A study on aerobic lipid substrate elimination by microbial consortium

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Abstract: The purpose of this study was to validate the applicability of specialized microbial consortium for the degradation of lipids in wastewater. An experimental model of the process is proposed that enables prediction of the required batch length. This model can be used for supervision of the process and to control cycles of the batch reactor. The study involved 4 reactors with microbial consortium obtained by inoculation from a commercially available biopreparate. Each reactor was fed a different load of lipid containing substrate. The biodiversity, settling characteristics and COD reductions were measured. The biodiversity of the microbial consortium changed within a range of ±15% depending on lipids concentration, as shown by the Shannon index and increasing amount of β-proteobacteria. Higher concentrations of lipids increased the biodiversity suggesting higher growth of microorganisms capable of utilizing lipids as energy and carbon source by producing lipid hydrolyzing enzymes. High lipid concentrations degrade the settling capabilities of the biomass. Higher lipid concentrations (0.5–2.0 [g/l]) increase the final COD (1445–2160 [mg O_2/l]). The time necessary for substrate degradation changes with the initial concentration and can be predicted using the proposed model. The study showed that specialized microbial consortium is capable of reducing the lipids containing substrate and maintains its biodiversity suggesting that utilization of such consortia in multiple cycles of a batch reactor is possible. Future research should concentrate on assessing the biodiversity and effectiveness of substrate reduction after an increased number of batch reactor cycles.

Introduction

Municipal and some industrial wastewaters, including food processing wastes, contain lipids (fats, oils and greases), which can cause severe environmental pollution (Alade et al. 2011). Those compounds can form an oil film on water surface, preventing the diffusion of oxygen into water leading to the death of aquatic life (Facchin et al. 2013) and promote growth of filamentous microorganisms which cause bulking and foaming (Martins et al. 2004).

Some industrial food processing wastewaters contain a high concentration of lipids, e.g. from dairy products industry over 300 mg/L, from edible oil refinery over 500 mg/L and from slaughterhouses over 2000 mg/L (Saifuddin and Chua 2006). Lipids can be removed from wastewater by physical or chemical methods, e.g. grease traps, DAF systems or coagulation, flocculation and neutralization by addition of chemical compounds (Willey 2001). The application of ultrasound in oily wastewater pre-treatment has also been investigated (Moysa-Łobos 2018). However, these compounds can pass through physicochemical treatment processes and contribute to the levels of BOD and COD in the effluents. Therefore, biological treatment process is commonly used to additionally remove lipids from lipid-rich wastewater.

In aerobic wastewater treatment systems, lipids are considered to be biodegradable. However, these compounds reduce the rates at which oxygen is transferred to biofilms, causing lower microbial activity (Becker et al. 1999). The usage of bacteria or mixed microbial cultures, which are able to biodegrade lipids, can be a good option for the treatment of lipid-rich wastewater (Brooksband et al. 2007) but also for antibiotics biodegradation (Jałowiecki et al. 2019). A number of studies dealt with anaerobic processes used for degradation of lipids (Fujihira et.al. 2018, Ning et. al. 2018).

There are many commercially available microbial biopreparates, containing selected microorganisms and/or enzymes (lipases) helpful in lipids biodegradation. However, the use of pure enzyme supplements is less attractive than the application of viable microorganisms. Microorganisms hydrolyze lipids as well as biodegrade them further to CO_2 + H_2O and have a greater tolerance to changes in environmental conditions. Additionally, many industrial wastewaters, including food processing wastes, invariably contain colloidal and particulate organics that must undergo hydrolysis prior to biodegradation.

Literature data show that bioaugmentation is not always successful (Herrero and Stuckey 2015). This may be due to insufficient knowledge of mutual dependencies among

culture microorganisms. It is probable that the incorporated microorganisms, despite having an effective enzymatic potential enabling the degradation of particular substrates, are not capable of populating certain ecological niches and die in the competition with the local microflora. Therefore, it is important to investigate the changes in microorganisms populations, both bred from a specific biopreparates and also in activated sludge bioaugmented with biopreparate. The objective of the presented research was to determine the effectiveness of selected lipid substrate elimination by a specially prepared biocenosis, bred from a biopreparate containing microorganisms predestined for degradation of lipids. Biocenosis changes and sedimentation characteristic of the microbial culture were also investigated. Additionally, a simplified model of the biodegradation batch process is proposed in order to enable prediction of the required single batch length.

