Importance and implications of respiration quotient (RQ): Bacterial experiments with *Pseudomonas nautica* and *Vibrio natriegens*

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Abstract

Respiratory metabolism in two different physiological states of acetate and pyruvate grown cultures of *Pseudomonas nautica* and *Vibrio natriegens* were compared. Here we analyze 35 hours experiments and 520 hours experiments in which time-courses of protein, pyruvate, acetate, respiratory CO_2 production (RCO_2), respiratory O_2 consumption (RO_2), isocitrate dehydrogenase (IDH) activity, and potential respiration were measured. The cultures were monitored through their exponential growth, their steady state, and their senescence phases. Respiratory quotients (RQs) were calculated from the ratio of the respiration rates (RCO_2/RO_2). Such RQs are widely used in ocean ecosystems models, calculations of carbon flux, and in evaluations of the autotrophic-heterotrophic nature of the ocean, its metabolic balance. In all cultures, regardless of bacterial species and carbon source, the RQ tended to rise nearly an order of magnitude from values below 1 during nutrient sufficiency to values close to 10 during nutrient deficiency. The respiration rates during the growth period paralleled the biomass increase but after the nutrients were exhausted the respiration rates fell. Through this same transition period the IDH activity and the potential respiration (ϕ) remained relatively high for first 10 hours of nutrient deprivation and then fell slowly, along with the biomass, as the nutrient deprivation continued. During this starvation period the biomass specific IDH and ϕ decreased. This finding challenges the idea that IDH and the respiratory electron transport systems (ETS) are constitutive and can be used for biomass proxies. We conclude that the physiological state of the bacteria affects the RQ. These results argue that many ecosystems models, oceanographic calculations of carbon flux, and evaluations of the ocean's metabolic balance need to be reconsidered in light of this newly discovered RQ variability.

Keywords: O_2 consumption, CO_2 production, IDH, potential respiration (Φ), growth

1. Introduction

Respiration (R) in the ocean has many facets. In the deep waters of the Black Sea it produces hydrogen sulfide, in the anoxic intermediate waters off the Peru coast it produced nitrite, nitrous oxide and nitrogen gas, in most of the world's ocean it simply consumes oxygen and produces water. However, in all cases, throughout the ocean respiration is ubiquitous and produces CO_2. Here, for the oxic parts of the world ocean, we focus on the relationship between CO_2 production and the concurrent consumption of O_2 (R) during aerobic respiration. R roughly balances oxygenic photosynthesis, production (P), in the marine carbon cycle, but until the Winkler O_2 technique was modified (Bryan et al., 1976) R was not well researched because it is so difficult to measure. With this improved technology, oceanographers began to note an excess of R over P in some parts of the ocean giving rise to the current controversy about the metabolic state of the ocean (Ducklow and Doney, 2013). The quandary is whether the oligotrophic ocean is autotrophic or heterotrophic. Williams et al. (2012) reported in situ observations where the results using natural seawater isotope composition clearly showed an autotrophic state whereby results from modified Winkler analysis on incubated seawater samples showed a heterotrophic one. One reason for this discrepancy could be the assumed value for the respiration quotient (RQ) used to convert R from the Winkler analysis into respiratory CO_2 production (RCO_2). Without this conversion respiration cannot be compared to photosynthesis or plankton productivity. Consequently, a deep understanding and careful measurement of RQ is essential for progress in resolving, not only this oceanographic quandary, but also for making many other marine ecosystems calculations, including carbon flux (Packard and Christensen, 2004; Steinberg et al., 2008; Packard and Gómez, 2013). Here, we report measurements of RQ in two species of marine bacteria growing separately on two different carbon sources, acetate and pyruvate. Furthermore, we show that RQ changes with the starvation level, the carbon source, and the bacterial species.

Via aerobic respiration marine organisms obtain the energy to live from a wide range of compounds that are reduced in different, but well coordinated biochemical pathways. Two of these key pathways are the Krebs or tricarboxylic acid (TCA) cycle and the respiratory electron transport system (ETS). The TCA cycle accepts pyruvate from glycolysis and acetate from β-oxidation of fatty acids, condenses them in separate reactions with oxaloacetate, and oxidizes

the resulting citric acids to different tricarboxylic acids such as glutamic, succinic, fumaric, and malic acid. This oxidation process yields three CO_2 from pyruvate, but only two from acetate. Concurrently pyruvate oxidation by the TCA yields 10 reducing equivalents ($10H^+$) to the ETS while acetate oxidation results in only $8H^+$. These reducing equivalents enter the ETS via complex I and complex II and are rapidly transferred to ubiquinone (Q), reducing it to ubiquinol (QH2). In the case of acetate, the combined stoichiometry of this sequence of reactions, glycolysis, TCA cycle and the ETS results in the production of two molecules of CO_2 and the consumption of two O_2 molecules. The ratio between the produced CO_2 and the consumed O_2, the RQ , would be 1 ($RQ= \Delta CO_2/- \Delta O_2= 1$). In the case of pyruvate, the combined stoichiometry of this sequence of reactions, glycolysis, TCA cycle and the ETS results in the production of three molecules of CO_2 and the consumption of 2.5 O_2 molecules. The ratio between the produced CO_2 and the consumed O_2, the RQ, would be 1.2.

The majority of studies of respiration in the aquatic ecosystems are based on assumed RQ values ranging from 0.7 to 1.2 (Berggren et al., 2012), however, depending on the nutrition, the RQ can vary over a broader range. For example, nutrition based on oxalic acid can result in an RQ of 4. To calculate a true RQ value it is important to accurately calculate CO_2 production. In the past this was difficult and expensive and so was rarely done. Now, with the development of the CO_2 optode, RCO_2 (Mills et al., 1992) measurements are more feasible (Berggren et al., 2012).

In Oceanography there are relatively few measurements of RQ (Oviatt et al., 1986; Robinson et al., 2002). As a result, RQs are calculated theoretically by analyzing the stoichiometry of the complete oxidation of substrates (Takahashi et al., 1985; Anderson, 1995; and Hedges et al., 2002). In this approach, tissues or whole organisms oxidizing pure carbohydrate have an RQ of 1.0, those oxidizing fat, an RQ of 0.7, and those oxidizing protein, an RQ of about 0.8 (Cantarow and Schepartz, 1967; Guyton, 1971; Hoar, 1975; Stanier and Forsling, 1990). Values outside this range (i.e. 0.7–1.0) are relatively rarely encountered, although RQs below 0.7 can be associated with gluconeogenesis, and transient increases above 1.0 can result from the conversion of carbohydrate into fat (Cantarow and Schepartz, 1967). This variability in RQ can indicate fundamental shifts in bacterial physiology and carbon

consumption that may occur along the environmental gradients and that cannot be deduced from other measurements (Berggren et al., 2012).

Here we show that RQ can range higher the range reported above. To investigate this wide range, we examine time-courses of the physiological respiration rates (RO_2 and RCO_2) and activities of the enzyme, isocitrate dehydrogenase (IDH) and the respiratory ETS (potential respiration, ϕ) in acetate and pyruvate-grown marine bacteria, *Pseudomona nautica* and *Vibrio natriegens* cultures.

2. Material and Methods

2.1 Experimental design.

To investigate the RQ in different bacterial growth stages, time-course experiments were run on batch cultures, maintained on pyruvate or acetate as described in Berdalet et al. (1995) and Packard et al. (1996[b]). Short term experiments were run for maximum 35 hours, long term ones were run for 2-3 weeks. The bacteria cultures were grown in 25 cotton-plugged 500 ml Erlenmeyer flasks containing 100 ml of media. The optical density at 550 nm (OD550) had an initial value after inoculation of 0,1 absorbance units. At 2 h intervals, 2 flasks were chosen randomly, 25 ml of culture were transferred to the Oxymax (respirometer) flasks, and the respiration was measured. Then culture samples were taken in duplicate for OD550 (turbidity measured as absorbance at 550 nm), protein, pyruvate, acetate, RCO_2, RO_2, IDH activity, and potential respiration (ϕ) as the cultures grew exponentially, reached steady state, and passed into senescence.

