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CYCLODEXTRIN-BASED TRAPS TO CONTROL COOPERATIVE ACTION OF BACTERIA

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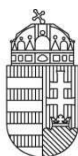

NATIONAL RESEARCH, DEVELOPMENT AND INNOVATION OFFICE
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31 October 2021

Introduction

Bacterial Quorum Sensing (QS) is a cell-to-cell communication process, in which, bacteria, performing cooperative behavior, produce and detect extracellular signaling chemicals, to monitor cell population density. Numerous bacterial processes including bioluminescence, virulence factor production, biofilm formation etc. are known to be influenced by this bacterial communication network. Interest in QS systems has emerged in response to the fact that these processes have significant impact on the environment, human health as well as agriculture. In this project as a novel approach for the attenuation of quorum sensing which involves selective sequestering of the signal molecules using various (native and designed) cyclodextrins was explored. Cyclodextrins-mediated quorum quenching (QQ) is an innovative concept and the available information about their effects is very scarce.

Our aim was to develop **cyclodextrin (CD)-based traps** for capturing the signaling molecules (AHLs) concentrating on the Gram-negative bacteria and thus hindering their cooperative action, e.g. biofilm formation, bioluminescence, and virulence. A library of CD derivatives: monomers and polymers with positive or negative charge, substituted with shorter or longer alkyl chains, labeled or not with fluorescent moieties were designed, synthesized and tested to select the most suitable ones. New long-chain sulfoalkyl CD derivatives were designed based on a Japanese publication on QQ activity of alkylamine CD (C7-C12) derivatives on various bacteria[2].

We planned to develop simple tests for quantitative characterization of QS using the bioluminescence of *Aliivibrio fischeri* and further tests with other bacteria and used various CDs including the well-known monomer and polymer derivatives and also the newly synthesized ones to clarify the structural criteria of the quorum quenching (QQ) effect of CDs.

In order to understand the mechanism how the CD-based traps work and to find the best fitting version concerning the cavity size, type of substituent, charge, etc. we planned to perform interaction studies between some AHLs with a series of CDs using various techniques.

1 SYNTHESIS AND CHARACTERIZATION OF CD DERIVATIVES

1.1 Synthesis of AHLs

We planned to synthesize the homoserine lactone (HSL) series but after some preliminary experiments we stopped this work and purchased the signal molecules for the bacteria used in our experiments (*Aliivibrio fischeri, Chromobacterium violaceum, Pseudomonas aeruginosa, Serratia marcescens*). The synthetic group concentrated on the preparation of the CD derivatives designed for QQ.

1.2 Synthesis of various CD derivatives

Several CD scaffolds were prepared in suitable amount for evaluating the CD complexing ability towards the signaling molecules and the inhibition activity of the macrocycles. In particular, a series of 6-monoalkylthio CDs were synthesized by reacting 6-monotosyl CD with alkylthiol. The βCD line was prepared in WP1 by using 6-monotosyl-βCD as starting material in one step reaction. The prepared 6-monosubstitued CD derivatives were further reacted with glycidyltrimethyl ammonium chloride in order to obtain permanently positive charged multifunctional scaffolds and to enhance the aqueous solubility. (*Fig. 1.1*). It has been already reported that CDs bearing positive charges can exhibit antibacterial activity via interacting with the negatively charged cell wall of bacteria so it was expected that the presence of quaternary amino groups will improve the antibacterial effect. The simultaneous modification of the CD scaffold with both positive charges and long alkyl chain was expected to result in a synergistic Quorum Quenching (QQ) effect.

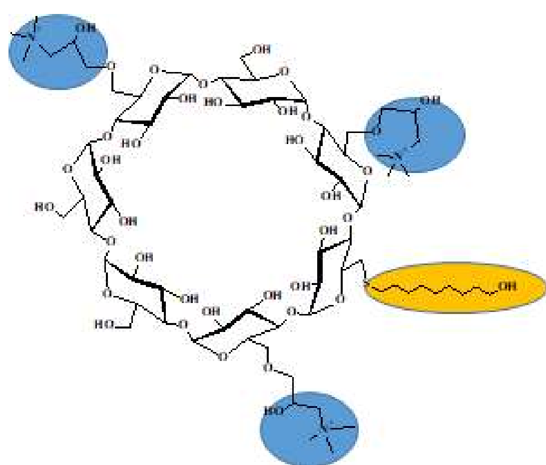


Fig. 1.1 *Structural formula of QA-(6mono-decanthiol)-BCD*

In the series of 6-monoalkylthio-beta-CD derivatives the following products were synthesized: 6-O-monotosyl-beta-CD was reacted with 1-decanthiol in dimethyl formamide using sodium methoxide under inert atmosphere to get **6-mono-decanethiol-beta-CD**. Similar reactions were performed to get **6-mono-O-dodecanethiol-** and **6-mono-hexadecanethiol-beta-CD**. The products were purified by chromatography and reacted with glycidyltrimethyl ammonium chloride to incorporate positive charge (quaternary amino group = QA) into the molecule to get **QA-6S-C10-BCD, QA-6S-C12BCD and QA-6S-C16-BCD**.

The preparation of the alpha-/gamma-CD series followed different reaction route:

Native CDs were reacted with N-bromosuccinimide and triphenylphosphine to obtain 6-monobrominated CDs. The strict control of the temperature allows the introduction of one single unit of halogen on the primary rim of the CDs. The 6-alkylthio CD derivatives were obtained by reacting 6-monobromo-CDs with the proper alkanethiol with Na methoxide in DMSO. By using an excess of thiol, the conversion was quantitative and the reaction crudes could be purified by methanol crystallization. This way, 6-monohexylthio, 6-monododecanethio and 6-monohexadecanethio ACD and GCD (10 g of each) were prepared. **Permanent positive charges** (quaternary amino, QA groups) were randomly installed on the 6-monothioether CD derivatives in alkaline conditions with glycidyltrimethyl ammonium chloride. The insertion of positive charges on the CD scaffolds increased remarkably the aqueous solubility of the small library of CD derivatives thus allowing an exhaustive purification by dialysis. QA-(6-monohexylthio), QA-(6-monododecanethio) and QA-(6-monohexadecanethio) ACD and GCD (**QA-6S-C6-ACD, QA-6S-C6-GCD, QA-6S-C12-ACD, QA-6S-C12-GCD, QA-6S-C16-ACD, QA-6S-C16-GCD,** 5 g of each) were obtained and their structure elucidated by NMR and MS in WP2. For comparison **QABCD and its polymer** were also prepared.

For preparing **6-oligosubstituted** CDs and **fluorescently labeled** 6-monoalkyl substituted derivatives (in WP3), we have developed direct, short and efficient primary-side difunctionalization strategies. Azidation led to the corresponding pure diazido regioisomers. Direct mono-tosylation of 6-monoazido-BCD or mono-azidation of the single regioisomers 6A,6X-ditosyl-BCDs afforded hetero-difunctionalized 6A-monoazido-6X-tosyl-BCDs in significant yields. Overall, the single regioisomers, 6A,6X-ditosyl-, 6A,6X-diazido- and 6A-monoazido-6X-monotosyl-BCD were prepared in one or two steps and purified in multigram scale. This work has been published in *Beilstein J. Org. Chem*[1]. These difunctionalization strategies were planned for the preparation of fluorescently labeled 6-monoalkyl substituted derivatives (*Fig. 1.2*).

