

Quantification of depth-dependent microbial growth in encapsulated systems

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Abstract

Encapsulated systems have been widely used in environmental applications to selectively retain and protect microorganisms. The permeable matrix used for encapsulation, however, limits the accessibility of existing analytical methods to study the behavior of the encapsulated microorganisms. Here, we present a novel method that overcomes these limitations and enables direct observation and enumeration of encapsulated microbial colonies over a range of spatial and temporal scales. The method involves embedding, cross-sectioning, and analyzing the system via fluorescence *in situ* hybridization, while retaining the structure of encapsulants and the morphology of encapsulated colonies. The major novelty of this method lies in its ability to distinguish between and subsequently analyze multiple microorganisms within a single encapsulation matrix across depth. Our results demonstrated the applicability and repeatability of this method with alginate-encapsulated pure (*Nitrosomonas europaea*) and enrichment cultures (anammox enrichment). The use of this method can potentially reveal interactions between encapsulated microorganisms and their surrounding matrix, as well as quantitatively validate predictions from mathematical models, thereby advancing our understanding of microbial ecology in encapsulated, or even biofilm systems, and facilitating the optimization of these systems.

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Keywords

Encapsulation, fluorescence in situ hybridization, cross-section, enumeration, colony, *Nitrosomonas europaea*, anammox

Abstract

Encapsulated systems have been widely used in environmental applications to selectively retain and protect microorganisms. The permeable matrix used for encapsulation, however, limits the accessibility of existing analytical methods to study the behavior of the encapsulated microorganisms. Here, we present a novel method that overcomes these limitations and enables direct observation and enumeration of encapsulated microbial colonies over a range of spatial and temporal scales. The method involves embedding, cross-sectioning, and analyzing the system via fluorescence *in situ* hybridization, while retaining the structure of encapsulants and the morphology of encapsulated colonies. The major novelty of this method lies in its ability to distinguish between and subsequently analyze multiple microorganisms within a single encapsulation matrix across depth. Our results demonstrated the applicability and repeatability of this method with alginate-encapsulated pure (*Nitrosomonas europaea*) and enrichment cultures (anammox enrichment). The use of this method can potentially reveal interactions between encapsulated microorganisms and their surrounding matrix, as well as quantitatively validate predictions from mathematical models, thereby advancing our understanding of microbial ecology in encapsulated, or even biofilm systems, and facilitating the optimization of these systems.

Introduction

Microbial encapsulation is a versatile environmental biotechnology with applications ranging from nutrient removal and resource recovery from wastewater to bioremediation of contaminants. The permeable matrices used as encapsulants not only selectively retain microorganisms, but also protect them against potential inhibitors in the external environment. Nevertheless, encapsulants create physical barriers to directly access microbes for analysis. Existing tools have diverse capabilities in microbial and morphological analysis but are limited when applied to elucidate the growth of multiple encapsulated microorganisms across a temporal and spatial span.

Due to the similarity between biofilm and encapsulated systems, microscopic methods are widely used in both systems to reveal the morphology and assembly of microbes. For instance, optical microscopy, optical coherence tomography, and electron microscopy have been used to reveal structures in biofilm systems, which are applicable to encapsulated systems as well. Optical and fluorescent dyes can be used to enhance the contrast between the encapsulant and encapsulated microorganisms. Live/dead staining is also available to identify living encapsulated cells. Meanwhile, encapsulants are inherently different from extracellular polymeric substances in biofilms. It is challenging to preserve their structure during the pretreatment of samples. To prepare samples for microscopy, fixation and dehydration procedures can cause shrinkage and even destruction of hydrogel-derived encapsulants. Electron microscopy can better preserve encapsulant structures and improve the resolution of submicron morphological details; it cannot, however, differentiate between microbial species.

In addition to microscopic methods, molecular and physio-chemical techniques are used to quantify the growth of encapsulated microorganisms. Measurable parameters include culturable colony forming units (CFU), DNA copies, cell protein content, and dry cell weight. Sequencing and quantitative amplification (e.g., quantitative polymerase chain reaction (qPCR)) of genetic material can further identify and quantify the targeted components of an encapsulated microbial community. Nevertheless, extraction of cell contents or DNA from encapsulated microorganisms often requires homogenization and removal of the encapsulant. As a result, the spatial information of cell distribution within the encapsulant is lost during such a process. Modifications of the procedure with selective shearing of surface layers and sorting of size fractions have been demonstrated with granular activated sludge; however, these modifications are difficult to apply on encapsulated systems, as encapsulants are much harder to selectively disassemble.

