

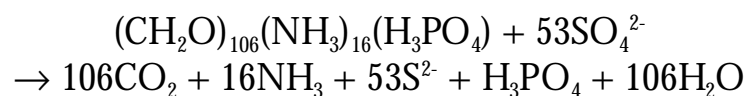
Part II:
Anoxic Decomposition of the Mucins of
The Marine Naticid Gastropod
Polinices duplicatus

Introduction

A basic understanding of the major decomposition pathway of mucus from marine gastropods by microbes is essential to infer the role such secretions might play in early diagenesis (Berner, 1981). The following section describes an attempt to elucidate overall rates of decomposition and also whether a natural microbial assemblage from a coastal estuary decomposes this complex carbohydrate-glycoprotein substrate homogeneously or by selecting certain portions over others. The approach used to investigate utilization of mucus takes advantage of the release of major soluble by-products of anaerobic decomposition and the rates with which they are released relative to one another, to calculate the approximate average stoichiometry of the decomposing organic molecules (e.g. Aller and Yingst, 1980).

Despite the simplicity of this approach, there are certain factors which have to be considered when designing these experiments, so that they are as realistic and as meaningful as possible:

Anaerobic decomposition - Polinices duplicatus, as most naticidae (Kabat, 1990; Weissberger, 1999), is infaunal, therefore it is most likely that the majority (if not all) of the mucus produced and secreted will be deposited in deeper, anoxic sediments. Therefore, decomposition under anoxic conditions is much more likely than under aerobic conditions. Most marine sediments are dominated by sulfate reduction (Berner, 1981). Sulfate reduction-mediated organic matter decomposition may be represented by the following chemical equation (Froehlich *et al.*, 1979):



It would, therefore, be reasonable to use conditions leading to sulfate reduction-mediated decomposition of mucus. This may be easily achieved by using pore water from sulfate-reducing sediments as an inoculum for an incubation. This pore water will presumably be relatively enriched in microbial assemblages which are predominantly characterized by this process.

Temperature - The temperature of decomposition is significant with regards to decomposition rates due to its effect on enzymatic activity. A lower temperature limit is set by the fact that below 10° C, this gastropod exhibits a rapid drop in metabolic rates, crawling activity, feeding rates and growth (Huebner, 1973; Edwards and Huebner, 1977; Huebner and Edwards, 1981), and consequently mucus deposition is naturally minimal at these temperatures. Although these experiments do not investigate decomposition rates *per se*, it should be deemed wise that they be carried out at a temperature resembling that of natural waters at a time period when mucus is deposited.

Surface Association and Interaction - Adsorption and surface-enzyme interactions (Mayer, 1989) are among the factors contributing to differences between rates of microbial decomposition of mucus between various marine regimes, e.g. soft sediments (such as in this case), rocky substrates (such as in the case of other marine gastropods, e.g. limpets) and the water column. Adsorption of organic substances to particles (such as sediment grains) might affect the availability of certain parts of the molecules to hydrolyzing enzymes, and result in different hydrolysis rates than those involving unbound molecules (Mayer, 1994; Rontani and Bonin, 2000). It is also suggested that the activity of proteolytic enzymes in sediments is associated more with particle surfaces than with pore-waters (Mayer, 1989). Therefore, a surface similar to a sedimentary one may be provided by using glass beads on to which mucus is deposited by animals.

However, in natural sediments, the substrate decomposition pattern may differ in the presence or absence of other constituents, such as phosphorus-rich minerals, charged clays or ambient organic matter of terrestrial or planktonic origin. This is briefly investigated here by separate incubations of mucus either alone or mixed with sediment.

Decomposition indicators - The decomposition indicators used in this study are the solutes ΣCO_2 and NH_4^+ , since they are general products of decomposition reactions (including fermentation). The possibility of these species being consumed during processes such as methanogenesis is ignored under the conditions of the experiment. The production of these solutes gives

an approximate indicator of the C:N composition of the decomposing organic matter (Berner, 1981).

