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COMPETITION BETWEEN *Nitrospira* spp. AND *Nitrobacter* spp.

IN NITRITE-OXIDIZING BIOREACTORS

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Running title: Competition between *Nitrospira* and *Nitrobacter*

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Summary

In this work the question was addressed if in nitrite oxidizing activated sludge systems the environmental competition between *Nitrobacter* spp. and *Nitrospira* spp., which only recently has been discovered to play a role in these systems, is affected by the nitrite concentrations. Two parallel chemostats were inoculated with nitrifying activated sludge containing *Nitrospira* and operated under identical conditions. After addition of *Nitrobacter* to both chemostats, the nitrite concentration in the influent of one of the chemostats was increased such that nitrite peaks in the bulk liquid of this reactor were detected. The other chemostat served as control reactor, which always had a constant nitrite influent concentration. The relative cellular area of *Nitrospira* and *Nitrobacter* was determined by quantitative fluorescence *in situ* hybridization. The nitrite perturbation stimulated the growth of *Nitrobacter* while in the undisturbed control chemostat *Nitrospira* dominated. Overall, the results of this experimental study support the hypothesis that *Nitrobacter* is a superior competitor when resources are abundant, while *Nitrospira* thrive under conditions of resource scarcity. Interestingly, the dominance of *Nitrobacter* over *Nitrospira* , caused by the elevated nitrite concentrations, could not be reverted by lowering the available nitrite concentration to the original level. One possible explanation for this result is that when *Nitrobacter* is present at a certain cell density it is able to inhibit the growth of *Nitrospira* . An alternative explanation would be that the length of the experimental period was not long enough to observe an increase of the *Nitrospira* population.

Key words: *Nitrospira*, *Nitrobacter*, activated sludge, competition, chemostat.

INTRODUCTION

Nitrite oxidation, catalyzed by chemolithoautotrophic bacteria, is a key process in most nitrifying wastewater treatment plants (WWTPs). Traditionally, *Nitrobacter* was considered to be the most important nitrite-oxidizer in WWTPs (Henze, 1995). Therefore, the finding that *Nitrobacter* could not be detected in various nitrifying WWTPs, using fluorescence *in situ* hybridization (FISH), with specific 16S rRNA-targeted oligonucleotide probes, came as a surprise (Wagner, et al. 1996). Using the full cycle rRNA approach, the occurrence of novel, yet uncultured, *Nitrospira*-like nitrite-oxidizing bacteria in nitrifying WWTPs could be demonstrated (Daims, et al. 2001; Juretschko, et al. 1998).

Microorganisms have evolved strategies that enable them to successfully survive and maintain themselves within communities. It has been hypothesized that the predominance of *Nitrospira* over *Nitrobacter* in most WWTPs is a reflection of their different survival strategies. The competition between two groups of microorganisms for one substrate is based on the rate of substrate utilization. In terms of the saturation kinetics, two types of strategy for winning this competition are distinguished in microbial ecology: *r*-strategy (high maximum specific growth and substrate utilization rates at high substrate concentrations) and *K*-strategy (high substrate affinity at low substrate concentrations) (Andrews and Harris, 1986). While *Nitrospira* are (according to data extracted from combined microelectrode-FISH analyses) *K*-strategists, and thus may possess a low maximum specific growth rate, but are well-adapted to low nitrite and oxygen concentrations, *Nitrobacter* was hypothesized to be a relatively fast-growing *r*-strategist with low affinities to nitrite and oxygen (Schramm, et al. 1999, Kim and Kim, 2006). Since nitrite concentration in most reactors from WWTPs is low, *Nitrospira* should outcompete *Nitrobacter* in these systems. In plants with temporally or spatially elevated nitrite concentration,

however, as it happens for example in nitrifying sequencing batch reactors, both nitrite-oxidizers should be able to co-exist. In fact the co-occurrence of *Nitrobacter* and *Nitrospira* in a nitrifying sequencing batch biofilm reactor has already been observed (Daims, et al. 2001).

Better understanding of such interrelationships between *Nitrospira* and *Nitrobacter* growth strategy and the concentration of nitrite in the environment might enable improvements in the operation of treatment facilities, such as:

- Selection of adequate inocula for fast start-up of nitrification reactors or their recuperation after toxic shocks;
- Optimization of nitrification reactor operation conditions, such as continuous versus discontinuous feed, dissolved oxygen concentration etc.

