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Is annual metabolic cycling in the unicellular microalgae *Chlorella* and *Isochrysis* coupled to the annual earth gravity cycle?

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Uptake rates of ^{14}C-labelled guanine by autospores of the unicellular green alga *Chlorella fusca* Shihira et Krauss were measured at different times over 21 months. The autospores were derived from synchronous cultures produced from stock cultures that had not been exposed to natural light the last six years before the experiments, nor during the 21 month long experimental period. The experiments were performed in Bergen, Norway (60°,23′ N; 5°,20′ E). Uptake rates showed distinct annual variations over the year, with lowest values during the winter and highest during the summer. The August : December : February ratios for the rates were 1.0, 0.70 and 0.43, respectively. Half saturating guanine concentration for the uptake was the same over the year, namely 0.24 μM. Growth rates of the unicellular marine flagellate *Isochrysis* sp. were measured in March, August and December, and the rates were distinctly different, with August : December : March ratios of 1.0, 0.41 and 0.64. The number of cells reached in the stationary phases of *Isochrysis* cultures showed similar time-of-the-year dependency with ratios of 1.0, 0.44 and 0.58 for August : December : March, respectively. These cells had not experienced day light for the two last years before the experiments.

Our results show the existence of annual rhythms in two microalgae that had not been exposed to natural light for a long time.

A persistent endogenous clock that was set when the cells lived under natural light conditions long ago may be one explanation for their behaviour; another one is a coupling to the sinusoidal and minute variation over the year of earth gravity. Hence the cells display maximal activity when gravity is at its lowest value during the summer in the Northern hemisphere, and lowest activity when the gravity is at is highest in the winter.

To our knowledge our results are the first experimental work that points to the possibility that cells may be influenced by the annual cycle of earth gravity.

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1 Introduction

Knowledge about annual rhythms in microalgae is almost non-existent. Using many different microalgae for experiments over more than 40 years in our laboratory, students and researchers have repeatedly experienced bad growth performance of the algae from November to February. Both stock cultures and experimental cultures were grown in artificial light and with no contact with natural light. Lotte Schwinck (1) working on photophosphorylation in *Chlorella* and *Scenedesmus* reported that her experimental organisms, cultivated yearlong in Germany under constant artificial illumination and at constant room temperature, grew faster in spring and early summer than in winter. Kessler and Langner (2), also in Germany, showed clear and more or less smooth annual cycles of hydrogenase activity in *Chlorella* cultured from stock cultures kept in a window, and hence exposed to natural light over the year. The enzyme activity was highest in September and October and lowest in March. In similar experiments on nitrite and nitrate reductase in *Chlorella* and *Ankistrodesmus*, Kessler and Czygan (3) found quite similar rhythms as these, now with maximal rates in June-July, and minimal ones in October-November. Langner (4) also studied annual periodicity of hydrogenase activity, as well as some other processes in *Chlorella*, using cells kept as stocks under constant light conditions and cells stored on agar in a window. He also found distinct annual rhythms, highest in summer and lowest in winter in the cultures that were inoculated with the "window-cells" and kept under constant condition during the experiments. Interestingly the algae that did not experience the daylight had similar, but less clear variations over the year.

In an article by Pettersen and Knutsen (5) on guanine uptake experiments in synchronized cultures of *Chlorella* it was briefly mentioned that an annual rhythm in the uptake rates had been detected. These uptake experiments were not aimed at finding annual rhythms, but seasonal variations became evident during the analysis of the uptake experiments. It is important to note that the stock cultures had not been exposed to daylight the last six years before the experiments, as well as during the 21 months of the experimental period, where the experiments were done under constant conditions. The guanine results corroborated the trends Langner found with his cultures that were inoculated from the stocks that were not exposed to daylight. We here report in a complete fashion the early findings on guanine uptake related to the time of the year. We also report on experimental results that show differences in growth rates and maximum culture cell numbers at three different times of the year of the marine flagellate *Isochrysis* sp. These results are taken from studies of its growth physiology, using cells that did not see daylight for two years before the experiments. Our results, taken together with those mentioned above, show that three unicellular algae, *Chlorella*, *Scenedesmus* and *Isochrysis*, have a mechanism that somehow can recognize the time of the year without being coupled to the annual variation of daylight. Based on our findings with *Chlorella* and *Isochrysis*, and published models of how cells can register and respond to subtle changes in gravity by Mesland (6), Kondepudi and Prigogine (7), Kondepudi and co-workers (8), Kondepudi (9)

and Albrecht-Buehler (10) we suggest that the annual earth gravity cycle causes the described annual metabolic cycles.