The main objective of this study was to develop a novel method for measuring spatial and temporal changes in the encapsulated growth of various microorganisms. Such a method can potentially provide information to validate predictions from mathematical models, allowing more predictable applications of microbial encapsulation technologies in environmental engineering.

Methods and Materials

2.1 Microbial encapsulation and batch test setup

A pure culture of *Nitrosomonas europaea* ATCC 19718^T and an enrichment culture of anammox bacteria were used as inocula for microbial encapsulation. The enrichment culture was granular in nature and collected from a bench-scale sequencing batch reactor under steady-state operation. An equal volume of 4% sodium alginate solution and the inoculum culture was completely mixed before crosslinking in a 4% calcium chloride solution to form spherical beads as described previously (Wang et al., 2022). The inocula were about 20 and 500 mgTSS/L for the *Nitrosomonas* culture and anammox enrichment respectively. Crosslinked alginate beads had diameters of 2.4 – 2.8 mm.

Batch bioreactors with encapsulated biomass were established as previously reported. For tests with encapsulated *Nitrosomonas*, ammonia (50 – 150 mgN/L) was added daily to the synthetic culture medium as NH₄Cl, whereas an equal amount of ammonia (as NH₄Cl) and nitrite as NaNO₂ (50 – 100 mgN/L each) was added to the medium for tests with the anammox enrichment culture. Triplicate batch reactors (200 mL) were placed on an orbital shaker at 100 rpm and operated for a week. Sponge caps were used to allow diffusion of air into the reactors, and dissolved oxygen concentrations were kept between 0.5 to 4.0 mg/L. Water samples (1.5 mL) were collected daily, filtered, and analyzed with spectrophotometric methods for ammonia, nitrite, and nitrate. Six beads were sampled (3 for imaging and 3 for DNA extraction) and processed immediately before and after each batch incubation.

2.2 Sample preparation, cross-sectioning, and FISH procedure

Alginate bead samples were fixed immediately after collection with 3% (w/v) paraformaldehyde. Each alginate bead was placed in the conical bottom of a 1.5-mL microcentrifuge tube and infiltrated stepwise with 25, 50, 75, and 100% glycolmethacrylate (GMA) as previously described. GMA-infused samples were thus molded into the tip of the microcentrifuge tube. Following the solidification of GMA, the bottom of the microcentrifuge tubes was cut open and placed on a microtome (Microm HM505E). Cross-section cutting (10-μm thickness) was conducted starting from the surface of each bead. Three consecutive sections were collected upon every 100-μm advancement until the entire bead was completely cross-sectioned (**Figure 1**). The collected sections were placed on multi-well slides pre-coated with a 0.1% poly-L-lysine solution. Fluorescence *in situ* hybridization (FISH) was conducted with oligonucleotide probes targeting ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB), and anammox bacteria (**Table 1**). The hybridization was conducted with 35% formamide at 46 °C for 2 hours. An antifade mountant with DAPI (ProLong Gold, Invitrogen) was used as a DNA counter stain. Alginate beads without microbial inoculum were analyzed by the cross-section FISH method as negative controls using each fluorescence probe to check for autofluorescence. The anammox enrichment culture was used as a positive control for each fluorescence probe, as it was assumed to contain AOB, NOB, and anammox bacteria. Prior to fixation, the granular anammox culture was homogenized for one minute with a handheld homogenizer to break apart large granules. The anammox biomass was then fixed and used for FISH as described above, without the embedding material present. GMA has been previously shown to not interfere with, but rather, to improve the sensitivity of FISH analysis.

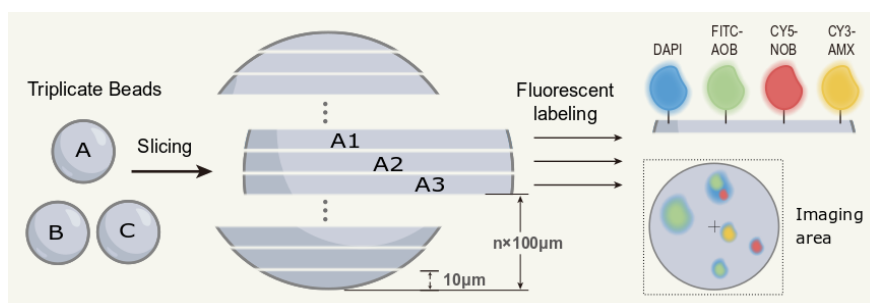


Figure 1. Overview of the cross-section and FISH procedures.

