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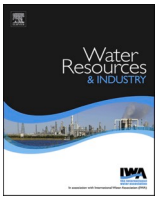

Investigating the biomass-specific inhibitory effect of benzalkonium chloride on anaerobic granules: A kinetic approach

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ABSTRACT

Benzalkonium Chloride (BAC), a quaternary ammonium compound commonly used in industrial disinfection processes, was studied for its biomass-specific inhibitory effect on the specific methanogenic activity (SMA) of anaerobic granules. Inhibition batch assays were conducted with varying BAC concentrations (5–40 mg/L), resulting in biomass-dependent inhibition. Considering its surface-active nature, a biomass-specific BAC load was a suitable parameter to determine IC_{50} values, ranging from 4.3 to 6.1 mg BAC/g VS. For predicting the corresponding biomass-specific inhibition constant in ADM1, the inclusion of an additional adsorption-inhibition term was required for a better validation of results. The model yielded a biomass-specific IC_{50} of 5.3 mg BAC/g VS. The results encourage a change in perspective on IC_{50} for surfactants by determining a biomass-specific IC_{50}, particularly in scenarios when surfactants accumulate within anaerobic reactors. Hereby, the reliability and practical relevance of IC_{50} is increased, driving the development of mitigation strategies.

1. Introduction

Anaerobic wastewater treatment is a technology applied worldwide, particularly for the pre-treatment of industrial wastewater with high organic loads [1]. The advantages of such processes include low energy consumption, low sludge production as well as methane (CH_4) production [2]. Specifically, granule-based reactor technology represents a significant milestone in the development of anaerobic wastewater treatment, providing the sustainable solution for the efficient treatment of industrial wastewater with high organic loads [1,3]. Anaerobic granules offer great advantages, including excellent settling properties, high biomass concentration, microbial diversity, efficient substrate mass transport and resilience to shock loads [4–8]. Both the performance and operational stability of high-rate anaerobic reactors is significantly dependent on the condition and activity of the granules [9–13]. Nevertheless, operational disturbances and instabilities occur in practice due to a reduced activity of the anaerobic biomass, which often results in a reduced performance accompanied by disintegration of granular sludge and excessive sludge washout. Such malfunctions are attributed to toxic and inhibiting effects of wastewater constituents utilized in individual applications [14,15] or to altered granule formation due to the composition of the treated wastewater [16,17]. Inhibitory substances are often the leading cause of reactor instability and failure. These substances comprise inorganic toxicants and organic toxicants as well as industrial chemicals used in the upstream production facilities as for instance complexing agents, disinfectants, surfactants, detergents and flocculants.

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Quaternary ammonium compounds (QAC) are extensively applied in industrial applications as surfactants, disinfectants, fabric softeners, emulsifiers, pesticides, biocides and corrosion inhibitors [18,19]. The surface-activity of QAC is caused by the presence of four functional groups (hydrophobic alkyl groups) bound covalently to a positively charged, hydrophilic central nitrogen atom [20]. Due to the high adsorption affinity, these cationic surfactants strongly adhere to negatively charged particles, suspended particulates and sludge [21].

Up to 75 % of the QAC used in domestic and industrial utilization are discharged and accumulated in wastewater treatment facilities [22]. The amount of QAC in sewage sludge samples ranges between 22 and 103 mg/kg dried weight [23]. Anaerobic digesters contain QAC concentrations that reach up to 4–50 mg/L and 4.0–10.5 mg/g dry sludge [19]. In fact, QAC concentrations might be higher in industrial wastewater treatment systems for food processing industries or beverage facilities [24,25]. In activated sludge treatment systems, QAC exhibit biological degradability with reported degradation rates >90 % [26,27]. As a result of the adsorptive affinity to (bio)solids, adsorption prevails biodegradation. Consequently, the transfer of QACs to the solid phase is probably the main removal mechanism. Besides, there are no relevant studies about QACs biodegradability under anaerobic conditions [19,24,28–30].

With respect to the inhibitory effect of QAC in anaerobic systems, few studies exist. Tezel et al. [24] determined the inhibitory effect of QAC on mixed methanogenic cultures in batch tests at a concentration of 25 mg QAC/L, above which methanogenesis was inhibited. Exposures of QAC in a fed-batch reactor resulted in accumulation of Volatile Fatty Acids (VFA) at 30 mg QAC/L.

Among QACs, Benzalkonium Chloride (BAC) is one of the most important cationic surfactants, whose diverse applications comprise surface disinfection in medical care facilities, food production and beverage industries. Zhang et al. [31] investigated the inhibition caused by BAC at concentrations ranging from 5 to 20 mg/L on different biomass concentrations in an activated sludge system by identifying respiratory enzymes and the half-saturation competitive inhibition constant, which was equivalent to half maximal Effective Concentration (EC_{50}). In that study, EC_{50} ranged between 0.12 and 3.60 mg BAC/L. It is important to notice that the higher EC_{50} reported values were obtained at higher biomass concentrations. Flores et al. [32] initially conducted anaerobic batch-tests, yielding a half maximal Inhibitory Concentration (IC_{50}) of 13 mg BAC/L. However, tests conducted with continuously operated reactors showed that exposure of the inhibitor at the previously determined IC_{50} concentration caused a complete failure of methane production. Given the surface-active, adsorptive nature of BAC, previously mentioned concentrations are neither practical nor universally applicable. Particularly in scenarios where surfactants accumulate in continuously operated anaerobic reactors due to their affinity for adsorption, conventional IC_{50} values fail in providing a reliable and transferable estimate of inhibitory effects.

From the modelling point of view, despite the extensive use of surface-active substances and the existent knowledge of their inhibition mechanism, the inclusion of a term that describes surface-active inhibition (including BAC) has not been deeply explored. The standard anaerobic digestion model ADM1 [33] only contains fundamental inhibition terms in anaerobic digestion, for example free ammonia and pH inhibition. Due to ability of surface-active substances to adhere on the biomass granules, the representation of the simultaneous inhibition/adsorption is not trivial. A simplified approach to include the simultaneous inhibition/adsorption in a modified ADM1 was reported by Palatsi et al. [34]. They successfully modelled long chain fatty acid inhibition kinetics within the ADM1 framework. The used inhibition/adsorption approach delivered better results than the ones obtained by applying a traditional ADM1 inhibition kinetics and by a model using Halden equation as inhibition kinetics.