Materials and methods

The experiments were carried out using 4 batch reactors (Fig. 1), referred to as RA, RB, RC and RD, filled at the beginning of the experiment with a microbial consortium fed with different loads of lipid containing substrate (Table 1). Chemical oxygen demand (COD) has been measured using the Hach COD reactor and DR2010 IR spectrophotometer.

Experimental setup
The mixture of microorganisms used in the experiments was first obtained from a commercially available biopreparate

stored in lyophilized form and designed to treat lipid-rich wastewater. The microbial culture was started by suspending 4 [g] of the biopreaprate in 3 [l] of water and feeding the breeding culture (BR) with oil of olive (250 [mg/l]), Tween® 80 (25 [mg/l]) and peptone (200 [mg/l]) once a week for 2 months to promote the growth of lipase secreting bacteria. For the next 3 months the culture was fed peptone only, until the startup culture (ST) was obtained and used in further studies. The microbial biopreparate contained lipase-producing bacteria, including *Bacillus* sp., *Pseudomonas* sp., *Lactobacillus* sp., *Lactococcus* sp., *Acinetobacbacter* sp., *Alcaligenes* sp. and other microorganisms involved in wastewater treatment process (*i.a. Nitrobacter* sp., *Nitrosomonas* sp., *Comamonas* sp., *Rhizobium* sp., *Saccharomyces* sp., *Deinococcus* sp.) according to the manufacturers specification. The plexiglass reactors were completely mixed, had a working volume of 1.5 [l] and were aerated through an air diffuser and maintained at room temperature. The microbial consortium for each reactor was taken from the ST culture, and the initial biomass concentration in RA, RB, RC and RD was 1.8 [mg/l]. Different loads of substrate (Table 1) were added to each reactor.

Biomass sampling, DNA extraction and PCR conditions
Biomass samples (volume of 50 ml) were collected at the beginning (BR) and at the end (ST) from the reactor used for growing of bacterial inoculum and from reactors RA, RB, RC and RD at the end of the experiment and stored at -45°C until DNA extraction. Total genomic DNA was isolated from
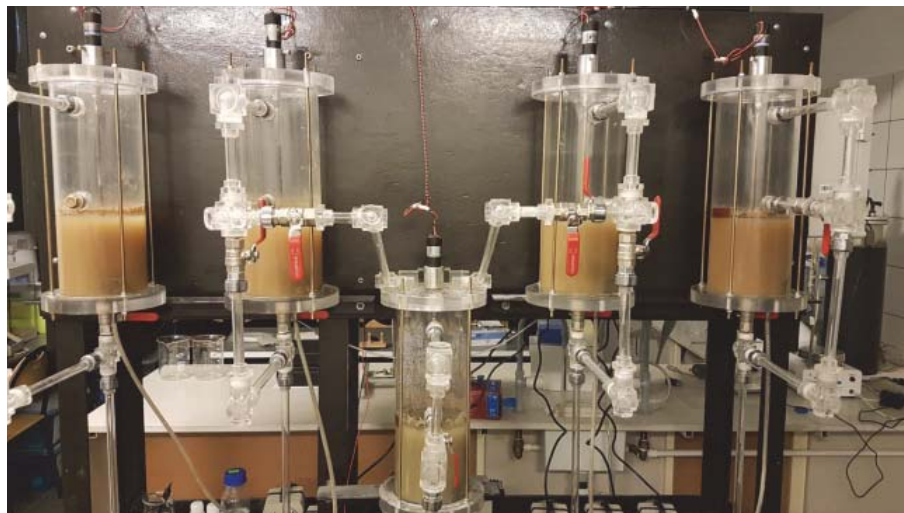

Fig. 1. Four batch reactors used in the presented studies
(the 5-th reactor at the bottom is used as a reservoir for biomass)

Table 1. Summary of substrate added to individual reactors at the beginning of the experiment

Batch name	Measured COD [mg O$_2$/l]	Peptone [g/l]	Tween®80 [µl/l]	Oil of olives [g/l]
RA	1445	0,8	50	0,5
RB	1662	0,8	100	1,0
RC	1920	0,8	150	1,5
RD	2160	0,8	200	2,0

