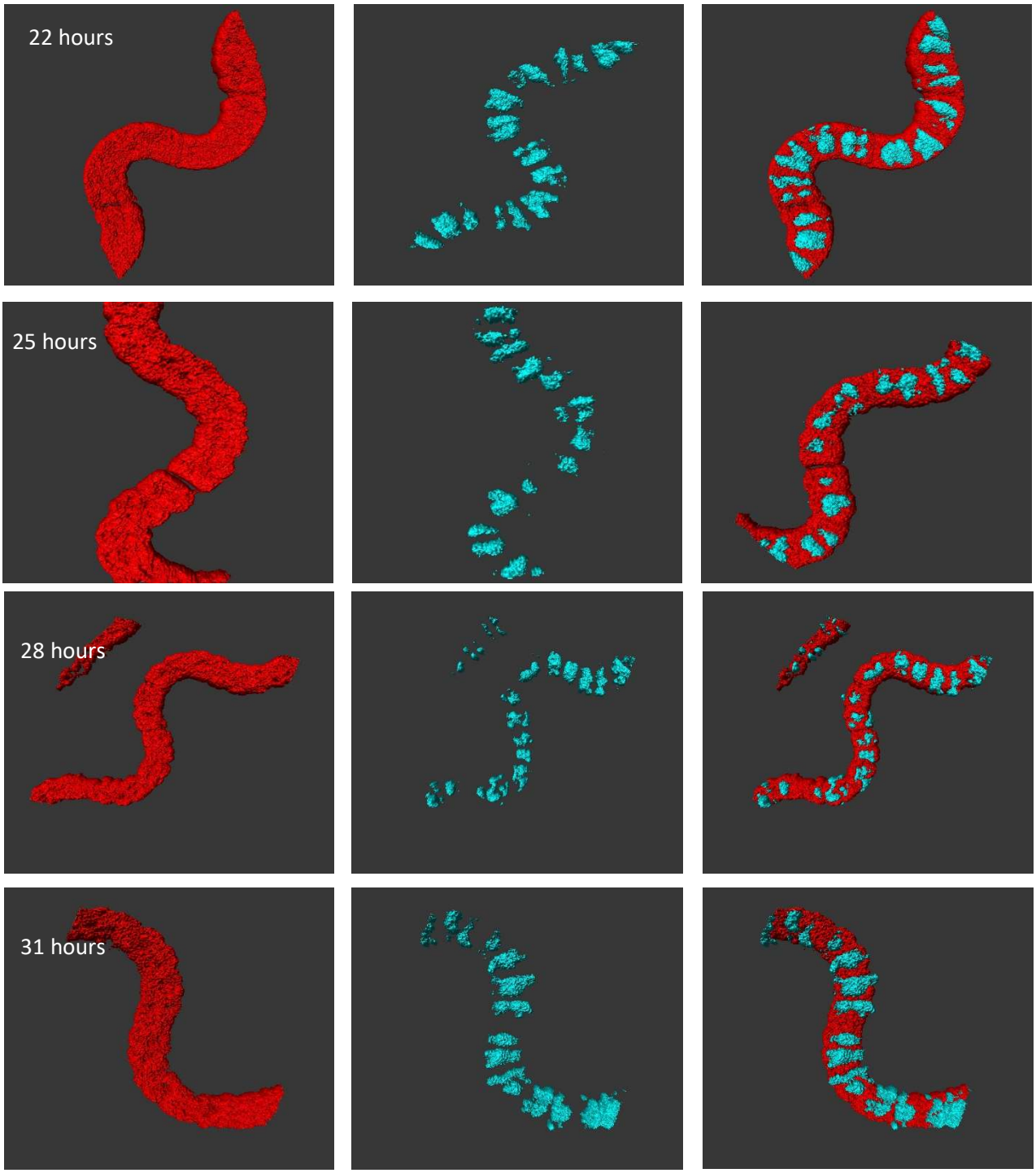


Figure 20- Thylakoids (left), DNA (middle), overlay image (right) of not-induced Deborah 0, 3, 6, 20, 22, 25, 28, 31 hours after beginning of the experiment. At time points 20, 22, 25, 28 and 31 hours, we removed some of the stacks by AMIRA software (right column) to show the location of DNA within the thylakoids.

3.2.2. Deborah; induced with MMC

Figure 21 shows induced Deborah with MMC at time point “25 hours”. The thylakoids are not consistent anymore and the network-like structure of thylakoids looked disturbed. The DNA is more located at the center or on one side of the cells.

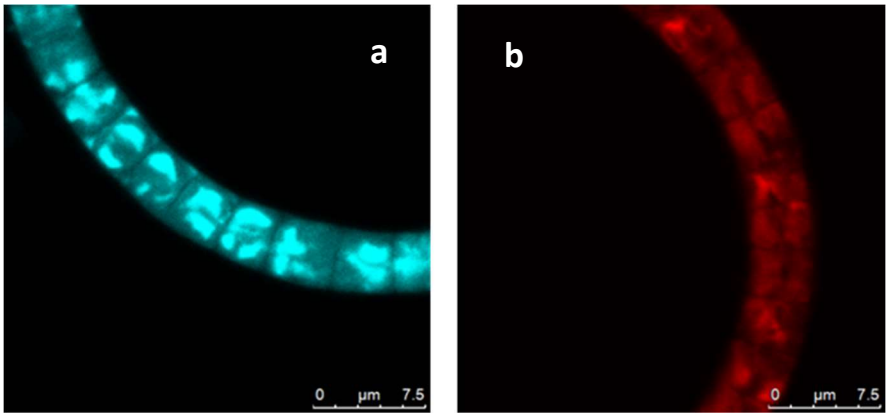
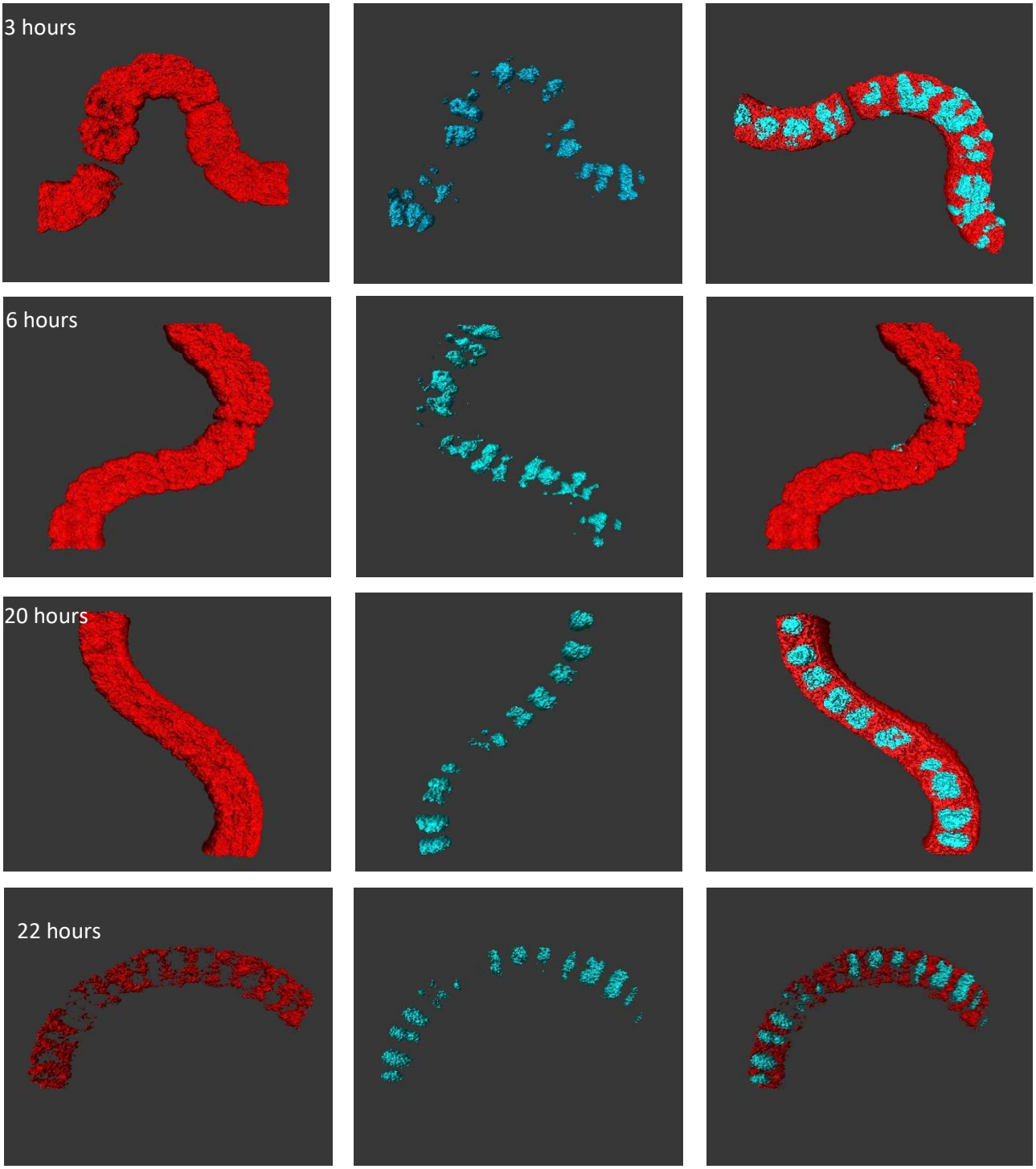


Figure 21- The confocal images of DNA (a) and thylakoids (b) of induced Deborah 25 hours after the treatment with MMC

As we did not expect any immediate effect of MMC, sampling of Deborah was started 3 hours after the treatment with MMC. Figure 22 show the changes of DNA and thylakoids over time in induced Deborah by MMC. At time point 20h, we can see significant alteration in the thylakoids so that the network of the thylakoids was disturbed with visible fractures through the whole structure. Macroscopically, the cultures have lost their characteristic green color over time. The DNA looked more compact and condensed in the middle or on one side of the cells. Interestingly, at time point 25h, the thylakoids looked reconstructed. The DNA can be found all over the cell, without less compression.


