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Executive summary

This deliverable outlines strategies for detecting and controlling microbial contamination in *Chlorella sorokiniana* closed cultivation systems, developed within the ALGAESOL project. Routine monitoring using light microscopy and flow cytometry was identified as the most effective approach, combining qualitative and quantitative detection of contaminants such as bacteria, protozoa, and ciliates. For sterilization, laboratory-scale autoclaving is reliable, while multi-stage filtration is preferred for large volumes or media containing organic components. Despite rigorous sterilization, ciliate contamination remains a challenge, especially at pilot scale. Initial attempts to isolate and study ciliates were hampered by culture loss, but reference strains have been acquired for ongoing research. The strategies presented here are practical and scalable, and further optimization will focus on industrial application and validation in pilot photobioreactors.

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1.Introduction

The objective of this deliverable is to develop and evaluate strategies to detect and mitigate microbial contamination in *Chlorella sorokiniana* cultures growing in closed cultivation systems. The deliverable is part of task T3.2 Microbial contamination control strategies in WP3 (Microalgae-based conversion route). Microbial contamination in microalgal cultures refers to the unintended presence, growth, or proliferation of unwanted microorganisms—such as bacteria, fungi, viruses, protozoa, or non-target microalgae—that negatively impact the desired microalgal species. These contaminants can outcompete or inhibit the growth of the target microalgae, alter the chemical composition of the culture (e.g., by consuming nutrients or releasing toxins), reduce biomass productivity, and compromise the overall quality and safety of downstream products.

The objective of this deliverable is to:

(i) Rapid and cost-effective detection of microbial contamination in microalgal cultures.

(ii) Evaluating sterilization efficacy across cultivation scales: heat treatment and filtration.

(iii) Isolation of ciliates from microalgal biomass.

2. Results and discussion

2.1 Rapid and cost-effective detection of microbial contamination in microalgal cultures

Light microcopy and flow cytometry stand out as particularly efficient methods for microbial contamination detection in microalgal closed cultivation systems. **Light microscopy** is cost-effective, quick to set up and provides direct, real-time visual feedback on the presence of unwanted microorganisms in the sample without extensive sample preparation. Sample preparation is straightforward (e.g., placing a small volume on a slide or examining a culture flask directly) and suitable for lab and pilot scale inspection. During optical inspection, we can distinguish different microalgal species, protozoa and zooplankton based on characteristics such as shape, size, motility and overall morphology (Figure 1). Light microscopes provide qualitative information that can be implemented in routine checks to quickly confirm or rule out microbial contaminations. However, it has limited resolution for small bacteria and involves a time-consuming process of quantifying both microalgae and microbial contamination.

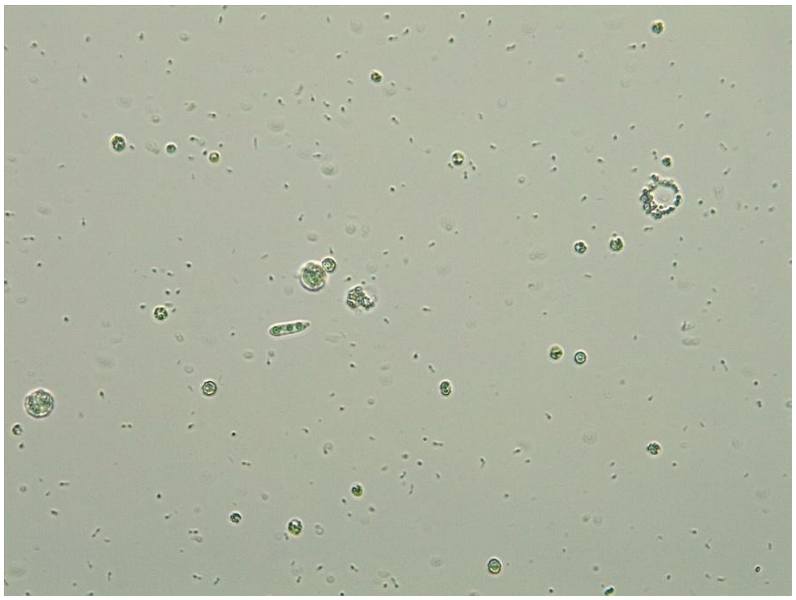

Figure 1. Microscopic image showing a field containing numerous small and mostly oval-shaped microalgal and protist cells.