The focus of this investigation is on identifying the biomass-specific inhibition concentration induced by BAC on the acetoclastic methanogenic activity of anaerobic granular sludge. Serum Bottle Tests (SBT) and Gas Endeavour System (GES) were used to assess BAC inhibition at different initial concentrations of acetate, biomass and BAC. To assure the comparability of the results the food/microorganism ratio (F/M) was kept constant for all the experiments. In addition, a modified version of the ADM1 was used that includes a term for the BAC inhibition. Using biomass-based inhibition kinetics, the type of observed inhibition was explored, and the value of the inhibition constant (K_i) was estimated based on the experimental results.

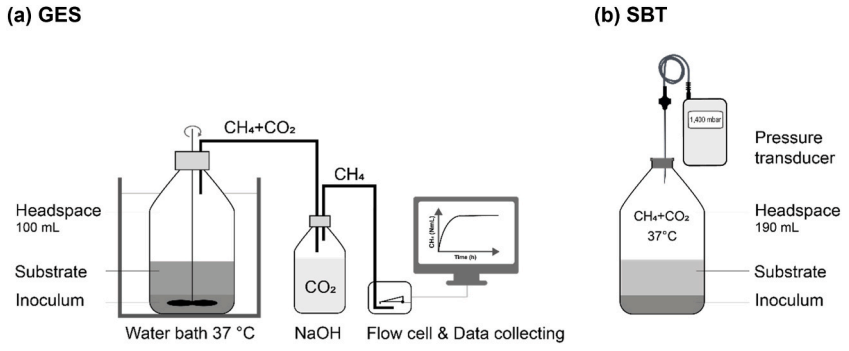

Fig. 1. Experimental setup of (a) volumetric method Gas Endeavour System (GES) and (b) manometric method Serum Bottle Tests (SBT).

2. Material and methods

2.1. Inoculum and substrate

Anaerobic mesophilic (37 °C) granular sludge obtained from a full-scale Upflow Anaerobic Sludge Blanket (UASB) reactor treating brewery wastewater was used as inoculum in all batch assays. The sludge had a Total Solids (TS) and Volatile Solids (VS) content of 8 % and 6.9 %, respectively. TS and VS content of the granular sludge were determined according to DIN EN standards [35,36]. The granular sludge was stored without substrate at 20 °C to limit residual endogenous activity.

For determination of the acetoclastic methanogenic activity, sodium acetate-3hydrate ($NaC_2H_3O_2{\cdot}3H_2O$, Merck, CAS number: 6131-90-4) was used in all batch assays as single carbon source. Since methanogens are classified as the most sensitive group of microorganisms in the AD process, $NaC_2H_3O_2{\cdot}3H_2O$ was selected as representative substrate. Two initial COD concentrations were evaluated: 2 and 4 g/L. Initial pH was adjusted to 7.0 ± 0.1 using 0.25 M NaOH (Carl Roth, CAS number: 1310-73-2).

2.2. Experimental set-up and biomass characterization

To determine the Specific Methanogenic Activity (SMA), the volumetric and the manometric method were applied for biogas and methane detection respectively. The experimental setup for both methods is shown in Fig. 1.

2.2.1. Volumetric method

The Gas Endeavour System (GES) (Bioprocess Control, Sweden) with a reactor volume of 500 mL was used as volumetric method. The reactor volume was divided into a 400 mL liquid phase and a 100 mL headspace. To achieve anaerobic conditions before transferring the substrate, all test vessels were flushed with nitrogen gas (N_2) for approximately 2 min.

Two different biomass concentrations were tested: 4 g VS/L and 8 g VS/L. For this purpose, the anaerobic granular sludge (size fraction: 1.5–2.0 mm) was transferred into the test vessels. To assure a F/M of 0.5 g COD/g VS initial COD concentrations of 2 and 4 g/L were selected. Acetate was used as single COD source i.e. reference substrate. Test vessels filled with substrate and sludge were placed in a temperature controlled thermostatic water bath (Grant Instruments, SAP18, 18 L), where the temperature was kept at 37 ± 0.2 °C.

Each reactor had an inbuilt overhead stirrer that was operated at 60 s intervals every hour to promote outgassing of the biogas, to increase the contact between substrate and biomass and to have a uniform mixing of the inhibitor. The stirrer speed was set at 50 rpm to provide a gentle mixing, reducing the mechanical stress applied to the granules. Produced methane was continuously measured after a CO_2 removal step in 3 M NaOH.

For each run, six series of batches were investigated at concentrations of 5, 10, 15, 20, 30 and 40 mg/L of BAC (50 % w/w aq. Solution, Alfa Aesar). Batch assays were performed in duplicate and were accompanied by blanks for subtracting the intrinsic methane production. Likewise, controls without inhibitor were also run. For the figures and for modelling purposes, the mean values of the results from the experiments were taken. All experiments were performed with the same sludge and substrate under identical temperature and pH conditions. The rest of the experimental conditions are presented in Table 1.

To assess possible irreversible inhibition of BAC, the test runs GES-I and GES-II were repeated. For this purpose, the runs were dismantled immediately after completion of the batch test. To carry out the replicates, the biomass previously exposed to BAC was washed at least 3 times with tap water and tested under identical test conditions, without adding further BAC. The repeated runs were named GES-I/I and GES-II/II as seen in Table 1.

2.2.2. Manometric method

For the manometric method - known as Serum Bottle Test (SBT) - 3 and 6 g (wet weight) of granular sludge (size fraction: 1.5–2.0 mm) were transferred to 250 mL serum bottle reactors, as described in Angelidaki and Sanders [37]. Bottle reactors were filled up to a volume of 50 mL with tap water. A concentrated acetate solution was added to achieve an initial COD of 2 or 4 g/L. To determine inhibition induced by BAC, batch assays were conducted without nutrient solution in order to avoid undesirable interaction with the inhibitor. The resulting effective headspace volume was 190 mL. Bottles were flushed for 2 min with N_2 to guarantee anaerobic conditions and immediately closed with rubber septa and aluminium caps (WICOM Germany GmbH). Afterwards the bottles were incubated without stirring at a mesophilic temperature of 36 ± 1 °C. The increase in pressure resulting from biogas production was discontinuously determined by using a pressure transducer (GMH 3156, Greisinger) with microneedles over a period of 2–5 days.

