Microelectrode Measurements of the Activity Distribution in Nitrifying Bacterial Aggregates

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Microelectrodes for ammonium, oxygen, nitrate, and pH were used to study nitrifying aggregates grown in a fluidized-bed reactor. Local reactant fluxes and distribution of microbial activity could be determined from the microprofiles. The interfacial fluxes of the reactants closely reflected the stoichiometry of bacterial nitrification. Both ammonium consumption and nitrate production were localized in the outer shells, with a thickness of approximately 100 to 120 μm, of the aggregates. Under conditions in which ammonium and oxygen penetrated the whole aggregate, nitrification was restricted to this zone; oxygen was consumed in the central parts of the aggregates as well, probably because of oxidation of dead biomass. A sudden increase of the oxygen concentration to saturation (pure oxygen) was inhibitory to nitrification. The pH profiles showed acidification in the aggregates, but not to an inhibitory level. The distribution of activity was determined by the penetration depth of oxygen during aggregate development in the reactor. Mass transfer was significantly limited by the boundary layer surrounding the aggregates. Microelectrode measurements showed that the thickness of this layer was correlated with the diffusion coefficient of the species. Determination of the distribution of nitrifying activity required the use of ammonium or nitrate microelectrodes, whereas the use of oxygen microelectrodes alone would lead to erroneous results.

In response to environmental problems caused by strongly increased ammonium emission by intensive agricultural and industrial activities, wastewater (19) and waste gas (8) purification plants have been designed and applied to reduce emission of nitrogen compounds. The ultimate removal of ammonium can be achieved by dissimilative reduction of nitrate to molecular nitrogen. Therefore, ammonium has to be oxidized to nitrate first, which can be achieved by autotrophic nitrification. The reaction for the oxidation of ammonium to nitrate is given by:

\[ \text{NH}_4^+ + 2\text{O}_2 \rightarrow + \text{NO}_3^- + 2\text{H}_2\text{O}^+ \]

Thus leading to a stoichiometry for \( \text{NH}_4^+ -\text{O}_2 -\text{NO}_3^- \) of 1:2:1. During biological nitrification, however, nitrogen is also incorporated in biomass (Nbio) and additional oxidizing equivalents are available from CO₂ fixation and reduction. For nitrification, including biomass synthesis, a stoichiometry for \( \text{NH}_4^+ -\text{O}_2 -\text{NO}_3^- -\text{N}_{\text{bio}} \) of 1:1.83:0.98:0.021 has been suggested (10).

Since the growth rates and biomass yields of nitrifying organisms are low, their application in continuous-flow processes requires efficient retention of biomass, and development of bacterial aggregates with good settling properties is needed. The design, scale-up, and operation of such bioreactors require a profound understanding of biofilm behavior. Therefore, information about the processes involved and the location of the processes in aggregates is required. Up to now, most efforts have been directed to mathematical modeling of nitrification by immobilized biocatalysts (11, 12, 22, 32). According to these studies, ammonium is rate limiting at bulk concentrations lower than 0.2 mM, for biofilms incubated in air saturated medium, because of diffusive resistances. At higher ammonium concentrations, oxygen is the limiting substrate. Evidence for the theoretical models has been provided by pilot studies and correlation of literature data (29). Microprofiles of the reactants inside the biofilm, however, give better insight into the factors governing the process. Therefore, measurements were done with ammonium (6) and nitrate (5) microelectrodes, enabling a spatial resolution in micrometers. With oxygen microelectrode measurements, microprofiles of all reactants and concomitant fluxes of the biological nitrification can be obtained. Moreover, direct measurement of the thickness of the active part of the biofilm is possible.

In a reactor with immobilized bacteria, substrate transport to the cells involves convective transport from the bulk liquid to the vicinity of the biofilm, convective and diffusive transport through a boundary layer, and diffusion through the biofilm to the cells; products follow the same route in opposite direction. The mass boundary layer δ is the zone near the biofilm, where the local concentration gradually changes from the bulk liquid level to the concentration at the surface of the particle. Because of this layer, the substrate and product concentrations at the surface of the biofilm may differ considerably from the bulk concentrations. The thickness of the mass boundary layer is dependent on the hydrodynamics of the liquid and the diffusion coefficient of the compound. The local substrate concentration in the biofilm is determined by the reaction rate and diffusion resistance. Therefore, transport through the mass boundary layer must be taken into account if it significantly contributes to the total diffusional resistance. With microelectrodes, δ can be measured directly.