Ecological studies performed in batch cultures demonstrated that *Nitrobacter* and *Nitrospira* cells could be enriched from activated sludge, when cultivated on media containing different initial nitrite-nitrogen ($C_{NO_2^-\text{-}N}$) concentrations, 406 mg L^{-1} and 40.6 mg L^{-1}, respectively (Bartosch, et al. 1999). Despite the great importance of using batch cultures to study the kinetic principles involved in the competition between *Nitrospira* and *Nitrobacter*, the utilization of continuous cultures is of a major practical importance, since most WWTPs have continuous activated sludge reactors. In these systems, growth is continuously limited by nitrite. Flow-through systems have been used in ecological studies as model systems for aquatic habitats, where nutrient concentrations are often growth rate limiting (Kuenen and Harder, 1982). The most commonly used flow-through system for ecological studies is the chemostat.

In this study, we investigated the competition between *Nitrospira* spp. and *Nitrobacter* spp. for nitrite in activated sludge, using chemostats. Microbial population dynamics in suspended

biomass was evaluated using fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes.

MATERIALS AND METHODS

Inocula. Nitrifying activated sludge from the Gut Marienhof WWTP (Germany) was used as initial inoculum in both reactors, containing 8.7 % of *Nitrospira* spp. (based on total bacteria FISH) and no detectable *Nitrobacter*.

Nitrobacter inoculum came from a nitrite-oxidizing culture in a laboratory chemostat fed with a mineral media (containing 250 mg L^{-1} of ammonium-nitrogen). The concentration was $2.5 \cdot 10^7$ cells mL^{-1}, with 53 % of *Nitrobacter* spp. and no detectable *Nitrospira*. A volume of 7.2 mL was added in each pulse.

Experimental design. Two parallel chemostats, with a working volume of 4.1 L, were inoculated with activated sludge containing *Nitrospira* bacteria. After an adaptation period of 12 d, *Nitrobacter* was added twice to both. In one chemostat, transient strong nitrite pulses in the influent were introduced to induce nitrite peaks in the bulk liquid. The other chemostat served as a control experiment, which always had a constant influent nitrite concentration. Both chemostats were operated at a dilution rate of 0.12 d^{-1}. The temperature was kept at 25 °C and the *pH* was adjusted to 7.5. Oxygen was supplied by pumping pre-humidified pressurized air through ceramic diffusers, and a constant dissolved oxygen concentration of 7 mg L^{-1} was kept in the chemostats. Mixing was provided by magnetic stirring (100 min^{-1}), as well as by aeration. The inorganic medium was composed of $NaNO_2$ (1.23 g L^{-1}), $NaHCO_3$ (20 mg L^{-1}), KH_2PO_4 (110 mg L^{-1}), $Mg(SO_4) \cdot 7H_2O$ (380 mg L^{-1}), $FeSO_4 \cdot 7H_2O$ (7.2 mg L^{-1}), EDTA (10.3 mg L^{-1}),

$ZnSO_4 \cdot 7H_2O$ (0.5 mg L^{-1}), $CuSO_4 \cdot 5H_2O$ (0.5 mg L^{-1}), and $NaMoO_4 . \cdot 2H_2O$ (0.5 mg L^{-1}), leading to a nitrite-nitrogen ($C_{NO_2^-\text{-}N}$) influent concentration of 250 mg L^{-1}. Nitrite was determined photometrically, in influent/effluent samples filtered with 0.22 μm membrane filters; suspended biomass in the chemostats was determined as Total Suspended Solids (TSS) (both according to APHA, 1995).

Fluorescence *in situ* hybridization (FISH). Samples of suspended biomass were fixed with 4 % paraformaldehyde solution, for *in situ* hybridization. FISH was performed using the hybridization and washing buffers as described previously (Manz, et al. 1992). The following 16S rRNA-targeted oligonucleotide probes were used in this study: (i) Ntspa712 and Ntspa662, which are specific for the phylum and genus *Nitrospira*, respectively (Daims, et al. 2001); (ii) Nit3, which is complementary to a sequence region of all *Nitrobacter* species (Wagner, et al. 1996); and (iii) EUB probe mixture, which consists of probes EUB338 (Amann, et al. 1990), EUB338-II, and EUB338-III (Daims, et al. 1999) covering the domain *Bacteria*. Probes for nitrite-oxidizing bacteria were used in a 1:1 ratio together with the specific probe competitors. The oligonucleotide probes were purchased as labeled derivatives (Interaktiva, Ulm, Germany). EUB338, EUB338-II, and EUB338-III were labelled with the fluorescent sulfoindocyanine dye Cy5, Ntspa712, Ntspa662, and Nit3 with Cy3. For simultaneous utilization of Ntspa712 and Nit3 the latter was alternatively labelled with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS).

Biomass samples from both chemostats were hybridized with probe NON338 labelled with FLUOS, Cy3 and CY5 to exclude non specific probe binding (Manz, et.al. 1992). In none of the samples was non-specific labelling of cells observed.

