Mosses as candidates for stratospheric biomarkers

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Abstract

Several plants are used to visualize ozone concentration in troposphere, however, there are no such biomarkers nor biological probes for ozone protective layer in stratosphere. Mosses - widespread, cosmopolitan, easy to handle and to analyse - are hereby proposed as suitable candidates for such a task for they can be used in a variety types of studies for large scientific community as well as for educational purposes. Three moss species representing two categories of ecological strategies typical for bryophytes (stress tolerator and ruderal), were applied for the first assessment of their ability to sustain conditions of the stratosphere. Samples of mosses were attached to balloon and lifted to stratosphere. Morphological changes and chlorophyll condition were observed to assess their survival abilities in chemically active ozone layer. *Orthotrichum anomalum* was found to be much more resilient than *Atrichum undulatum*. Our study revealed ozone-sensitive moss species and deepened the knowledge of plant survival strategies after exposure to rapid environmental changes.

Key words: Bryophytes, *Orthotrichum anomalum*, *Atrichum undulatum*, stress

Introduction

Biomonitoring of atmospheric chemistry is the best method to reveal visible effects of air quality on living organisms. Its also the best evidence to convince citizens and policy makers to find the ways to reduce pollution or activate climate protection programs. Chemical components of the atmosphere such ozone, nitric oxide and sulphur dioxide harm plants, animals, humans, and entire ecosystems. Several plants are sensitive to elevated ozone levels and show signs of visible injury. Conifers and broad-leaf trees, shrubs, lichens and mosses, domesticated crops and grasses all have member species susceptible to ozone injury. The Aura Ozone Monitoring Garden in the NASA Goddard Space Flight Center has the ozone monitoring garden full of plants that scientists have found to be ozone-sensitive. Each of these plants showed damage of their leaves after the exposure to high levels of ozone characterized by fine coloured spots on the upper surfaces of their leaves called stipples, and some leaves also turn yellow. Ozone enters plants through leaf stomata. Oxidized tissue changes biochemical and physiological processes. Both visible injury (e.g. stipples and chlorophyll degeneration) and growth effects (e.g. premature leaf loss, reduced photosynthesis and reduced leaf, root and total dry weights) can occur in sensitive plant species (Skelly 2000).

Since the stratospheric ozone is one of the most crucial component for life on our planet, we wanted to find easy visual method of ozone detection which brings down to earth, what remote sensing observations are telling us.

This study was the first attempt to use biomonitoring in the second major layer of Earth's atmosphere (10-50km).

Although in their scientific stratospheric mission, the Indian Space Research Organization (2009) revealed several metabolically active bacteria and fungi species in the stratosphere, it is impossible to use them for biomonitoring studies because of lack of „*in situ*" qualitative or quantitative detection methods. Therefore the main aim of our work was to select appropriate species living on Earth, which can resist harsh environmental factors and which can be used to monitor the chemistry in the stratosphere - its protective ozone layer located about 25 km above our planet surface. We selected 2 moss species known to be resistant to cold temperatures, low pressure levels and UV light radiation.

Materials and Methods

Moss material

Species *Orthotrichum anomalum* (Hedw.) and *Atrichum undulatum* (Hedw.) were utilized to assess their survival skills after exposure to the stratospheric conditions. These species represent bryophytes rather in the stress tolerator category. *O. anomalum* was proved to be desiccation tolerant (Alpert and Oliver. 2002). *A. undulatum* as a species of Polytrichales has a rather different water physiology - it is not ectohydric but mixohydric as well as it shows some characteristics of a Competitor category sensu Grime (1977). Both *O. anomalum* and *A.undulatum* are fairly abundant and cosmopolitan. Dark-green fresh gametophytes of *O. anomalum* with shoots 1.5 cm tall, with straight leaves 2.5-4 mm long were collected in Ostravian region (Czechia) a week before the experiment from its natural habitat on basic rocks. *A. undulatum* with 3 cm tall dark green leaves were collected in Kraków from the meadow in forest. Additionally we tested *Physcomitrella patens*, representing the more ruderal - ephemeral species. Our previous tests on this moss revealed very weak resistance to stratospheric conditions (not published data), so here we only added this organism to show chloroplast degradation. *P. patens* is widely used as a model organism in genetics thus well known in regards to its autecology and physiology. *P. patens* strain was purchased from the International Moss Stock Center, Freiburg, Germany (Reute-K1 Cat. No 40040) and the sample consisted of young, 0.5cm tall gametophytes of the light-green moss with star-shaped rosettes of leaves. All plants were washed in distilled water, dried on a paper towel and separately placed inside the 2 ml plastic eppendorf test vials (Sarstedt, Cat. Nr 72.694.106) 24 hours before the day of experiment. Total number of the experimental vials attached to the balloon was 32 (16 per species) and 32 control vials were kept on the ground. All vials were coded for further analysis. Opened and airtight closed samples, all in two copies (sustaining pressure and chemical components), were considered. P. patens was placed in one closed vial in the capsule and one on the ground. After the return of capsules to Earth, mosses in test vials were kept all under the same conditions and transported back to the laboratory (another 24 hours) for analysis. Additional control test was made by freezing mosses in -20 °C.