Flow cytometry enables rapid, quantitative analysis by measuring thousands of cells per second, distinguishing various populations based on size, fluorescence, and optical properties. By measuring forward scatter (FSC) and side scatter (SSC), the cytometer extracts information on cell size and internal complexity, allowing initial differentiation among distinct microalgal cell populations. This allows us to detect different size populations and detect the presence of microbial contaminants that are outside the size range of the cultivated microalgae. During analysis, suspended microalgae cells pass one by one through a laser beam, where the emitted fluorescence signals are recorded by detection channels at wavelength ranges. The natural autofluorescence of microalgal chlorophyll is excited by a blue laser and the red emission is collected by a red channel and for a routine inspection of a sample, staining is not required. In parallel to the natural pigment emissions, signals from nucleic acid-binding dyes are used to label bacteria or other microorganisms. In order to detect bacteria and heterotrophic flagellates, SYBR Green is used as DNA stain to detect bacteria in the microalgae cultures and the instrument is configured at different configurations to distinguish microalgae from bacteria and heterotrophic flagellates exhibiting different morphological and/or spectral signatures (Figure 2). Although flow cytometry instrumentation involves a higher initial investment, once established, it enables rapid and high-throughput sample analysis, making it a cost- and time-efficient method for routine quantifying and monitoring the growth of microalgae and the presence of microbial contamination.

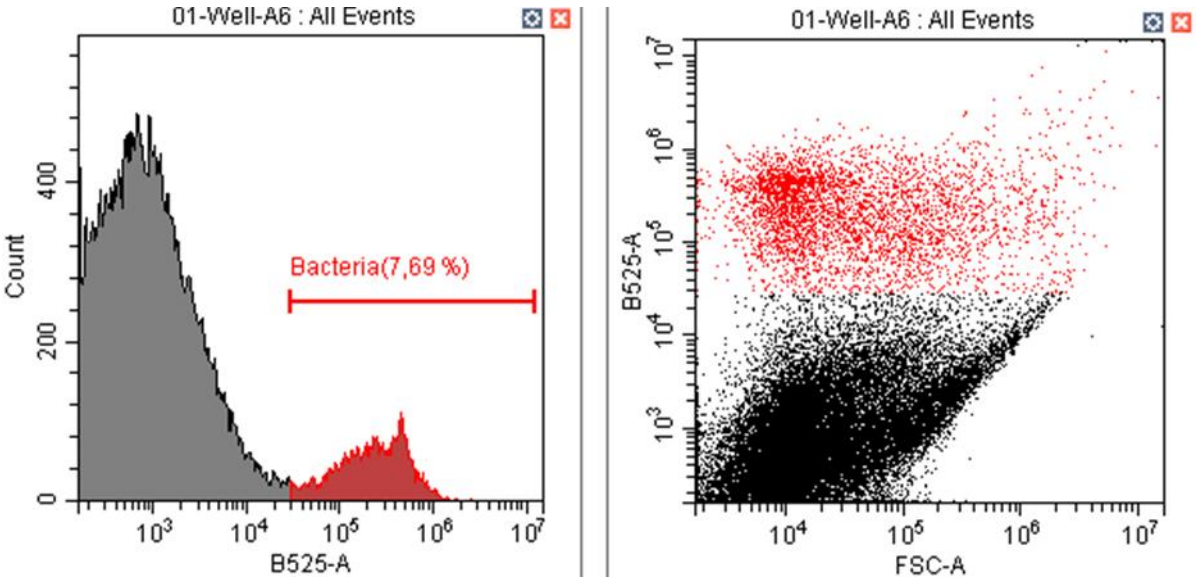

Figure 2. The histogram and dot plot represent the bacteria population stained with SYBR green where B525-A is the detection channel for the green fluorescence.

