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Doctoral thesis

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Cell inclusions in microscopic eukaryotes

Buněčné inkluze v mikroskopických eukaryotech

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Praha 2023

DECLARATIONS / PROHLÁŠENÍ

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Praha, 5. 5. 2023

Peter Mojzeš

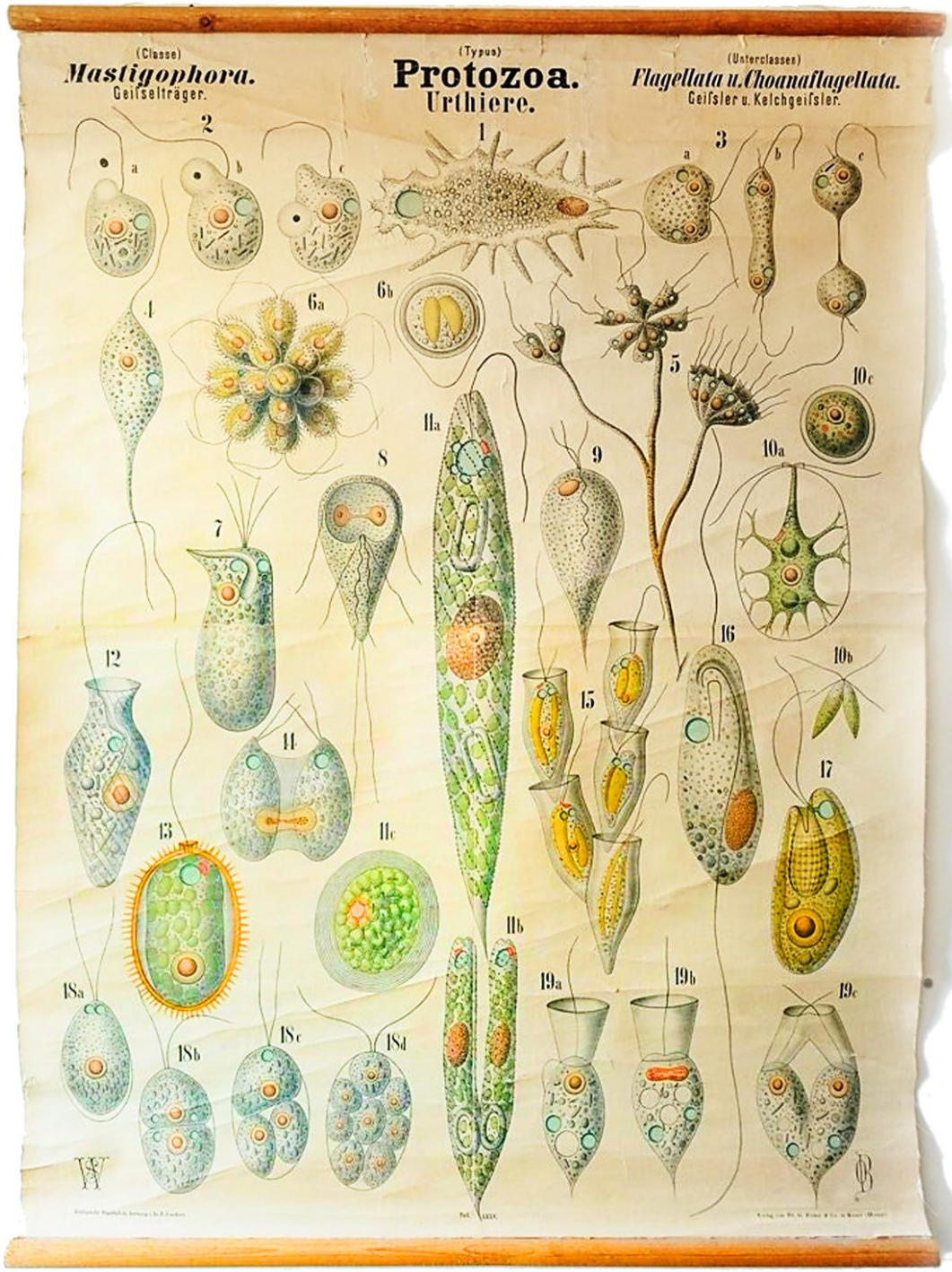
Ars longa, vita brevis aut Ars longa, Ph.D. brevis „skillfulness takes time and life is short“

Vita brevis,
ars longa,
occasio praeceps,
experimentum periculosum,
iudicium difficile.

Life is short,
and art long,
opportunity fleeting,
experimentations perilous,
and judgment difficult.

… Hippocrates

Enigmatic cell inclusions have been spotted by first biologists and pioneering microscopists although they have not been identified since then. Thus, this task waits for the new generation. “Crystalline inclusions” as well as “reddish globules” are nicely depicted in a variety of protists of this ancient educational artwork – providing its inspirational potential in our academic club the Dead Fish (Mrtvá Ryba) even 150 years after its original publication.



“Urthiere – Protozoa” by Rudolf Leuckart, the "father of parasitology" (1822–1898), Hinrich Nitsche (1845–1902), and marine biologist Carl Chun (1852–1914), published by Th. G. Fischer & Co (Kassel and Leipzig), printed by Hermann Richter & Co in Leipzig between 1877 and 1892.

Source: *https://www.vintagewallcharts.com/listing/775182420/original-antique-rudolf-leuckart*

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I am eminently lucky to add a warm thanks to the Biology Centre of the Czech Academy of Science, namely to Julius Lukeš and Daria Tashyreva for bringing me to the exciting world of diplonemids, Ansgar Gruber and Miroslav Oborník for the same attraction to a one-of-a-kind emerging model *Chromera velia*, and to Hendrik Küpper for introducing me to X-ray fluorescence and synchrotron measurements. Working as an innovation scout spread my horizons and enabled me to help other scientists in consulting their patent files and the application potential of their research in the real world, thus I gratefully worked with colleagues at the Centre for Knowledge and Technology Transfer and Charles University Innovations Prague.

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Microbes under the spotlight of Raman microscopy

Every time I crane my neck over a microscope to spot the limelight on all the hustle and bustle of astounding protists teeming with life throughout the field of my vision, I am searching whether they are carrying a piece of quintessential adornment inside of them. And they are! A breathtaking joy to behold that almost defies description. The mesmerizing tell-tale sign of purine microcrystals inside the cells of various protists is their birefringence visible in a polarizing microscope as little shining pieces of gemstones. Eclectic light-polarizing cell inclusions and reflective pieces of glooming treasure have been spotted throughout centuries and only recently did it become obvious that it might be present throughout all the groups of living protists.

https://y2u.be/UtygkzDmz8U

Abstract

This thesis reports, for the first time, a systematic study identifying various cell inclusions across the broad diversity of all major eukaryotic supergroups. Raman microspectroscopy technique was employed as a powerful technique, which is becoming the method of the first choice for such studies.

In Chapter 1, the spatial reconstruction of chloroplasts of the unicellular zygnematophyte *Cylindrocystis* sp. (Streptophyta) revealed "empty spaces" inside. Subsequent analysis showed massive accumulations of polyphosphate, which is not located in the cytoplasm as in other species, but inside the chloroplasts. This newly discovered ability of the zygnematophytes, a sister group of terrestrial plants, to accumulate polyphosphate leads us to speculate about a possible adaptation to extreme Arctic conditions or even a preadaptation leading to plant terrestrialization.

Chapter 2 summarizes the serendipitous discovery of purine crystalline inclusions in dinoflagellates and other microalgae. Investigated dinoflagellate species comprised zooxanthellae, the endosymbionts of reef-building corals, and the species causing toxic algal blooms. Purine crystals were further shown to act as dynamic high-capacity nitrogen storage. To show how widespread purine inclusions are, a revision of crystalline inclusions in all major eukaryotic supergroups was conducted. The representatives among free-living protists and algae, parasites, and endosymbionts, both model species, and environmental samples, were selected. Apart from being widespread in microscopic eukaryotes, purine crystals are possibly the most ancient cell crystalline inclusions, likely present in the last eukaryotic common ancestor. In comparison to the formerly described oxalates, calcite, or silica, this paradigm of eukaryotic biocrystallization could be shifted in favor of purines. Moreover, the first hypothetical scheme of purine crystal metabolism inside the membrane-bounded compartment was introduced. Finally, the phylogeny of important transporters and metabolic enzymes was analyzed to address the most plausible pathways.

Chapter 3 scrutinized the great collection of deep-sea diplonemid protists isolates for cellular composition. They were only recently found to be one of the most abundant and diversified protists in the oceans. Herein, they were shown to be the long-missing hyperaccumulators of Ba and Sr, which may explain Ba/Sr correlations with marine productivities, the proxies for the palaeoceanographic reconstructions of the past climate.

Chapter 4 was inspired by questioning the chemical composition of “red droplets” in the newly described species *Synura rubra*. We found the storage polysaccharide of chrysolaminarin to be potentially misinterpreted based on ultrastructures to be lipid droplets since the species descriptions in the 1970s. “Red droplets” appeared to emit intense fluorescence that spectrally shifted over the course of the measurement. Fluorescence prevented Raman analysis and the unstable pigments and compounds of “red droplets” could not be identified by HPLC MS either. The potential occurrence of anthocyanins based on the analogous spectral behavior was discussed.

Abstrakt

Tato práce přináší systematickou studii, která identifikuje různé buněčné inkluze napříč širokou diverzitou všech hlavních eukaryotických domén. Technika Ramanovy mikrospektroskopie byla použita jako metoda první volby pro studie, které vyžadují mikroanalytický přístup.

V **kapitole 1** odhalila prostorová rekonstrukce chloroplastů jednobuněčné spájivky *Cylindrocystis* sp. (Streptophyta) "prázdné prostory". Následná analýza prokázala masivními akumulace polyfosfátu, který není jako v jiných druzích v cytoplazmě, ale právě uvnitř chloroplastů. Tato nově objevená schopnost zygnematofyt, sesterské skupiny suchozemských rostlin, nás vede ke spekulacím o možné adaptaci na extrémní arktické podmínky nebo dokonce preadaptaci vedoucí k terestrializaci rostlin.

Kapitola 2 shrnuje náš náhodný objev purinových krystalických inkluzí u obrněnek (Dinoflagellata, Alveolata) a dalších mikrořas. Nejprve byly zkoumány druhy způsobující toxické vodní květy a zooxantely, endosymbionti korálů budujících útesy. Dále bylo prokázáno, že purinové krystaly fungují jako dynamická vysokokapacitní zásobárna dusíku. Aby se ukázalo, jak jsou purinové inkluze rozšířené, byla provedena revize krystalických inkluzí ve všech hlavních eukaryotických skupinách. Byli zkoumáni zástupci z řad volně žijících prvoků a řas, parazitů i endosymbiontů, a to jak modelové druhy, tak vzorky z prostředí. Kromě toho, že jsou purinové krystaly u mikroskopických eukaryot velmi rozšířené, jsou pravděpodobně nejstaršími buněčnými krystalickými inkluzemi, které se pravděpodobně vyskytovaly už u posledního společného předka eukaryot. Ve srovnání s dříve popsanými oxaláty, kalcitem nebo oxidem křemičitým, bychom tak mohli stávající paradigma eukaryotické biokrystalizace změnit ve prospěch purinů. Navíc jsme představili první hypotetické schéma metabolismu purinových krystalů uvnitř váčků čili membránou ohraničených kompartmentů. Nakonec fylogenetická analýza důležitých přenašečů a metabolických enzymů ukázala na ty z nejpravděpodobnějších komponent tvorby purinových krystalů.

V **kapitole 3** bylo analyzováno složení buněk izolátů hlubokomořských protist – diplonem. Teprve nedávno bylo zjištěno, že se jedná o jedny z nejhojnějších a nejrozmanitějších prvoků v oceánech. Zde se ukázalo, že jsou dlouho chybějícími hyperakumulátory Ba a Sr, které mohou vysvětlit korelace Ba/Sr s mořskou produktivitou, proxy pro paleoceánografické rekonstrukce klimatu v geologických érách.

Kapitola 4 byla inspirována otázkou o chemickém složení "red droplets" u nově popsaného druhu *Synura rubra*. Zjistili jsme, že zásobní polysacharid chrysolaminarin je od popisů druhu v 70. letech 20. století potenciálně nesprávně interpretován na základě ultrastruktur jako lipidové kapénky. Ukázalo se, že "red droplets" emitují intenzivní fluorescenci, která se v průběhu měření spektrálně posouvá. Fluorescence bránila analýze Ramanových spekter a nestabilní pigmenty a sloučeniny "red droplets" nebylo možné identifikovat ani pomocí HPLC MS. Nakonec jsme věc uzavřeli na základě analogického spektrálního chování „red droplets“ a antokyanů s potenciálem příčinné souvislosti.

List of abbreviations

ADP – adenosine diphosphate
AMP – adenosine monophosphate
ATP – adenosine triphosphate
cAMP – cyclic adenosine monophosphate
CARS – coherent anti-Stokes Raman spectroscopy
cGMP – cyclic guanosine monophosphate
CNT – concentrative nucleoside transporter
DNA – deoxyribonucleic acid
ED – electron diffraction
EDX – energy dispersive X-ray spectroscopy
EELS – electron energy loss spectroscopy
EFTEM – energy filtered transmission electron microscopy
ENT – equilibrative nucleoside transporter
GC – gas chromatography
GDP – guanosine diphosphate
GMP – guanosine monophosphate
GTP – guanosine triphosphate
HGPT – hypoxanthine-guanine phosphoribosyl-transferase
HPLC – high-performance liquid chromatography
ICP – inductively coupled plasma
IMP – inosine monophosphate
IR – infrared
KEGG – Kyoto Encyclopedia of Genes and Genomes
LECA – last eukaryotic common ancestor
MS – mass spectrometry
NanoSIMS – nanoscale secondary ion mass spectrometry
NAT – nucleobase-ascorbate transporter
NCS1 – nucleobase-cation symporter 1
polyP – polyphosphate
rbcL – ribulose-bisphosphate carboxylase gene
rDNA – ribosomal deoxyribonucleic acid
RNA – ribonucleic acid
SAR – Stramenopiles Alveolata Rhizaria (supergroup)
SBF – serial block face
SEM – scanning electron microscopy
SERS – surface-enhanced Raman spectroscopy
SLC – solute carrier protein
SRS – stimulated Raman spectroscopy
TEM – transmission electron microscopy
TESR – tip-enhanced Raman spectroscopy
UV – ultraviolet

List of publications attached

1. Barcytė, D., **Pilátová**, J., Mojzeš, P., Nedbalová, L. (2020). The arctic *Cylindrocystis* (Zygnematophyceae, Streptophyta) green algae are genetically and morphologically diverse and exhibit effective accumulation of polyphosphate. *Journal of Phycology*, 56(1), 217–232. See **Chapter 1**.

2. Mojzeš, P., Gao, L., Ismagulova, T., **Pilátová, J.**, Moudříková, Š., Gorelová, O., Solovchenko, A., Nedbal, L., Salih, A. (2020). Guanine, a high-capacity and rapid-turnover nitrogen reserve in microalgal cells. *Proceedings of the National Academy of Sciences*, 117(51), 32722–32730. See **Chapter 2**.

3. **Pilátová, J.,** Pánek, T., Oborník, M., Čepička, I., Mojzeš, P. (2022). Revisiting biocrystallization: purine crystals are widespread in eukaryotes. *The ISME Journal*, 16(9), 2290–2294. See **Chapter 2**.

4. **Pilátová, J.,** Tashyreva, D., Týč, J., Vancová, M., Bokhari, S. N. H., Skoupý, R., Klementová, M., Küpper, H., Mojzeš, P., Lukeš, J. (2022). Massive accumulation of strontium and barium in diplonemid protists. *mBio,* just accepted. See **Chapter 3**.

5. Škaloud, P., Škaloudová, M., Jadrná, I., **Pilátová, J.**, Shin, Woonghi, Kopecký, J. Unravelling the hidden complexity in diversity and pigment composition of a colonial flagellate *Synura sphagnicola* (Chrysophyceae, Stramenopiles). *Fottea*, just accepted. See **Chapter 4**.

Popularizing article

Pilátová, J. (2022). Revisiting biocrystallization: Purine crystals are widespread in eukaryotes. Paradigm shift in eukaryotic biocrystallization: purine biocrystals are likely an ancestral type of cellular inclusions in eukaryotes. Cell inclusions in the spotlight of Raman microspectroscopy. Nature Community Microbiology – available online. See **Chapter 2.**

Introduction

This thesis is a summary of my work during my doctoral studies. Most of the work presented herein have been published in peer-reviewed journals, although much of other work remains to be finished in near future. The published articles are attached to the thesis. It was a great privilege to be able to learn from experts in their respective fields and also to use this knowledge in helping to find new phenomena for biology. In this work, the roots of the research disciplines providing an inspiring basis for this work were scrutinized in the context of our country. Further, this work contains a summary of various encounters with cell inclusions in unicellular protists that also challenged their metabolic functions on the cellular level or their impact in the global cycles of elements in the ecosystems level. The newly emerging scientific questions and future research plans are outlined as well. All in all, the contribution of this work to the current state of knowledge and the emerging superior microanalytical tool of Raman microspectroscopy have been discussed as a central method of this thesis enabling new insights in the research of cell inclusions with its advantages and limits. Finally, the research outreach as a communication tool of the scientific results with the general public has been enclosed at the end of the thesis.

Standing on the shoulders of giants of the Czech microbiologists and microscopists

Once there is a limited space in this work to provide a comprehensive introduction to the basis of the main discoveries starting new fields of research as well as methodological approaches that occurred in our country, it cannot list all the important scientists that assisted to develop the respective fields. This is an apology for not including them all and mostly for omitting the great majority of the currently operating brilliant minds that create the future of Czech science.

Since the beginning of microscopy, microbiology started to boost our knowledge of the diversity of unicellular eukaryotes and their cellular architecture. In the mid-19th century in Prague at Charles University, the innovative application of microscopy laid the important milestones to establish new fields of animal and plant physiology, histology, and “cell biology” in the hands of Jan Evangelista Purkyně (1787–1869) and his student Julius Sachs (1832–1897) (Žárský 2012). Followed by their students, *i.e.,* Bohumil Němec (1873–1966), the founder of the Department of Plant Physiology in 1901, dean of the Faculty of Science and rector of Charles University and a presidential candidate (Longinov 2020), became one of the most influential personas of his era, propelling the scientific continuity in a forthcoming generation.