Table 1. Oligonucleotide probes used for FISH. (Adopted from Shi et al., 2016)

Target Group	Probe	Dye	Sequence 5'3'
NOB	Ntspa662	Cy5	GGA ATT CCG CGC TCC TCT
	Ntspa712	Cy5	CGC CTT CGC CAC CGG CCT TCC
AOB	Nso1225	FAM	CGC CAT TGT ATT ACG TGT GA
	NEU	FAM	CCC CTC TGC TGC ACT CTA
	Cluster6a 192	FAM	CTT TCG ATC CCC TAC TTT CC
Anammox bacteria	Amx820	Cy3	AAA ACC CCT CTA CTT AGT GCC C

2.3 Image processing and data analysis

FISH images of cross-sections were obtained using an Olympus IX-81 inverted microscope equipped with a long working distance (WD 2.7-4.0) 40x objective lens (NA-0.6) and a DP73 camera (Olympus, Japan). Image processing was carried out on the Fiji software (v 2.10.0). Rolling ball background subtraction (30 pixels) and particle size ($>2 \times 2$ pixels) and circularity (>0.1) filters were used to correct for uneven background and filter out false positives from the textures left on the slices by the microtome slicing. The size and count of fluorescent colonies from each section were recorded. Colony counts were normalized to the volume of detection, as calculated by the area of each image (1.47 mm^2) and the thickness of the cross-section ($10 \text{ }\mu\text{m}$). Box plots were constructed in R with a median represented by a centerline, a 25th to 75th interpercentile range represented by a shaded box, and smallest/largest values within the 1.5 times interquartile range represented by upper/lower error bars. Statistical analyses were performed in R (v 4.2.2). Non-parametric tests were used to compare particle sizes. The Kruskal-Wallis one-way ANOVA was conducted on triplicates at each depth, whereas the Wilcoxon signed-rank test was conducted between paired samples before and after each incubation. Student t-tests were performed on qPCR results to compare gene copy numbers before and after incubation.

2.4 DNA extraction and qPCR

To extract DNA, an entire alginate bead was dissolved in 1 mL of sodium citrate (55 mM) and EDTA (30 mM) solution. The dissolved bead solution was centrifuged at $4 \text{ }^\circ\text{C}$ to obtain cell pellets, which were washed twice and resuspended in buffer solution (10 mM Tris, 0.1 mM EDTA) for storage at $-20 \text{ }^\circ\text{C}$. The PowerSoil DNA extraction kit (Qiagen) was used to extract DNA from the pellet in the buffer. qPCR was used to quantify total bacteria, AOB, anammox bacteria, and NOB with 341F and 805R primers, amoA_1F and amoA_2R primers, hzsA_1597F and hzsA_1857R primers, and nxrB169f and mxrB638r primers, respectively (Table S1). The qPCR was performed on a StepOnePlus Real-Time PCR System (Thermo Fisher) with the standard curve method as described previously. The amplification efficiencies for all primer sets were $>90\%$, except for the 16S rRNA gene ($>85\%$).

Results and Discussion

Method validation with encapsulated *Nitrosomonas*

The background noise of FISH probes and autofluorescence of alginate embedded in GMA were first examined using abiotic alginate beads to which no microbial inoculum was added. No fluorescence was detected for all the probes used, except for CY3. The texture of GMA-embedded alginate sections emitted fluorescence within the filtered wavelength of CY3 (Figure S3). The observed texture could have resulted from the tearing of GMA and embedded alginate during cross-sectioning. Such CY3 signals (i.e., false detection of anammox colonies) was successfully eliminated during data processing with size and circularity filtering because the autofluorescent textures were large non-circular objects along the direction of cutting.

The specificity of FISH probes was verified using a pure culture of *Nitrosomonas* and an anammox enrichment culture. FISH probes successfully detected AOB and anammox bacteria in both suspended and encapsulated

samples (Figure S4). No NOB colonies were detected in the granular, non-encapsulated anammox enrichment culture. This is probably because low concentrations of NOB were present in the culture, which could have resulted in no observable colonies in the $5 \mu\text{m}^2$ area under the microscope. Successful detection of NOB colonies was demonstrated in experiments with encapsulated biomass by both qPCR and FISH (Figure S5), indicating that NOB must have been present in the initial culture, just at a low density (see below).