Table 1
Experimental conditions of the batch assays with sodium acetate-3hydrate salt as COD source with the methods SBT (Serum Bottle Test), GES (Gas Endeavour System) and their replicates GES-I/II and GES-II/II.

#run	c_0 (kg COD/m^3)	Inoculum(g VS/L)	F/M	Addition of BAC	Monitored variables
GES-I	2	4	0.5	X	V_{CH4}
GES-II	4	8	0.5	X	V_{CH4}
GES-I/II	2	4	0.5	–	V_{CH4}
GES-II/II	4	8	0.5	–	V_{CH4}
SBT-I	2	4	0.5	X	p_{Biogas}
SBT-II	4	8	0.5	X	p_{Biogas}

Biogas samples of 5 mL from the headspace were taken at the end of the experiment (no further pressure increase in 3 consecutive days as stop criterion). The composition of the produced biogas was determined using gas chromatography (Agilent Technologies, Type 6890 N) equipped with flame ionization detector and thermal conductivity detector. An overview of the SBT test runs and the experimental conditions is given in Table 1.

The biogas production was determined by means of the ideal gas law. The methane concentration measured at the end of the experiments was considered constant as recommended by Angelidaki and Sanders [37]. Based on this recommendation methane production was calculated. The experiments were conducted in duplicate, and the outcomes were averaged and corrected by the atmospheric pressure as well as the residual methane production (blank experiments). Methane production was normalized to standard conditions (STP: 273.15 K; 1013.25 hPa).

2.2.3. Determination of specific methanogenic activity and inhibition

SMA can be determined directly as the ratio of the produced methane volume difference (ΔV) and the chosen time interval (Δt). Acetate is immediately converted to methane by methanogens, resulting in a linear increase in methane production in the selected time interval. The SMA was calculated by application of following equation:

$$SMA = \frac{V(t_2) - V(t_1)}{(t_2 - t_1) \bullet m_{VS}} \quad (1)$$

where

SMA Specific methanogenic activity (NmL CH_4/g VS/d or g COD/g VS/d; with theoretical methane yield of 350 NmL CH_4/g COD)
$V(t_2)$, $V(t_1)$ Cumulated CH_4 volume at t_1 and t_2, subtracting endogenous activity (NmL CH_4)
t_2, t_1 time 1; time 2 (h; d)
m_{VS} Mass of VS in the batch (g VS)

For comparison of the SMAs between different batch assays, the time of 10–15 h was selected. At this time, all assays linearly produced methane. The inhibition of SMA (I) is defined as the decrease in SMA with respect to a certain BAC concentration (SMA_{Ci}) in comparison to the SMA examined in control vessels (SMA_{max}), in correspondence with **Equation (2)**:

$$I = \frac{SMA_{max} - SMA_{Ci}}{SMA_{max}} \bullet 100 \quad (2)$$

2.3. Kinetic studies

A model based on ADM1 [33] was implemented in Aquasim 2.0 [38], with the objective of simulating both the volumetric and manometric methods. The model was used for the estimation of two unknown parameters: the fraction of acetate degraders present in the sludge (f_a) and the BAC inhibition constant (K_I).

ADM1 was adapted to the specific conditions of the experimental set-ups using the following assumptions:

- Since acetate is the single substrate present in the system, the only relevant transformation process is the uptake of acetate by the acetate degraders and the corresponding biomass decay process.
- Only a fraction of the biomass (measured as VS) corresponds to the acetate degraders. The fraction f_a quantifies the acetate degraders' biomass in the model and was fitted for all runs.
- The inhibitory effect of the BAC is considered to be a non-competitive one and it is described by a biomass specific inhibition function as proposed in Palatsi et al. [34]; This approach accounts in a simplified manner for the adsorption of the inhibitor on the granule's surface for a given granule size as present in the inoculum.

Table 2
Model application: Kinetic, liquid-gas transfer and stoichiometric parameters.

Kinetic parameters		Units	Value	Reference
K_S	Half saturation value for acetate	g COD/L	0.15	[33]
$k_{m,Ac}$	Specific maximum uptake rate for acetate	(g_{COD} ac/L)(kg_{COD} X/L))/h	0.16	This study
$k_{dec,XAc}$	Decay rate	1/h	0.00083	[33]
K_i	Biomass specific inhibition constant	mg BAC/gCOD	8.0	This study
		mg BAC/gVS	5.3	
Liquid-gas transfer parameters				
k_La	Liquid-gas transfer coefficient	1/h	0.27	[33]
k_{H_CH4}	Henry's law constant for methane	mol/m^3Pa	1.4×10^{-5}	[60]
Stoichiometric parameters				
f_A	Fraction of acetate degraders	–	0.25	This study
Y_{Ac}	Yield of biomass on acetate	g COD X/g COD ac	0.05	[33]

- The system can be represented using a liquid compartment where the transformation processes take place and an inert gas compartment that receives the produced methane. The liquid-gas transfer parameters are based on Henry's law.

Table 2 lists the values used for all the model parameters. The resulting model is presented in the stoichiometric matrix in Table 3.

2.4. Parameter estimation

The parameter estimation tool offered in Aquasim 2.0 was used to estimate the values of the parameters of interest: f_A, $k_{m,Ac}$ and K_I. The experimental dataset from the experiments GES-I and GES-II were used for the fitting. The value for f_A and $k_{m,Ac}$ were estimated using the assays without inhibitor. The obtained value for f_A and $k_{m,Ac}$ were then fixed for the estimation of the inhibitory constant (K_I). The estimated parameters were selected taking into account Xi^2 calculated directly in Aquasim. The lower the Xi^2 value the better the fit between experimental and modelling results.

In addition, the goodness of fit between experimental and simulated data was quantified using the Theil's Inequality Coefficient (TIC) and the Root Mean Square Error (RMSE). A TIC value < 0.3 ensures a good agreement between experimental and simulated data. The lower RMSE value the better model performance.

