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Maintenance Energy Demand and Starvation Recovery Dynamics of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* Cultivated in a Retentostat with Complete Biomass Retention

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***Nitrosomonas europaea* and *Nitrobacter winogradskyi* (strain "Engel") were grown in ammonia-limited and nitrite-limited conditions, respectively, in a retentostat with complete biomass retention at 25°C and pH 8. Fitting the retentostat biomass and oxygen consumption data of *N. europaea* and *N. winogradskyi* to the linear equation for substrate utilization resulted in up to eight-times-lower maintenance requirements compared to the maintenance energy demand (*m*) calculated from chemostat experiments. Independent of the growth rate at different stages of such a retention culture, the maximum specific oxygen consumption rate measured by mass spectrometric analysis of inlet and outlet gas oxygen content always amounted to approximately 45 μmol of O_2 mg^{-1} of biomass-C · h^{-1} for both *N. europaea* and *N. winogradskyi*. When bacteria were starved for different time periods (up to 3 months), the spontaneous respiratory activity after an ammonia or nitrite pulse decreased with increasing duration of the previous starvation time period, but the observed decrease was many times faster for *N. winogradskyi* than for *N. europaea*. Likewise, the velocity of resuscitation decreased with extended time periods of starvation. The increase in oxygen consumption rates during resuscitation referred to the reviving population only, since in parallel no significant increase in the cell concentrations was detectable. *N. europaea* more readily recovers from starvation than *N. winogradskyi*, explaining the occasionally observed nitrite accumulation in the environment after ammonia becomes available. From chloramphenicol (100 μg · ml^{-1}) inhibition experiments with *N. winogradskyi*, it has been concluded that energy-starved cells must have a lower protein turnover rate than nonstarved cells. As pointed out by Stein and Arp (L. Y. Stein and D. J. Arp, Appl. Environ. Microbiol. 64:1514–1521, 1998), nitrifying bacteria in soil have to cope with extremely low nutrient concentrations. Therefore, a chemostat is probably not a suitable tool for studying their physiological properties during a long-lasting nutrient shortage. In comparison with chemostats, retentostats offer a more realistic approach with respect to substrate provision and availability.**

Bacteria in natural habitats are faced with fluctuating nutrient availabilities with a temporary excess supply followed by various periods of nutrient deficiency (21, 35). Under these conditions, a successful life strategy depends not only on the fast and effective uptake and conversion of nutrients, as measured and described in terms of μ_{max} and μ_{max}/K_s (18, 28), but also on an appropriate starvation survival strategy. In nonsporulating bacteria, this strategy should include a maintenance energy demand that is as low as possible while remaining ready for a fast response to nutrient upshifts.

In the life and survival strategies of nitrifying bacteria, well-balanced activities between ammonia oxidizers and nitrite oxidizers are crucial for the complete conversion of ammonia to nitrate. Incomplete nitrification occurs in activated sludge plants (26), wastewater reservoirs (2), and water distribution systems (13), and it is mainly caused by reduced or missing activity of nitrite-oxidizing bacteria such as *Nitrobacter* spp. (4). This may be due to inhibitory effects, low affinity to oxygen (19), or tardy starvation recovery of *Nitrobacter* spp. after nitrite upshifts following prolonged periods of starvation. Knowledge concerning starvation survival in autotrophic nitrifying bacteria is scarce, and studies were almost exclusively done with cells from batch cultures (see, for example, reference 30) in biofilms (see, for example, reference 5) and soil columns (40). As pointed out by Mason and Egli (20), the nature of the transition period from feast to famine may greatly influence the long-term survival and, in natural environments, one would expect to see a gradual transition from the exponential to the stationary phase rather than a sudden change. This gradual transition could be obtained for suspended cultures by decreasing dilution rate stepwise in continuous cultures (chemostats). However, extremely low dilution rates in chemostats ($\ll$0.05 h^{-1}) give rise to inhomogeneities due to mixing problems and low steady-state biomass concentrations (9). This problem can be overcome by using bioreactors combined with filtration devices to retain the biomass (retentostats or recyclostats) (11, 36). Such retention culture systems have also been successfully applied in studies of different physiological properties of microorganisms associated with slow growth, e.g., stringent response in *Escherichia coli*, *Bacillus* spp., and *Paracoccus denitrificans* (1, 9, 11, 15, 38); growth and product formation characteristics in *Aspergillus niger* at different growth rates (27,

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39); or the maintenance energy demand of different prokaryotes, including *Nitrosomonas* spp. (6, 31, 32). In heterotrophic prokaryotes two (or three) growth domains could be distinguished during growth in retentostats (11, 38). The first growth domain could be described with a Pirt-type equation, and growth characteristics were similar to those found in continuous cultures; the second domain can be characterized by the onset of the stringent response, while growth seemed to be linear, resulting in a low yield and extremely low maintenance requirement values. The first objective of the present work was to show that, in accordance with an appropriate starvation-survival strategy, the maintenance energy demand of nitrifying bacteria is lower in nutrient-poor conditions compared to well-nourished cultures. A second objective of this study was to show that there is a difference in the time course of recovery between ammonia oxidizers and nitrite oxidizers after starvation as a possible explanation for the transient nitrite build-up in ecosystems. We used the retentostat culture system for the reasons given above and to have enough biomass for an in situ determination of oxygen consumption rates, thus avoiding contaminations and disturbances of the culture due to sampling.