0.2 g of biomass samples using FastDNA® SPIN KIT FOR SOIL (MP Biomedicals, USA) according to manufacturer's instructions and stored at -20°C until PCR amplification. Primers: 338f-GC (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG 3') and 518r (5' ATT ACC GCG GCT GCT GG 3') were used for partial (ca. 180 bp) 16S rRNA bacterial gene PCR amplification. PCR was carried out in a 30 µl (total volume) reaction mixture containing 19 µl sterile MiliQ water, 6 µl PCR buffer (GoFlexi TAQ, Promega), 2.4 µl MgCl2 (2 mM), 0.25 µl of both primers (5 pmol/µl), 1.3 µl dNTPs (20 pmol/µl), 0.5 µl of genomic DNA and 0.3 µl Taq DNA polymerase (1.5 U) (Muyzer 1993). PCR amplification was performed using an Eppendorf thermal cycler and the following steps: (1) the initial denaturation step (10 min at 95°C); (2) 30 cycles, each single cycle consisting of denaturation (1 min at 95°C), annealing (1 min at 53°C), and elongation (2 min at 72°C); and (3) the final extension step (12 min at 72°C). The products were evaluated in agarose gel (0.8% w/vol agarose, 1 × TBE buffer), stained with ethidium bromide (1% w/vol) in MiliQ water and photographed under UV light.

DGGE denaturing gradient gel electrophoresis

The DGGE of PCR products obtained in reaction with primers 338f-GC/518r were performed using the Dcode Universal Mutation Detection System (BioRad). The polyacrylamide gel (8% [v/v] with a gradient of 30–60% denaturant) was run for 13 h at 55 V in a 1 × TAE buffer at a constant temperature of 60°C (Muyzer 1993). The gel was stained with SYBR GOLD (1:10 000, Invitrogen) in MiliQ water for 20 min and washed in MiliQ water twice for 15 min, then visualized under UV light and photographed.

Analysis of the DGGE fingerprints

The DGGE banding patterns with PCR products were analyzed using Quantity One 1D Software (BioRad). The structural diversity of the bacterial community was estimated on the basis of the Shannon-Weaver diversity index, H (Eichner et al. 1999, Luxmy et al. 2000), estimated from the relative band intensities obtained from the DGGE fingerprints.

FISH methodology

In order to assess the general biocenosis of the microbial consortium, the Fluorescent In-Situ Hybridization protocol was employed according to (Daims et. al. 2005). Universal bacterial probe Eubmix, and probes for α-, β- and γ-proteobacteria were used. Additionally, probes indicating bacteria important for nitrogen processing were added: NIT3, NSO1225, NTSPA.

Results and discussion

Fig. 2a presents changes in measured COD. The effectiveness of lipid substrate elimination, as estimated by COD measurements, was roughly the same, despite different initial substrate concentrations (Table 1). After 5 days, the concentrations of COD in all reactors reached the steady state, although for lower initial COD concentrations (especially reactors RA and RB) steady state was reached after 3 days. Those results show that the biocenosis of microbial consortium is characterized by the ability to effectively use lipid substrate as a source of carbon and energy.