The reproducibility of the cross-section results was examined by comparing three consecutive 10- μm sections from the same alginate bead. The sample was collected after 5 days of aerobic incubation of encapsulated *Nitrosomonas*. Cross-sections 100 μm apart were collected for the entire bead. Both the area and count of FISH-detected AOB colonies increased with depth from the surface from 0 to 300 μm , followed by a decrease as one moved further toward the center of the bead (1500 μm), indicating preferred growth of AOB near the surface (**Figure 2**). One would have expected the microbial counts and areas to be roughly symmetrical around the core of the bead (e.g., 0 to 1500 μm mirrored in the 1500 to 3000 μm slices). This, however, was not the case. Indeed, the quality of the sections obtained worsened as the slices were taken from deeper than the core of the bead. The integrity of the slices at large depths (>1500 μm) could therefore not be used for analysis. This was thought to be caused by the fact that the GMA embedding material was molded into the conical shape of the microcentrifuge tube (Figure S6). At the tip of the tube, the slices consisted primarily of the bead itself, but as the cross-sectioning went deeper into each sample, the slices consisted of more of the GMA surrounding the bead. It was thought that as a result, greater friction occurred between the GMA/sample and the blade, compared to the sample only and the blade, resulting in tearing and poor sample quality. To ensure the quality of our analysis, we thereafter performed cross-sectioning only up to the center of each alginate bead sample. This is an important limitation of this technique, as it is predicated on the assumption that microbial growth is symmetrical about the core of a bead. If investigating other embedded geometries or systems where growth might not be symmetrical (larger beads in a high-rate packed bed for example), more samples of embedded microorganisms will need to be analyzed to obtain a clear picture of growth.

When one looks at the 0-1500 μm depths, the largest and densest AOB colonies were observed around 300 μm from the surface of the alginate bead. The areas of AOB colonies were statistically compared among the three neighboring sections, with the three consecutive sections at depth $D \pm 10 \mu\text{m}$ treated as technical triplicates representing a single alginate bead at depth D . No significant differences were observed for 11 sets of sections (Kruskal-Wallis test, $p > 0.05$), demonstrating that these three neighboring slices could indeed act as replicates. Nevertheless, four sets of slices, out of 15, showed significant differences among the three “replicate” slices (at 300, 400, 1100, and 1200 μm). Part of this appears to be the result of the larger colony areas and larger variations in colony area observed at depths 300 and 400 μm where most of the growth occurred.

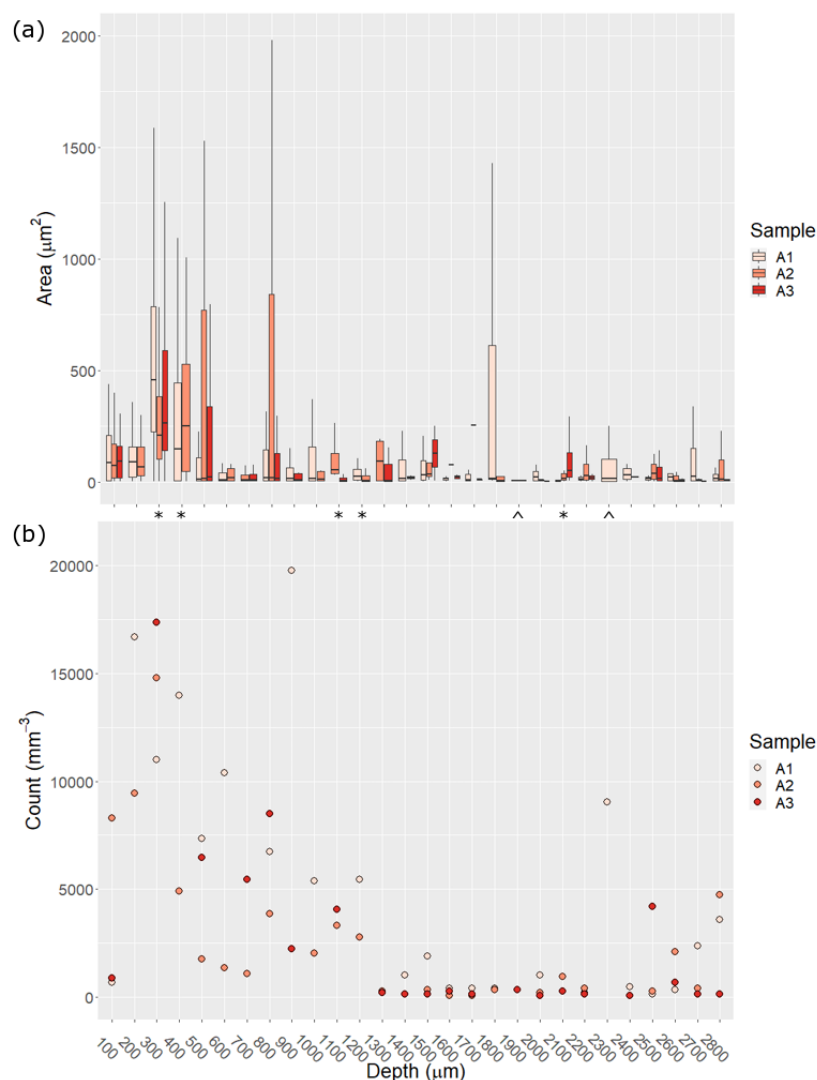


Figure 2. (a) Size and (b) count of detected AOB colonies in three consecutive cross-sections (A1, A2, and A3) from the same alginate bead after a 5-day batch incubation. The sets of cross-sections with p-values < 0.05 from a Kruskal-Wallis test are marked with asterisks. Depths with only one sample are marked with carets and excluded from a Kruskal-Wallis test. Error bars indicate the 95% confidence intervals. Here a depth of 0 and 3000 represent the outer surface of the bead, with 1500 μm equaling the center/core of the bead.

