DIPLOMARBEIT

Titel der Diplomarbeit

„Resource stoichiometry and the growth rate hypothesis in Verrucomicrobium spinosum“

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)

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Wien, 27. Okt. 2008
ABSTRACT:

In this study I tried to evaluate the applicability of the growth rate hypothesis (GRH), a main idea within the framework of biological stoichiometry, on Verrucomicrobium spinosum — a bacterium that is commonly found in various environmental ecosystems. In order to do so, I further developed a method to quantify DNA and RNA within the cell using a single fluorescence dye (RiboGreen). Furthermore, I assessed under which circumstances the GRH is applicable on V. spinosum and tried to set these constraints in context with its environmental applicability. I showed that the GRH is applicable to our model organism under P limitation, and the occurrence of a decoupling effect if another nutrient becomes limiting. Furthermore, I discuss the applicability of the GRH on natural environments and the adherent problems.
INTRODUCTION

When biologists look at ecosystems, they mostly concentrate on different types of organisms or interactions, depending on their discipline. In order to get a more complete view of the ecosystem and its functions, it is essential to measure and evaluate across multiple scales with a single and versatile currency. With this goal in mind, Sterner and Elser developed ecological stoichiometry and defined it as: “…the study of the balance of multiple chemical elements in ecological interactions” (Sterner & Elser 2002). Ecological interactions follow the same fundamental rules as all chemical reactions, as chemical rearrangements are made, mass must be conserved and elements can neither be created nor destroyed. Stoichiometry varies among different organisms, species, nutritional modes, trophic levels and the chemical composition of different organisms is also constrained to different degrees (Sterner & Elser 2002).

Ecological stoichiometry provides the basis for explicit links between the elemental compositions of organisms, their macromolecular composition and physiology. The growth rate hypothesis (GRH) presents an idea within the framework of ecological stoichiometry and hypothesizes that an increase in growth rate requires increased ribosome content and therefore ribosomal RNA (rRNA). RNA is rich in phosphorus (P), therefore a positive linear relationship between growth rate and cellular P – content exists (Sterner & Elser 2002).

Although the positive relationship between rRNA and growth rate has been confirmed for many organisms, growing at their maximum growth rates during development or reduced growth rates under P-limitation, e.g. crustaceans, insects, zooplankton (Elser et al. 2003), phytoplankton (Dorch et al. 1983), single cell bacteria (Fagerbakke et al. 1996) as well as bacterial communities (Makino & Cotner 2004) there are also limits to its applicability. For example, a decoupling of
GRH parameters has been observed when growth is determined by other factors (starvation, or N as limiting nutrient) or when P is present in excess (e.g. luxury uptake) (Elser et al. 2003). Furthermore, the relationship can uncouple after repeated feast and famine cycles (Frigon et al. 2006), and at extreme low growth rates of anaerobe ammonium oxidizers (Schmid et al. 2001) and nitrite and ammonium oxidizers (Morgenroth et al. 2000).

Most of GRH studies on microorganisms have been done on freshwater isolates (Makino & Cotner 2004) and on model species, such as *E.coli* (Bremer & Dennis 1996). Collectively, these studies support that the GRH does not apply to all tested species and strains. Therefore, I tested the GRH on *Verrucomicrobium spinosum* (phylum Verrucomicrobia) that is common in terrestrial and aquatic ecosystems. I also tested if the predictions of the GRH apply across a wide range of resource stoichiometries for *V. spinosum*. Further I discuss advantages and shortcomings of the GRH framework and suggest possible modifications to broaden its applicability.
MATERIALS AND METHODS:

Cell Cultures and Resource Treatments

*Verrucomicrobium spinosum* (DSM 4136, phylum Verrucomicrobia) a ubiquitous freshwater gram-negative bacteria (Schlesner 1987) was grown in batch cultures under 12 unique C:N:P resource ratios consisting of two quantities of carbon, each with three levels of nitrogen (high, medium, low) and two levels of phosphorus (Figure 1).