MATERIALS AND METHODS

Organism, medium, and cultivation system. *Nitrobacter winogradskyi* (strain "Engel"; obtained from H.-P. Koops, University of Hamburg) was grown in a completely mixed bioreactor (920 ml) with external biomass retention mediated by a stirred microfiltration cell (volume, 110 ml; polyvinylidene difluoride filter, 0.22-μm pore size; Millipore) in a bypass of the reactor. The culture suspension was pumped with a high flow rate through the microfiltration cell, causing a mean residence time of only 30 s for the bacteria within the filtration unit. Therefore, both units were considered a one-stage bioreactor with respect to the process kinetics. The whole system was set up by the mechanics workshop of the Institute of Biotechnology at Forschungszentrum Jülich GmbH.

The technical features of the filtration unit were in accordance with the features described elsewhere (32). The only difference was a reduced volume and the renunciation of two baffles. A conductivity contact positioned below the lid of the fermentor triggered a peristaltic pump that removed the filtrate at a rate equivalent to the substrate provision rate. The substrate provision rate was 1.42 mmol of $NaNO_2 \cdot h^{-1}$ at a hydraulic retention time of 0.1 h^{-1} and 7.1 mmol of $NaNO_2 \cdot h^{-1}$ for the same retention time during the starvation and reactivation experiments in order to achieve higher respiration rates for the mass spectrometric oxygen uptake determinations. The whole system was darkened to prevent photoinhibition, and the bioreactor was kept at 25°C by means of a temperature-regulated water jacket. Inlet and outlet gas tubings were made from stainless steel, and the other tubings consisted of silicone and polytetrafluoroethylene. An aeration rate of 10 liters of compressed air per h kept the dissolved oxygen (DO) (measured by a DO Probe; Ingold) at between 70 and 100% saturation and was sufficiently high for the mass spectrometer. The continuous addition of CO_2 (5% [vol/vol]) in the inlet gas ensured a pH of 8.0 in the fermentor. The fermentor setup for *Nitrosomonas europaea* was as described previously (27); however, instead of a recycling finger, a ceramic bottom plate (0.2 μm) was used. The setup was very similar to the FZ-Jülich bioreactor. The culture volume was about 0.5 liter; ammonium concentration in the medium was 5 mM; and the hydraulic retention time (D) was ca. 0.11 h^{-1}, resulting in substrate provision rates of about 0.3 mmol of ammonium h^{-1}. The pH was kept at 8.0 by adding 5% (wt/vol) Na_2CO_3.

The inorganic medium contained $NaNO_2$ or $(NH_4)_2SO_4$ as indicated in the Results section and also (per liter) 80 mg of KCl, 50 mg of $MgSO_4 \cdot 7H_2O$, 100 mg of $CaCl_2 \cdot 2H_2O$, 100 mg of $Na_2HPO_4 \cdot 12H_2O$, 1,130 mg of $NaHCO_3$, 0.71 mg of $FeCl_2 \cdot 4H_2O$, 1.35 mg of $Na_2EDTA \cdot 2H_2O$, and 1 ml of trace element solution as described by Tappe et al. (32). Na_2HPO_4 and $NaHCO_3$ solutions were autoclaved separately and added after cooling. All retentostat cultures were regularly (weekly) checked for heterotrophic contaminations by incubating samples in tryptic soy broth and also by plating them on 10% nutrient broth agar plates. Turbidity or CFU values after 1 or 2 weeks of incubation, respectively, were assessed as heterotrophic contaminations. Results derived from contaminated runs as well as from runs with bacteria attached to the reactor walls were not taken into consideration.

BioCord (TBR Corp., Ltd.), a nylon cord with woven nylon attached to it, was used for immobilizing the bacteria. This BioCord has been successfully used as a bioscreen in rivers and wastewater treatment plants in Japan.

Analytical procedures. Oxygen consumption kinetics were measured with a mass spectrometer (MM8-80F; VG Gas Analysis Systems). During continuous operation, the outlet gas passed through a 250-ml water-cooled condensing flask before it entered the mass spectrometer. When nitrite pulses were supplied during short-term shift-up experiments, the outlet was switched to a stainless steel tubing leading directly to the spectrometer to obtain a faster time-dependent signal resolution.