2.2 Bacterial cultures.

Vibrio natriegens (ATCC 33788) and *Pseudomona nautica* (Strain 617 from Dr. P. Bonin, Université de la Méditerranée, Marseille, France) were used for these experiments. The culturing has been described by Berdalet et al. (1995) and Roy et al. (1999). Before an experiment, the bacteria had been adapted for a minimum of 15 generations to the experimental media (pyruvate or acetate). To inoculate the experiments exponential or

early stationary phase were used. Cultures were continuously agitated on an orbital shaker at 100 rpm at 22^{o}C and growth was followed spectrophotometrically at 550 nm (OD550).

2.3 Culture media.

Pseudomona nautica was cultured according to Packard et al. (1996[a]) and the medium for *Vibrio natriegens* was developed from the media of Niven et al. (1977), Baumann and Baumann (1981), King and Berman (1984) and Nissen et al. (1987). Later, the optimal conditions for growth of *Vibrio natriegens* were established after the experiments with: 400 mM NaCl, 10 mM $MgSO_4 \circ 7H_20$, 10 mM $CaCl_2 \circ 2H_20$, 10 mM KCl, 25 mM NH_4Cl, 0.33 mM phosphate buffer, 0.01 mM $FeSO_4 \circ 7H_20$, and 30 mM sodium acetate or 20 mM pyruvate. Reagents for the culture media were obtained from Sigma-Aldrich Co. All components were dissolved in 0.22 µm filtered deionized water, excepting $FeSO_4 \circ 7\ H_20$ and phosphate buffer and pH was adjusted to 7.5 with 1 N NaOH. To remove particles the solution was filtered through a GF/F glass fiber filter. Later the solution was autoclaved for 45 min at 121°C. Then, to avoid precipitation during the autoclaving, the phosphate buffer (0.67 M, pH 7.5) and the iron sulphate solution ($FeSO_4 \circ 7H_2O$, 0.1 mM) were prepared separately. The PO_4 buffer was sterilized by autoclaving and $FeSO_4$ solution was filtered through 0.22 µm acrodiscs. Finally, both solutions were kept frozen and were added to the culture medium before use.

2.4 Protein measurements.

The bacterial pellets were defrosted, well mixed, and analyzed for protein in aliquots of 0.5 ml by the Lowry Method (Lowry et al., 1951) according to Berdalet et al. (1995). If the absorbance at 750 nm of the samples exceeds 0.4 the homogenates were diluted and analyzed again. For standardization, duplicate measurements of Bovine Serum Albumin (BSA) from Sigma Chemical Company were used.

2.5 Respiration measurements.

Micro-Oxymax respirometer (Columbus Instruments International Corporation, Columbus, OH, USA) measured changes in the concentration of CO_2 and O_2, in the head space of the experimental flasks with an oxygen detector based on the principle of a PbO_2

fuel cell. This instrument has an infrared detector (sensitive to the 2000 µm absorption peak of CO_2), a multiple sample chamber (for up to 20 channels), a reference chamber and a computerized data acquisition and analysis system. Micro-Oxymax works in aerobic conditions because the apparatus can change the air in the head space if the O_2 level fell below 19,3 %. Respiration is given as µmol O_2 /min /l. A normal measurement takes about 30 min. The calibration of the oxygen detector was done with high precision gas standards. The respirometry for all the original measurements was described by Berdalet et al. (1995).

2.6 Biochemical parameters.

Duplicate samples were taken for acetate and/or pyruvate from every flask. Later 5 to 10 ml of culture, depending of the level of biomass, was centrifuged at 10000 x *g* for at 4°C for 15 min. The supernatant fluid was collected in an acid-rinsed Corex tube and then stored in liquid nitrogen for acetate or pyruvate analysis (Ahmed et al., 1976).

High Performance Liquid Chromatography (HPLC) was used by J-P Gagné as described by Berdalet et al. (1995) and Packard et al. (1996a), to separate and quantify acetate and pyruvate in their acid.

2.7 In vitro IDH activity (A_{IDH}).

IDH assay was determinate spectrophotometrically at 340 nm following the NADPH production (Reeves et al., 1971, 1972; Holms & Bennett, 1971; Berdalet et al., 1995). This is the reaction revealing the CO_2 produced from the isocitrate oxidation.

$$\text{Isocitrate} + NADP^+ \leftrightarrow \alpha\text{-ketoglutarate} + NADPH + CO_2;$$

Results are given as µm CO_2 $(min)^{-1}$ (liter of culture)$^{-1}$. The reaction was started by addition of $NADP^+$. The IDH activity is calculated from the regression line of OD340 versus time. NADPH is used as the standard for converting OD340 to CO_2 (µmol) because, from the equation above, NADPH production is stochiometrically equal to CO_2 production.

2.8 Potencial respiration (ϕ).

Depending on the biomass, samples from 5 to 10 ml of culture were centrifuged at 10,000 ×g for 15min at 4 °C and the pellets were stored in liquid nitrogen. Later they were resuspended at 0 to 4 °C in 2 ml of the homogenizing buffer, and measured kinetically for ETS activity with a modification of the Packard and Williams (1981) as described in Packard and Christensen (2004). Final results are converted from ETS units of µmol $e^{-}min^{-1}$ l^{-1} to potential respiration units in µmol O_2 min^{-1} l^{-1} of culture dividing by 4 ($4e^{-}+4H^{+}+O_2 \rightarrow 2H_2O$).

2.9 Statistical analysis

Data were analyzed using the program R from the R Development Core Team 2010 (R Foundation for Statistical Computing, Vienna, Austria). Relationships between R_{CO2}/R_{O2} and IDH/Φ in different time scales (short and long-term experiments) were obtained from the regression equations, using confidence limits of 95% and the Pearson correlation coefficients. ANCOVA were applied to determine statistical differences between slopes and ordinates in the regression lines. Normality of residuals was confirmed by Shapiro-Wilk test.

3. Results

3.1 Time course of respiration, enzyme activity, biomass, and carbon source.

Two types of experiments were analyzed: Short term (Day) experiments that could extend to 35 hours and long term (2-3 week) experiments that ranged between 330 and 520 hours. In those experiments we work with two bacteria species, *Ps. nautica* and *V. natriegens* growing on two different substrates (acetate and pyruvate). In Figure 1 measurements of substrate (carbon source), protein, enzyme activity (IDH and ϕ), physiological respiration (RCO_2 and RO_2) are shown. On the other hand we have the same measurements for long term experiments in Figure 2. Measurements are represented on the same scale, so that conversion factors have to be applied. As with many of the results, the graphics are plotted with different scales to focus on the behavior of the variables over time.