The biological fluidized-bed process is a relatively new technique in wastewater treatment. Applications have been developed for anaerobic and aerobic processes, e.g., methanogenesis, acidification (14, 19), and oxidation of sulfide to sulfate and of ammonium to nitrate (21). For the development of the nitrifying bacterial aggregates used in this study, a two-phase (liquid-solid) fluid-bed nitrification reactor with an external aerator was operated successfully. Due to the relatively low shear forces, as compared with those in three-phase systems, aggregates developed without addition.
interface, liquid profiles were almost recirculated (1.85 mM), CaCl$_2$ (8.36 × 10^{-2} mM), MgCl$_2$ (1 × 10^{-2} mM), and NaMoO$_4$ (4 × 10^{-3} mM). The influent flow rate was 2.14 × 10^{-2} ml/s. The liquid phase of the reactor was recirculated at a rate of 1 ml/s via a 700-ml aeration tank. The temperature was maintained at 30°C, and the pH was kept at 8. The reactor was inoculated with activated sludge from a municipal wastewater treatment plant (R.W.Z.I.-oost, Amsterdam). The reactor was covered with black paper to prevent growth of algae.

**Microelectrode preparation and microprofile measurements.** Ammonium, nitrate, and pH microelectrodes with tip diameters of 1 μm were prepared as described previously (5–7). Oxygen electrodes with internal reference and a tip diameter of 10 μm were prepared (25). Measurements were performed in a flow cell as described previously (6) at 30°C with a flow rate of 5 × 10^{-2} ml/s, resulting in an average liquid velocity of 0.5 mm/s. The medium used for profile measurements consisted of NH$_4$Cl (0.3 mM), NaNO$_3$ (0.1 mM), Na$_2$HPO$_4$ (5.7 × 10^{-3} mM), MgCl$_2$ (8.36 × 10^{-2} mM), FeCl$_3$ (1.85 × 10^{-1} mM), CaCl$_2$ (2 × 10^{-2} mM), MnCl$_2$ (1 × 10^{-2} mM), NaMoO$_4$ (4 × 10^{-3} mM), and EDTA (2.7 × 10^{-1} mM) adjusted to pH 8. Potassium ions were not added to avoid interference with the ammonium electrode. The electrodes penetrated the aggregates 90 to 110° from the front stagnation point of the flow on the particle; the direction was almost perpendicular to the flow. Aggregates were sampled from approximately 0.25 m of the reactor base. All microprofiles were recorded in different aggregates. The biofilm-liquid interface was determined by using a dissection microscope, with a small depth of field, focused on the edge of the aggregate. The microelectrode was brought into the focal plane and advanced axially until it contacted the aggregate edge.

**MATERIALS AND METHODS**

**Reactor operation.** The reactor setup is presented schematically in Fig. 1. A conical 360-ml continuous-flow reactor with a height of 0.8 m and internal diameters of 1 cm at the bottom and 3 cm at the top was used. The reactor influent contained NH$_4$Cl (5.6 mM), Na$_2$CO$_3$ (30 mM), Na$_2$SO$_4$ (1 mM), K$_2$HPO$_4$ (5.7 × 10^{-1} mM), MgCl$_2$ (8.36 × 10^{-2} mM), FeCl$_3$ (1.85 × 10^{-1} mM), CaCl$_2$ (2 × 10^{-2} mM), MnCl$_2$ (1 × 10^{-2} mM), and NaMoO$_4$ (4 × 10^{-3} mM). The influent flow rate was 2.14 × 10^{-2} ml/s. The liquid phase of the reactor was recirculated at a rate of 1 ml/s via a 700-ml aeration tank. The temperature was maintained at 30°C, and the pH was kept at 8. The reactor was inoculated with activated sludge from a municipal wastewater treatment plant (R.W.Z.I.-oost, Amsterdam). The reactor was covered with black paper to prevent growth of algae.

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**Analytical methods.** Nitrate and nitrite concentrations were determined by high-pressure liquid chromatography (Chrompack Ionospher tm A column; Rl detector; ERC 7510 ERMA). Ammonium was determined colorimetrically (Spectroquant 14752; Merck). Nitrogen incorporated in biomass was estimated from the dry weight washed out from the reactor by using a microbial element analysis (27).