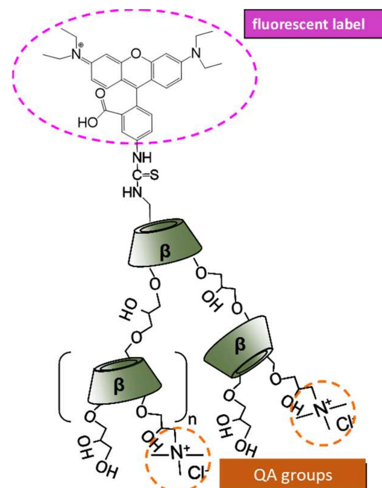


Fig. 1.2 *Scheme of fluorescently labelled CD polymer*

However, as the long chain 6-monoalkylthio derivatives had disappointing QQ activity, these syntheses were cancelled from the research plan. Also the fluorescent labeling of 6-mono-S-alkyl substituted CD was cancelled due to the same reason. The effective derivatives (BCD and RAMEB) were fluorescently labelled to be used in the biological tests. Also, fluoresceinyl BCD polymer was prepared in WP3.The same synthetic strategies developed for functionalization of the CD monomer were adapted to CD-based epichlorohydrin cross-linked polymer of low molecular weight. These highly water-soluble architectures were partially halogenated and/or azidated in order to prepare the versatile intermediates necessary for further functionalization. Then the partially halogenated polymer was used as substrate for the fluorescent labeling. In some cases, also QA groups were built in. The molecular weight of these polymers was characterized by static light scattering measurements.

1.3 Analysis

MALDI-TOF mass spectra were recorded on a Bruker Microflex LRF system (*Fig. 1.3*).

The microflex LRF operated in positive ion mode using the linear detector. Ion generation was achieved using a 60 Hz N2-Cartridge-Laser including variable power attenuator and UV optics. The laser operated at 337 nm and 2,5-dihydroxybenzoic acid (DHB) was used as matrix. For sample preparation 2,5-dihydroxybenzoic acid was used as matrix.

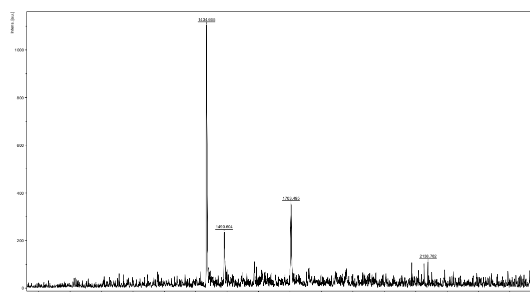


Fig. 1.3 *MALDI TOF chromatogram of* ***QA-6S-C12-BCD***

Their aggregation behavior (nanoparticle formation) was studied by photon correlation spectroscopy (dynamic light scattering) using Malvern Zetasizer Nano ZS (Malvern Instruments, UK) equipment using 1% aqueous solution. The size distribution curves according to the volume of particles are illustrated in *Fig. 1.4* for an ACD and 2 BCD derivatives. The size of the monomer CDs is approx. 1.5 nm. In the solutions of the QA-alkylthio-CD derivatives no monomers can be identified. The new products show aggregation depending on the size of the alkyl chain. The QA-6S-C10-ACD formed small-sized aggregates of 8-10 nm (average 8.2 nm). The QA-6S-C10-BCD showed bimodal distribution of aggregates: in addition to the small particles of 8–10 nm average diameter also larger particles of approx. 50–500 nm size (average 163 nm) can be found. The QA-6S-C16-BCD sample exhibited even higher aggregation: only large-sized aggregates of 50-500 nm (average 111 nm) can be found. The GCD series showed similar behavior: increasing affinity to aggregate with increasing alkyl chain length.

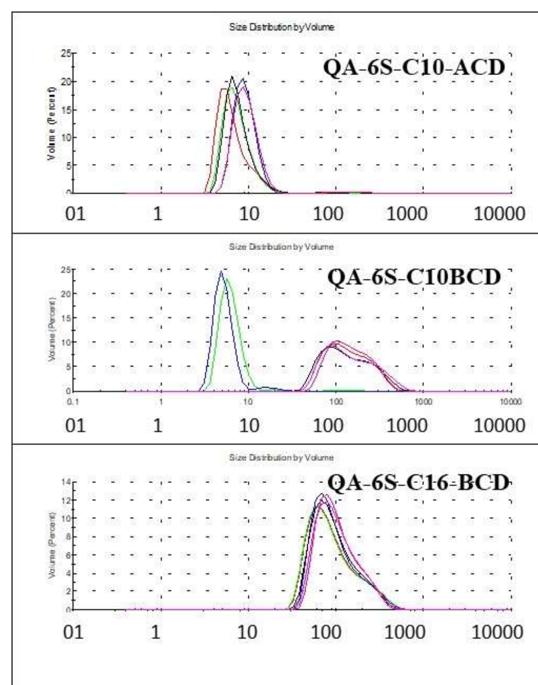


Fig. 1.4 *Size distribution of aggregates in 1% solutions of the new BCD derivatives (5 parallel measurements)*

The C16 derivatives of ACD and GCD aggregated so much that no clear solutions could be obtained in water. The scheme of aggregates is shown in *Fig. 1.5*. The other derivatives showed similar size distribution curves. It should be noted, however, that the derivatives with longer alkyl chain often gave hazy solutions due to too high aggregation.

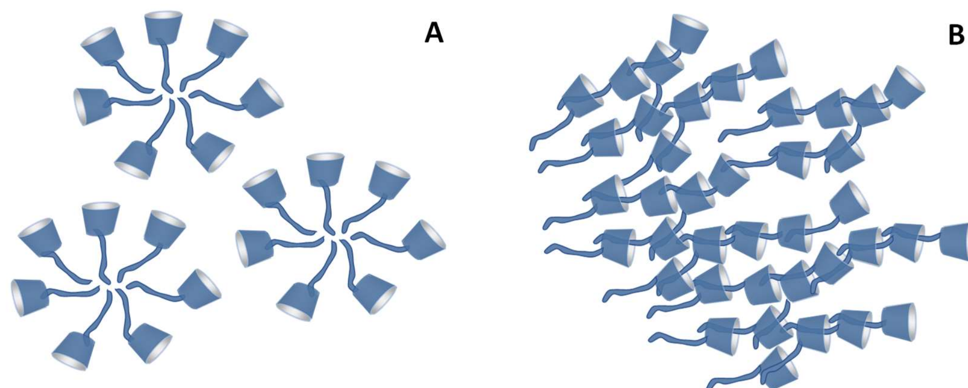


***Fig**. 1.5 Self-assembly of monosulfoalkyl derivatives into small micelle like aggregates (A) and via supramolecular interactions into larger sized aggregates (B)*

1.4 Interaction studies

Their interaction with the AHL series was planned be studied by capillary electrophoresis or competitive binding experiments. As AHLs have no ionizable groups their capillary electrophoretic study was conceivable only with ionic CD derivatives. These measurements however did not result in peak shapes suitable for evaluation, so we tried the competitive complex formation. This method is based on complexing phenolphthalein by the CD then adding the competitive guest, which dispels phenolphthalein from the CD cavity and thus the color (light absorption) is changed which can be measured by photometry. The competitive complexation method suggested in the paper of Morihoshi et al.[2] for AHL/CD systems, however, did not work, as it requires two competitive guests interacting with CDs only but not with each other. We, however, found that this requirement is not met: the phenolphthalein solution faded upon adding AHL, too. As the absorbance of phenolphthalein solution decreased in the presence of AHLs even in the absence of CDs this method could not be used either.