Methods

Experimental Procedure

Mucus used in the incubations was collected using acid-rinsed, combusted glass beads of average diameter 175 μm . Snails were transferred for several minutes into a container of well-aerated, 0.2- μm filtered seawater and rinsed, and then were allowed to crawl over the beads in 0.2 μm -filtered seawater for several minutes. This ensured that the substrate present is gastropod mucous secretions. Anoxic sediment was collected from Flax Pond, a coastal estuary whose sedimentary diagenetic environment is dominated by sulfate reduction (Swider and Mackin, 1989). The sediment was sieved through a 0.5 mm sieve in a glove bag under N_2 . Seawater, also collected from Flax Pond, was filtered twice through 0.2 μm filters, and was deoxygenated with a gas mixture of N_2 and CO_2 , to maintain natural pH.

Commonly, experiments of two types were performed, usually in parallel with the same materials, consisting of a total of five separate incubation treatments:

- (a) Plain mucus incubations, where the treatments included: (1) mucus-coated glass beads, (2) clean glass bead controls, and (3) de-oxygenated seawater controls; and
- (b) Sediment-mucus incubations, where the treatments included: (1) sediment mixed with mucus-coated glass beads, (2) sediment mixed with clean glass beads control, and (3) de-oxygenated seawater controls, as in (a).

Treatments were set up in triplicate with an average of five separate times. All vials containing substrates were filled to the top with de-oxygenated seawater, to provide additional solvent for solute analysis. The treatments in plain mucus incubations were inoculated with 50 μl of porewater from the anoxic sediment, which acted as an inoculum of natural bacterial assemblages. The vials were stored in O_2 -impermeable bags with O_2 scrubbers, which in turn were placed in an incubator set at 14 $^\circ$ C, and removed at pre-set time intervals. All vials were weighed as each component was added so that compositions were known and could be calibrated for.

At each time interval, the contents of the vials were well mixed by shaking gently for several seconds. They then were centrifuged at 1,000 rpm for 10 minutes so that the particulate portion would settle. The supernatant was removed, filtered through a 0.2 μm filter and analyzed for ΣCO_2 , NH_4^+ (see below; Hall and Aller, 1992) and phosphate (Presley, 1971). Although the amount of P in mucus was shown to be small (see Part I), this analysis was run due to the importance of the element in sediment mineral and organic matter cycling. The particulate portion was used to determine porosity using wet-dry mass balance, assuming densities of 2.6 g/ml for sediment and 1.03 g/ml for seawater.

Analytical Methods

Total Carbon Dioxide (ΣCO_2). ΣCO_2 was measured using a technique developed by Hall and Aller (1992) which allows for the use of very small (10-40 μl) volumes of sample. This technique depends on the fact that the stable form of ΣCO_2 in an acidic solution is gaseous. In this case, a solution of 30 mM HCl carried the sample over a gas-permeable, teflon-coated membrane. On the other side of the membrane, a basic solution (in this case, 10 mM NaOH) flowed in the same direction and received the transferring solutes of interest. The basic solution is then passed through a conductance meter where the amount of the solute was quantified. Standards were prepared in an NaCl solution, since dissolved salts seem to have a small but detectable effect on the conductivity measured. They consisted of solutions of NaHCO_3 of concentrations between 1.25 – 5 mM. The correlation of conductivity and concentration in this range was linear and gave a r^2 value typically greater than 0.995 when a sample size of 20 μl was used.

Ammonium. Ammonium was measured using the same method as ΣCO_2 , but by reversing the pH of the carrier solutions. The initial carrier in this case was a 10 mM NaOH/0.2 M Sodium Citrate solution, and the receiving carrier was a 50 μM HCl solution. Standards were made using NH_4Cl in the concentration range of 10 - 250 μM , above which the relationship between conductivity and concentration is not linear with sample sizes of 20 μl .

