Microalgae communities – assay for in-vivo effects of hybridosomes from spruce needle homogenate

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Results

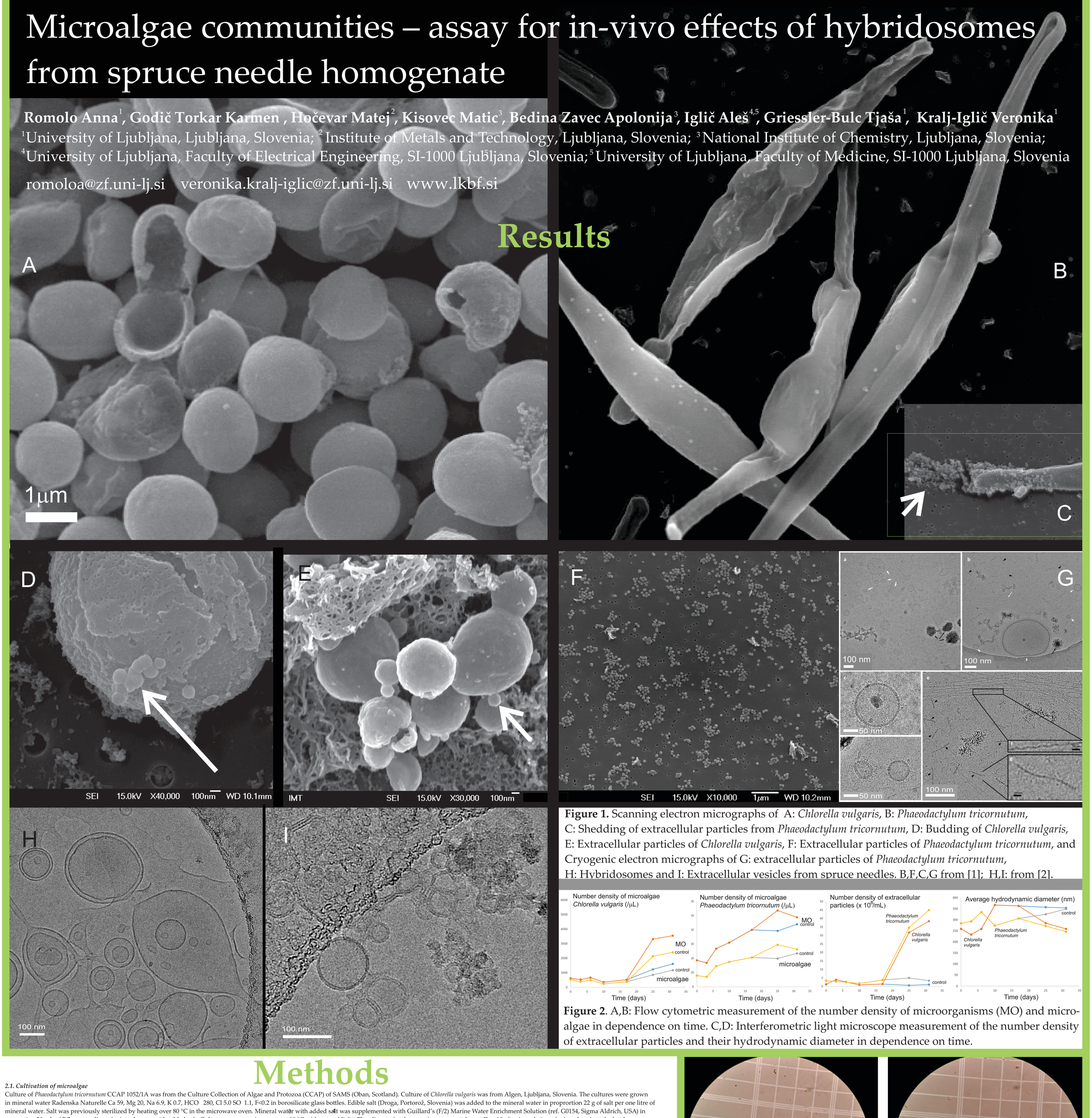

Figure 1. Scanning electron micrographs of A: *Chlorella vulgaris*, B: *Phaeodactylum tricornutum*, C: Shedding of extracellular particles from *Phaeodactylum tricornutum*, D: Budding of *Chlorella vulgaris*, E: Extracellular particles of *Chlorella vulgaris*, F: Extracellular particles of *Phaeodactylum tricornutum*, and Cryogenic electron micrographs of G: extracellular particles of *Phaeodactylum tricornutum*, H: Hybridosomes and I: Extracellular vesicles from spruce needles. B,F,C,G from [1]; H,I: from [2].