Microscopy and digital image analyses. Fluorescence signals were recorded with an LSM 510 confocal laser scanning microscope (Zeiss, Germany) equipped with two HeNe lasers (543 nm and 633 nm) for detection of Cy3 or Cy5 and one Argon laser (450 nm to 514 nm) for detection of FLUOS. For quantification of probe-targeted bacteria, simultaneous hybridizations were performed with a Cy3 labeled specific probe and Cy5 labeled EUB probe mixture. For each hybridization experiment at least 20 randomly chosen confocal images (thickness 1 µm) were recorded. The objective used was Plan-Neofluor objective (63×/1.25 oil). For each confocal image, the relative cellular area (*RCA*), defined as the ratio of the area of those cells labeled by the genus specific probe to the area of all bacteria stained by the EUB probe mixture, was determined semi-automatically using the procedure described previously (Schmid, et al. 2000). *RCA* of probe targeted bacteria was calculated as an average of 20 values ± 95% confidence interval. Size of cell aggregates (considered as the length between the two most distant points within the aggregate) were determined by using the measurement tools of the software delivered with the CLSM (LSM 510, version 2.01). More than 20 aggregates were measured and an average values ± 95% confidence interval was calculated.

RESULTS

We have been primarily concerned in this investigation with the selection of an adequate dilution rate to cultivate *Nitrospira* spp.. Previous work (data not published) showed that *Nitrobacter* spp., in contrast to *Nitrospira*, was enriched in a chemostat at a dilution rate of 0.25 d^{-1}. On the other hand, the condition to achieve nitrification in WWTPs is to operate the activated sludge tank with a dilution rate between 0.1 d^{-1} and 0.2 d^{-1}, for a temperature higher than 10 °C (Jansen, et al. 1995). Based on these data, a dilution rate of 0.12 d^{-1} was chosen.

Nitrospira was the dominant genus of nitrite-oxidizers in the activated sludge from the WWTP in Gut Marienhof, used as inoculum. *Nitrobacter* was not detected by FISH. The nitrite-nitrogen concentration ($C_{NO_2^-\text{-N}}$), measured in the activated sludge tank, was very low, 0.012 mg L^{-1}, indicating a very good nitrification performance.

Biomass concentration was determined at the end of the experimental period to be 5.8 mg/L for chemostat Control and 5.3 mg/L for chemostat Test. Simultaneously the specific nitrite oxidation rate constant $k_{N\text{-}NO_2}$ (zero order) was determined to be $1.52 \cdot 10^{-4}$ kg·kg^{-1}·s^{-1} in chemostat Control and $1.48 \cdot 10^{-4}$ kg·kg^{-1}·s^{-1} in chemostat Test by individual batch experiments, where 4 mg L^{-1} of $N\text{-}N^{-}O_2^{-}$ were added and the subsequent substrate consumption rate analyzed.

Population dynamics

Microbial population dynamics and nitrite concentration in both chemostats, obtained during the 100 d of operation, are depicted in Fig. 1. The adaptation period, following inoculation of both chemostats with activated sludge containing *Nitrospira*, was characterized by an increase of *Nitrospira*'s relative cellular area and a low concentration of nitrite-nitrogen ($C_{NO_2^-\text{-N}}$) in the bulk liquid (0.026 mg L^{-1} ± 0.015 mg L^{-1} for the chemostat Control and 0.065 mg L^{-1} ± 0.028 mg L^{-1} for the chemostat Test). This result indicated that *Nitrospira* spp. were able to grow at the chosen dilution rate (0.12 d^{-1}).

Nitrobacter spp. growth was detected in chemostat Test, where nitrite peaks in the bulk liquid were induced (up to 80 mg L^{-1}), 13 d after its first inoculation (Fig. 1, panel A). In the undisturbed chemostat Control, *Nitrobacter* spp. growth was not detected, after the same period of time (Fig. 1, panel B). Subsequently, *Nitrobacter* spp. grew continuously in chemostat Test, as opposed to *Nitrospira* spp. that decreased, until it became the dominant population (Fig. 1, panel

A). The dominance of *Nitrobacter* spp. over *Nitrospira* spp. could not be reverted by lowering the available nitrite concentration in the bulk liquid to the original level (Fig. 1, panel A). The nitrite concentration in the bulk liquid of chemostat Control, which had been always constant, presented an unexpected oscillation between days 35 and 75, with a maximum nitrite peack concentration of 5 mg L^{-1}. This transient higher nitrite concentration coincided with the detection of *Nitrobacter* spp. growth in chemostat Control (Fig. 1, panel B). Unlike chemostat Test, *Nitrospira* spp. were the dominating population in chemostat Control during the period of operation with higher nitrite concentration. However, it should be stressed that the nitrite concentrations attained in this chemostat were considerably lower than the ones in the chemostat Test. Afterwards, the nitrite concentration decreased to the initial value, and the population dynamics presented an inversed trend (Fig. 1, panel B).