Encapsulated growth of *Nitrosomonas* colonies

Initial bead samples and bead samples taken after one day of incubation were analyzed for bacterial numbers and colony area (Figure S7). Under aerobic batch incubation in triplicate reactors, the encapsulated *Nitrosomonas* consumed ammonium at $42 \pm 9 \text{ mgN/L-d}$ and produced nitrite at $39 \pm 13 \text{ mgN/L-d}$ ($n = 3$). Encapsulated AOB colonies were initially distributed evenly throughout the depth range of 0 to 1500 μm (bead core) with an average colony size of $20 \mu\text{m}^2$ and an average density of $1,360 \text{ colonies mm}^{-3}$. Such AOB distribution was expected as the alginate and bacterial culture were homogenized before crosslinking.

No significant change in distribution was observed after the 1-day incubation of encapsulated AOB under pairwise comparison (Wilcoxon test, $p > 0.05$). This is also expected, because the doubling time of encapsulated *Nitrosomonas* around 0.5 d ; therefore, one day of incubation is likely to be insufficient to develop clear differences in colony morphology and number.

The same experimental setup was used to incubate encapsulated *Nitrosomonas* for 5 days. Three different beads were collected and cross-sectioned at the end of the incubation. Three consecutive slices (at depth $D \pm 10 \mu\text{m}$, where D is every 100 μm until 1200 μm) from the same bead were analyzed and used as replicates to represent the colony profile at each depth D . As observed previously, the initial AOB numbers and colony sizes were evenly distributed through the bead depth of 0 to 1200 μm (Figure S7). Ammonium consumption and nitrite production was also similar to that previously observed (Figure S2). In contrast to the 1-day incubation, however, a different spatial distribution of encapsulated AOB colonies was observed after the 5-day incubation, as plenty of time had been given for the microorganisms to grow (**Figure 3**). Preferential growth of encapsulated colonies was detected near the surface (300-500 μm), shown by increased size and density of AOB colonies at those depths. Some variations existed between the depth profiles of AOB colonies obtained from different bead samples, but overall, the trends were observable, quantifiable, and reproducible. Of note is the fact that the data in Figure 3 represents triplicate slices in three separate beads. Size of colonies were statistically different from each other in the three beads (Kruskal-Wallis test, $p < 0.05$), however, the colony counts were not significantly different among the three beads (Kruskal-Wallis test, $p > 0.05$).