**Calculations.** Fluxes ($J_s$) through the interface of aggregates with radius $r$ were calculated by using Fick’s first law:

$$J_s = -D_s(dS/dr)_{r=R}$$

where $D_s$ is the molecular diffusion coefficient of compound S in the liquid phase, $r$ is the distance from the center, and $(dS/dr)_{r=R}$ is the concentration gradient in the boundary layer at the biofilm-liquid interface. The diffusion coefficients of oxygen, ammonium, and nitrate at 30°C were taken at 2.72 × 10^{-5}, 1.8 × 10^{-9}, and 1.6 × 10^{-9} m^2/s, respectively (1).

Mass transfer between flowing medium and a solid surface is determined by the thickness of the mass boundary layer and therefore is dependent on both the diffusion coefficient of the compound and the hydrodynamics. The hypothetical boundary layer $\delta_h$, the zone in the near vicinity of the biofilm where mass transfer is governed by diffusion, is defined by the slope of its concentration profile at the surface of the biofilm (16):

$$\delta_h = (S_b - S_i)/(dS/dr)_{r=R}$$

where $S_b$ is the bulk concentration at an infinite distance and $S_i$ is the concentration at the biofilm-liquid interface. When the relationship between mass transfer to both hydodynamic and diffusivity is expressed as the Sherwood number (Sh), the empirical equation for spheres in flowing liquid is (24):

$$Sh = 2 + 0.59 \text{Re}^{1/2} \text{Sc}^{1/3} = k d_p D_s$$

where Re is the Reynolds number ($\rho u_d d_p / \eta$), dimensionless number defining the hydraulic regime, $\text{Sc}$ is Schmidt number ($\eta / \rho D_s$, dimensionless number relating the hydrodynamic and the mass boundary layer), $\rho$ is the liquid density, $\eta$ is the dynamic liquid viscosity, $k$ is the mass transfer coefficient, and $d_p$ is the diameter of the particle. $\delta_h$ is calculated from:

$$k = D_s / \delta_h$$

An analytical approximation was used for calculation of the local mass boundary layer thickness $\delta$, which is the distance from the surface where the concentration equals 90% of the bulk concentration (18):

$$\delta = 1.266(D_s R^2/\mu)^{1/3} \theta - (\sin \theta)/2)^{1/3}/\sin \theta$$

where $\theta$ is the angle in radians from the front stagnation point of the flow on the particle. This equation is only valid for spherical particles under creeping-flow conditions (Re $\ll$ 1), i.e., when the streamlines on both sides of the equator perpendicular to the flow direction are completely symmetrical.

**RESULTS**

**Reactor performance.** During start-up, the original sludge used as the inoculum disappeared and disc-shaped, smooth aggregates with a diameter of 1 to 3 mm developed (Fig. 2).
Initially, the nitrite concentration was low, whereas conversion of ammonium to nitrate was optimal after 2 months and remained stable for at least 9 months. The influent contained 5.6 mM ammonium; in the steady state, 10.26 mol m⁻³ oxygen was consumed, as calculated from the concentration difference over the bed and the recirculation time. The effluent contained approximately 0.1 to 0.3 mM ammonium, ≤ 0.05 mM nitrite, 0.24 mM biomass nitrogen, and 5.2 mM nitrate. Thus, the conversion of ammonium to nitrate amounted to 93%, and approximately 4% of the ammonium was incorporated in biomass. The amount of ammonium stripped out of the reactor was negligible. With an oxygen microelectrode lowered carefully in the reactor, the oxygen concentration at the sampling height was determined to be approximately 0.1 mM. Occasionally, we observed protozoa, which were probably predating on the nitrifying population; these protozoa were killed by an anaerobic treatment of the reactor for 1 h. After resumption of aeration, nitrification proceeded as before.