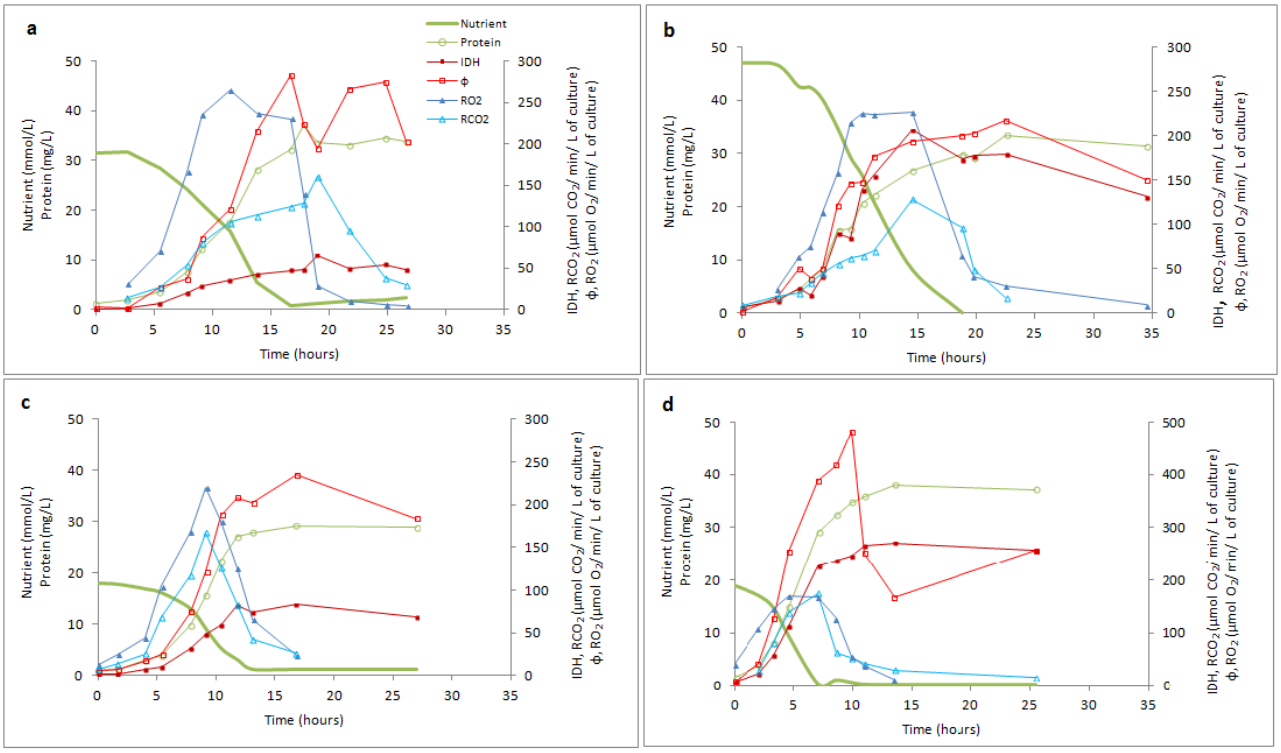

Figure 1. Time courses of respiration, enzyme activity, biomass and carbon source in the short term experiments. The key in panel **a** explains the curves. A. *Ps. nautica* in acetate. B. *V. natriegens* in acetate. C. *Ps. nautica* in pyruvate. D. *V. natriegens* in pyruvate. Note that, depending on the experiment, the cultures are nutrient-sufficient in the first 5-15 h and nutrient-limited (starved) afterwards. To fit this graph the protein data has been divided per 10, RCO_2 and RO_2 has been multiplied per 5.

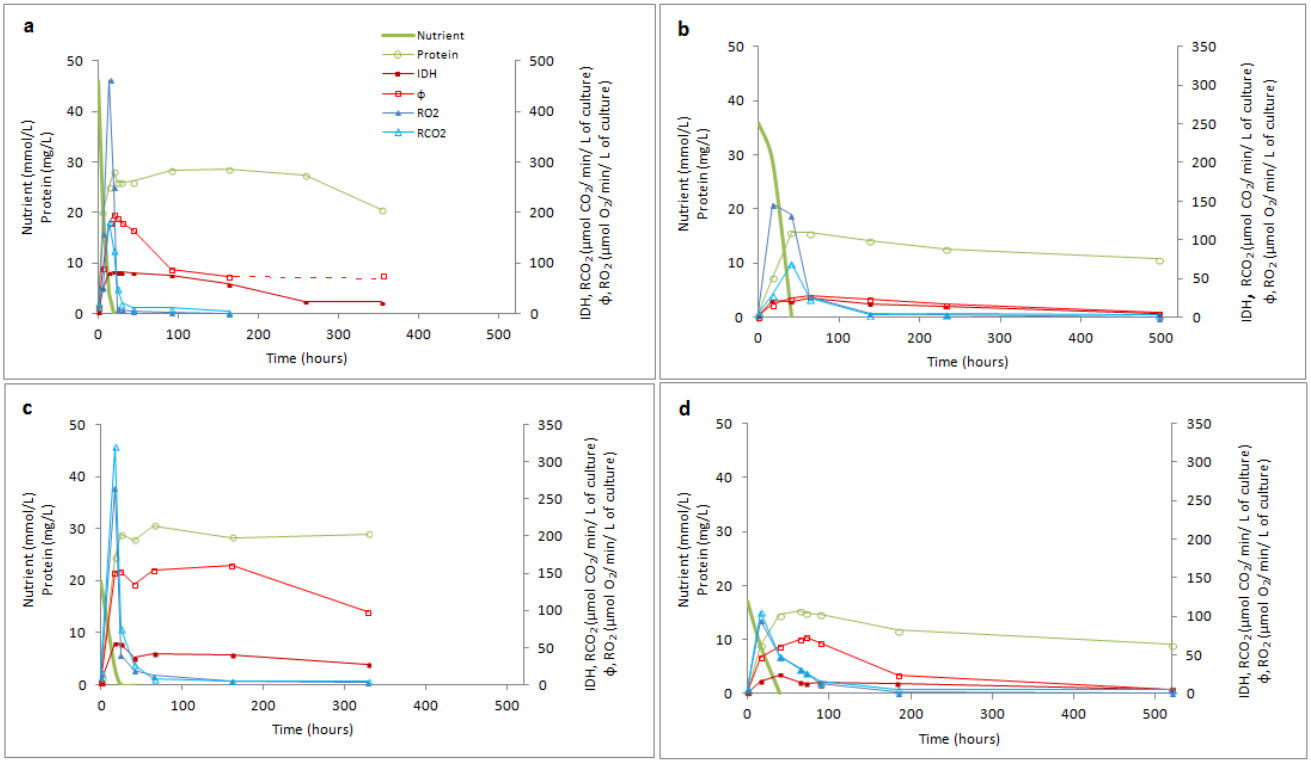

Figure 2. Time courses of respiration, enzyme activity, biomass, and carbon source in the long term experiments. Note that except for the first 30-35 h, the cultures are continually starved for carbon. They are nutrient-limited. During this period the biomass and the enzyme activities decline while the respiration ($RO_2$and RCO_2) is barely measureable. To fit the data on this graph, the protein data has been divided by 10, while the respiration (RO_2 and RCO_2) has been multiplied by 10. Dotted lines symbolize absent data. Dotted lines symbolize absent data.

3.2 Respiration quotient

Short term experiments

RQ measurements of cultures in the short term experiments are shown in Figure 3. When the cultures are nutrient-sufficient the RQ values are low, but when nutrient limitation begins the RQ values rises dramatically in both *Ps. nautica* and *V. natriegens* in acetate. While nutrietion is available RQ values remain constant, but when nutrient limitation sets in RQ jumps to high values. (Figure 3 a,b). For *Ps. nautica* in acetate (Figure 3 a) RQ is more or less constant but after starvation has set in, the RQ rises. In *Ps. nautica*

on pyruvate we can see the RQ rise only at the end of the experiment (Figure 3 c). In contrast to the smooth pre-starvation RQ signals in the first three experiments, in the *V. natriegens* culture growing on pyruvate (Figure 4d) the signal displays noticeable noise between the 7th and the 12 h of the experiment. Afterwards it rises to an RQ of 1.4 at the 25h. In summary, these short-term experiments show a distinct shift in the RQ when either bacteria species is growing on acetate (Figs. 3a and b). In contrast when either species is growing on pyruvate the shift, although real, is much less pronounced (Figs 3 c and d).

