Technical Note
Granulation of a mixture of suspended anaerobic and aerobic cultures under alternating anaerobic/microaerobic/aerobic conditions: a preliminary study
Tuba H Ergüder and Göksel N Demirer*
Department of Environmental Engineering, Middle East Technical University, İnönü Bulvari, 06531, Ankara, Turkey

Abstract: This paper focuses on a new granulation study area where a mixture of suspended anaerobic and aerobic cultures was used as seed. Mixed suspended anaerobic and aerobic cultures exposed to alternating anaerobic/microaerobic/aerobic conditions developed granulates up to 2.5 mm in size. The granules developed displayed both oxygen uptake (3.83–8.75 mg dissolved oxygen g \(^{-1}\) volatile suspended solids h \(^{-1}\)) and methanogenic activities (7.5 cm\(^3\) CH\(_4\) d \(^{-1}\)) comparable to those of aerobic and anaerobic control cultures, respectively. Oxygen doses of 60, 100 and 120% of the total chemical oxygen demand added (23, 38 and 45 cm\(^3\)) and ethanol (among ethanol, glucose, acetic acid, and glucose+acetic acid) resulted in the largest and the most stable granules.

Keywords: aerobic; anaerobic; combined systems; granule; microaerobic; oxygen

INTRODUCTION
Combined reactors containing both anaerobic and aerobic cultures are of great interest. The co-existence of these two different types of cultures in the same reactor is attributed to either intrinsic tolerance of anaerobic cultures or formation of anaerobic niches (shielding effect).\(^1\) Barriers such as biofilms, settled layers of particles or soil aggregates almost exclusively limit the convective mass transport of oxygen supply to deep positions. In addition to barriers, the excess amount of readily metabolizable substrate compared with the oxygen supply might lead to formation of anaerobic environments in the systems or inside the particulate matrices located even in highly aerated environments.\(^2\) Several anaerobic nuclei were identified deep inside the flocs of originally aerobic sludge.\(^2\) The formation of anaerobic zones in the aerobic biofilms was used for the co-immobilization of anaerobic and aerobic cultures on Ca-alginate beads.\(^3,4\) The aerobic cultures grew in the oxygen-sufficient surface area, while the anaerobic ones grew in the oxygen-deficient central part of the gel beads.\(^4\) The co-existence of anaerobic and aerobic cultures was also demonstrated in packed-bed bio-barriers,\(^5\) soil slurry\(^6\) and upflow sludge blanket reactors.\(^7\) In these combined reactors with either free or co-immobilized cultures (of anaerobes and aerobes), dissolved oxygen (DO) concentrations display alternating conditions from aerobic (DO > 2 mg dm\(^{-3}\)) and/or microaerobic (DO < 1 mg dm\(^{-3}\))\(^8\) to anaerobic (DO = 0 mg dm\(^{-3}\)) through the reactor contents or from bulk liquid to the depths of the immobilized co-cultures, and thus develop supportive living conditions for each culture type. The co-existence and operation of anaerobes and aerobes in the same environment (ie combined reactor) might be advantageous compared with conventional anaerobic and aerobic systems in terms of energy requirement, initial investment cost and recovery from organic shock loads.\(^9,10\) Also, recalcitrant xenobiotic aromatic compounds requiring sequential anaerobic and aerobic/anoxic treatment could be completely mineralized in the same reactor.\(^3,5–7\) However, the possible applications of combined systems are still not well known. To the best of our knowledge, despite the advantages of treatment with granular cultures, granulation by using these two cultures has not been studied so far except by Ferguson\(^11\) who investigated the degradation of perchloroethylene and benzene, but observed pelletization.
In this study, combined systems were established to investigate the granulation of mixed anaerobic and aerobic cultures (without any supporting media). This paper, therefore, focuses on a new granulation study area, where a mixture of suspended anaerobic and aerobic cultures was used as seed and exposed to alternating cyclic anaerobic and aerobic (in turn their transient microaerobic) conditions. Alternating conditions were achieved by modifying the experimental procedure of Ferguson. Being a preliminary study, it was primarily aimed at investigating the possibility of granule development with the mentioned cultures. The effects of oxygen dose, substrate type, initial amount of biomass and the operational mode on granulation were investigated.