At the end we used HPLC method. The complex association constants (K) of caproyl and octanoyl homoserine lactone (3-oxo-C6-HSL and C8-HSL, resp.) with the three different native cyclodextrins (ACD, BCD, GCD) were determined by HPLC with a previously described method[3]. Waters Symmetry C18 (5µm 4.6x250mm) column was used in Agilent 1100 LC system with UV detection. The mobile phase was acetonitrile:purified water with dissolved CD (10 mM BCD, 15 mM ACD or GCD); 0,8 mL/min flow. Stock solution from the AHLs was ~ 1 mg/mL (dissolved in pure ACN). The samples were diluted to ~ 0,1 mg/mL with 50% ACN. Injection volume was 10 µl.

Changes in retention times as a function of cyclodextrin concentration have been quantitatively treated to obtain the K values. Adding CD to the eluent in increasing concentration the retention time is shifted and from the change of capacity factors (k) the complex association constant can be determined.

As an example, the chromatograms of octanoyl homoserine lactone in the presence of various concentrations of ACD are demonstrated in *Fig. 1.6* and the K values are listed in *Table 1.1*.

***Table 1.1** Complex association constant values (M^{-1}) for AHLs complexes with native CDs*

	ACD	BCD	GCD
3-oxo-C6-HSL	5.9	10.0	<2
C8-HSL	11.8	26.2	<2

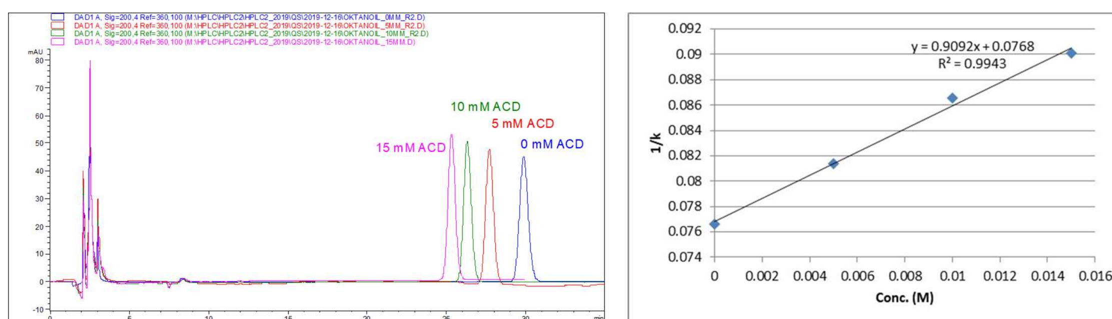


Fig. 1.6 *Representative HPLC chromatograms of C8-HSL with ACD (left) and reciprocal capacity factor vs concentration curve (right) for determination of association constant (in this case 11.8 M-1)*

These two examples suggest that BCD has higher affinity than ACD, while GCD is ineffective similarly to the values obtained by NMR for C4-HSL, typical signaling compound of *Pseudomonas aeruginosa*: 18.9 M-1 and 23.8 M-1 for ACD and BCD, respectively, while GCD could not form inclusion complex with this autoinducer either[4]. These NMR studies revealed that the acyl chain of HSL is included in the CD cavity (*Fig. 1.7*). The acyl chain is the part of HSL which proved to be the most important interacting site for the 3-OXO-C8-HSL signal to the cell membrane in case of *Agrobacterium tumefaciens* mutant cells[5]. Including this acyl chain into the cavity hinders the activity and this is claimed to be the mechanism how CDs inhibit quorum sensing.

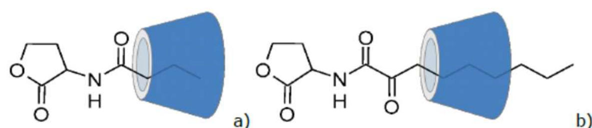


Fig. 1.7 *Scheme of CD complex with C4-HSL (a) and C8-OHSL (b) N-acyl-L-homoserine*

1.5 Method development of AHL analysis in biological samples

HPLC method was be developed for the analysis of AHLs in biological samples. but the sensitivity was not enough for determination of the extremely low concentrations of AHLs therefore an HPLC-ESI-MS method was also developed. Using SIM (selected ion monitoring) mode, only the compounds of predetermined molecular weight can reach the detector making possible the sensitive determination of AHLs at low concentrations.

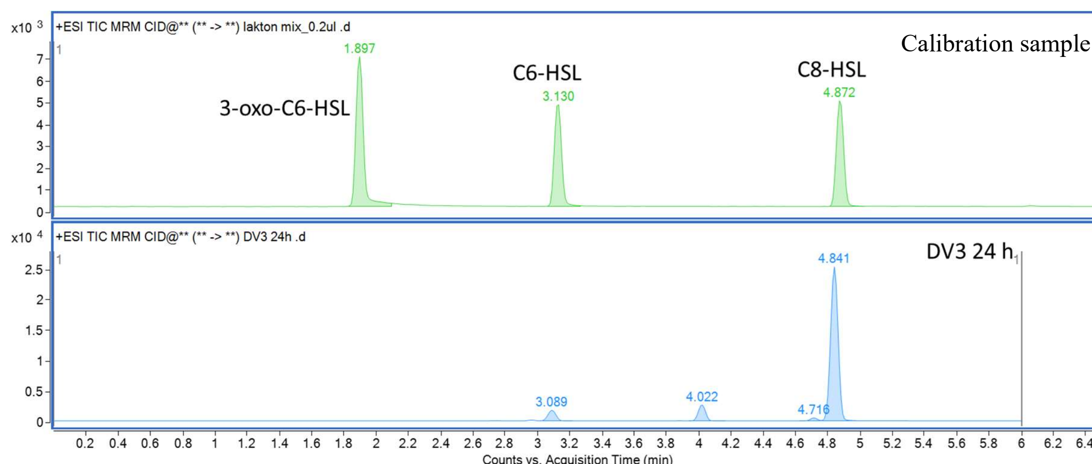


Fig. 1.8 *Total ion chromatogram of a calibration sample (top) and a biological sample from Aliivibrio fischeri culture (bottom)*

For sample preparation the biological samples were centrifuged and 5 mL withdrawn from the bottom layer. Chloroform (5 mL) was added and stirred overnight. The chloroform phase was sampled (1 mL) and this sample was dried then redissolved in acetonitrile (1 mL). These solutions were injected into the HPLC. Calibration samples were prepared from culture medium extracted similarly and adding proper amounts of AHLs to the acetonitrile solutions (1 mL). Illustrative chromatograms are shown in *Fig. 1.8*.