Morphology of *Nitrospira* and *Nitrobacter* aggregates

In both chemostats *Nitrospira* spp. formed clusters with densely packed cells (Fig. 2 A, Fig. 3 A,), which were labeled simultaneously by probes Ntspa712 and Ntspa662. As the respective areas were identical, only the one labeled with Ntspa712 was used for quantification purposes. *Nitrobacter* spp., which were mainly present as individual cells and some small cell aggregates, in the culture used as inoculum, formed increasingly bigger aggregates in chemostat Test, during the period of operation with nitrite peaks in the bulk liquid (Fig. 2, panel B) while the relatively low nitrite concentration in chemostat Control supported less of their growth in considerably smaller aggregates (Fig. 3, panel B). When the available nitrite concentration was reduced to the original value again, the previously formed *Nitrobacter* spp. population decreased in both chemostats (Fig. 2, panel C, Fig. 3, panel C), but in the case of chemostat Test still constituted

the dominant species whereas in chemostat Control *Nitrospira* spp. had always remained dominant.

Nitrospira aggregates presented a cyclic growth pattern, as shown in Figure 1, panel B, for chemostat Control. The periods with increasing size of *Nitrospira* aggregates coincided with constant and low nitrite-nitrogen concentration (C_{NO2^-}-N < 0.1 mg L^{-1}), while nitrite peaks (C_{NO2^-}-N < 5 mg L^{-1}) were observed throughout the decreasing part of the cycle.

Some of the *Nitrospira* spp. aggregates had a spherical appearance, but most of them were shaped more irregularly. The smallest and largest size of the aggregates, observed during chemostat Control operation, were 10.59 μm ± 0.89 μm and 26.38 μm ± 1.26 μm, respectively. The cells within the aggregates usually were uniformly packed. Only in the enriched cultures of *Nitrospira* spp. (i.e. where up to 80 % of all bacteria were detected with probe Ntspa712, Figure 4) appeared aggregates with internal substructures, but in a small proportion.

DISCUSSION

It has been demonstrated in the experiments described above that nitrite concentration affected the competition between *Nitrospira* spp. and *Nitrobacter* spp.. Transient elevated nitrite concentrations stimulated the growth of *Nitrobacter* spp., while in the undisturbed chemostat Control *Nitrospira* spp. dominated. These results are consistent with the above mentioned *K*/*r*-hypothesis. *Nitrobacter*, as an *r*-strategist, through a rapid growth rate, takes over and dominates situations in which resources are temporarily abundant, while *Nitrospira*, which grows more slowly, characteristic of *K*-strategists, tend to be successful in resource-limited situations.

This experimental set-up simulated successfully the nitrite-nitrogen concentration ($C_{NO_2^-\text{-}N}$) existing in real WWTPs (0.012 mg L^{-1} in the activated sludge tank of Gut Marienhof), where *Nitrospira* are the dominant nitrite-oxidizers.

The experiment designed to work as a control (chemostat operated at a constant influent nitrite concentration) failed, due to the unexpected oscillation of nitrite concentration in the bulk liquid, which stimulated *Nitrobacter* spp. growth. This result independently confirms the different growth strategies of *Nitrospira* spp. and *Nitrobacter* spp..

Interestingly, the dominance of *Nitrobacter* spp. over *Nitrospira* spp. in chemostat Test, caused by the transient elevated nitrite concentration, could not be reverted by lowering the available nitrite concentration to the original level. One possible explanation for this result is that *Nitrobacter* spp., if present at a certain cell density, is able to inhibit the growth of *Nitrospira* spp.. A direct way to verify this hypothesis might consist in adding spent medium from a *Nitrobacter* culture to the feed of a *Nitrospira* culture and observe any inhibitory effects of it.

Another possible explanation for this result is related to the factor time (length of the experimental period). In a chemostat, the rate at which a population becomes dominant over the other depends in the first place on their differences in growth rate (Kuenen and Harder, 1982). Considering a small difference in growth rates between the two nitrite-oxidizers in chemostat Test, *Nitrospira* spp. will require long periods of time to become dominant in the mixed population.