Balloon mission

Samples of mosses were launched to the stratosphere on 25th of April 2015 on specially designed for the mission capsule, from the Aeroclub in Kruszyn, Poland (52° 35'09.79'' N 19° 00'43.38''E). The main part of the capsule consisted of the cylindrical eppendorf holder protected from mechanical damage by light flexible carbon rods. 300g heavy capsule together with additional capsules for telemetry, UV-light measurements and video cameras were attached to balloon according to the **Fig.1.left.** Balloon has reached the stratosphere, samples of mosses were thus subjected to changes in altitude from 0 km to 28 km, and temperatures ranging from 27 °C on ground to -32 °C at the highest altitudes. The mission took about 2h, 82 min of ascent and 40 min of descent (**Fig.1.right**). After landing, capsules were collected by the members of the stratospheric mission (about 15 min. after landing). Balloon position was tracked using the flight tracker service at www.spacenear.us.

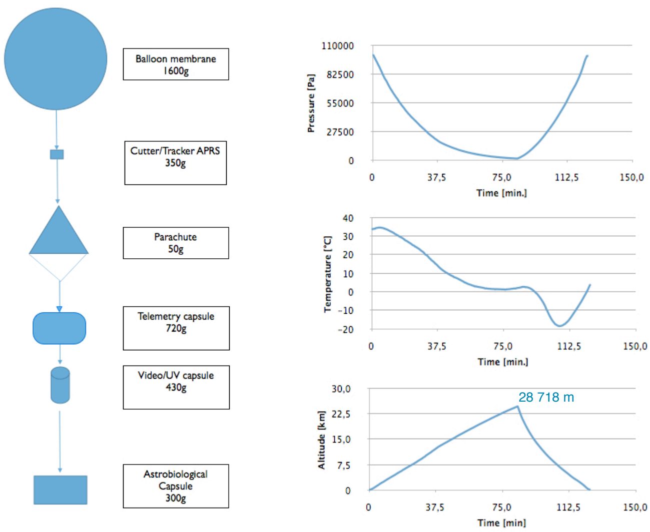


Fig.1. The scheme of balloon assembly (left), and the basic graphs from the Pt1000 temperature detector and BMP085 sensors (altitude and pressure), recorded during the balloon flight.

Cell imaging

Leaves in the third row from the top of each plant were cut and photographed with Axio Vision camera under the microscope. Cellular damage and cell wall deformations were detected under the bright light Nicon Microphot EPI-FL313682 microscope (10x objective). Changes in chloroplast morphology and chlorophyll autofluorescence were visualised using 100x oil immersion objective lens under the Leica fluorescent microscope (exciting light 546 nm). Obtained microscopic images were analysed and processed in Image J Software, version 1.44g (Schulze et al., 2011), using Wacom Bamboo tablet. Cell surface and mean grey value measurements were performed on three separate leaves in each moss sample as an indicator of dehydration. Each measurement consisted of randomly chosen 10 cells taken from the middle part of left side of the leaf. Measurements were repeated by the same person, using the same tools and software.

HPLC analysis

Vitamin C (ascorbic acid), is a key low molecular weight antioxidant in the plant cell. Ascorbic acid can directly react with reactive oxygen species like singlet oxygen, ozone, superoxide, and hydroxyl radical. To measure the concentration of L-ascorbic acid in tested samples we used high-performance liquid chromatography. Mosses were rubbed in a mortar with 2,5ml of 3% metaphosphoric acid. Obtained solution was sonificated for 15 min., filtered and analysed in chromatograph HPLC-DAD (Column: RPC18 3,5µm 4.6X150mm, Eluent: MeOH:100mM KH_2PO_4 (10:90), Flow: 0,5ml/min., Wavelength: 245nm).

Statistical analysis

Data was analysed using Hierarchical Clustering on Principal Components to find general trends in mosses response and Factorial Logistic regression to reveal main dependencies throughout the statistical sample. All analyses and plots were created using statistical tool R (R Development Core Team 2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Results