![Figure 1: Experimental design to achieve 12 unique C:N:P treatments](image)
The used minimal media (Table 1) was based on the DSMZ complex media for *Verrucomicrobium spinosum* (DSMZ Medium 607: M13, [www.dsmz.de](http://www.dsmz.de)). Alterations to the complex media were made, to be able to independently alter quantities of C (glucose), N (NaNO₃) and P (Na₂HPO₄). The original recipe was modified using NaMoO₄ as the molybdenum source instead of (NH₄)MoO₇O₂₄ and CoSO₄ as the cobalt source instead of Co(NO₃)₂. Moreover peptone, yeast extract, glucose and vitamin solution were excluded to make it easier to manipulate the defined C:N:P ratio (Table 2).

### Table 1: Composition of the minimal medium for *Verrucomicrobium spinosum*

| Minimal media | Verrucomicrobium sp. | Tris-HCl pH7.5 | 5mM | Na₂B₄O₇ | 0.0464mM | CaCl₂ | 2.936mM | Na₂MoO₄ | 0.898mM | CoSO₄ | 0.088mM | Na₂SO₄ | 6.894mM | CuSO₄ | 0.157mM | NaCl | 0.100mM | FeSO₄ | 8.920mM | Na₂EDTA | 0.855mM | H₃BO₃ | 0.105mM | NaF | 0.018mM | KBr | 0.013mM | NaHCO₃ | 0.571mM | KCl | 2.227mM | Na₂EDTA | 1.046mM | MgCl₂ | 6.125mM | SrCl₂ | 0.038mM | MgSO₄ | 2.410mM | ZnSO₄ | 3.808mM | MnSO₄ | 0.911mM |

### Table 2: 12 unique C:N:P – ratios according to the experimental design, two quantities of carbon, with three levels of nitrogen, each with two levels of phosphorus

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C [mM]</th>
<th>N [mM]</th>
<th>P [mM]</th>
<th>C:N</th>
<th>C:P</th>
<th>N:P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>150</td>
<td>12.5</td>
<td>0.25</td>
<td>12</td>
<td>600</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>150</td>
<td>12.5</td>
<td>0.025</td>
<td>12</td>
<td>6000</td>
<td>500</td>
</tr>
<tr>
<td>3</td>
<td>150</td>
<td>1.25</td>
<td>0.25</td>
<td>120</td>
<td>600</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>150</td>
<td>1.25</td>
<td>0.025</td>
<td>120</td>
<td>6000</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>150</td>
<td>0.125</td>
<td>0.25</td>
<td>1200</td>
<td>600</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>150</td>
<td>0.125</td>
<td>0.025</td>
<td>1200</td>
<td>6000</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>30</td>
<td>5</td>
<td>0.1</td>
<td>6</td>
<td>300</td>
<td>50</td>
</tr>
<tr>
<td>8</td>
<td>30</td>
<td>5</td>
<td>0.01</td>
<td>6</td>
<td>3000</td>
<td>500</td>
</tr>
<tr>
<td>9</td>
<td>30</td>
<td>0.5</td>
<td>0.1</td>
<td>60</td>
<td>300</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>0.5</td>
<td>0.01</td>
<td>60</td>
<td>3000</td>
<td>50</td>
</tr>
<tr>
<td>11</td>
<td>30</td>
<td>0.05</td>
<td>0.1</td>
<td>600</td>
<td>300</td>
<td>0.5</td>
</tr>
<tr>
<td>12</td>
<td>30</td>
<td>0.05</td>
<td>0.01</td>
<td>600</td>
<td>3000</td>
<td>5</td>
</tr>
</tbody>
</table>
The main experiment was conducted using 2 L Erlenmeyer flasks containing 400 ml of minimal media combined with a defined resource level (C:N:P). Each resource level was made in triplicates with one extra bottle to control the performance of growth over time (according to pre-measured growth curves) and to check the right time for harvest. Each 2 L Erlenmeyer flask was inoculated with pre-cultures grown on minimal media with 25mM: 2.5mM: 0.25mM of CNP respectively. All pre-cultures were inoculated with Verrucomicrobium spinosum grown on agar plates (1.5% agar) containing medium 607:M13, initiated from a single colony stored at 4 °C (grown at 30°C for 5 days).