MATERIALS AND METHODS

Seed cultures and basal medium (BM)
Mixed anaerobic digester sludge and aerobic activated sludge, that were used in the experiments as seed cultures, were obtained from anaerobic sludge digesters and the recycle line of activated sludge units of the Greater Municipality of Ankara Domestic Wastewater Treatment Plant, respectively. Basal medium (BM) containing the necessary micro- and macro-nutrients was used in the experiments. The BM constituents and concentrations of each (given in parentheses as mg dm$^{-3}$) are as follows: NH$_4$Cl (400), KCl (400), (NH$_4$)$_2$HPO$_4$ (80), CaCl$_2$ (80), CoCl$_2$.6H$_2$O (10), Na$_2$PO$_4$.12H$_2$O (10), KI (10), CuCl$_2$.2H$_2$O (0.5), ZnCl$_2$ (0.5), AlCl$_3$.6H$_2$O (0.5), Na$_2$MoO$_4$.2H$_2$O (0.5), H$_3$BO$_3$ (0.5), NiCl$_2$.6H$_2$O (0.5), Na$_2$WO$_4$.2H$_2$O (0.5), Na$_2$SeO$_3$.5H$_2$O (0.76), cysteine (10), NaHCO$_3$ (6000), yeast extract (50) and Resazurin (provided by Dr Daniel H Zitomer, Marquette University, Milwaukee, Wisconsin) (1.04) as an oxidation–reduction indicator dye.

Experimental procedure
To investigate the possibility of granulation and the factors affecting granulation, experiments were conducted as ‘screening studies’ which were composed of sets of reactors. All the reactors (110 cm$^3$) with an effective volume of 50 cm$^3$ were seeded with a mixture of anaerobic digester sludge and aerobic activated sludge (50:50 v/v) and BM was added. The reactors were flushed with N$_2$/CO$_2$ (70/30) gas mixture for 4 min, sealed with septa and then maintained on a continual two-day schedule (wasting/feeding cycle) (Fig 1). At the start of the schedule (Day 1, odd days), the excess headspace gas was released using a 100 cm$^3$ gas-tight syringe. Additionally, 10 cm$^3$ of mixed liquor was wasted and 10 cm$^3$ of BM was added. Sludge wasting and subsequent BM addition were only done once in two days (odd days). Primary substrate was daily fed to the reactors achieving a chemical oxygen demand (COD) loading rate of 500 mg dm$^{-3}$ d$^{-1}$ (or 25 mg COD d$^{-1}$). At the start of Day 2 (even days) (Fig 1), after measuring and releasing the headspace gas, pure oxygen was injected into the headspace of the reactors using a syringe fitted with a pressure-locked valve. Oxygen doses were based on the percent of the total COD added (50 mg COD) ranging from 10 to 120% (corresponding to volumes of 4 to 45 cm$^3$ at standard temperature and pressure, STP).

All the reactors were set up in duplicate. Strictly anaerobic (no oxygen and seeded with only anaerobic digester sludge) and aerobic control (maximum oxygen dose and seeded with only aerobic activated sludge) reactors were also set up. The reactors, except the controls, are referred to as test reactors from this part on. All the reactors conducted for the screening studies were visually monitored for the changes in the color of the supernatants and cultures, and in the sizes of the seed cultures. The differences among the set-ups and experimental procedures of the screening studies are given in Table 1. Three sets (Sets A, B and C) of reactors were conducted for the second screening study to investigate the effect of substrate type. By comparing Sets A and B, and Sets B and C, the shaking effect and the sludge wasting effect, respectively, were determined. In the first and fourth screening study, the oxygenation effect and the microbial activity of the developed granules were investigated, respectively.

![Figure 1. Schematic diagram of the continual two-day schedule (wasting/feeding cycle).](https://example.com/fig1.png)
The third screening study was conducted to investigate the effect of the ratio (R) of anaerobic biomass to aerobic biomass on granulation (ie the relationship between the anaerobic and aerobic cultures in terms of mixed liquor volatile suspended solid (MLVSS) concentration). Different R-values (1.7, 2.9, 4.6, 5.7 and 7.5) were achieved by changing the initial amount of each culture while setting the initial average mixed liquor suspended solids (MLSS) and MLVSS concentrations of the mixture constant at approximately 7600 and 3200 mg dm\(^{-3}\), respectively.

To investigate the effect of total biomass concentration on granulation, the anaerobic and aerobic seed sludge concentrations (given in parenthesis in Table 1) were doubled for two R-values (4.6 and 7.5).