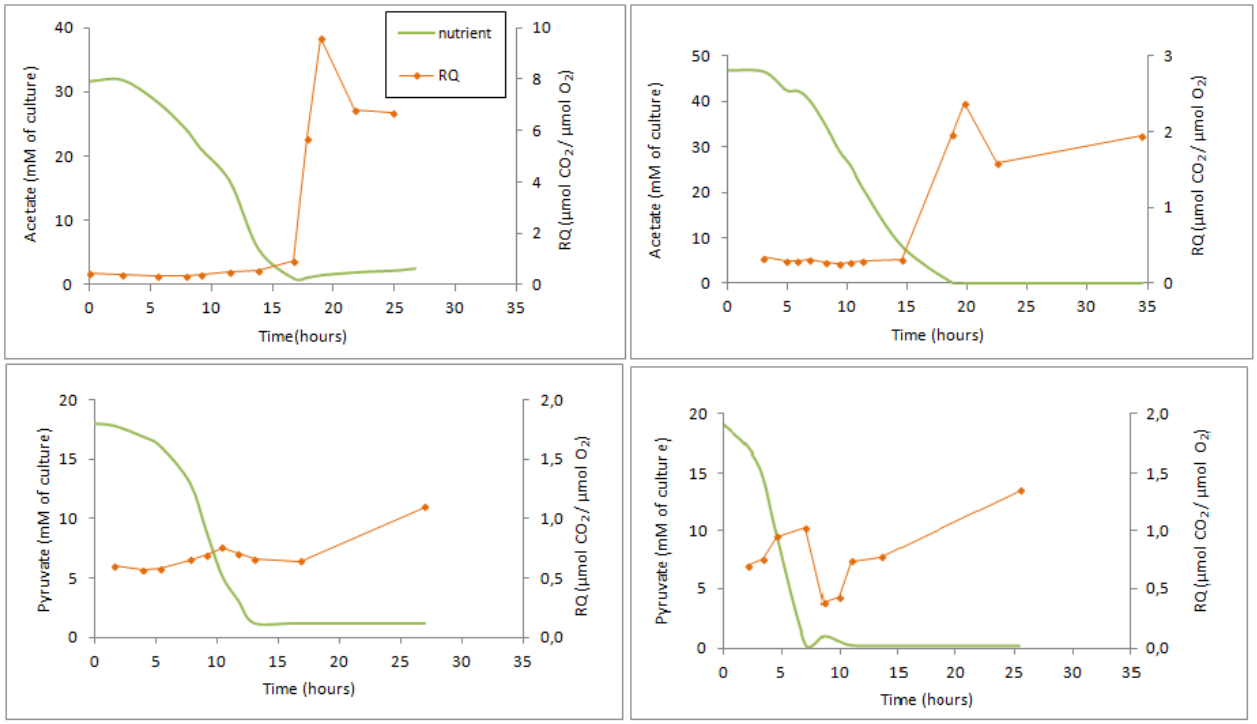

Figure 3. Short term experiments. RQ and substrate (carbon source) versus time. Note the nutrient sufficiency on the left and nutrient depletion (starvation) on the right. The top panels are acetate-based cultures; the bottom panels are pyruvate-based cultures. Left panels are *Ps. nautica.* Right panels are *V. natriegens.* In all experiments RQ rises during starvation.

Long term experiment

For the long term experiments (Figure 4) we see the same behavior as for the short term experiments (Figure 3). In all cases nutrient limitation begins before the first 50 first hours. *Ps. nautica* in acetate (Fig 4 a) is the experiment which has the most similar behavior to the results in the short term experiments. We see an RQ jump when nutrient limitation is reached with a subsequent rise to the end of the experiment (Fig.4a). In *Ps. nautica* growing on pyruvate (Figure 4 c) there is initial noise in the RQ signal, but after nutrient limitation sets in the RQ rise is steady. In *V. natriegens* growing on acetate RQ rises to 1.2 after acetate exhaustion(Figure 4b), but this RQ is much less than the high value (8.0) found in the *Ps. nautica* culture growing on acetate and the high value (4.5) in the *V. natriegens* culture growing on pyruvate (Figure 4 d).

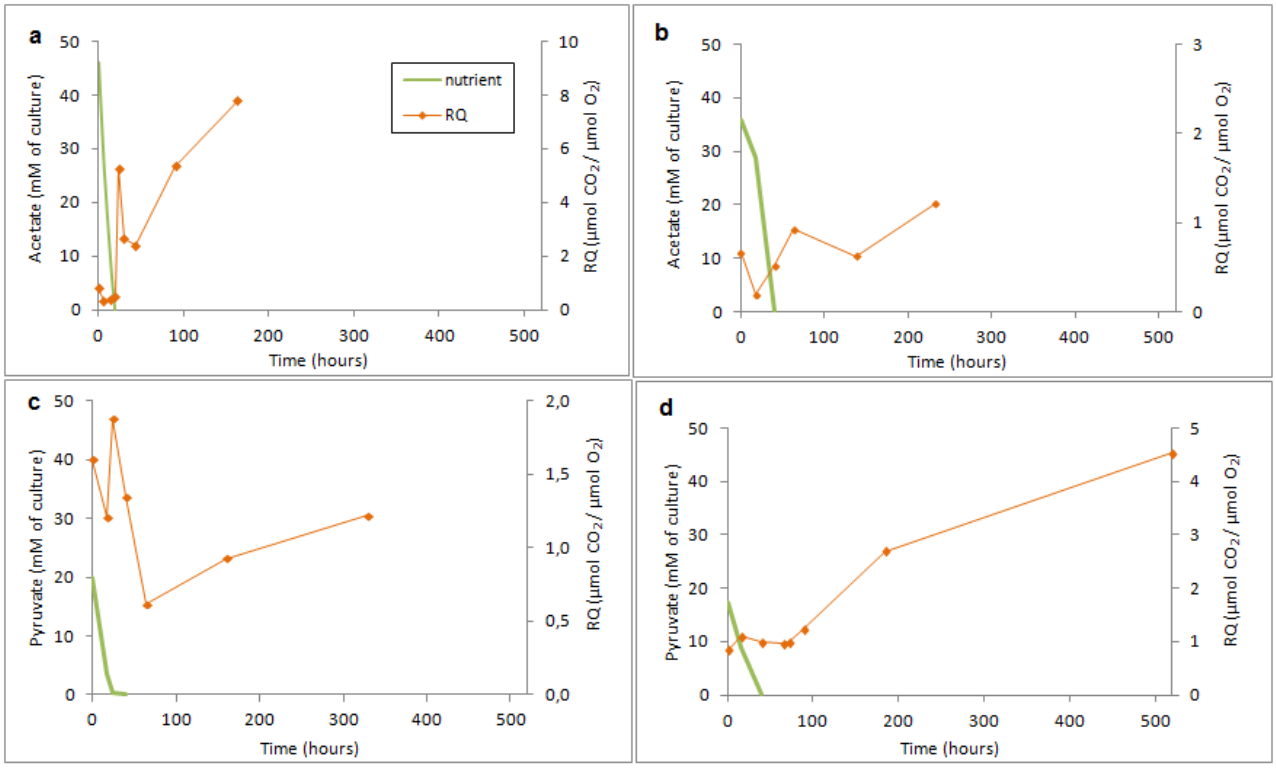

Figure 4. Long term experiments. RQ and substrate versus time. A. *Ps. nautica* in acetate. B. *V. natriegens* in acetate. C. *Ps. nautica* in pyruvate. D. *V. natriegens* in pyruvate. In all panels RQ increases during the late starvation period.

3.3 Physiological measurments: RCO_2 and RO_2.

Short term experiments

Physiological measurments of RCO_2 and RO_2 in time are shown in Figure 5. In all experiments RO_2 is higher than RCO_2 before starvation is reached. We observe that experiments with *Ps. nautica* and *V. natriegens* on acetate are similar. The two curves are out of phase; peaks are displaced in time. In these experiments RO_2 rises rapidly, but as starvation begins, around hour 15, the RO_2 falls. During this fall peaks and then rapidly declines (Figure 5 a and b). In the two pyruvate-based experiments pyruvate decreases from a initial level of 20 mmol/l nearly to zero after about 10 h (Figure 5 c and d). *Ps. nautica* growing on pyruvate exhibits a perfect parallel rise and fall in both forms of respiration (RCO_2 and RO_2).(Figure 5 c). The decrease in respiration occurs before nutrient limitation. However, in *V. natriegens* growing on pyruvate (Figure 5 d), the respiration signals are out of phase but not so much as they were in the both acetate-based cultures.