The pre-cultures were grown in 250 ml Erlenmeyer flasks with 50 ml of media for 67h at 30°C (OD$_{450}$ of 0.4). After the pre-cultures reached an OD$_{450}$ of 0.4, cultures were centrifuged (10,800xg for 15min), biomass pellets collected, resuspended, merged and washed in C; N; P free minimal medium. Thereafter it was pelleted and resuspended again in C; N; P free minimal medium. Each 400 ml media (one 2 L Erlenmeyer flask) was inoculated with 8 ml of pre-culture. All flasks were shaken on orbital shakers at 170 rpm and 22°C until they reached the pre-determined active growth and stationary growth phase. They were then harvested, washed and weighed.
Sampling

To evaluate differences between actively growing and stationary phase populations cells were harvested in both mid-log and during stationary phase. In order to determine, the time point indicating mid-log or the beginning of stationary phase, growth curves were recorded for all 12 resource ratios in a preliminary experiment. With this data growth curves were modeled using the logistic growth model,

\[ N(t) = \frac{k}{1 + \left(\frac{k-n}{n} \times e^{-rt}\right)} \]

\( k \) ... carry capacity
\( n \) ... cell density at time zero
\( r \) ... intrinsic growth rate
\( N(t) \) ... cell density at time \( t \)

The parameters \( n \), \( r \) and \( k \) were estimated using Delta Graph, which uses an iterative algorithm with the Levenberg-Marquardt method (Red Rock Software Inc.). Growth curves were used to estimate growth rate and to determine the harvesting time points (Figure 2). During the main experiment, an additional 2 L Erlenmeyer bottle was used to follow these predetermined growth curves by OD measurements and to ensure that my sampling times coincided with the intended growth phase. At the selected time points (mid-log; stationary phase) the cultures were harvested (16,900xg for 45 minutes). Afterwards the supernatant was discarded and the pellet was resuspended and washed in 130 mmol NaCl. Following the second centrifuging step (36,700xg for 20min) it was aliquoted, resuspended and temporarily stored in RNA-later® at 4°C for 2 to 3 hours too make sure RNA-later® reaches and stabilizes the RNA (in order to avoid any shift in RNA-content), and finally stored at -80°C pending final analysis.
Measurement of RNA and DNA: the microplate assay

RNA and DNA were measured by fluorometry using the fluorescent stain RiboGreen® after the methods of (Makino & Cotner 2004).

Preexperiments for the fluorescence double digestion method.

RiboGreen® stains both RNA and DNA. Initially I tested a single digestion method (Dell'Anno et al. 1998, Makino & Cotner 2004), in which the complete fluorescence signal for both nucleic acids had been determined first, followed by an enzymatic digestion of RNA. The amount of RNA was then determined by the decrease of the fluorescence signal after enzymatic digestion. The amount of DNA was then assumed to be proportionate to the remaining fluorescence. However this approach assumes that the non-nucleic acid fluorescence is an insignificant component of the residual fluorescent signal. To determine whether the background fluorescence was constant for our 12 C:N:P - treatments, I performed a pre-experiment.
measuring the fluorescence signal of all 12 treatments with and without digestion of nucleic acids (i.e. DNAse and RNAse treated). The variance of the background fluorescence, i.e. post RNAse and DNAse digestion fluorescence, in the majority of the treatments was negligible, though it varied between treatments and in some treatments it constituted as much as 15 – 20% of the total fluorescence signal (Figure 3). To address this problem I used a double digestion method following the method of Wagner et al. (1998) to quantify DNA and RNA independently.

![Figure 3: Pre-experiment on background fluorescence before (black) and after (white) nucleic acid digestion](example.png)

**Sample preparation and the double digestion method.** The -80°C stored samples were first thawed (at room temperature) and centrifuged (32,200xg, 15min) to separate the pellet from the viscous RNA-later® liquid. Then I removed the supernatant weighed 1 mg of pellet in a 2 ml Eppendorf tube (micro test tube, Eppendorf, cat no.:...
0030120.086), and resuspended it by shaking and vortexing in Standard buffer (SB). In addition to the detergent n-laurylsarcosine (Bentle et al. 1981) I sonicated the resuspended sample using an ultrasonic bath (frequency 35kHz) for 8 minutes. The samples and controls were aliquoted in 1.5 ml Eppendorf tubes (Flex-Tube®, Eppendorf, cat no.: 022364111) and diluted to previously determined dilution factors, between 1:10, 1:50; dependent on the estimated amount of nucleic acids in each sample. I was able to perform each step at room temperature, because in absence of external added DNase and RNAse, no change in the quantity of nucleic acids within the sample over the period of analysis was observable (e.g. due to indigenous enzyme activity).