Jan Evangelista Purkyně, Julius Sachs as well as Bohumil Němec, and others combined all the virtues of scientists, artists, science popularizers, and influential participants of social and political life. At the beginning of the 20th century, prominent phycologists and protistologists published ingenious pioneering studies well-cited until now – Adolf Pascher (1881–1945) and Ernst Georg Prinsheim (1881–1970). The latter started the first culture collection worldwide at our university (Neustupa 2017, 2018). The next generations of phycologists, being originally trained by plant physiologists (Silvestr Prát), dared to start a field of biotechnology in Třeboň established by Ivan

Šetlík (1928–2009) in the 1960s (Prášil 2009). And more research units spread around the country in the hands of Šetlík's students – *e.g.*, Martin Trtílek's and his successful biotechnology company Photon Systems Instruments in Drásov.

Meanwhile, protozoology and parasitology pioneer Otto Jírovec (1907–1972) described a plethora of new species of parasites and resolved their life cycles and fostered the parasitology community to flourish. His students continued building the strong reputation of our parasitology research through ground-breaking discoveries of new types of organelles derived from mitochondria. Miklós Müller and Jaroslav Kulda with their colleagues (Donald Lindmark, Apolena Čerkasová, Jiří Čerkasov) reported the first description of hydrogenosomes in trichomonads and Jiří Vávra (1933–2018) described mitosomes in microsporidia, both published in the 1970s (Tachezy 2008). The current generation brings new completely unexpected discoveries, such as the first amitochondriate eukaryote *Monocercomonoides* sp. by Anna Karnkowska and Vladimír Hampl (Karnkowska et al. 2016) shifting the paradigm of our understanding of the cellular metabolism, organization, and functioning.

Electron microscopy brought more detailed insights into our understanding of cellular and organellar architecture by providing us with tremendous resolution on the level of cellular (ultra)structures. The development of electron microscopy by Armin Delong (1925–2017) and his colleagues in Brno in the 1950s enabled to build upon this invention both – successful business and scientific careers with a long-standing tradition that brought many new advances and applications to the field of electron microscopy (Delong and Lencová 2021). Furthermore, the recent advances in holographic microscopy with quantitative phase imaging have their Czech roots in Brno as well (Strbkova et al. 2020). Back to electron microscopies, analytical electron microscopy, *e.g.*, energy dispersive X-ray spectroscopy (EDX), electron energy loss spectroscopy (EELS), energy-filtered transmission electron microscopy (EFTEM) provides us with an elementary analytical tool with a great spatial resolution (Shebanova et al. 2017, Ismagulova et al. 2018). Another elementary analysis that added a possibility to detect isotopically labeled cells that are prepared in the same way as for electron microscopy is the nanoscale secondary ion mass spectrometry (NanoSIMS), which has been also applied in the research of cellular inclusions in protists (Kopp et al. 2013, Krueger et al. 2018). Nevertheless, imaging methods using elementary analysis still cannot distinguish complex molecules.

Thus, another powerful tool for biologists, Raman microspectroscopy, is a game-changer in our understanding of chemical composition in biological samples *in vivo* and *in situ*. It is a combination of laser scanning confocal microscopy with Raman vibrational spectroscopy which yields in chemical imaging of cellular compartments. The principle of inelastic Raman scattering got its name in honor of its discoverer and a Nobel Prize laureate Sir Chandrasekhara Venkata Raman (1888–1970) (Raman 1928, Raman and Krishnan 1928). The physics of the principle of Raman scattering and the applications of emerging variety of Raman spectroscopies have been completely explained in detail elsewhere (Long 2002, Shipp et al. 2017, Lee et al. 2021). The Czech footprint in the history of discoveries of Raman scattering dates to the laboratory of Adolf Smekal (1895–1959) who theoretically predicted Raman scattering. He is naturally considered an Austrian physicist being raised in Olomouc and Brno, during Austro-Hungarian Empire and working in Vienna and Berlin. The subsequent empirical proofs were accomplished by Chandrasekhara Venkata Raman in 1930 for which he was awarded Nobel Prize in 1970 (Singh 2002, Momber 2010). The optical phenomenon is coined Raman scattering or Smekal-Raman scattering. Sir Raman who visited Jaroslav Heyrovský at Charles University in 1958 built a personal friendship and scientific collaboration with him; Heyrovský was awarded a Nobel Prize the following year. In the 1960s, Raman spectroscopy was started and

developed by Bohuslav Strauch (1929–2017) in the Department of Inorganic Chemistry of the Faculty of Science, Charles University, bringing new important insights into the vibrational spectral analysis of inorganic and biologically relevant molecules (Vlčková et al. 2018).

Interestingly, the first biological material measured by the first Raman microscope constructed by Michel Delhaye and Paul Dhamelincourt in 1975 was microalgal species *Botryococcus braunii* (Delhaye and Dhamelincourt 1975, Largeau et al. 1980). Since then, Raman microspectroscopic measurements of photosynthetic microorganisms remained almost silent for three decades with a sporadic publication record (Moudříková 2018). The reason is probably that the highly fluorescent chlorophyll hampers collection of Raman signal that is less intense by orders of magnitude. Moreover, pigment-caused absorbance of high-intense laser beams brings irreversible thermal changes to highly absorbing samples. However, the Czech team made a great leap forward in developing an elegant solution of chlorophyll photobleaching that fast degrades the major source of autofluorescence and enables the measurements of photosynthetic microalgae and cyanobacteria (Moudříková et al. 2016). This great advancement opened a new era of applications of Raman microspectroscopy in the biology of photosynthetic unicellular eukaryotes (Moudříková et al. 2017a, 2017b, 2021, Zachleder et al. 2018, Mojzeš et al. 2020, Pilátová et al. 2022, 2023).

The method of the first choice for microanalytical studies of cell compartments *in vivo*

To address the chemical composition of an unknown cell inclusion is a very challenging task even with the vast portfolio of analytical techniques available. Each group of compounds requires a suitable methodological approach for its isolation or extraction, separation, and detection, to be analyzed properly. The investigation of cell inclusions in protists and their biological importance has been impeded considerably by the lack of specific methods for their characterization *in situ* and *in vivo*. In the sense of the popular motto “seeing is believing”, the modern microscopic techniques combining analytical approaches enable us to address these structures in more detail combining sensitive analytical tools. Among these microscopic methods, Raman microspectroscopy occupies an important place, because it provides spatially resolved information about the molecular composition of the object under investigation without requiring any specific staining or labeling.

Defining the main scope of the work – cell inclusion, compartment, and organelle

Once this thesis is devoted to the research of subcellular structures, one can question the particular meaning of terms, such as cell inclusions, compartments or organelles, used in this work. Cell inclusions are mostly defined as membrane-free subcellular structures that hold storage or secretory material emerging in the process of phase separation. In contrast, organelles are mostly defined as a membrane-bounded compartments acting in a specific biological function (Alberts et al. 2015). Compartments of all kinds usually refers to more general meaning than the organelles, *e.g.*, cytoplasmic compartment is bounded by the cytoplasmic membrane, but is not described as an organelle. The emphasis on the presence of the membrane is justified because the term then automatically reflects the mode of operation and the biosynthetic origin of each of them. However, simple rules usually fail in categorizing highly variable biological structures. For instance, lipid droplets are commonly classified as cell inclusions although they are covered by a single layer of the outer membrane derived from endoplasmic reticulum (Cottier and Schneiter 2022). Storage

polysaccharides, *e.g.*, starch, glycogen, chrysolaminarin, are likewise put into the cell inclusion category, although in different species their location varies. In animals, glycogen is supposed to be a cell inclusion *sensu stricto* unless some pathological states referring to possess a membrane over the glycogen granule (Matsui et al. 1983). In plants and green algae, starch is present inside the chloroplast, inside the endosymbiotic organelle (van den Hoek et al. 1995). In Stramenopiles, namely Eustigmatophyceae, chrysolaminarin, is questioned to form ultrastructures of lamellate vesicles that are arguably covered by the double-layer membrane (Schnepf et al. 1996). Polyphosphate granules are mostly described as cell inclusions despite being membrane bounded once in the cytoplasm. The synonym acidocalcisomes, which implies the organellar architecture by the ending of the word "-some" or body favors its organellar structuring and functioning (Kornberg 1999, Lander et al. 2016). Simultaneously, we have described the as-yet-unknown localization of polyphosphate inside the chloroplasts of zygnematophyte algae. Therefore, if the condensate of material is present inside another compartment or organelle, how should we call it then? Being aware of discrepancies the misuse of a well-defined term may cause, here the rather intuitive use of the term 'cell inclusion' *sensu lato* in a generalized way refer to any type of a condensed material regardless its precise localization within a membrane bounded compartment or even an organelle. The Raman microspectroscopy as a central method used in this work usually does not allow to discern such a level of detail.

Methods used in this work

In this work, a combination of micro and macro analytical techniques has been performed with the focus on analytical imaging. Optical microspectroscopic approach was emphasized including techniques newly established in biology, such as Raman microspectroscopy, and holographic microscopy or quantitative phase imaging. The author designed experiments and conducted data acquisition, processing, and interpretation of all the optical microspectroscopic methods applied. The author participated on sample preparation and data acquisition of both transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The microanalytical approaches used in this work comprise TEM EDX, electron diffraction (ED), serial block face (SBF) SEM and SEM EDX. These more advanced techniques were supervised and fully processed by experts, *i.e.*, Mariana Klementová, Marie Vancová, Jiří Týč and Radim Skoupý. Compared to microanalytical techniques and imaging, the qualitative and quantitative macro analytical methods have been applied in order to examine the bulk elemental composition of the samples. In this respect, inductively coupled plasma (ICP) MS was run by its operator Syed Nadeem Hussain Bokhari. The bioinformatic approach has been conducted by experts in the field of molecular phylogenomics, *i.e.*, Tomáš Pánek and Miroslav Oborník. The particular author's contribution to each of the studies is specified in every chapter.

Aims of the thesis

Long-time questioned nature of various cell inclusions in unicellular eukaryotes as depicted in the historic poster "Urtiere, Protozoa" by Rudolf Leuckart, Hinrich Nitsche, and Carl Chun (see above), oftentimes remain without the proper attribution. Research of many biologically relevant substances has been impeded considerably by the absence of methods for their effective identification. Raman microspectroscopy provides a remarkable explanatory power to address the chemical nature of cell inclusions. The aim of this work is to address the chemical nature and functional properties of the

understudied cell inclusions in unicellular eukaryotes using the powerful tool of Raman microspectroscopy accompanied by other imaging and analytical techniques.

Aim 1: Chloroplast morphology reconstruction of newly described strains of Arctic microalgae *Cylindrocystis* sp. (Zygnematophyceae, Streptophyta) using bright field, fluorescence and Raman microspectroscopy; revisiting the nature of Kopetzky-Rechtperg bodies.

Aim 2: Chemical identification of purine crystals, revisiting their distribution in eukaryotes and analysis of their metabolism in the model system of a marine dinoflagellate *Amphidinium carterae* (Dinoflagellata, Alveolata, SAR).

Aim 3: Characterization of the chemical composition of crystalline inclusions in diplonemids (Euglenozoa, Excavata) using Raman microspectroscopy and electron microscopy; further conceptualization of the potential role of diplonemids in Ba-Sr cycling and palaeoceanographic records.

Aim 4: Analysis of autofluorescence spectra and chemical composition of the prominent cell structures in *Synura sphagnicola* and newly described species *S. rubra* (Chrysophyceae, Stramenopiles, SAR).

Structure of the dissertation thesis

This dissertation is divided into four stand-alone chapters that correspond to aims of the work. Each chapter contains a summary or an abstract of the published article that is attached to this work. Each chapter further contains a short introduction to the topic, a discussion of published results, the merit of its significance and/or the next perspectives. The general discussion aims at the assessment of the research of cell inclusions in a variety of biological systems using Raman microspectroscopy; it is a critical scrutiny of its advantages and limits including raising awareness of this method among broad spectrum of biologists and challenging the spectroscopic community to improve the tool to another level of current standards. Conclusions summarize the main findings published in this thesis, research outreach, and the acknowledgement of many new collaborations including raising mutual grants and exciting projects.

Chapter 1 – Polyphosphate inside chloroplasts of streptophytes

This chapter summarizes aim 1 and the published results with the focus on author's contribution to the study. The article is enclosed in the attachments. It has become a highly cited paper of Journal of Phycology. This work has been awarded as the best students' talk at the 60th Czech Phycology Society Meeting in 2019.

Full citation

Barcytė, D., **Pilátová**, J., Mojzeš, P., Nedbalová, L. (2020). The arctic *Cylindrocystis* (Zygnematophyceae, Streptophyta) green algae are genetically and morphologically diverse and exhibit effective accumulation of polyphosphate. *Journal of Phycology*, 56(1), 217–232. IF = 3.173

Author's contribution

JP designed the Raman measurements and experiments including testing the origin of Kopetzky-Rechtperg bodies, performed Raman measurements and fluorescence imaging, processed and interpreted spectroscopic and microscopic data, conceptualized the results, provided reagents and expertise, and got involved in substantial part of manuscript writing.

Summary

The green algal genus *Cylindrocystis* is widespread in various types of environments, including extreme habitats. However, very little is known about its diversity, especially in polar regions. In the present study, we isolated seven new *Cylindrocystis*-like strains from terrestrial and freshwater habitats in Svalbard (High Arctic). We aimed to compare the new isolates on a molecular (*rbc*L and 18S rDNA), morphological (light and confocal laser scanning microscopy), and cytological (Raman microscopy) basis. Our results demonstrated that the Arctic *Cylindrocystis* were not of a monophyletic origin and that the studied strains clustered within two clades (tentatively named the soil and freshwater/glacier clades) and four separate lineages. Morphological data (cell size, shape, and chloroplast morphology) supported the presence of several distinct taxa among the new isolates. Moreover, the results showed that the Arctic *Cylindrocystis* strains were closely related to strains originating from the temperate zone, indicating high ecological versatility and successful long-distance dispersal of the genus. Large amounts of inorganic polyphosphate (polyP) grains were detected within the chloroplasts of the cultured Arctic *Cylindrocystis* strains, suggesting effective luxury uptake of phosphorus. Additionally, various intracellular structures were identified using Raman microscopy and cytochemical and fluorescent staining. This study represents the first attempt to combine molecular, morphological, ecological, and biogeographical data for Arctic *Cylindrocystis*. Our novel cytological observations partially explain the success of *Cylindrocystis*-like microalgae in polar regions.

Commentary on polyphosphate rediscovery and homologues (volutin, acidocalcisome)

Polyphosphate has been morphologically described as volutin in a Gram-negative bacterium *Spirillum volutans* for the first time (Meyer 1904). The chemical composition of volutin was identified to be polyphosphate in yeast cells (Wiame 1947). Arthur Kornberg, a Nobel laureate for his work on understanding the DNA and RNA synthesis, rediscovered polyphosphate for science refusing the intellectual vacuum that objected the research of its functional roles in cells expressed as disgraceful description of polyphosphate being a "molecular fossil" (Kornberg 1995). During further proceedings, polyphosphate has been recognized as a multifunctional structure in all varieties of living organisms ranging from bacteria and cyanobacteria to all species of eukaryotes including human blood platelets (Docampo et al. 2005). Docampo focused his research on studies of the polyphosphate biological roles in various models – *Trypanosoma*, as well as in *Chlamydomonas* or yeasts (Docampo et al. 2005, Docampo and Lukeš 2012, Lander et al. 2016). He prefers the name acidocalcisome for the structure bearing negatively charged polyphosphate together with the counterions of calcium that gave the structure its name. Indeed, this terminological trichotomy using "volutin", "polyphosphate" and "acidocalcisome" survived in many ways into currently published research articles.

Polyphosphate structures and methods of analysis

Polyphosphate has variable contents with respect to chain length ranging from three units to thousands and/or branched or cyclic chains, hence, it is not easy to study its detailed structural features (Sanz-Luque et al. 2020). To quantify or address the chemical nature of polyphosphate various methods have been employed: ^{31}P nuclear magnetic resonance spectroscopy, chromatography, electrophoresis, Fourier-transform infrared spectroscopy, Raman spectroscopy, electron-spray ionization mass spectrometry, and colorimetric enzyme assays, *e.g.*, malachite green assay determination of PO_4^{3-} released from hydrolyzed polyphosphate (Christ et al. 2020, Sanz-Luque et al. 2020, Lapointe et al. 2022).

Polyphosphate in chloroplasts and potential functions

In brief, we found polyphosphate inside chloroplasts of zygnematophyte *Cylindrocystis* sp. while searching for the substance filling the chlorophyll-free areas appearing on their three-dimensional reconstruction using confocal laser-scanning fluorescence microscopy. Based on personal communication with Roberto Docampo, an expert on polyphosphates, he confirmed our finding of polyphosphates in chloroplasts to be the first record in the literature. Diversified functions of polyphosphate comprise phosphorus and divalent cations storage (Harold 1966, Siderius et al. 1996), osmoregulation and calcium signaling (Lander et al. 2016), pH buffering (Pick et al. 1991), energy storage in the form of macroergic bonds (Kornberg 1999), and antioxidative role (Gray and Jakob 2015). Moreover, it can act as inorganic chaperone against protein aggregation due to its negative charge binding water and solubilizing proteins (Xie and Jakob 2019). For now, we may only speculate about the actual functioning in *Cylindrocystis* sp. – whether it is an adaptation to harsh conditions of High Arctics or even a preadaptation for land territorialization that occurred in the ancestors of zygnematophytes and land plants.