Cell damage as a marker

Extreme stratospheric environment is lethal for nearly all living organisms because of simultaneous presence of three harsh physical factors: low temperature, pressure closed to 0 Pa and high-energetic solar and cosmic radiation. While temperature decreases and reaches freezing levels, appeared crystals of ice may cause mechanical damage inside plant cells. Another deleterious factor present in stratosphere is exposure to high-energetic radiation such UV light and gamma rays. Ozone layer, as additional chemical factor, may influence generation of free radicals and reactive oxygen species (ROS), leading to cell necrosis. One of the characteristic damage caused by ozone are stipples - single dark brown damaged cells. **Fig. 2** shows effects of stratosphere (S) compared to ground controls (C), revealed in cellular morphological changes in three tested moss species: *O. anomalum*, *P. patens* and *A. undulatum*. *Orthotrichum* was the less sensitive for stratospheric conditions, while Physcomitrella was too delicate. *Atrichum* appeared to show the most diverse cellular rearrangements. Green chloroplasts are equally dispersed inside the cells in control sample F, while after the stratospheric exposure cells changed color from green to brown and chloroplasts became rearranged and stuck to cellular walls (B bottom). Opened samples exposed for low pressures and ozone penetration revealed chloroplasts condensed in the centre by shrunk cellular membranes (B up and C, D). Blue arrow on D shows typical stipple while purple arrow reveals and island with still active chloroplasts. Cell necrosis is noted by blue arrow on E. **Table 1** reveals data from the mission: Averages of all dead cells (brown colour with still visible chloroplasts) and stipples (very intensive dark brown cells with destroyed chloroplasts) counted from images. Interestingly we observed that covering plants with aluminium foil for 48h strongly influenced chloroplast degradation in both types of analysed tissues (experimental and controls).

Stratosphere	Dead cells	Stipples
closed A	1	0
opened A	25	6
darkclosedA	85	0
darkopenA	45	2
closed O	0	0
opened O	0	0
darkclosedO	0	0
darkopenO	0	5
Control	**Dead cells**	**Stipples**
closed A	0	0
opened A	0	0
darkclosedA	61	0
darkopenA	14	0
closed O	0	0
opened O	0	0
darkclosedO	0	0
darkopenO	0	0

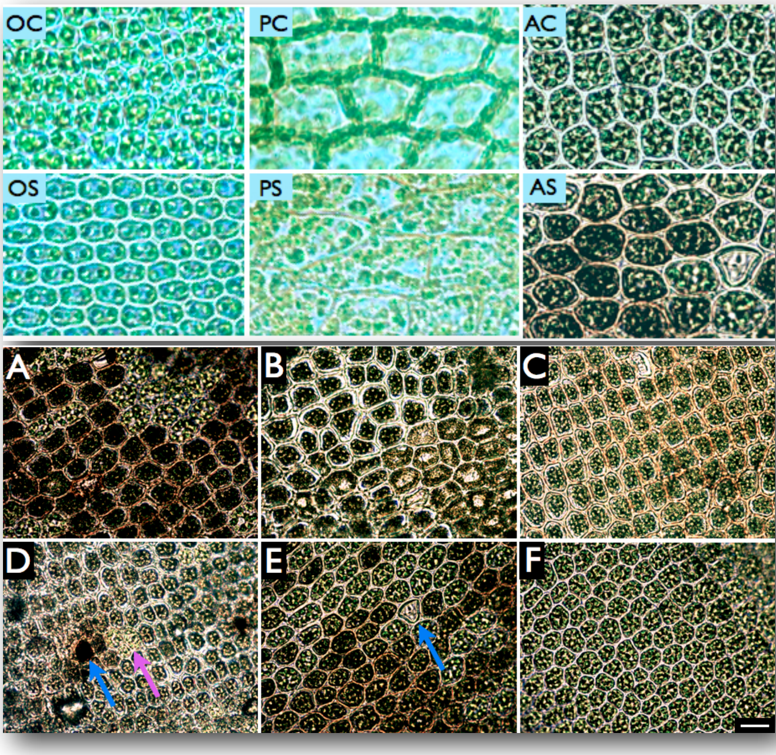


Fig. 2. Microscopic images of leaves of three types of tested mosses (O-*Orthotrichum*, P-*Physcomitrella*, A-*Atrichum*). Green active chlorophyll is clearly seen in control samples (C) while chloroplast colour and location change after the exposure to the stratosphere. *Atrichum* sp. exhibited the most diverse types of cellular damage: dark brown stipples (A and blue arrow in D), dense cytoplasm and chloroplast condensation (B up), chloroplast edging (B down), cytoplasm shrinkage (B up, C and D). F-control with not damaged cells. Scale-50 microns.

Table 1. Averages of all dead cells and stipples from obtained microscopic images. Stipples appeared only for opened samples suggesting that this damage might be caused by ozone.

Cell size and chlorophyll intensity change in stratosphere

Since ice crystals and low pressure may affect cell size, we analyzed this parameters in tested samples (**Fig. 3** and **Fig. 4**). Factorial logistic regression was used to assess the effect of the treatment and environmental factors on the mean grey value (MGV), and cell area values: significant dependencies ($p < 0.05$) of all the factors on the observed values was founded, with the species and environment being, not surprisingly, the key factors. When the data were assessed species-wise, the effect was most prominent in case of MGV in *Atrichum* where all the factors and their interactions were significant even among controls; cell area of *Atrichum* was affected by the stratospheric conditions and by treatment in interaction with the those conditions. *Orthotrichum* was significantly affected by the stratospheric conditions as well, but once in the stratosphere, the treatment (closed or opened lid) played no role. The response of the species, though in both significant, is rather diverse as is apparent from **Fig. 4.** Whilst *Atrichum* cells demonstrated increase in MGV after being subjected to stratospheric conditions – both in closed and opened vials, the response of *Orthotrichum* cells was contrary (decrease in MGV) and not quite as prominent.