3. Results and discussion

3.1. Effects of BAC on methane yield and methanogenic activity

The effects of BAC on anaerobic granular sludge were studied in terms of Biochemical Methane Potential (BMP) and SMA. Table 4 sums up the results with respect to BMP obtained in the GES-I and GES-II as well as SBT-I and SBT-II runs. Despite increasing inhibitor concentration, acetate was entirely consumed in all batch activity tests and methane production reached a plateau level close to the theoretical value. The results presented in Table 4 show the range of BMP values obtained from the GES-I and GES-II approaches to be between 157–163 NmL CH_4/g VS and 161–170 NmL CH_4/g VS, respectively. The observed deviations from the theoretically attainable BMP value of 3–10 % were calculated based on the acetate quantities of 1.7 g and 3.4 g, corresponding to 0.8 g COD and 1.6 g COD ($BMP_{theoretical} = 176$ NmL CH_4/g VS). With a deviation from the theoretical value of 2–5 %, the SBT-I and SBT-II approaches yielded BMP values within the ranges of 165–174 NmL CH_4/g VS and 168–171 NmL CH_4/g VS.

As seen in Fig. 2, with increasing BAC concentration, the level of inhibition increased. This was reflected in the deceleration of the methane production and in the increasing time required for full acetate utilization. In the GES-I runs, the acetate in the control assay was almost completely consumed after a period of 30 h. A similar conversion rate was observed in the batches with 5, 10 and 15 mg BAC/L. From 20 mg BAC/L onwards, only 85 % of the substrate was used after 30 h. The batches with a BAC concentration of 30 and 40 mg/L showed conversion rates of 66 and 45 %, respectively, after a period of 30 h. These results suggested that with increasing inhibitor concentration, inhibition effects became more pronounced. In the GES-II assays, both the amount of biomass and the substrate were doubled, resulting in an identical F/M of 0.5 as in GES-I, nevertheless, the inhibiting effects were not as marked as in GES-I. After a period of 25 h, the substrate in the control assay as well as in the assays with BAC concentration between 5 and 30 mg/L was almost completely consumed. Only the addition of 40 mg/L of BAC led to a lower conversion rate at the specific time point (25 h), namely 80 %. The lower inhibitory effect of BAC in the GES-II assay can be explained by the reduction in the biomass-specific inhibitor concentration. In this assay more biomass and thus a bigger surface was available for the BAC to adsorb on. BAC only affected the granules, onto which it adsorbs, therefore at same BAC concentrations a reduced inhibition is measured when more biomass is present in the system.

Tezel et al. [24] investigated the inhibition of methanogenesis by QAC by observing a significant reduction in methane production at concentrations of 25 and 37.5 mg QAC/L. It was noteworthy that at the highest concentration of 40 mg/L tested in this study, complete COD conversion was maintained despite a slower conversion rate. No complete collapse of the anaerobic process was observed at the concentrations investigated, which also reflected the results reported by Tezel et al. [24]. Under the scenario of single exposure in anaerobic inhibition batch tests, the results indicate that BAC had a decelerating effect on the acetoclastic methanogenesis. Nonetheless, these findings imply that the transferability to continuously operated reactor systems and batch systems with prolonged exposure to BAC is limited.

The SMA in both control batches (GES-I and GES-II) was 0.90 gCOD/(g VS·d). For the GES-I batches containing 1.6 g VS, the addition of 5, 10 and 15 mg BAC/L resulted in activity rates of 0.80, 0.63 and 0.51 gCOD/(g VS·d), which corresponds to an inhibition

Table 3
Petersen matrix for soluble and particulate components.

j	Component → i / Process ↓	1 S_{Ac}	2 S_{CH4}	3 X_{Ac}	4 X_C	Rate
1	Uptake of acetate (non-competitive Inhibition)	−1	1 - Y_{Ac}	Y_{Ac}		$k_{m,Ac}\left(\frac{S_{Ac}}{K_S + S_{Ac}\left(1+\frac{S_I}{K_I X_{Ac}}\right)}\right)X_{Ac}$
2	Decay of X_{Ac}			−1	1	$k_{dec,XAc}X_{Ac}$

Table 4
Biochemical methane potential (BMP) as a function of induced Benzalkonium Chloride (BAC) concentrations (0–40 mg/L) of the runs GES-I + GES-II and SBT-I + SBT-II.

BAC (mg/L)	#runs	BMP (NmL CH_4/g VS)	#runs	BMP (NmL CH_4/g VS)
0	GES-I	159.2	SBT-I	164.8
5		159.1		165.1
10		157.0		166.9
15		158.1		166.3
20		158.4		173.7
30		159.4		170.8
40		163.3		163.7
0	GES-II	161.7	SBT-II	167.9
5		164.9		170.1
10		161.1		169.6
15		162.1		170.7
20		167.7		170.6
30		164.3		169.4
40		169.9		170.9

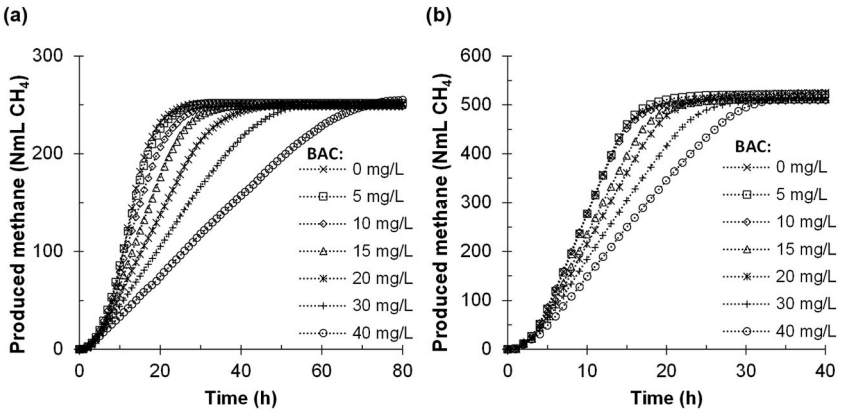

Fig. 2. Cumulative methane production of test runs GES-I (a) and GES-II (b) under exposure of Benzalkonium Chloride (BAC).

of acetoclastic methanogenesis of 10, 30 and 43 %, respectively. In the case of higher BAC concentrations of 20–30 mg/L, higher inhibition levels of 58 % and 68 % were observed. The highest tested inhibitor concentration of 40 mg BAC/L ultimately led to a drastic inhibition of 77 %. In the GES-II runs, the inhibiting effect of BAC appeared to be lower. None or only slight inhibitory effects were found at BAC concentrations of 5 and 10 mg/L, whereas a reduction in SMA of only 19–27 % was observed at 15 and 20 mg/L of BAC. Doubling the inhibitor concentration to 40 mg BAC/L amounted to an inhibition of 50 %, which corresponds to a SMA of 0.46 gCOD/(g VS·d) (Fig. 2).