1.6 Complexes of quorum sensing inhibitors

Two quorum sensing inhibitors (triclosan and limonene) were selected for detailed studies concerning their complexation with various CDs and after preparing the complexes for examining how complexation influence their QQ effects.

Triclosan is a cheap, broad spectrum antibiotic with low water solubility (10 mg/L) and low toxicity. Applying at low concentration, it does not kill bacteria but is able to inhibit the communication between bacterial cells. Fidaleo et al. found that complexation with hydroxypropyl and methyl BCD (HPBCD and RAMEB) can solubilize triclosan increasing its quorum sensing inhibitory activity on *C. violaceum* model system.[6]

Phase solubility studies were performed to select the best complex forming partner among 14 CDs. *Fig. 1.9* shows the solubilizing power of various ACD and BCD derivatives on triclosan. The solubility was not enhanced by GCD and its derivatives. Both polymers (ACDPS and BCDPS) were very effective followed by RAME- and SBE- derivatives.

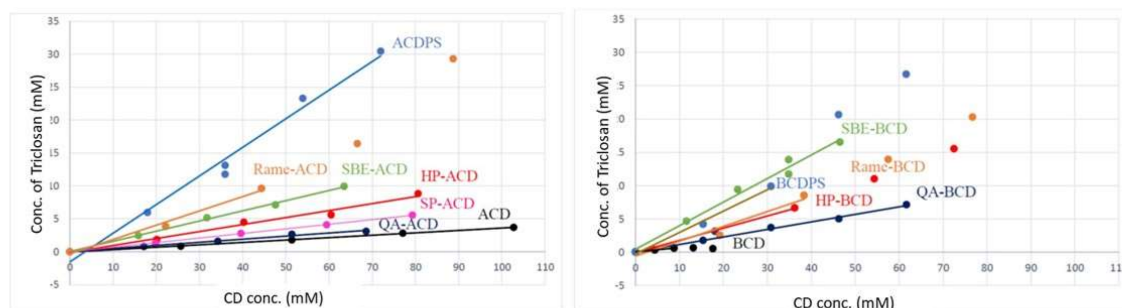


Fig. 1.9 *Solubility isotherms of Triclosan with various ACD and BCD derivatives in water at room temperature*

The K values calculated from the initial linear part of solubility isotherms follow the order of ACDPS > SBE-BCD > BCDPS >RAMEB > RAMEA > SBE-ACD, etc.

Four complexes were prepared using the 2 polymers and 2 RAME-CD derivatives and kneading technique to obtain about 10% active ingredient content. The release of triclosan from the complexes was fast with $t_{1/2}$ of about 5 min for the polymers, and about 30 min for the RAME-CD derivatives[7].

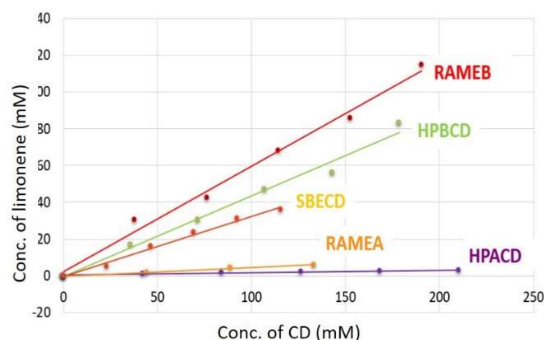
After the experiments with triclosan in WP1, **limonene** was selected as another QS inhibiting agent to be complexed with CDs and studied in microbiological models. Limonene is a water-insoluble terpene of high volatility abundant in essential oils, such as orange, lemon and mint oil. These oils inhibit quorum sensing and also limonene has antimicrobial and quorum quenching effect[8].

The 3 native CDs and their typical random substituted derivatives were investigated in phase solubility measurements (*Fig. 1.10*). The most proper cavity size for complexation of limonene proved to be that of BCD. The highest solubilizing effect was obtained for its random methylated, hydroxypropyl and sulfobutyl ether derivatives (RAMEB > HPBCD > SBEBCD).

The complex association constants follow the order of slopes of the solubility isotherms. The BCD derivatives are superior compared to ACD derivatives, while GCDs are ineffective.

Limonene is highly volatile compound. *Fig. 1.11* illustrates how the volatility is reduced in the presence of various CDs due to complexation as determined by gas chromatography. The BCD and its derivatives are superior to ACD and GCD in this respect, too.

Complexes of 5–10% limonene content were prepared and used in in vitro experiments[9].



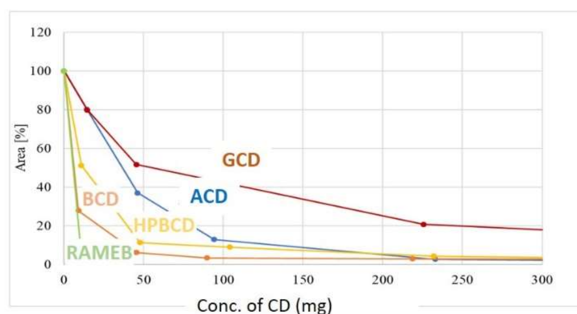


Fig. 1.10 *Solubility isotherms of limonene in aqueous solutions of various CDs (only the most effective CD derivatives are shown)*

Fig. 1.11 *Decrease of limonene volatility in the presence of various CDs measured by head space gas chromatography*

2 MICROBIOLOGICAL SYSTEMS AND METHDOLOGY FOR ASSESSING THE CD-MEDIATED MODULATION OF QUORUM SENSING MECHANISMS

2.1. Introduction and objectives

Our comprehensive integrative research involved the assessment and characterization of the concentration- and time-dependent effect of CDs on QS mechanisms in various bacterial systems.

The following selected microbiological model systems - with different endpoint applying Gram-negative bacteria including opportunistic human pathogens - were used, the methodology specifically modified and optimized then applied for studying CDs effects on QS:

- *Aliivibrio fischeri* (NRRL B-11177, DSM 7151) – testing bioluminescence inhibition;
- *Chromobacterium violaceum* (DSM 30191, ATCC 12472) – testing growth and pigmentation (violacein production);
- *Serratia marcescens (*DSM 46342, ATCC 27117*)* – testing growth and biofilm formation moreover prodigiosin production;
- *Pseudomonas aeruginosa* (DM 1117, ATCC 27853) – testing growth and biofilm formation;
- *Pseudomonas aeruginosa PAO1* (DSM 22644, ATCC 15692) – testing swarming, growth, pigmentation and biofilm formation.

Various cyclodextrins in various concentrations were applied in order to find the feasible cyclodextrin trap hindering the cooperative action of bacteria (*Table 2.1*).

In all model systems besides the testing of specific endpoints such as bioluminescence, biofilm formation, pigment production and swarming, viability of cells and proliferation of these bacteria were also studied by conventional microbiological methods including optical density, culture-based plate counts or specific enzymatic activities. Statistical analysis of variance (ANOVA) by STATISTICA 13.1® software was used for identifying significant effects ($p<0.05$).