An aliquot of the diluted sample was distributed to 1.5 ml Eppendorf tubes for digestion. Each digestion was carried out in the dark at room temperature for 1h with a surplus of enzyme. Both the length of the experiment and the enzyme activity were shown through previous experiments (data not shown) to be sufficient to maximize nucleic acid degradation. After digestion 100 µl of each sample were transferred into 96 well black polystyrene micro plates (greiner®, cat no.: 655077). I added 100µl of 200x diluted RiboGreen® to each sample aliquot and kept the entire plate in the dark for 5 minutes (see RiboGreen® RNA Quantitation Reagent And Kit manual; MP 11490). I then measured the fluorescence in each with a TECAN® infinite M200 fluorescence analyzer (Excitation wavelength: 480 nm; Emission wavelength: 520 nm1). After the measurement, the obtained fluorescence numbers were recalculated in actual amounts of nucleic acids using internal standard curves as described below.

1 Wavelengths selected according to Quant-iT™ RiboGreen® RNA Regent and Kit – manual
Standards and controls:
RNA concentrations were derived from fluorescent units using a RNA standard provided by the Invitrogen Quant iT™ RiboGreen® Reagent Kit (Invitrogen™, cat no.: R11491). As DNA standard I used the Lambda DNA standard also from Invitrogen™. With these standards concentration curves were made at dilution rates of 20, 50, 100, 250, 500, 900 ng nucleic acid/ml (Figure 4). For diluting the nucleic acid standards I used MgCl₂ – Buffer, which enabled me to perform both digestions (DNase and RNase) in the same buffer. The used standard curves behaved simiarily to those described by the manufacturer with an $R^2 \geq 0.99$ (Jones 1998), which proved a high level of accuracy.

![Concentration curves for DNA and RNA standard](image)

Figure 4: Concentration curves for DNA and RNA standard, to recalculate the relative fluorescence units into actual amounts of nucleic acid.

Measurement of biomass C, N and P content
The P content was determined through acid extracts with 65% HNO₃ and 70% HClO₃ (4:1) on a heating plate with a defined heating procedure (100°C for 15 min, 120°C for 15 min, 160°C for 60 min, 180°C and 200 °C for 15 min followed by a constant temperature of 250°C till the sample is confined to small volume). After cooling down to room temperature the residual volume was filled up with distilled
water and subsequently measured by photometric phosphate analysis according to (Schinner et al. 1993). The C and N content was determined by drying the samples and analyzing them with continuous-flow gas isotope ratio mass spectrometry. The elemental analyzer (EA 1110, CE Instruments, Milan, Italy) was interfered via a ConFlo II device (Finnigan MAT, Bremen, Germany) to the gas isotope ratio mass spectrometer (DeltaPlus, Finnigan MAT, Bremen, Germany) (McKee et al. 2002, Watzka et al. 2006).