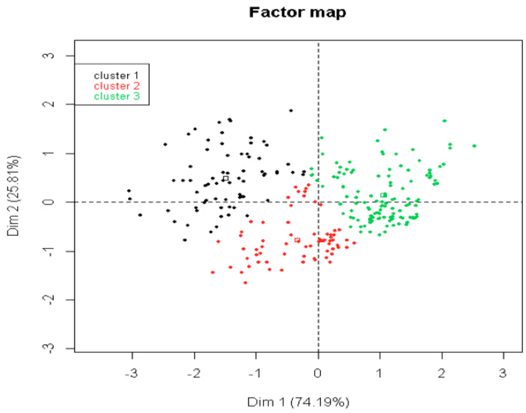


Fig. 3. The Hierarchical Clustering on Principal Components performed on the data on cell area, MGV (mean grey value), species, treatment and environment showed that there is indeed a distinction apparent in the data by separating it to three clusters two canonical axes accounting for most of the variability in the data (~100 %). However, although the PCA model was significant for all the variables ($p < 0.05$), their representation in the clusters was mostly species-specific and clear differentiation in the environmental factors was not found.

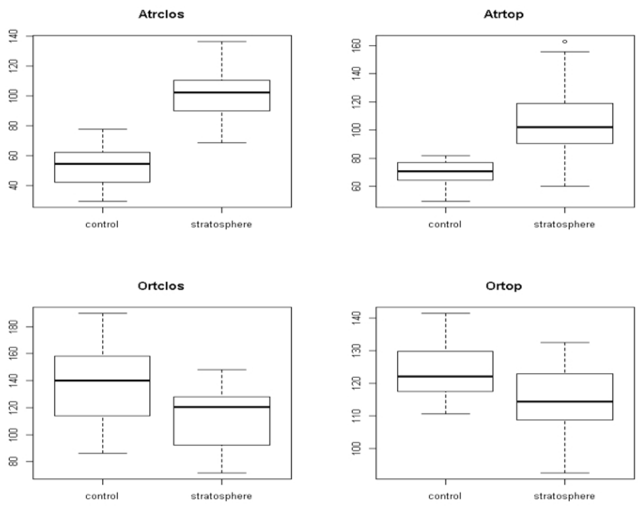
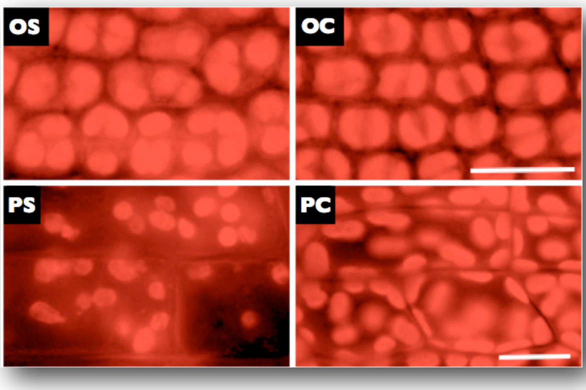


Fig. 4. Boxplots with mean grey value distribution in analysed groups of mosses: *Atrichum* and *Orthotrichum*, in two conditions: with opened and closed eppendorfs in the stratosphere and control samples. In case of *Atrichum*, increase in MGV was observed in both cases. No such effect, even slight decrease was observed in *Orthotrichum*.

Chlorophyll degradation

Active chlorophyll exhibits autofluorescence, which intensity is relative to amount of its concentration in the tissue. UV light may strongly reduce chlorophyll activity. During performed mission we detected the UV irradiance, with maximal value 6,222 mW/cm^2 at 24 km above the Earth (**Fig.5. right**). Strong differences in chlorophyll autofluorescence intensity between *O. anomalum* and *P. patens* were observed, either in initial state (C) or after the treatment in stratosphere (S) (**Fig.5. left**).In *P. patens* significantly lower chlorophyll autofluorescence was observed in the samples subjected to mission ($p < 0.001$ compared to the control). In the end, *P. patens* samples showed lower autofluorescence then *O. anomalum* control despite the initial state, hence the drop in fluorescence after the exposure to the stratospheric conditions is much more dramatic in *P. patens*.