Dry weight was determined as described by Bulthuis et al. (10). Cell numbers and cell sizes were regularly determined with a Coulter Counter (Type Multisizer II, 20-μm orifice) and, in order to avoid large sampling volumes for dry-weight determinations, the biomass was largely calculated from the biovolume (mean cell size multiplied by the cell concentration) and from the relation between particulate organic carbon, the biovolume, and the dry weight. The particulate organic carbon content was measured with a Total Organic Carbon Analyzer (Dohrmann DC-190) as the difference between untreated and the 0.2-μm-filtered sample.

NO_2^- and NO_3^- were checked with Merckoquant test strips (Merck GmbH) and analyzed with an Autoanalyzer (Cenco B.V.). Starvation conditions were achieved simply by stopping the medium flow and filtrate removal pump while the stirrer and the aeration were kept running. Nitrite or ammonium pulses were added with a sterile syringe directly into the reactor via the sample port. The inlet of the sample port ended up next to the stirrer to ensure fast and thorough mixing.

Equations describing biomass production and oxygen consumption in the retentostat. The full derivation of the equations is given by van Verseveld et al. (38). The basis of the model is formed by the carbon and energy balance for growth and product formation: CH_mO_l (substrate) + aNH_3 + bO_2 → y_c $CH_pO_nN_g$ (biomass) + $zCH_tO_sN_t$ (product) + cH_2O + dCO_2. The subscripts stand for fractional or whole numbers, depending on the ratio of H, O, and N to one C atom in the respective molecules. For example, if glucose were the substrate, the subscripts "m" and "l" became "2" and "1." Then:

$$r_s = m_s x_t + \frac{r_x}{Y_{xsm}} \quad (1)$$

in which r_s is the substrate provision rate (in moles per hour), m_s is the maintenance requirement (in moles of substrate per gram [dry weight] of biomass per hour), x_t is biomass present at time t (in grams [dry weight]), r_x is the rate of biomass formation (in grams [dry weight] of biomass per hour), and Y_{xsm} the growth yield corrected for the maintenance requirements (in grams [dry weight] of biomass per mole of substrate). Rearrangement leads to $r_x = (r_s - m_s x_t)Y_{xsm}$, and integration yields equation 2, when r_s is a constant, as is the case in a retentostat:

$$x_t = \frac{r_s}{m_s} + \left(x_0 - \frac{r_s}{m_s}\right) e^{-m_s Y_{xsm} t} \quad (2)$$

in which x_0 is the biomass concentration at time zero.

Equation 3 shows the rate of oxygen consumption (r_{O_2} [in moles of O_2 per hour]), when no product is formed, and is obtained from the energy balance ($b = 0.25[\gamma_s - y_c\gamma_x]$), $y_c = Y_{xs}/M_x$, $(Y_{xs}/M_x)/r_s = r_x/M_x$, and $r_{O_2} = br_s c$, in which c is the amount of carbon atoms in the substrate.

$$r_{O_2} = 0.25\left(\left[\frac{\gamma_s}{Y_{xsm}} - \frac{\gamma_x}{M_x}\right] r_x c + \gamma_x m_s x_t c\right) \quad (3)$$

in which γ_s and γ_x are, respectively, the degree of reduction of substrate (for nitrifiers taken as NH_3CO_2 and HNO_2CO_2, as a combination between the energy source and CO_2 and thus, respectively, as 6 and 2) and the degree of reduction of biomass (all on the C_1 base). M_x is the molecular weight of 1 C_1-mol of biomass. Data of retention experiments can be fitted nonlinearly by using equations 2 and 3 as explained below.

Data analysis. As described in van Verseveld et al. (38), a computer program was developed that optimizes the best-fitting curves by means of nonlinear least-squares analysis. The program (PFIT) searches for optimal values for the variable parameters in the given functions by minimizing the sum of squared differences between experimental and predicted points divided by the number of data points (i.e., the SS value). Data were normalized because of the different orders of magnitude among the variables.

RESULTS AND DISCUSSION

***Y_{xsm}* and *m_s* of *N. europaea* and *N. winogradskyi* in retentostat culture.** *N. europaea* was batch grown in a mineral medium containing 5 mM $(NH_4)_2SO_4$ and, after the consumption of all ammonia, was switched to retentostat mode.