Changes in biomass concentration, measured using the dry-weight method, are presented in Fig. 2b. For higher substrate concentrations, higher biomass concentrations have been achieved. Fig 2c presents O_2 saturation values. At the experiment commencement, dissolved oxygen concentrations dropped due to high oxygen caused by high substrate concentration. After 2 days, a constant level of oxygen saturation was established, denoting a depletion of major substrate concentrations in all reactors. Fig. 2d shows pH changes in the respective reactors. The initial pH in all four reactors was at the level of 7.5 and reached stable end values after about 4–5 days. For the reactor containing the lowest substrate concentrations, the pH changes throughout the experiment have been the smallest, exhibiting a relation between the amount of substrate processed and the corresponding pH changes. To assess the settling properties of the biomass the volume of sludge after 30 [min] sedimentation (Fig 2e) and the sludge volume index (SVI), defined as the volume occupied by 1g of the settled sludge (Fig 2f), are presented. The settling characteristic of the biomass was worst at the beginning and achieved a satisfactory level of 50–100 [ml/g] (Cheremisinoff 2002) after about 4–5 days with the exception of the reactor RD having the highest lipids concentration, thus suggesting that the presence of lipids deteriorates the settling properties of the biomass. While the removal of COD is correlated with the improvement of the settling characteristic of sludge and suggests that this improvement may be indicative of the lipids removal, further investigations are needed to confirm a direct link between settling characteristic and lipids concentration in the mixed liquor. Fluorescent *in-situ* hybridization analysis was used to assess major changes in the biodiversity of bacterial