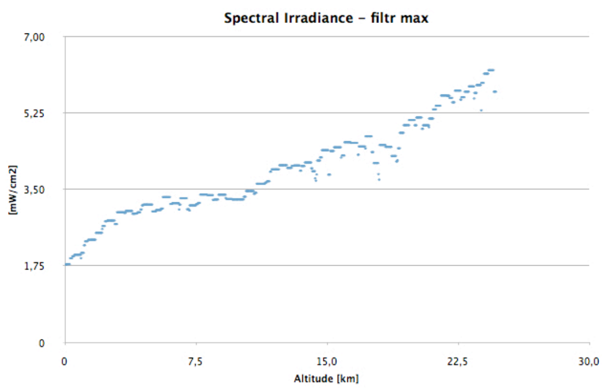
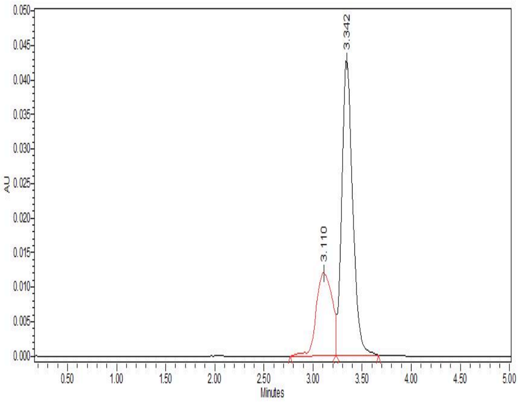


Fig. 5. left. Autofluorescence of the chlorophyll in *Orthotrichum anomalum* (O) and *Physcomitrella patens* (P) in S (stratospheric) and C (control) conditions. Scales for rows of images: 20 microns. **Fig.5.right.** Spectral irradiance data filtered for the maximal value in relation to increasing altitude during JADE mission balloon flight.

Vitamin C may not be directly involved in stress responses in tested mosses

Using sensitive chromatography method we obtained unexpected result, since we were not able to detect and quantify vitamin C in mosses. Spectra of all eight extracted moss samples did not reveal a pick characteristic for vit. C (**Fig. 6 left**). This result (8 samples containing less than 10 µg/g), indicates, that either our method was not sensitive enough, or that vitamin C is not directly involved in this kind of stress responses.



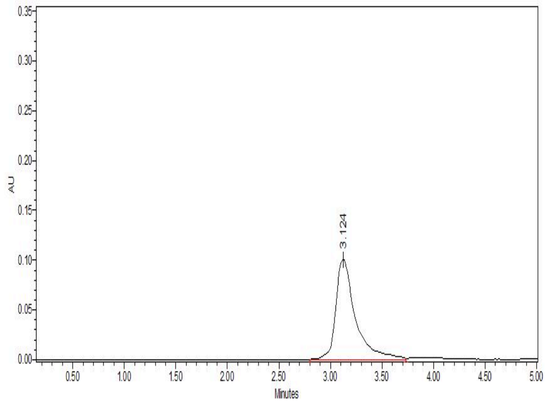


Fig. 6. Spectra from HPLC analysis. **Left**: Spectrum characteristic for the vitamin C (black) and **right**: example of analyzed moss sample without the characteristic spectrum. The signal present in both plots comes from small amount of contamination.

Conclusions

This work presents results from the first attempt to use plants for biomonitoring in the stratosphere.

Three moss species represent different stress responses to the harsh fast-changing environmental conditions reflected in chloroplast quality, chloroplast morphology and cellular damage but not in vitamin C concentrations. This features can be used for future biomonitoring studies.

Knowledge about mosses physiology after exposure to fast environmental changes may be useful to study plant resistance in seed design and production of resistant vegetables, fruits and cereals.

Mosses are cosmopolitan and their leaves consist of single cellular layer, what makes microscopic study easy to handle. This plants are great model organisms for educational work.

In particular, *Atrichum* sp. seems to be the best biomarker for the ozone layer in the stratosphere, since it is resistant to low temperatures and reveals several morphological changes at cellular level, especially characteristic stipples.

Acknowledgements

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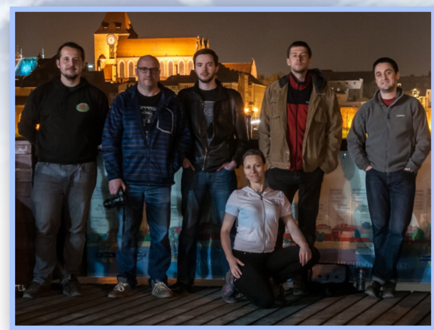
Appendix with photo-report included below:

JADE Mission

Team members:
1. Jędrzej Górski - team leader, UV light detection
2. Maciej Jakimiec - balloon launching and flight control
3. Daniel Kaczmara - telemetry, ground station, 3D projections of flight
4. Daniel Karczmit - chemistry, expert in tree climbing
5. Dr Agata Kołodziejczyk - astrobiology, scientific project design
6. Jędrzej Kowalewski - photography and video, car driver and scout