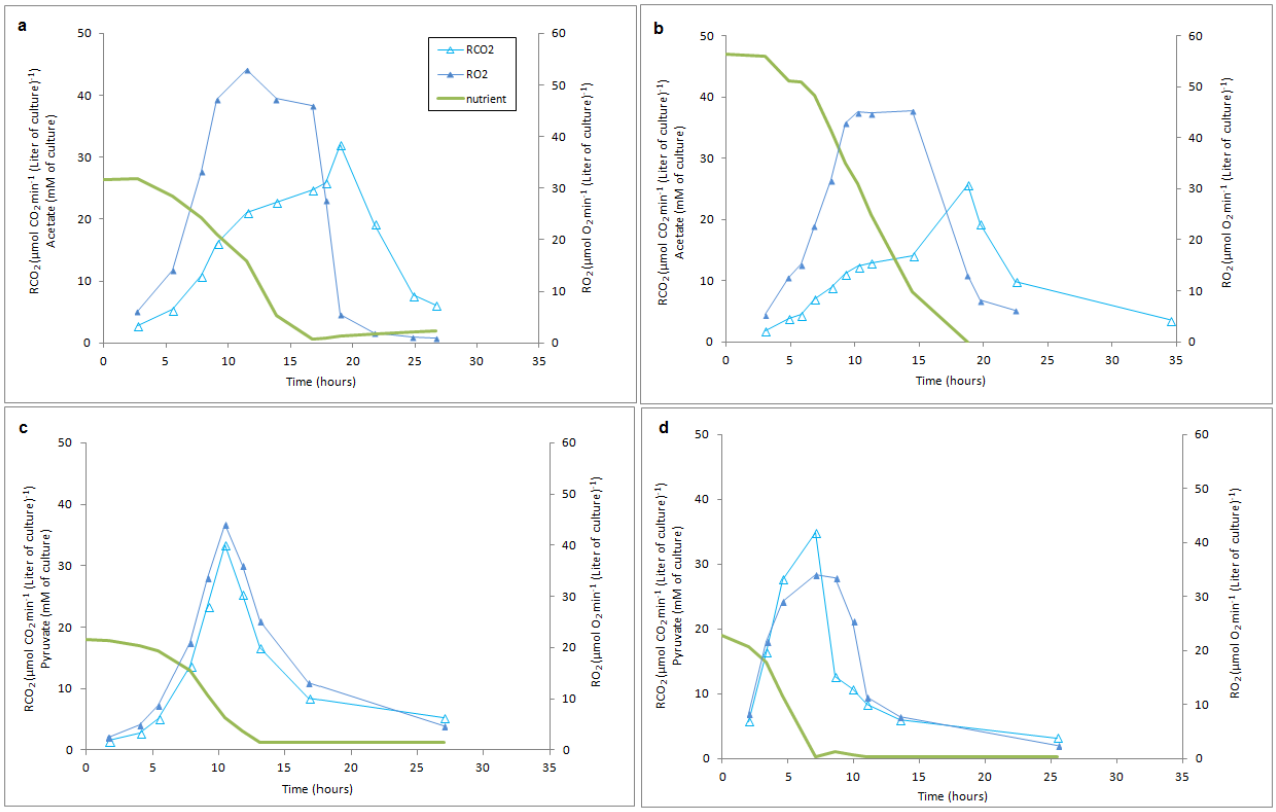

Figure 5. R_{CO2}, R_{O2}, and substrates versus time. A. *Ps. nautica* in acetate. B. *V. natriegens* in acetate. C. *Ps. nautica* in pyruvate. D. *V. natriegens* in pyruvate. As in the

previous Figs. nutrient sufficiency is on the left and nutrient deficiency is on the right. Note that for the cultures growing on acetate, RCO_2 and RO_2 appear out of phase. The RCO_2 is lower than the RO_2 during nutrient sufficiency, but the reverse during nutrient deficiency. This should cause RQ to shift from a low value during nutrient sufficiency to a high value during nutrient depletion in the acetate-based cultures.

Values of RCO_2 and RO_2 for short term experiments are shown in Figure 6. We see that only *Ps. nautica* on pyruvate has a good correlation. (Note the behavior of this experiment in Fig 5c.) In the other three experiments we do not see the same mathematical behaviour in the pooled data. There appears to be no relationship between RCO_2 and RO_2 values. For example, in case of *Ps. nautica* growing on acetate, for an RO_2 of 6 µmol O_2 min/l we obtain a value for RCO_2 of about 3 µmol CO_2 min/l and 33 µmol CO_2 min/l (Fig. 6). However, if the nutrient-starved and the nutrient-sufficient data are considered separately, two different relationships appear in each experiment. Lines indicating low RCO_2/RO_2 ratios occur during well-fed, nutrient sufficient conditions and lines indicating high RCO_2/RO_2 ratios occur in starvation.

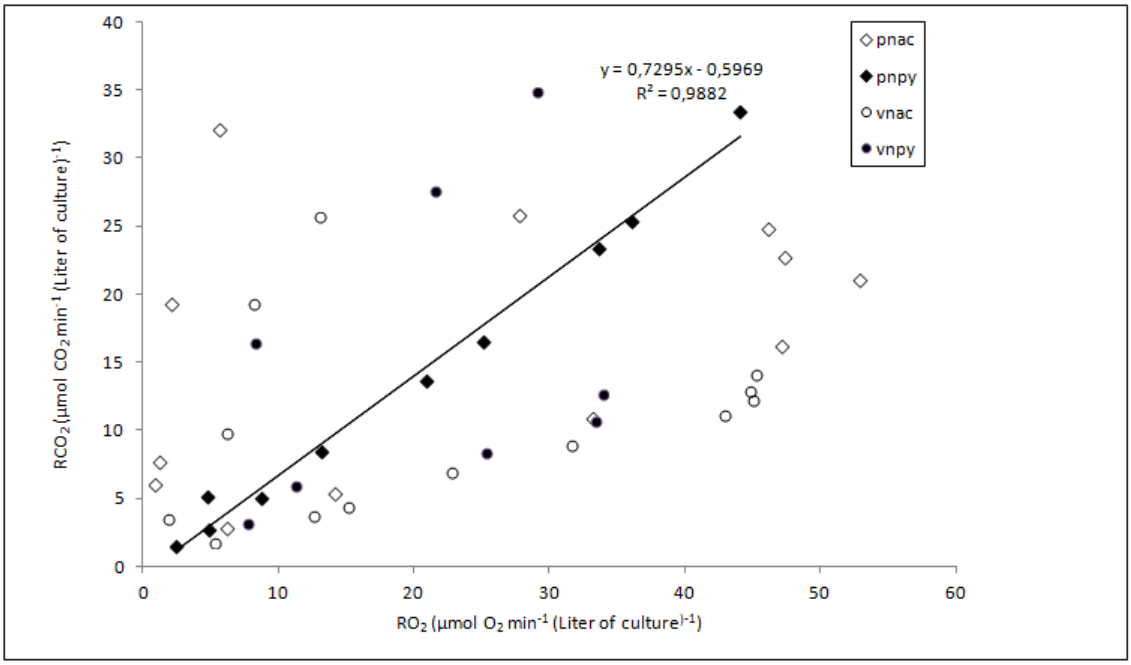

Figure 6. Respiratory CO2 production versus respiratory O2 consumption in four short term experiments. Pnac = *Ps. nautica* in acetate, pnpy = *Ps. nautica* in pyruvate, vnac = *V. natriegens* in acetate, vnpy = *V. natriegens* in pyruvate. The regression line holds for the

pnpy data; the equation is: R_{CO_2}= 0,7295 R_{O_2} -0,5969, (R^2=0,9882, n=10). The slope of the equation (0,7295) is the mean RQ value. For the acetate-based cultures, the data below the line are nutrient sufficient; the data above the line are nutrient depleted, equations for starvation conditions are shown in Table 1. From the equations in Table 1, it is clear that the starved acetate-based cultures have different RQs (slopes) than the starved pyruvate-based cultures. The difference between the two starved acetate-based cultures is more subtle, but ANCOVA analysis revealed that at $p<0.05$, the slopes, and the RQs were different except for bacteria grown on pyruvate in starrved conditions.