Phosphate. Samples fixed with concentrated HCl were tested colorimetrically (Presley, 1971) to determine dissolved inorganic phosphate concentrations in porewater. Standards consisted of 0 – 50 μM solutions of KH_2PO_4 .

Results

Plain mucus incubations

Typical results of one experiment are shown in Figure 1. In general, controls did not exhibit any change in solute concentrations. The only exception was observed in the concentration of ΣCO_2 in clean glass bead incubations, and that presumably indicates the decomposition of DOC in the organic-rich estuarine water of Flax Pond by the inoculum, enhanced by the increased surface area provided by the spheres. In the experimental treatments, a clear increase in both solutes is observed over time. No change greater than the analytical error was observed in phosphate in any treatment.

The results of all the experiments are pooled together in Figure 2 in a stoichiometric plot. Significant increase in solute concentrations evidently takes place only in the case of the experimental treatments and thus may be safely attributed to the decomposition of mucus. A linear regression through the experimental points gives a slope which corresponds to a ΣCO_2 to NH_4^+ ratio of 4.8. No correction for adsorption was made.

Sediment-mucus incubations

The results for these experiments are shown in Figure 3, in a stoichiometric plot. The major complication with this set of experiments is the large signal given off by sedimentary organic matter decomposition. Therefore, while there is seemingly a difference in the signal given by the release of ΣCO_2 and NH_4^+ ($\Sigma\text{CO}_2/\text{NH}_4^+ = 12.2$ in control versus 8.3 in experimental), when one combines the experimental and control data, one obtains an almost equally significant correlation ($r^2 = 0.845$) with a slope value approximately averaged between the two (10.2).

A null hypothesis of the slopes of the two data sets being equal was tested using a procedure described by Bethea *et al.* (1995). The following statistic was calculated:

$$T = \frac{\beta_1 - \beta_2}{\sqrt{S_{\beta_1}^2 + S_{\beta_2}^2}}$$

where β_i = the slope of each linear regression
 $S_{\beta_i}^2$ = the variance of each data set

$$= \frac{\hat{\sigma}^2}{\sum (X_{ij} - \bar{X}_i)^2}$$
 \bar{X}_i = mean of data group

$$\hat{\sigma}^2 = \frac{SSE_1 + SSE_2}{n_1 + n_2 - 4}$$
 SSE_i = error sum of square for each data set

$$= \sum (Y_{ij} - \bar{Y}_i)^2 - \beta_i^2 \sum (X_{ij} - \bar{X}_i)^2$$
 n_i = degrees of freedom

The null hypothesis, that $\beta_1 = \beta_2$, is accepted if $t_{v, \alpha/2} < T < t_{v, 1 - \alpha/2}$, where $v = n_1 + n_2 - 4$ degrees of freedom, in this case 25. At $\alpha = 0.02$ (i.e. a 98 % confidence interval), $t_{25, 0.01} = -2.49$, and $T = -2.66$. At $\alpha = 0.01$ (i.e. a 99 % confidence interval), $t_{25, 0.005} = -2.79$. Therefore, the hypothesis is accepted at 99 %, but at a 98 % confidence interval the two relationships are shown to be different.

Furthermore, a logarithmic relationship of the form $y = m \cdot \ln(x) + c$ gives an even better fit than a linear regression does (Figure 4). This fact suggests that there could be a change in the response of the incubating assemblages to the available substrate, switching into a different pool richer in N. Alternatively, it could be an artifact resulting from the precipitation of CaCO_3 due to quite high ΣCO_2 concentrations in the interstitial spaces. As a result, only NH_4^+ changes were detected. The statistical test described above was applied in this case by letting $\ln(x) = X$, therefore $y = mX + c$. The calculated T equals -4.91, constituting the difference between the two data sets statistically significant even at the 99 % interval, whereas the linear relationships were not.