Our findings may point for the first time at the existence of a universal coupling between the annual gravity cycle affecting all organisms, since the mechanisms of gravity detection and response systems referred to above are based on universal cellular properties.

2 Materials and methods

2.1 Organisms and their cultivation

For studies of guanine uptake we used *Chlorella fusca* Shihira et Krauss 211-8b (*Chlorellaceae*) obtained in 1962 from The Algal Collection, University of Göttingen, Germany. It was kept in our laboratory during the following years as stock cultures on agar and in liquid media and only in continuous light from fluorescent tubes. All the experiments were performed in our laboratory situated at 60°22′59.61″ N and 5°19′55.60″ E. The cells used for experiments during 1968–69, were grown and synchronized in the mineral medium by Kuhl and Lorenzen (11) prepared from glass distilled water and autoclaved mineral stock solutions stored in refrigerators. Culturing temperature was 30°C and the irradiance 20 klux (about 350 μmol m^{-2} s^{-1}). Culturing glass tubes of 38 mm inner diameter and with conical bottom, equipped with an aeration tube extending to the bottom through a polypropylene cap, were used for synchronized growth. Complete tube assemblies containing 250–300 mL medium were autoclaved. During culturing the tubes were standing in a thermostatted water bath placed in front of a bank of 5 parallel fluorescent tubes (3 Philips TL 40W/32 and 2 TL 40W/55, Philips, Holland). The cultures were bubbled with air containing 1.5% CO_2. The air, piped from a central compressor (thereby sterilizing the air at about 200°C) was filtered through activated charcoal, humidified by bubbling through autoclaved distilled water, and again filtered through a cotton plug in each aeration tube. Synchronization was according to the repeated light : dark method of Lorenzen (5, 11, 12) with dilution of the culture to a standard cell number at the end of each dark period. A high degree of synchrony was obtained using 1.6 million cells/mL as daily starting cell density and 14 : 10 hours light : dark cycles. With this system highly synchronous cultures were produced daily for weeks, and under our culturing conditions sporangia with 16 and 32 spores were produced, giving daily cell number increase of 25. Sporulation started at the 19th hour of the cycle, and within 2 hours all spores had been released as illustrated in Fig. 1. More than 99% of the cells in this culture are newly formed autospores.

The marine flagellate *Isochrysis* sp. (*Prymnesiophyceae*), used for growth studies, was isolated on April 1st in 1995 from the marine location Puddefjorden in Bergen (60°, 22′, 95″ N; 5°, 19′, 55″ E), and kept in our algal collection. Stock cultures and experimental cells were grown in Conway medium (13) consisting of filtered and autoclaved sea water that had been diluted to 80% strength with sterile distilled water. Before autoclaving the sea water was filtered through a membrane filter with 0.2 μm pores. Thereafter minerals were added from stock solutions. The sea water was taken from the sea water supply of the Bergen High Technology Centre, coming from 90 m

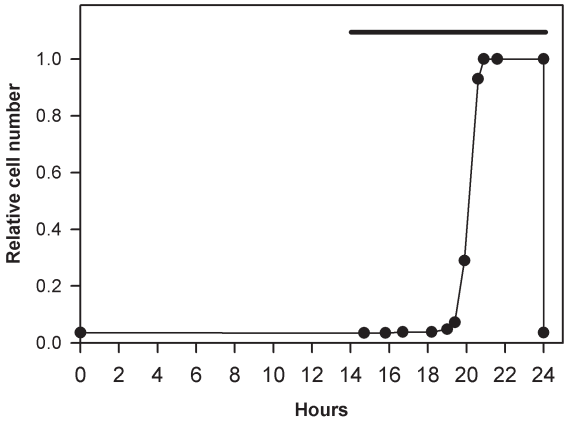


Figure 1. Cell number in the culture as function of cultivation time in the 14 : 10 Light-Dark cycle of the synchronized culture, illustrating the sudden and concentrated release of the autospores.