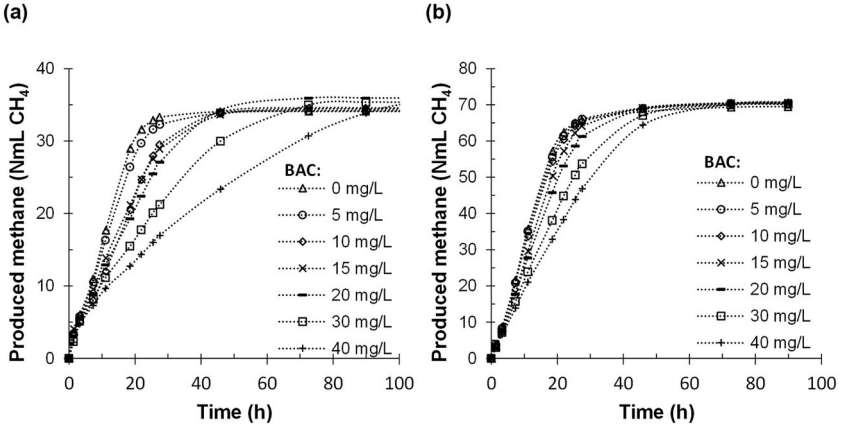

Fig. 3. Cumulative methane production of test runs SBT-I (a) and SBT-II (b) under exposure of Benzalkonium Chloride (BAC).

Results of the test runs SBT-I and SBT-II are shown in Fig. 3. A SMA of 0.52–0.53 g COD/(g VS·d) was calculated for the control batch assays. Similar to the GES-I and GES-II approaches, a detrimental effect of BAC addition on SMA was detectable. For the SBT-I test runs (0.2 g VS) 5, 10 and 15 mg BAC/L already resulted in an inhibition of 11, 27 and 35 %, whereas SMA is halved at 20 mg BAC/L. The highest investigated concentration of 40 mg BAC/L had a drastic effect on the SMA reducing it to 0.16 gCOD/(g VS·d) and causing an inhibition of 71 %. In the case of SBT-II tests (0.4 g VS) minor inhibition phenomena was observed for BAC concentrations between 5 and 15 mg/L. At inhibitor concentrations of 20 mg/L and 30 mg/L, SMA was reduced to 0.41 g COD/(g VS·d) and 0.33 g COD/(g VS·d), respectively, which translated into inhibition levels of 22 % and 38 %. An inhibition of 50 % was attained first at a concentration of 40 mg/L. Although the experimental setups and hydrodynamic conditions differed between the SBT and GES approaches, the inhibitions observed with increasing BAC concentrations were comparable between the two methods.

Based on the results presented in Figs. 2 and 3, the determination of IC_{10}, IC_{30} and IC_{50} values for SBT-I, SBT-II, GES-I and GES-II was feasible. Specifically, the IC_{10} and IC_{30} values for SBT-I and GES-I corresponded to 5 mg BAC/L and 10 mg BAC/L, respectively. Moreover, the IC_{50} value for SBT-I ranged from 20 mg BAC/L and 30 mg BAC/L, resulting in inhibition levels of 43 % and 60 %. Similarly, IC_{50} for GES-I ranged from 15 mg BAC/L to 20 mg BAC/L, corresponding to inhibitions of 43 % and 58 %. The SBT-II and GES-II batch tests, which involved a doubling of the biomass examined, exhibited IC_{10}, IC_{30} and IC_{50} values that were approximately two-fold higher than those of SBT-I and GES-I. Regarding SBT-II, 10 % and 30 % inhibition occurred at 10 mg BAC/L and 20–30 mg BAC/L respectively, whereas IC_{10} and IC_{30} for GES-II were between 10 mg BAC/L and 15–20 mg BAC/L. Both methods resulted in the determination of an IC_{50} of 40 mg BAC/L. With increasing inhibitor concentration, no adverse effects on BMP could be deduced. However, higher inhibition of SMA was identified. Thus, BAC exposure elicited a performance degrading effect that initially decreased the acetoclastic methane conversion rate. Based on these results, non-competitive inhibition could be assumed.

Flores et al. [32] conducted batch inhibition assays with anaerobic granules, revealing values for IC_{10} and IC_{50} of 1.3 mg BAC/L and 13 mg BAC/L, respectively. In contrast, the IC_{10} and IC_{50} values of 5 mg BAC/L as well as between 20 and 30 mg BAC/L determined in this study are significantly higher than those from Flores et al. [32]. Consequently, specifying a biomass-specific inhibitor concentration is of practical relevance to strengthen the importance, transferability, and comparability of IC_{50} values.

Our results show a strong dependency of the IC_{50} value on the amount of biomass used in the assays. Therefore, inhibition tests need to be analyzed taking the biomass amount into account. For example, the GES-I run had a biomass amount of 1.6 g VS and an IC_{50} value of approximately 20 mg BAC/L, whereas for the GES-II, with 3.2 g VS, 20 mg BAC/L only caused an inhibition of SMA around 27 %. An increase in the biomass quantity clearly limited the inhibitory effect of BAC. A higher amount of biomass resulted in an increase of the available adsorption area for the inhibitor and therefore the system was able to tolerate higher inhibitor concentrations before a considerable reduction in the SMA was observed. Due to this effect, biomass-specific values of the critical inhibition concentrations e.g. IC_{50} are clearly better indicators when dealing with surface-active inhibitors that are capable of binding on the granule surface such as BAC. The ratio of BAC to biomass then plays a pivotal role in the performance evaluation of systems exposed to BAC.