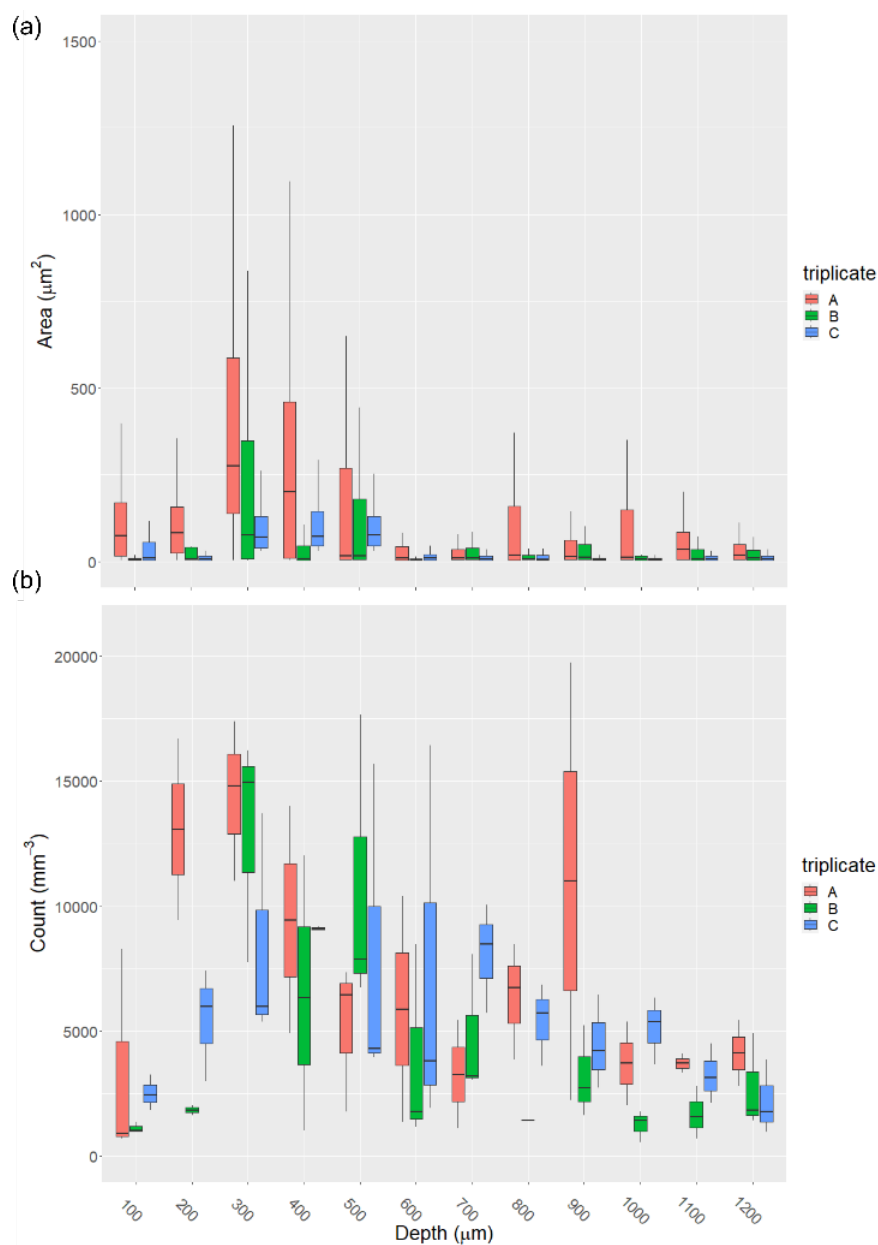


Figure 3. The distribution of (a) size and (b) count of AOB colonies detected by FISH across depth in three different alginate beads (A, B, and C) after a 5-day batch incubation. Error bars indicate the 95% confidence intervals.

In contrast to our microtome cross-sectioning and FISH method, qPCR could only provide average results for an entire bead, and as a result, could not provide information on changes in the spatial distribution of encapsulated colonies over time (**Figure 4a**). Since DNA was extracted after the dissolution of the entire alginate bead, the qPCR results served as an average count across the depth profile for all encapsulated cells. No significant change was observed in either the 16S rRNA gene or *amoA* copy numbers after the 1-day incubation ($p < 0.05$, student t-test), which agrees with the results from cross-sectioning and FISH methods (Figure S7). In contrast, significant increases in copy number were observed for both genes after

the 5-day incubation ($p < 0.05$, student t-test). Increase in the copy number *amoA* again agrees with the cross-section FISH results showing the growth of AOB colonies near the bead surface (**Figure 3**). The difference between increases in the 16S rRNA gene and *amoA* copy numbers could be attributed to the potential growth of heterotrophs within and on the surface of alginate beads. These heterotrophs may have grown by using carbon fixed and secreted by AOB and/or by degrading alginate. The cross-sectioning and FISH method presented herein, however, provides much more detailed data and enables a much clearer understanding of growth in an encapsulated system. The details of these results also facilitate the validation of mathematical models (e.g., Wang et al., 2022).

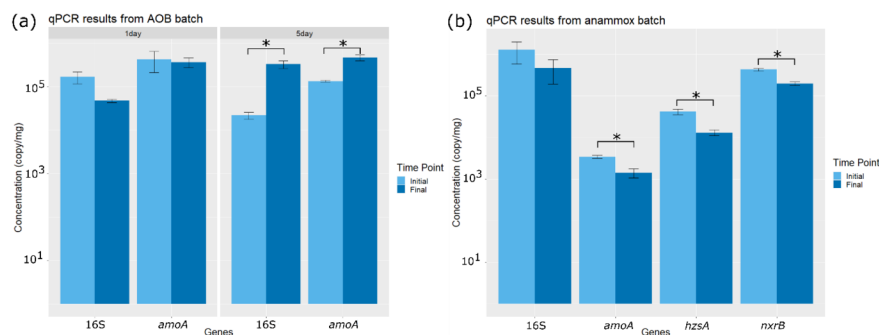


Figure 4. Quantities of the 16S rRNA gene and *amoA* in the beads collected from (a) encapsulated AOB and (b) encapsulated anammox batch reactors before and after incubation. Error bars indicate the variations between triplicate alginate beads collected at each time point. Asterisks indicate statistical differences by pairwise student t-tests ($p < 0.05$).