For day-to-day routine checks in microalgae cultivation, light microscopy provides immediate results, while flow cytometry offers rapid quantitative screening of bacterial and protozoan contamination and microalgae health. Other methods, such as plate counting and PCR-based screening, can be considered under specific circumstances or to complement these primary methods. However, those methods are not typically feasible for routine, frequent testing because plate-based methods require hours to days for microbial growth and not all microorganisms are culturable on standard media. PCR-based screening methods require DNA, extractions, preparation of samples for large datasets that require bioinformatics expertise and time for processing.

2.2 Evaluating sterilization efficacy across cultivation scales: heat treatment and filtration

When cultivating microalgae and maintaining sterile growth conditions, one of the key considerations is media sterilization, an essential step for preventing contamination and ensuring stable growth. At the laboratory scale, autoclaving is straightforward: relatively small culture volumes (typically in the range of 100 mL to a few liters) can quickly be heated to 121 °C for 15–20 minutes at an elevated pressure of about 1.05 bar. This process effectively denatures proteins and disrupts membranes of most microorganisms, yielding sterile media for inoculation. Lab-scale autoclaves are relatively cost-effective and require minimal labor. In comparative studies, a non-autoclaved batch is often maintained as a control to assess sterilization efficacy (Figure 3). Once the media is sterilized, the inoculation of a microalgae culture and handling of cultures takes place in a sterile bench.

Figure 3. Six plates arranged in two rows. Each row contains three plates representing three types of culture plates: Luria–Bertani (LB), Tryptone Yeast Extract (TYA), and Tryptic Soy (TS). The top row shows plates that were spread with BG-11 media subjected to standard autoclaving conditions (121 °C, 15 min) and no visible bacterial colonies are present on the plate surface while the bottom row shows the non-autoclaved BG-11 media exhibits bacterial colonies on all three plate types.

When cultivating microalgae in media containing organic molecules such as glucose or in complex matrices such as recirculating aquaculture system water (RAS water)**,** sterilization is a critical step to avoid contamination by unwanted microorganisms. However, autoclaving may not be the most appropriate sterilization method for these types of media. This is because autoclaving involves exposing the medium to high temperature (typically 121 °C) and high pressure, which can chemically degrade or alter the composition of many organic molecules. In this case the most suitable strategy for sterilization of the media is filtration. When filtering microalgae growth media, usually pre-filtration is not necessarily due to the absence of particles that can cause clogging of the filter. For removing most of the bacterial and fungal contamination, 0.2 µm–0.45 µm filters are used. This is convenient when media contains glucose for heterotrophic cultivation in order to eliminate fungi. In order to maintain filtration sterile and prevent from reintroducing contaminants in the media is important to perform the filtration in the sterile bench. Sterile filtration was highly efficient in case of complex matrixes such as RAS water (Figure 4). In some cases, microbial contamination may still occur even after filtration of the medium, since salt used for microalgal cultures can be a contamination source due to manufacturing irregularities or tiny defects in the filters that allow resting cysts to pass through, that is mostly the case for ciliates and some bacteria contaminations.

Figure 4. Six plates arranged in two rows. Each row contains three plates representing three types of culture plates: Luria–Bertani (LB), Tryptone Yeast Extract (TYA), and Tryptic Soy (TS). The top row shows plates that were spread with RAS was filtered and no visible bacterial colonies are present on the plate surface. Meanwhile, the bottom row shows non-filtered RAS water, which exhibits bacterial colonies on all three plate types.

Bacteria are typically not a problem for phototrophic cultivation when the media contains only inorganic nutrients even if bacteria are always present in the cultures. Checking bacteria during a cultivation cycle is good strategy to predict bacterial concentrations and potential negative impact on the biomass production. Bacterial contamination is a problem during heterotrophic cultivation when organic carbon is used as a carbon source and both, the microalgae and bacteria are competing for the same source. Further to bacteria, during heterotrophic cultivation another risk of contamination is with fungi which develop fast and compete with microalgae. In this case, the culture has to be regrown. At pilot microalgae cultivation , the sterilization relies on two-steps filtration, which is more practical at large volumes and cost-effective approach than autoclaving big volumes (Figure 5.).