Table 1. Regression lines and r^2 for short term starvation conditions(*) shown in Fig.6. pnac= *Ps. nautica* on acetate, vnac= *V. natriegens* on acetate, pnpy= *Pseudomnas nautica* on pyruvate, vnpy= *V. natriegens* on pyruvate.

Strain	Equation	r^2
pnac*	y= 5,2692x + 3,4891	0,92
vnac*	y= 2,0564x - 0,4225	0,95
pnpy*	y= 0,5675x + 1,95	0,98
vnpy*	y= 0,5737x + 1,7177	0,98

Long term experiment

In Figure 2 we see that all physiological respiratory activity (RCO_2 and RO_2) ends within the first 100 hours of the experiment. We can see different shifts in the data as nutrient limitation sets in. *Ps. nautica* in acetate (Figure 2a) has higher values of RO_2 until starvation begins. Then, at the moment of starvation, RO_2 doubles, from~25 to 50µmol O_2/min/l, but after this rise, the RCO_2 values are larger than RO_2. In the case of *V. natriegens* in acetate all RCO_2 and RO_2 values are almost the same and only the RCO_2 rises at the moment that nutrient limitation begins. *Ps. nautica* in pyruvate is the only long term experiment where a jump in the RCO_2 and RO_2 co-occurs at starvation point (Figure 2c). *V. natriegens* on pyruvate jumps before starvation begins (Figure 2 d). Experiments on pyruvate show higher RCO_2 values than RO_2 during the experiments (Figure 2 c and d).

All RCO_2/RO_2 for the long term experiments are shown in Figure 7, we see a good correlation in all cases except in *V. natriegens* on acetate (Table 2). The slope of these functions are the general RQ, this is a quantitative way to estimate RQ. However, it must be remembered that each point represents a physiological measurement made at different time in the life of the culture. ANCOVA analysis (Table 2) between cultures which were fed with the same nutrient (i.e. *Ps. nautica* or *V. natriegens* on acetate) shows that the slopes are not significant different ($p>0.05$). On the other hand, for the same strain, but for different substrates (i.e. *Ps. nautica* on acetate or pyruvate) the slopes appear to be significantly different ($p<0.05$). Comparing slopes (RQs) in Fig. 7 during starvation conditions finds all of them to be significantly different ($p<0.05$) (Table 2).

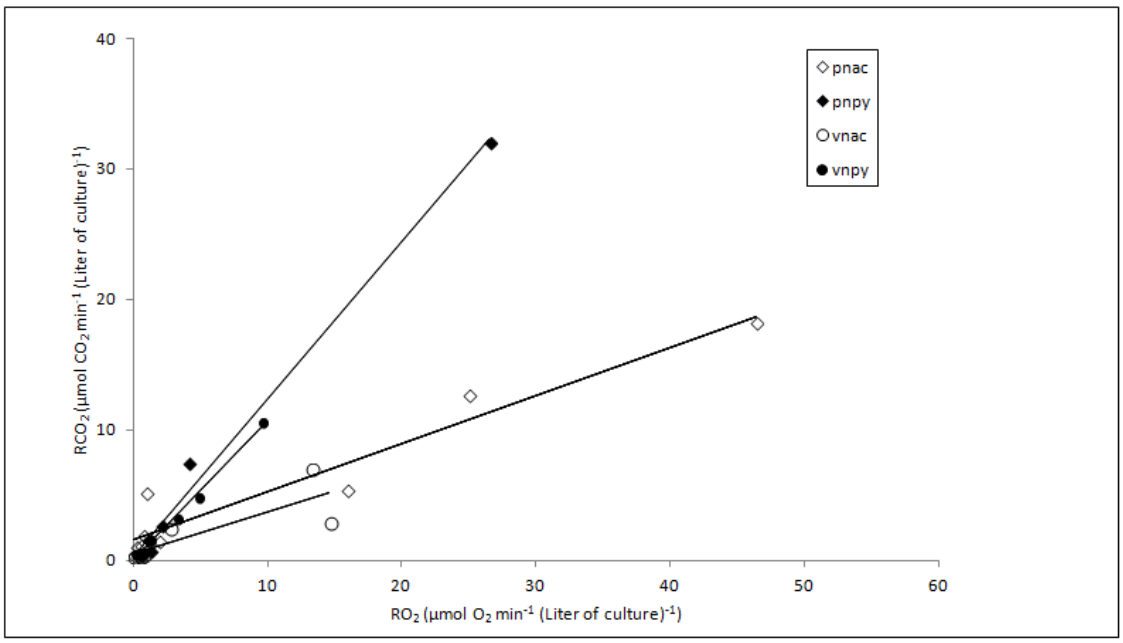

Figure 7. Long term experiments. pnac= *Ps. nautica* in acetate, pnpy= *Ps. nautica* in pyruvate, vnac= *V. natriegens* in acetate, vnpy= *V. natriegens* in pyruvate. Note that the carbon sources dictate the parallelism of the curves, not the bacterial species. In addition, note that the RQ (slope) is higher for the three-carbon substrate (pyruvate) than for the two carbon one (acetate).

Table 2. Regression lines and r^2 for long term experiments shown in Fig. 7. Note that pnac = *Ps. nautica* on acetate, vnac = *V. natriegens* on acetate, pnpy = *Pseudomnas nautica* on pyruvate, vnpy = *V. natriegens* on pyruvate. *Starvation conditions in long term experiments.

Strain	Equation	r^2
pnac	y= 0,3681x + 1,5454	0,93
pnac*	y= 4,2758x – 0.1633	0,76
vnac	y= 0,3152x + 0,5364	0,68
vnac*	y= 0,8582x + 0,1265	0,97
pnpy	y= 1,204x + 0,3718	0,99
pnpy*	y= 1,204x + 0,3665	0,99
vnpy	y= 1,0624x + 0,0701	0,99
vnpy*	y= 1,0541x + 0,1333	0,99

3.4 IDH and Φ normalized by biomass (protein)

Short term experiments

Time courses of protein-specific enzymatic activity are shown in Figure 8. Enzymatic activity is present even during starvation. Φ in *Ps. nautica* on acetate (Figure 8a) is higher than the corresponding IDH values. IDH decreases before starvation begins, but Φ holds almost constant. Again, IDH rises in acetate–*based cultures of V. natriegens* before nutrient limitation begins (Figure 8b) as we saw in *Ps. nautica* on acetate (Figure 8a). In this case, values of IDH and Φ are more or less similar. *Ps. nautica* experiments display a better correlation in acetate and in pyruvate for IDH and Φ. In *Ps. nautica* on pyruvate, we see again higher values for Φ. In the case of *Ps. nautica* in pyruvate (Fig. 8c) have the most similar values for IDH and Φ and before starvation begins IDH rises and Φ activity falls. *V. natriegens* on pyruvate displays an IDH rise until starvation begins and then a slow drop (Figure 8 d), Φ activity rises too and has a more dramatic drop in nutrient limitation conditions. In all cases except *V. natriegens* in pyruvate (Fig 8 d) we see IDH and Φ activity

rises before nutrient limitation begins. According to the ANCOVA analysis the difference between IDH and ϕ is significant ($p<0.05$).