culture. For α- and γ-proteobacteria, no noticeable differences have been observed. For β-proteobacteria, a considerable proliferation has been observed in all cultures with respect to the ST culture as shown by representative results in Fig. 3. Fig. 3a presents a representative region of the sample indicating all bacteria (EubMIX probe, Fluos dye; green color) while Fig. 3b presents only β-proteobacteria (β-proteobacteria probe, Cy3 dye; orange-red color) in the same representative region. NIT3, NSO1225 and NTSPA probes gave negative results, suggesting that nitrogen processing bacteria failed to develop in the used cultures. The fingerprints obtained from DGGE separation of bacterial 16S rRNA gene fragments are shown in Fig. 4(a). Based on the obtained fingerprints that visualize the biodiversity of the microbial consortium, it is possible to show bands common to all reactors (frames 1,2,3) that stopped at the same heights. The presence of those bands suggests the presence of common genotypes both in the ST culture fed with easily biodegradable substrate (peptone) and in batch systems fed with different concentrations of slowly biodegradable substrate (lipids). Those bands stopped relatively high in the polyacrylamide gel, which suggests high concentration of AT pairs in the analyzed sequence. Additionally, in the breeding culture (BR), a band not present in other cultures has been identified (frame 4). This band stopped very low in the gel, which suggests high concentration of GC pairs in the analyzed sequence. In the start-up culture
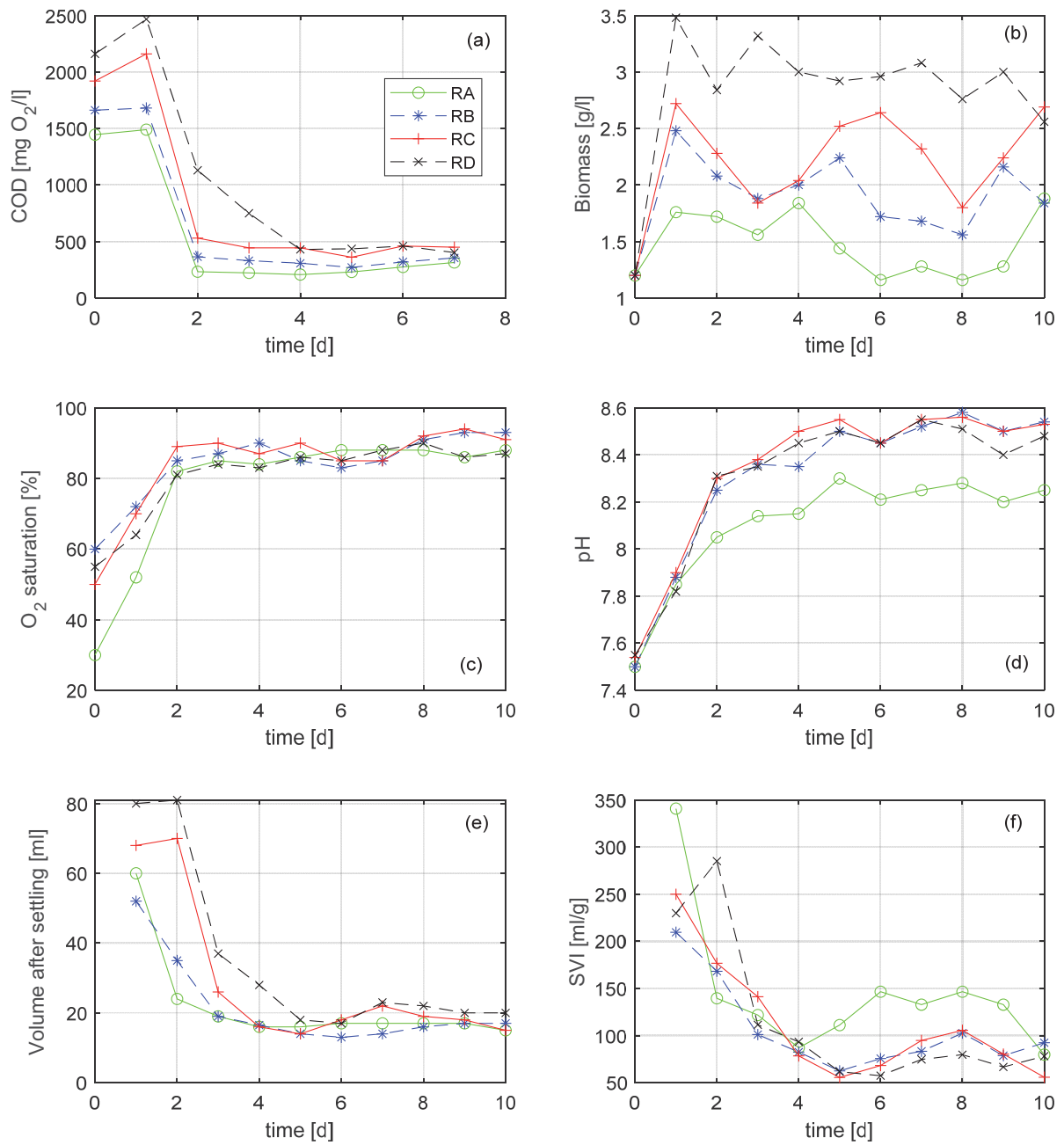
Fig. 2. Experiment results. (a) Changes in COD [mg O$_2$/l]. (b) Biomass concentrations [g/l]. (c) Dissolved oxygen saturation [%]. (d) pH changes. (e) Volume after 30 minutes of settling [ml]. (f) Sludge volume index [ml/g]
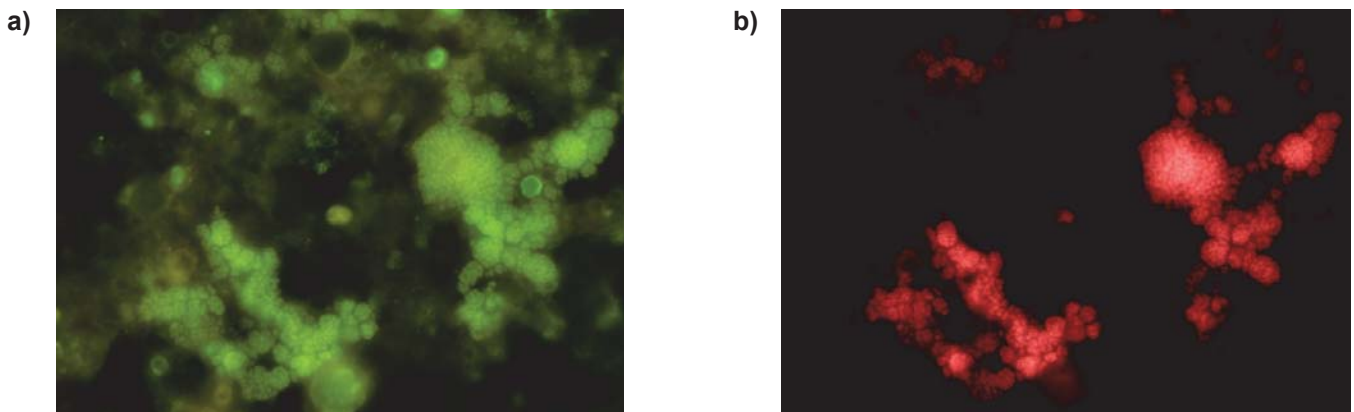