Figure 2. A,B: Flow cytometric measurement of the number density of microorganisms (MO) and microalgae in dependence on time. C,D: Interferometric light microscope measurement of the number density of extracellular particles and their hydrodynamic diameter in dependence on time.

Methods

2.1. Cultivation of microalgae
Culture of *Phaeodactylum tricornutum* CCAP 1052/1A was from the Culture Collection of Algae and Protozoa (CCAP) of SAMS (Oban, Scotland). Culture of *Chlorella vulgaris* was from Algen, Ljubljana, Slovenia. The cultures were grown in mineral water Radenska Naturelle Ca 59, Mg 20, Na 6.9, K 0.7, HCO 280, Cl 5.0 SO 1.1, F<0.2 in borosilicate glass bottles. Edible salt (Droga, Portorož, Slovenia) was added to the mineral water in proprortion 22 g of salt per one litre of mineral water. Salt was previously sterilized by heating over 80 °C in the microwave oven. Mineral water with added salt was supplemented with Guillard's (F/2) Marine Water Enrichment Solution (ref. G0154, Sigma Aldrich, USA) in proportion 20 mL of F/2 per one litre of mineral water with added salt. Culture was grown in a room at 18 °C with natural light. The aliquots for the experiment were taken at Day 18 after inoculation of microalgae into the bottle.

2.2. Design of the experiment with microalgae
Microalgae cultures were aliquoted into 250 mL glass bottles (6 aliquots from each culture). From these, 3 aliquots were added hybridosomes from spruce needles and 3 aliquots were used as controls. At day 18 after the inoculation of microalgae into the bottles we have added 1 mL of hybridosome solution to the samples. The number density of hybridosomes was 1.01 (1 ± 0.05) x 10^9/mL. The boxes were placed in a room at 18 °C and exposed to natural light. For assessment, we gently mixed the samples by circular motion of the boxes and took 200 μL of the conditioned media for measurements of microorganisms (**MO**) by FCM and extracellular particles (**EPs**) by ILM. We have replenished the volume of the samples by ultraclean water with added salt and f/2. After day 31, samples were examined for microbiological content.

2.3. Preparation of spruce needle homogenate
Branches were cut from the *Picea abies* tree and used immediately. Branches were immersed into 1.5 L of water at 30 oC with 10 mL of sodium hypochlorite (NaClO, 0.1 %) for 1 hour. The branches were rinsed with water. The needles were cut off from the branches. 50.0 g of wet needles were immersed in 300 mL of ultraclean water and stirred for 1 minute in KOIOS 850W Smoothie Bullet Blender (KOIOS, Neweg, USA). The homogenate was filtered through 0.5 mm nylon net cloth to remove larger particles.

2.4. Isolation of extracellular vesicles (EVs) from spruce needle homogenate
EVs were isolated by differential centrifugation, using a protocol widely used for the isolation of small extracellular vesicles (Mantille et al., 2022). Briefly, the cells and larger particles were removed by low-speed centrifugation (300 g, 10 min, 4°C, centrifuge Centric 260R with rotor RA 6/50 (Domel, Slovenia)), using 50 mL conical centrifuge tubes (ref.S.078.02.008.050, Isolab Laborgeräte GmbH, Germany); and 2000 g, 10 min, 4°C (Centric 400R centrifuge with rotor RS4/100 (Domel, Slovenia)), using 15 mL conical centrifuge tubes (ref. S.078.02.001.050, Isolab Laborgeräte GmbH, Germany). Each step was repeated twice. Then, the cell-depleted medium was centrifuged at 10 000 g and 4°C for 30 min (Beckman L8-70M ultracentrifuge, rotor SW55Ti (Beckman Coulter, USA)), using thin-wall polypropylene centrifuge tubes (ref. 326819, Beckman Coulter, USA). The pellet was resuspended in 50 μL of ultraclean water.