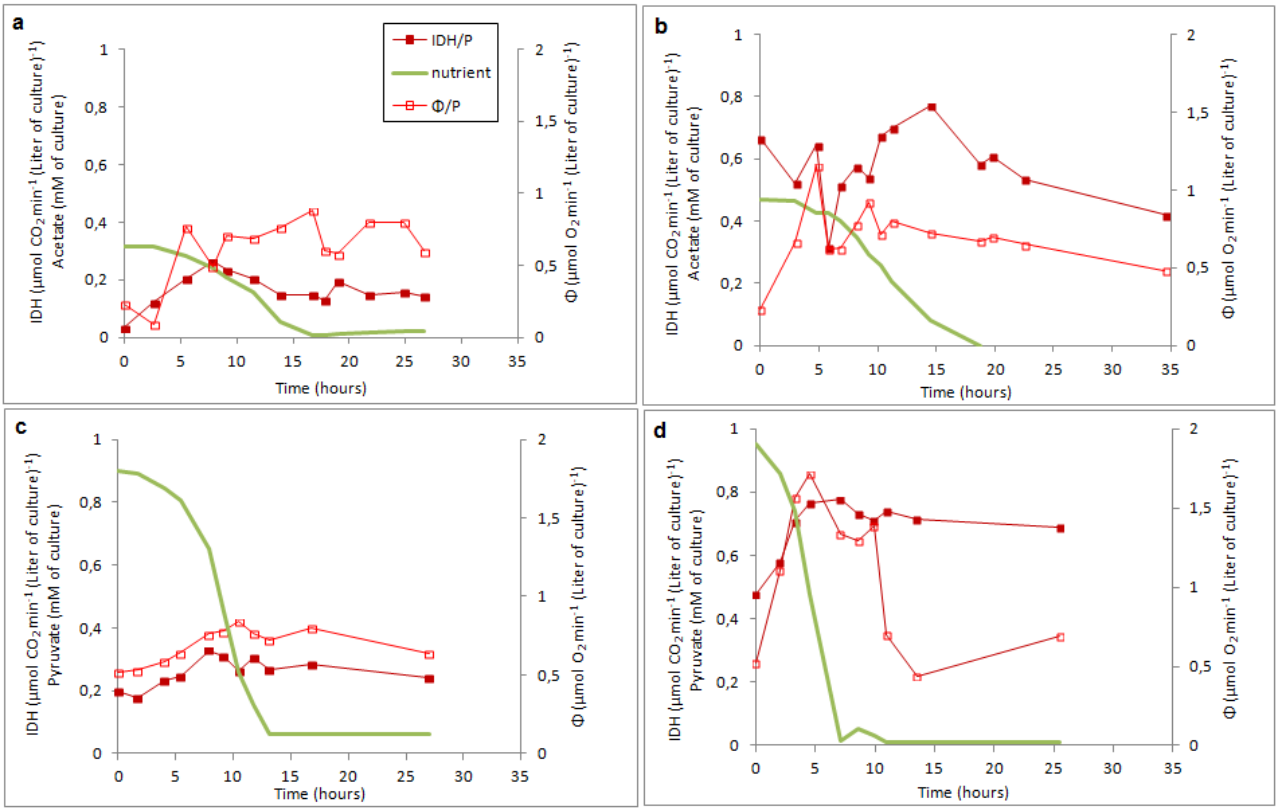

Figure 8. Time courses in the short-term experiments of enzyme activities normalized by biomass (IDH/protein and ϕ/protein). A) *Ps. nautica* in acetate , b) *V. natriegens* in acetate. c) *Ps. nautica* in pyruvate d) *V. natriegens* in pyruvate. As with the other time-course Figures, the nutrient-sufficient condition is on the left while the nutrient-deficient condition is on the right. Note that, if these enzymes were good proxies for protein biomass or if they were constituent parts of the bacterial cell, both IDH/protein and ϕ/protein would tend to be straight lines, independent of the culture age.

Long term experiments

We see that enzymatic processes in the long term experiments (Figure 9) vary less than in the short term experiments (Figure 8). In all experiments we see enzymatic activity even during nutrient limitation. Almost all IDH and Φ values are similar. In *Ps. nautica* on acetate (Figure 9 a) we see a little IDH jump after nutrient limitation is reached and then it decreases until ~ 350 h where it starts to increase with Φ. *V. natriegens* on acetate jumps in IDH and Φ after nutrient limitation and then the two enzymatic activities decrease together to levels lower than 0,1 µmol CO_2/min/l and µmol O_2/min/l (Figure 9 b). In experiments on pyruvate we observe that Φ activity rises after nutrient limitation conditions and that IDH activity drops (Figure 9 b and d). In *Ps. nautica* on pyruvate Φ and IDH rise at~250 h and later they drop together to lower values (Figure 9 c). *V. natriegens* on pyruvate is the only experiment where Φ activity rises from 0 µmol O_2/min/l (before 100h of experiment is reached) to 0,45 µmol O_2/min/l (~200h) (Figure 9 d). Here again comparison of the slopes of IDH and ϕ by ANCOVA analysis reveals a significant different ($p<0.05$).

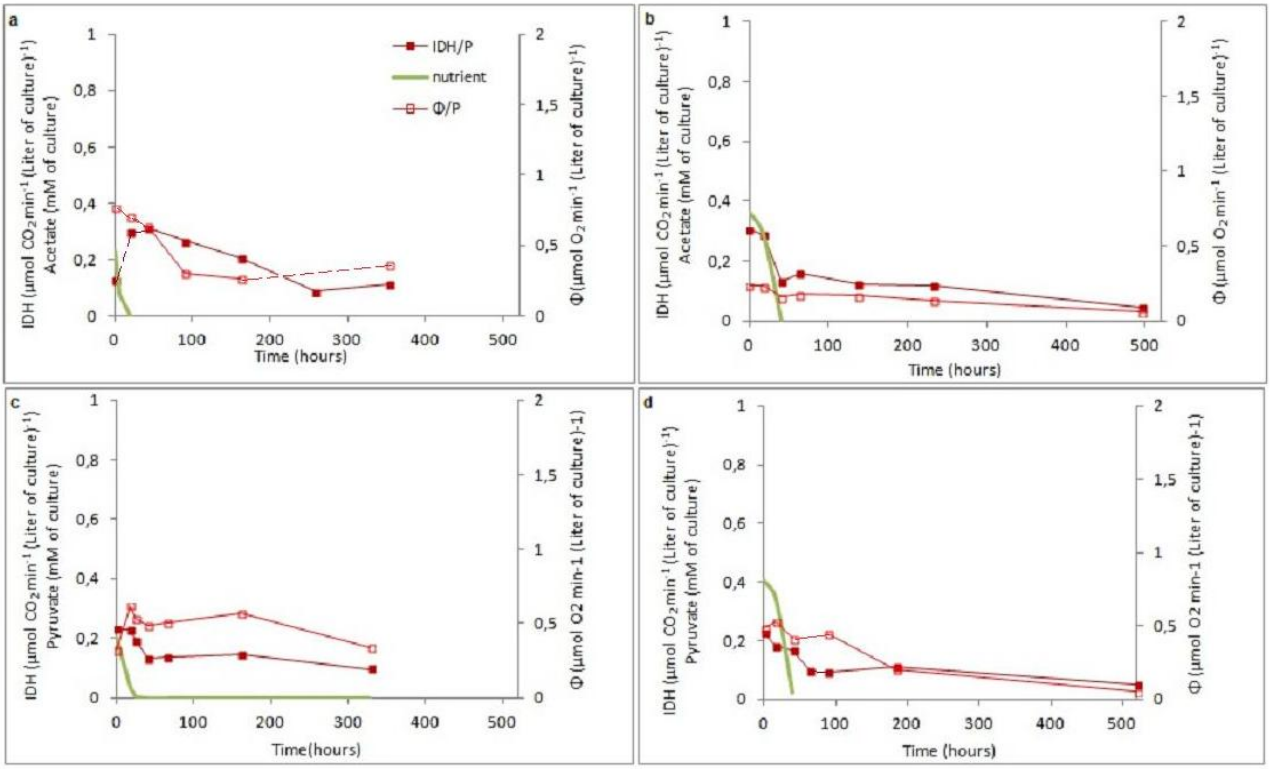

Figure 9. Time courses in the long-term experiments of IDH/protein and ϕ/protein. A) *Ps. nautica* in acetate, b*) V. natriegens* in acetate, c) *Ps. nautica* in pyruvate, and d) *V.*

*natriegen*s in pyruvate . Note that the nutrient-sufficient condition terminates on the left when a projection of the green line intersects with the abscissa. Nutrient-deficiency characterizes most of the culture's life. Note that, both IDH/protein and ϕ/protein tend to decrease with time as the bacterial cultures starve.