Kopetzky-Rechtperg body abolished

One of the reviewers of our manuscript pinpointed a potential connection of polyphosphate and earlier described Kopetzky-Rechtperg bodies. These structures were defined based on toluidine blue staining in different zygnematophyte genera and species: *C. brebissonii, Closterium, Cosmarium, Netrium, Zygnema, Spirogyra, Mougeotia* (Kopetzky-Rechtperg 1949, Höfler and Schindler 1950, Hirn 1953). The attribution to polyphosphate was mostly hypothetical with other candidate interpretations including pectins or tannin (Kopetzky-Rechtperg 1931, Hirn 1953). In particular, toluidine blue is used as non-specific staining or contrasting of the cell wall and mucilage sheet, nucleus, mitochondria, polyphosphate granules, lysosomes, Nissl bodies, rosettes of ribosomes attached to the rough endoplasmic reticulum, etc. (Love et al. 1955, Brook 1992, Chelvanayagam and Beazley 1997, Gomes et al. 2013, Zhang and Tung 2018). By colocalization of fluorescently labeled cell compartments, we disproved the existence of Kopetzky-Rechtperg bodies and suggested them to be a complex of other organelles – most likely mitochondria, lysosomes, and others (Barcytė et al. 2020).

Chapter 2 – Purine inclusions: widespread in eukaryotes and versatile in their functions

This chapter stems from our two publications (Mojzeš et al. 2020, Pilátová et al. 2022) that are attached to the thesis and completes aim 2. As a major part of the thesis, a broader background to the topic of eukaryotic biocrystallization with the focus on purine inclusions is provided. The summary of the studies of our predecessors is completed with our new insights and the future research challenges aimed at functional studies. Rediscovering purine crystals as an important and versatile cellular structure is an analogous situation with polyphosphate from the previous chapter, but unlike polyphosphate, the research of purine crystals has not been compiled in a comprehensive review. Therefore, here I provide a brief overview of the pioneering works in the newly emerging field of research to establish a basis for our contributions to it or the future review article. This work has been awarded for the best student's talk in 16th Students' Day of Plant Biology in 2021 and best students' talk at the 60th Czech Phycology Society Meeting in 2019.

Full citation

Mojzeš, P., Gao, L., Ismagulova, T., Pilátová, J., Moudříková, Š., Gorelová, O., Solovchenko, A., Nedbal, L., Salih, A. (2020). Guanine, a high-capacity and rapid-turnover nitrogen reserve in microalgal cells. *Proceedings of the National Academy of Sciences*, 117(51), 32722–32730. IF = 13.450

Author's contribution

JP participated in designing and performing experiments, data processing and interpretation, conceptualizing the results and writing the manuscript.

Summary

Nitrogen (N) is an essential macronutrient for microalgae, influencing their productivity, composition, and growth dynamics. Despite the dramatic consequences of N starvation, many free-living and endosymbiotic microalgae thrive in N-poor and N-fluctuating environments, giving rise to questions about the existence and nature of their long-term N reserves. Our understanding of these processes requires an unequivocal identification of the N reserves in microalgal cells as well as their turnover kinetics and subcellular localization. Herein, we identified crystalline guanine as the enigmatic large-capacity and rapid-turnover N reserve of microalgae. The identification was unambiguously supported by confocal Raman, fluorescence, and analytical transmission electron microscopies as well as stable isotope labeling. We discovered that the storing capacity for crystalline guanine by the marine dinoflagellate *Amphidinium carterae* was sufficient to support N requirements for several new generations. We determined that N reserves were rapidly accumulated from guanine available in the environment as well as biosynthesized from various N-containing nutrients. Storage of exogenic N in the form of crystalline guanine was found broadly distributed across taxonomically distant groups of microalgae from diverse habitats, from freshwater and marine free-living forms to endosymbiotic

microalgae of reef-building corals (*Acropora millepora*, *Euphyllia paraancora*). We propose that crystalline guanine is the elusive N depot that mitigates the negative consequences of episodic N shortage. Guanine may act similarly to cyanophycin granules in cyanobacteria. Considering the phytoplankton nitrogen pool size and dynamics, guanine is proposed to be an important storage form participating in the global N cycle.

Full citation

Pilátová, J., Pánek, T., Oborník, M., Čepička, I., Mojzeš, P. (2022). Revisiting biocrystallization: purine crystals are widespread in eukaryotes. *The ISME Journal*, 16(9), 2290–2294, IF = 13.214

Author's contribution

JP conceived the study, designed and performed the key experiments, processed and interpreted data, conceptualized the results, provided reagents and expertise, wrote the manuscript, first and the corresponding author.

Summary

Despite the widespread occurrence of intracellular crystalline inclusions in unicellular eukaryotes, scant attention has been paid to their composition, functions, and evolutionary origins. Using Raman microscopy, we examined >200 species from all major eukaryotic supergroups. We detected cellular crystalline inclusions in 77 % species out of which 80 % is composed of purines, such as anhydrous guanine (62 %), guanine monohydrate (2 %), uric acid (12 %) and xanthine (4 %). Our finding shifts the paradigm assuming predominance of calcite and oxalates. Purine crystals emerge in microorganisms in all habitats, *e.g.*, in freshwater algae, endosymbionts of reef-building corals, deadly parasites, anaerobes in termite guts, or slime molds. Hence, purine biocrystallization is a general and ancestral eukaryotic process likely present in the last eukaryotic common ancestor (LECA) and here we propose two proteins omnipresent in eukaryotes that are likely in charge of their metabolism: hypoxanthine-guanine phosphoribosyl transferase and equilibrative nucleoside transporter. Purine crystalline inclusions are multifunctional structures representing high-capacity and rapid-turnover reserves of nitrogen and optically active elements, *e.g.*, used in light sensing. Thus, we anticipate our work to be a starting point for further studies spanning from cell biology to global ecology, with potential applications in biotechnologies, bio-optics, or in human medicine.

Biocrystallization – an intrinsic biological phenomenon

Biocrystallization or biomineralization, independent on a particular terminology or definitions, are hugely widespread among organisms, they impact the biosphere and global cycles of elements. The term "biocrystal" was first coined by Ernst Haeckel, the founder of modern protistology and ecology, in his synthetic monography "Crystal Souls: Studies of Inorganic Life" published in 1917 (Haeckel 1917). Since then, the knowledge about the basic types of minerals produced in living organisms grew in numbers over time. In total, around 60 different types of minerals have been described in organisms from prokaryotes to animals, being deposited both inside and outside cells (Lowenstam and Weiner 1989). Back then, most of the studies that focused on biomineralization did not take organic compounds into account with an exception for calcium oxalate that can also form a geological mineral called whewellite. As the term "mineral" traditionally refers to inorganic

compounds in geology, the usage of "biocrystal" instead might prevail for its broader meaning encompassing both organic and inorganic crystals formed by organisms, although it does not fit perfectly for description of amorphous matter. Nowadays material scientists working on the fast-growing field of organic biocrystallization of animals actually promote the term "organic biomineralization" (Palmer et al. 2018, Wagner et al. 2021, Pinsk et al. 2022). Historically, silica and calcium bearing biominerals were supposed to form the majority of all the existing forms of biominerals in protists as well as in animals as estimated previously by Lowenstrum and Weiner (1989). Similarly to other authors, they omitted to mention the purine crystals reported in the 1960s. It was commonly assumed that biocrystals comprise mostly calcite or aragonite ($CaCO_3$), silica in the form of opal (SiO_2), and oxalates, among which there were a few exceptions, such as barite ($BaSO_4$), celestite ($SrSO_4$), exceptionally purines (Haeckel 1917, DeSa and Hastings 1968, Raven and Knoll 2010). The following brief summary of biominerals is not intended to present the detailed list of all findings on eukaryotic biocrystallization, as we want to emphasize mainly the newly found particularities with the main focus on the up-to-now overlooked purine crystals.

Calcite and aragonite. In protists, the process of calcification is mostly studied in extracellular scales and shells of ecologically important representatives of coccolitophorids (Haptophyta) and foraminiferans (Rhizaria), although some rhodophytes and green algae (Archaeplastida) can also get incrusted with carbonates (Lowenstam and Weiner 1989). Recently, some dinoflagellates were found to produce calcite (Jantschke et al. 2020) and interestingly also diatoms that are otherwise specialists on opal incrustation (Ehrlich et al. 2016, Pilátová et al. 2022).

Opal. Silica scales, shells, lorices, spicules and frustules are mostly ornamented skeletal constructions that emerged more than ten times independently in various groups of microscopic eukaryotes in diatoms (Bacillariophyceae, Stramenopiles, SAR), photosynthetic and heterotrophic chrysophytes (Chrysophyceae, Stramenopiles, SAR), testate ciliates (Alveolata, SAR), mixotrophic marine radiolarians and testate eugliphids (Rhizaria, SAR), and predatory freshwater heliozoans (Centroplasthelida) (Lowenstam and Weiner 1989, Raven and Knoll 2010).

Barite. With the rise of microanalytical techniques, disentangling the chemical identification of some inorganic biocrystals was enabled. For instance, barite crystals have been thoroughly documented in many species of zygnematophyte microalgae (Kreger 1957, Kreger and Boeré 1969, Brook et al. 1980, 1988, Krejci et al. 2011). Some Xenophyophorea (Rhizaria, SAR), benthic macroscopic multinucleate relatives of foraminiferans thriving in deep oceans, were found to produce intracellular barite apart from calcified extracellular tests (Gooday and Nott 1982). Marine haptophytes are widespread and diversified ecologically important group of microalgae building their ornamented calcified scales. Rarely, *Pavlova* sp. is reported to possess intracellular barite crystals (Fresnel et al. 1979). Finally, ciliate *Loxodes* sp. (Ciliophora, Alveolata, SAR) form barite as statocysts in its vacuolar graviperceptive apparatus (Hemmersbach and Häder 1999).

Celestite. Acantharea (Rhizaria, SAR) are well-known representatives of mixotrophic plankton hosting endosymbiotic microalgae that, compared to other Rhizaria, produce their ornamented extracellular skeleton from celestite (Bütschli 1906, Odum 1951). Only recently, diplonemids were found to produce massive amounts of celestite and barite inside their cells (Pilátová et al. 2023).

Oxalates. Calcium oxalates are mostly known from plant tissues or human pathologies (Franceschi and Nakata 2005, Nakata 2012). Similarly, they are repeatedly found in microalgae of green and red lineage: ulvophytes including cladophorids, some streptophytes, and rhodophytes (Archaeplastida) (Pueschel 1995, 2012, Leliaert and Coppejans 2014).

Crystalline biopolymers. Among organic biocrystals, biopolymers can also crystallize. For instance, some proteins naturally crystallize inside bacteria, plant, and animal cells. They have been recently reviewed alongside with their functions ranging from nutrition storage to virulence factors (Schönherr et al. 2018). The best-known examples are proteinaceous inclusions of insulin or Bt-toxin named after the original producer *Bacillus thuringiensis.* The (micro)crystalline character of the most common polysaccharide in the world, cellulose, determines its physicochemical properties, mostly mechanical strength (Medronho et al. 2012).

Historic overview of early discoveries of purine crystalline inclusions in protists

The serendipitous discovery of purine biocrystals in microalgae (Moudříková et al. 2017a) triggered many questions about how widespread this type of crystalline inclusions is (Jantschke et al. 2019) and also why they were neglected for so long. The first question has been addressed by the recent work showing that purine crystals are the most widespread type of crystalline inclusions in eukaryotes and potentially the most ancestral type of biocrystals (Pilátová et al. 2022). The latter question about why this type of material was too difficult to analyze in the past, might be addressed by a simple claim about the lack of a suitable (micro)analytical technique to be applied. *Nota bene*, using macroanalytical methods is even more demanding for choosing the right one as they are specific to the targeted group of compounds in case of both – various inorganic, or organic compounds, such as minerals, lipids, sugars, phenolics, various types of different pigments, purines, etc. Thus, it is necessary to guess what the crystals are made of first prior to picking the right analytical tool. Still, in some cases, purine inclusions have been identified during the last sixty years. The first such identification was motivated by a completely different interest while lacking knowledge about the presence of crystalline inclusions in the cells (Roush 1961). In the old literature, there are numerous reports of crystals of the exact structures we later found to be formed by purines. Back then, their composition was either unknown or was erroneously misinterpreted as it now appears in the light of our new findings. In this chapter, the summary of previous findings on intracellular crystalline inclusions that are likely formed by purines is presented. Additionally, the suspected source of potential flaws in the used methodological approaches have been discussed.

As soon as the discovery of microscopy brought its powerful insights into the hands of biologists, they started a completely new field of microbiology, including the separate disciplines of protistology, protozoology, phycology, and others. The greatest pioneers of modern biology encountered biocrystals during their very first microscopic endeavors. The founder of modern protistology and phycology, Christian Gottfried Ehrenberg, was mostly concerned about the extracellular frustules, lorices, thecas or shells as a central point of his great monography Mikrogeologie. There is not much evidence that he encountered the intracellular crystals in hundreds of the microalgal and protists species he described in the first half of the 19th century (Ehrenberg 1854). Charles Darwin explicitly noted the crystalline inclusions dropping out of the bursting cells of flagellates, most likely, dinoflagellates or ciliates, of the red tide he encountered on Beagle nearby Chile coast (Darwin 1840). Ernst Haeckel coined the term “biocrystal” that has been used since then describing the silica or calcite scales of flagellates, frustules of diatoms, and sophisticated and highly ornamented skeletons of foraminifers and radiolarians (Haeckel 1872, 1917). In mid-19th century, crystals in amoebae have been described for the first time (Auerbach and Page 1856). Naturally, researchers focused on particular species of algae or protists. In the 20th century, scarce comparative studies suggested similarities in crystal morphology (**Figure 1**) and chemical properties in ciliates, amoebae, or microalgae (Bernheimer 1938, Hausmann 1982). Correlative observations of crystals’

appearance ("Lithosomen" or "Steinkörper") concerning the trophic level in ciliates, cryptophytes, foraminiferans, and amoebae (rhizopods) brought important insights into the contents and functions of the crystalline inclusions although their final identification remained for future research.

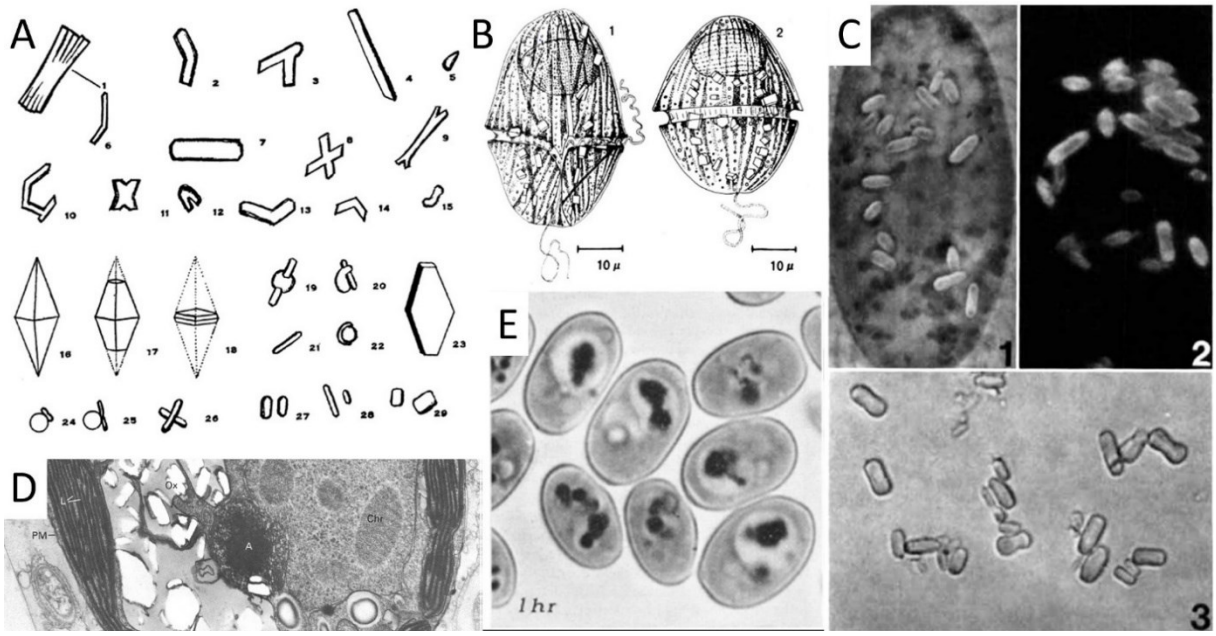

Figure 1: The illustrations of crystalline inclusions morphology and chemical nature. A – Types of crystals found in various protozoa. 1–15 *Paramecium multimicronucleatum*; 16–18 *Trichamoeba villosa* and *Chaos diffluens*; 19–22 *Halteria grandinella*; 23 – *Cochliopodium bilimbosum;* 24–26 *Mayorella* sp.; 27–29 *Chilomonas paramecium* (Bernheimer 1938). B – dinoflagellate *Gymnodnium alaskensis* n. sp. (1) ventral view of long form, showing chromatophores, nucleus, and anterior and longitudinal flagellum, (2) dorsal view of the short form (Bursa 1963). C – Phase contrast photomicrographs of ciliate *Parauronema acutum* with refractile bodies (Soldo et al. 1978). D – Transmission electron micrograph of dinoflagellate symbiont of Foraminifera showing holes after dropping off the crystalline inclusions in vacuoles as well as inside the accumulation body (A) – misinterpreted to be formed by calcium oxalate (Ca) (Leutenegger 1977). E – UV microscopy applying 260 nm excitation on yeast *Candida utilis* after 1-hour guanine incubation (Svihla et al. 1963).

Amoebae. As early as in 1856, crystalline inclusions of amoebae (Amoebozoa) were first described (Auerbach and Page 1856). A series of attempts to analyze their contents brought different conclusions, *i.e.*, allantoin-like compounds $(C_5H_9N_4O_4)_x$ / $C_4H_6N_4O_3$ or triuret (carbonyl diurea), by employing IR spectroscopy and X-ray diffraction (Grunbaum, B. W., Max Møller, K. M., Thomas 1959, Griffin 1960, 1961, Carlström and Møller 1961). Although since the 1960s, crystalline inclusions in amoebae have not been re-examined, we later found that the original attempt for their identification was close to our recent findings of guanine ($C_5H_5N_5O$) and xanthine ($C_5H_4N_4O_2$) crystalline inclusions (Pilátová et al. 2022). Experimentally removed crystalline inclusions described as "refractile bodies" were quickly rebuilt pointing to their storage function (Hausmann and Walz 1979, Hausmann 1982).