Nitrospira's spp. growth in clusters has been frequently observed in activated sludge and biofilm systems (Daims, et al. 2001; Juretschko, et al. 1998). Clustered growth could serve to decrease the impact of the loss of catabolic and anabolic intermediates to the environment on the biomass

yield (Strous, 2000). In the clusters, each cell would benefit from its neighbor's losses. Interestingly, *Nitrobacter* spp. formed clusters mainly during the period of operation with nitrite peaks in the bulk liquid. Substrate limitation is one of the many environmental conditions that can stimulate bacteria to aggregate (Wimpenny, et al. 1997), but such explanation is inappropriate for the case of *Nitrobacter* spp.. One possible explanation for this result is that *Nitrobacter* spp. bacteria formed aggregates to protect themselves from the possible inhibiting effect of a high nitrite concentration in the bulk liquid.

What remains to be discussed is the unexpected occurrence of nitrite peaks in the bulk liquid of chemostat Control, between days 35 and 75. It is tempting to speculate that this event is related with the size of *Nitrospira* spp. aggregates. As the aggregate grows, the average mass transfer rate from the bulk liquid to individual bacteria inside the aggregate decreases. Consequently, the substrate concentration inside the aggregate decreases below the threshold level at which cells are just able to pay their maintenance costs, and only a thin outer layer of the aggregate is metabolically active. This reduces degradation rates compared with that from smaller aggregates, and might explain the increase of nitrite in the bulk liquid. The cyclic growth pattern of *Nitrospira* spp. aggregates might be explained by the model proposed by Brandt *et al.* (2000), for the life cycle of a microbial aggregate: the aggregate starts to grow, develops a dead kernel and disintegrates. The biomass in the living layer re-distributes in new aggregates.

For the present case the aplicability of this hypothesis may be supported by the fact that the observed aggregate diameters were considerably larger than the critical value calculated by Matson and Characklis (1976), above which nitrite diffusion into the aggregates becomes a limiting factor for the bacteria's metabolic activity:

$$d_{crit} = \sqrt{\frac{3 \cdot D_{eff} \cdot C^{S}_{N\text{-}NO_2^-}}{2 \cdot \rho_a \cdot k_{N\text{-}NO_2^-}}}$$

in which D_{eff} is the effective diffusivity of nitrite, $C^{S}_{N\text{-}NO_2^-}$ is the surface concentration of nitrite, ρ_a is the microbial aggregate density, and $k_{N\text{-}NO_2^-}$ is the zero order rate constant. The following values were used for the present conditions: D_{eff} is $1 \cdot 10^{-9}$ $m^2 \cdot s^{-1}$ (it was considered 80 % of the diffusion coefficient of nitrite in liquid), $C^{S}_{N\text{-}NO_2^-}$ is $6.5 \cdot 10^{-5}$ $kg \cdot m^{-3}$ (nitrite-nitrogen concentration in chemostat Control), and ρ_a is 30 $kg \cdot m^{-3}$ (Beccari, et al. 1992). As stated earlier, $k_{N\text{-}NO_2^-}$ in chemostat Control was determined to be $1.52 \cdot 10^{-4}$ $kg \cdot kg^{-1} \cdot s^{-1}$. The resulting critical diameter d_{crit} is 5 μm and thus considerably smaller than that of the *Nitrospira* spp. aggregates found in chemostat Control, which ranged between 10 μm and 25 μm, thus suporting the hypothesis that the aggregates' increased size was reducing the metabolic efficiency of the *Nitrospira* spp. population in the chemostat.

Competitive exclusion precludes two populations from occupying exactly the same niche. Multiple populations, however, may coexist within one chemostat if the environmental conditions vary in time (concentration oscillations, Atlas, *et al.* 1993) or space (local concentration gradients as may be provoked by bacterial growth on the walls). Therefore, it is tempting to speculate that *Nitrospira* spp. and *Nitrobacter* spp. would continue to coexist in chemostat Control, under the experimental conditions.

Understanding the strategies of bacterial competitiveness (competition) at low versus high resource concentrations may have practical applications, as for example, for the start-up or

breakdown remediation of WWTPs and for bioremediation of soils and groundwater contaminated by nitrogen compounds. That is, successful implementation and operation of biotechnological processes may depend on identifying bacterial strains whose growth parameters are well suited to wastewater composition or site-specific conditions in terms of resource concentration.

Nitrospira has only recently been discovered to play a role in nitrite oxidation in engineered systems. Therefore the ecophysiology of these bacteria is still far from complete. Overall, the results of this experimental study support the existence of different survival strategies for *Nitrospira* and *Nitrobacter* (*K*/*r*-hypothesis). However, to confirm this result, there is a need for more fundamental studies on the kinetics *Nitrospira* growth.