depth in a well mixed fjord (Byfjorden, Bergen), pumped 5 km, filtered through a 60 μm filter and treated with UV-light before storage in a central distribution tank. The water we used was taken from the tank and then "aged" by storage in the dark for several weeks before being used in the experiments. Until the start of the reported experiments in 1998 the cells were kept as stock cultures at 20°C in liquid cultures and on agar slants under constant illumination from fluorescent tubes, and without being exposed to daylight. Stock cultures for the experiments were produced using inocula from these cultures and growing them in 50 mL Erlenmayer flasks kept at 24°C in continuous artificial light of 50 μmol m^{-2} s^{-1} at the front of the flasks, and without exposure to natural light at all. These cultures were transferred to fresh media every 14 days, and used to start growth experiments with the same glass tube and lighting system as employed for *Chlorella*.

2.2 Guanine experiments with Chlorella

The following standard uptake measuring procedure was used in all our experiments, always using autospores harvested on membrane filters at the end of the dark period of the synchronous cycle. The spores were re-suspended in fresh medium of 30°C to give the appropriate experimental cell number, and then kept aerated in darkness at this temperature until the start of experiments. Twenty mL of cell suspension were transferred to a black glass tube standing in a water bath of 30°C and temperature equilibrated for 5 min and with aeration. The incubation with radioactive guanine was started by rapidly adding and mixing the appropriate volume of radioactive guanine solution to the algal suspension. Sampling of the incubation mixture started less than 5 seconds after the addition of the radioactive compound by rapidly pipetting and rapidly filtering 1.0 to 2.0 mL cell suspension (always less than 6 million cells/mL) onto 21 mm diameter membrane filters (0.45 μm pore size). The cells were quickly separated from the radioactive solution by suction and then washed without delay on

the filter by passing 1 mL distilled water through the filter. From each incubation at least 4 samples were taken, one at each of 20, 60, 105 and 150 seconds. Filtering half the sample volume took 3–6 seconds, which was included in the sampling time. After drying filters and cells, the radioactivity on the filters was measured by scintillation counting. The initial transport rate, V_{in}, was estimated from the time course of the accumulation of the radioactivity in the cells, by drawing the tangent to the uptake curve through zero time.

Measurements of uptake rates involved cell counting, pipetting, filtering and radioactivity measurements, and control testing of these steps gave the following standard deviations: ± 2.5 % standard deviation for counting 5 million and more cells per mL, and from ± 2.0 to 4.5 % standard deviation (SD) for pipetting, filtering and counting. The first value refers to samples with 1000 counts per minute (cpm) and the second one to samples with 100 cpm (i.e. for rates determined with the lowest guanine concentrations). Calculating the SD for the rate determinations with the two situations gives SD of ± 3.2 and 5.1, respectively.

2.3 Growth experiments with Isochrysis

The three experiments reported on here were originally done to see what effects varying nitrogen supply had on the growth of this alga. They were done in March, August and December of 1998. Cultures with differing starting concentrations of nitrate were prepared by spinning down the cells of a starter culture, washing the cells by suspending them in N-free medium, followed by centrifugation and suspending the cells in N-free medium to give the desired starting cell density. From this culture 250 mL portions were transferred to the growth tubes and appropriate volumes of nitrate stock solution were added to give nitrate concentrations in the 0.6 to 38.4 mM range. The cultures were standing in a thermostatted water bath at 24°C and illuminated by a bank of 5 parallel fluorescent tubes (2 Osram 58W/21, 2 Philips TLD 58W/95 and 1 Philips 58W/83RS), giving 300 μmol m^{-2} s^{-1} irradiation at the front of the culturing tube. Growth was followed by counting and sizing the cells in samples taken at the same time of the day for 9 days.