Yeasts. The first correct finding of purine crystals occurred in opportunist pathogenic yeast *Candida utilis* (Opisthokonta, Obazoa) (Roush 1961) (**Figure 1**). The discovery of crystalline purines inside vacuoles of *Candida* happened due to the interest in purine as a source of nitrogen in yeast nutrition with the more detailed study on the particular traits of purine transport (Roush et al. 1959, Roush 1961). Cells were successively fed with seven types of purines with simultaneous measurement of their consumption from the medium by absorption spectroscopy; while the deposition of large amounts of purines inside the cells was demonstrated by analysis of their extracts subjected to

enzymatic assays using guanase, uricase, xanthine oxidase and adenase (Roush et al. 1959). Purine crystals formation within the vacuole was then shortly reported in Nature journal (Roush 1961) by Roush in 1961 and further documented using ultraviolet microscopy two years later (Svihla et al. 1963). As in case of other unicellular eukaryotes, the follow-up studies are missing, and the purine crystalline inclusions escapes researchers' attention since then.

Dinoflagellates. Independently to previously mentioned cases, many attempts to characterize crystalline inclusions in microalgal dinoflagellates (Alveolata, SAR) have occurred with more divergent conclusions as an example of how challenging this task was. All that started with an assignment to find the source of bioluminescence in *Gonyaulax polyedra* and to isolate and characterize the so-called "scintillons" or flashing particles (DeSa et al. 1963, Sweeney and Bouck 1966). However, the analysis of cell fractions by absorption spectroscopy resulted in identification of guanine crystals often forming chevron-shaped twinned crystals (DeSa and Hastings 1968). However, those appeared even more numerous in non-luminiscent strains (*Gymnodinium nelsoni* and *Prorocentrum micans*) (Sweeney and Bouck 1966), or completely missing in some bioluminescent species (*Noctiluca miliaris*) (Fogel et al. 1972). Using the analogy from purine crystals functioning as nitrogen storage in *Candida* led to the adoption of the same hypothesis in case of dinoflagellates. Simultaneously, the theory that inclusions function as photonic mirrors was raised and doubted without its further testing (Schmitter 1971). Another work, (Taylor 1968) misinterpreted guanine crystals in *Symbiodinium microadriaticum* as calcium oxalate based on silver staining omitting chemical analysis at that point. This conclusion was disseminated by other citing authors (Kevin et al. 1969, Fankboner 1971, Leutenegger 1977). Another work misidentified the guanine crystals as endosymbiotic bacteria moving fast inside the vacuoles (Silva 1967, Gold and Pollingher 1971). All this points to the fact that addressing the nature of the crystalline inclusions was not trivial and many scientists struggled with such a task. Further works brought more discrepancies in purine crystals analysis, claiming them to be uric acid, which has been retained in the following references until now (Clode et al. 2009, Yamashita et al. 2009, Kopp et al. 2013, Rosset et al. 2017, Krueger et al. 2018). In this case, the primary work has been done on dinoflagellate zooxanthellae isolated directly from marine anemone *Aiptasia* sp. that was analyzed by gas chromatography mass spectrometry (GCMS) finding uric acid as the main constituent of crystalline inclusion of the symbiotic dinoflagellates (Clode et al. 2009). This interpretation was adopted in the study of eyespots in *Symbiodinium* sp. possessing a crystalline reflective layer that was claimed to consist of uric acid as well (Yamashita et al. 2009). Kopp et al. (2013) used GCMS for purine inclusions analysis and the isotopically sensitive elementary analysis NanoSIMS for pulse-chase experiments showing the dynamics of nitrogen uptake and usage directly in the zooxanthellae inside the coral hosts. They confirmed the presence of uric acid in their samples (Kopp et al. 2013). The study of zooxanthellae ultrastructures via TEM under different nitrogen and phosphorous availability (Rosset et al. 2017) showed an increase in the crystals stored in high nitrogen and low phosphorous conditions, while the chemical composition of the crystals was cited from the previous works to be uric acid (Clode et al. 2009, Kopp et al. 2013). Another NanoSIMS analysis of both ^{13}C and ^{15}N partitioning in the coral host tissues and symbiont cells upon autotrophic and heterotrophic feeding (Krueger et al. 2018) referred to uric acid storage crystals retrieved elsewhere (Clode et al. 2009, Kopp et al. 2013).

Our results speak against the occurrence of uric acid in dinoflagellates as the long-term nitrogen storage purine form. Although we documented uric acid crystals in some diatoms or green algae, we never identified it in dinoflagellates in spite of the fact that we frequently worked with various species of zooxanthellae and free-living dinoflagellates. In our experiments we always found solely guanine crystals as the only constituent of the crystalline inclusions by a direct analysis using

Raman microspectroscopy in living cells *in situ*. The authors claiming the presence of uric acid did not use a direct analysis but extracted the compounds from the complex tissue of an anemone *Aiptasia* sp. (Clode et al. 2009) or a reef-building coral *Pocillopora damicornis* (Kopp et al. 2013). We suggest that previous observations were caused by contamination, because crystals of uric acid are present in the anemone's tissue – as anemones possess excessive amounts of uric acid themselves (Fox and Pantin 1941, Shick 1991). Moreover, uric acid is more soluble than guanine (Yalkowsky et al. 2010) and therefore it may have overridden guanine in the analysis due to its favorable chemical properties. Another argument may be that GCMS is not the preferable analytical tool for analyzing purines that are used as standards in liquid chromatography (Traut 1994), but Raman microspectroscopy has recently enabled unambiguous identification of purine inclusions *in vivo* and *in situ*, which helped to shed more light on this intriguing issue in dinoflagellates and other protists (Jantschke et al. 2019, Mojzeš et al. 2020, Pilátová et al. 2022).

Ciliates. Before the identification of the chemical composition was accomplished, it was hypothesized that the crystalline inclusions in ciliates served as "Exkretkörner" for excretion of unwanted waste (Schewiakoff 1893). However, this was later disproved by functional studies. For example, *Paramecium* species possess mainly small granular crystals when they are fed with bacteria, or they form numerous large crystals after the addition of a surplus protein sources. These structures disappear over the period of starvation (Hausmann 1982). Based on such observations, an alternative hypothesis that they serve storage function was adopted. The chemical identification of crystals in a model ciliate *Paramecium tetraurelia* (Alveolata, SAR) was done by a thorough combination of analytical approaches using X-ray powder diffraction and SEM EDX on isolated crystals. The first results suggested that they are composed of calcium struvite $(Mg,Ca)NH_4PO_4 \cdot 6H_2O$ (Grover et al. 1997). Five years later, Creutz et al. (2002) reanalyzed the same species and mutants using similar methods adding HPLC MS and they got completely different results. The morphology, X-ray diffractograms, as well as mass spectrometry data, confirmed the presence of a purine mixture consisting of guanine, hypoxanthine, and xanthine (Creutz et al. 2002). Since the isolation procedure of the crystals is not described in the publication of (Grover et al. 1997), we may only speculate about the primary cause of the first erroneous chemical identification of biocrystals in *Paramecium* such as contamination of samples by inorganic salts from the medium.

Foraminifera. The documented ultrastructures of susceptible microcrystalline inclusions in Foraminifera (Rhizaria, SAR) dates back to the golden ages of electron microscopy in the 1960s (Hedley and Bertaud 1962). These inclusions were named "stercomata" as a specific cellular compartment. They possess a typically "marbled" or "cracked" texture that is characteristic for ultra-thin sections through crystals in geology as well as in biocrystals. Although "stercomata" remain a not-yet-identified structures, we suspect them to be very likely crystalline inclusions. In Foraminifera there are always more possibilities because they possess ornamented biomineralized shells made of calcite (Weiner and Addadi 2011), thus, "stercomata" might be the vesicles delivering the calcified material to the cell surface. Another option is that "stercomata" represent purine crystals that happen to have similar patterns on TEM sections unless they drop out leaving a hole apparent in the micrograph instead. In our revision of eukaryotic diversity using Raman microanalysis, we found calcite particles inside the fixed cells of an old specimen preserved in formaldehyde (Pilátová et al. 2022). Still, we cannot exclude the occasional presence of purine crystals, as many eukaryotes can simultaneously produce more types of different crystalline inclusions. These speculations are further supported by published observation about the relation of crystals appearance after feeding with copepods or infusoria, whereas they completely disappeared upon feeding only with diatoms

(Hausmann 1982), similar situation from observation on ciliates as mentioned above was later shown to be correlated with the presence of guanine crystals (Creutz et al. 2002, Pilátová et al. 2022).

Studies of purine uptake

Alternatively and complementary to the research of crystal chemical composition, cellular metabolism and nutrients uptake of the crystal-forming substrates is very helpful for the better understanding of the biocrystallization process. Originally, metabolic studies motivated the first ever reported identification of purine intracellular crystallization (Roush et al. 1959). By a lucky coincidence, in *Candida utilis* the authors found that the purine uptake was unexpectedly enormous, exceeding the solubility levels of purines, eventually concluding about their crystallization in vacuoles (Roush 1961). In other unicellular eukaryotes, several studies on guanine uptake were performed using ^{14}C-radiolabeled substrate. Guanine transport in autotrophically grown synchronous *Chlorella fusca* was shown to be constitutive, active, and with high affinity (Pettersen and Knutsen 1974). Once accumulated, the intracellular guanine was proven not to leak out nor be exchanged with the exogenous one. After passing through a "metabolic compartment", the solid-state guanine was proposed to be stored in a "nonmetabolic compartment", probably the vacuoles, and metabolized only at growth conditions with high nitrogen demand (Pettersen 1975). On the other hand, using the same autoradiographic method, (Shah and Syrett 1982) demonstrated that several nitrate-grown diatoms (*e.g., Phaeodactylum tricornutum*) and chlorophytes (*e.g., Chlamydomonas reinhardii*) lack the ability to take up guanine but can do so after a period of N-deprivation. Further electrophoretic and chromatographic analyses concluded that guanine accumulated by *P. tricornutum* was most probably stored as methylated hypoxanthine (Shah and Syrett 1984). Unfortunately, such studies have not been further developed and the scientific interest in this field is about to rise again after forty years.

Revisiting eukaryotic biocrystallization with the focus on protists

Raman microscopy in hands of successful pioneers of this method for microalgal microanalysis approached the refurbishing the old studies using a new powerful method. By a coincidence, they found guanine crystals in a model green alga *Desmodesmus quadricauda* and in yellow-green alga *Trachydiscus minutus* (Moudříková et al. 2017a). The hypothesis of nitrogen storage in the form of crystalline purines has been further developed in the following study devoted mostly to dinoflagellates – both free living and endosymbionts of reef-building corals. With one exception, guanine crystals were reported in all tested species of dinoflagellates as well as other microscopic algae from marine, freshwater and terrestrial habitats of diversified taxonomic origin (Mojzeš et al. 2020). The exceptional finding of uric acid inclusions in a streptophytic alga *Klebsormidium flaccidum* pointed at the chemical diversity among purine inclusions. To disentangle always questioned chemical nature of various cell inclusions in unicellular protists, we conducted comprehensive screening of over 200 species of single-celled eukaryotes representing ca. 70 % of eukaryotic diversity, finding that 80 % of the crystalline inclusions consist of purines (Pilátová et al. 2022). Purine crystals are present in all kinds of biotechnologically exploited organisms, medically important parasites, and environmental samples from a broad variety of habitats. The diversity of tested representatives is documented via polarization microscopy that enables to clearly see the birefringent crystalline inclusions we found in the cells under polarization microscopy. Those crystals are moving by Brownian motion inside their membrane-bounded compartment as depicted on a

video: https://y2u.be/UtygkzDmz8U. Apart from anhydrous guanine (Hirsch et al. 2015, Pinsk et al. 2022), several previously uncharacterized organic biocrystals were found, including guanine monohydrate, xanthine, and uric acid or their mixtures (Pilátová et al. 2022, Pinsk et al. 2022). Eventually, we outlined an evolutionary scenario for purine crystal formation in unicellular eukaryotes based on highly conserved purine transporters, which provided the first phylogenetic study and suggested that purine crystal formation was likely a feature of the last eukaryotic common ancestor (LECA). This represents a paradigm shift in our understanding of eukaryotic biocrystallization.

Why are purines used to form biocrystals in cells?

Diverse roles of purines in the cells

Purines are known to be one of the preliminary organic compounds that could be synthesized from inorganic precursors, being the oldest biomolecules and important building blocks of the emerging life forms on Earth (Enchev and Slavova 2021). Purines are part of nucleic acids, *i.e.*, DNA, RNA, and nucleosides and nucleotides that serve as a component of nucleic acids and the source of chemical energy in cells (GTP, GDP, ATP, ADP) and signaling molecules (cAMP, cGMP) (Denninger and Marletta 1999). Their metabolism is targeted in cancer therapies and virostatics (Gazivoda et al. 2005). In humans, hyperuricemia (excess of uric acid in the bloodstream) causes severe pathologies, such as nephropathy and gouty arthritis (Moe 2010).

Purine crystals in nitrogen metabolism

In the form of crystals though, we are still just at the beginning of our understanding of the multitude of potential functions that are predetermined by their special physicochemical features. The excessive amounts of various nitrogen sources (nitrates, ammonia etc.) is usually toxic to multicellular organisms – both plants and animals (Britto and Kronzucker 2002, Camargo and Alonso 2006). In invertebrate and vertebrate animals, purines serve as the degradation product of nitrogen metabolism in excretion (Wright 1995). This was the first alleged function of crystals in ciliates, still, these “Exkretkörner” remained of unknown composition (Schewiakoff 1893). The feeding experiments then helped to reject the hypothesis of crystals functioning in excretion and adopted an alternative hypothesis that they rather represent a storage compartment (Hausmann 1982). Crystals always disappeared upon starvation and then usually reappeared after feeding on bacteria or protein-rich media with an exception on feeding by diatoms that did not provoke crystals formation (Hausmann 1982). The later explanation of purine crystals, with purines being the richest biomolecules in nitrogen, thus, serving as nitrogen storage prevailed (Creutz et al. 2002, Clode et al. 2009, Kopp et al. 2013, Moudříková et al. 2017a, Rosset et al. 2017, Jantschke et al. 2019, Mojzeš et al. 2020).

Physicochemical features of purines

Nitrogen proportion per molecule. Purines are biomolecules that are the richest in nitrogen content per molecular weight (**Table 1**, **Figure 2**) with 52 % and 46 % of nitrogen per molecule in adenine and guanine, respectively. It is half as much as in arginine, an amino acid with the highest proportion of

nitrogen per molecule having 32 % nitrogen. Guanine scores double to triple more nitrogen than its derivatives comprising GMP, GDP and GTP, similarly to average protein or nucleic acid containing 17 and 15 % of nitrogen per molecule, respectively (Sterner and Elser 2002).

Table 1: The overview of nitrogen proportions in the common nitrogen-containing biomolecules with the stress on purines; the data on average protein and nucleic acid are reported elsewhere (Sterner and Elser 2002). Number of nitrogen atoms per molecule is expressed as **#N.**

Compound	#N	Mw (g/mol)	N (%)	Compound	#N	Mw (g/mol)	N (%)
Adenine	**5**	135.13	51.8	AMP/ADP/ATP	5	–	20/16/14
Guanine	5	151.13	46.3	GMP/GDP/GTP	5	–	19/16/13
Hypoxanthine	4	136.11	41.2	Average protein	–	–	17
Xanthine	4	152.11	36.8	Average nucleic acid	–	–	15
Uric acid	4	168.11	33.3	Aspartate	1	133.11	10.5
Arginine	4	174.20	32.2	Tyrosine	1	181.19	7.7
Cyanophycin (unit)	5	307.31	22.8	Chlorophyll *a*	4	893.51	6.3
				Phosphatidylcholine	1	314.25	4.5

Solubility. Compared to nucleotides and nucleosides, the solubility of purines is very low (**Table 2**). In principle, guanine is one of the least soluble organic compounds with the solubility comparable to the inorganic minerals, such as barite, the mineral well-known for its extreme low solubility (Nordstrom et al. 1990, Yalkowsky et al. 2010). Finally, the saturation level of guanine in neutral pH was measured to be 25 μM (Darvishzad et al. 2018). Assessing the solubility of guanine became challenging as it depends on pH, temperature, and addition of other salts, but mostly because of its spontaneous nano-crystallization in the saturating conditions (Darvishzad et al. 2018). This phenomenon may bring us another clue to better understand, why guanine is the most favorable purine in the formation of biocrystals. Both, the natural formation of anhydrous crystals and high nitrogen contents, altogether, makes purines a high-capacity nitrogen storage (Mojzeš et al. 2020).

Table 2: The summary of water solubility data of purines, nucleosides, and nucleotides (Yalkowsky et al. 2010).