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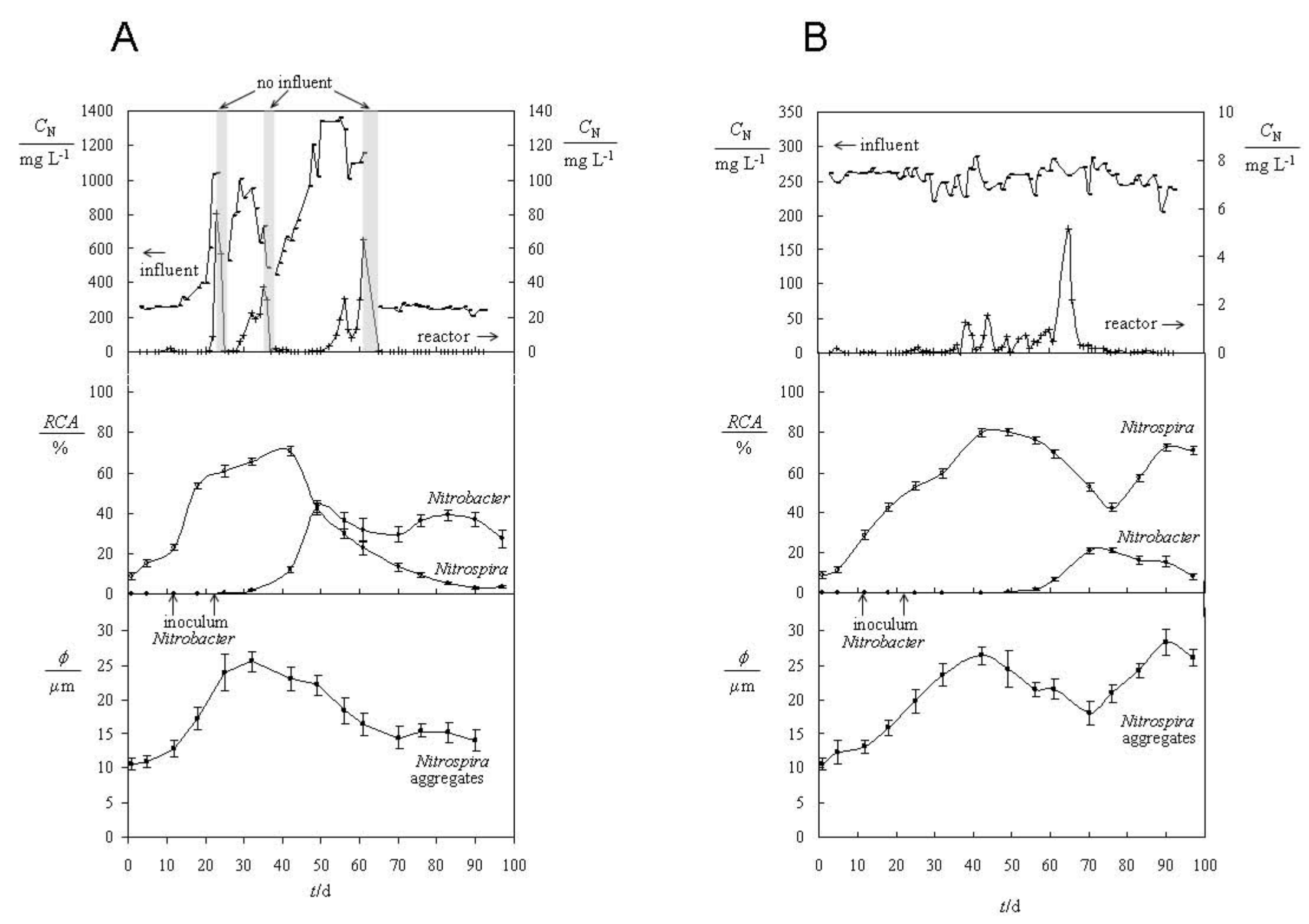
FIGURE LEGENDS

Figure 1. Population dynamics of nitrite-oxidizers, quantified as relative cellular area (*RCA*), nitrite-nitrogen concentration ($C_{NO_2^-\text{-}N}$) in the bulk liquid and size of *Nitrospira* spp. aggregates (ϕ) as a function of time of operation (t): A) chemostat Test (shaded areas indicate periods in which the influent was discontinued in order to avoid excessive nitrite accumulation within the reactor); B) chemostat Control (constant influent nitrite concentration during all experimental period). Error bars present 95% confidence interval.

Figure 2. Population dynamics of nitrite-oxidizing bacteria in chemostat Test. Confocal laser-scanning micrographs of nitrite-oxidizing biomass hybridized with Cy3-labeled probe Ntspa712, specific for the detection of *Nitrospira* (in red), and FLUOS-labeled probe Nit3, specific for the detection of *Nitrobacter* (green). Scale bars are 10 μm: A) t = 18 d, B) t = 49 d, and C) t = 83 d. Error bars present 95% confidence interval.