2.4 Cell counting and sizing

Chlorella cells were counted using a Celloscope particle counter (Ljungberg Co, Sweden) with 100 μm orifice and with filtered 0.9% NaCl as counting electrolyte. *Isochrysis* growth was followed by cell counting and sizing using a Coulter ZM/Channelyzer particle analysing system (Coulter Electronics Limited, Luton, England), with 100 μm orifice and filtered growth medium as counting electrolyte. Standard deviation of the cell counting was ± 2.5%.

2.5 Biovolume

Biovolume is the total cellular volume in a sample, calculated by multiplying the average cellular volume (μm^3) with cell number (cells/mL) of the sample.

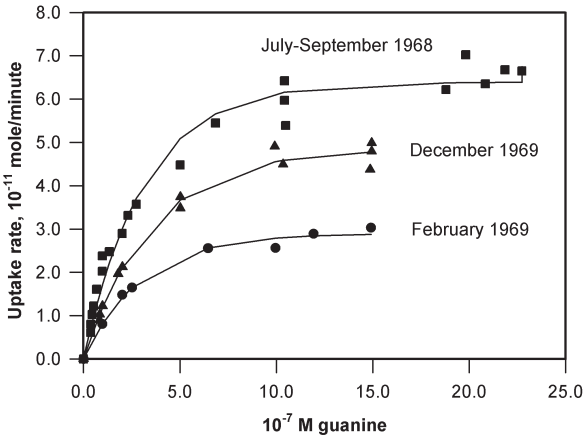


Figure 2. Initial rates of guanine uptake into 2.5 million *Chlorella* autospores as function of concentration, measured at 3 times of the year.

2.6 Radioactive solutions and activity measurements

(8-^{14}C) guanine sulphate was from The Radiochemical Centre, Amersham, England. The two batches used had specific activities of 36.1 and 57 mCi/mmol, respectively. Stock solutions from 2 to 500 μM were made by dissolving (^{14}C) guanine in 0.01 N HCl. In experiments with higher guanine concentrations than 2 μM, we used a stock solution of 1.0 mM made by adding radioactive guanine to an unlabelled solution, which reduced the specific activity to 1/50. The radioactivity of cells on membrane filters was measured with a Tri Carb liquid scintillation counter using Instagel as counting solution (both from Packard Instrument Co., USA).

3 Results

3.1 Chlorella *experiments*

Uptake experiments were done in July–September 1968 (pooled results), February 1969 and December 1969 to describe the dependency of initial uptake rate, V_{in}, on guanine concentration, employing concentration series from 0.035 to 2.3 μM (July–September) and 0.035 to 1.5 μM (February and December). The three curves of Fig. 2 were made with the program Excel (Microsoft Office Excel) and the curves drawn using its simplex curve fitting feature. As said before the standard deviation was less than $\pm$ 5.1% for all points of the graph. The graph shows that the initial rate V_{in} was concentration dependent and constant above 1.5 μM guanine, thus giving the maximal initial rates, V_{inmax}, measured during each of the three time periods.

As can be seen at all of the concentrations the uptake rates were much higher in the summer than in winter. Relative to V_{inmax} of July/September, being 1.00, the December and February rates were 0.76 and 0.45, respectively. The half saturation concentration of the uptake, describing the affinity of the uptake system for guanine,