The substrate provision rate (r_s) was 0.299 mmol of NH_4^+ h^{-1}. Figure 1 shows the steady increase in biomass even after 1,600 h in the retentostat mode. Neither different modes of growth (12, 22, 34, 37) nor a linear increase of biomass or zero growth (as described by Panikov [24] for *Pseudomonas* and *Bacillus* spp., respectively) was indicated. The mean cell volume of batch-grown *N. europaea* reached 0.7 μm^3 and was reduced only to 0.5 μm^3 when batch-grown cells were subse-

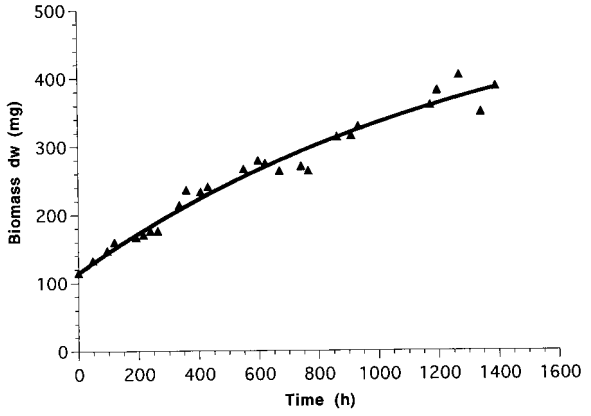

FIG. 1. Biomass concentration in milligrams (dry weight) of *N. europaea* in continuous culture with 100% biomass retention (r_s = 0.299 mmol of NH_4^+ h^{-1}). Data show the increase of biomass after reaching 100 mg in the retentostat mode. The solid line represents the best fit for X_t, a value obtained by using the equations 2 and 3 as described in Materials and Methods. The results of one experiment are shown.

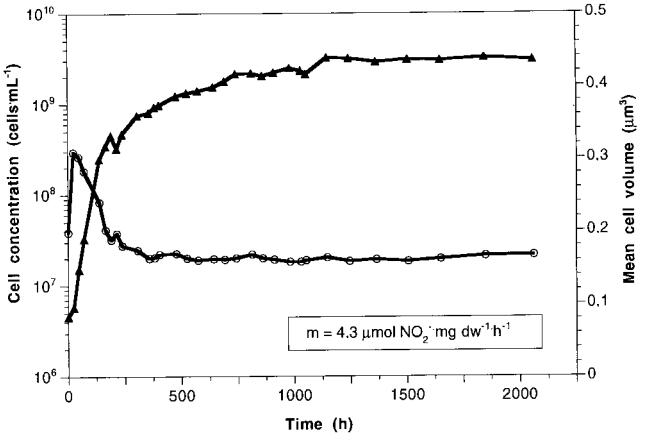

FIG. 2. Cell concentration in cells per milliliter (triangles) and mean cell volume in cubic micrometers (circles) of *N. winogradskyi* in continuous culture with 100% biomass retention (r_s = 1.42 mmol of $NaNO_2$ h^{-1}). Cells were batch grown during the first 80 h. From 1,100 h onwards, the biomass concentration remained at ca. 332 mg (dry weight) per liter. The ratio of r_s to steady-state biomass concentration reflects a maintenance energy demand (m) of 4.3 μmol of NO_2^- per mg (dry weight) per hour. The results of the longest of three similar experiments are shown.

quently starved for 7 weeks. In contrast, the retentostat-grown cells were reduced to a mean cell volume of 0.22 μm^3 (data not shown) but were still bigger than dwarf cells, which are defined by Bakken (3) as cells with volumes of <0.07 μm^3. This finding probably reflects the impact of the culture's "history" on its actual physiological state (see reference 20). The oxygen consumption was measured continuously, and the consumption rate of 0.44 mmol $O_2 \cdot h^{-1}$ at an r_s of 0.299 mmol of NH_4^+ h^{-1} reflects the demand of 1.5 mol of O_2 per mol of NH_4^+ oxidized to NO_2^-.

N. winogradskyi was batch grown with 14 mmol of NO_2^- $liter^{-1}$ and switched to retentostat mode after all of the nitrite was consumed. Figure 2 shows the longest-lasting retentostat run (over 2,000 h) of three runs as an example of apparently reaching zero growth. The biomass concentration ended up at 332 mg (dry weight) $liter^{-1}$. As seen in Fig. 2 and as mentioned for *N. europaea* above, mean cell volumes reached a maximum of about 0.3 μm^3 initially and decreased slowly to a minimum value (0.17 μm^3) with decreasing growth rate. For *Nitrobacter*, the measured O_2 consumption was also consistent with the stoichiometric demand of 0.5 mol of O_2 per mol of NO_2^- oxidized to NO_3^- (data not shown). Since the substrate provision rate was kept constant at 1.42 mmol of $NaNO_2 \cdot h^{-1}$ (r_s) during the whole run, the maintenance energy demand, if "zero growth" is assumed, can be calculated by the ratio of r_s to the biomass (dry weight). This ratio was 4.3 mmol of nitrite per g (dry weight) per h.

If all three retentostat runs with *Nitrobacter* were combined, the increase of biomass dry weight (Fig. 3) clearly leads to the conclusion that, as with *Nitrosomonas*, a true steady-state or zero growth could be approached but was practically not reached.