Microprofiles. The smooth surface of the aggregates facilitated accurate determination of the biofilm-liquid interface. Simultaneous measurement of ammonium, oxygen, and nitrate profiles in one aggregate is difficult for technical reasons; therefore, three to five profiles of each reactant were measured in separate aggregates. Some of these microprofiles, measured in air-saturated medium with an ammonium concentration of 0.23 mM, are presented in Fig. 3. Clearly, the ammonium and oxygen concentrations in the aggregates decreased gradually going from the bulk liquid to the center of the aggregates, whereas the nitrate concentration increased on the same route. Ammonium and nitrate profiles indicated that under these conditions the nitrifying activity was located mainly on the outside of the aggregate as a shell approximately 100 to 120 μm thick. The oxygen concentration at the 100-μm depth was low (0.04 mM). Therefore, the oxygen supply seemed the limiting factor for nitrification, although oxygen was present at a depth of 300 μm. From these profiles, the fluxes of the various compounds through the interface were calculated; their mean values are presented in Table 1. The oxygen fluxes from the nitrifying outer shell to the deeper non-nitrifying zone were calculated by assuming that the intraparticle diffusion coefficient was equal to the molecular diffusion coefficient. Oxygen fluxes through the interface were corrected by subtracting the fluxes from the active zone to the center, resulting in the oxygen consumption in the nitrifying zone (Table 1). The ratio of the uncorrected fluxes for NH₄⁺-O₂-NO₃⁻ was...

FIG. 2. Photograph of nitrifying aggregates.

FIG. 3. Microprofiles of ammonium (+), oxygen (○), and nitrate (□) in nitrifying bacterial aggregates with a diameter of 2 mm in air-saturated medium. The dotted line indicates the 100-μm depth in aggregates.
TABLE 1. Fluxes of ammonium, oxygen, and nitrate through the aggregate-bulk liquid interface.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Flux rate (10^{-7} \text{ mol/m}^2 \text{ s}) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\text{NH}_4^+)</td>
</tr>
<tr>
<td>Air saturated</td>
<td>13.8 (3.4)</td>
</tr>
<tr>
<td>Oxygen saturated</td>
<td>8.9 (1.8)</td>
</tr>
</tbody>
</table>

\(^a\) Numbers within parentheses indicate 90\% probability intervals \(t\) test of significance.
\(^b\) Values corrected for oxygen flux to non-nitrifying center.

1:2.00:0.83, whereas the ratio for the corrected fluxes was 1:1.80:0.83.

Mass boundary layers were observed outside the aggregate mass, and the ammonium and oxygen concentrations at the surface of the aggregate were considerably lower than the bulk concentrations. Mean values of measured \(\delta_n\) for oxygen, nitrate, and ammonium and values calculated by using equations 3 and 4 are summarized in Table 2. Values for \(\delta\), measured as the distance from the aggregate surface where the local concentration was 90\% of the concentration difference between the bulk and the interface \((S_b - S_i)\) are presented in Table 2. The Reynolds numbers in the flow cell were calculated to be 5 for hydraulic flow and 2 for the submerged aggregate placed in the flow cell. Therefore, the flow regime in the cell and around the aggregate was laminar.

This was confirmed by observation of the pattern of ink leaking out of ink-saturated spherical agar beads with a diameter of 2.5 mm.

To investigate whether oxygen is indeed the limiting factor for the ammonium oxidation, experiments were performed in medium conditioned with pure oxygen at concentrations of up to 1.15 mM. Some of the microprofiles of the reactants measured under high-oxygen conditions and after an incubation period of at least 1 h are presented in Fig. 4. Oxygen penetrated the whole aggregate; however, the nitrification zone was still restricted to the outer 100 \(\mu\text{m}\) of the aggregate, as shown by the ammonium and nitrate profiles. The concomitant mean fluxes of the reactants through the interface and those of oxygen corrected for the uncorrected fluxes to the non-nitrifying center (Table 1) did not increase when the oxygen concentration in the bulk liquid was maintained at the maximal level for 2 h. On the contrary, the activities of the aggregates and the ammonium and nitrate fluxes were significantly lower than those of aggregates under atmospheric oxygen conditions, with a probability of more than 0.95 \(t\) test of significance). The oxygen flux through the interface did not decrease significantly, according to this test, because of the increased flux to the non-nitrifying center. However, the net consumption in the active zone, obtained after correction for the efflux to the non-nitrifying center, did decrease significantly. The ratio of \(\text{NH}_4^+\) to \(\text{NO}_3^-\) for the uncorrected fluxes was 1:2.8:0.99, whereas that for the corrected fluxes was 1:1.79:0.99.