3 hours
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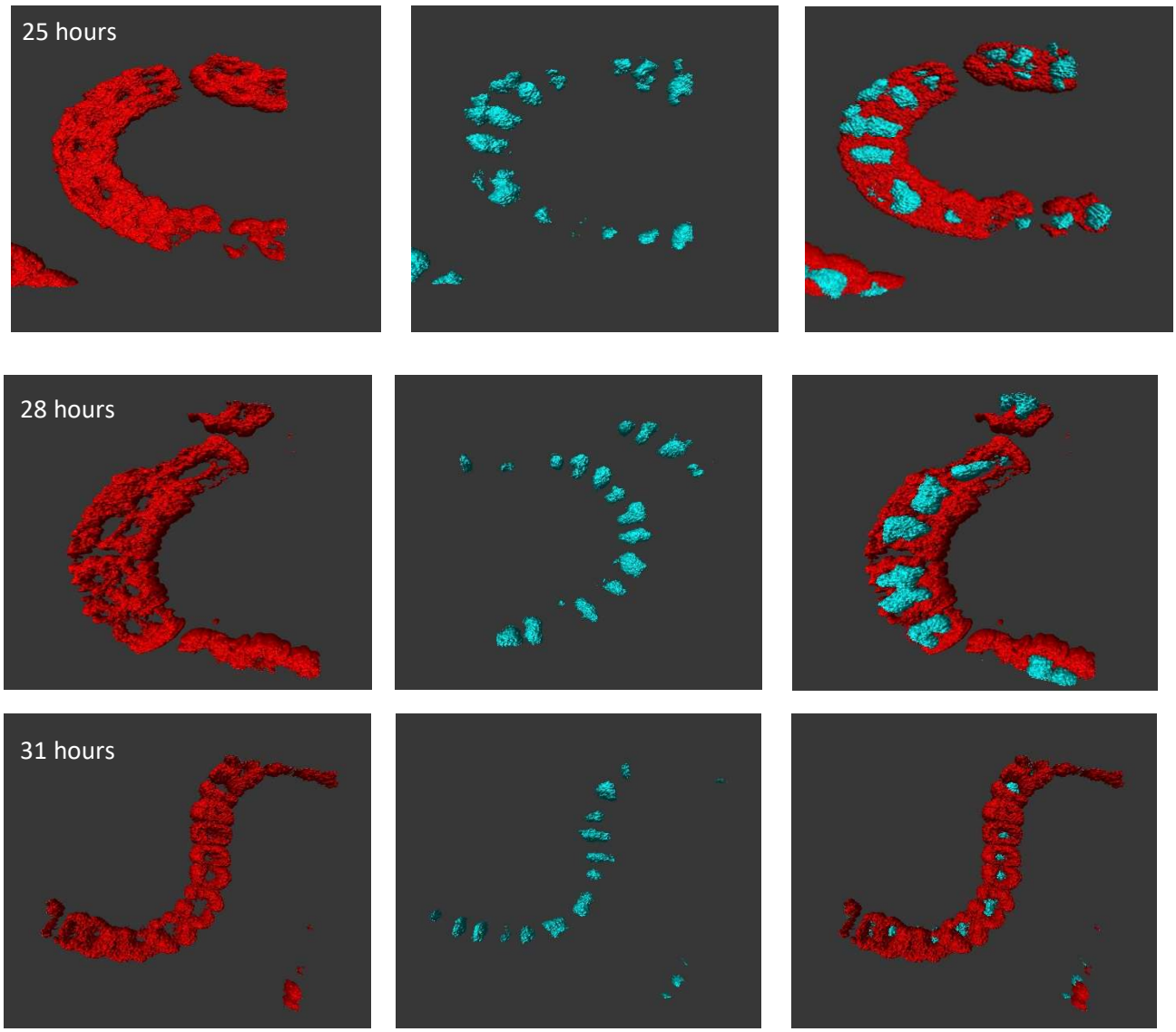


Figure 22- Thylakoids (left), DNA (middle), overlay image (right) of induced Deborah with MMC 3, 6, 20, 22, 25, 28, 31 hours after beginning of the experiment.

3.2.3. Nakuru incubated under constant light

Figure 23 demonstrates “raw images” of DNA and thylakoids of Nakuru, grown under constant light, taken by confocal microscope at time point “0 hours”. interestingly, thylakoids formed a continuous network at the periphery of the cells. The DNA distinctively expand over the cells.

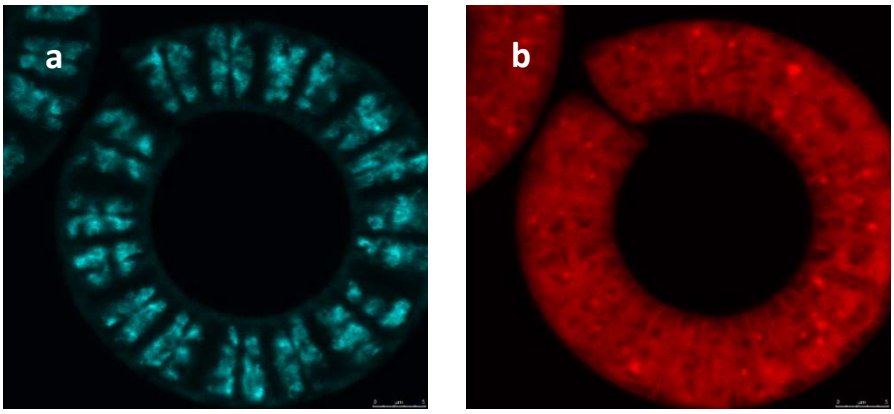
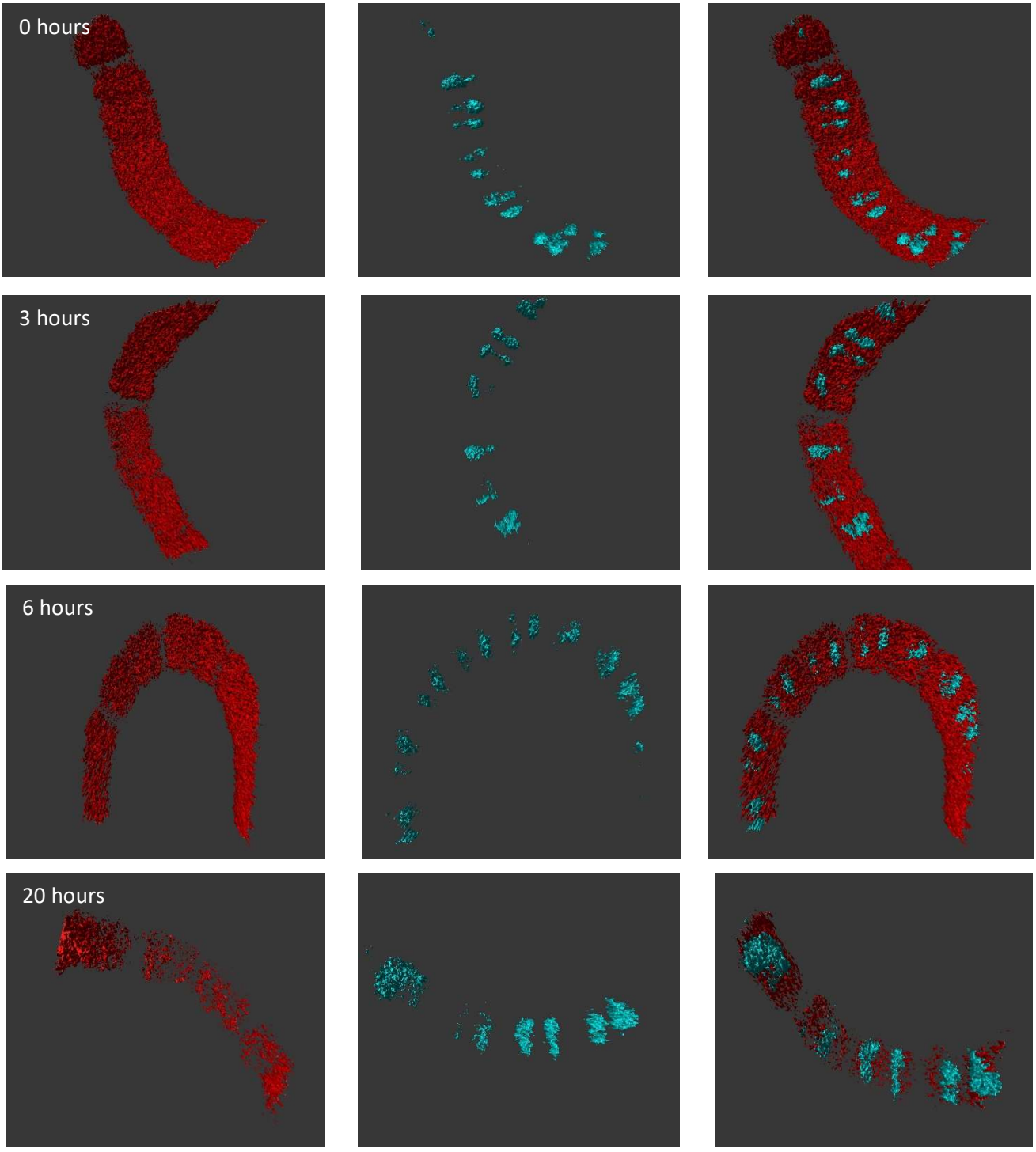


Figure 23- The confocal images of DNA (a) and thylakoids (b) of Nakuru under constant light

In the figure 24, the 3D segmentation of DNA and thylakoids of Nakuru incubated under constant light is visible. The structure of thylakoids is changed 20 hours after exposure to the constant light. The network-like structure of thylakoids looked disturbed and compare to the raw image (Figure 23), the consistency of the structure is less visible. The sample was pale green in color after running the experiment. The DNA is also clearly distinguishable. In overlay images, it is obvious that the DNA dispersed almost homogenously through the cell. But, at time point 25h, the DNA looked denser at the center or one side of the cells. At time point 28 and 31h, the DNA returned to its initial state and dispersed again.