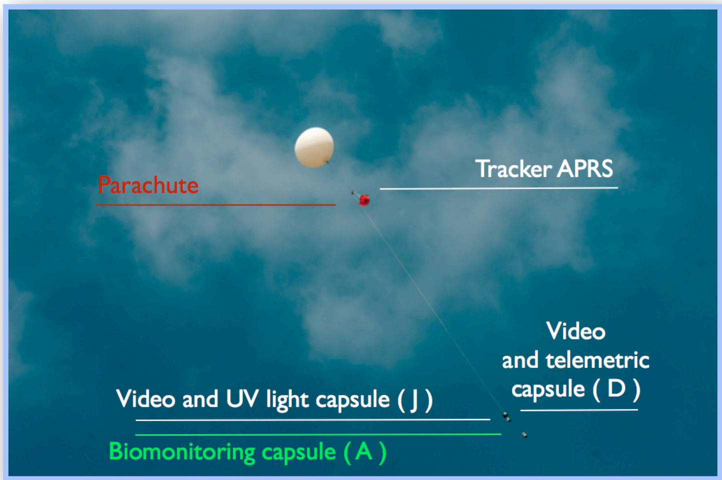
Our collaborator: Dr Oldřich Motyka - ecologist, statistician



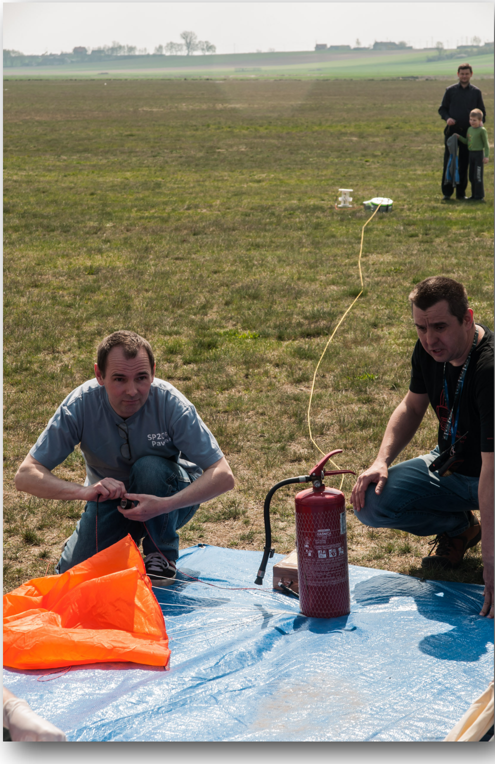
Challenge for the best scientific experiment: Biomonitoring in the stratosphere

Objective: to make invisible stratospheric ozone visible

Mission parameters:
Name: JADE = Jędrzej, Agata, Daniel Experiment
Date: 25.04.2015
Time of launching: 10:29 UTC
Time of landing: 12.32 UTC
Location: Aeroclub in Kruszyn, Poland
(WGS-84) 52° 35'09.79'' N 19° 00'43.38''E
Ballon type: light
Ballon diameter: 2-16m
Ballon gas: Hydrogen
Max. height of flight: 28 718 m
Temp.min: -32°C (APRS Data), -17°C
Flight distance: about 100km, 42km
from the launching site (in stright line)
Cameras: GoPro, Mobius Camera,
HD wing camera
Detectors: Pt1000, BMP085, photodiod SG-01S
Telemetry: URCEP - Universal Remote Control Experimental Platform