**Analytical methods**

Aqueous effluent samples and the granules were analyzed for their MLSS/MLVSS contents and SOUR (Specific Oxygen Uptake Rate, mg DO g\(^{-1}\) VSS h\(^{-1}\)) activities, respectively.\(^{13}\) The methanogenic activity (MA) of the granules was measured following the procedure of Zitomer and Shrout.\(^{10}\) As substrate, acetic acid (HAc) (COD of 1500 mg dm\(^{-3}\)) was used. Methane content determination was accomplished by using KOH stock solution.\(^{14}\) DO concentrations of the reactor contents were measured by a DO meter (9071 Model, Jenway Ltd, Essex, UK) before SOUR and MA analyses.

Throughout the screening studies, the granules developed in the reactors were observed and compared in terms of size and durability. The optimum conditions required for granulation were defined as the conditions leading to granules of maximum sizes without any disintegration until the end of the experiment. Because the continual two-day schedules were maintained, the reactors could not be opened for granule sampling and in turn for the particle size analysis during the experiments in order not to disturb the headspace gas content. Therefore, during the experimental run, granulation processes in the reactors could only be observed visually.

**RESULTS AND DISCUSSION**

Screening studies indicated that alternating anaerobic/aerobic (in turn their transient microaerobic) conditions could be achieved in reactors by the continual two-day schedule. The reducing and oxidizing conditions in the test reactors were verified by the color exhibited by the Resazurin dye in the BM. Resazurin exhibits no color at an oxidation–reduction potential relative to a standard hydrogen electrode (Eh) below approximately −50 millivolts, but is pink under more oxidized conditions.\(^{15}\) The test reactors fed with oxygen doses above and equal to 60, 100 and 120% of the total COD added were typically pink most of the times during even days and colorless during odd days of the schedule. The 10 and 30% oxygen-fed test reactors displayed anaerobic conditions most of the time (colorless supernatant); however, aerobic conditions were also observed during short periods of approximately 15 min after oxygen addition by the pink supernatant (first screening study).

It was found that granules up to 2–2.5 mm could be developed from a mixture of anaerobic and aerobic suspended cultures via the continual two-day schedule. Mixed suspended cultures followed a granulation process which might be described in five main phases: Phase 0: all cultures in suspended solid (SS) form, no pellets; Phase 1: mainly suspended cultures, very few pellets; Phase 2: notable removal of SS, pelletized cultures (loose and soft appearance) with diameter (d\(_p\)) < 0.3 mm; Phase 3: no visible SS, d\(_p\) ≤ 1 mm; Phase 4: no visible SS, d\(_p\) ≤ 2–2.5 mm. The cultures reaching Phases 3 or 4 had a more compact structure and, thus, were accepted as granules. Granulation was highly related to the peculiarity of the schedule and the applied oxygen doses. Substrate type, shaking, sludge wasting and total biomass affected

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**Table 1. The differences among the set-ups and experimental procedures of the screening studies**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>First</th>
<th>Second</th>
<th>Third</th>
<th>Fourth</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameter</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>O(_2) doses (%)</strong></td>
<td>10, 30, 60, 100, 120</td>
<td>60, 120</td>
<td>120</td>
<td>60, 120</td>
</tr>
<tr>
<td><strong>O(_2) volumes (cm(^3))</strong></td>
<td>4, 12, 23, 38, 45</td>
<td>23, 45</td>
<td>45</td>
<td>23, 45</td>
</tr>
<tr>
<td><strong>Substrate</strong></td>
<td>Ethanol, Glucose + HAc(^{a})</td>
<td>Ethanol, Glucose + HAc, Glucose, HAc</td>
<td>Ethanol</td>
<td>Ethanol</td>
</tr>
<tr>
<td><strong>Shaking(^{b})</strong></td>
<td>Yes</td>
<td>Only for Set A</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Incubated in</strong></td>
<td>Shaker</td>
<td>Shaker (Set A), TCR(^{c}) (Sets B, C)</td>
<td>Shaker</td>
<td>Shaker</td>
</tr>
<tr>
<td><strong>SW and BMA(^{d})</strong></td>
<td>Yes</td>
<td>Only for Set C</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>MLSS (mg dm(^{-3}))</strong></td>
<td>8636 ± 300</td>
<td>16 990 ± 247</td>
<td>7600 (15 500)(^{e})</td>
<td>19 280 ± 350</td>
</tr>
<tr>
<td><strong>MLVSS (mg dm(^{-3}))</strong></td>
<td>4588 ± 57</td>
<td>7842 ± 106</td>
<td>3200 (6400)(^{e})</td>
<td>7980 ± 71</td>
</tr>
<tr>
<td><strong>R</strong></td>
<td>2.5</td>
<td>6.6</td>
<td>1.7, 2.9, 4.6, 5.7, 7.5</td>
<td>5.2</td>
</tr>
<tr>
<td><strong>Experimental run</strong></td>
<td>38 days</td>
<td>28 days</td>
<td>42 days</td>
<td>50 days</td>
</tr>
</tbody>
</table>

\(^{a}\) Glucose + HAc (1:1).