The fitted values of several runs, calculated by using the fit program as described in Materials and Methods, are shown in Table 1. The m_s value for *Nitrobacter*, fitted by nonlinear analysis based on equations 2 and 3 (see Materials and Methods) was 2.84 mmol of nitrite per g (dry weight) per h, a value somewhat lower than the m_s obtained simply by dividing r_s and the biomass concentration at "zero growth" (4.3 mmol of nitrite per g [dry weight] per h). This result supports the assumption that either the experiments did not last long enough to achieve a real steady state or the extremely slow biomass increase was compensated for by removing small amounts of biomass during sampling.

From Table 1 it is also clear that biomass yields of *N. europaea* are higher than those of *N. winogradskyi* and, more importantly, that the maintenance requirements of *N. winogradskyi* are higher than those of *N. europaea*. Although there are relatively high variations for m_s between the duplicates, the mean values for m_s for the *Nitrosomonas* and *Nitrobacter* spp. are distinctly different, being three to four times higher for the latter organism. The higher Y_{xsm} for *Nitrosomonas* is consistent with the higher molar energy yield per mole of ammonia oxidized compared to nitrite oxidation.

By comparing chemostat and retentostat data for *N. europaea*, Tomaschewski (33) showed that at lower growth rates less substrate was needed for maintenance requirements than at relatively higher μ values. For *N. winogradskyi*, we also determined the maintenance demand from steady-state bio-

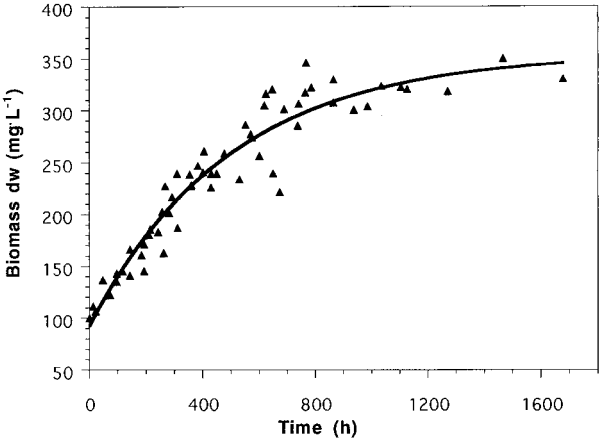

FIG. 3. Biomass concentrations in milligrams (dry weight) per liter of three retentostat runs with *N. winogradskyi* (r_s = 1.42 mmol of $NaNO_2^- \cdot h^{-1}$ in all runs). The data show the increase in biomass after it reached 100 mg (dry weight) per liter in the retentostat mode. The solid line represents the best fit. The fitted value for m was 2.8 μmol of NO_2^- per mg (dry weight) per h.

TABLE 1. Fitted retentostat data of *N. europaea* and *N. winogradskyi* grown under ammonium- and nitrite-limited conditions, respectively[a]

Organism	Retentostat data					
	Y_{xsm}			m_s		
	Expt 1	Expt 2	Avg	Expt 1	Expt 2	Avg
N. europaea	1.33	1.09	1.21	0.56	1.27	0.92
N. winogradskyi	0.3	0.3	0.3	4.27	2.84	3.6

[a] Two experiments were carried out independently with each strain, and Y_{xsm} and m_s are given as mean (average) values as well as for each run. The Y_{xsm} is the maximum yield corrected for maintenance requirements in grams (dry weight) of biomass per mole of substrate; the m_s is the maintenance requirement in millimoles per gram (dry weight) of biomass per hour.

mass yields in chemostat cultures at different dilution rates by the method of Pirt (25), and this analysis yielded a three- to fourfold-higher value than in the retentostat cultures (results not shown). Other maintenance measurements (17) estimated values of 30 and 50 mmol g^{-1} h^{-1}, respectively, for ammonia and nitrite oxidizers; these values were at least 10 times higher than our retentostat values. This result once more confirms that maintenance requirements are not constant but will depend on the actual growth rate, as has been shown for several heterotrophic organisms (1, 10, 11, 31, 36, 38). However, in heterotrophic organisms it has been concluded that the seemingly lower maintenance requirements are due to regulation induced by the stringent response, resulting in an apparently linear time-dependent increase in the biomass.

Homogeneity of "nongrowing" nitrifiers in the retentostat. When bacteria are growing very slowly, either at low dilution rates in the chemostat or in a retentostat with biomass feedback, it has to be verified whether the whole population contributes to the total substrate uptake or whether one part of the population is dormant while the other part is responsible for all of the activity. Since the determination of viable counts of nitrifiers by most-probable-number techniques or direct plate counts is time-consuming and very inaccurate, the reactivity and homogeneity of the *N. winogradskyi* population was determined as the change in cell volume distribution after a nitrite upshift. It has been demonstrated for *N. europaea* (32) and *P. fluorescens* (42) that the pattern of changes in cell volume distribution after upshifts in energy availability can be used as an indicator for the physiological homogeneity or at least for the homogeneous or inhomogeneous reactivity of a culture. An undistorted shift to larger mean cell volumes (log-normal distribution) after an upshift in energy availability indicates that a culture is homogeneously reactive. In contrast, a bimodal shift in cell volume distribution indicates that a population consists of subpopulations with different reactivities (42).