	Purine	Solubility (mol/l)	Solubility (g/l)	t (°C)
1	Guanine	3.97E-05	5.90E-03	25
2	Uric acid	2.10E-04	3.50E-02	25
3	Xanthine	2.50E-04	3.70E-02	21
4	Uric acid (dihydrate)	3.40E-04	6.90E-02	25
5	Guanosine	4.30E-03	1.20E+00	25
6	Hypoxanthine	5.30E-03	7.20E-01	25
7	Adenine	6.60E-03	8.90E-01	25
8	cAMP	2.40E-02	7.80E+00	20
–	Caffeine	4.90E+00	9.60E+02	25

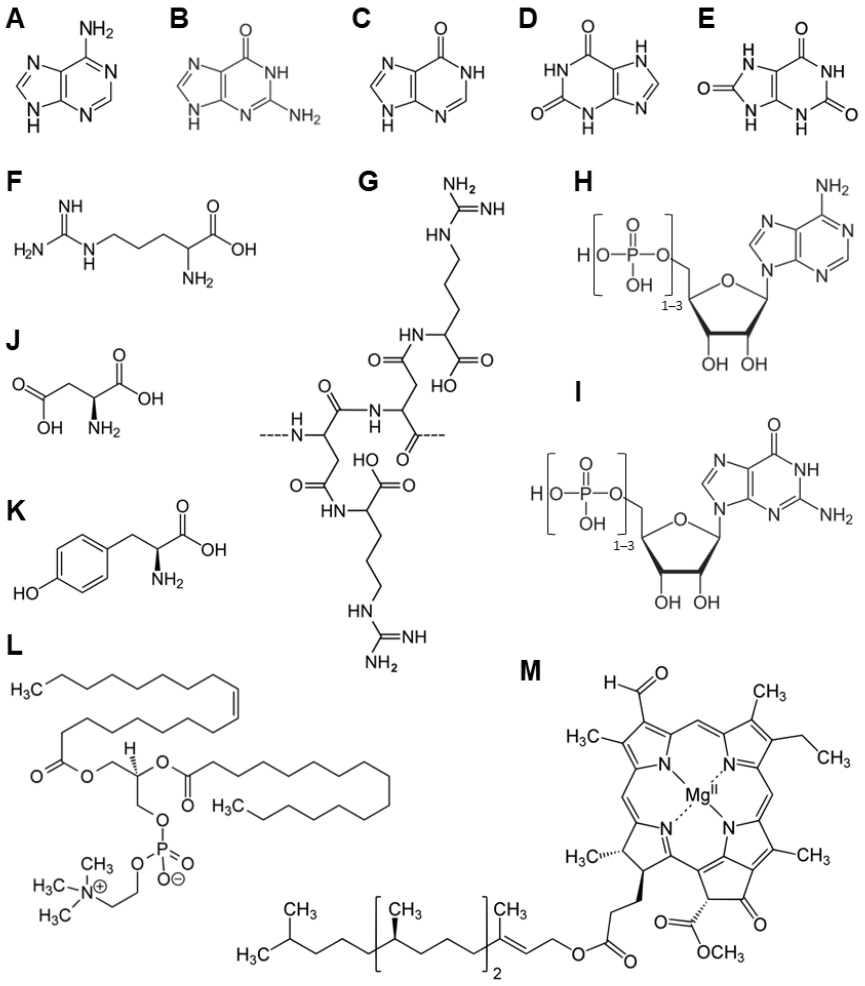

Figure 2: Main representatives of the most prominent nitrogen-containing compounds. *Purines:* A – adenine, B – guanine, C – hypoxanthine, D – xanthine, E – uric acid; *nucleotides:* H – adenosine (mono-, di-, tri-)phosphate, I – guanosine (mono-, di-, tri-)phosphate; *amino acids:* F – arginine, G – cyanophycin; J – serine, K – tyrosine; *phospholipids:* L – phosphatidylcholine; *pigments:* M – chlorophyll *a.*

Physiological quantities of purines and their derivatives. The metabolism and transportation of purines, nucleosides, and nucleotides are tightly regulated in all living cells. In some cells, free nucleobases occur in non-detectable traces (Pastor-Belda et al. 2022) compared to nucleosides and nucleotides with higher concentrations. Another complication for detection is low solubility of free nucleobases (Table 2) which may hamper their detection. The greatest survey on nucleobases, nucleosides and nucleotides contents in different cell types and species exhibit some general trends (Traut 1994). ATP occurrence in millimolar ranges is 10 times more concentrated compared to ADP and 100 times to AMP; this means that ATP:ADP:AMP contents are far from equilibrium (Hardie 2018). Furthermore, ATP concentration highly exceeds the energy consumption of cells, and thus, may act as a potent solubilization agent of proteins (Patel et al. 2017).

The concentrations of GTP are also usually higher than GDP and GMP, which are comparable to concentrations of other nucleotides in model yeast cells (Osorio et al. 2003). Nevertheless, in some microbes, the levels of GDP and GTP may rise towards the senescent growth phase as well as

the whole adenylate pool (Graven et al. 2014). This illustrates how variable the levels of nucleotides and nucleosides can be over cellular growth. Still, because of the statistically constant contents of ATP in cells, it appeared relevant to assess the ATP levels as a biomass proxy – advantageous for demanding environmental sampling including marine sediments or activated sludge (Karl 1980, Bochdansky et al. 2021). The knowledge of the proportion of purine crystals in the overall nucleobases, nucleosides, and nucleotides pool is currently missing, as well as a reliable method for their detection.

Presumed processes of purine crystals formation and degradation

In order to uncover the process of purine crystals formation and degradation, here we will limit ourselves on guanine crystals in particular (Figure 3). Localization of purine crystals inside membrane-bounded compartments implies the need for transporters to provide purine biocrystallization with its substrate inside the vesicle. Additionally, certain metabolic enzymes (*e.g.*, of the salvage pathway) should be employed in the purine crystal formation and/or degradation by producing the crystal-forming substrate either in the cytoplasm or inside the crystal-bearing vesicle, as well as by recycling the purines into other metabolites. The source of energy to power the transport of purines, nucleosides or nucleotides into the vesicle is great puzzle, as it must be a secondary active transport enabling to push the crystal-forming substrate against the chemical concentration that should be supposedly higher in the crystal-bearing vesicle. In theory, the process of guanine crystals degradation is easier to explain by involving proton pumps, *e.g.*, H^+-ATPase and PPase. Their function in decreasing pH influences the solubility of guanine (Darvishzad et al. 2018). Protons may also act in the guanine symport into the cytoplasm, as most of the transporters have bidirectional mode of operation, in this case the motion would likely follow the concentration gradient (Figure 3).

The research of purine, nucleoside and nucleotide transporters is important in the cell metabolism and cancer research as many of the cytostatic drugs are nucleobase derivatives (Gazivoda et al. 2005). The scrutiny of various possible scenarios of employment of either purine, nucleoside or nucleotide transporters in the crystal-bearing vesicle yielded in suggesting the main candidates among transporters and metabolic enzymes to be experimentally tested in the future. Although some of the transporters were reported to be omnipresent in all the major eukaryotic supergroups (Kourkoulou et al. 2018), the later analysis of published genomes and transcriptomes showed it is not the case (Pilátová et al. 2022). In fact, purine transporters of a wide specificity for various purines and pyrimidines represent a complex of multiple paralogues that evolved independently, i.e., nucleobase-cation symporter 1 (NCS1), NCS2 (or nucleobase-ascorbate transporter – NAT), AzgA. In case of NCS1, the paneukaryotic phylogenetic screen revealed eight susceptible paralogues, *i.e.*, Fcy, Fur, algal, plant, and four newly found NCS1 A, B, C and D. Plus, a couple of independent episodes of horizontal gene transfer was detected: from bacteria in case of diatoms, dinoflagellates, chrompodellids, and fungi; or from fungi in oomycetes. Furthermore, susceptible sequence contamination occurred in sequencing data from Cercozoa in the case of "new NCS1 C".

NCS2 consists of at least four paralogues with a potential contaminant sequence in the case of NAT A in rhodophytes and dinoflagellates, moreover, with an occurrence of horizontal gene transfer from bacteria to metamonads and fungi, and from fungi to eumycetozoans (Pilátová et al. 2022). The evolution of AzgA witnessed at least one duplication yielding in two main paralogues of this hypoxanthine-adenine-guanine-transporter. The overlap of detected purine biocrystallization with the occurrence of NCS1, NCS2 and AzgA transporters appeared incomplete with missing NCS1 in

16, NCS2 in 13 and AzgA in 12 out of total analyzed number of 28 purine-crystal-forming clades. Conversely, these transporters are present in some of the clades that were not proved to produce purine biocrystals yet, such as Rhodophyta, Phaeophyta or Phytomyxea, that were not found to produce purine biocrystals. All in all, the new phylogenetic analysis of nucleobase transporters did not propose any concrete candidate in the metabolism of purine crystalline inclusions.

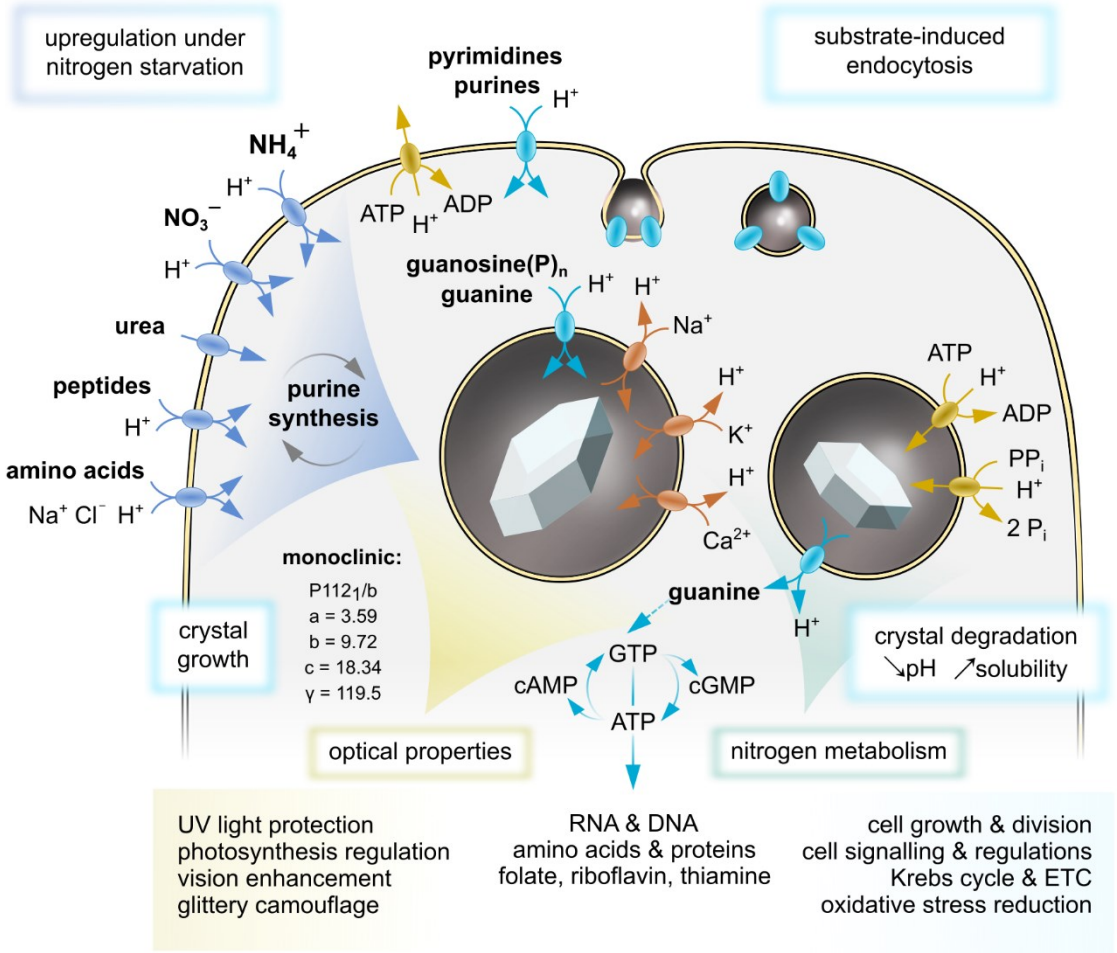

Figure 3: Hypothetical scenario of biogenic guanine crystal formation and degradation inside membrane-bounded compartments of eukaryotic cells. Initial source of nitrogen is transported into the cell via transporters of ammonia, nitrate, nitrite, urea, peptides, and amino acids (in blue). Nitrogen starvation causes the upregulation of the transporters of all nitrogen-rich compounds, after exposure to its substrate, some of them are internalized into a vesicle through the negative feedback regulation, lowering their number on the plasma membrane, as is documented on the example of purine transporters (Kourkoulou et al. 2018). Transporters of purine precursors (in cyan) could target guanine, guanosine, or guanosine phosphates inside the vesicle, acting as uniporters or proton and sodium symporters powered by presumable secondary active transport of cation antiporters (in orange) or H^+-ATPase and PPase (in yellow) that may be employed in the process of crystal degradation provoked by lowering the pH and thus increasing guanine solubility as well as powering the export. Guanine is a great high-capacity nitrogen storage useful in the metabolism not just purines, but also photosynthesis, pigments production and others. The biochemical pathways recycling guanine were based on KEGG. Guanine crystals can act as photonic mirrors due to their immense optical properties, *e.g.*, refractive index 1,84. The crystallization is likely regulated by the nucleation center and potentially other agents. The biogenic anhydrous β-guanine crystal lattice parameters and predicted functions are based on Hirsch et al. (2015), Jantschke et al. (2019) and complemented with our hypotheses.

Further comparative genomics of concentrative and equilibrative nucleoside transporters (CNTs and ENTs) or solute carrier 28 and 29 (SLC28 and SLC29), respectively, showed the common appearance of ENT in all 57 tested eukaryotic genomes representing the broad diversity of eukaryotes. ENT was suggested as a promising candidate for future experimental testing and thorough phylogenetic analysis. Despite its name, ENT has a broad substrate specificity encompassing nucleosides and nucleobases and rather diverse mode of operation of either proton- and sodium-dependent symporters or cation-independent uniporters (Boswell-Casteel and Hays 2017). The metabolic enzyme hypoxanthine-guanine phosphoribosyl-transferases (HGPT) is another hot candidate to be tested in purine crystal formation and metabolism. HGPT is a crucial point in the salvage pathway, and thus, it is present in all eukaryotes. Nevertheless, its sequence is not long enough to provide reliable phylogenetic information about its evolution (Pilátová et al. 2022).

In order to complete the complex picture of guanine biocrystallization, one needs to take into account another assumption based on the knowledge of different biocrystallization systems. The biocrystallization occurs in the supersaturated solution that accumulates in the membrane-bounded vesicle and requires the presence of a nucleation center that triggers and regulates the process of biocrystallization. The biogenic guanine crystals have a well-defined shapes and structures that are completely different from guanine precipitates *in vitro* that suggest such a regulatory machinery (Hirsch et al. 2015). Although these mechanisms are vastly unknown, the new findings point to the potential analogy to melanosome formation via fibrillar macromolecular templates (Wagner et al. 2023).

The further purine crystals metabolism is necessarily interconnected with other metabolic pathways in the cells. As was experimentally proven, guanine crystals in cells of a marine dinoflagellate *Amphidinium carterae* can supply the nitrogen consumption in order to cover three consecutive cell divisions yielding in 8 daughter cells (Mojzeš et al. 2020). This means that guanine is metabolically transformed into other nitrogen-rich molecules, such as amino acids forming proteins, chlorophylls, vitamins, etc. Indeed, according to the database Kyoto Encyclopedia of Genes and Genomes (KEGG) the purine metabolism is directly adjacent to other metabolic pathways, such as anabolism of amino acids, *e.g.*, and thus protein synthesis, cell growth and cell division. It is also directly connected with vitamin productions, such as thiamine (vitamin B1), riboflavin (vitamin B2) important cofactors for enzymes of Krebs cycle, pentose cycle and ATP synthesis, or even folate (vitamin B9) with its role again in pyrimidine and purine synthesis.

Optical properties of purine biocrystals studied in animals so far

Recently, a resurgence of interest in purine biocrystallization has emerged at an interdisciplinary interface of material science, biology, and bio-optics. In animals, purines are not just metabolic end-products for the excretion of surplus (Wright 1995). They are responsible for a wide variety of optical phenomena in animal coloration and vision. Biogenic purine crystals have a highly reflective, but thermodynamically unstable face that is crystallographically defined as “(100)” with the highest refractive index of 1.83 (Hirsch et al. 2015). Conversely, when guanine is re-crystallized from an aqueous solution, this optically active face is not pronounced. Highly reflective guanine crystals are the building blocks of photonic mirrors in eyes, used in the image formation and photon capture (Wagner et al. 2021). Also, the silvery iridescence of fish scales and the tunable colors of planktonic crustaceans and chameleons are caused by photonic guanine assemblies. A striking feature of these systems is the extraordinary variety of crystal morphologies that organisms produce from the same

molecule, indicating exquisite control over the crystallization process (Hirsch et al. 2015). The tightly regulated mechanisms of purine biocrystallization is vastly unknown, although, the new findings point to the potential analogy to melanosome formation via fibrillar macromolecular templates (Wagner et al. 2023).

Research outreach

Based on an invitation to popularize our findings to a broader spectrum of readers earning an impact outside academia, we wrote a layman summary of our findings. We published our new conceptualization of purine biocrystallization delineating an overlap with other fields and potential future applications at Nature Community Microbiology, sections “Behind the paper” and “Under the microscope” (attached to the thesis). The article was shared by the group “Journey to the Microscosmos” that has 780 thousand followers on their youtube channel, the International Society of Evolutionary Protistology. Other popularizing articles have been published in Czech as well.

Future challenges

The extraordinary control that organisms exert over these crystallization processes raises key questions in the field: *How do organisms control the formation of molecular crystals? What biomolecules and genes are responsible for regulating the crystal nucleation and growth process?* To address these, single-celled model eukaryotes might be of great advantage due to the immense control over their cellular processes achievable by the established techniques of molecular biology.

Chapter 3 – Diplonemids' unexpected role in Ba^{2+} and Sr^{2+} cycling

This chapter summarizes our new insights into the potential role of diplonemids (Diplonemae, Excavata) in Ba^{2+} and Sr^{2+} accumulation and cycling in the ocean, and thus, fulfills aim 3. The published article is attached to the thesis (Pilátová et al. 2023). The publication process of this work required argumentation with the promotors of the "bacterial hypothesis" of particulate barite and celestite formation. This chapter reflects the series of argumentation in favor of either diplonemid or bacterial hypotheses as well as deciphering the missing piece of evidence. Neither of them can currently fully explain the observed phenomena. The argumentation exchange summarized in this chapter may act as a basis for a future opinion article.

Full citation

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Author's contribution

JP participated in conceiving the study, designed the experiments, performed the key experiments, processed and interpreted data, conceptualized the results, provided reagents and expertise, wrote substantial part of the manuscript, first and the corresponding author.