0 hours
3 hours
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20 hours

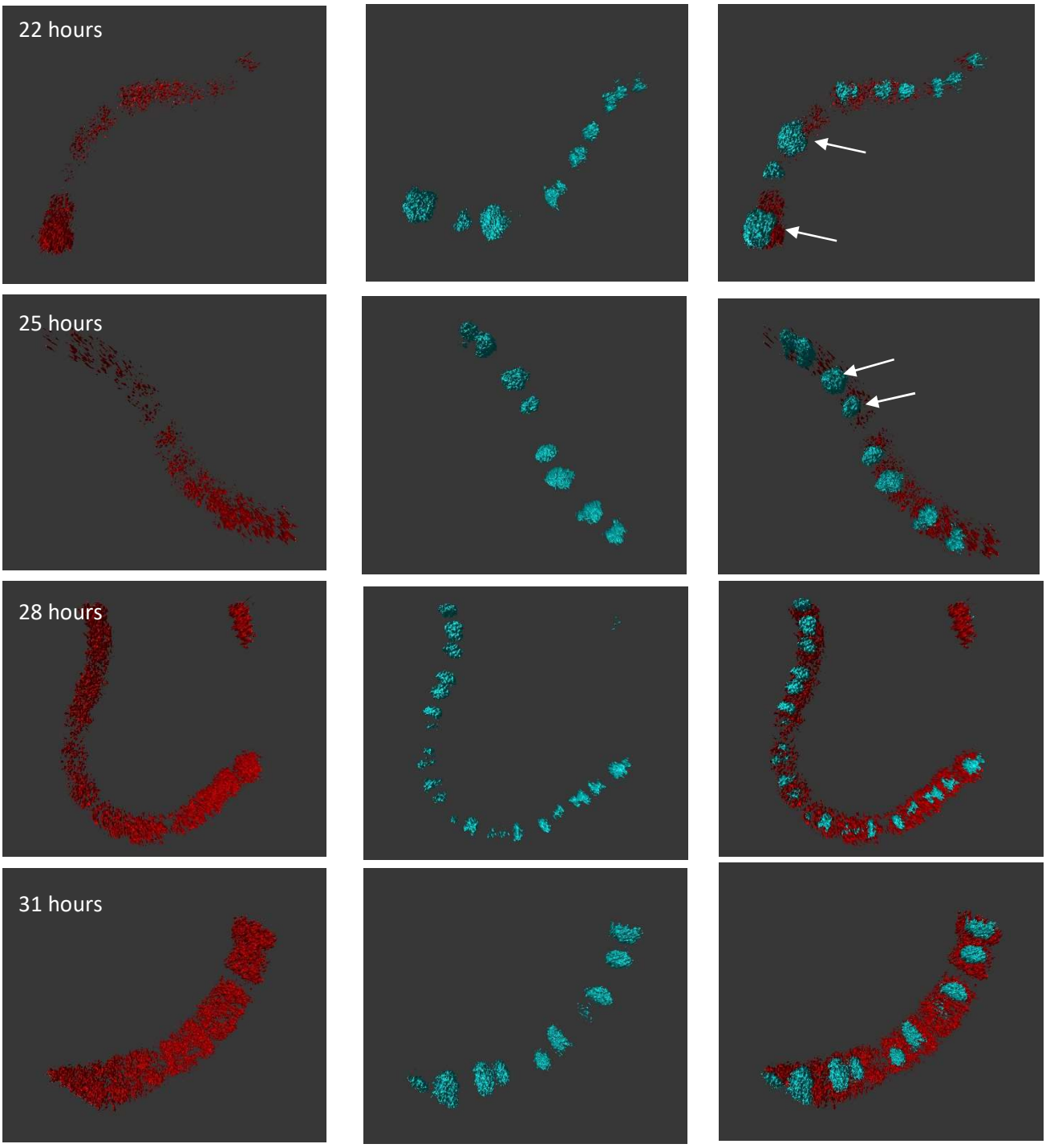


Figure 24- Thylakoids (left), DNA (middle), overlay image (right) of Nakuru incubated under the constant light 0, 3, 6, 20, 22, 25, 28, 31 hours after exposure to the permanent light. Arrows show the DNA which is located on one side of the cell.

3.2.4. Nakuru incubated under constant darkness

The DNA and thylakoids of Nakuru grown at full darkness are presented in figure 25. The structure of thylakoids has changed so that there are holes and disconnection in the thylakoids network. The membrane of thylakoids also bleached much faster. The DNA has changed so that, it seemed more compact and shifted towards one side of the cells.

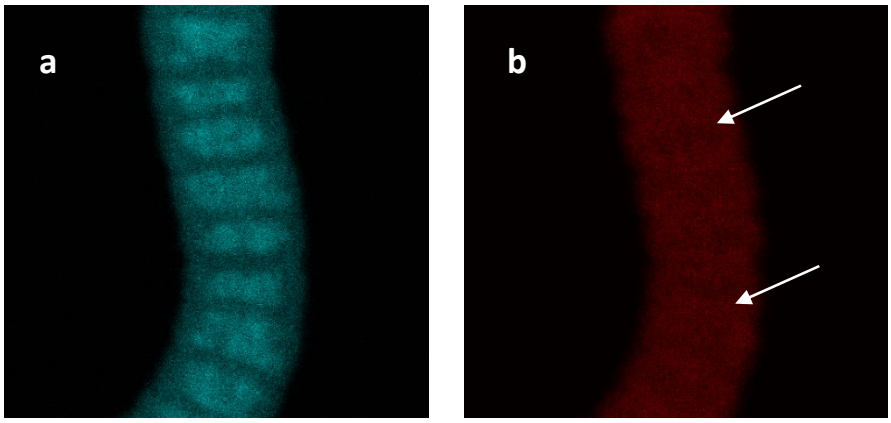
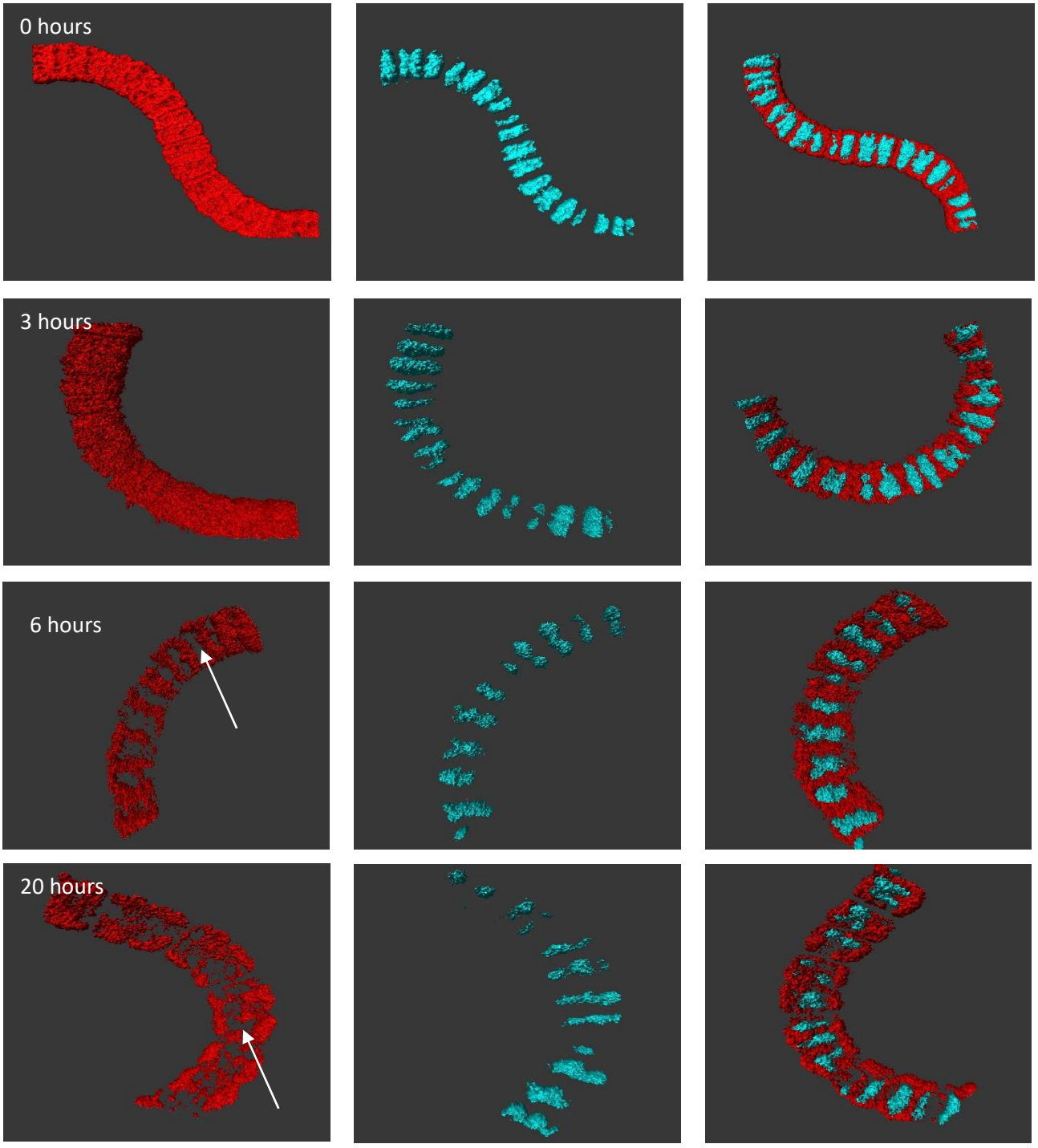


Figure 25- The confocal images of DNA (a) and thylakoids (b) of Nakuru stored at darkness for 25 hours. Arrows in the image b pointed out the holes in the thylakoids.