Cultures of *N. winogradskyi* or *N. europaea* which were maintained in the retentostat for several weeks with the continued addition of an energy source, but apparently without net growth, both responded to a substrate pulse with an undistorted, homogeneous shift to larger cell volumes. From these findings, we conclude that the whole population responded actively in this state of approximately no growth, and we never found any indication of the existence of a dormant fraction in the fermentor.

In contrast, when *N. winogradskyi* was starved in the absence of an external energy source for several weeks, a structured population with respect to cell volume distribution became visible after nitrite was once again supplied. In this case, a bimodal distribution could be interpreted based on a fraction of small nongrowing or slowly growing cells (a dormant fraction?) and a proportion of larger and fast-proliferating cells. The same behavior was shown for *N. europaea* (ATCC 25196T) cultures when starved for 3 months (33).

Respiratory activity and resuscitation of *Nitrosomonas* and *Nitrobacter* after different periods of starvation. Further experiments with both nitrifiers with the retentostat mode of growth were carried out to establish the maximal activity and reactivity values after starvation. These cultures were used to determine the specific maximum activities of *N. europaea* and *N. winogradskyi* at different specific growth rates, as well as the reactivities after different energy starvation time intervals. To monitor oxygen consumption rates and consumption kinetics, ammonium or nitrite pulses were added to the reactor. Because of the impact on the culture during starvation and reactivation experiments, the actual growth rate at the moment of pulse addition could only be estimated roughly (in contrast to the almost undisturbed runs depicted in Fig. 1, 2, and 3). However, independent of the actual growth rate, at any state of the cultivation between μ_{max} and very slow growth in the later stages of the run, the specific maximum oxygen uptake rates amounted to 40 to 46 μmol O_2 · mg of biomass-C^{-1} · h^{-1} and 43 to 47 μmol O_2 · mg of biomass-C^{-1} · h^{-1} for *N. europaea* and *N. winogradskyi*, respectively. Based on dissolved oxygen measurement, a response of the bacteria to the interrupted medium supply as well as the ammonia or nitrite pulse was detectable within a few seconds. The oxygen concentration in the outlet gas flow detected by the mass spectrometer was, of course, slightly retarded because of the phase transfer of oxygen from water to gas phase and the hysteresis due to mixing conditions within the gas phase above the culture suspension. But the pulsed amount of ammonia or nitrite and the time response of the system was always sufficient to detect the maximum oxidation rate. This result was verified with different concentrations of ammonia or nitrite added to ensure that the amount supplied was not limiting for the respiratory activity. Likewise, the maximum oxygen consumption rate gave the same results whether measured during continuous feeding or during disrupted feeding, provided that the respiratory activity exceeded the oxygen consumption rate necessary to consume the continuously supplied ammonia or nitrite (Fig. 4, data

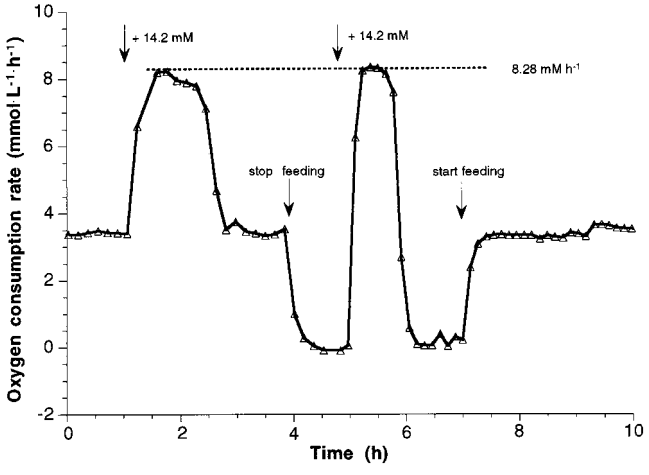

FIG. 4. Oxygen consumption rate of *N. winogradskyi* responding to a pulse of 14.2 mM nitrite during continuous and disrupted feeding in a retentostat run with 100% biomass retention. The same specific maximum oxygen consumption rate of 44 μmol O_2 · mg of biomass-C^{-1} · h^{-1} was reached after the substrate pulse in both cases at a biomass concentration of 188 mg of biomass-C · $liter^{-1}$.