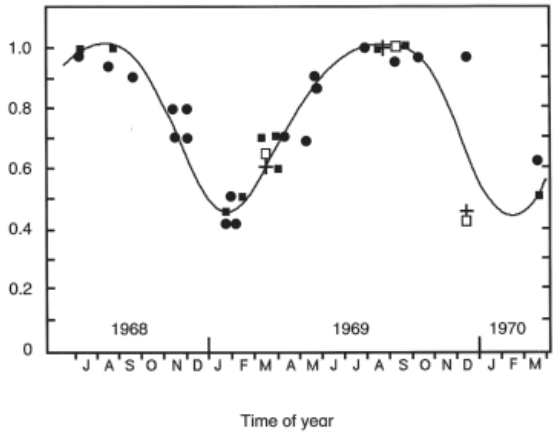


Figure 3. Relative values of initial uptake rates of guanine into *Chlorella* autospores as function of time of the year from July 1968 to March 1970: •, V_{in} measured at 0.1 μM guanine; ■, V_{inmax} measured at 1.5 μM. □, Relative growth rates of *Isochrysis* at saturating nitrate concentration, measured at 3 dates in 1998; +, relative maximal cell number obtained in cultures with 14.4 mM nitrate. These values are also calculated relative to their corresponding August values set like 1.0.

was calculated from double reciprocal plots of these data to be 0.24 μM for all three times of the year. Furthermore, during the whole experimental period of 21 consecutive months, from June 1968 to March 1970, many additional measurements of initial rates were performed at 0.1 μM (nineteen $V_{\rm in}$ values) and at 1.5 μM (nine $V_{\rm inmax}$ values). Relative rates are calculated for both data sets and with their respective August values set like 1.0. The two data sets are plotted together against the dates of the rate measurements and shown in Fig. 3. It can be seen that both types of uptake rates ($V_{\rm in}$, $V_{\rm inmax}$) describing the same cellular process, fluctuated strongly over the year in a smooth and continuous sinus-like mode, and with highest values in August/September and less than half of those in January/February.

3.2 Isochrysis experiments

Figure 4 is given as an example (December) of the kind of results obtained from growth experiments with this alga cultured at differing nitrate concentrations. The two growth curves describe the development over time of cell numbers and biovolumes in the culture. Maximal growth rates based on cell numbers and biovolumes were estimated from these curves. For the curves in Fig. 4 the maximal rates were $4.5 \cdot 10^6$ cells mL^{-1} h^{-1} and $0.18 \cdot 10^8$ μm^3 mL^{-1} h^{-1} calculated from day 4 to day 6. Similarly maximal growth rates were obtained from three experiments using growth-saturating nitrate concentrations that were performed in August, December and March, respectively. Calculated relative to the August rate (1.0) the December rate was 0.41 and the March rate 0.64 for cell number accumulation and 1.0, 0.36 and 0.64 for the biovolume accumulation rates, respectively. This clearly demonstrates that the growth of *Isochrysis* also differed during the year, growing much faster in summer than in

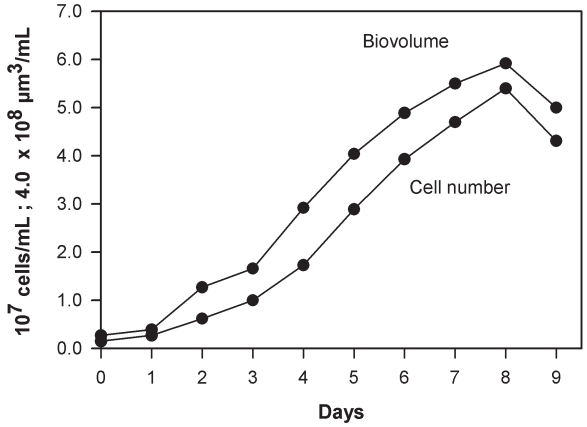


Figure 4. Accumulation of dry weight and biovolume of *Isochrysis* grown in medium with 12.0 mM nitrate, shown as function of cultivation time. From an experiment in December 1998.