In the figure 26, the images of thylakoids and DNA of Nakuru, grown in the darkness, are provided. Confocal images showed a significant change in the structure of thylakoids over time. The first significant sign of change was detectable after 6 hours. The thylakoids do not look like a connected network and there are visible changes through the entire chain. At this time, the color of the culture changed from dark to pale green. There are no detectable alterations in the DNA during the first 20 hours. But at next time points (time point 22h), DNA seemed to be compressed and accumulated on one side of the cells.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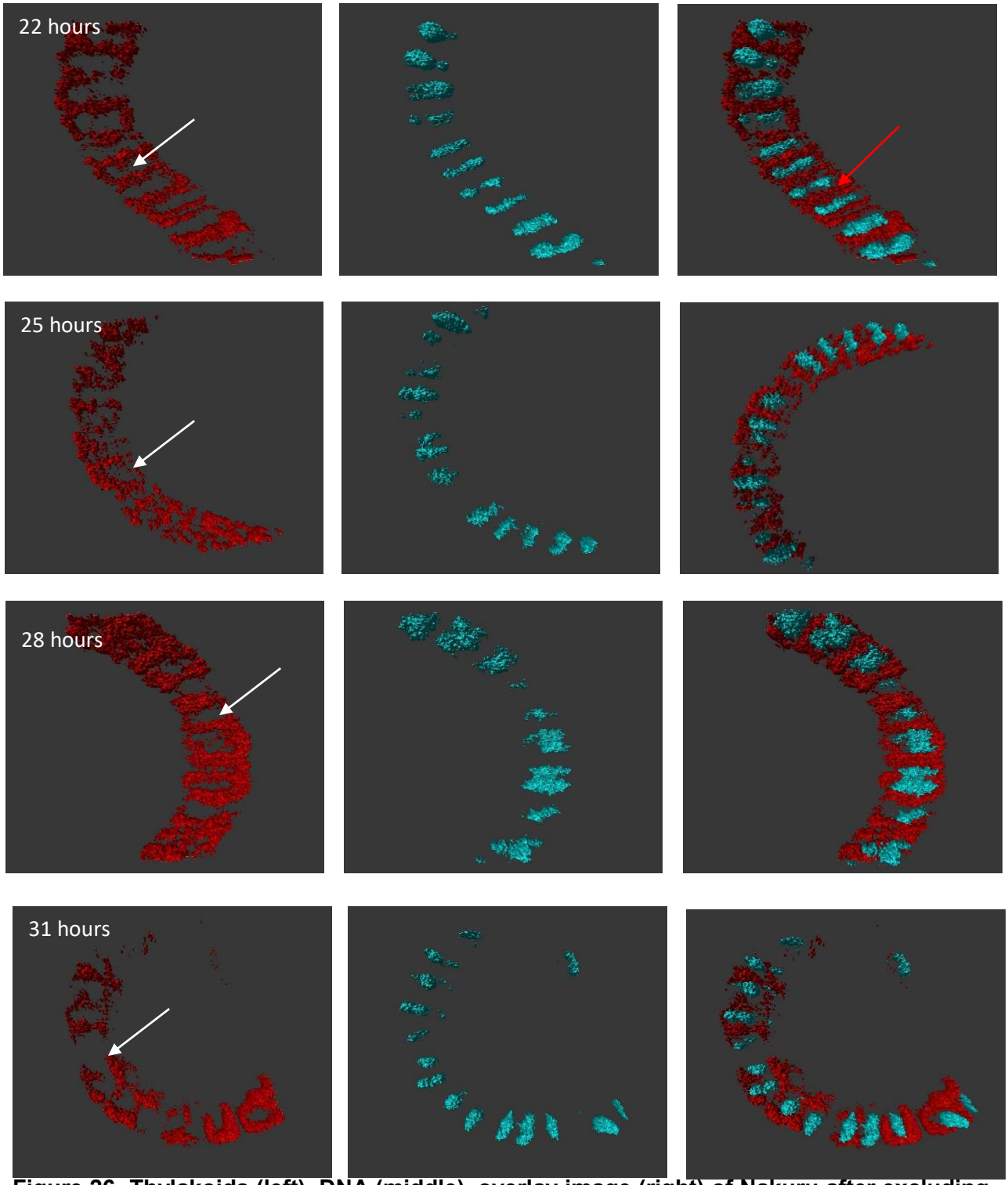


Figure 26- Thylakoids (left), DNA (middle), overlay image (right) of Nakuru after excluding the light 0, 3, 6, 20, 22, 25, 28, 31 hours after beginning of the experiment. White arrows show the changes and disconectivity of the network of thylakoids. Red arrow shows the compressed and localized DNA.

3.2.5. Oloidien incubated under constant light

Figure 27 demonstrates the DNA and thylakoids of Oloidien cultured under the constant light, taken by confocal microscope at time point “25 hours”. There is visible abnormality in the DNA and dissociation in the thylakoid structure. There are detectable holes in the DNA and the DNA is not distributed homogenously anymore. The structure of thylakoids looked continuous and as we can see in figure 27b, thylakoids are located at the periphery of the cells like a network.

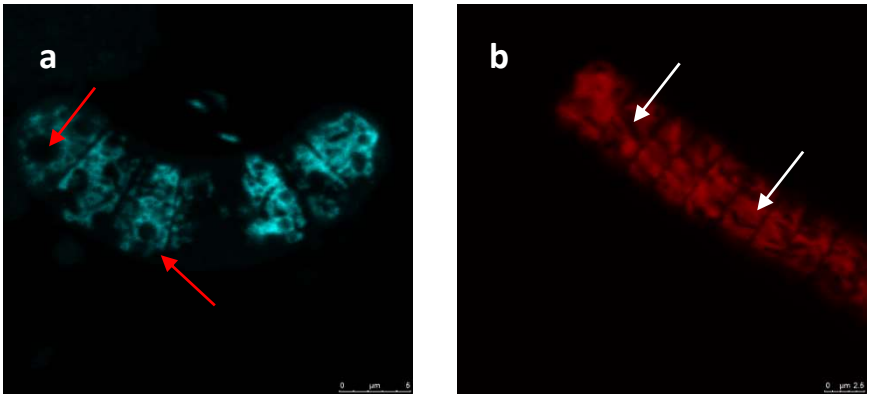
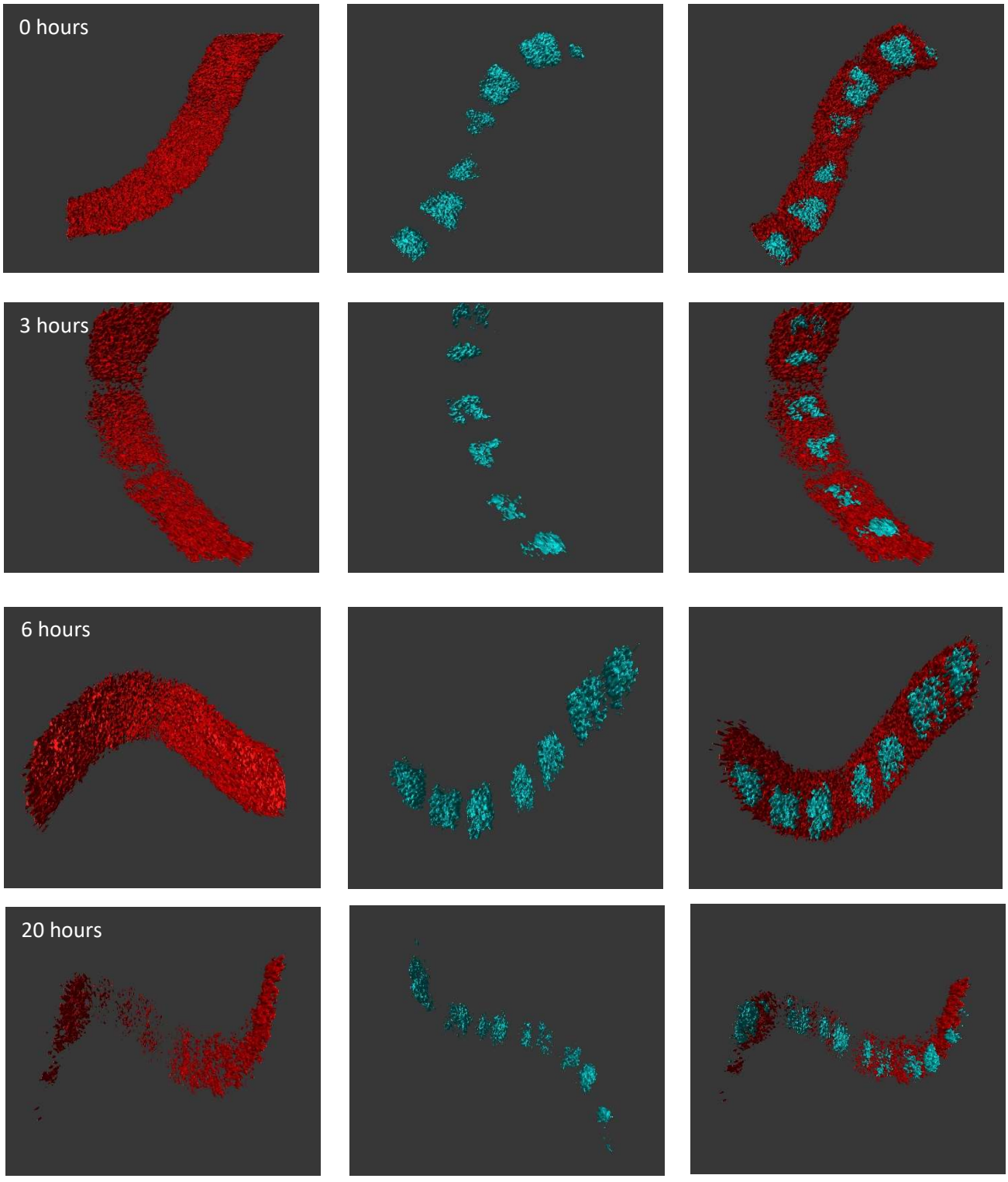