\(^{b}\) Shaking on shaker table at 35 ± 1°C at 160 rpm.

\(^{c}\) TCR: Temperature-controlled room at 35 ± 2°C.

\(^{d}\) SW: Sludge wasting, BMA: BM added.

\(^{e}\) To determine total biomass effect MLSS and MLVSS contents were doubled for two R-values (4.6 and 7.5).
the granulation period, the size and the amount of granules.

Effect of oxygen dose
The granulation phases observed in the reactors of the first screening study are given in Fig 2. Independent of the substrate type, in all of the test reactors fed with oxygen doses of 60, 100 and 120% of the total COD added, granules up to 1 mm and 2–2.5 mm developed (Phases 3 and 4). As the oxygen dose increased, the color of the granules changed from brown to light brown (60% and 120% oxygen-fed ones, respectively). The time required to achieve granules of 2–2.5 mm decreased with the increase in the oxygen dose from 60 to 120% (Fig 2(a)). The oxygen doses less than 60% were inadequate for granulation (10% oxygen), but resulted in formation of loose flocs (30% oxygen) (Fig 2). Granulation was also observed in the aerobic controls in 4–6 days, while it was not observed in anaerobic controls during most of the operational periods (Fig 2). However, granules in aerobic controls got smaller in time (Fig 2), while the ones in test reactors fed with the same oxygen dose (120%) and substrate type did not disintegrate until the end of the experiment. Therefore, it can be stated that aerobic cultures might have been triggering the granulation in the test reactors, while anaerobic cultures might have strengthened the developed granules and increased their stability.

Effect of substrate type
The first granulation was observed in the reactors fed with glucose, which was then followed by the reactors fed with glucose + HAc, HAc, and ethanol, respectively (second screening study). However, the granules fed with ethanol grew to larger sizes, while the ones fed with other substrates became smaller and disintegrated and the amount of SS in the reactors increased until the end of the experiment. Similar disintegration was also observed in the first screening study for glucose + HAc and 100% oxygen-fed granules (Fig 2(b)). Therefore, among the four substrates, ethanol resulted in the formation of the largest and most stable granules.

Effect of initial $R$-value and total biomass
$R$-values did not affect the granule sizes. Independent of the $R$-value, granules of almost 2–2.5 mm were developed in the test reactors with initial MLSS concentrations of 7600 mg dm$^{-3}$ (third screening study, Table 1). However, the test reactors with same $R$-value but higher MLSS content (>15 000 mg dm$^{-3}$) had pelletized cultures with smaller (<0.5 mm) and fewer particles, which could not be visually observed due to the dense sludge bed, but were detected at the end of the experiment by opening the reactors. Development of granules with smaller sizes might be due to the high seed content, which might have been a barrier to oxygen transfer and in turn provided resistance to its mass transport$^1$ and growth of the cultures.

Effect of shaking and sludge wasting
Due to the dense sludge bed in the test reactors of the second screening study (MLSS: 16 990 mg dm$^{-3}$, Table 1), it was hard to observe the granulation process. Thus, aerobic controls where granulation could easily be monitored due to the low aerobic seed content (MLSS: 1440 mg dm$^{-3}$) were used to examine the effect of shaking and sludge wasting. The first granules in the aerobic controls of Sets A (shaking, no wasting) and B (no shaking and no wasting) were developed on Days 2–4 and 10, respectively. Granules of Set A (up to 2.5 mm) were larger than those of Set B (up to 1 mm). Thus, the shaking process was postulated to decrease the period required for granulation via increasing the gas–liquid interfacial area, the oxygen transfer rate$^1$ and in turn the growth of cultures. The sludge wasting process resulted in the continuous, and even the total, loss of the seed cultures if applied without shaking, as in the aerobic controls of Set C (no shaking, but wasting) (Table 1). However, on the other hand, sludge wasting might supply a competitive advantage for the granule-forming cultures by removing the ones not occupied in the granulation and in turn increasing the substrate and oxygen load that the granule-forming ones are exposed to.