Similar to this study, research on encapsulated *Nitrobacter* in carrageenan showed that the maximum biomass concentration and colony size were found near the encapsulant surface. These phenomena can be explained and modeled by oxygen and ammonium diffusion limitation. In addition, in the research described herein, the diffusion of calcium cations into the alginate matrix was used to crosslink the encapsulant, which can produce a harder layer near the surface; This could explain the lack of obvious colony eruption observed by others that was not observed herein, perhaps as a result of a harder outer layer of alginate, which may have provided resistance for encapsulated colonies to erupt out of the encapsulant, or even perhaps to grow.

Extended application with encapsulated Anammox enrichment

Our new method was applied to analyze anammox bacteria co-encapsulated with *N. europaea* to demonstrate its ability to simultaneously track the growth of multiple microorganisms. The inoculum consisted of a granular anammox enrichment culture and the pure suspended culture of *N. europaea*. The difference in the original biomass forms (granular sludge vs. suspended cells) most likely resulted in distinct characteristics in colony size and count observed between encapsulated AOB and anammox bacteria before incubation (**Figure 5**). AOB colonies were smaller and evenly distributed across the bead depth (**Figure 5a & 5b**), whereas anammox bacteria colonies were larger in size and exhibited large variations in colony count among different beads sampled (**Figure 5e & 5f**).

After incubation, nitrite and ammonium were both consumed after each addition (Figure S1). Encapsulated AOB increased in colony counts, particularly in the samples analyzed from 0 to 700 μm in depth. This is consistent with what was observed with the AOB-only encapsulants, with growth near the surface of the bead. One of the sampled beads (sample C) deviated significantly from the other two beads in colony counts across depth (**Figure 5c & 5d**). For NOB, a slight increase in colony size and count was also observed from 0-700 μm in depth after incubation (Figure S5). Meanwhile, the colony size of anammox bacteria decreased slightly after the 5-day incubation period. Less variation in the colony count of anammox bacteria was observed among sampled beads after the incubation, with the largest number of colonies seen around 300-500 μm in depth (**Figure 5g & 5h**). Similar to the experimental results with only encapsulated *N. europaea*, qPCR

did not provide information on the changes in the spatial distribution of encapsulated colonies (**Figure 4b**). No significant change was detected in the 16S rRNA gene copy numbers before and after the incubation; however, the copy numbers for *amoA*, *hzsA*, and *nrrB* all decreased significantly ($p < 0.05$, student t-test), indicating a decrease in average concentrations of AOB, NOB, and anammox bacteria. One sees much more nuance in the cross-sectioning and FISH results, where some beads showed growth, including growth at the bead surface, and one bead (B) showed an overall decrease in colony counts after incubation.

Similar to this study, reported the stratification of anammox bacteria and comammox bacteria in a polyvinyl alcohol-alginate matrix. The co-encapsulation system was also used as an “engineered biofilm” to study the ecological cooperation between a pure culture and an anammox enrichment. Spatial segregation of comammox in the outer layer and anammox in the inner layer was driven by oxygen and nutrient concentration gradients across depth. FISH analysis was performed to qualitatively determine the growth of encapsulated microbes near the surface. Our method provides quantitative information about the size and number of encapsulated colonies at a given time and depth, which reflects the growth of microbial populations. The depth at which microbes grow within the encapsulant can be influenced by a combination of factors, including microbial growth kinetics, mass transfer of substrates, and competition and/or synergistic relationships between different groups of microorganisms. Mathematical models are useful to simulate the combined effect of such complex interactions; nevertheless, the predictions from modeling need to be experimentally validated. The method developed in this study should be extremely useful for this purpose. In conjunction with other analytical tools, such as microsensors that provide the concentration gradients of substrates, our method should be able to advance our understanding of microbial ecology in encapsulated systems. Due to the resemblance between biofilms and encapsulated systems, our method is also potentially applicable to biofilm systems for quantifying colonies across depth. Such advances could greatly enhance our understanding of microbial colony formation across spatial and temporal scales, expanding predictable environmental engineering applications of both encapsulated and biofilm systems in contaminant remediation, resource recovery, and cell preservation and transportation.