Figure 27- The confocal images of DNA (a) and thylakoids (b) of Oloidien under constant light at time point 25 hours. White arrows show the fractures in thylakoids and red ones show the abnormality of DNA.

In the figure 28, the 3D visualization of DNA and thylakoids of Oloidien, under constant light is provided. There are visible alterations in the thylakoids structure so that it did not extend continuously at the periphery of the cells. Over time, the alteration (detected fractures) expanded and the thylakoids lost their expected structure. At time points 25 and 28h, we could see the most prominent changes in the thylakoids. The DNA also looked different. It was less visible and localized at the center of the cells at time points 22, 25 and 28 hours and expanded at the other time points.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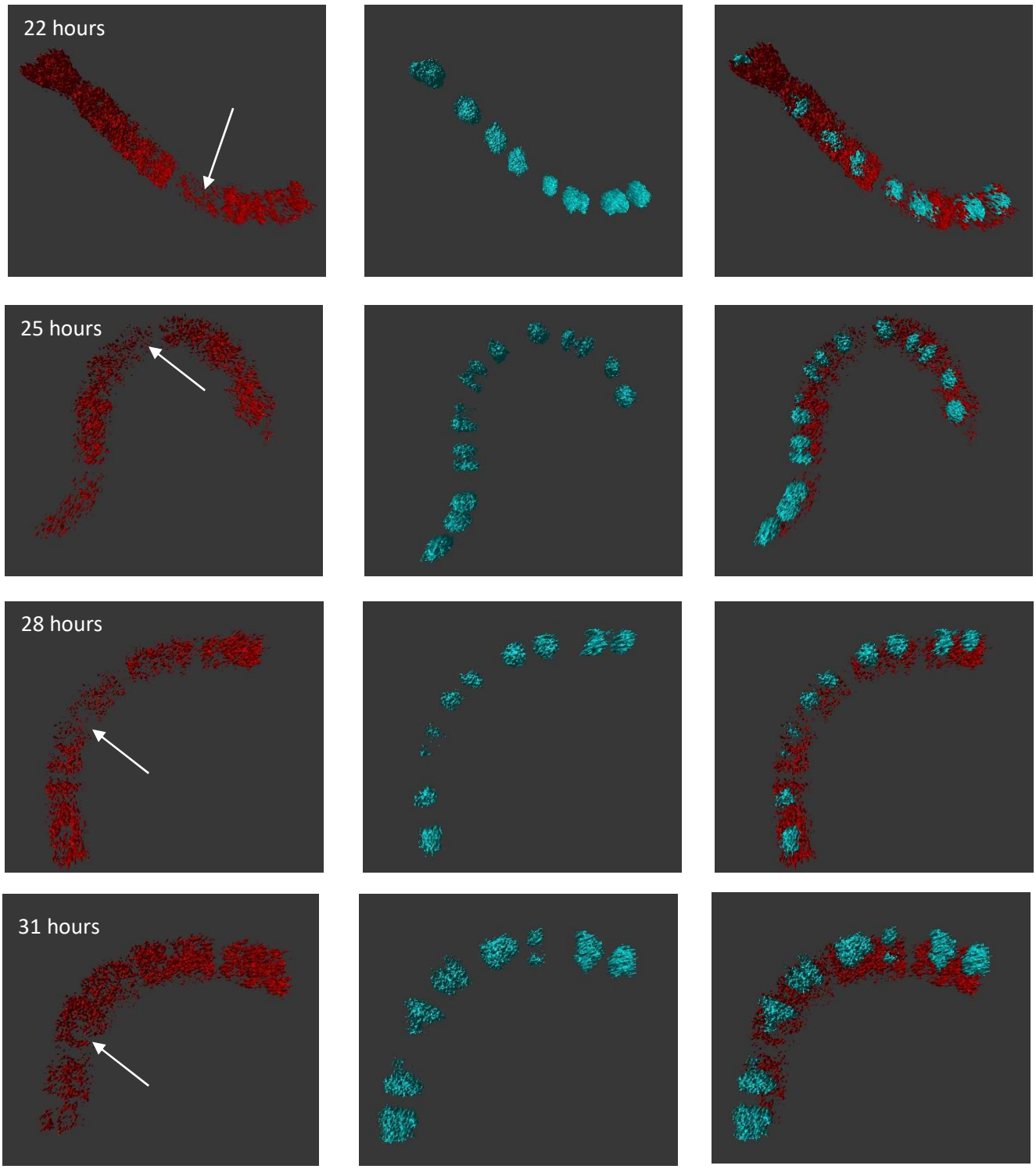


Figure 28- Thylakoids (left), DNA (middle), overlay image (right) of Oloidien under the constant light 0, 3, 6, 20, 22, 25, 28, 31 hours after beginning of the experiment. Arrows show the alteration of thylakoids.

3.2.6. Oloidien incubated under constant darkness

The DNA and thylakoids of Oloidien grown under 24 hours' darkness are presented in figure 29. There are visible holes in the DNA in some cells, the DNA looked reduced in content. The disturbance of the thylakoids is significant (Figure 29b). The thylakoids were no longer detectable as a network and looked like shattered.

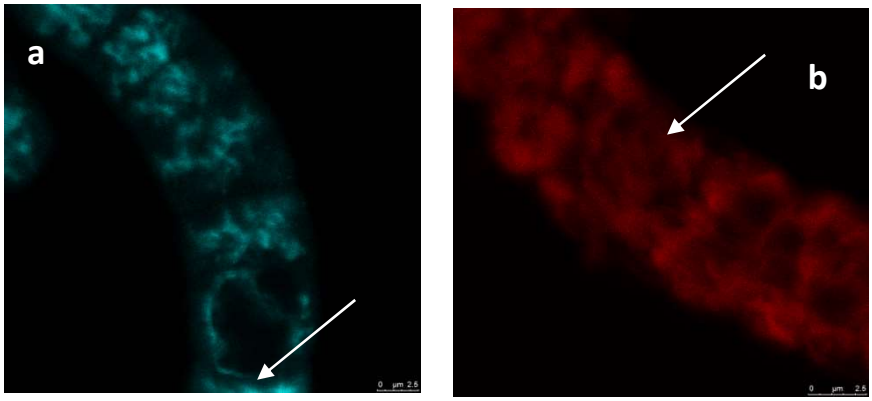
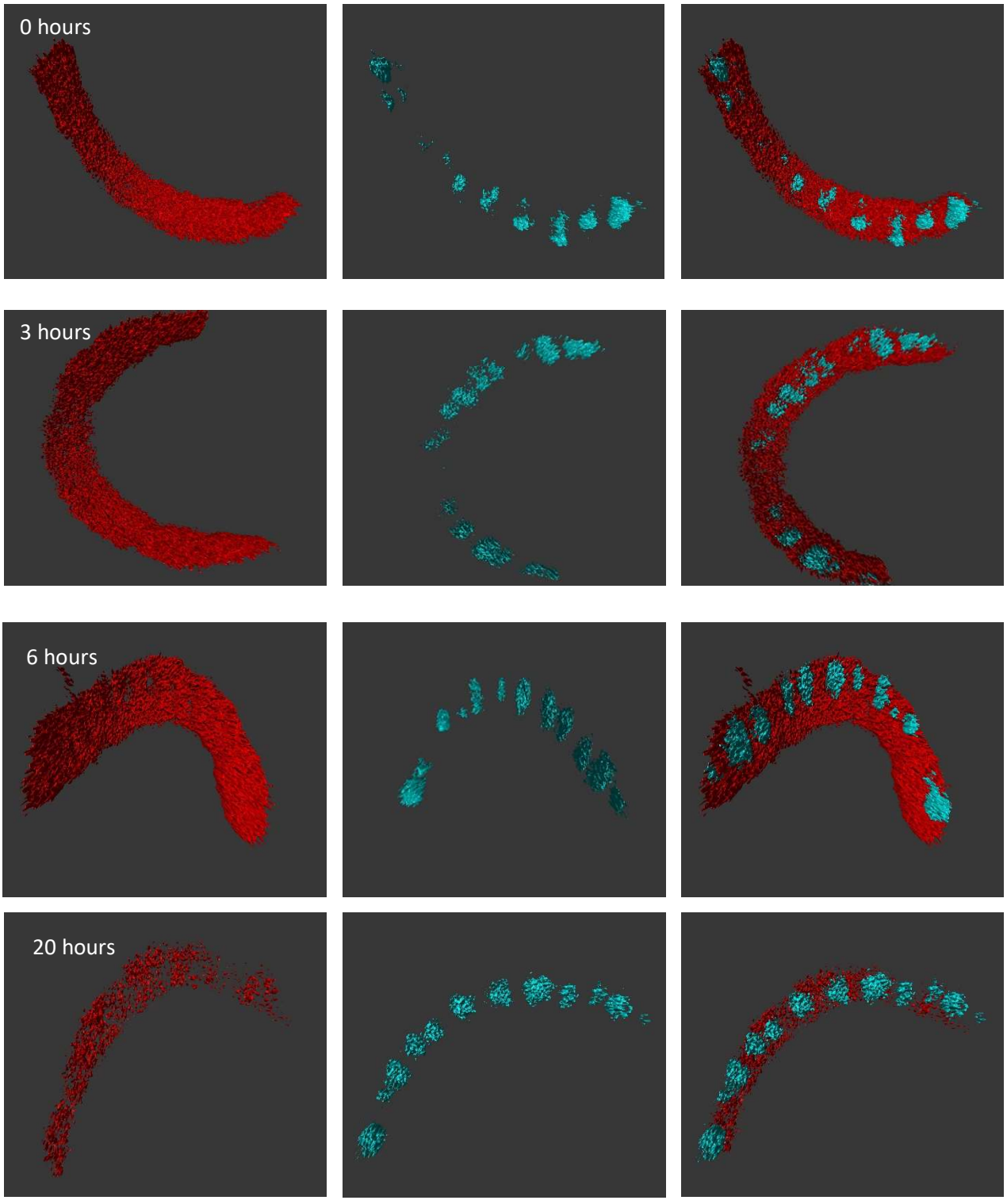