We conducted very detailed and complex research with the *Aliivibrio fischeri* model system. The prominent role of this bacterial model system was primarily due to the methodological advantages. This test system has a number of well-measurable endpoints and provides fast and reliable information on the effects of the test substances.

Since the chemical signals of *Aliivibrio fischeri* could influence the behavior of many other bacterial and plant test systems, the results could serve as a basis for further research on other model systems of human and environmental significance, in which these chemical signals might influence the physiology of the organisms.

Table 2.1 *Abbreviations (with the degree of substitution (DS) given in parenthesis) and molecular weights (with the solubility in parenthesis) of tested cyclodextrins.*

	α-CD		**β-CD**		**γ-CD**	
	Abbreviation	Molecular weight (Solubility g/L)	Abbreviation	Molecular weight (Solubility g/L)	Abbreviation	Molecular weight (Solubility g/L)
Native CDs	ACD	972 (145)	BCD	1135 (18)	GCD	1297 (232)
Hydroxypropyl CDs	HPACD (4.6)	1240 (>500)	HPBCD (4.5)	1396(>500)		
Random methylated CDs	RAMEA (11)	1127 (>500)	RAMEB (12)	1300 (>500)	RAMEG (12)	1464 (>500)
Trimethyl-aminopropyl CDs	QAACD (4.5)	1655 (>500)				
Sulfobutyl ether CDs			SBEBCD (6.4)	2147 (>500)		
CD polymers	ACDPS	1390* (>500)	BCDPS	1621* (>500)		

** The molecular weight of a unit containing one CD molecule.*

Contrary to the original research plan, we did not perform any research with the *Agrobacterium tumefaciens* model system, instead we carried out extensive studies with two *Pseudomonas aeruginosa* species to investigate the CD-affected QS in order to apply the knowledge connected to the practical problems of the health care as well as environmental and industrial technologies. Besides, *Pseudomonas aeruginosa* is an important opportunistic pathogen, and a frequently applied model organism for biofilm studies. Microbial biofilm formation has brought vast amounts of problems to our everyday life, and costs billions of dollars worldwide in medical infections in hospitals, product contamination, equipment damage etc. So novel strategies based on the quenching of the biofilm formation are urgently needed.

2.2. Results

2.2.1. The effect of cyclodextrins and its derivatives on the proliferation and bioluminescence of Aliivibrio fischeri

We selected *Aliivibrio fischeri*, a bacterium, producing light, based on Quorum Sensing, to be the first to investigate the cyclodextrins' effect on this bioluminescence (*Fig. 2.1*)

The *Aliivibrio fischeri* bioluminescence test is a rapid, simple and cost effective method for testing the bioluminescence and may serve as a good model for studying the QS regulated processes. The emitted light, the so called bioluminescence, is directly proportional to the metabolic activity of the bacterial community, and any inhibition of the interaction between the chemical signals and the receptor, as well as the cytotoxicity may cause a parallel decrease in the bioluminescence.

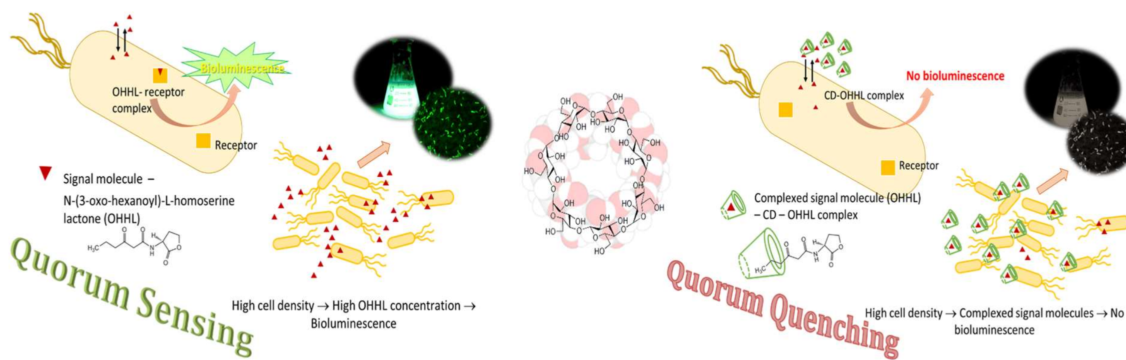


***Fig. 2.1**. Main concept of QQ in Aliivibrio fischeri with cyclodextrins*

The effect of native α-, β- and γ-cyclodextrins (ACD, BCD and GCD), 2-hydroxypropyl (HPACD, HPBCD), random-methylated (RAMEA, RAMEB, RAMEG), trimethylaminopropyl (QAACD), sulfobutyl ether (SBEBCD) derivatives and their epichlorohydrin-crosslinked polymers (ACDP and BCDP) on the bioluminescence and viability of *A. fischeri* cells was tested in a series of experiments at 0.156–10 mM concentration range.

In order to differentiate between the direct inhibition of bacterial communication and the cytotoxic effect, both optical density (OD) and tetrazolium reduction activity (TRA) were determined.

Our main objectives were 1) to test the applicability of *A. fischeri* bioluminescence assay as a high-throughput screening tool in assessing the efficiency of CD-mediated QQ, 2) to investigate the potential concentration- and time-dependent quorum quenching effect of the three most common native cyclodextrins and their most widely used derivatives on the bioluminescence intensity, 3) to compare the QQ effectiveness of the different CD molecules by determining Effective Concentration (EC_{20}) and Minimum Inhibitory Concentration (MIC).

According to our results, especially high quorum quenching effect was found for ACD: 10 mM ACD at 120 min contact time caused ~64% inhibition of bioluminescence[10]. Experiments with the co-administration of ACD and N-(3-oxohexanoyl)-L-homoserine lactone, the signaling molecule of *A. fischeri* clearly showed, that the stimulating effect of this signal was diminished by ACD suggesting, that complexation was responsible for the observed *QS* suppression.

Although BCD and its hydroxypropyl derivative significantly inhibited bioluminescence at as low as 0.156 mM concentration (*Table 2.2*), their efficiency did not reach the level of ACD.

***Table 2.2** Effective concentrations causing 20% inhibition of bioluminescence (EC_{20}) and the Minimum Inhibitory Concentration (MIC) for ACD, BCD and their derivatives observed at 120 min contact time*

	EC20 and MIC values [mM]									
	ACD	RAMEA	HPACD	QAACD	ACDP	BCD	RAMEB	HPBCD	SBEBCD	BCDP
EC_{20}	2.300	3.200	3.500	6.000	7.390	3.240	6.790	4.080	7.390	10.000
MIC	0.625	0.625	0.625	2.500	2.500	0.156	2.500	0.156	0.625	0.625

To demonstrate that the inhibition of bioluminescence by CDs is the consequence of capturing the signal molecules via inclusion complex formation and thus, reducing the apparent signal molecule concentration to give false information to bacteria on the cell density, we measured the effect of the simultaneously added CD and signal compound. The most widely recognized signal molecule of *A. fischeri*, the 3-oxo-C6-HSL was used in these experiments.