Figure 3. Population dynamics of nitrite-oxidizing bacteria in chemostat Control. A) t = 18 d, B) t = 49 d, and C) t = 83 d. All conditions correspond to those in Figure 2.

Figure 4. Confocal laser-scanning micrograph of nitrite-oxidizing biomass hybridized with Cy3-labeled probe Ntspa712, specific for the detection of Nitrospira spp. (in red). Distribution of cells within the aggregates: A) cells uniformly packed, B) cells with internal microstructures. The scale bar is 10 ⌈m.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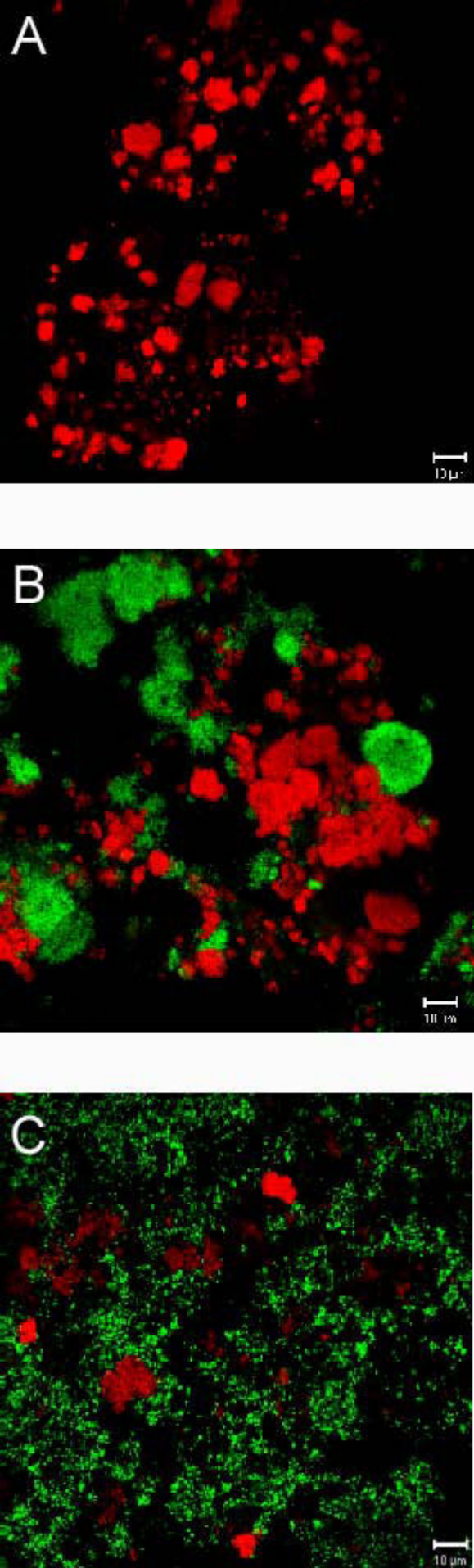
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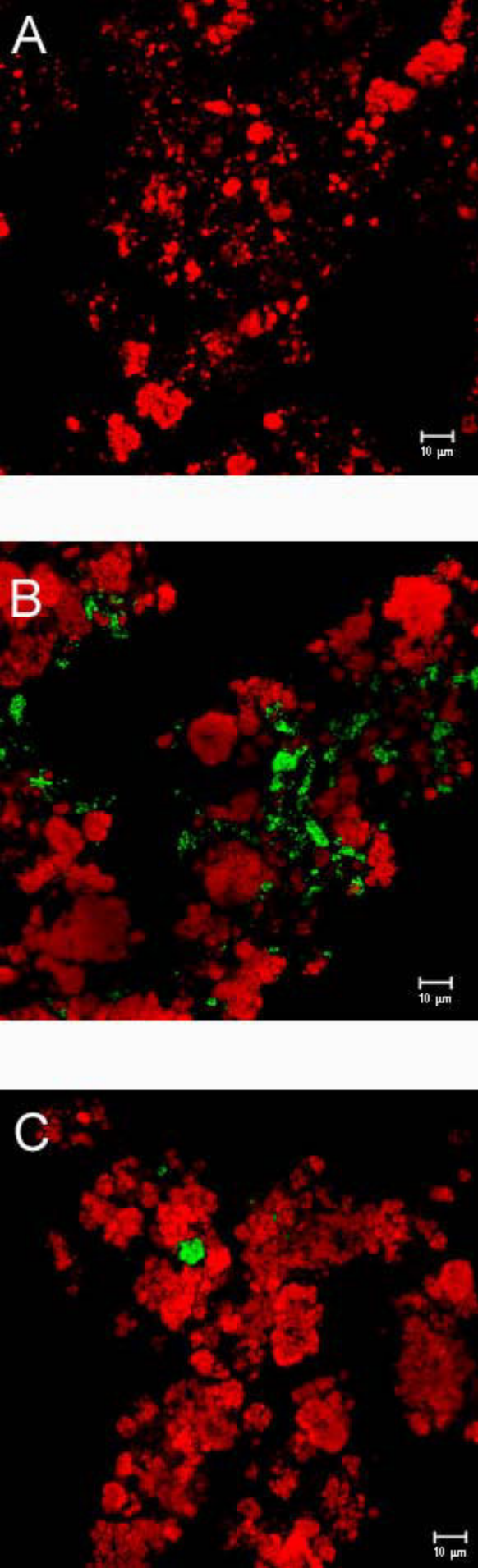
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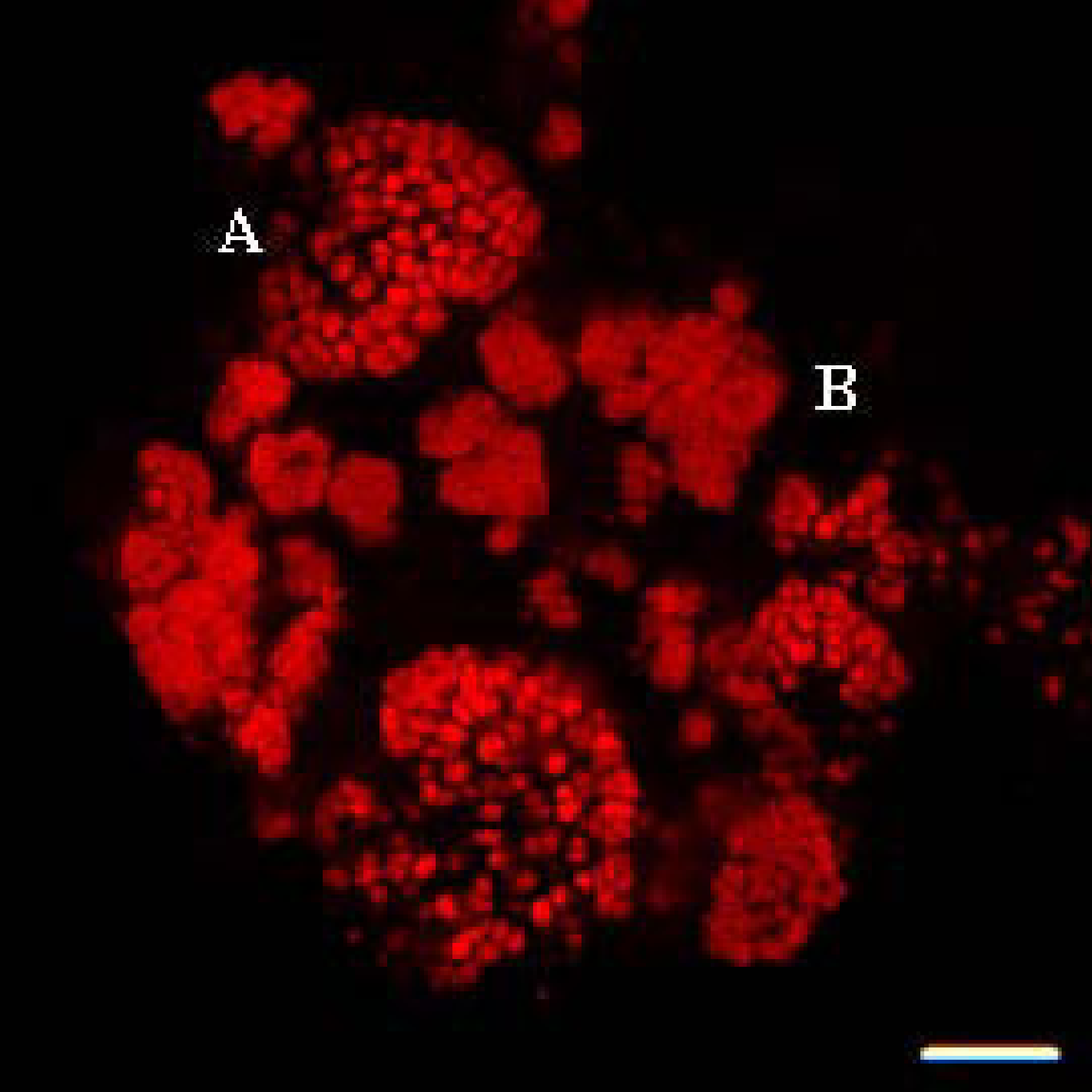
C
10 µm


A
B