Figure 2. Granulation in the reactors of first screening study fed with (a) ethanol, (b) Glucose + HAc.
Table 2. Results of the SOUR and SMA analyses

<table>
<thead>
<tr>
<th>Reactor type</th>
<th>OUR$^a$</th>
<th>DO$^b$</th>
<th>SOUR$^c$</th>
<th>MA$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R^2$</td>
<td>(mg dm$^{-3}$)</td>
<td>(mg DO g$^{-1}$ VSS h$^{-1}$)</td>
<td>(cm$^3$ CH$_4$ d$^{-1}$)</td>
</tr>
<tr>
<td>Aerobic control</td>
<td>0.999</td>
<td>3.7</td>
<td>3.224 ± 0.459</td>
<td>—</td>
</tr>
<tr>
<td>60% oxygen-fed test</td>
<td>0.995</td>
<td>0.5</td>
<td>8.749 ± 0.779</td>
<td>7.5</td>
</tr>
<tr>
<td>120% oxygen-fed test</td>
<td>0.9988</td>
<td>3.3</td>
<td>3.827 ± 0.028</td>
<td>7.5</td>
</tr>
<tr>
<td>Anaerobic control</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>8.4</td>
</tr>
</tbody>
</table>

$^a$ OUR: Oxygen uptake rate (mg DO dm$^{-3}$ min$^{-1}$).
$^b$ DO was measured at the end of the even days of the schedule.

Microbial activity
MA and SOUR analyses (Table 2) performed for the granules (2–2.5 mm) developed in the test reactors of the fourth screening study demonstrated the co-existence of viable anaerobic and aerobic cultures in these granules. As seen in Table 2, microaerobic and aerobic conditions were prevailing in the 60% and 120% oxygen-fed reactors at the end of the even days of the schedule, respectively, which would probably alternate to anaerobic conditions by the end of the odd days (colorless Resazurin). Despite the alternating conditions from aerobic to microaerobic during even days of the schedule, methanogens in the developed granules were protected and displayed significant methane production (7.5 cm$^3$ d$^{-1}$), comparable to that of anaerobic cultures (8.5 cm$^3$ d$^{-1}$). The possible survival and operation of anaerobes in the developed granules might be explained by their location at the inner parts (ie by the shielding effect of aerobic cultures surrounding the anaerobes). A similar survival mechanism due to oxygen transfer limitation was demonstrated in gel beads, where anaerobic cultures grew mainly in the oxygen-deficient central part while aerobic cultures were located at the outer parts of the beads surrounded with a DO of 7 mg dm$^{-3}$.$^4$ Granules fed with 60 and 120% oxygen also exhibited significant SOUR values of 8.749 ± 0.779 and 3.827 ± 0.028 mg DO g$^{-1}$ volatile suspended solids (VSS) h$^{-1}$, respectively, comparable to that of aerobic cultures (3.224 ± 0.459 mg DO g$^{-1}$ VSS h$^{-1}$) (Table 2). The higher SOUR of 60% oxygen-fed granules compared with the 120% oxygen-fed ones might be attributed to the duration of the oxygen limitation. The 60% oxygen-fed granules or the aerobic cultures in these granules might have been exposed to prolonged microaerobic conditions compared with 120% oxygen-fed ones and responded metabolically to environmental changes by increasing their SOUR activities. The increase in the affinity for oxygen utilization with the decrease in the initial DO concentrations from 1 to 0.05 mg dm$^{-3}$ has been reported.$^{16}$

CONCLUSIONS
This study demonstrated the possibility of the granulation of a mixture of anaerobic and aerobic suspended cultures exposed to alternating anaerobic/microaerobic/aerobic conditions. The granules developed displayed significant oxygen uptake and methane production activities, indicating that they were composed of both anaerobic and aerobic cultures. Aerobic cultures supported under oxidized conditions might have triggered the granulation, while anaerobic cultures might have achieved the stability of the developed granules by locating at inner oxygen-free parts as a survival mechanism. Therefore, formation of the living/growth conditions for both anaerobic and aerobic cultures, their cultivation, adaptation and granulation was highly related to the peculiarity of the alternating cyclic conditions. Nevertheless, a detailed study (including image analyses, particle size distribution, physical characterization and change in the SOUR and MA activities through granulation) should be further performed to verify the hypothesized granulation and survival mechanisms. This study is significant for representing the possible combined living of anaerobic and aerobic cultures in granular form. In addition to the advantages of granules in treatment technology, systems processing with the developed granules might have advantages of both anaerobic and aerobic systems. The application of alternating anaerobic/microaerobic/aerobic conditions in a bioreactor would decrease the oxygen requirement as well as the operating and associated energy costs relative to a corresponding aerobic bioreactor as already stated by Peng et al.$^{17}$

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