While the cross-section method developed in this study shows promise as a tool to quantify encapsulated growth of microbial colonies, there are still some limitations to the method and potential improvements needed. For instance, contamination during slicing is difficult to avoid, resulting in the carryover of cells to deeper cross-sections. Additionally, the low image quality of the cross-sections deeper in the beads (1500 to 3000 μm) may be solved by improvements in the durability of the embedding materials, molding techniques, and microtome slicing procedure. This, for spherical beads, would essentially serve as another replicate, increasing the power of one’s observations in a symmetrical system by enabling data from 0-1500 μm to be augmented by data from 1500-3000 μm . Confocal fluorescence microscopy may also be used to improve the resolution of cross-section FISH images. Future work could focus on improving the method to allow for a more comprehensive analysis of the growth and behavior of microorganisms within encapsulant matrices.

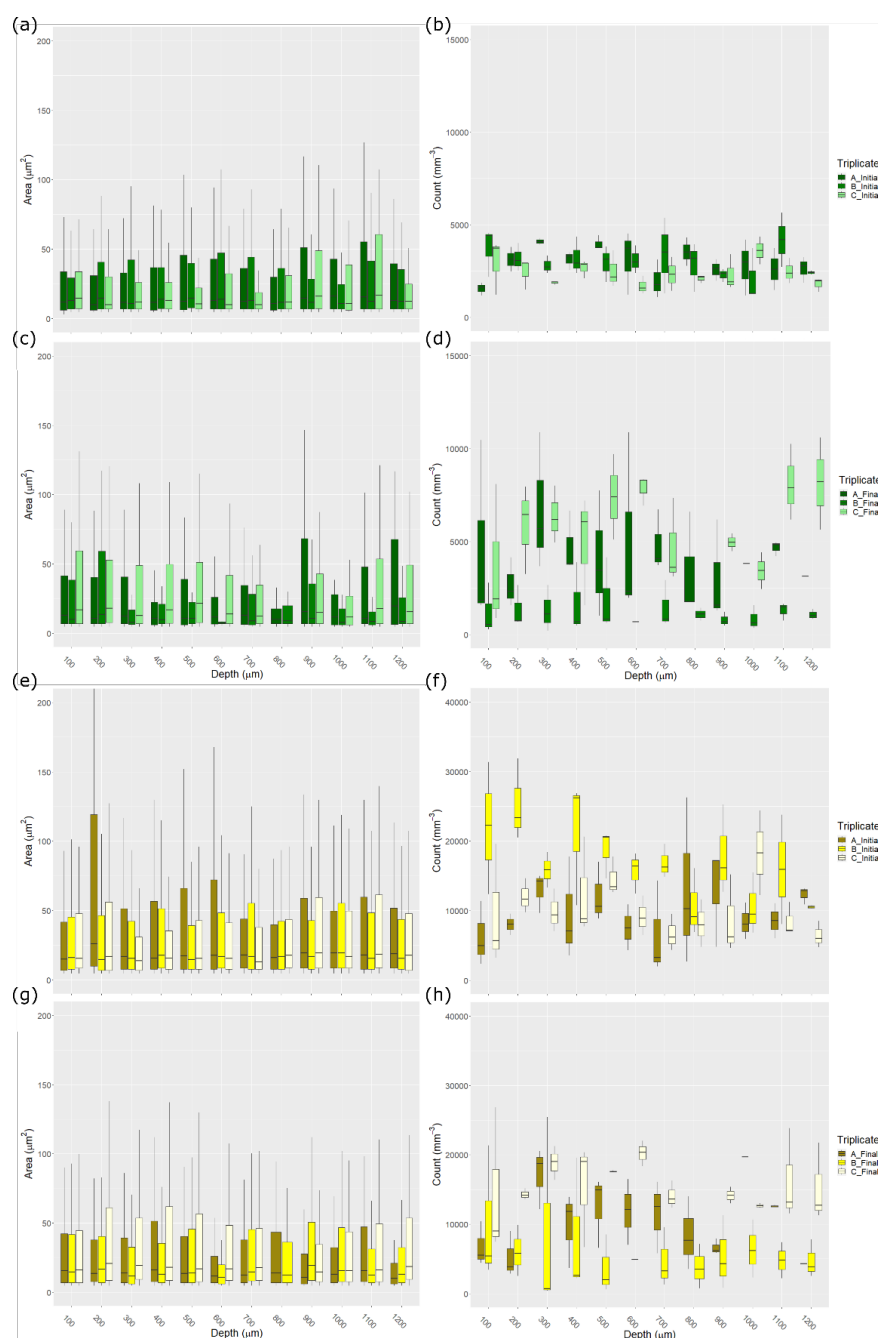


Figure 5. The distribution of size and count of AOB colonies across depth in three different alginate beads before (a, b) and after (c, d) the 5-day incubation. The distribution of anammox bacteria colonies in each corresponding bead before (e, f) and after (g, h) the 5-day incubation.

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Reference