Indeed, adding 3-oxo-C6-HSL alone to the culture media, the stimulation was huge, and when adding ACD alone, the inhibition was significant as shown in *Fig. 2.2*. When both 3-oxo-C6-HSL and ACD were present simultaneously, ACD compensated the effect of the signal molecule. Our results supported the idea that ACD could efficiently complex the signal molecule. Tests with the co-administration of ACD and 3-oxo-C6-HSL confirmed both the role of 3-oxo-C6-HSL in the *A. fischeri* QS regulated processes (concentration-dependent stimulation) and the 3-oxo-C6-HSL-ACD complexation, which was responsible for the observed QS suppression.

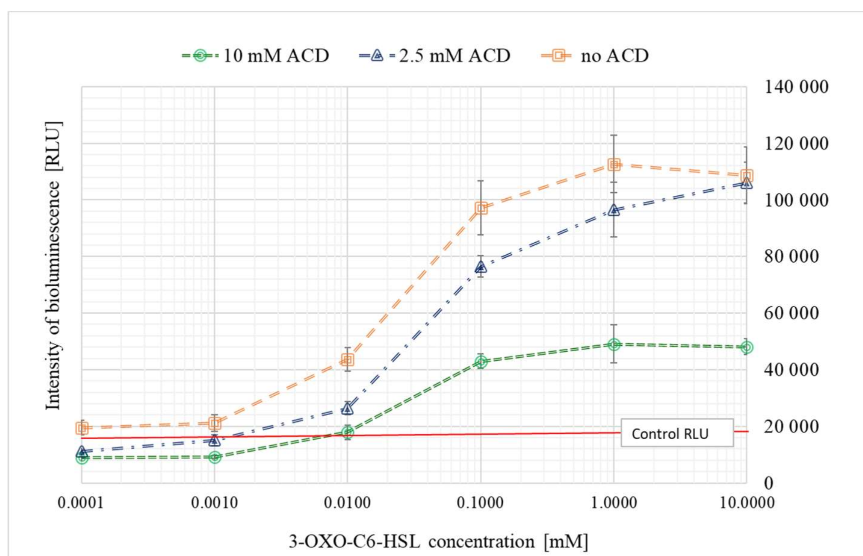


Fig. 2.2. *The effect of 2.5 mM and 10 mM ACD on bioluminescence of. A. fischeri (expressed in Relative Light Unit, RLU)*

Cyclodextrin-induced alterations in the tetrazolium-reduction based enzyme activity (proportional with the number of living bacteria) were observed only in the case of ACD, but only after 120 min at the two highest ACD concentrations. Enzyme activity was inhibited by 24% and 29%, upon 5 mM and 10 mM ACD addition, respectively. These results clearly demonstrated that the inhibitory effect on bioluminescence (~64% after 120 min at 10 mM ACD) stemmed mainly from the effect on quorum sensing, presumably due to the complexation of the signal molecule(s), and in case of ACD the contribution of a slight cytotoxic effect —at least at the higher concentrations— cannot be excluded.

Testing of new CD derivatives with different alkyl chain lengths (QA-6S-C6-ACD (CYL-4780), QA-6S-C6-BCD (CYL-4756), QA-6S-C12-BCD (CYL-4757), QA-6S-C16-BCD (4758), QA-6S-C6-GCD (4781)) was also carried out in the *A. fischeri* model system. Contrary to our expectations and the literature[2], we did not find significant inhibitory effects on bioluminescence. The QQ-effect of these CDs could be prevented by the fact that the formation of the inclusion complex between the CDs and signal molecule was sterically inhibited by the alkyl chain (the alkyl chain of the adjacent CD competed with the acyl chain of the signal molecule for the CD cavity). The inclusion of the alkyl chain by another CD was suggested based on the aggregation behavior of these compounds.

Long-term, complex, scale-up studies with *Aliivibrio fischeri* were also carried out to determine the potential long-term QQ effect of selected CDs (ACD, HPACD, BCD, RAMEB, SBEBCD, ACDPS) measuring the bioluminescence to characterize and confirm the stabile effects of CDs on QS mechanisms (*Fig. 2.3*).

The results of the comprehensive evaluation demonstrated the outstanding and long-term quorum quenching effect of ACD and its derivatives (*Fig. 2.3*). The highest long-term effect was exhibited by RAMEA; 12.5 mM RAMEA after 26 hours and 38 hours inhibited bioluminescence by 84% and 78%, respectively.

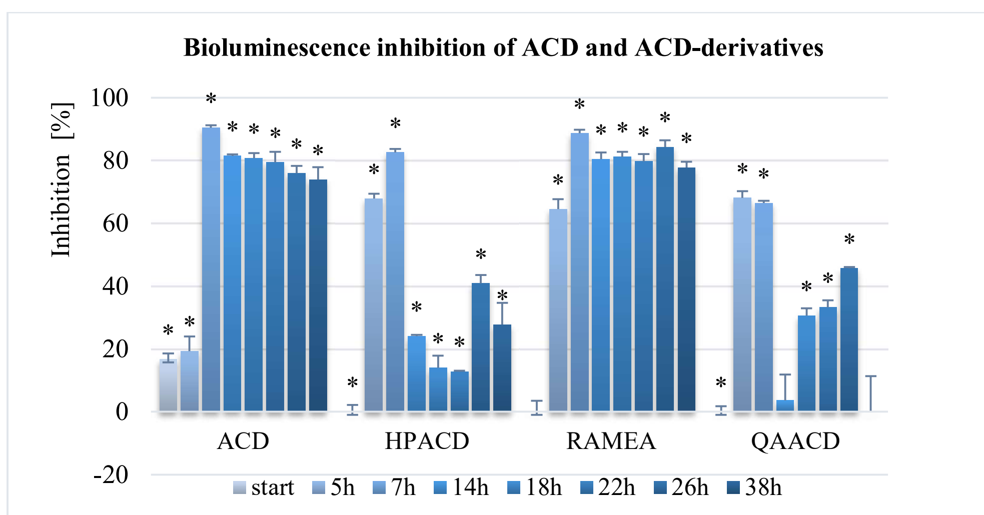


Fig. 2.3 *The effect of 12.5 mM ACD and its derivatives on the bioluminescence on long term*

2.2.2. Effect of triclosan complexes on the bioluminescence and viability of A. fischeri test organism

Triclosan is a widely used antibiotic with recently recognized QS-inhibiting effect. It has low solubility in water, which can be highly improved by complexation. During the last decades some scientists have explored the possibility of incorporating triclosan into modified cyclodextrins to enhance stability and water solubility.

Effect of triclosan and various triclosan-CD complexes on bacterial communication was studied by *A. fischeri* bioluminescence model system as well. The concentration – response curves were recorded to calculate the values of EC_{20}, EC_{50} (the concentration inducing 20% and 50% of maximal response) and LOEC (lowest effect concentration). The results listed in *Table 2.3* clearly show that complexation with CD decreased the effect triclosan on *A. fischeri* bioluminescence except SBEBCD complex.