Discussion

From the plain mucus incubations, it is evident that at 14° C mucus is decomposed readily over several days by natural microbial assemblages. In

addition, from the ratio of ΣCO_2 to NH_4^+ released (Figure 2), it appears that a nitrogen-rich portion of the mucous substrate is being preferentially decomposed. Whereas the C to N ratio of *P. duplicatus* pedal mucus has been measured to be approximately between 10 and 11.6, the signal given here is half of that, suggesting that primarily the protein moiety is being decomposed first.

This inference also depends on possible simultaneous reactions. Adsorption of NH_4^+ can occur, although it has been shown that, in the case of the glass beads used in these experiments, it is very little (R.C. Aller, unpublished data). In the present case, any adsorption would only accentuate the conclusion that a nitrogen-rich substrate is decomposed. Another pool where solutes might end up and not be detected is microbial biomass. In such sealed incubations, it is rarely in steady state, and commonly increases (Fenchel *et al.*, 1998). Again, assuming that microbes will be assimilating matter at a C:N ratio of approximately 5.5 (Fenchel *et al.*, 1998), this could only shift the ratio of the released solutes in favor of ΣCO_2 . If that is indeed the case here, then the substrate C:N ratio is lower still.

The results of the sediment-mucus incubations also suggest that preferential decomposition of a nitrogen-rich moiety within the mucus takes place. Adsorption of ammonium in such sediments is expected to be substantial, therefore driving all the slopes to lower values when incorporated into the relationships. Since it is not known here, the C:N ratios of 12.2 and 8.3 given by the slopes of the linear fits should be referred to with caution, since they are relative to one another but not absolute. There appears to be a latent response to the presence of a substrate richer in nitrogen than the ambient one, and this might be explained by postulating that although hydrolysis of these large mucopolysaccharides might be rapid (Arnosti *et al.*, 1994; Arnosti, 1996), the evolution of the ultimate decomposition products, such as NH_4^+ , is gradual (Arnosti and Repeta, 1994). This is expected if large mucopolysaccharides are hydrolyzed to smaller units which, nonetheless, need further hydrolysis to reach a size small enough to enter the microbial cell, where they will be respired and converted to these decomposition products.

In any case, due to this latent response, a logarithmic regression provides a better fit through either data set (Figure 4). This, in essence, is a demonstration of a better fit of a multiple G model than a single G model (Berner, 1981). Possible explanations for this do not involve only the mechanics of decomposition of the substrate by bacteria, but also artifacts of the experimental method which encourage certain species to accumulate in high concentrations, so as to induce processes which might not be expected

otherwise. For example, these closed incubations force ΣCO_2 concentrations high enough, in ranges where CaCO_3 might precipitate (Aller *et al.*, 1996), and thus remove ΣCO_2 and result in the slope observed.

Conclusion

When mucus of *P. duplicatus* is exposed to natural sedimentary microbial assemblages, it is decomposed steadily over a period of several days. It appears that the nitrogen-rich protein pool of the mucus is decomposed first since the solutes under study give a very low $\Sigma\text{CO}_2/\text{NH}_4^+$ signal, approximately half of that measured by CNS directly on mucus (see Part I). Any effects on mucus decomposition by sedimentary matrices such as minerals and ambient organic matter were not resolved in detail in this study.