Working reagents
RNA-free water was made by adding 1ml of DEPC (Diethylpyrocarbonate \(\equiv 0.1\%\)) to 1 Liter purified water (MilliQ) and left to fumigate in the extractor hood for 24 h before autoclaving. I made a 10x MgCl₂ buffer by combining 100 mM TRIS with 25 mM MgCl₂ and 1 mM CaCl₂ followed by filling it in an RNAse free bottle (has been heated on 200°C for 3h). Standard – Buffer (SB-buffer) was made by adding 0.167 % n-lauryl sarcosine (Bentle et al. 1981, Makino & Cotner 2004) the MgCl₂ – Buffer. TE – Buffer was produced by first making a 10mM TRIS solution with a pH of 7.5 and then adding 1 mM EDTA and filling it in a RNAse free bottle (according to RiboGreen® RNA Quantitation Reagent And Kit manual; MP 11490). RiboGreen® is a nucleic acid stain which stains DNA and RNA in the same way. I used the high range essay recommended by the RiboGreen® manual, in order to determine concentrations from 0 to 1000ng RNA or DNA. Therefore I stained our sample with a 200 times diluted (with TE) RiboGreen® stock solution (www.invitrogen.com). The DNAse I used for DNA digestion was a bovine pancreas DNAse (fermentas DNAse I; #EN5210, 1u/µl). One unit of enzyme completely degrades 1µg of plasmid DNA in 10 min at 37°C (www.fermentas.com). The applied RNAse was a stock solution with 10mg RNAse/ml which I diluted to a
working solution of 3.75 µg/ml (concentration determined by pre-experiments). RNAlater® is an aqueous tissue storage reagent that rapidly permeates most tissues to stabilize and protect RNA in fresh specimens (www.ambion.com).

Statistical analysis
Statistical analysis was performed using SPSS. I measured growth rate [h⁻¹], the RNA:DNA ratio [ng ml⁻¹(ng ml⁻¹)⁻¹] and the quantity of C, N and P per unit dry mass (C DM⁻¹, N DM⁻¹, P DM⁻¹). These variables were tested on normal distribution using the K-S test. Regressions between Growth rate and RNA:DNA ratio, and between Growth rate C, N, P, C:N, C:P, N:P were performed.
RESULTS:

According to the performed Kolmogorow-Smirnov test all used variables were normally distributed (Table 3).

<table>
<thead>
<tr>
<th>GR</th>
<th>RNA:DNA</th>
<th>N DM(^{-1})</th>
<th>C DM(^{-1})</th>
<th>P DM(^{-1})</th>
<th>P/C</th>
<th>N/P</th>
<th>C/P</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-S Z</td>
<td>0.660</td>
<td>0.504</td>
<td>0.509</td>
<td>0.940</td>
<td>0.461</td>
<td>0.416</td>
<td>0.553</td>
</tr>
<tr>
<td>Sig(P)</td>
<td>0.777</td>
<td>0.961</td>
<td>0.958</td>
<td>0.341</td>
<td>0.984</td>
<td>0.995</td>
<td>0.920</td>
</tr>
</tbody>
</table>

I found a significant positive correlation between growth rate and the RNA:DNA ratio through all 12 resource treatments ($R^2 = 0.50; P = 0.01$), as predicted by the GRH (Figure 5).

![Figure 5: Relationship between growth rate and RNA:DNA ratio, using a linear regression model with CI = 95 (dashed line)]
Moreover a significant positive correlation ($R^2 = 0.40$, $P < 0.05$) between the P content (% P of dry mass) and the RNA:DNA ratio, also in agreement with the predictions made by the GRH (Figure 6) has been observed.

![Figure 6: Relationship between the P content (per gram drymass) and RNA:DNA ratio, using a linear regression model with CI = 0.95 (dashed line)](image)

Finally a significant positive correlation ($R^2 = 0.60; P < 0.01$) between biomass P and growth rate supporting the presence of the GRH mechanism in *V. spinosum* under these conditions has been found (Figure 7).

In order to address if these relationships were present under all resource stoichiometries I separated the data into high P and low P treatments (see Figure 1; even vs. odd numbered treatments). I found a positive significant correlation between biomass P:C and RNA:DNA ratio for the low P treatments ($R^2 = 0.58; P < 0.05$), but no significant relationship for the high P treatments as predicted by the GRH (Figure 8).
Figure 7: Relationship between the P content (per gram dry mass) and growth rate, using a linear regression model with CI = 0.95 (dashed line).

Figure 8: Influence of different P levels on the relationship between biomass P:C and RNA:DNA ratio.
At low and medium N levels the organisms were N limited, because biomass yields were consistently lower with decreasing N while both C and P were the same between treatments (Figure 9).