Figure 29- The confocal images of DNA (a) and thylakoids (b) of Oloidien prevented from light at time point 25 hours. The arrow in figure a shows a hole in the DNA and in figure b shows the destruction if thylakoids.

Figure 30 shows the DNA and thylakoids of Oloidien grown under consistent darkness. The alteration of thylakoids started 6h after excluding light, there were significant changes in the thylakoids at time points 20h. These changes included fractures through the network, disconnectivity and the lack of thylakoids in some parts of the cells. The thylakoid alterations got sever continuously and no "reconstruction" of the thylakoids was not detected. The DNA was also changed. At time point 28h, it looked more compact at the center of the cells. The DNA expanded all over the cell at other time points, however, compare to the "raw image" (Figure 29) the content of the DNA looked reduced and detection of DNA was harder 20 hours after incubation.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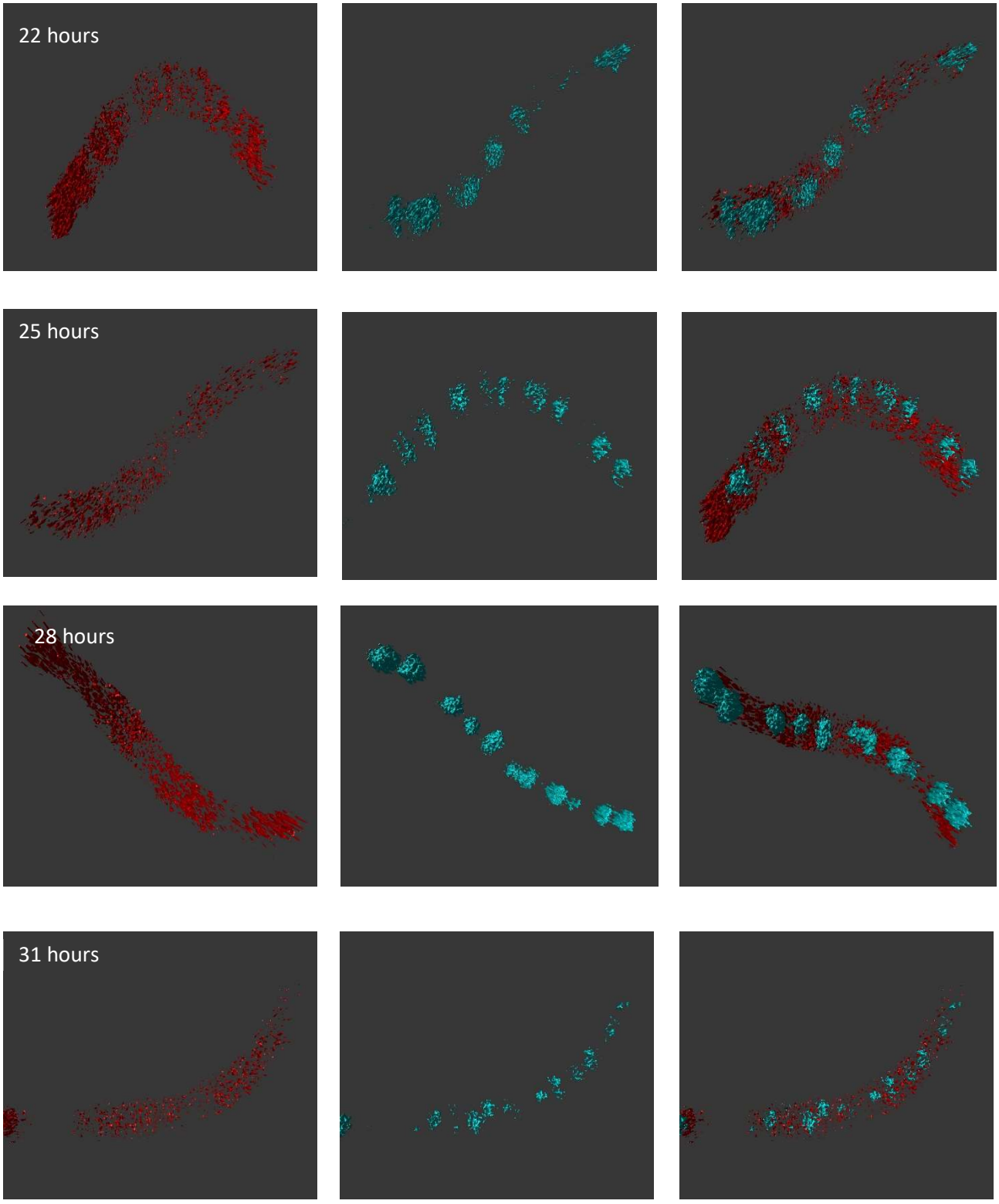

Figure 30- Thylakoids (left), DNA (middle), overlay image (right) of Oloidien after excluding the light. Arrows show the DNA which is pushed to one side of the cell

4. DISCUSSION

Arthrospira fusiformis is one of the main ecological drivers in soda lakes as it is resistant to the harsh conditions of these ecosystems. Many other species such as Lesser Flamingo depend on it for nutrition. *Arthrospira* is also known for its high nutritional value and considered as one of the most important industrially cultivated microalgae (Vonshak & Tomaselli, 2007). Thereby, it is of great interest to know any factors such as virus attacks which may affect *Arthrospira* in both the field and in cultures. In this survey, we used flow cytometry to detect cyanophage infection in three strains of *A. fusiformis* and then investigated temporal alteration in the DNA and thylakoids of *A. fusiformis* by means of confocal microscopy.

4.1. Flow cytometry revealed oscillation in the number of viruses in all samples

Peduzzi et al (2014) have discussed trophic cascade effects caused by cyanophage infection as the main reason for the significant reduction in the number of flamingos in the Lake Nakuru (Kenya). The authors illustrated that *A. fusiformis* blooms are interrupted at irregular intervals through cyanophage infection (Figure 33). They also measured the highest densities of suspended viruses ever recorded in a natural aquatic system. It was concluded that the virus infection affects the bottom-up trophic cascade, the food chain and the final consumers (Schagerl & Peduzzi, 2014). In transmission electron micrographs, they also detected visible signs of viral infection in *A. fusiformis* cells (Figure 34).