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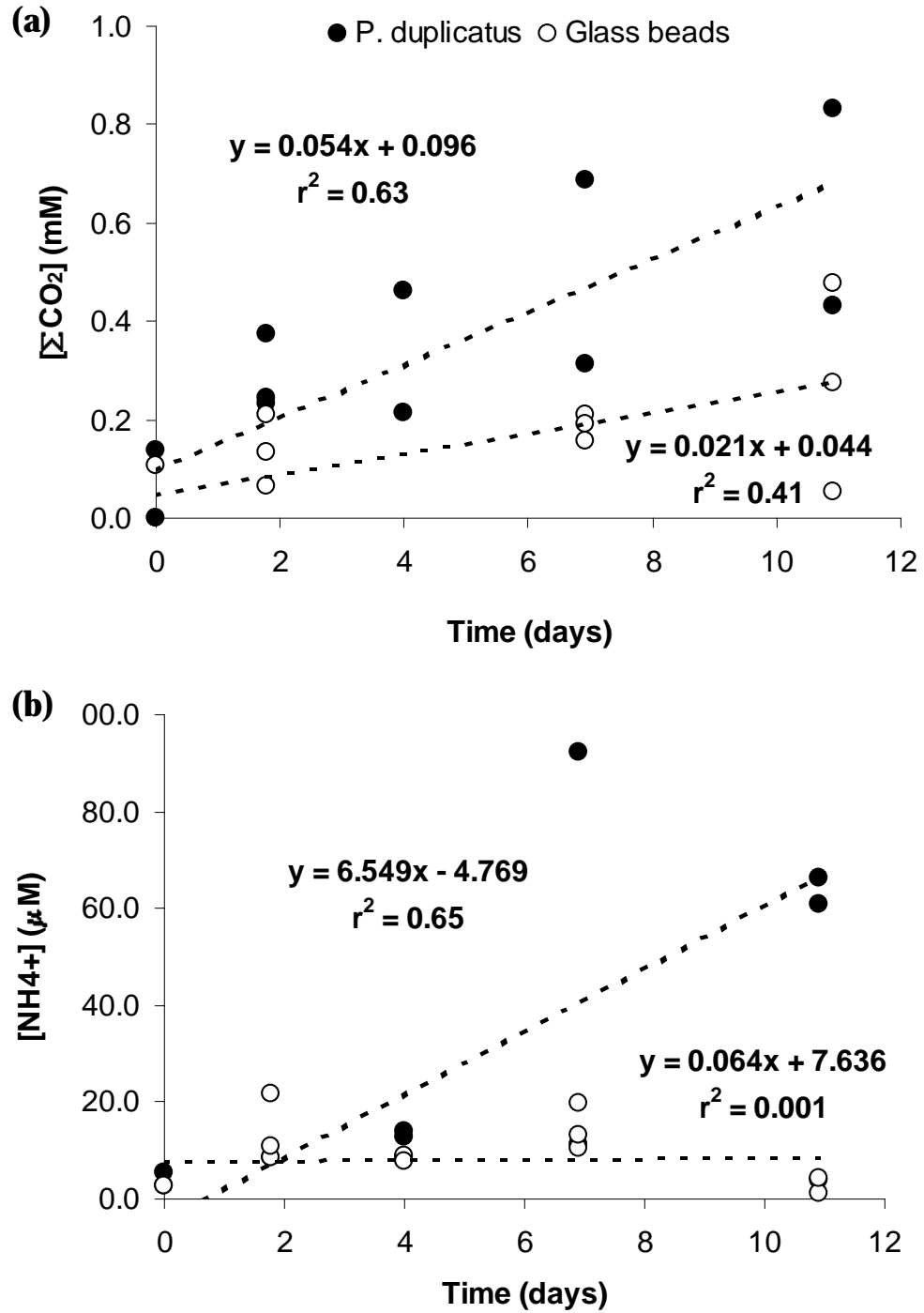


Figure 1. The change of **(a)** ΣCO_2 , and **(b)** NH_4^+ concentrations with time during a plain mucus incubation experiment. Linear regressions are illustrative of the general trends of concentration change over time.