![Figure 9: Influence of limiting nutrients (P and N) on the biomass](image)

I found an increase in the coefficient of correlation from a $R^2 \approx 0.5$ (data not shown) to a $R^2 \approx 0.9$ of the significant positive correlation ($P = 0.01$) between P:C and growth rate, when the data points at N limitation treatments are excluded (Figure 10). Resource quantity had no significant effect on the GRH relationships (growth, RNA/DNA, P content) (Figure 11).
Figure 10: Influence of different N levels on the relationship between biomass P:C and growth rate

\[
y = 0.74x -0.01 \\
R^2 = 0.88 \\
P = 0.01
\]

Figure 11: Influence of different C levels on the relationship between P content growth rate
DISCUSSION:

Fluorescence measurements and tests on the GRH

The main goal of this study was to test the applicability of the GRH for *V. spinosum* and to illuminate for which resource stoichiometries the GRH proves true. In order to achieve this goal it was necessary to further develop an existing assay (Makino & Cotner 2004) to quantify DNA and RNA independently within the cell with a single fluorescent dye. Instead of only digesting RNA and recalculating the amount of DNA, I digested both RNA and DNA and therefore directly measured both nucleic acids. This was necessary, because the former method relied on the assumption of a constant blank, which was not fulfilled in this study.

In my analysis, 0.1% sarcosyl was present (SB), which as shown with other detergents, could decrease the fluorescence signal of RiboGreen® (Jones 1998). However, this does not influence our ability to quantify nucleic acids, because the standards and all the compared samples have been treated identically.

The preparation and storage procedure of the samples were critical steps, because it is well known, that indigenous nucleases (especially RNAse) are very aggressive and can digest significant amounts of RNA and DNA during preparation time. However, when I tested sample degradation in the absence of added nucleases (data not shown) no significant decrease in nucleic acid fluorescence could be detected over time. This suggests that *in situ* nucleic acid digestion did not significantly affect my results.

I found a significant positive correlation between the RNA:DNA ratio and growth rate (Figure 5) and between the RNA:DNA ratio and cellular P content (Figure 6). I also found a significant correlation between growth rate and cellular P content supporting that the GRH
mechanisms are present in *Verrucomicrobium spinosum* (Figure 7). In order to predict the impact of stoichiometric patterns on ecosystems, it is important to know the response of the indigenous community. To predict the response of the indigenous community on different stoichiometric patterns it is important to know the behavior of the different organisms the community is based on. Therefore the applicability of the GRH on *V. spinosum* constitutes another part in a puzzle, to predict community and furthermore ecosystem response on stoichiometric patterns.

However, because this was a controlled experiment we were also able to evaluate under which resource stoichiometries, the GRH was operating. When treatments were separated by the relative level of P (Figure 1; i.e. odd vs. even numbered treatments), I found that the GRH was only applicable to the low P yet not to the high P treatments (Figure 8). Therefore, my data suggest that the applicability of the GRH depends on the phosphorus in the biomass. This agrees with the GRH (Elser et al. 1996): it is hypothesized to occur due to luxury uptake of phosphorus and the accompanied relative shift of the main cellular P compartment from rRNA to storage compounds. This pattern also appeared during tests on other protozoan and metazoan organisms and constitutes a major confinement to this hypothesis (Elser et al. 2003). But on the other hand, it also assumes that P is not present in excess which also appears in natural environments such as in freshwaters.

A significant part of the scatter between growth rate and P content is due to the N limited treatments. The N – limitation of the treatments is visible in Figure 9 where biomass yield is basically following the trend of N supply until it reaches a medium level and also changes in P are visible influencing the biomass yield. This constitutes another border of the GRH, and is in agreement with previously published results found on Daphnia (Elser et al. 2003).
The present results also highlight that carbon quantity did not have a strong influence on any of the GRH couplings (Figure 11). This might occur due to the fact, that in our experimental setting carbon quantity did not, compared to the other nutrients (N and P) constitute a limiting nutrient.

This study not only refined a method to quantify RNA and DNA within the cell, to circumvent the problems with extraction and background fluorescence. I also showed under which circumstances the GRH is applicable to a heterotrophic bacterium (V. spinosum) common in many environments.