2.5. Preparation of hybridosomes
Hybridosomes were prepared by mixing appropriate proportions of liophylized soya lecithin granules, glycerol and supernatant of isolation of EVs from spruce needle homogenate, at room temperature. Soyabean lecithin granules were placed into the falcon tubes. Supernatant was added and the suspension was left at room temperature for 1 hour. Glycerol was added and the samples were mixed mechanically (manually) with metallic stick until the ingredients formed a uniform cream-line consistence. The samples were kept at room temperature.

2.6. Flow cytometry
The microalgae number densities were measured by flow cytometry by a MACSQuant Analyzer flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) and the related software. The following instrument settings were employed: FSC: 458 V; SSC: 467 V with a trigger set to 1.48, B3: 300 V; R1: 360 V. Non-fluorescent particles corresponding to other microorganisms and cell debris were detected from the forward (FSC) and side scatter parameter (SSC), as they were not autofluorescent. The microalgal cells were identified based on chlorophyll autofluorescence (AFP), detecting red emission (channels B3: 488 nm/655–730 nm, and R1: 635 nm/655–730 nm). Samples were mixed by pipetting before measurement and 20,000 events per well were acquired.

2.7. Interferometric light microscopy (Romolo, A. et al., Assessment of Small Cellular Particles from Four Different Natural Sources and Liposomes by Interferometric Light Microscopy. Int. J. Mol. Sci. 2022, 23, 15801)
The average hydrodynamic diameter (D_h) and the number density of EPs were determined by ILM using Videodrop (Myriade, Paris, France). Signal from the medium (physiological saline) was under the detection limit. The threshold value of 3.8 was used. Seven microliters of sample were placed between cover glasses and illuminated by 2 W of blue LED light. The light scattered on the particle was imaged by a bright-field microscope objective and allowed to interfere with the incoming light. The image was recorded by a complementary metal-oxide-semiconductor high-resolution high-speed camera. Interference enhances the information in the scattered light. The contribution of the incident light was subtracted from the detected image. The obtained pattern, which includes contrasting black and white spots, was recognized as a particle, and its position in the sample was assessed. The number density of the particles is the number of detected particles within the detected volume, which depends on the microscope characteristics and the particles' size. The typical detection volume was 15 pL. Hydrodynamic diameter D_h was estimated by tracking the position of the imaged particle within the recorded movie. The diffusion coefficient D of the motion of the particle is taken to be proportional to the mean square displacement d of the particle between two consecutive frames taken in the time interval Δt, $\langle d^2(\Delta t)\rangle = \langle 4D\,\Delta t\rangle$, while the hydrodynamic diameter was estimated by assuming that the particles were spherical and using the Stokes–Einstein relation $D_h = kT/3\pi\eta D$. Each particle that was included in the analysis was tracked and processed individually, and the respective incident light signal was subtracted from each image. Processing of the images and the movies was performed by using the associated software, QVIR 2.6.0 (Myriade, Paris, France). We measured each sample 3 times. Samples were measured undiluted.

2.8. Scanning Electron Microscopy
Samples were placed on 0.05-micron mixed-cellulose-esters' filters (Sterlitech, Auburn, AL, USA) and incubated in 39.3 mM double distilled water solution of OsO_4 for 2 h. Then they were washed 3 times with distilled water (10 min each), dehydrated in graded series of ethanol (30%, 50%, 70%, 80%, 90%) and absolute ethanol, each step 10 min. Absolute ethanol was replaced twice. Then they were washed in hexamethyldisilazane (mixed with absolute ethanol; 30% and 50%) and in absolute hexamethyldisilazane, each step 10 min. The samples were left to dry in air overnight. For examination under JSM-6500F Field Emission Scanning Electron Microscope (JEOL Ltd., Tokyo, Japan), the samples were sputtered with Au/Pd (PECS Gatan 682).