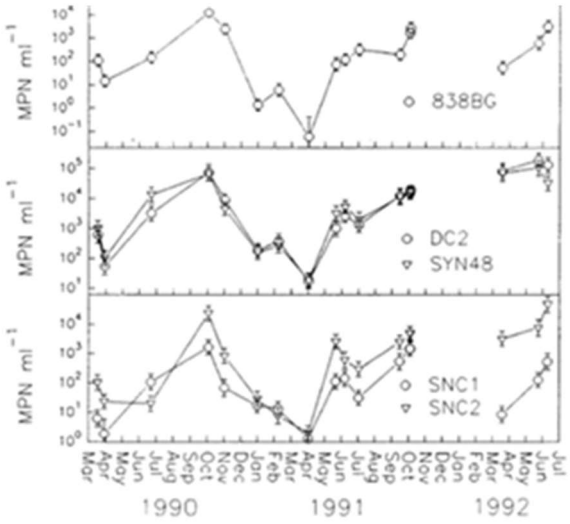

Figure 31- The number of cyanophages infecting five marine *Synechococcus* strains (838BG, DC2, SNC1, SNC2, SYN48) in coastal seawater of the western Gulf of Mexico (Suttle & Chan, 1994)

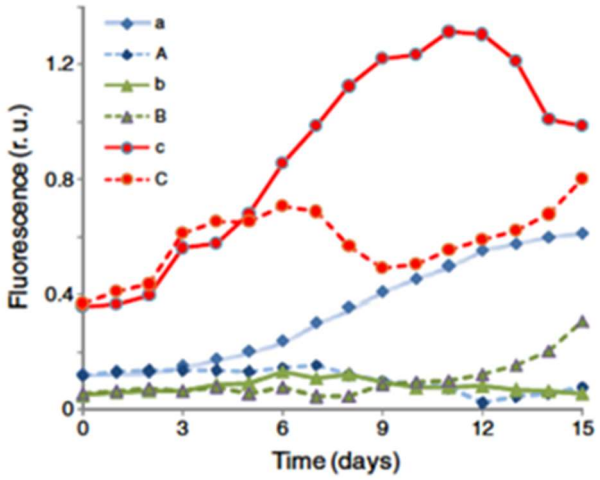

Figure 32- variable responses of different *Arthrospira platensis* strains incubated with cyanophages. A, B, C: infected; a, b, c: not infected cultures. Lines represent the cyanophage induction and colors represent different strains. In one strain (green line), cyanophage induction did not affect the cyanobacteria but in two other strains, cyanophage induction caused high mortality (Jacquet et al, 2013).

Peduzzi et al (2014) have discussed trophic cascade effects caused by cyanophage infection as the main reason for the significant reduction in the number of flamingos in the Lake Nakuru (Kenya). The authors illustrated that *A. fusiformis* blooms are interrupted at irregular intervals through cyanophage infection (Figure 33). They also measured the highest densities of suspended viruses ever recorded in a natural aquatic system. It was concluded that the virus infection affects the bottom-up trophic cascade, the food chain and the final consumers (Schagerl & Peduzzi, 2014). In transmission electron micrographs, they also detected visible signs of viral infection in *A. fusiformis* cells (Figure 34).

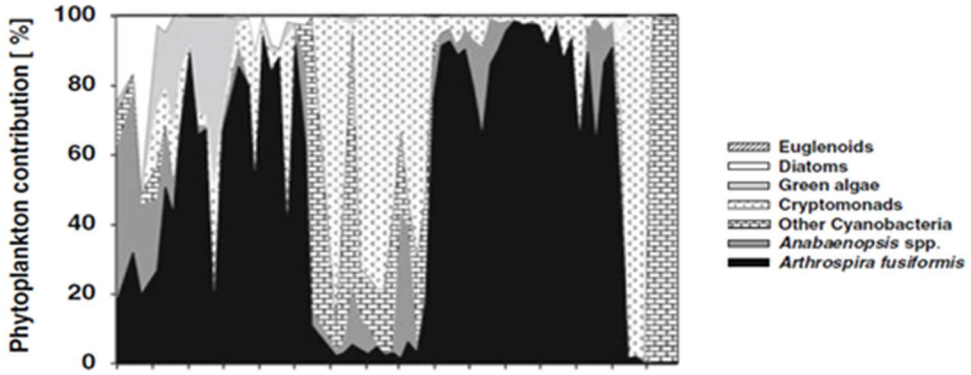

Figure 33- the breakdown of *Arthrospira fusiformis* biomass (Schagerl & Peduzzi, 2014).

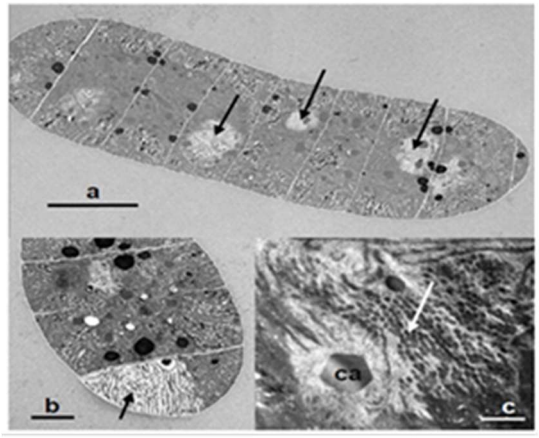

Figure 34- Ultrastructure of "cyanophage infection in *A. fusiformis* cells. (a) longitudinal section through a coil of a cell filament; arrows point to infected areas, which appear bright and disarranged, scale bar 5mm; (b) close up of an infected area (arrow) with signs of already assembled capsids, scale bar 2mm; (c) area containing already visibly developed cyanophages (white arrow)" (Schagerl & Peduzzi, 2014)

In the case of Deborah, we saw different patterns in FCM diagrams between the samples treated with and without MMC. It has been shown in other studies that treatment with MMC causes the induction of prophages in various cyanobacteria (Chen et al, 2006; Jacquet et al, 2013; Raya & Hbert, 2009; McDonald et al, 2010). In our experiment, treatment with MMC caused a significant increase in the number of viruses, relative to the control without MMC reaching a peak at 20 hours after induction with MMC.

FCM analysis of Nakuru and Oloidien did not show a significant difference in the number of viruses between MMC-treated and the control sample without MMC. Thus in cultures of these two strains we did not find an indication for MMC-inducible prophages. However, in both, MMC-treated and untreated controls we found oscillations in the number of viruses every 22 to 25h hours. Since *A*. *fusiformis* is by far the most prominent component in our cultures and cyanophages are known for diurnal patterning of virus-release (Welkie et al. 2018, Yoshida et al. 2018) we assumed this increase was attributed to cyanophages of *A. fusiformis*. Cyanophages show diurnal rhythm so that the adsorption of some cyanophages or their replication is affected by light (Ni & Zeng, 2016). Liu et al (2019) showed that the cyanophages infecting *Prochlorococcus* sp. and *Synechococcus* sp. have diurnal infection rhythm (Liu et al, 2016). On the other hand, Jia et al (2010) showed that the adsorption of cyanophage S-PM2 to the *Synechococcus* sp. is not light-dependent (Jia et al, 2010). The observed oscillation in the number of viruses in both dark and light incubation suggests that within the time period tested the respective cyanophages in Nakuru and Oloidien do not depend on light. Interestingly, contrary to our expectation significantly more phages were released in Nakuru incubated in the dark. In Oloidien incubated in the dark phages occurred earlier than in light treated samples. This relates to two important parameters of phage infections: burst size (i.e. the number of viruses released per infected cell and generation time (i.e. the time the phage needs to complete the cycle from infection to virus release). Burst-sizes can be lower and generation times higher when host-fitness is impaired. In Cyanobacteria excess of reactive oxygen species (ROS) generated during photosynthetic light reactions needs to be detoxified in the dark (Welkie et al, 2019). Since our cultures were grown under light conditions without dark period one possible explanation for this finding is that, light incubated Nakuru and Oloidien samples were stressed by ROS. On the other hand, dark

incubated cultures had the chance to recover from this stress and produced more phages (Nakuru) respectively produced them earlier (Oloidien). This fits to the observations that under these conditions our cultures initially grew fast but suddenly collapsed after reaching higher densities.

4.2. Confocal microscopy revealed the alterations in the ultrastructure of *A. fusiformis*

The images obtained from confocal microscopy showed that the alteration in thylakoids started 20 hours after induction with MMC in Deborah strain which coincided with the highest number of viruses. Sherman and Haselkorn (1970) investigated the effects of cyanophage infections on the ultrastructure of the filamentous Cyanobacterium *Plectonema boryanum* using electron microscopy. They observed invagination of the thylakoids in the first 3 hours. It was also shown that cell lysis begins at 7 hours and by 15 hours very few cells remain unlysed (Figure 35).

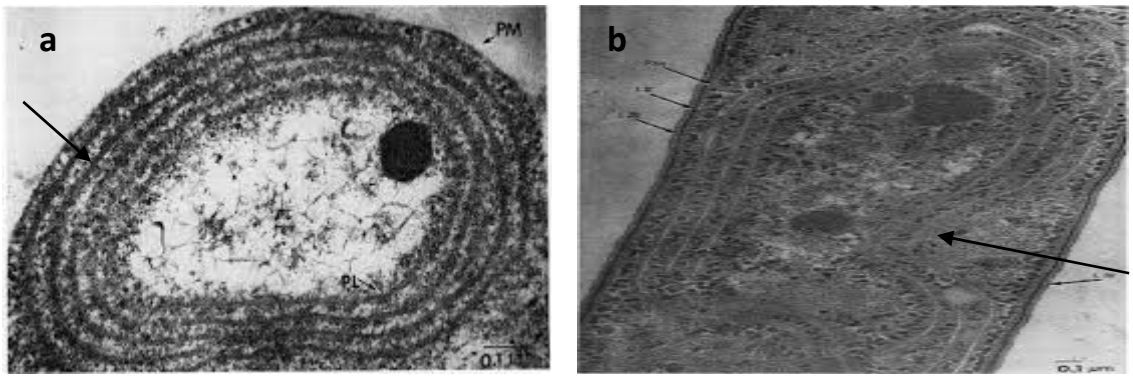

Figure 35- Uninfected *Plectonema boryanum* cell (figure a); photosynthetic lamellae shown with an arrow at periphery of the cell. Infected *Plectonema boryanum* cell (figure a); invagination of photosynthetic lamellae (Sherman & Haselkorn, 1970)

The structural dynamics of thylakoid membranes was studied by Iwai and colleagues (2014) using confocal microscopy. In this study, confocal microscopy allowed visualizing the thylakoid membrane in the moss *Physcomitrella patens* and showed that although the

entire structure of thylakoids is stable, the individual dot-like structure and thread-like structures are not completely fixed and showed some random oscillations (Figure 36).