The next step, after testing the GRH on an environmental organism, would be to apply the knowledge gained on a single strain on natural environments. Therefore, it is necessary to critically review the GRH and its accompanying problems.

First I will give an overview on the general applicability to microorganisms, discuss the problems arising and suggest concepts to solve them (dynamic energy budget theory, disposable soma theory). Then I will discuss the challenges faced when applied to communities to finally provide an outlook for the application on different systems.

Discussing the GRH:

**General applicability to microorganisms.** In the past, many tests on the GRH with various organisms and on various organizational levels have been done. This undermines its importance for linking resource stoichiometry with a physiological response (e.g. consumer growth) to predict large scale ecosystem level changes. An evaluation of GRH studies on various organisms seems to lead to a consistent pattern. The GRH is supported by findings from typical model organisms like *E. coli* (Bremer & Dennis 1996, Fagerbakke et al. 1996), *Vibrio natrigens* (Aiyar et al. 2002) and *Salmonella sp.* (Schaechter et al. 2002).
However there are also exceptions: For instance, at low growth rates, *E.coli* showed a relatively constant rRNA content (Binder & Liu 1998). Studies on *Vibrio fischerii* revealed the same pattern showing a retained residual amount of ribosomes under slow growing conditions, resulting in a decoupling of RNA, biomass P and growth at low growth rates (Flärdh et al. 1991). This pattern was also shown in additional studies on bacteria (*E.coli, Streptomyces coelicolor, Mycobacterium bovis, Selenomonas ruminantium*), budding yeast (*Saccharomyces cerevisiae*), fungi (*Neurospora crassa*), algae (*Prototheca zopffi*) and various metazoans (Vrede et al. 2004, Karpinets et al. 2006).

These observations, lead me to the assumption that at low growth rates different cellular processes may interfere with the mechanisms underlying the GRH. In order to find a conceptual framework to explain microbial behavior at low growth rates, as well as at medium and high growth rates, two attempts were made by enhancing the disposal soma theory (Nyström 2004) and the dynamic energy budget (DEB) theory (Kooijman et al. 2000) and linking it to the GRH (Vrede et al. 2004).

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The growth rate hypothesis (GRH) “It has been proposed, that an increase in growth rate requires an increase in protein biosynthesis and, therefore an additional allocation of cellular resources to synthesis of ribosomes and ribosomal RNA, which comprise the main part of cellular RNA. As a result of this allocation, the P:N ratio in the cell increases. This idea is referred to as the growth rate hypothesis.” (Karpinets et al. 2006)

The dynamic energy budget (DEB) theory aims to identify simple quantitative rules for the organization of metabolism of individual organisms. It delineates reserve, as separate from structure. Reserves are synthesized from environmental substrates for the purpose of somatic maintenance, growth and reproduction. Reserves as well as structure are to be general compounds, i.e. mixtures of a large number of compounds, which do not change in composition (Kooijman et al. 2000).

http://www.bio.vu.nl/thb/deb/index.html
Each of these hypotheses states that there are basically two types of readily distinguishable life strategies in microorganisms: growth/reproduction and maintenance/survival. The consensus between these theories states the existence of a conservative core of the cellular molecular machinery in all biota responsible for the housekeeping functions. It regulates the growth and protein synthesis in response of environmental change, and therefore causes unicellular organisms to conserve cellular resources under stressful conditions by directing them to maintenance instead of reproduction. Specifically, under resource limitation (i.e. shortage in mineral P supply) it increases the protein synthesis rate per ribosome to maintain a given growth rate, while on the other hand, it minimizes the allocation to P-rich rRNA (Vrede et al. 2004, Karpinets et al. 2006). These theories later help define the applicability of the GRH and its explanatory power, and they also explain the above stated cases at low growth rates, in which the GRH relationships decouple.

This suggestion of a unified cellular machinery looks like a promising way to describe organismal behavior with all its implications and broad scale influences, but is unfortunately not yet fully understood. One advantage is that it is not only applicable to heterotrophic copiotrophic bacteria as shown in lab experiments, but also is present in various protozoa and metazoan (see above).