Microprofiles of the pH in aggregates incubated under low- and high-oxygen conditions are presented in Fig. 5. In air-saturated medium containing 0.23 mM oxygen, the pH in the biofilm decreased to 6.7 within the outer layer of 100 \(\mu\text{m}\). In oxygen-saturated medium containing 1.15 mM oxygen, the pH decrease in the biofilm was less pronounced, because of decreased nitrification activity and levelled off at a depth of 200 \(\mu\text{m}\) to a value of 6.85.

**DISCUSSION**

The steady-state stoichiometry for nitrification and biomass synthesis in the fluid-bed reactor \((\text{NH}_4^+\text{-O}_2\text{-NO}_3^-\text{-NH}_4^+)\) was 1:1.84:0.94:0.04. The measured biomass yield was higher than expected; however, within the limits of experimental accuracy the values found are close to those sug-

### FIG. 4. Microprofiles of ammonium (+), oxygen (○), and nitrate (□) in nitrifying bacterial aggregates with a diameter of 2 mm in oxygen-saturated medium. The dotted line indicates the 100-\(\mu\text{m}\) depth in aggregates.

### FIG. 5. pH microprofiles measured in nitrifying bacterial aggregates incubated in air-saturated (+) and oxygen-saturated (□) medium. The dotted line indicates the 100-\(\mu\text{m}\) depth in aggregates.
gested previously (10). The ammonium removal was almost complete and amounted to 98.2 to 99.5%. The nitrification capacity of the reactor amounted to $3 \times 10^{-4}$ moles/m² s, which is lower than the reported performance in a gas-lift reactor ($1.5 \times 10^{-3}$ moles/m³ s) (19). The high recirculation/influent ratio of 47, needed to supply sufficient oxygen for complete ammonium oxidation, makes this reactor type unattractive for industrial applications, since a comparable system with pure oxygen and a recirculation ratio of 10 was also rejected (19). Moreover, the possibilities for upsizing the reactor are limited by the oxygen depletion at a reactor height of less than 1 m. For industrial purposes, three-phase fluidized bed reactors, with direct aeration of the reactor content, have been designed and applied (19). However, the reactor is highly suitable as a laboratory device because the low shear forces enable the development of large aggregates. Also the absence of carrier material that may damage the fragile electrode tips is of considerable advantage for microelectrode studies.

From the microprofiles measured in air-saturated medium (Fig. 3), it can be observed that the active nitrifying zone was limited to the outer 100 to 120 µm of the aggregates. Oxygen was not depleted completely at 100 µm depth but penetrated into the non-nitrifying center of the aggregate, where it was consumed by a process other than nitrification. As a result, the oxygen flux through the aggregate interface was 15% higher than that expected from the stoichiometry of the nitrification reaction and the fluxes of ammonium and nitrate. Oxygen may have been limiting for the nitrification rate, since the oxygen concentration at 100 µm depth came close to its reported $K_0$ ($2 \times 10^{-2}$ mM [29]). In that case, an increase of the oxygen bulk concentration should result in an increase of nitrifying activity. Microprofiles measured in medium saturated with pure oxygen did not show an increase of ammonia and nitrate fluxes or an increase in the thickness of the active zone. On the contrary, a decrease of the fluxes of the reactants was observed. Nitrifying activity was still limited to the outer 100-µm zone of the aggregates, although oxygen and ammonium concentrations in the whole aggregate were high. The centers of the aggregates were obviously inactive, whereas the active nitrifying bacteria were located exclusively in the outer 100 µm.

The distribution of activity might be explained by the history of the aggregates in the reactor. The thickness of the active zone of the sampled aggregates can be approximated from the local reactor circumstances by assuming zero-order kinetics for oxygen as the limiting substrate. Then the relation between the interface concentration $S_i$ and penetration depth $\lambda$ is given by (23):

$$\lambda = (2D_{\text{eff}}S_i/V_0)^{0.5} \tag{6}$$

where $D_{\text{eff}}$ is the effective diffusion coefficient in the biofilm and $V_0$ is the volumetric reaction rate. Aggregates were sampled from the lower part of the reactor (0.25 m), where the mean liquid velocity was $5 \times 10^{-3}$ m/s. According to equation 3, $Sh$ was 16 and the resulting mass transfer coefficient $k$ for oxygen amounted to $2.16 \times 10^{-5}$ mol/m² s.