Fig. 4 shows the link between the SMA (a) and the resulting inhibition (b) as function of a biomass-specific inhibitor load, for both SBT-I + II and GES-I + II. During SBT-I and SBT-II runs, SMA inhibition level of 30 % was observed with a biomass specific BAC load between 3.0 and 3.1 mg BAC/g VS. Regardless of the amount of biomass used, a reduction of 50 % of the SMA from 0.53 to 0.29 gCOD/(g VS·d) was achieved when a biomass specific BAC load between 5.0 and 6.1 mg BAC/g VS was applied. These results could also be verified with the volumetric methane measurement in GES-I and GES-II runs, which provides method-independent validation of the results. In the case of GES-I and GES-II biomass-specific loads between 2.5 and 2.9 mg BAC/g VS led to an inhibition of 30 %, whereas biomass-specific loads between 3.5 and 4.0 mg BAC/g VS resulted in an inhibition rate of 40 %. Similarly, as is the case of the SBT experiments, increasing the biomass specific BAC load to 4.3–5.0 mg BAC/g VS resulted in an inhibition level of 50 %. Despite the deviating hydrodynamic experimental conditions resulting from the different experimental setups, the biomass-specific BAC load at which 50 % inhibition occurred was comparable for both methods. The impact of hydrodynamics on substrate transport was more pronounced than the transport of the inhibitor to the biomass, as it was evident from the difference in the SMA of the references of both methods. Convective transport mechanisms were of little significance with respect to the inhibitor transport to

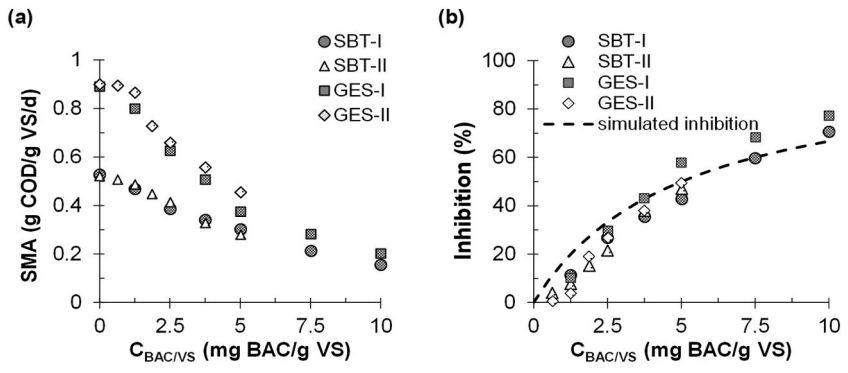

Fig. 4. (A) SMA (Specific Methanogenic Activity) and (b) Inhibition of test runs SBT-I and SBT-II as well as GES-I and GES-II; with corresponding biomass-specific BAC (Benzalkonium Chloride) load per g VS. Dashed line: simulated inhibition.

the biomass, enabling the determination of similar inhibition and biomass-specific BAC loads independently of the applied methods. The transport of the inhibitor to the biomass seemed to be predominantly diffusion-driven.

To date, only one study on the biomass-specific inhibitor load is known in the literature. He et al. [39] investigated the response of anaerobic digestion of excess sludge to BAC stress, by means of batch inhibition assays. An inhibition of 52.6 % was observed at BAC levels of 15 mg/g TSS. In this study, an inhibition of 77 % was observed at the highest investigated biomass-specific inhibitor load of 10 mg BAC/g VS, corresponding to 8.7 mg BAC/g TS. Due to the flocculent structure of excess sludge and its higher specific surface, notable disparity in biomass-specific loads were perceived compared to the values determined for anaerobic granules. This highlights the significant role of not only the biomass concentration, but also the biomass type and characteristics, particularly with respect to their ability to adsorb and accumulate surface-active inhibitors. In practical terms, there is a compelling need to identify relevant IC_{50} values for anaerobic reactors employing granular biomass as well as those utilizing suspended biomass, enhancing the understanding of inhibitor interactions and therefore establishing strategies for preventing operational malfunctions.

Fig. 4 (b) also depicts an initial, nearly linear dependence between biomass-specific inhibitor load and the inhibition level that continued up to the previously determined critical inhibitor load of approximately 5 mg BAC/g VS. From this critical point on the curve flattened. The pattern of the inhibition curve indicated that inhibition was probably characterized by two mechanisms. First, the inhibitor acted adsorptively, adhering to the surface of the biomass and then penetrating the biomass cells and disorganizing active enzyme sites involved in biochemical substrate conversion processes [31,40–43]. A linear relationship between biomass-specific inhibitor load as well as inhibition was evident until the critical load was reached, suggesting that the initial inhibition effects were not only affecting but also were limited by the available specific surface area. The flattening of the curve suggests that adsorptive saturation of the surface must have been reached, which is why a second, diffusive inhibition mechanism emerged with increasing inhibitor load. After adsorptive inhibition mechanism, BAC penetrated through pores and channels of the granules into the interior, where a further activity reducing interaction with acetoclastic methanogens took place.

Fig. 5 depicts the relationship between the biomass-specific inhibitor load and the resulting inhibition levels for the test runs GES-I + II as well as their replicates, denoted GES-I/II and GES-II/II. Here, the ratio between the SMA determined in the first and second runs and the SMA of the respective reference was calculated. The results shown in Fig. 5 clearly indicate the irreversibility of the inhibitory effects induced by BAC.

In the GES-I/II and GES-II/II (2. run) experiments SMA was determined anew immediately after the first exposure to BAC. The replicates were conducted with additional acetate as substrate, but without further inhibitor addition. Nearly the same SMA results were obtained for the first exposure studies as well as for the replicates. In the case of GES-I/II, for the biomass first exposed to a specific BAC load of 2.5 mg BAC/g VS an inhibition of 30 % was measured in the first exposure runs, as was also the case for the replicate experiments. Based on a SMA of both references of 0.89 g COD/(g VS·d), SMA_{BAC}/SMA_0 ratios for GES-I and GES-I/II were 0.70 and