Fig. 3. FISH results. (a) Fluos (green) indicating all bacteria. (b) Cy3 (orange-red) indicating β-proteobacteria

(ST), bands have been detected (frames 5) that are not present in particular batch reactors, which can suggest that the particular genotypes declined due to changed substrate conditions (addition of lipids and the slowly biodegradable fraction). Frames 6 and 7 denote bands that were present in the start-up culture and also in the batch reactors, suggesting the presence of genotypes that remained in the microbial consortium despite changes in substrates. In the batch cultures containing different concentrations of slowly degradable fraction, common bands have been detected (frames 8,9 and 10) that were not present in culture fed with easily degradable substrate. That can suggests a change in the biocoenosis due to changes in substrate conditions. The mentioned bands stopped relatively low in the gel suggesting high concentration of GC pairs in the analyzed sequence, containing a triple bond that is more difficult to break than a double bond of the AT pair. On the other hand, in the samples drawn from the reactors with the highest concentration of slowly degradable lipid substrate, certain bands have been detected that were not present in the culture (ST) fed only with easily degradable substrate (peptone), nor in the cultures fed with lipids in low concentrations. This probably suggests a selective influence of the forced substrate conditions. Those bands stopped relatively high in the polyacrylamide gel, which suggests high concentration of AT pairs in the analyzed sequence.

The numerical analysis of DGGE was carried out with Quantity On 1D Software (BioRad). Bacterial diversity was calculated based on the differences in the light intensity of bands being part of DGGE bands patterns (densitometric analysis) as a Shannon index (Shannon and Weaver, 1963). This index statistically describes the biodiversity in the ecosystem and its value is in the range between 0 and 4.5. Lack of significant changes in the biodiversity index suggests a stable biocoenosis, which in turn suggests non changing and optimal environmental conditions. Significant changes of the index suggest the presence of factors disturbing the particular biocenosis. The lowest value 2.04 of the Shannon index (Fig. 4b) was obtained for the breeding culture (BR) but rose to 2.57 in the ST culture. Depending on the concentration of lipids in the substrate, the Shannon index value was different in each batch reactor. The lowest value was noted in the reactor being fed the lowest concentration of lipids (2.28 in RA), and the highest value was noted in rectors with the highest concentration of lipids (2.92 in RC and RD). Therefore, the biodiversity increased for increasing the concentration of lipids in the substrate. Higher concentrations of lipids were advantageous for the biocoenosis. However, this information should be correlated with other physicochemical measurements (the effectiveness of lipids removal).

Based on the presented molecular analysis it can be concluded that the biocoenosis changed due to differences in the substrate concentration, and in particular that concentration of slowly degradable lipid fraction influenced the biodiversity of the microbial consortium.

Mathematical model for prediction and control

In the experimental setup presented, the main goal of the process is to reduce substrate S expressed as COD [mg O_2/l] that consists of high concentration of lipids but also other non-lipid organic matter. Fig. 2a suggests that once the predicted level of COD drops below a predetermined threshold, the process can be terminated, enabling a higher multi-cycle throughput of the batch processing system. The proposed model determination method is to obtain two calibration batches corresponding to the minimum and maximum predicted loads. COD for those two batches should be measured every 24 h. Therefore, batch RA and batch RD have been selected to act as the calibration batch experiments. The model should predict any batch, knowing only its initial substrate concentration S_{in} within the range of S_{inA} and S_{inD}. All other model parameters
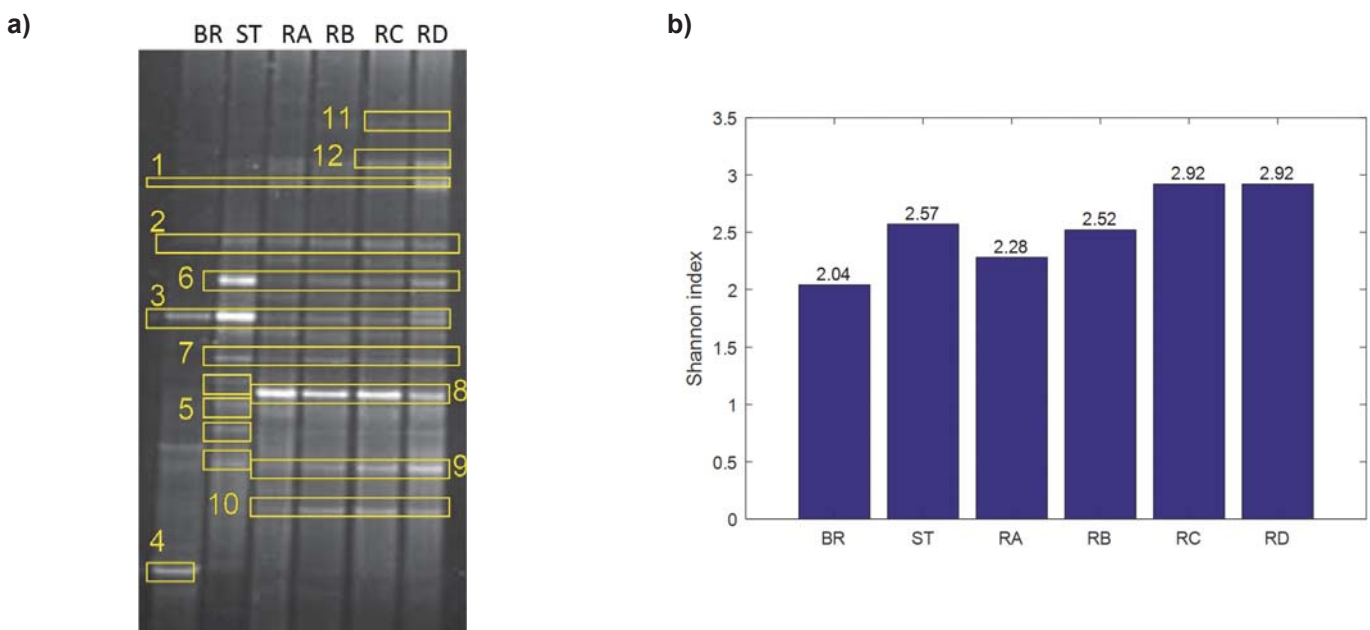
a)