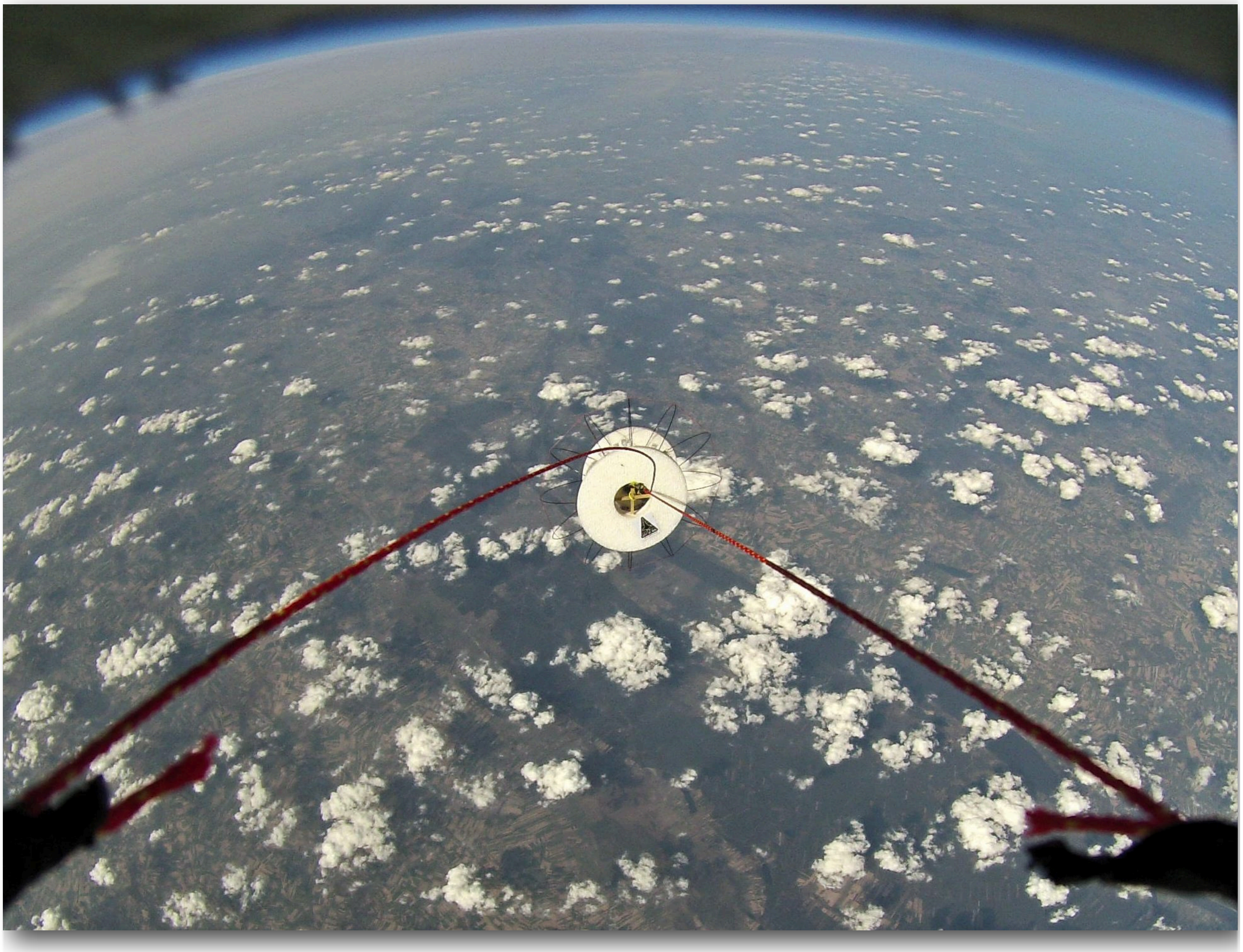


1. Launching

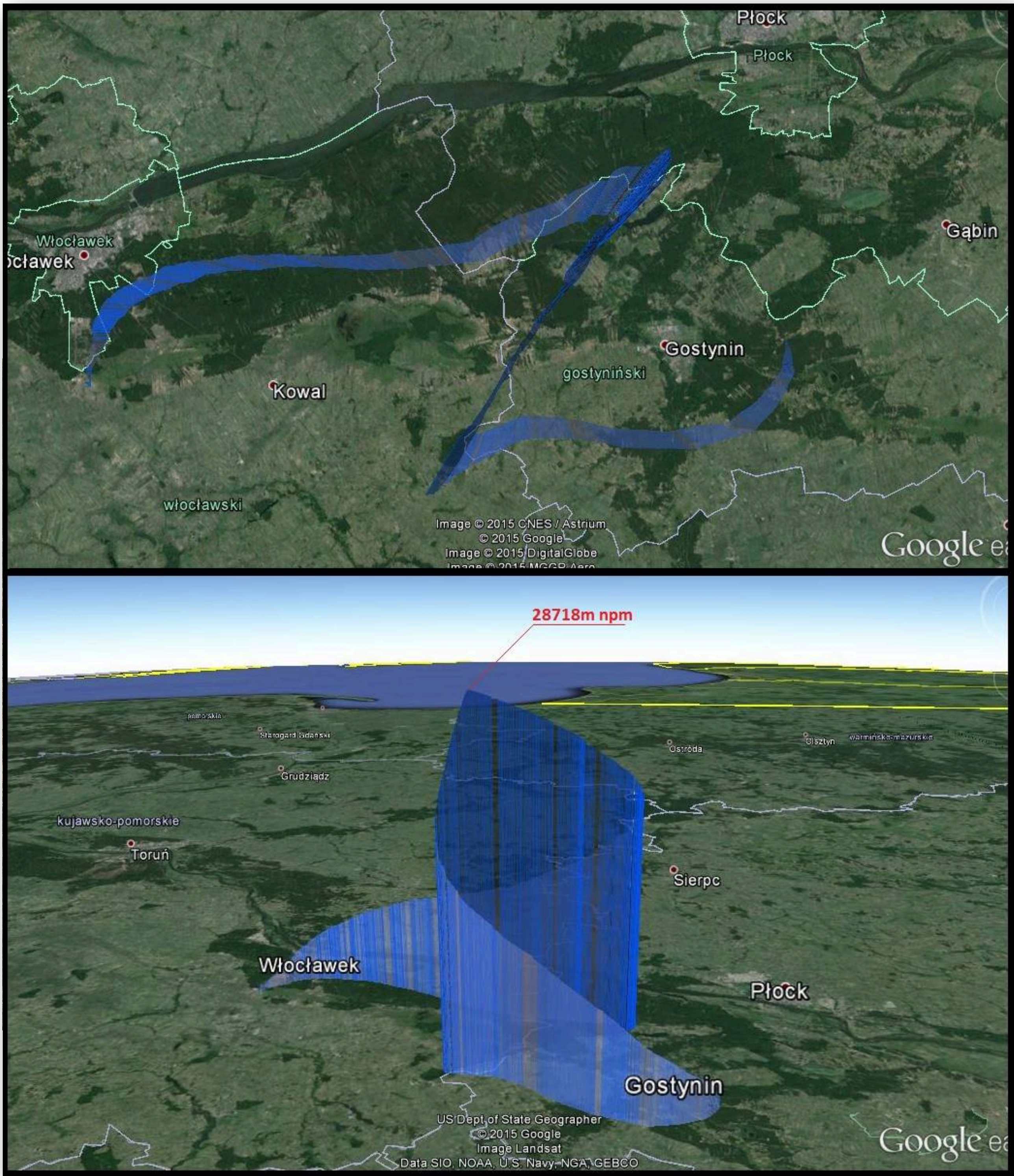


Preparing assembly....