Summary

Barium (Ba) and strontium (Sr) are often used as proxies for the reconstruction of past marine productivity and global climate. The ability to accumulate Ba^{2+} and Sr^{2+} in the form of crystals is rare among eukaryotes. Here we report that unicellular heterotrophs called diplonemids (Euglenozoa), one of the most abundant groups of marine planktonic protists, accumulate conspicuous amounts of these trace elements in the form of intracellular barite ($BaSO_4$) and celestite ($SrSO_4$) crystals, in concentrations greater than in other known Ba/Sr-accumulating organisms. Moreover, these flagellates can uptake Sr^{2+} exclusively or together with Ba^{2+} and form $(Ba,Sr)SO_4$. One species, *Namystinia karyoxenos*, is naturally capable of intracellular accumulation of Ba^{2+} and Sr^{2+} 42,000 and 10,000 times relative to the surrounding medium. Altering the amounts of both elements in the medium resulted in corresponding changes in the quantity and composition of crystals. Planktonic copepods fed with diplonemids produce celestite-rich fecal pellets, which facilitate the deposition of these minerals on the seafloor. We propose that diplonemids, which emerged during the Neoproterozoic era, qualify as impactful players of Ba^{2+}/Sr^{2+} cycling in the ocean that possibly contributed to sedimentary rock formation over long geological periods.

Diplonemid protists (Euglenozoan) are marine heterotrophic biflagellates (Tashyreva et al. 2022). Only recently, based on global sequencing projects of Tara oceans expedition and improved metabarcoding techniques collecting samples from different depths all around the world, diplonemids appeared to be one of the most abundant and diversified planktonic organisms in the ocean (Vargas et al. 2015, Flegontova et al. 2016). Environmental sequencing data further uncover their lifestyle being adapted to heterotrophy and low oxygen conditions in deep waters below the photic zone, known as mesopelagic zone, but still could be found in the whole water column (Flegontova et al. 2020, 2023). Other ecological functions are predicted not excluding parasitism (Kostygov et al. 2021). Diplonemid cells are fragile and naked – lacking the cell wall. Due to the nature of their microscopic translucent pigment-free cells, they are almost invisible, and unfortunately, impossible to be isolated directly from the oceans. Around twenty currently described species are maintained in culture; however, more than tens of thousands species are predicted based on metabarcoding data. All available cell cultures come from surface waters or aquaria of mostly unknown origin concerning the depth of their prevailing habitat, and they were isolated only after lengthy and focused cultivation efforts (Tashyreva et al. 2022). To the best of our knowledge, we analyzed the biggest collection of currently available diplonemid cultures.

We found that diplonemids accumulate copious amounts of strontium (Sr) and barium (Ba) in the form of intracellular celestite ($SrSO_4$), barite ($BaSO_4$), and strontiobarite ($(Ba,Sr)SO_4$) crystals (Pilátová et al. 2023). Although Ba and Sr are trace elements in natural seawater, certain diplonemid species are capable of their enormous concentration. A hyperaccumulating diplonemid, *Namystinia karyoxenos*, contains up to 10,000× and 42,000× higher content of Sr and Ba, respectively, relative to the surrounding medium, with strontiobarite representing 90 % of its dry weight (Pilátová et al. 2023). To confirm our findings, we employed various techniques: Raman microspectroscopy, SEM/TEM EDX ED, 3D reconstruction of crystalline morphology via SBF SEM and consecutive quantification by ICP MS which revealed Sr and Ba enormous concentrations. To assess the dry mass, gravimetry could not be applied because of the marine salts present in the cultivation medium and the cell fragility of diplonemids disabling their washing from the salty medium. Instead, we used quantitative phase imaging by holographic microscopy providing us with the information about the dry mass on a single cell level. Describing elements and their ions, we use the following chemical notation: Ba^{2+} or Sr^{2} as the ionic forms of the elements Ba and Sr are to be distinguished due to the applied analytical methods providing us with information about either the elementary composition, *e.g.*, ICP MS or TEM EDX, or crystal contents, *e.g.*, TEM ED, Raman microscopy. On the way for experimental testing of the hypothesis of whether celestite biocrystals can get to fecal pellets of zooplankton, we visited Observatoire Océanologique de Villefranche sur Mer. It was a great honor to meet a protist expert John Dolan, a zooplankton expert Fabien Lombard and a former director of the institute, and a founder of the Tara Oceans expedition Gabriel Gorsky.

We proved that diplonemid crystals' structure and composition are correlated with particulate barite and celestite found in the world's oceans as depicted by the seminal work of Dehairs et al. (1980). We also found the predators of diplonemids are another important addition to the puzzle. Filter-feeding zooplankton, such as copepods, can harvest diplonemids similarly to microalgae and the undigested remnants of the cells together with the biocrystals are excreted as fecal pellets. These are big enough to sediment fast and to transfer biominerals to the sea floor sediments. Interestingly, the control conditions for measuring fecal pellets produced by zooplankton fed on microalgae showed microplastics among the organic and inorganic undigested residuals. Microplastics are becoming a great topic of many environmental studies worldwide in respect to the pollution of our environments, mostly the oceans, and how this affects our lives (Ripken et al. 2021).

In the context of paleoceanography and biogeology, our findings about diplonemids' Ba/Sr hyperaccumulation is a potential clue to the long-questioned correlation of marine productivity and Ba/Sr in the biogenic sediments. The predicted bio-accumulator of barite/celestite could be diplonemids, the long-overlooked although widespread protists.

Particulate barite and celestite of biogenic origin are omnipresent in the oceans (Dehairs et al. 1980) with their saturation levels culminating in the deep waters (Monnin and Cividini 2006). Particulate barite and celestite correlate with marine productivity used for climate reconstructions in paleoceanography as they reflect the temperature changes based on the amount of fixed carbon that was transferred from the atmosphere and deposited on the sea floor (Carter et al. 2020). The substantial and still highly cited studies (Dehairs et al. 1980, Bishop 1988) established the basis for the modern research of this interesting correlation questioning the causality behind co-occurrence of fixed organic carbon and particulate barite and celestite of apparently biogenic origin. This biological driver remained unknown for decades, hence, nobody could discover an organism responsible for intracellular biomineralization suiting well the incremental knowledge about environmental particulate barite/celestite structures. Indeed, the above-mentioned pioneering works documented the environmental particulate barite and celestite with micrographs of crystals attached to desiccated organic or even cellular matter. Our findings of the exceptional capacity of diplonemids for intracellular Sr/Ba accumulation and biomineralization are the first viable alternative to the currently discussed hypotheses of chemical precipitations and bacterial origin of particulate barite. To the best of our knowledge, diplonemids meet all the observations of particulate barite/celestite occurrence in the oceans due to the following series of evidence:

i. diplonemids are capable of enormous active accumulation of Sr^{2+} and Ba^{2+} even from minute concentrations in barite/celestite unsaturated waters.
ii. intracellular crystals of celestite and barite formed by diplonemids correspond in size and shape to the structural properties of environmental particulate barite and celestite crystals (Dehairs et al. 1980).
iii. the variable proportions of Ba^{2+} and Sr^{2+} contents have been documented in diplonemids as well as in environmental samples of particulate barite and celestite as previously reported (Dehairs et al. 1980).
iv. as heterotrophs, diplonemids are not restricted to the photic zone, but they are widespread in the oceans, mostly in the mesopelagic zone, in astonishing abundance and diversity (Flegontova et al. 2016, 2020). This corresponds with the distribution of sampled particulate barite/celestite (Dehairs et al. 1980).
v. filter-feeding zooplankton can feed on diplonemids, fecal pellets are filled with undigested celestite originally formed by diplonemids, thus, transporting them into geological sediments. Particulate barite and celestite has been found in association with such aggregates, mostly formed by fecal pellets (Dehairs et al. 1980, Bishop 1988).
vi. occurrence of barite/celestite among unrelated diplonemids may involve more yet-undescribed species and copy similar pattern known from the patchy distribution of barite in freshwater zygnematophyte microalgae (Brook et al. 1988, Hall et al. 2008, Niedermeier et al. 2018). This is the weakest point of our study, because little is known about the biology of diplonemids. Most of their diversity has not been described yet, and thus, we lack crucial knowledge about how common their ability to produce barite/celestite is.

Recent studies of the bacterial-triggered formation of extracellular particulate barite on the extracellular polysaccharide structures would be a universally valid explanation (Martinez-Ruiz et al.

2018). Our data do not challenge this hypothesis, but we are cautious about interpretations that are disputable due to the lack of sufficient experimental proofs. In bacteria, barium was shown to be associated with phosphorous based on the elementary analysis of the environmental samples from marine aggregates (Martinez-Ruiz et al. 2018). This is a proof bacteria can accumulate barium, probably incorporated in polyphosphate granules. Direct evidence of its potential transformation to barite is currently missing. Another study tested the effect of the increase of the local concentration of sulfates produced by sulfur-oxidizing bacteria to provoke the barite crystallization (Gonzalez-Muñoz et al. 2012). This experiment used with cultivation media high in Ba^{2+} concentrations of 2 to 20 mM $BaCl_2$ which is around 5 orders of magnitude higher compared to reported environmental concentrations. Up to now, we do not have relevant data about the actual concentrations of either Ba, Sr or sulfates in the microenvironment of marine aggregates. The co-localization of particulate barite or celestite with bacteria in fecal pellets or marine aggregates is not surprising and may not be causally related. (Martinez-Ruiz et al. 2019). Hypothetically, we can imagine that Ba/Sr-(poly)phosphates storing bacteria (Ruiz-Agudo et al. 2021) can be ingested by diplonemids as far as they can feed on bacteria (Prokopchuk et al. 2022), and hence, they can further transform it into barite. For an evaluation of any of the above-mentioned possibility, experimental testing is required in a follow-up study.

In oceans, the majority of the particulate barite and celestite dissolve back in free ionic forms. However, still, the overall Ba-Sr influx from aerial deposition and estuaries is balanced by the same amount of material that sediments (Dehairs et al. 1980, Carter et al. 2020). Biomineralization in diplonemids best corroborates the findings on environmental particulate barite and celestite. Also, the concentrations of Ba^{2+} and Sr^{2+} found in our conditions are significantly higher than those previously reported for any other microorganisms, such as cyanobacteria, microalgae, and yeasts (Fisher et al. 1991, Paytan and Griffith 2007, Cam et al. 2016, Pilátová et al. 2023). Other important producers of celestite exoskeletons are represented by Acantharea (Rhizaria) which dissolve upon cell death in the upper 400 m of the sea (de Deckker 2004). Acantharea are mixotrophs hosting endosymbiotic microalgae that thrive in the euphotic zone, possessing huge ornamental shells with numerous spines reaching up to 500 µm in diameter, thus, they are not found in fecal pellets as they are too big to be ingested by filter-feeding zooplankton. Consequently, they are not transported to the sediments and are not preserved over vast geological eras (de Deckker 2004).

In summary, our findings provide an alternative hypothesis for explaining the long-questioned correlation between Ba^{2+} and Sr^{2+} and organic carbon in sediments, which acts as an important proxy for the reconstruction of past marine productivity and global climate. Our primary observation of trace elements hyperaccumulation raises questions about the mechanisms of intracellular barite/celestite biomineralization as well as the reasons to perform such energetically unfavorable metabolic activities. As far as the barite/celestite crystals are about four times denser than marine water or other cellular components, they might be used in graviperception to navigate in the water column or to make the cell relatively heavier to accelerate passive sedimentation. So far, all attempts other than isolation and description of new species from the photic zone failed, so with diplonemids, we are still not at the stage to deal with single-cell genomics or biomineralization studies. Unfortunately, extracting unicellular fragile transparent diplonemids from the deep sea remains a challenging task.

Research outreach

The layman summary of our research article has been published by Czech television news stream and prirodovedci.cz. This was followed by an interview in the show "Laboratory" (Laboratoř) at Czech radio station (Český rozhlas Plus).

Chapter 4 – “Red droplets” in *Synura* species

The main focus of the original article (Škaloud et al., 2023, just accepted) is taxonomic description of the new species and subspecies stemming from the complex of *S. sphagnicola* (Chrysophyceae, Stramenopiles, SAR), establishing new distinct species *S. rubra* and seven subspecies *sphagnicola*, *agila, bethyla* (within *S. sphagnicola*), *rubra, ampla, bella,* and *caelica* (within *S. rubra*). The spectroscopic work comprised characterization of autofluorescence spectra of various cellular structures, such as red droplets, flagella, plastids and a new as-yet-unknown structure. Due to the narrowing of the article scope, further fluorescence and Raman analyses were not included in the publication. The as-yet-unpublished results are summarized and discussed in this chapter in order to fulfill aim 4.

Full citation

Škaloud, P., Škaloudová, M., Jadrná, I., Pilátová, J., Shin, Woonghi, Kopecký, J. Unravelling the hidden complexity in diversity and pigment composition of a colonial flagellate *Synura sphagnicola* (Chrysophyceae, Stramenopiles). *Fottea*, soon to be accepted. IF = 3.242

Author’s contribution

JP designed and performed the spectroscopic experiments, processed and interpreted spectroscopic data, conceptualized the results, provided reagents and expertise, and got involved in manuscript writing.

Summary

In this study, we aimed to taxonomically evaluate a unique example of cryptic diversity in a freshwater protist species *Synura sphagnicola*, occurring at two evolutionary levels. First, we characterized two evolutionary old lineages that evolved approx. 14 Mya ago as *S. sphagnicola* and *S. rubra*, sp. nov. These species are morphologically well differentiated by the morphology of silica scales. Second, we proposed seven evolutionary young, but ecologically and geographically well differentiated lineages as separate sub-species *sphagnicola*, *agila*, *bethyla* (within *S. sphagnicola*), *rubra*, *ampla*, *bella,* and *caelica* (within *S. rubra*). In addition, we examined the autofluorescence and pigment composition of two selected strains, identifying fucoxanthin as a predominant carotenoid. We further showed that the red droplets in the cytoplasm, a prominent feature of both species, are formed by a previously unknown pigment. Finally, we identified chlorophyll c2 in *S. sphagnicola*. Since the lack of this pigment represents a major distinguishing characteristic to discriminate between classes Synurophyceae and Chrysophyceae, we formally synonymize these taxa.

Cellular contents in *Synura sphagnicola* and *S. rubra* with a focus on "red droplet"

A newly described species of *S. rubra* is a distinct ribotype species in a complex of *S. sphagnicola*, both taxa are distinguishable from others due to the presence of "red droplets". Questioning the contents of "red droplets" initially motivated our study employing Raman microspectroscopy. In the old literature, "red droplet" of *S. sphagnicola* is described to contain "haematochrome" possibly consisting of carotenoids – providing it with characteristic red color (Korshikov 1929). The alternative hypothesis might be that the "red droplet" typical for *S. sphagnicola* is an analogous structure to the "reddish globule" described in Eustigmatophyceae (Škaloud, personal communication). This may make sense since Chrysophyceae and Eustigmatophyceae are sister clades among Stramenopiles (Archibald et al. 2017). The separate study focused on "reddish globule" follows in next chapter.

Our experimental data support neither hypothesis. Based on our up-to-now indirect series of evidence, we suggest that the "red droplet" contains most likely anthocyanin-like phenolics (**Figure 4**). First, we documented autofluorescence of the "red droplets" with its emission in the red region of the wavelength that changes upon photooxidation to orange color, which corroborates with published data on similar behavior of plant anthocyanins (Brouillard 1983). Second, we tested the pH dependency of "red droplet" color under bright field. Again, the change from red to blue upon pH increase support our hypothesis about the presence of anthocyanin-like compounds – find details below. Third, the identification of the "red droplet" composition using conventional Raman microscopy with excitation at 532 nm was not successful due to its intense autofluorescence that hindered our measurement.

On the other hand, we could address the chemical composition of the other cellular inclusions using Raman microscopy. Interestingly, the most prominent cytoplasmic structures appearing as translucent granules represent lipid droplets instead of previously suggested chrysolaminarin (Hibberd 1978). The function of these lipid droplets is probably to provide energy storage in the form of triacylglycerols (**Figure 5**). It is worth noting that fragile naked *Synura* cells are susceptible to irreversible changes fast propagating to the cell death just a few minutes after transferring them onto the microscope slide. Hence, sample handling is demanding and must be done fast with freshly prepared samples, and the interpretation of spectroscopic and chemical properties must be always critically assessed.

Fluorescence properties of *Synura* species

To address the spectral properties of autofluorescence structures in *Synura*, emission spectra were measured by a laser scanning confocal microscope with 442nm laser excitation (Škaloud et al., just accepted). In laser scanning microscope, the enigmatic blue autofluorescence occurs in the center and/or periphery of the cell (**Figure 4**). The source of this autofluorescence is currently hard to address. It is emitted by various substrates in different compartments. Based on the literature, blue autofluorescence is a distinct feature of compounds possessing conjugated systems of double bonds or aromatic rings in their structures under UV excitation, *i.e.,* pyridine nucleotides (*e.g.,* nicotine-amide-adenine-dinucleotide (phosphate)), flavins (*e.g.,* flavin adenine dinucleotide), pterins, phenolics (*e.g.,* flavones, coumarins, etc.), alkaloids, tyrosine oxidation products, amyloid, and non-amyloid protein aggregates, etc. (Huang et al. 2002, Roshchina 2012, Fricano et al. 2019, Donaldson 2020). Thus, the source of blue autofluorescence needs to be analyzed further.

The emission spectra of “red droplets” were spectrally distinguishable from the plastids. They were ranging from 580 to 710 nm (expressed as the full width at half-maximum) with a peak of 640 nm. Over the course of observation, the fluorescence intensity increased and shifted its peak maxima from 640 nm to an orange-yellow of 580 nm (**Figure 4**). Simultaneously, during the observation, “red droplets” moved across the cell and grew bigger by fusing and/or with lipid droplets. Their overall number decreased while increasing their volume. The most probable reason for the emission shift was likely photooxidative chemical changes. Based on this observation and the literature search, we suspected “red droplets” were small vacuoles filled with anthocyanin-like phenolics (Roshchina 2012). Anthocyanin fluorescence emission depends on the particular composition, excitation wavelength, and pH (Drabent et al. 1999, Agati et al. 2020). Similar spectral shifts of plant anthocyanins upon their chemical oxidation have been recently measured via fluorimetry (Bartosz et al. 2020). Our data are in correspondence to the published parameters: wide band peaking around 630 nm of low-intensity changes due to oxidation into a more intense peak around 580 nm (Bartosz et al. 2020). Herein, we first show such a spectral shift of fluorescence caused by photooxidation *in situ* and *in vivo* compared to chemically oxidized anthocyanin extracts.