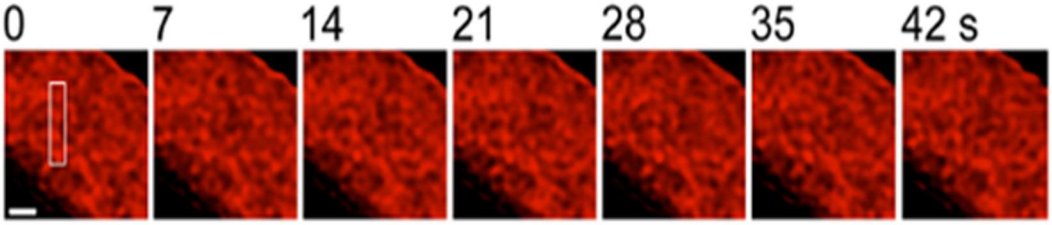

Figure 36- Random changes of the dot-like structures in the thylakoids of *Physcomitrella patens* (Iwai et al. 2014)

Skaloud and Radochova (2004) used confocal microscopy to investigate the number and morphology of chloroplasts in numerous genera of coccal green algae essential for species identification. Viruses may cause damage to the photosynthetic apparatus and its thylakoids membranes. It was shown that viral infection can lead to the invagination and deformation of thylakoids (Hewson, 2000). As shown by Sherman and Haselkorn (1970) viruses can disturb Photosystem II and cause breakdown of the electron transport to photosystem I. The resulting free oxygen species may cause damages to the photosynthetic apparatus (Hewson, 2000).

Confocal microscopy was also used to visualize the response of DNA to environmental factors (Murata et al, 2016) or to investigate DNA replication cycles (Manders et al, 1992). The DNA of non-induced Deborah was expectedly organized at periphery of the cells and showed uniform DNA labeling throughout the cells. The DNA of samples treated with MMC was more compact. In a study on the infection of *Synechococcus cedrorum,* the cyanophage AS-1M caused DNA degradation almost 3 hours after infection (Sherman & Pauw, 1976). We didn't observe the disappearance of the DAPI-DNA signal. However, if the breakdown of host DNA is tightly coupled with the replication of viral DNA this could easily be overlooked considering our rather long steps in between measurements.

In our confocal microscopy investigation, the timing of changes observed in thylakoids and DNA corresponded to the timing of peaks of viral abundance in FCM-cytograms. First visible changes in Nakuru, incubated under permanent light was observed at time point 20h. at this time point, there is almost no lytic infection. Moreover, there is no visible sign

of alteration in DNA. We may conclude that the alteration in thylakoids at time point 20h is due to the stress of high radiation. The most significant alteration in the thylakoids occurred at time point 25h with the highest number of viruses. This result is consistent with the study of Choi et al (2010) showed cell lysis of *Nitrosospira multiformis* after application of chromium and potassium cyanide resulting in prophage induction. At time points 22 and 25h, with highest number of viruses, DNA looked more compact and located on one side of the cell.

3D reconstruction of Nakuru incubated in the darkness showed the first signs of alteration in the thylakoids at time point 20h coincided with the increase in the number of viruses. The changes in the thylakoids continued even after the reduction in the number of viruses. Frohns and colleagues (2005) showed that viral infection of *Chlorella* caused membrane depolarization and alteration in K^+ channels. They suggested that the membrane depolarization stopped the metabolism of the host. Although alteration in K^+ channels did not change membrane organization, it decreased host growth and modified the photosystem II/photosystem I ratio (Checchetto et al 2012). The alteration in host metabolism, specifically changes in the lipid and protein content of the thylakoids (Vothknecht & Westhoff, 2001) may therefore be considered as a cause of thylakoids alteration. The most significant changes in the DNA was observed at time points 20, 22 and 25h which was coincided with the increasing number of viruses. DNA looked dense and located on one side of the cell.

First visible changes in Oloidien, incubated under permanent light was observed at time point 20h. at this time point, the number of viruses is increasing. The most significant increase in the number of viruses occurred at time point 28h with significant alteration in thylakoids. Moreover, we only observed the alteration of DNA at 25h and 28h therefore, we concluded that the changes in thylakoids in other time points cannot be due to lytic infection. Like Nakuru incubated under constant light, we assumed that the changes in thylakoids are as a result of high light stress. Changes in the cell volume and the related capacity for thylakoid membrane content, can be a response and adaptation to environmental changes. Montgomery (2014) concluded that the morphological alterations may regulate photosynthetic membrane capacity in response to changes in the external environment. In our study, we observed that constant radiation caused an alteration in

the photosynthetic membrane which is considered as an adaption to high light intensity and a pathway to optimize photosynthetic efficiency.

The number of viruses in Oloidien incubated under constant darkness increased constantly between time points 3h and 22h. The highest number of viruses occurred at time point 25h. However, the visible alteration of thylakoids started at time point 20h and severed at time point 25h. After this time point the structure of thylakoids didn't show visible changes. We concluded that the increasing lytic infection caused changes in thylakoids and DNA however, the changes in the DNA was visible only at the highest number of viruses (time point 25h).

4.3. Different phenotype in Oloidien

On the other hand, in the Oloidien culture, we have observed different phenotype of a cyanobacterium (Figure 37). This unknown phenotype in Oloidein sample occurred in lower pH medium (less than 12). Different environmental conditions favor different phenotype within the obtained cultures (Calvo-Díaz et al, 2011). This refers to the plasticity genes which means that regulatory loci respond to a specific environmental and causes morphogenical changes. Moreover, there is an evidence implying that light causes morphological alterations in cyanobacteria. Morphological changes eventually may result in alteration of photosynthetic membrane (Montgomery, 2014). Walters et al (2013) showed that red light and increased light intensity induced accumulation of reactive oxygen species which eventually led to a shift to a more spherical cell shape in cyanobacterium *Fremyella diplosiphon*. Pattanaik et al (2012) hypothesized that the prevalent wavelengths of available light and light intensity caused morphological changes. They showed that under red light, *F. diplosiphon* cells are blue-green in color, and the shape of cells are short and rounded. Under green light and the light intensity less than 10%, *F. diplosiphon* cells are red in color, and are longer and brick-shaped. The occurrence of different phenotypes in Oloidien due to changing its morphology in response to environmental factors calls for more investigations about the cultures obtained from Oloidien, Kenya.

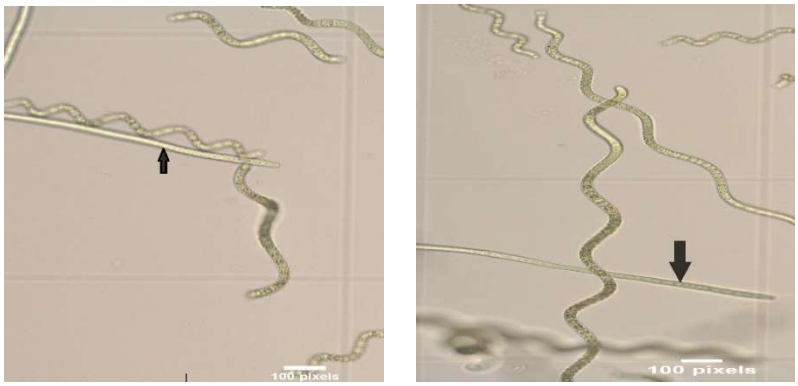

Figure 37- Arrows point to unknown, straight cyanobacterium occurring in Oloidien culture along with *Arthrospira fusiformis*

5. CONCLUSION

The results of this study, led us to conclude that confocal microscopy is a proper technique to follow temporal changes in ultrastructure of *A. fusiformis*, caused by cyanophage infection. By this technique, we are able to target specific structures of thylakoids and DNA and to follow their alteration over time. The data obtained from Deborah samples are more reliable as the outcomes of flow cytometry analyses are more consistent with confocal microscopy. The consequence of the investigation on Nakuru and Oloidien cultures proved the occurrence of light- independent cyanophages in these cultures. Moreover, we could show the effect of light stress on thylakoids of *A. fusiformis*. The effects of lysogenic induction can be distinguished from light-caused alterations by investigating the DNA. In this study, we showed that the light stress didn't alter the DNA but, during lytic phage the DNA looked more localized and compact. It is more precise to screen the cultures for the occurrence of any other cyanobacteria and use cultures without any contamination or cell debris.

For further investigations, we also suggest studying the effect of fixation on ultrastructure of cyanobacteria. Gasol and Giorgio (2000) mentioned that although glutaraldehyde can protect cells against lysis during freezing with liquid nitrogen and long term storage, it may cause artifact in confocal microscopy by producing auto fluorescence signals. Because various cyanobacteria or strains may respond to environmental factors or chemicals differently (Dadheech et al, 2013), it is better to investigate the effect of fixation on every strain separately. We also suggest taking more time points with shorter intervals for both flow cytometry and confocal microscopy. Because SYBR Green has a high sensitivity for dsDNA and it is especially useful to distinguish RNA or ssDNA from dsDNA. With exceptionally low background fluorescence and spectral characteristics that closely match light sources and filter sets in existing instruments, SYBR Green is ideal for use with laser scanners. Therefore, it is suggested to conduct a survey to compare the effectiveness of different fluorescent stains in representing DNA alterations.

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