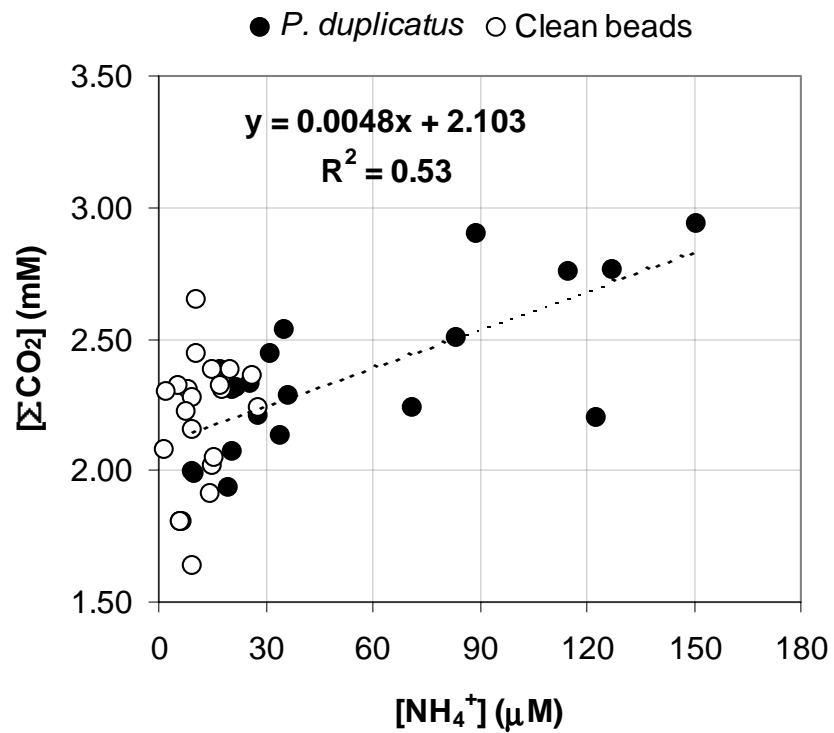


Figure 2. Results of plain mucus incubation experiments. Concentrations of ΣCO_2 and NH_4^+ in all vials are plotted against one another. The slope of the linear regression through the experimental values corresponds to a ratio of ΣCO_2 to $\text{NH}_4^+ = 4.8$.