Figure 5. This image shows a dual-stage water filtration system installed in a pilot facility for cultivation of microalgae in tubular photobioreactors. The system features two blue filter housings (1 and 0.3 micron) connected to input and output hoses, ensuring efficient water purification.

Despite the efforts to maintain the equipment and microalgal culture free of microbial contaminants, ciliates contamination is often observed at pilot scale.

2.3 Isolation of ciliates from microalgal biomass

Isolating natural contaminants such as ciliates, common grazers in microalgae cultures and studying strategies for their control and removal is a crucial step in microalgal biotechnology. A freeze-dried biomass was rehydrated in the BG-11 media and placed in the dark on a shaker for a few days to facilitate the revival of resting cysts. Under a microscopic inspection, motile ciliates were observed in the sample (Figure 6).

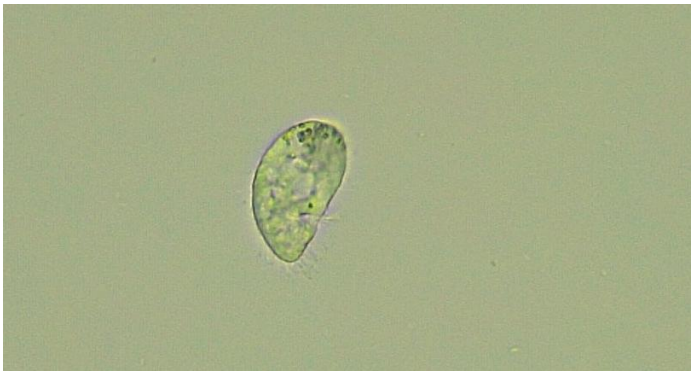

Figure 6. This image displays a ciliate observed under a light microscope.

The ciliates were carefully isolated using a micropipette under sterile conditions and transferred to a separate culture in PPY medium (Proteose Peptone Yeast extract), a nutrient-rich medium supporting ciliate growth. Although initial proliferation of the ciliate population was observed, the culture ultimately collapsed, resulting in the death of all ciliates. This crash could be attributed to several factors, including exhaustion of nutrients in the PPY medium, and accumulation of metabolic waste products.

3. Conclusion

In this deliverable, we concluded that light microscopy and flow cytometry are the most common, practical, and efficient strategies for day-to-day routine detection of microbial contamination in microalgal cultures at both lab and pilot scales. Choosing an appropriate sterilization method depends on medium composition, scale of operation, and availability of equipment. At laboratory scale, autoclaving remains a convenient choice, but as cultivation volumes increase or media become more complex (e.g., containing organic carbon sources), filtration often becomes more practical. Despite the effectiveness of both methods, regular monitoring of cultures—using rapid detection tools (e.g., light microscopy and flow cytometry)—is essential to confirm sterility and detect any emerging contamination (e.g., ciliates, bacteria) before it significantly impacts culture performance.

4. Degree of progress

The degree of fulfilment of the task activities, as outlined in the Description of Action (DoA), is currently only partially completed. This is primarily due to the loss of the ciliate cultures originally isolated from the microalgal biomass. This event necessitated the postponement of several planned experimental activities which will be reported in D3.2 in M21. To address this challenge and ensure continuity of experimental work, we ordered *Colpoda steinii* and *Vorticella microstoma* from the Culture Collection of Algae and Protozoa (CCAP). We will establish a continuous culture of the reference strains for further experiments and efforts to isolate natural contaminants from microalgae culture systems will continue. Furthermore, the scalability of these control strategies will be investigated by transferring the most promising approaches from bench-scale to pilot-scale photobioreactors to evaluate their effectiveness and feasibility under industrially relevant conditions.