Table 2.3 *Effective Concentration and Lowest Observed Effect Concentration values of triclosan (TRC) complexes with various CD derivatives determined in bioluminescence test system after 60 min contact time**

	EC_{20} [mg/L]	EC_{50} [mg/L]	LOEC [mg/L]
TRC	0.34	>0.81	0.20
TRC/SBE-BCD	5.30	9.20	3.90
	(0.27)	*(0.47)*	*(0.20)*
TRC/ACDP	16.80	43.90	15.60
	(1.90)	*(4.97)*	*(1.77)*
TRC/BCDP	23.10	46.80	12.50
	(1.85)	*(3.74)*	*(1.00)*
TRC/RAMEA	27.70	68.90	15.60
	(2.73)	*(6.79)*	*(1.54)*
TRC/RAMEB	59.10	95.70	31.30
	(6.06)	*(9.82)*	*(3.21)*

**The triclosan concentration of the complexes at the given effective concentrations are in parenthesis*

Thus, instead of the expected synergetic effect of triclosan and CDs the complexation reduced the efficiency via reducing the free triclosan concentration available for the bacteria[7].

2.2.2. The effect of cyclodextrins on the growth and pigment production of Chromobacterium violaceum

Chromobacterium violaceum produces the violacein pigment, a water-insoluble purple pigment with antibacterial activity in response to the presence of the N-hexanoyl homoserine lactone (C6-HSL) in the quorum sensing modulated process. As an endpoint for testing QQ effect, we studied violacein pigment production in the presence of cyclodextrins and compared it to the control.

Numerous experiments were carried out to develop a method for efficient violacein extraction. In the first phase of the experiments, we tested the required number of extraction steps for the most efficient extraction of the violacein pigment from the cells. The efficiency of 96% ethanol and butanol was also compared. Each measurement was performed in three replications and repeated three times. In these growth-experiments, we had the opportunity to test the effect of cyclodextrins in higher concentrations in the form of a suspension.

According to the results, the three subsequent extraction steps and application of ethanol proved to be the most efficient, so we continued to work accordingly during our further research (*Fig. 2.4*). The sampling and the pigment extraction was performed after 24, 48 and 72 hours of exposure time.

Fig. 2.4 *Violacein extracted from the cells in ethanol (Eppendorf tubes and microtiter plates)*

The 50 mM stock suspensions of CDs were tested for comparative evaluation of CDs-mediated effects in connection with violacein production. The CDs were added to the culture medium, their effect on the growth and pigment production was tested. In order to investigate whether the CD types affected cell growth, the optical density (at 630 nm) of the cell suspensions was measured. To examine whether the different CDs affected cell viability, the microculture tetrazolium and resazurin reduction assay was applied using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The measurements were performed after 24, 48 and 72 hours of exposure time as well. The ethanol phase containing the violacein was collected and its absorbance measured at a wavelength of 544 nm. The optical density of the original bioassay culture was also measured at a wavelength of 630 nm.

Based on the experience gained during the violacein extraction experiments we managed to develop a usable methodology; fast and simple extraction procedure applying ethanol for determination of produced violacein via QS providing a reliable quantitative bioassay for monitoring of QQ was worked out.
The method demonstrated the differences in the efficiency of the CDs tested (*Table 2.4*). Highest QQ efficiency in connection with violacein production (after 24 hours) was found for ACD and BCDPS with ~95% and ~85% inhibition, respectively. However, due to the large standard deviations, the extraction method needs to be further developed. Besides, further experiments are in progress to explore the concentration-dependent effects of cyclodextrins.

***Table 2.4**. CD-mediated effects on pigmentation of C. violaceum - inhibition of violacein production*

Cyclodextrins [50 mM]	Inhibition [%, 24 hours]		Inhibition [%, 48 hours]	
	Average	SD	Average	SD
ACD	94.6	0.2	90.7	0.9
RAMEA	72.3	1.6	43.5	5.2
ACDPS	76.4	1.3	66.8	1.2
HPACD	40.6	6.8	30.5	13.3
BCD	40.3	10.5	57.6	6.6
RAMEB	-3.6	5.1	12.8	25.8
HPBCD	10.	17.8	-22.4	31.8
SBEBCD	52.2	6.3	16.5	13.4
QABCD	69.0	3.9	62.7	1.7
BCDPS	85.2	1.5	64.9	4.4

2.2.3. The effect of cyclodextrins on the growth and pigment production of Serratia marcescens

Testing biofilm formation and pigmentation (prodigiosin production) for investigation of the CD-mediated QQ has been also performed by the *Serratia marcescens* model system.

Despite the numerous experiments performed with the bacterial strains available to us, we were not able to achieve adequate sensitivity in terms of either biofilm formation. In the case of pigment production, we found slight responses upon cyclodextrin addition, as the following table (*Table 2.5*) illustrates for ACD and HPACD.

Summarizing the result obtained in the *Serratia marcescens* model system, small inhibitory effects were obtained with large standard deviations.

***Table 2.5** The effect of ACD on prodigiosin production*

	ACD - 24 h		ACD - 48 h	
Cyclodextrin concentration	Inhibition [%]	*SD [%[*	Inhibition [%]	*SD [%[*
0.049	6.4	*6.9*	4.9	*8.4*
0.097	7.5	*6.6*	6.4	*9.0*
0.195	8.1	*3.1*	5.3	*7.5*
0.391	6.9	*2.9*	7.5	*7.3*
0.781	8.5	*8.5*	16.2	*5.8*
1.563	14.4	*7.0*	14.4	*11.5*
3.125	13.0	*8.8*	10.4	*10.2*
6.25	18.8	*6.7*	19.1	*7.6*
12.5	26.2	*3.7*	23.6	*14.4*

These studies will be continued with additional *Serratia marcescens* species and new experimental design to increase the sensitivity of the method.

2.2.4. Quorum quenching effect of cyclodextrins on the pyocyanin and pyoverdine production of Pseudomonas aeruginosa

The study aimed at investigation of *P. aeruginosa* pyocyanin production demonstrated that the conventional method using chloroform can be simplified by eliminating the step of chloroform extraction of pyocyanin pigment molecules resulting in a more environmental- and health-friendly method. But in small-volume test system (200 µL) pyocyanin was not produced, therefore using a small-volume test system proved to be a dead end. In the increased volume test system, ACD significantly inhibited the production of pyocyanin (27% and 39% inhibition at 5 and 10 mM concentrations, respectively)[11].

Pyoverdines, the fluorescent siderophores produced by *P. aeruginosa* are also important virulence factors. Their contributions to bacterial pathogenesis include providing a crucial nutrient (i.e., iron), regulation of other virulence factors and supporting the formation of biofilms. According to our results ACD and its derivatives (RAMEA, HPACD, QAACD) as well as BCD efficiently modulated the pyoverdine production of *P. aeruginosa* within the 0.195−6.25 mM concentration range in a small volume (200 µL) model system.

Based on the MIC values (*Table 2.6)* BCD was the most effective causing significant decrease of pyoverdine production with 0.195 mM MIC value.

Table 2.6 *Inhibition, Effective Concentration (EC) and Minimal Inhibitory Concentration (MIC) values of the tested α- and β-CD and their derivatives in the P. aeruginosa model system based on pyoverdine production*

[mM]	Inhibition [%] (at 6.25 mM)	EC_{20}	EC_{50}	MIC
ACD	55 ±4	1.211	3.309	0.391
RAMEA	54 ±12	1.015	3.524	0.391
HPACD	40 ±13	2.983	n.a.	3.125
QAACD	44±2	0.706	n.a.	0.781
BCD	30 ±4	0.187	n.a.	0.195
RAMEB	9 ±9	3.113	n.a.	3.125
SBEBCD	30 ±6	1.940	n.a.	1.563

Highest QQ efficiency in connection with pyoverdine production was found for ACD and RAMEA with ~55% inhibition at 6.25 mM concentration.