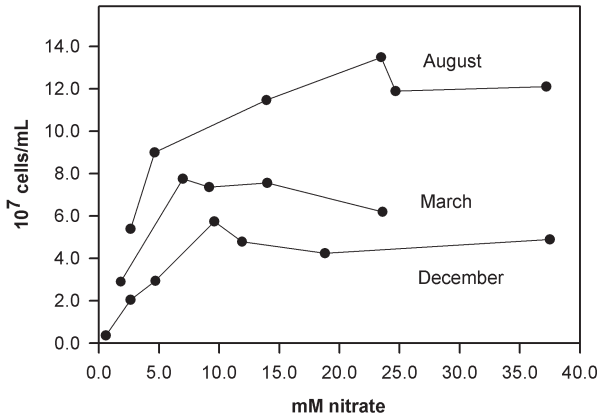


Figure 5. Maximal cell numbers obtained in *Isochrysis* cultures with differing initial nitrate concentrations measured at 3 times of the year in 1998.

spring and winter. Furthermore, maximal cell numbers obtained in each of the three experiments varied in the same manner as can be seen from the plots of cell number against nitrate concentrations in Fig. 5. At all nitrate concentrations also the maximal cell numbers were highest in August, lowest in December and somewhat higher than this in March, their relative values being 1.0, 0.44 and 0.58 (calculated for 14.4 mM nitrate). These results on maximal growth rates and cell production show the same clear differences between the summer, spring and winter culture values as found for the relative guanine uptake rates in *Chlorella*. This has been clearly visualized by co-plotting the relative *Isochrysis* data with the uptake rates from the *Chlorella* experiments in Fig. 3, demonstrating how closely they fit in with the *Chlorella* data.

4 Discussion

Firstly we have shown that clear differences exist between uptake rates of guanine in the freshwater alga *Chlorella* at three times of the year, and also that the rate changes smoothly over the year, being about twice as high in summer as in winter. Furthermore, we recorded very similar differences of growth rate and maximal cell production at three different times of the year in the marine *Isochrysis* sp. With both algae and for all the processes we measured their summer/winter ratios were about 2. It is most important to realize that the algae used in the experiments had not experienced daylight for a long time before and during the experiments, which was more than six years for the *Chlorella*. Hence these two microorganisms somehow "know" the time of the year without being coupled to natural daylight, which would be the most obvious annual time setting principle for an alga in Nature. As far as we can see our results are not caused by systematic annual cycling in culturing conditions. The main chemical factors that could have caused the variations are water for the culturing media and the air supply. Referring to what we have written about water and air in the Methods section, we think it is unlikely that the process variations we found in the algae were caused by annual variations of the quality of the water used for culturing media or the air used for culture mixing and carbon dioxide addition. With respect to *Isochrysis* growth, the concentration of the minerals in the culturing medium was never growth-limiting, meaning that any fluctuation over the year in the relatively insignificant mineral contents of the sea water used for media preparation together with the added amounts of mineral salts had no influence on the growth rates. Also, we find it unlikely that the distilled water used for *Chlorella* in 1968–69 and the seawater used thirty years later for *Isochrysis* should harbour one or more compounds that affect the two different cell types in the same manner, and in addition change systematically in concentration over the year. However, we cannot completely rule out that one of our stock cultures accidentally had been exposed to low intensity of natural daylight by being left on a lab bench for less than a day. But in that case one can wonder how the algae, from a glimpse of daylight, could distinguish chronologically enough between, for example, a grey day in April and an equally grey day in September to align the metabolism to the "right" time of the year. One would assume that in order to align the metabolism to the yearly light cycle long-time continuous exposure is required. On these grounds we hold that most probably our results show that these two algae are able to "know" the time of the year without being exposed to natural light. Our results thus quantitatively support the impressions students and researchers in our and other laboratories (1) have had about the differences in growth capacity of different microalgae in winter and summer in the Northern hemisphere. They also support the results and conclusion of Langner (4) from *Chlorella* stored in artificial light. The studies on hydrogenase activity by Kessler and Langner (2) in *Chlorella* and nitrate and nitrite reductase activities in *Ankistrodesmus braunii* by Kessler and Czygan (3), showed strong annual variations with highest rates in summer and lowest rates in winter. However the light conditions used under cell storage by these authors are unknown, so therefore one can only state that they demonstrated the existence of a timing system that relates metabolism to the time of the year. This could be a part of the system we have uncovered, or a different mechanism depending directly on daylight. The regulatory