Since the active zone is small compared with the radius of the particle, a flat film approximation can be used to calculate $V_0$, for which it can be deduced that

$$J = V_0 \lambda \tag{7}$$

In the aggregates investigated, $J$ was approximately $25 \times 10^{-7}$ mol/m² s (Table 1) and $\lambda$ was $10^{-4}$ m; therefore, $V_0$ for oxygen consumption is $2.5 \times 10^{-2}$ m/M. At steady state the flux through the interface equals the consumption in the aggregate:

$$kmd_{\text{eff}}(S_b - S_i) = V_0md_{\text{eff}}^\lambda \tag{8}$$

This, in combination with equation 6, results in

$$k(S_b - S_i) = (2D_{\text{eff}}V_0)^{0.5}(S_i)^{0.5} \tag{9}$$

At the relevant reactor height the liquid bulk oxygen concentration $S_b$ was approximately 0.1 mM; therefore, from equation 9 it follows that $S_i$ is approximately $2.5 \times 10^{-4}$ mM. According to equation 6, the penetration depth $\lambda$ for oxygen is 73 µm under reactor conditions. This value might be a slight underestimation, because the assumption of zero-order kinetics; however, it is close to the observed thickness of the active nitrifying zone. Under the assumption that ammonium is the limiting substrate, the same calculation results in an ammonium penetration depth of 103 µm for the lowest observed ammonium concentration in the reactor bulk liquid of 0.1 mM. A comparison of the calculated penetration depths of oxygen and ammonium shows good evidence that oxygen is the limiting substrate. The thickness of the active nitrifying zone is determined by the oxygen supply to the aggregate, and the center of the aggregate consisted of biomass inactivated by oxygen depletion. The center probably consists of lysed cells, since it is relatively flaccid compared with the outer shell and is easily washed away from broken aggregates (Fig. 2).

For the flow cell, it is concluded that nitrification is limited by the oxygen transport rate at ammonium concentrations higher than 0.1 mM. When the dissolved oxygen concentration is lower than the air-saturated value, as occurs under reactor conditions, it can be expected that the bulk liquid concentration at which ammonium becomes limiting for nitrification will even be lower than 0.1 mM. With the calculated oxygen penetration depth of 73 µm described above, ammonium becomes limiting below a bulk liquid concentration of 0.09 mM, when $S_i$ is 0.034 mM. The observation of a significant mass boundary layer during the microprofile measurements complicates the translation of the presented results to reactor circumstances. In the reactor, liquid velocities are higher than those applied in the flow cell, and consequently the mass boundary layer is smaller. In the reactor, therefore, the concentrations of reactants at the aggregate surface are closer to the bulk concentrations. It is clear that the mass boundary layer may influence the conversion rate of nitrifying aggregates strongly. This phenomenon should be taken into account in the design of experiments and reactors.

There is no general consensus in the definition of $\delta$, which has been defined as the distance from the surface where the concentration equals 100% (17, 28), 99% (26), and 90% (18) of the bulk concentration. For the last case, an analytical approximation for the local $\delta$ was found for spherical particles under creeping-flow conditions, when $Re \ll 1$ (equation 5). The thus calculated values for $\delta$ (Table 2) exceed the measured values. This could be explained by the fact that equation 5 is derived for creeping-flow conditions, whereas the $Re$ of aggregates in the flow cell was 2. An additional explanation for the differences may be found in the geometry of the aggregates. Calculations are based on the assumption of perfect spheres, whereas the aggregates were irregularly shaped and thus the flow pattern was influenced. Nevertheless, calculations lead to estimations that are correct within 1 order of magnitude. Furthermore, the prerequisite of creeping flow may be to rigid, and equation 5 may be a
sufficient approximation for flow conditions at which the flow does not detach from the sphere; namely, for Re < 20 (4).

A different interpretation is given by the definition of the hypothetical boundary layer δb: a zone in the vicinity of the biofilm in which mass transfer is governed by diffusion only (equation 2). This definition leads to a considerably smaller thickness of δb and was relevant for the stagnant liquid conditions prevailing, as apparent from oxygen microelectrode measurements (16). In this study, the estimation of δb from equations 3 and 4 was higher than the observed values. It should be stressed that δb thus calculated for aggregates in flowing media represents a mean value for the whole aggregate, whereas the actual local δb depends on the distance from the front stagnation point of the liquid on the aggregate.