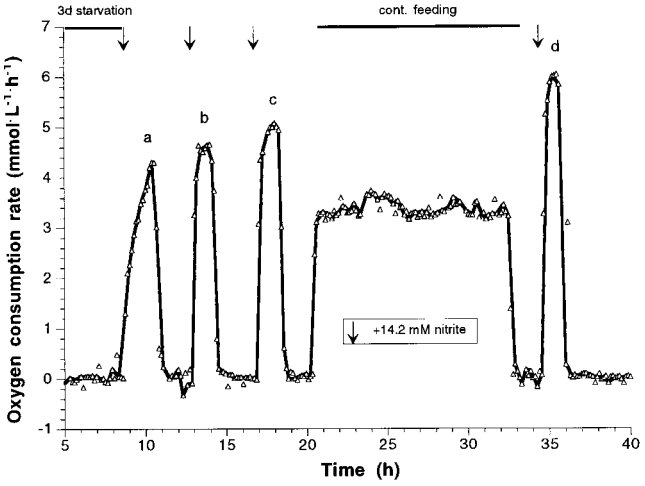

FIG. 5. Resuscitation of *N. winogradskyi* after 3 days of nitrite depletion. Definitions: ↓ = nitrite pulses of 14.2 mM; biomass concentration = 137 mg of biomass-C · liter^{-1}; maximum oxygen consumption rates 30 (a), 33 (b), 36 (c), and 44 μmol O_2 · mg of biomass-C^{-1} · h^{-1}. The maximum activity (see Fig. 4) was reestablished at the latest after 12 h of continuous feeding and the fourth pulse.

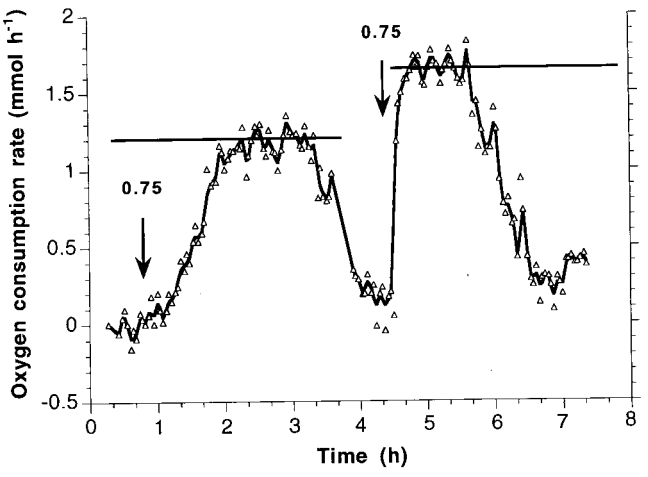

FIG. 6. Resuscitation of *N. europaea* after 17 days of ammonia depletion. Definitions: ↓ = ammonia pulses of 0.75 mM; biomass concentration = 138 mg of biomass-C · liter^{-1}; maximum oxygen consumption rate of the first pulse = 22 μmol of O_2 · mg of biomass-C^{-1} · h^{-1}.

shown for *Nitrobacter*).

Spontaneous respiratory activity and resuscitation after repeated ammonia or nitrite pulses and continuous feeding were measured after different time periods for *N. europaea* and *N. winogradskyi. N. europaea* showed fast resuscitation after 3 days of starvation. After two successive pulses of ammonia, the original activity was established again, while the first pulse yielded ±70% of the activity of the unstarved population (data not shown). When *N. winogradskyi* was starved for 3 days, the maximum oxygen consumption rate still was 66% of the activity of the unstarved population (Fig. 5). The shape of the first peak (Fig. 5, peak a) clearly indicates that the maximum activity is not attained readily (apart from the hysteresis of the system as mentioned above), as happens in unstarved cells (see Fig. 4). Additional pulses gave rise to an increasing maximum respiratory activity, and it took 14 h of continuous feeding until the original activity was reestablished.

When *N. winogradskyi* was starved for 6 days, the response in activity was only 25% compared with the maximum respiratory activity of unstarved cells, but it increased to 80% after a second pulse and continuous feeding for 15 h. For these cells it took more than 3 h to attain the 25% maximum activity compared to unstarved cells during the first nitrite pulse after starvation (data not shown).

N. europaea started to show a somewhat retarded resuscitation after 17 days of starvation (Fig. 6). After a first pulse of 0.75 mM ammonia, the population reached 50% of the maximal oxygen consumption rate within 1 h, in contrast to *N. winogradskyi*, which was less active already after 6 days of starvation. After three pulses maximal activity was reached again. Consequently, the resuscitation of *Nitrosomonas* occurs much more quickly than the resuscitation of *Nitrobacter*.