2.9. Cryogenic Transmission Electron Microscopy
C-flat™ 2/2, 200 mesh holey carbon grids (Protochips, Morrisville, NC, USA) were glow discharged: 20 mA, 60 s, positive polarity, air atmosphere (GloQube® Plus, Quorum, Laughton, UK). Then, 3 μL of the sample was applied to the grid, blotted, and vitrified in liquid ethane on Vitrobot Mark IV (Thermo Fisher Scientific, Waltham, MA, USA). Vitrobot conditions were set to 100% relative humidity, 4 °C, blot force: 2 and blot time: 7 s. Samples were visualized under cryogenic conditions using a 200 kV Glacios microscope with a Fal-con 3EC detector (Thermo Fisher Scientific, Waltham, MA, USA).

References
1. Božič D, et al. Ultrastructure and stability of cellular nanoparticles isolated from Phaeodactylum tricornutum and Dunaliella tertiolecta conditioned media Open Res Europe 2022, 2:121
2. Romolo, A et al. Assessment of Small Cellular Particles from Four Different Natural Sources and Liposomes by Interferometric Light Microscopy. Int. J. Mol. Sci. 23, 15801.

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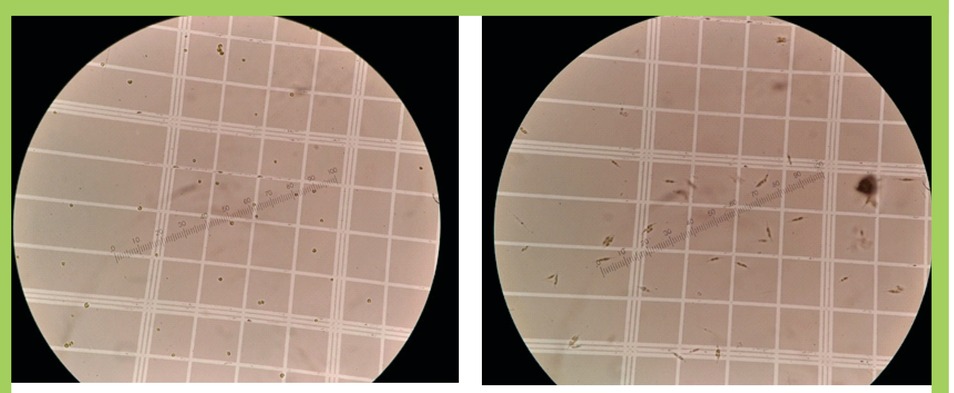

Figure 3. A: Joined samples of Chlorellae vulgaris and B: of Phaeodactylum tricornutum in Burker-Turk chamber. We found monocultures, respectively, with number densities cca 10^7/mL, and other microorganisms (Table 1).

Table 1. Microbiological analysis of the *Chlorellae vulgaris* and *Phaeodactylum tricornutum* societies

Sample	N_{MO} at 22 °C (CFU/mL)	N_{MO} at 37°C (CFU/mL)	N_{fungi} (CFU/mL)	$N_{Bacillus\ spp.}$ (CFU/mL)	$N_{c.b.\ \&E.coli}$ (CFU/100 mL or CFU/50 mL)*	$N_{Pseudomonas\ spp.}$ (CFU/100 mL or CFU/50 mL)*
1	100	150	2	0		
2	400	300	2	0	Samples 1-6**	Samples 1-6**
3	150	150	1	0		
4	200	250	0	0		
5	100	100	1	0	50 CFU/100 mL	50 CFU/100 mL
6	300	350	0	0		
7	100	200	0	0	Samples 7-12**	Samples 7-12**
8	100	300	9	0		
9	100	200	2	0		
10	100	400	2	0	150 CFU/100 mL	50 CFU/100 mL
11	100	150	3	0		
12	100	350	0	0		

CFU – number of Colony Forming Units. * 50 mL of the sample was filtered. ** Aliquots were joined.

In the samples treated with hybridosomes we observed notable increase of the number density of microorganisms and speciffically, microalgae. The proportion of microalgae to other microorganisms remained largely unchanged. Microalgae communities proved a convenient system for *In vivo* testing.