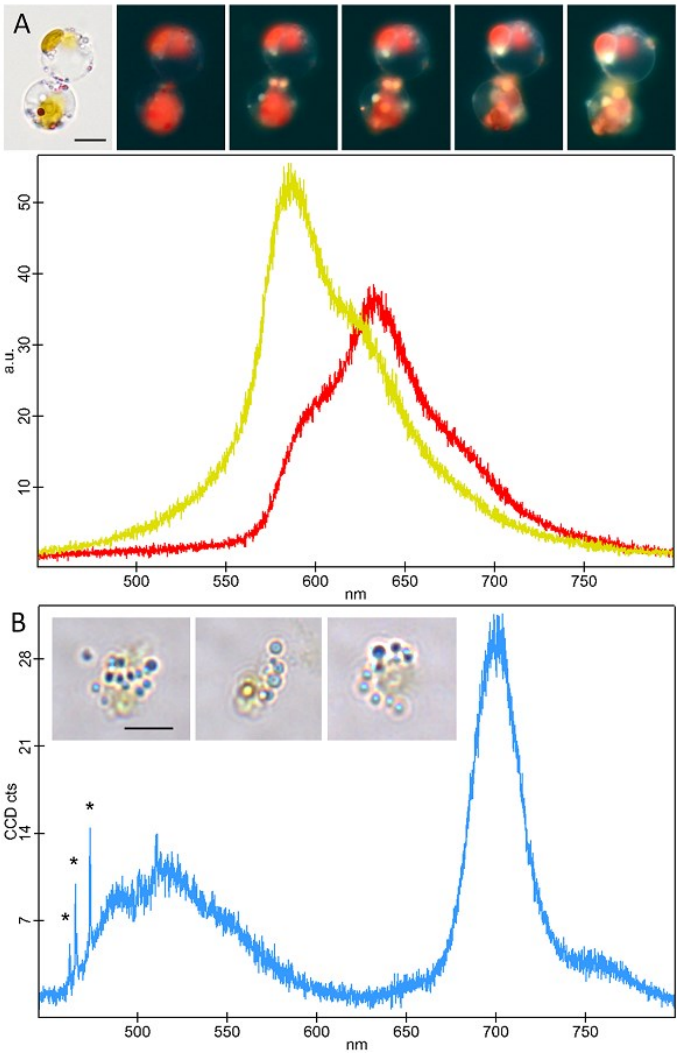

Figure 4: Color and fluorescence change during photooxidation and after application of strong basis.
A. Fluorescence microscopy of *S. rubra* supplemented by fluorometric measurement of “red droplets” under 442nm excitation in process of photooxidation change from red to yellow.
B. The color change of the “red droplet” turns blue in 1M NaOH in *S. sphagnicola.* Respective fluorometric measurements represent the whole cells – blue droplet and chlorophyll, with first sharp peaks (*) referring to resonance-enhanced Raman spectrum of carotenoids (1005, 1155, and 1525 cm^{-1}). Scale bars represent 10 µm.

"Red droplets" most likely contain anthocyanin-like compounds

To further test the hypothesis of anthocyanin contents of "red droplets", we exposed *Synura* cells to high pH upon the addition of 1M NaOH. This resulted in an immediate color change from red-violet to blue, which corroborates our assumptions (**Figure 4**). The visible color shift of the content of "red droplets" from fuchsia pink-red in low pH to blue in neutral/alkaline pH corresponds to the reported color shift of anthocyanin-like phenolic compounds (Brouillard 1983). Anthocyanins are rather chemically labile secondary metabolites protecting cells against oxidative stress caused by environmental factors, such as intense irradiation by UV and visible light, cold temperatures, and osmotic stress. If not needed they are enzymatically degraded (Chalker-Scott 1999, Zipor et al. 2015). This is congruent to our observations in the *Synura* species, in which case "red droplets" appear preferentially in the senescent cultures facing various stressors.

Anthocyanins are commonly found in vacuoles of higher plants (reference). They are usually not reported in Stramenopiles in which case they are either not produced or have not been detected due to their chemical instability. Lacking the genomic data of *Synura* species, we cannot search for potential biosynthetic traits of anthocyanin-like phenolics. However, the basic anabolism of phenolic compounds is commonly present in Stramenopiles together with other algae (Goiris et al. 2014). Interestingly, only a few genera of *Synura* species contain "red droplets". Here, we may speculate whether other species bear the colorless form of anthocyanins (Brouillard 1983).

Raman microspectroscopic analysis of cell compartments

The chemical composition of *Synura* species cellular compartments was analyzed by Raman microspectroscopy using a 532nm excitation laser as described elsewhere (Moudříková et al. 2016, Pilátová et al. 2022). In *S. sphagnicola* and *S. rubra*, rounded translucent granules are morphologically prominent cellular structures in the cytoplasm. These structures were previously reported to contain chrysolaminarin (Hibberd 1978). However, we could not find a matching Raman spectrum attributed to chrysolaminarin. The original interpretation of chrysolaminarin was based on light polarization which is not reliable, as lipid droplets or guanine crystals can also appear light polarizing (Pilátová et al. 2022). For a rigorous assessment of the chrysolaminarin production in *Synura* species, it would require a systematic microanalytical revision of the chemical composition of cell inclusions over different growth phases, preferentially as a comparative study of different species accompanied by their ultrastructures.

Additionally, Raman microscopy provided evidence specific spectral characteristics of other cell structures (**Figure 5**). Lipid droplets were confirmed to contain triacylglycerols possessing ester bonds (1740 cm^{-1}) and a high proportion of unsaturated fatty acids due to a 2-fold ratio between 1660 cm^{-1} and 1440 cm^{-1}. We obtained a complex fingerprint of plastids, which shows the major protein peak caused by aromatic amino acids, *i.e.,* phenylalanine and tyrosine (1002 cm^{-1}), and complex signal of thylakoids (around 1650 cm^{-1}). We detected polyphosphate (1160 cm^{-1}) in some of the cells. We also tried to characterize the "red droplet" composition using Raman microscopy. However, "red droplet" did not show any Raman fingerprint due to the high fluorescence background up to the saturation level of the detector.

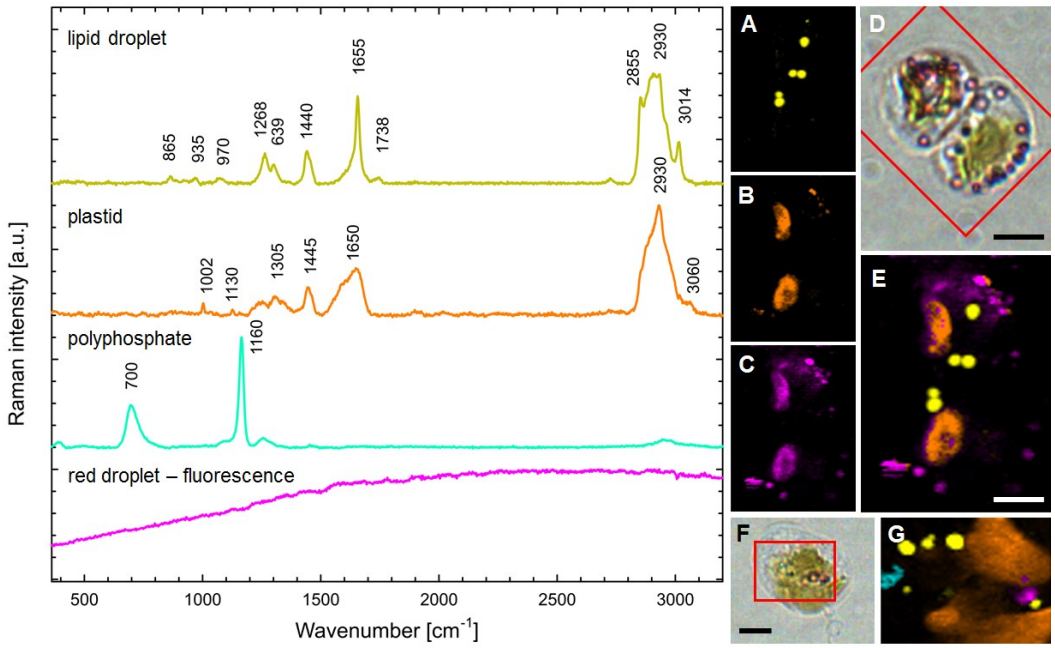

Figure 5. Raman microscopy analysis of *S. sphagnicola* (A–E) and *S. rubra* (F–G) using 532nm excitation laser (20 mW). A – fluorescence of lipid droplets, B – plastid, C – autofluorescence of red droplets and plastids, D – bright field with the red-lined region of interest, E – merged A–C, F – bright field with red-lined ROI, G – merged spectral map in the same color coding as Raman spectra depicted on the left. The fluorescence spectrum of red droplets (C) ranging from 1000 to 3000 cm^{-1} equals 560 to 630 nm, respectively. Scale bars represent 5 µm.

Employing Raman microscopy, we failed to measure the vibrational spectra of anthocyanins due to the intense fluorescence background of "red droplets". Unfortunately, the protocol of photobleaching prior to Raman measurements (Moudříková et al. 2016) in order to diminish autofluorescence while saving the main cellular structures did not work in this case, because it disrupted the fragile cells. As mentioned above, anthocyanin-like pigments or related phenolics are unstable compounds undergoing fast degradation (Bartosz et al. 2020). We did not find any anthocyanins during HPLC measurements either (methods stated in the manuscript Škaloud et al., just accepted). This can be explained by their degradation during the cell lysis step of pigment extraction. Similar problems were reported elsewhere (Brouillard 1983).

General discussion

Cellular inclusions, compartments, and organelles are long-researched structures in plant, animal, and human cells as well as in unicellular eukaryotes. The knowledge about their particular organization or building plans, functioning, and biogenesis is mostly developed in yeast, animal, or plant models (Alberts et al. 2015). So far, fewer research capacities have been invested in their investigation in the ever-growing field of unicellular eukaryotes (phycology and protistology). In this thesis, the application of Raman microspectroscopy has been promoted to address several types of cellular inclusions *sensu lato*, as defined in the introduction. Cell inclusions are found as a condensed material of either organic or inorganic matter, both of single-substance or a complex mixed composition, in the solid or liquid phase. The critical assessment of the advantages and limitations of Raman microspectroscopy are added, as it is a powerful microanalytical method enabling biologists to address the composition of various cell inclusion *in vivo* and *in situ* almost real-time. Here is a brief summary of the main findings delivered in this thesis together with the main questions that are stemming from the new piece of knowledge brought to the scientific discussion in variety of distinct topics.

The advantages and limitations of Raman microspectroscopy

In this work, confocal Raman microspectroscopy is promoted as a kind of chemical imaging based on molecular specificity of vibrational spectra. It may become the first-choice method for *in situ* studies of chemical composition of living cells (Moudříková et al. 2016, Mojzeš et al. 2020, Pilátová et al. 2022). The Raman microspectroscopy is a combination of confocal laser scanning microscopy with the resolution limited by diffraction limit depending on the used excitation wavelength and numeric aperture of the objective, roughly 200 nm in the *x*- and *y*-axis and 1 µm in the *z*-axis. The principle of Raman inelastic scattering has been discovered by Sir C. V. Raman (Raman and Krishnan 1928) and comprehensively explained many times after that (Shipp et al. 2017, Lee et al. 2021). As a label-free technique, it does not need any staining, cell fixation, or other treatments prior to the measurement, which makes it fast and versatile in its usage. The intensity of Raman spectra is directly proportional to the concentration of the measured chemical entity in the samples which makes this technique a (semi-)quantitative tool. In contrast, other microanalytical techniques are mostly limited to elementary analysis, such as SEM/TEM EDX, EELS, EFTEM, or NanoSIMS, in which case we miss the information on the chemical composition on the molecular level (Kopp et al. 2013, Shebanova et al. 2017, Ismagulova et al. 2018). Moreover, Raman microspectroscopy brings great advantages compared to time-consuming and laborious macroanalytical approaches using significant quantities of bulk biomass of (mili)grams of dry weight, extensive cell homogenization, and extraction protocols specific for different biologically relevant compounds (Moudříková 2018). The advantage of Raman microspectroscopy over other microanalytical techniques is the versatility of its use for the analysis of variable chemical compounds at once in a diversity of samples of various cell types or tissues. Raman microspectroscopy is therefore an irreplaceable tool enabling to combine optical microscopy and spectroscopy.

Raman microspectroscopy has been originally developed and used for other purposes while its usage in biomedical research is still at its beginning. For instance, Raman (micro)spectroscopy is well established in material science, geology, in antiquity restoring, in industrial quality control as well as in military and security forces for detection of explosives, flammable chemicals or drugs (Mogilevsky et al. 2012, Košařová et al. 2016, Schulze et al. 2018, Culka and Jehlička 2019, Dispas et al. 2022). The numerous applications of Raman microspectroscopy in various fields are illustrated as a word cloud reflecting the distribution of field-specific keywords occurring in the publication database Pubmed (Figure 6). Raman spectroscopy enables the investigation of the geology and remnants of life on Mars by the latest rover Perseverance equipped with SHERLOC or "Scanning habitable environments with Raman and luminescence for organics and chemicals" (Bhartia et al. 2021). The Raman microspectroscopy has also contributed to the development of lithium-ion batteries, for which gentlemen Goodenough, Whittingham, and Yoshito were awarded the Nobel Prize in Chemistry in 2019 (Burrows et al. 2019). The endless applications are just started to be employed in biomedical research in diagnostics of bacterial infections or cancer tumor histopathology (Shipp et al. 2017). The great potential for direct chemical analysis of living cells and biological tissues together with their morphology by Raman microspectroscopy has witnessed a series of cumulative advancements including the development of data-analytical tools (Storozhuk et al. 2022).

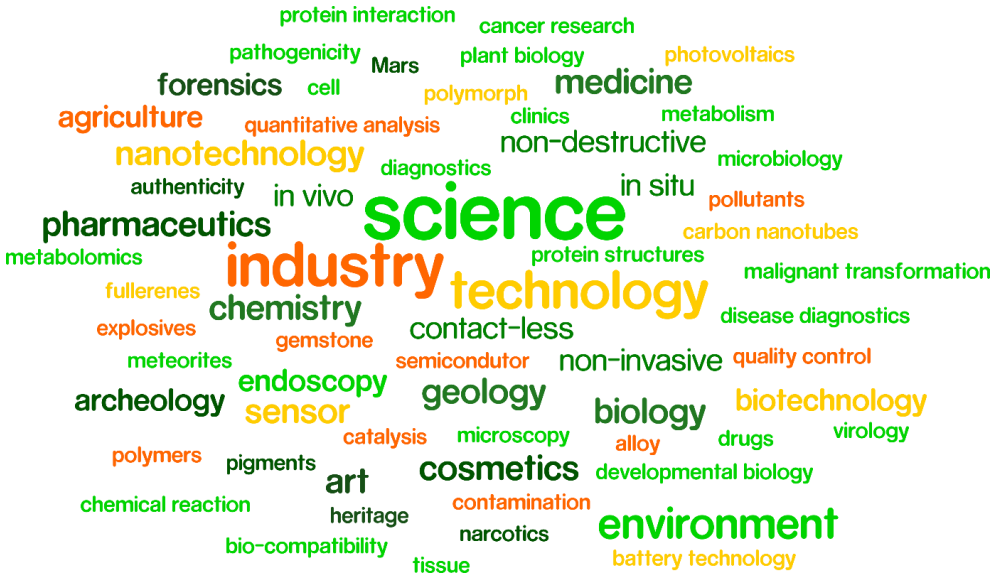

Figure 6: Word cloud of applications using Raman (micro)spectroscopy. The distribution of various applications and key words associated with Raman (micro)spectroscopy based on the publication numbers reported by the Pubmed database.

Although the first usage for analysis of microalgae has been reported about forty years ago (Largeau et al. 1980), the development of other microanalytical techniques, such as fluorescence probes and all the fluorescence microscopic applications improved faster and spread wider compared to Raman microspectroscopy. The general application of Raman microspectroscopy remains challenging because of various causes: i. autofluorescence features of the biological samples, ii. light induced photochemical or photophysical deterioration of instable compounds, iii. time-consuming sample scanning, iv. limited sensitivity of spontaneous Raman scattering, v.

demanding data-processing of multidimensional spectral images, vi. lack of suitable spectral databases for spectral identification.

The greatest obstacle in Raman measurements is the (auto)fluorescence that is by orders of magnitude more intense compared to Raman scattering and thus obscuring its weaker signal. In some cases, together with the light absorbance of the sample, it leads to local overheating around the laser beam or even burning of the sample. One of the possibilities to be applied is using the laser of longer wavelengths (typically red lasers, *e.g.*, 785 nm) that are outside the absorption range of the common fluorophores. Another option is applying a recently established photobleaching protocol. As a matter of fact, the chlorophyll-containing samples exhibit intense autofluorescence that can be diminished upon exposing the sample of microalgae or cyanobacteria to an unfocused laser beam over the course of tens of seconds up to one minute, see the details in Moudříková et al. (2016). This recent breakthrough for the applications of Raman microspectroscopy in photosynthetic microorganisms opened new possibilities in their research.

Another source of inconvenience emerging during the use of high-intensity laser excitation is its deteriorating effect on photo-labile molecules that are unstable in high light, and thus, undergoing photochemical or photophysical changes, commonly yielding in photo-oxidation. For instance, during the study of biocrystals (Pilátová et al. 2022), we encountered decomposition of lipophilic crystals, such as mixed sterol-carotenoid crystals, or crystals of uric acid. Both examples changed their structure over the course of measurement and their signal disappeared.

The time-consuming process of laser-scanning data acquisition prevents the analysis of fast-moving objects or limits the measurement of the statistically relevant number of samples in real-time. Timescales of sample measurements by conventional Raman microspectroscopy detecting spontaneous Raman scattering last tens of minutes to hours depending on the size of the sample and the number of cells measured. Nowadays, there are also more advanced techniques that are not as widely available yet, but speed up the measurements in a way it is possible to capture moving cells. For instance, chemical time-lapse imaging of swimming flagellates was acquired in the green alga *Haematococcus pluvialis* by coherent anti-Stokes Raman spectroscopy (CARS) (Barlow et al. 2014), or in *Euglena gracilis* by stimulated Raman spectroscopy (SRS) (Wakisaka et al. 2016). Both advanced techniques are comprehensively explained in Moudříková (2018) or the recent review (Shipp et al. 2017, Lee et al. 2021).