system connected with the time-keeping system of our cells affects the whole cell, which is evident from the differences in growth of *Isochrysis*, growth being the overall integrating process of the alga. This also holds for our *Chlorella*: Since the cells in our experiments grew photoautotrophically they had no need for an external guanine supply, hence the constitutive uptake system for guanine was without importance for the cells under this condition. Similarly this is also the case for the hydrogenase activities described above. The rhythms revealed by us could be caused by a long-time persistent internal rhythm set by daylight when the cells last experienced daylight long enough to perceive the time of year. Notably our *Chlorella* culture had not been exposed to daylight for more than six years, while *Isochrysis* was closer, about 24 months, to its last exposure, which was in the seawater the day of its isolation. Alternatively an external natural force varying in intensity in a cyclic manner over the year may cause the observed variations. Such a force must be detected by the cell and its intensity registered and transduced to affect one or more of the processes that regulate cellular activities. Earth gravity might be this controlling force since its intensity varies over the year in a cyclic manner according to Zerbini and co-workers (14), with maximal values in January and minimal ones in August in the Northern hemisphere and the opposite pattern in the Southern as held by Barkin and Ferrandiz (15). The cyclic gravity pattern is just the opposite to our results, meaning that the cellular activities are highest when gravity is lowest, and *vice versa*.

However the gravitational variations are very small and one can question if cells can perceive such small gravity variations at all, and if they can possibly affect the cells. Mesland (6) proposed how gravity might affect small biological systems, basing his idea on the works of Kondepudi and Prigogine (7) and Kondepudi and co-workers (8). They hold that in steady state systems that are far from thermodynamic equilibrium small perturbations of the system might have significant effects. Their main idea is that some of the systems have so-called bifurcating points where the system has to choose between two pathways. At the bifurcating point the system is extremely sensitive to external stress, like gravity.

A mechanism for gravity perception by individual cells has been proposed by Kondepudi (9). According to him the cell can detect and respond to weak gravity signals over random thermal noise by a dynamic non-equilibrium mechanism that performs "signal averaging" over long timescales. He holds that through this process a cell is able to respond to a small systematic force embedded in a large randomly fluctuating force. The non-equilibrium state of the living cell is crucial for the mechanism to work.

Albrecht-Buehler (10) presented a possible cellular mechanism for gravity sensing and response by cells in his article entitled "Possible Mechanisms of Indirect Gravity Sensing by Cells": "Cells may be able to sense certain environmental changes caused by gravity and thus sense indirectly gravity amplitudes." We will not go into this in great detail, but present its main elements here. He holds that the most promising cellular gravity detection principles are cell to cell contact and contact between subcellular organelles and the cytoskeleton. The latter is relevant for our unicellular organisms. The reduction of high frequency noise required for signal averaging can be realised due to the viscoelastic property of the cytoplasm or via membrane potentials. According to the model the relatively small gravity change translates to changes in contacts and membrane potential, which are un-amplified signals. He proposes

that these signals are then strongly amplified at a critical value by their action on a membrane parameter that can have two or more alternative states, i.e. according to the bifurcating principle described above. The parameter is then switched into one of the other states resulting in a strongly amplified signal. This leads next to action changes in the cytoplasm integration system, which in turn makes changes in the cytoskeleton and thereafter changes in cell functions.

The presence in our cells of an annual rhythm which is not at all coupled to daylight, is fundamentally important and deserves further research. The fundamentally different explanation we here offer, namely that the rhythm can be caused by the annual gravity cycle, is based on published theories on how single cells can register subtle changes in gravity and respond to them. To our knowledge our results are the first experimental work that points to the possibility that cells may be influenced by the annual cycle of earth gravity.

This gravity dependency will have wide implications about the understanding of the life of micro algae, with respect to their cell biology, physiology and ecology. Annual fluctuations in growth capacity can have practical implications for algal utilization like commercial algal biomass production, whether it will be for aquaculture feed, energy biomass or for production of bioactive substances and fine chemicals.

However our findings may point to the existence of a universal coupling of the annual gravity cycling to all organisms, since the mechanisms of gravity detection and response systems described above are based on general cellular properties.

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