When *N. winogradskyi* has been starved for 35 days (Fig. 7) and pulsed with nitrite, no spontaneous oxygen consumption was detectable by mass spectrometric measurement of the difference in oxygen concentration of inlet and outlet gas for ca. 5 h. Nevertheless, within a few minutes, the dissolved oxygen concentration dropped to slightly below 100% saturation, indicating a very low level of immediate activity. Subsequently, it took ca. 60 h to oxidize the whole amount of nitrite added; this was accompanied by a steady increase in the oxygen consumption rate. Finally, a consumption rate of 17 μmol of O_2 · mg of biomass-C^{-1} · h^{-1} (37% activity of unstarved cells) was attained. Another 20 h in the absence of nitrite again caused a drop in activity that was compensated for and even surpassed during the following pulse. Altogether, after four pulses of 14.2 mM nitrite, *N. winogradskyi* had recovered to 50% activity with respect to the maximum oxygen consumption rate of unstarved cells. In order to inhibit de novo protein synthesis, the next nitrite pulse was added to the fermentor together with chloramphenicol to give a final concentration of 100 μg · ml^{-1} (this concentration has been found to be sufficient to inhibit the growth of *N. winogradskyi* in a batch culture). Thereafter, the medium (plus 100 μg of chloramphenicol per ml) was continuously supplied again. After an even slightly higher spontaneous response in oxygen consumption rate compared to the

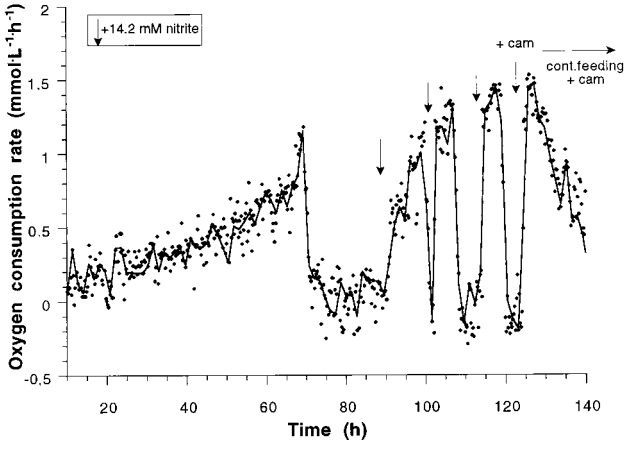

FIG. 7. Resuscitation of *N. winogradskyi* after 35 days of nitrite depletion. With the last pulse, the maximum specific activity amounted to 50% of the activity of unstarved cells (Fig. 4). Together with the last pulse, chloramphenicol was added to give a final concentration of 100 μg · ml^{-1}. At the same time, the substrate supply was switched to continuous feeding, also including chloramphenicol at 100 μg · ml^{-1} to maintain the concentration. (Note that the absolute activity is lower compared to Fig. 4 and 5 due to the removal of biomass for another experiment.)

former pulse (without nitrite), a decrease in activity could be seen, and the oxygen consumption rate dropped with 50% within the next 20 h. This suggests that protein synthesis is indispensable for recovering the original activity and further suggests that even the present physiological status cannot be maintained without synthesizing new proteins. Obviously, the protein turnover rate will be higher in the presence of an energy-delivering substrate because the spontaneous maximum oxygen consumption rates after several days of nitrite starvation were always higher than expected from the protein turnover. According to Bock et al. (7), both inactivation of the nitrite oxidoreductase and changes in the fine structure of the cells, especially with respect to the multiple intracellular membranes, take place during starvation. Therefore, resuscitation will be accompanied by similarly complex different rearrangements as well as by the synthesis of new cellular components depending on the duration of the time period of starvation. It is worth mentioning that a significant decrease in cell numbers during starvation was never observed. Even the mean cell volume remained constant during prolonged time periods in absence of nitrite. This finding is also in agreement with other studies (8, 42), and it confirms the challenge to improve the integral analysis of bacterial populations and the need to get more insight into the individual activity distributions to better understand the life strategies of these organisms.

The findings of a faster resuscitation of *Nitrosomonas* in pure culture were checked in a mixed-culture experiment with both strains, which were additionally immobilized on BioCord. *N. europaea* and *N. winogradskyi* were immobilized on BioCord and kept at 4°C for 3 and 6 months. The recovery experiments confirmed the above-mentioned observations that *N. europaea* can be resuscitated more easily than can *N. winogradskyi*. After the BioCord was added to ammonia-containing growth medium at 25°C, nitrite accumulated immediately both on the 3- and 6-month-old samples, thus showing that *N. europaea* was able to immediately use available ammonia, whereas *N. winogradskyi* needed time to revive before it could use the accumulated nitrite.

If the above-mentioned findings, together with the finding that *N. europaea* is a better competitor for limiting amounts of oxygen than *N. winogradskyi* (19), are generally valid for the physiological responses of ammonia and nitrite oxidizers, it would explain the occasionally observed nitrite accumulation in surface waters (29), wastewater treatment plants (2, 26), and even soils (unpublished observations of soil columns of acid pine forest soils that were exposed to 0.5 mM ammonium sulfate after 3 weeks of starvation).

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