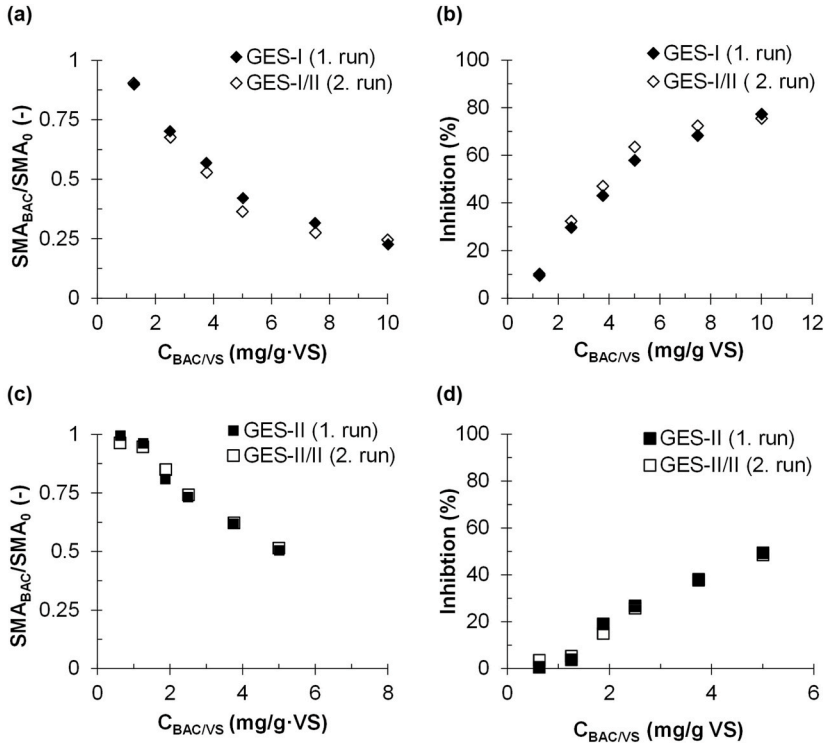

Fig. 5. SMA_{BAC}/SMA_0 and inhibition as function of biomass-specific BAC (Benzalkonium Chloride) load for test runs GES-I (a+b) and GES-II (c + d) with their corresponding replicates GES-I/II (2. run) and GES-II/II (2.run) in absence of BAC.

0.68, respectively (see Fig. S1). Likewise, for the replicate assay of first run which had been originally exposed to a 5 mg BAC/g VS inhibition load, the calculated SMA was equal to 0.31 g COD/(g VS·d), which corresponds nearly to the SMA of the original experiment of 0.37 g COD/(g VS·d) and yielded SMA_{BAC}/SMA_0 ratios between 0.42 and 0.36. Finally, the highest investigated inhibitor load of 10 mg BAC/g VS showed the same SMA of 0.21 g COD/(g VS·d) for both first exposure and replicate assays.

Similarly, in the GES-II/II experiments comparable results were observed. Given to the SMA_0 of 0.9 g COD/(g VS·d) and 0.7 g COD/(g VS·d) in GES-II and GES-II/II, respectively, a BAC load of 2.5 mg BAC/g VS resulted in an inhibition level of 26 % and 27 %, in both first and second replicate runs. Although lower initial SMA was determined in GES-II/II than in the first runs of GES-II, the respective ratios SMA_{BAC}/SMA_0 under inhibitor exposure were almost identical, namely 0.73 and 0.74. In the same manner, the biomass exposed to a BAC load of 5 mg BAC/g VS showed a decrease in the SMA from 0.89 to 0.45 g COD/(g VS·d) in the first run, whereas in the replicate experiment (GES-II/II) the SMA decreased from 0.7 to 0.35 g COD/(g VS·d). In both cases the SMA_{BAC}/SMA_0 ratio equaled 0.51.

It should be noted that these exposures and replicates were conducted as one-time events, which reveals the limitations of batch assays. The results obtained in this study appear to contradict the findings of Tezel et al. [24], who observed a recovery of the methanogenesis following a BAC exposure. The reported recovery may be due to the growth of uninhibited methanogens in a prolonged incubation period of 100 days. In a continuously operated reactor, additional factors such as biomass regrowth will influence the BAC inhibition of methanogens and its potential reversibility. These effects will likely differ from results of the batch-tests conducted in this study.

As already derived from the inhibition function in Fig. 4, the inhibitory effect of BAC on methanogenesis is explainable by two main mechanisms: adsorption of BAC on the granules‘ surfaces and diffusion that comprised further activity reducing interactions [31, 40–43].Given the granular nature of the investigated biomass, adsorption played an important role and was the first step of the inhibition process. BAC has a positive charge and it is therefore affine to negatively charged surfaces, such as anaerobic sludge [44]. A hypothesis was proposed that BAC bound onto the surface of the granules, effectively blocking it. This blockage may have hindered the substrate transport into the interior as well as gas transfer to the outside of the granules and consequently caused a retardation of the methane production rate. The BAC irreversibly bonded to the granules surface and could not be removed by standard operation procedures [45–48].

With increasing BAC load, the second, diffusive inhibition mechanism may have set in. Consequently, BAC penetrated further into the interior of the granules, where predominantly methanogens are located [49]. When interfering directly with the cytoplasmic membrane of archaea and bacteria, BAC leads to the disturbance of several cellular processes [50,51]. In general, the bactericidal effects of cationic agents include the destruction of the lipid bilayer of the bacterial cell membrane, the dissipation of the proton motive force, and the disruption of membrane-bound enzymes [52–55]. It has been reported that toxicity and/or inhibition induced by QACs has strong effect on methanogens (archaea) due to their membrane structure. Methanogens lack an outer membrane [56], which facilitates the access of QACs to the cytoplasmic membrane [24]. Furthermore, QACs also impact the energy generation of methanogens by interfering with the proton motive force, which is responsible for ATP production of methanogens [57]. These effects usually lead to cell death. The results obtained from the replicates not only confirmed the irreversibility of the inhibition induced by BAC, but also demonstrated the participation of effects leading to cell death of methanogens.

3.2. Parameter estimation of acetate degradation kinetics

Using the datasets of the experiments without BAC addition, the parameters f_A (acetate degraders fraction) and $k_{m,Ac}$ (specific maximum uptake rate for acetate) were estimated.

The value estimated for $k_{m,Ac}$ (0.16 (g_{COD} ac/L)(kg_{COD} X/L))/h) was within the range suggested in the literature [33], however it was located rather at the lower end of the range. It is assumed that the $k_{m,Ac}$ reflects to a certain extend the diffusion processes that took place within the granules and are not explicitly included in the model. The lumping of diffusion effects (which limit the reaction rates) in kinetic parameters is well known and is often used to model systems with granular biomass [58].