The slopes of the microprofiles in the boundary layer and in the outer zone of the aggregate were approximately the same, indicating that the effective diffusion coefficients in nitrifying aggregates were close to the molecular diffusion coefficients. The values in the aggregates may have been slightly lower than the theoretical molecular diffusion coefficients. Therefore, the oxygen fluxes to the non-nitrifying center might be overestimated by 10 to 20%.

Microprofiles measured in medium saturated with pure oxygen did not show the expected increase of ammonium and nitrate fluxes or increase of the thickness of the active zone. On the contrary, a decrease of the fluxes of the reactants was observed. A reduced nitrifying activity was found in the outer 100-μm zone of the aggregates, whereas the aggregate center obviously consisted of inactive bacteria that may have died by starvation. Moreover, high oxygen concentrations appeared to inhibit nitrification. This is contrary to the results obtained with a Warburg respirometer in a study in which the rates of ammonium oxidation by biofilm material were measured under oxygen and normal air (13). However, since the contents of the Warburg vessel were not agitated, a considerable drop in the oxygen concentration in the mass boundary layer might have protected the cells from inhibition by oxygen. Inhibition of growth of Nitrobacter cultures was observed under elevated oxygen concentrations (9). Rapid ammonium accumulation upon a sudden increase of the oxygen concentration was observed during mixed culture experiments; however, acclimatization of nitrifiers to high oxygen concentrations was observed (3, 15).

The mechanism of the observed inhibition is unknown, but it might be speculated that accumulation of free radicals plays a role. Therefore, adaptation of biofilms to high oxygen levels may involve development of protective mechanisms such as superoxide dismutase and catalase. The applied incubation time in our studies (1 to 2 h) clearly is not sufficient for these adaptations.

In air-saturated medium, the ratio of the reactant fluxes approximated the stoichiometry of the nitrification reaction, whereas in oxygen-saturated medium the oxygen flux was relatively high. Approximately 30% of the oxygen flux in aggregates incubated in oxygen-saturated medium can be attributed to processes in the center of the aggregates, most probably oxidation of dead biomass. Therefore, the thickness of the active nitrifying zone cannot be determined from oxygen microprofiles under increased oxygen tensions. Moreover, because of this phenomenon the results obtained with biological oxygen monitors and Warburg vessels may lead to erroneous conclusions.

The reaction equation for nitrification indicates that H₂O₂ is one of the products. Previous measurements in which a pH microelectrode was mounted to the back of a nitrifying biofilm showed that the pH decreased as a result nitrifying activity (30). Indeed, the pH microprofiles presented in this report show a substantial decrease of the pH in the aggregates. The thickness of the active zone, as determined from the pH microprofile measured with air-saturated medium, also approximated 100 μm. The profiles measured in oxygen-saturated medium were less pronounced than those measured in air-saturated medium. It can be concluded that the extent of the pH profiles reflected the nitrifying activity.

The unknown oxidation processes in the center of the aggregates occurring under high oxygen tension had little influence on the local pH. The decrease of the pH is not sufficient for inhibition of nitrification, since nitrification will proceed uninhibited in the pH range of 5.5 to 7.3 (2). Therefore, the explanation for the restriction of activity to the outer 100-μm layer must be the absence of nitrifying organisms in the centers of the aggregates.

In some other studies indications of heterogeneous activity distribution in nitrifying aggregates were found. Development of a heterogeneous biomass distribution was found in an artificial medium system, namely, 2-mm spherical gels containing immobilized Nitrobacter agilis cells with an initially homogeneous distribution. After a steady state was reached, 90% of the immobilized cells were localized in the outer shell of 140 μm, as shown by image analysis (31). Another study showed that the specific nitrifying activity of biofilms decreased during biofilm growth; this decrease was attributed to development of heterotrophic organisms, diffusion limitation, or segregation of microbial communities (20). The critical biofilm thickness was reported to be 0.1 mm, however, without supporting data (19). These observations are confirmed, however, by the direct measurements presented in this study, which underlines the value of the microelectrode technique.

REFERENCES
ACTIVITY DISTRIBUTION OF NITRIFYING AGGREGATES

9:561-566.