2. Flight: mosses in stratosphere



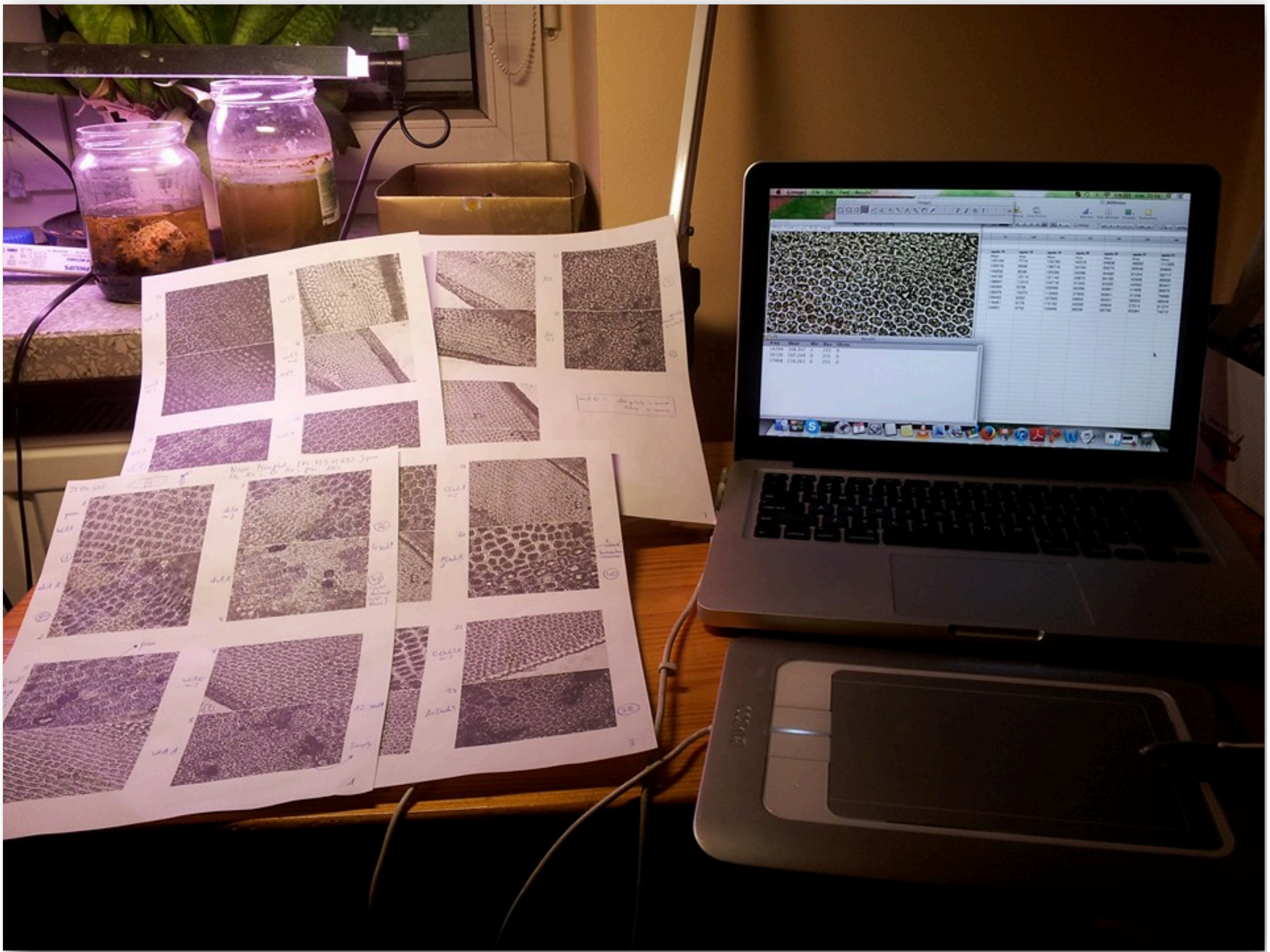
3. 3D projection of the balloon flight:



4. Landing: happy team just after finding the capsules:

5. Moss experiments: Oldrich preparing eppendorfs with mosses.

Boarding completed...



Cell surface and mean grey value meassurements on the tablet.

More info: video: https://youtu.be/wPQe8jPW1cA

E-mail: fichbio@gmail.com