4. Discussion

4.1 RQ variability and carbon limitation.

In this study we observed high variability in RQ values. In fresh water lakes and ponds of Quebec, Berggren et al. (2012) found RQs varying from 0.25-2.26 and argued that the common assumptions of 0.8-1.0 RQ values are not justified by the literature. Furthermore, they argue that the use of a constant RQ depresses estimations of the metabolic balance between respiration and photosynthesis, i.e., P/R ratio. They also argued that high RQs could be physiologically explained by biogeochemical pathways of DOC degradation. However, it is not obvious how the community-level RQ could rise to values as high as 10 in response to cell biochemistry as our study shows (Figure 1 a). Starvation, in the form of carbon-source deprivation, had a strong impact and before our study, this variable and its impact on RQ had not been well studied. For example, Figure 5 a and b shows how RQ rises during starvation though a combination of a slight R_{CO2} rise and a dramatic drop in R_{O2}. Again In Fig. 6, the RQ shifts up from nutrient sufficiency to nutrient limitation. Lower RQ levels occur in the first phase of the experiments when the bacteria were well fed, later the RQ rises as carbon starvation sets in.

4.2. Carbon source and RQ

In long term experiments comparing RCO_2/RCO_2 ratios we found out that two different bacteria grown on the same substrate are related, ANCOVA-test showed that in these cases there is no significant difference between Ps. nautica and V. natriegens related to their slopes (Table 2). It is clear from Fig. 3 that *Ps. nautica* and *V. natriegens* cultures grown on acetate or pyruvate RQ´s are completely different. Acetate and pyruvate are products of lipid and

carbohydrate metabolism, respectively; the results suggest that paired measurements of RCO_2 and RO_2 could serve as a diagnostic for detecting different types of metabolism in situ studies (Roy et al., 1999). High RQ would indicate carbohydrate metabolism which indicates a strong presence of carbohydrates, otherwise low RQ would indicate lipid metabolism.

Our results suggested that, in *Ps. nautica* cultures, the RQ is always lower than 1.0 before acetate or pyruvate is exhausted. However, *Ps. nautica* in the acetate-grown culture, when acetate was exhausted, the RQ rose above 1.0 and reached a value higher than 10. That increase of RQ was caused by a fast reduction of RO_2, after acetate exhaustion when RCO_2 was still high (Figure 5 a). Again, the time variations of RQ while the cultures passed from a well-nourished state to a nutrient-starved state argue for a reconsideration of the practice of assuming a constant RQ of 1.0. In figure 7 we deduce from the slopes that acetate gives a similar RQ for the two bacteria and like on pyruvate grown cultures, the same was observed from Berdalet et al. 1995.

4.3 RQ and biochemical pathways.

The RQ is theoretically regulated by biochemical pathways of metabolism, in this way Krebs-cycle CO_2 production is the result of three enzymes: isocitrate dehydrogenase (IDH, EC 1.1.1.42), α-ketoglutarate dehydrogenase (α-KGDH, EC 1.2.7.3), and pyruvate dehydrogenase (PDH, EC 1.2.2.2) (Walsch and Koshland 1984; Holms 1986 a,b; Packard et al. 1996 a). The Krebs cycle is nearly universal in microbes. In our results we see that enzymatic activity still works in starvation conditions (Figure 8). In nature micro-organism are able to react quickly to environmental changes, adapting their metabolic profiles to organic inputs (Martinez et al., 1996; Mudryk and Donderski, 1997; Sala and Gude, 2004; Mudryk and Skorczewski, 2006) in this study we suggest an adaptation of enzymatic activity of the physiological states of bacteria. Caruso et al. (2013) pointed out that heterotrophic metabolism and Bacterioplancton respiration shows different seasonal and interannual cycles. Variations in the microbial activity levels are frequent along trophic gradients (Hoppe et al., 1998; Caruso et al., 2005; Chróst and Siuda, 2006; Williams and Jochem, 2006; Cunha et al., 2010). Perhaps considering these cycles we can understand how enzymatic activity of microbes is able to adapt into different conditions

in an in-vitro experiment. Also (Berggren et al., 2012) argues that natural Bacterioplancton utilize complex substrate mixtures and the cells themselves have a wide range of physiological states or it also can be explained that high enzymatic activity rates can regenerate nutrients from dissolved substrates, an making available C, N, P which are needed to fulfill microbial metabolic demand (Berdalet et al., 1995).

4.4. IDH/P and RQ variability

Acetate, a 2 carbon molecule, is transformed into acetyl-CoA and loses both carbons when cycled through the TCA. During growth on acetate IDH is partially inactivated (El-Mansi et al., 1985) in order to allow the operation of the glyoxylate cycle. While acetate serves to repress IDH activity, pyruvate (a 3 carbon molecule) serves to activate it (Holms et al., 1971), so IDH activity on acetate-based cultures should be lower than on pyruvate cultures, and lower IDH means lower CO_2 production and lower RQ but in our results we have not observed this behavior (Figure 3a and 8a and figure 1 b and d) instead of that we see that IDH reaches higher values for experiments of *Vibrio natriegens* (Figure 8b and d). In long term experiments we do not see this behavior, values of IDH are more or less constant. IDH is the responsible enzyme for the flow of carbon through the Krebs cycle to produce energy and CO_2, and for the flow of carbon through the glyoxylate cycle to produce cell constituent and it is known that many bacteria strains are able to maintain their enzymatic machinery when the carbon source is exhausted (Roy et al. 2001). The IDH/P activity and Φ/P activity ratios did not ascend like the RQ did when the nutrient was limited (Figure 8 and 9). El-Mansi et al. (1985) in his study showed that, during growth on acetate, IDH is partially inactivated to facilitate glyoxylate cycle activity. This behavior of IDH activity/Φ activity in acetate and pyruvate experiments suggests that respiratory enzyme concentrations are not responsible for RQ differences observed in nutrient limitation.

5. Conclusion

1. Respiration under starved and well-fed conditions of acetate and pyruvate-based cultures of *Pseudomonas nautica* and *Vibrio natriegens* show that respiration rates during nutrient-sufficiency parallels the biomass increase, but that after nutrients are exhausted, the respiration rates fall.
2. IDH activity and potential respiration can maintain high levels as nutrients are depleted, but after 10 h of starvation they, and the cell-protein fall slowly, as nutrient-deficiency persists.
3. During starvation biomass-specific IDH and ϕ decreases and this latter finding challenges the idea that IDH and the respiratory electron transport systems (ETS) are constitutive and can be used for biomass proxies.
4. RQs, calculated from the respiratory data, rise nearly by a factor of 10 during the shift from nutrient-sufficiency to nutrient-deficiency. Because of this, it is clear that the nutritional conditions of the bacteria greatly changes the RQ.
5. The fact that RQ varies so much will greatly impact ecosystems models, ocean carbon flux calculations, and predictions of the balance between ocean-autotrophy and heterotrophy.

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