b)

Fig. 4. (a) DGGE pattern of bacterial 16S rRNA genes fragments with a size *ca.* 180 bp amplified using DNA obtained from activated sludge samples. Frames indicate bands discussed in the text. (b) Biodiversity of activated sludge defined by the Shannon index in the breeding culture (BR), the start-up culture (ST) and in batch cultures RA, RB, RC and RD

for the particular S_{in} are derived from the calibration batches corresponding to S_{inA} and S_{inD}.

In Fig. 2a, the measured COD first rises and reaches a peak value after the first day. Afterwards, the measured COD decreases exponentially and reaches a final value. The initial rise is believed to be due to adaptation of the biomass and higher production of soluble material that is not settling before COD samples were being taken. Since the nature of this increase is complex and the model should be as simple as possible, the model lumps all the phenomena involved in the initial increase into a single rate parameter. Therefore, it is assumed that the initial rise of substrate is linear and reaches a maximum value after 24h. In the second phase, when the biomass is adapted to the high lipids load, an exponential function is chosen to describe the degradation of substrate. Fig. 5 presents the general concept of the mathematical model.

The proposed model is given by the following equation:

$$S(t) = \begin{cases} S_{in} + (S_1 - S_{in})t & for\ t \leq 1 \\ (S_1 - S_f)e^{-(t-1)/T} + S_f & for\ t \geq 1 \end{cases} \quad (1)$$

Where:
S_{in} is the initial substrate load for a particular batch,
S_1 is the substrate concentration after the first phase of the process,
S_f is the final substrate concentration,
T is the time constant associated with the second phase of the batch process [day] and t is time [day].
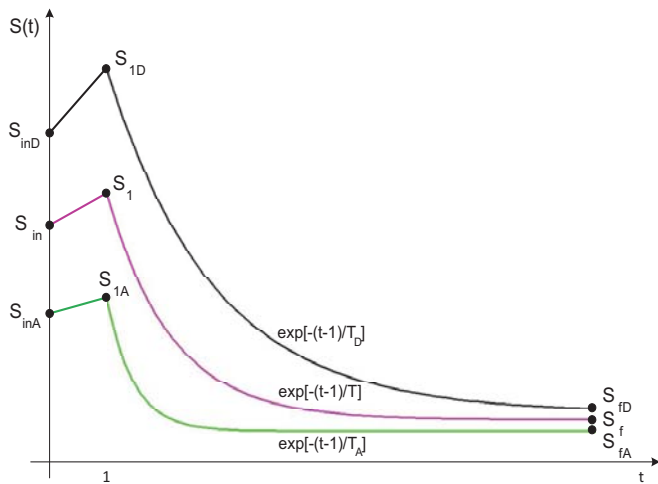
For any batch, the following values need to be determined: S_{in}, S_1, S_f and T. For RA and RD batches, those values are determined experimentally. The final substrate concentrations are computed by averaging a couple of final data points. In order to obtain the necessary values for any other middle batch, the following scaling factor is defined:

$$x = \frac{S_{in} - S_{inA}}{S_{inD} - S_{inA}} \qquad x \in [0,1] \quad (2)$$

Based on eq. (2), the model parameters are given by the following equations:

$$S_1 = S_{1A} + (S_{1D} - S_{1A})x = S_{1A} + \frac{(S_{1D} - S_{1A})(S_{in} - S_{inA})}{S_{inD} - S_{inA}} \quad (3)$$

$$S_f = S_{fA} + (S_{fD} - S_{fA})x = S_{fA} + \frac{(S_{fD} - S_{fA})(S_{in} - S_{inA})}{S_{inD} - S_{inA}} \quad (4)$$

$$T = T_A + (T_D - T_A)x = T_A + \frac{(T_D - T_A)(S_{in} - S_{inA})}{S_{inD} - S_{inA}} \quad (5)$$

It may be verified that relations (1)–(5) are adequate for any initial substrate concentrations S_{in} within the assumed range. Fig. 6 presents the obtained model results (lines) and the measurements (data points) for comparison, while Table 2 details the obtained parameters.

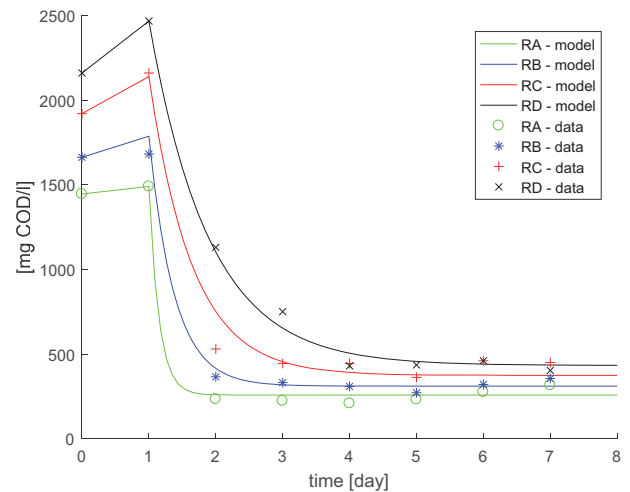Fig. 5. Model for predicting the batch process

Fig. 6. Comparison between the measured values and model predictions

Table 2. Model parameters for calibration (A and D) and predicted (B and C) batches

Batch name	S_{in} [mg O_2/l]	X (scaling factor)	S_1 [mg O_2/l]	S_f [mg O_2/l]	T [day]
RA	1445	0	1490	257	0,15
RB	1662	0,303	1786	310	0,38
RC	1920	0,664	2139	373	0,65
RD	2160	1	2467	432	0,90

The model predicts somewhat higher values of substrate concentration in the degradation phase. In general however, once calibration batches are obtained, and the initial concentration of lipid-rich substrate is known, the model enables the degradation of COD to be predicted, and the termination time to be computed given a desirable percentage removal of COD.

Conclusions

The biodiversity of the microbial consortium changes within a range of ±15%, as shown by the Shannon index and proliferation of β-proteobacteria. It appears that higher concentrations of lipids positively influence the biodiversity (Shannon index) which is consistent with the higher lipase secreting bacteria that are probably the cause of the higher Shannon index for higher lipids loads. In order to verify the applicability of the cultures under experiments in long term biodegradation of lipids, future research should include investigating biodiversity changes after a prolonged period of time and after a substantial number of batch cycles.

The introduction of lipid rich substrate causes a deterioration of biomass settling capabilities. In most cases settling properties returned to an acceptable range after about 4 days with some long lasting deterioration for the highest lipids concentration. It is therefore assumed that lipids have been degraded but high concentrations of lipids affect the settling properties of biomass.

It is possible to model the degradation process using a simple model given by equation (1). The model only predicts the conversation of overall COD in the reactors, without differentiating between lipids and other organic carbon sources. Additionally, the changing settling characteristic of the microbial culture correlates to some degree with the removal of lipids and this correlation can be the subject of separate investigations.

Acknowledgements

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