**Application of the GRH and its enhancements to natural communities.** In order for a GRH type relationship to be applicable on an ecosystem scale one must consider its’ applicability to phototrophic
organisms as they contribute a significant part of the total biomass. Previous research argues that these theories (GRH, disposal soma theory and the DEB) are not applicable to phototrophs, because they show large variations in N:P biomass stoichiometry due to changes in rubisco and pigment content varying with light and nutrient supply (Vrede et al. 2002, Makino et al. 2003, Karpinets et al. 2006). This argument may apply to terrestrial phototrophs (e.g., angiosperms), but perhaps not to aquatic autotrophs (e.g., cyanobacteria). On the other hand, studies on P limited cyanobacteria (e.g., Anabaena) and on green algae (e.g., Scenedesmus) showed that growth rate correlated with both cellular RNA and P (Healey & Hendzel 1975). Furthermore, a study on a marine Synechococcus strain revealed that rRNA and growth rate were correlated at intermediate growth rates ($\approx 0.7$ to $1.5$ d$^{-1}$). However, at low growth rates ($\approx 0.2$ to $0.5$ d$^{-1}$) rRNA cell content remained almost constant and at high growth rate ($\approx 1.6$ to $1.8$ d$^{-1}$) rRNA cell content did not increase with growth rate most likely because of saturation effects (Binder & Liu 1998). In this study different growth rates were achieved by altering light intensities. So although this organism is phototrophic, its behavior is explainable by the GRH for intermediated growth rates and by changes in ribosome efficiency at both high and low growth rate as hypothesized by the DEB and the disposal soma theory.

Although these examples provide no prove for the applicability of the GRH on aquatic phototrophs. Because the relationship becomes more complex if more than one environmental variable is considered simultaneously (e.g. resource stoichiometry and light density). But it gives an insight of possible problems when applied to natural communities and shows on the other hand the hidden potential within these concepts for future research.
**The GRH in different habitats.** It has been observed that the GRH mechanisms are problematic in nitrite and ammonium (aerobe (Morgenroth et al. 2000); anaerobe (Schmid et al. 2001)) oxidizing organisms and after repeated feast and famine cycles (Frigon et al. 2006). These studies were performed with wastewater bacteria, where bacterial communities are shaped by different biotic and abiotic factors compared to natural environments. But also in P-limited freshwater and marine ecosystems the above stated theories did not fully predict the performance of growth at different stoichiometric settings (Kemp et al. 1993, Makino & Cotner 2004). This observation leads to the question whether the GRH, DEB and disposable soma theory are applicable to all types of ecosystems in consideration of their community structure. And if not, what implications does it have on the general application to natural environments. Such as predictions can only be made for relationships till community level. But not for interactions between different communities for which in some cases the above stated ideas are not applicable.

**Synthesis.** My data suggest, that the GRH is applicable to *V. spinosum* an abundant bacterium in natural environments under low P ratios. Furthermore, there is enough evidence to argue that the GRH is a useful tool to connect physiology with ecosystem processes. The improvement of the GRH with the consideration of what influences ribosomal efficiency could prove to be even more robust. Yet there are still questions to be answered. For instance, how do single species and community structure influence growth in communities? Or, how general are these observed patterns and to which organizational level can we apply them (single habitats or whole ecosystems).
ACKNOWLEDGEMENTS:

Funding for this work came from the MICDIF project. I want to thank Margarete Watzka for providing the C, N analysis data, Katharina Keiblinger for the P analysis data. Ieda Nunez Hämerle and Marvin Pölzl for their help in cultivating and growing *V. spinosum*, and Edward K. Hall for calculating the growth rate models and his continuous support and advice.
REFERENCE:


ANHANG:

ZUSAMMENFASSUNG:


Ich konnte zeigen, dass die GRH im Fall der Phosphatlimitierung auf *V. spinosum* anwendbar ist. Und dass diese Beziehung verloren geht, falls ein anderer Nährstoff zum limitierenden Faktor wird. Ergänzend beschäftigte ich mich mit den weiteren Auswirkungen unserer Ergebnisse in Anwendung auf natürliche Ökosysteme und den damit einhergehenden Problemen.
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