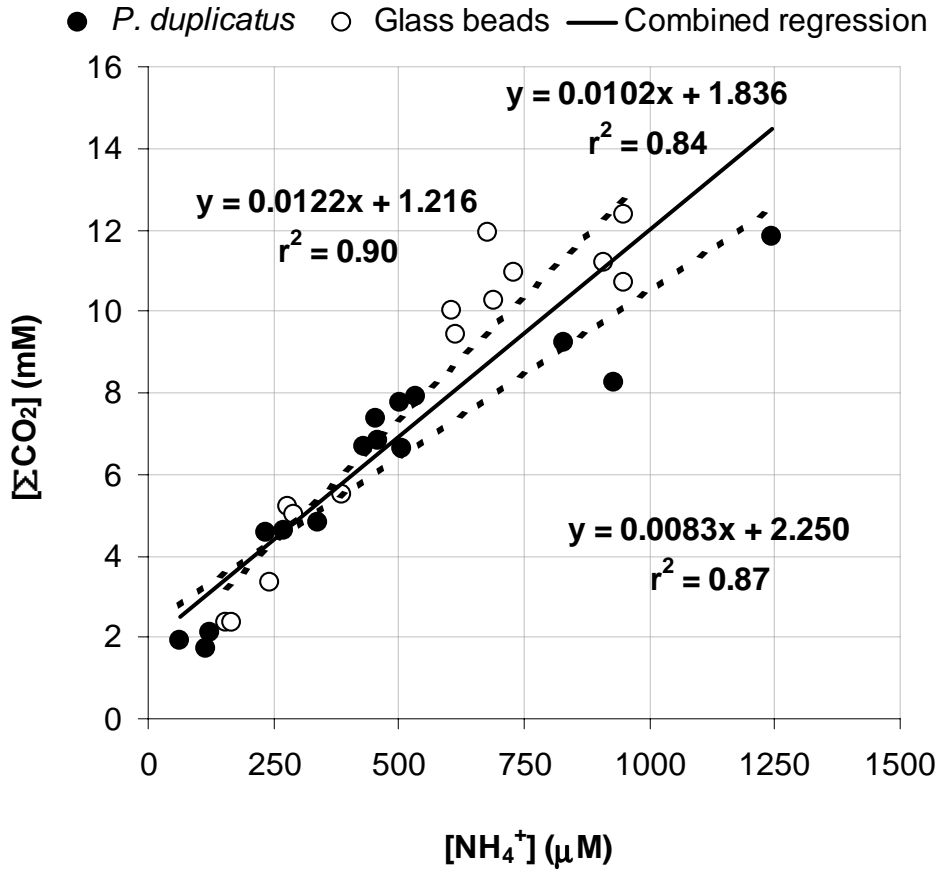


Figure 3. Results of sediment-mucus incubation experiments - linear regressions. Regressions indicate a different ΣCO_2 to NH_4^+ ratio released between the two treatments, consistent with decomposition of a nitrogen-rich substrate in mucus. Although a regression through both data sets is almost equally significant, a test of this hypothesis shows that the two linear relationships are statistically different.

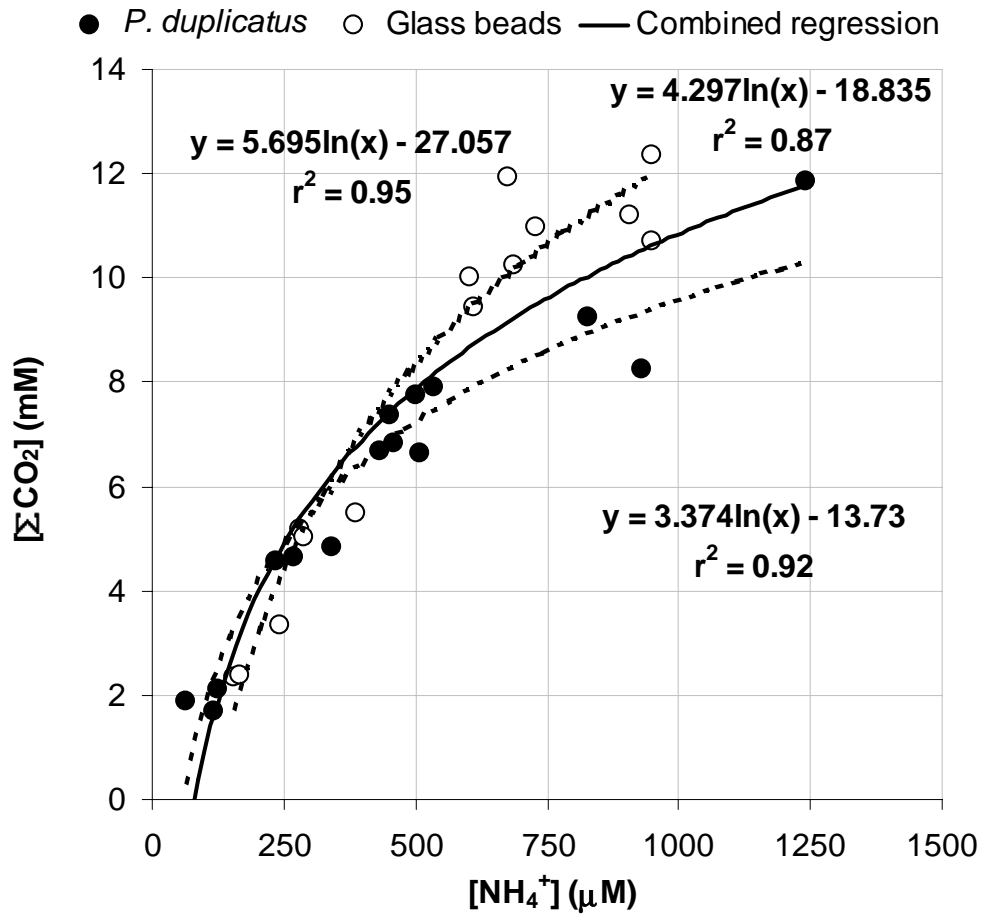


Figure 4. Results of sediment-mucus incubation experiments - logarithmic regressions. These regressions give better fits than linear ones, suggesting either a change in the sedimentary pool decomposed by the assemblages or experimental artifacts, most likely CaCO_3 precipitation.