The sensitivity of spontaneous Raman scattering in an analysis of chemical compounds in low concentrations is limited by its weak signal. This obstacle can be overcome by a special technique employing surface-enhanced Raman spectroscopy that can enhance the signal up to 5 orders of magnitude using special metal-doped colloids or metal-coated substrates (Lee et al. 2021). This technique has been applied for the research of extracellular polysaccharides of microalgal biofilm (Ramya et al. 2010). The modified version with higher spatial resolution combining atomic force microscopy with surface-enhanced Raman spectroscopy (SERS) called tip-enhanced Raman spectroscopy (TERS) has not been reported with respect to applications on microalgal samples.

The endless possibilities of applications of Raman microspectroscopy in biology or biomedical research are still scarcely used due to the lack of its promotion among researchers. The recent comprehensive review scrutinized all the late technical advancements in application of Raman microscopy in microbiology (Lee et al. 2021). However, there is a more serious impediment to its wide and high-throughput application in these fields due to the missing software tools for automated data processing of Raman multi-dimensional hyperspectral chemical maps. The commercially

available software does not provide the users with all the tools needed for rigorous data processing or statistical assessment of the measurements. This became a great bottleneck for Raman users worldwide. The initiative to build software for Raman spectra deconvolution in single-cell measurements together with a pipeline for data processing was raised in order to develop a reliable tool for medical diagnostics by a German team led by Thomas Bocklitz and Jürgen Popp in Jena (Storozhuk et al. 2022). In the case of chemical mapping, the missing tools for simpler and faster data processing in the multidimensional Raman scans of the complex biological samples alongside with the quantitative statistical assessments of the results triggered a collaboration with Filip Peška and Martin Pilát from the Department of Theoretical Computer Science and Mathematical Logic, Faculty of Mathematics and Physics. RamAIn software (Figure 7) has been developed as a part of the student's project and the current state of the work with the detailed technical documentation has been published in the form of bachelor thesis (Peška 2022). The software has a simple user-friendly interface with a function to build an automated data-processing pipeline including spectral cropping, cosmic ray removal, background removal, spectral deconvolution, and data export in the form of chemical maps as well as processed spectral datasets. The next step is to optimize spectral deconvolution and develop an automated statistical analysis of the spectral distribution in the chemical maps.

Figure 7: RamAIn software under development. The schematic distribution of higher-level widgets in RamAIn application documented in the bachelor thesis of Filip Peška (Peška 2022)

Another important missing part for effective data interpretation is the lack of an initiative for a worldwide open-source database collecting all published spectral data from optical spectroscopies, *i.e.*, Raman, infra-red, fluorescence, and absorbance spectroscopies. The expensive Raman spectral databases are tailored for industrial purposes and commonly lack the biologically relevant molecules and they are not updated in the real-time. The existing call for such a platform emerges in the literature with hardly any response from the Raman spectroscopy community (De Gelder et al. 2007, Movasaghi et al. 2007, Guo et al. 2020). The currently most comprehensive free Raman databases are focused on geology or mineralogy, *e.g.*, RRUFF (rruff.info). Another initiative to collect Raman spectra globally is "ramanbase.org" built by colleagues at the Institute of Scientific Instrumentation Czech Academy of Science. My work on this project has been discontinued. Apart from these, there is

not an existing open platform storing optical spectral data in its variety. Also, there in not any formal publication protocol that would demand standardized measurements using calibrations (Bocklitz et al. 2015, 2016) or data collection in an open format by the journal editorial policies. The former is as big issue as the latter. Raman spectra reflect the particular spectrometer configuration and results in a huge variation among different laboratories and/or instruments measuring the same standard that was illustrated in detail by an initiative formed by 15 institutes from 7 European countries (Guo et al. 2020). The lack of solution for a reliable standardized protocols as well as the lack of an open database are the great impediment for data search as well as interpretation of the biologically relevant molecules (De Gelder et al. 2007).

Cell inclusions in the spotlight of Raman microspectroscopy

Raman microspectroscopy enabled the unambiguous interpretation of various cell inclusions in this thesis. Others will need to be examined by multiple analytical tools in order to complete the identification of their complex composition, such as the pigment composition of "red droplets" of *Synura sphagnicola*. Based on the new insights in the chemical composition of the cells, we could establish new hypotheses on ecophysiology adaptations in arctic microalgae (Chapter 1), on different levels of cellular metabolism of nitrogen (Chapter 2), global cycling of elements (Chapter 2 and 3), or revisiting cell inclusions first described 50 years ago (Chapter 4).

Crystalline cell inclusions

Crystalline inclusions were vastly revisited in unicellular eukaryotes of all the major supergroups. In the contrast with the reported prevalence of calcium-containing crystals, such as calcium oxalate, calcite, aragonite, hydroxyapatite etc., organic purines were found to be dominantly present in all the major eukaryotic supergroups. Additionally, on top of purines and calcium-containing biocrystals, various cells can form strontianite, barite, celestite, and other organic crystals that are vastly unknown from cells, *i.e.*, free saturated fatty acids, sterols, carotenoids, occasionally in mixtures of sterols and carotenoids. In some clades, purine crystals have been independently described few times earlier, but the research attention was discontinued for decades (Roush 1961, DeSa and Hastings 1968, Creutz et al. 2002, Moudříková et al. 2017a, Jantschke et al. 2019, Pilátová et al. 2022). In most microscopic eukaryotes, their presence has not been found yet until our pioneering work. Among various types of purine crystalline inclusions, guanine is well characterized in animals together with its photonic features (Gur et al. 2017, Wagner et al. 2021). Compared to unicellular eukaryotes, animals excrete guanine or uric acid as an end-product of their nitrogen metabolism (Wright 1995). This is not the case in protists and microalgae in which case nitrogen is a limiting element. Conversely, in microbial eukaryotes, purine crystals act as dynamic high-capacity nitrogen storage that was experimentally supported (Mojzeš et al. 2020). Moreover, we found monocrystalline guanine monohydrate and xanthine to form biogenic crystals in organisms for the first time including best-studied animal models (Pilátová et al. 2022). The chemical structures or process of purine biocrystal formation are of limited understanding, although interesting for their potential usage in bio-optics due to the immensely effective refractive index usable in photonic mirrors. Understanding the process of purine biocrystallization – the nucleation centers or crystal degradation process can help in the treatment of people suffering from gouty arthritis or kidney stones (Bove et al. 2017). The lack of knowledge about nitrogen deposition in cells limits our understanding of nitrogen metabolism from the cellular level to the level of entire ecosystems.

Surely there are interesting research questions and challenges spanning from cell biology to materials science or global ecology.

In the case of diplonemid protists, newly found inorganic crystals of celestite, baritocelestite, strontiobarite, and barite were shown to be a result of active bioaccumulation of both trace elements, Ba and Sr, in the greatest reported amount (Pilátová et al. 2023). In the context of paleoceanography, this may provide a new alternative insight into the emergence of particulate barite and celestite in the world oceans (Dehairs et al. 1980). Barium and strontium are commonly used as proxies for organic carbon flow or estimates of marine productivity and eventually in past climate reconstructions (Dymond et al. 1992, Dymond and Collier 1996). Although the correlation of marine productivity and Ba and Sr contents in the sediments are well documented, there are only hypothetical scenarios of the origin of particulate barite and celestite in the water column: i. chemical precipitation in the microenvironment of the aggregates (Horner et al. 2017), ii. chemical precipitation triggered by extracellular polymeric structures of the bacterial cell wall (Martinez-Ruiz et al. 2018), iii. active formation of biocrystals in diplonemids (Pilátová et al. 2023).

Each of the hypotheses lacks an important piece of evidence hampering its full support as the methods used could be interpreted only within the limits they mount. First, we miss the direct experimental evidence or measurements of microenvironmental conditions enabling to simulate such conditions in the laboratory (Horner et al. 2017). Second, in some bacteria, Ba was shown to bioaccumulate intracellularly in an amorphous phosphor-rich phase, while consecutive direct evidence for barite formation occurring extracellularly attached to the cell wall is missing, instead, sulfur-oxidizing bacteria were shown to enable barite precipitation from the solution containing 100 000 times higher concentrations of Ba compared to the environmental conditions (Martinez-Ruiz et al. 2018). Third, the diversity of diplonemid is still mostly unknown including the piece of information about their capacity for biocrystallization, although there are a couple of species forming massive amounts of celestite, it may not be the case for others (Pilátová et al. 2023). Our current knowledge about how and why particulate barite occurs in the sea needs to be improved by further in-depth studies.

Inclusions as a part of other organelles

Although cell inclusions are mostly localized directly in the cytoplasm or in a membrane-bounded compartment of its own, there are more types of such inclusions that are present inside organelles and those are even more challenging to be assessed directly. Inside chloroplasts, there could be pyrenoids, starch granules or plastoglobuli (van den Hoek et al. 1995). Newly found massive accumulations of polyphosphate inside chloroplasts of zygnematophyte microalgae, *i.e.*, *Cylindrocystis* spp., has not been reported until now (Docampo et al. 2005, Lander et al. 2016, Barcytė et al. 2020). This new discovery provokes questions about how general this phenomenon is and what consequences it has for the cell physiology and ecophysiology. The finding of polyphosphate in other zygnematophytes besides the genus *Cylindrocystis*, e.g., *Spirogyra* sp. (unpublished results), raises questions as to how widespread this phenomenon is in other streptophytes or even chlorophytes. The questions raised for future research about the accumulations of polyphosphate may encompass its presumable role in the cell metabolism especially stress mitigation, its physiologic relevance for adaptations to the harsh Arctic climate going as far as the speculation on its potential role in the evolutionary important process of terrestralization in the common ancestor of zygnematophytes and land plants (Anydrophytes) (Žárský et al. 2022).

Pigmented cell inclusions and other cell compartments in *Synura* species

In *Synura sphagnicola* and newly described *S. rubra* species, the analysis of "red droplets" and other cell compartments was only partially included in the original publication. The new insights based on the measurements of the autofluorescence and Raman spectra in *Synura* species challenged the original description and/or interpretation of some of them. According to our data, we do not support the original description of chrysolaminarin granules (Hibberd 1978), still, questioning the righteous description of chrysolaminarin contents would require a systematic revision of different species over different growth phases. Examination of the presumable anthocyanin-like composition of "red droplets" would require the more advanced approach of either time-resolved Raman microscopy or employment of a longer wavelength for excitation to avoid the intense autofluorescence that is harmful to the sample and Raman spectra collection. HPLC MS may not be useful in deciphering the composition of the enigmatic "red droplet" due to the instability of the pigments upon cell disruption as observed under bright field microscopy.

In general, Raman microspectroscopy has proven to be a very useful technique for identifying cellular inclusions. Main advantages are discussed alongside with the main challenges considering hardware limitations and missing data-processing tools altogether hampering the vast usage of this powerful qualitative and quantitative method. To overcome such limitations will help the users in all variety of biomedical fields to employ routinely this powerful microanalytical technique. In this work, numerous cell inclusions were identified using Raman microspectroscopy with some exceptions that were too complex and needs to be examined by a combination of other analytical techniques, such as HPLC MS or nuclear magnetic resonance. The identification of cell inclusions allowed us to establish new hypotheses regarding cellular metabolism, environmental adaptations, or even the global cycling of elements. Raman microspectroscopy has an irreplaceable position among other microscopic or microanalytical techniques empowering biologists with a tool wita great potential for bringing new insights to a variety of studies that are by far not limited to cell inclusions only.

Conclusions, outlooks, and future research

Main findings

- I found polyphosphate inside chloroplasts of a zygnematophyte alga *Cylindrocystis* sp. (Streptophyta, Archaeplastida) and hypothesized about its role in adaptation to extreme environment of high Arctic or in land terrestrialization.
- I abolished Kopetzky-Rechtperg body as a non-specific term for a variety of different organelles in *Cylindrocystis* sp. by comparing toluidine-blue staining with more specific fluorescent dyes.
- I helped to address guanine accumulation in marine dinoflagellate *A. carterae* (Alveolata, SAR) as well as symbiotic zooxanthellae of reef-building corals with the quantification of guanine accumulation.
- I proved the widespread occurrence of purine crystals in diversified microscopic eukaryotes in all major groups of eukaryotes living in various habitats.
- I raised a series of hypotheses about the potential functions of purine crystals in the microalgae and protists.
- I found that some diplonemids are hyperaccumulators of Ba/Sr. This made me establish an alternative explanation of the phenomenon of particulate barite/celestite appearance in the oceans. This is important for our understanding of why Ba/Sr in correlated with marine productivity.
- I determined the fluorescence characteristics of subcellular structures of a newly described species *Synura rubra* compared to *S. sphagnicola* (Chrysophyceae, Stramenopiles, SAR) and further analyzed them.
- The microscopic world of protists has endless potential for scientific explorations and with the help of newly emerging technique enabling single-cell approaches we can get closer to the essence of their biology. In this respect, Raman microspectroscopy is an invaluable tool.

Acknowledgements – tribute to my predecessor and collaborators

In my doctoral thesis, I focused on cell inclusions in single-celled eukaryotes. I have discovered the nature of several of them while working with species coming from all the major eukaryotic supergroups, including samples from various kinds of habitats ranging from snow algae to coral reef endosymbionts. Samples were acquired from public and private culture collections as well as environmental sampling. The new asset that was brought to the research of microscopic eukaryotes is Raman microspectroscopy – a powerful tool for nondestructive label-free studies of cell inclusions *in vivo* and *in situ*. This technique is newly pioneering biology while allowing direct chemical analysis of intact cells that cannot be obtained by any other analytical approach. This brought us completely new insights into the protist world on a single-cell level. In this place, the tribute needs to be paid to the substantial work of Šárka Moudříková who established an efficient photobleaching protocol that

enabled us to overcome the strong chlorophyll autofluorescence otherwise hindering Raman spectra capture (Moudříková et al. 2016).

Apart from my work included in this thesis, I worked in collaboration with colleagues in the Institute of Scientific Instrumentation of the Czech Academy of Science in Brno, namely Zdeněk Pilát and Sylvie Bernatová. Therein, Raman tweezer was developed as a tool of high-throughput single-cell spectral analysis with an application in real-time bacteriophage infection diagnostics. In this project with a published outcome, I helped with data interpretation (Pilát et al. 2020).

In order to establish new model systems in the research of purine crystals, we started a collaboration with Ansgar Gruber and Miroslav Oborník (Laboratory of Evolutionary Protistology at Biology Centre, Czech Academy of Science) which was supported by the Czech Science Foundation in a mutual project called "Guanine crystals as nitrogen storage compartments in *Chromera velia*".

The work on diplonemids stemmed into a new project employing synchrotron measurements. Thanks to Daria Tashyreva and Julius Lukeš (Laboratory of Molecular Biology of Protists, Biology Centre, Czech Academy of Science, České Budějovice) and Hendrik Küpper (Institute of Plant Molecular Biology, Biology Centre, Czech Academy of Science, České Budějovice), we applied for beamtime at the European Synchrotron Radiation Facility, Grenoble, France, to address the subcellular allocation of Sr and Ba in diplonemids. The data will be processed, interpreted, and published in the upcoming years.

The inspiring potential of eustigmatophytes brought more questions about an unknown source of green autofluorescence of the lipid droplets in some of the species and the yellow autofluorescence of the "reddish globule". Rigorous spectroscopic exanimation ended up in the plausible explanation that both structures exhibit variable spectral properties of carotenoids emitted in a different chemical environment. Meanwhile, the unexpected signal in cathodoluminiscence on freshly prepared freeze-fractured samples on TEM has been detected. This project spun out as a diploma thesis of Anna Rosenkrancová in the Faculty of Mathematics and Physics also in collaboration with Marie Vancová (Laboratory of Electron Microscopy at Biology Centre in České Budějovice), Radim Skoupý (DELMIC B.V., Delft, Netherlands) and Vladimír Krzyzanek (Institute of Scientific Instrumentation of Czech Academy of Science, Brno).

Scientific meetings brought more collaborations on the identification of cellular crystalline inclusions in various algal species. Antonio Calado (University of Aveiro, Portugal) was intrigued by the crystalline inclusions in the complex stigmata of dinoflagellates (Craveiro et al. 2010). Those have been lately reported to contain uric acid (Yamashita et al. 2009) which was not supported by our preliminary data (manuscript under preparation). Frederik Leliaert (Meise Botanic Garden, Belgium) initially described seven types of crystals in marine Cladophorophyceae (Leliaert and Coppejans 2014). The revision of their chemical nature via Raman microspectroscopy is currently in progress (manuscript under preparation).

The fruitful collaboration with computer scientists Filip Peška and Martin Pilát from the Department of Theoretical Computer Science and Mathematical Logic, Faculty of Mathematics and Physics was established in order to create a new user-friendly software for automation of Raman spectral data processing. This work was supported by TAČR GAMA 2. The next step would be to initiate the worldwide open-source database collecting all published spectral data from optical spectroscopies, *i.e.*, Raman, infra-red, fluorescence, and absorbance.

Also, I helped to initiate the creation of an open Raman database “ramanbase.org” with colleagues at the Institute of Scientific Instrumentation Czech Academy of Science. My work on this project has been discontinued.

Research outreach

The science communication with public is getting more important over time. Sharing the cutting-edge expertise with the colleagues as well as the broad public is an ever-growing trend. The three pillars of a modern academia are shifting the emphasis from “teaching” and “research” towards the “transfer” of newly earned knowledge to the society for its direct benefits. Besides my role as a technology scout at the faculty, I actively participated in popularization of science in science festivals and shows, delivered presentations, performed one-woman shows, and taught classes of secondary school students as well as bachelor and master students. I wrote popular research articles and finally could help to popularize my own results. I did so through the platform of Nature Community – the article is attached to the thesis.

Felix qui potuit rerum cognoscere causas.

Virgilius, Georgica

…I guess learning is and is not a pleasant experience, but still, under all the favorable and unfavorable circumstances I feel that I was lucky

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Attachment – original publications