Table 5 shows the RMSE values, as measure of the model's goodness of fit, for the experiments without BAC addition (0 mg BAC/L). For both GES-I and GES-II experiments, the calculated RMSE was 0.03 g CH_4-COD, which was acceptable taking into account the methods, limitations and the assumptions included in the model. Figs. S2 and S3 in the supplementary information display a graphical comparison of the simulated and experimental results. As Fig. S2 shows for GES-I, the model slightly overestimated the methane production rate throughout the investigated period. In the case of GES-II a better fit could be achieved and only a marginal underestimation appeared around the hour 17 and 24 of the assay.

Table 5
RMSE values for the runs GES-I and GES-II under exposure to BAC = 0–40 mg/L.

Run	BAC (mg/L)	BAC (mg/g VS)	RMSE (g CH_4-COD)	Run	BAC (mg/L)	BAC (mg/g VS)	RMSE (g CH_4-COD)
GES-I	0	0.00	0.03	GES-II	0	0.00	0.03
	5	1.25	0.02		5	0.63	0.06
	10	2.50	0.03		10	1.25	0.09
	15	3.75	0.02		15	1.88	0.07
	20	5.00	0.02		20	2.50	0.08
	30	7.50	0.03		30	3.75	0.07
	40	10.00	0.08		40	5.00	0.07

The biomass-specific non-competitive inhibition model could be reasonably fitted to the experimental results, so an initial mathematical analysis was performed. The expression for non-competitive inhibition reported in Ref. [59] is shown in **Equation (3)**, where S_I and K_I are the respective inhibitor concentration and inhibitory constant:

$$\%\text{Inhibition} = \frac{\frac{S_I}{K_I}}{1 + \frac{S_I}{K_I}} \tag{3}$$

Equation (3) was adapted for the case of a biomass specific inhibition using a biomass-specific inhibitor concentration that is defined as $\frac{S_I}{X_B}$, with X_B as the biomass concentration. **Equation (4)** is derived:

$$\%\text{Inhibition} = \frac{\frac{S_I}{X_B K_I}}{1 + \frac{S_I}{X_B K_I}} \tag{4}$$

According to **Equation (4)** a plot of the % of inhibition as a function of the inhibitor biomass specific load (mg BAC/g VS) should result in a Monod-like curve, in which the IC_{50} value could be directly read, analogous to the determination of a half-saturation constant for a traditional Monod expression. Fig. 4 (b) shows the plot for the estimation of the biomass specific inhibition constant (K_I). This figure shows that experimentally determined IC_{50} took values between 4.3 and 6.1 mg BAC/g VS.

The developed model was applied to independently estimate the value of K_I that describes best the experimental results of the GES-I and GES- II set of assays. The value that minimized the average RMSE of all the 12 experiments (with BAC addition) was found to be 8.0 mg BAC/mg COD_x, which equals a specific inhibitor load of 5.3 mg BAC/g VS. In the case of GES-I, the model delivered good results for almost all the tested BAC concentrations (1.5–7.5 mg BAC/g VS). In this range of inhibitor concentrations, the model prediction presented minor deviations from the measured methane production, however at a concentration of 10 mg BAC/g VS the model differed significantly from the experimental results. This was confirmed by the RMSE values in Table 5, where the experiment with an inhibition load of 10 mg BAC/g VS had a RMSE twice as high as the other experiments. A significant deviation observed at higher inhibitor load potentially indicated a limitation of the model approach applied. As previously explained, two mechanisms of inhibition may be assumed, based on the findings from Fig. 4. With increased inhibitor load, diffusive inhibition mechanisms became more prominent. However, the inhibition-adsorption model used in this study had certain limitations and was not able to completely encompass the second, diffusive mechanism. These results emphasize the importance of developing more extended models that can accurately capture the complexity of inhibitions mechanisms. For the GES-II runs, the model underestimated the methane production rate for all the investigated BAC concentrations. The RMSEs ranged between 0.06 and 0.09 g CH_4-COD, and no evident difference between the experiments could be noted. In Table S4 of the supplementary information the values of the TIC for all the simulations are provided, as support of the results presented in Table 5.

The model results can be transferred to the plot % inhibition vs. specific BAC load. In Fig. 4 (b) the dashed line represents the % of inhibition predicted by the model using the estimated value of K_I (5.3 mg BAC/g VS). The model slightly overestimated the inhibition caused by specific BAC loads lower than K_I. In contrast, it underestimated the inhibition that results from specific BAC loads higher than K_I. The increased actual inhibition levels at inhibitor loads bigger than approx. 7 mg BAC/g VS most probably indicated that BAC was able to directly interact with the acetoclastic methanogens further constraining the methane production. This effect led to deviations from the model predictions, which did not include such interactions. However, to confirm this hypothesis and thoroughly explain this phenomenon further studies are needed.

4. Conclusions

The results of the present work provided additional evidence for the nature of inhibitory effects of BAC on anaerobic granules. BAC exposures significantly affected the SMA, indicating the presence of a non-competitive inhibition. Moreover, the practical importance of specifying the inhibitory concentration as a biomass-specific inhibitor load was demonstrated. Biomass-specific BAC loads between 4.3 and 6.1 mg BAC/g VS led to a 50 % inhibition of SMA (IC_{50}). An ADM1 based model was employed to effectively describe the experimental results. The inclusion of a biomass-specific inhibition term aligned with the experimental data and served as a simplified approach to represent the adsorption of a surface-active inhibitor onto the surface of anaerobic granules. Model fit for all experiments resulted in a biomass-specific IC_{50} of 5.3 BAC mg/g VS. Due to one-time exposures in the batch assays, the generalization of the results as well as the transferability to continuously operated reactors is subject to certain limitations. Still, results for biomass-specific IC_{50} are much more convincing than using inhibitor bulk mass concentrations. Further research should focus on examining long-term effects on anaerobic granules and verify the identified inhibitory biomass-specific load for continuous systems.

CRediT authorship contribution statement

Sinem Fundneider-Kale: Conceptualization, Methodology, Validation, Formal analysis, Resources, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. Vanessa Acevedo Alonso: Methodology, Validation, Software, Formal analysis, Resources, Writing – review & editing. Markus Engelhart: Writing – review & editing, Supervision, Funding acquisition.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Prof. Dr.-Ing. Markus Engelhart reports financial support was provided by Willy-Hager-Stiftung, Stuttgart. Prof. Dr.-Ing. Markus Engelhart reports a relationship with Willy-Hager-Stiftung, Stuttgart that includes: funding grants.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wri.2023.100237.

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