In summary, our results show that the highest QQ efficiency in the tested test systems was achieved in most cases by the use of ACD. However, the MIC values for BCD and its derivatives (RAMEB, SBEBCD) were lower in the most model systems tested. The background concerning these results is still need to be explored.

2.2.5. The effect of cyclodextrins (CD) and CD derivatives on the biofilm formation of Pseudomonas aeruginosa

Our research targeted the comprehensive study of bacterial communication in the *P. aeruginosa* model system applying different endpoints and the assessment of the effect of cyclodextrins on quorum sensing. The focus of our work was development of an efficient methodology for studying the swarming motility of *P. aeruginosa* and the biofilm formation, because this mechanism has outstanding importance both from human and environmental point of view since bacteria in biofilms are responsible for several infectious diseases.

Because s*warming* is of huge importance in the first stage of biofilm formation we dedicated special attention to its study during our research. In order to find the most favorable experimental set-up we studied and changed many parameters during our experimental series planned for methodological development aiming the study of *swarming* (*Fig. 2.5*).

Amongst the applied test organisms, the PAO1 strain of *P. aeruginosa* proved to be suitable for testing *swarming*. This strain was cultured on various media (LB, Nutrient, BM2, M8, M9, PGM) and we tried to „trigger" its dendrite formation.

We observed that the swarming is clearly dependent on the carbon source in the media. From the tested media the BM2 and M8 contributed to dendrite formation, and colony growth. The swarming phenomenon needs a half-fluid substrate which is favorable for the cells in this form of motion. For this reason, the substrate may contain low amounts of agar.

Test organisms
- *P. aeruginosa 1117*
- P. *aeruginosa* PAO1

Media
- M8
- M9
- BM2
- PGM
- LB

Agar concentration
- 0.6%
- 0.7%
- 0.8%
- 1.0%

Duration of drying
- 10 min
- 30 min

Inokulum administration
- toothpick insertion
- pipetting

Temperature of incubation
- 22°C
- 30°C
- 37°C

Duration of exposure
- 24 h
- 48 h
- 72 h

Fig. 2.5 *Tested parameters in the development of a plated-based assay for swarming motility in Pseudomonas aeruginosa*

Based on the information and experience gained during the motility experiments and using the recommendations of the scientific literature we managed to develop a usable methodology. Based on our results we recommend the following methodology for the motility experiments: *Pseudomonas aeruginosa* PAO1 strain, on BM2 type 0.8% substrate, dried for 30 minutes, the inoculum inserted by toothpick, incubated at 22 °C and 30 °C for 24 and 48 hours.

The effects of cyclodextrins (ACD, RAMEA, QAACD, HPACD, ACDPS, BCD, RAMEB, QABCD, HPBCD, BCDPS) were also studied with the developed methodology.

It can be concluded, that the ACD and RAMEA had the highest inhibitory effect both on colony growth and dendrite forming (*Figure 2.6–2.7*), and in addition, the latter was inhibited also by HPBCD. The α-cyclodextrin hydroxypropyl and its quaternary-amino derivative stimulated dendrite forming, resulting in nice, long appendices/extensions (*Figure 2.8*), thus, increasing also the diameter of the colony. In addition, BCD, BCDPS and the QABCD had similar effect. This methodology, even if in a semi-quantitative way, may become a good screening tool during preliminary assessment.

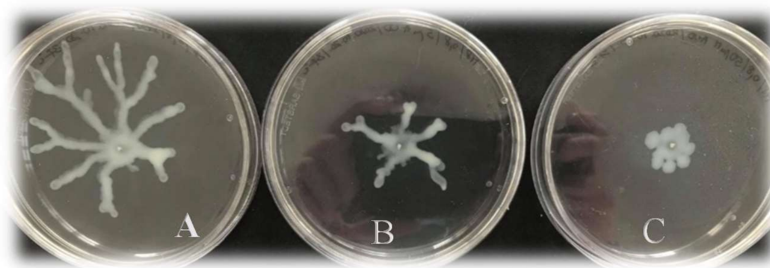


Fig. 2.6 *Effect of ACD on swarming after 48h (A – control (no ACD), B - 5 mM ACD, C -50 mM ACD)*

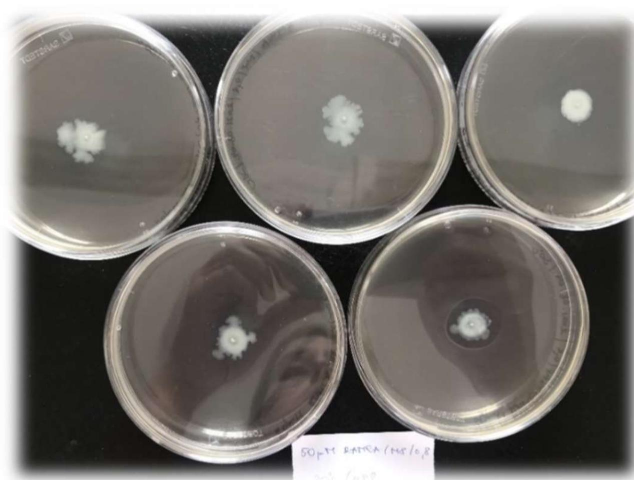


Fig. 2.7 *Effect of 50 mM RAMEA on swarming (5 parallels, 48h)*

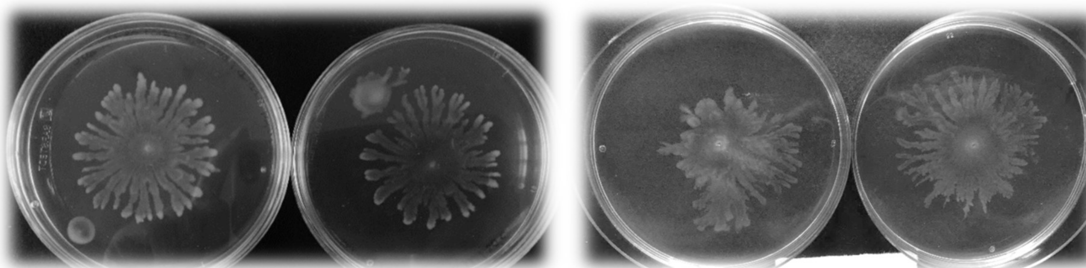


Fig. 2.8 *Effect of 50 mM QAACD (left) and 50 mM QABCD (right) on swarming (2-2 parallels, 48h)*

Subsequent to this, we studied the concentration- and time-dependent effect of various cyclodextrins and their specific derivatives on biofilm formation in a microtiter plate system of high permeability targeting also methodology development.

Within the quorum sensing–based biofilm formation study (at 22 and 30 ºC) we tested with *Pseudomonas aeruginosa* bacterium the effect of eight cyclodextrins (ACD, RAMEA, QAACD, ACDPS, BCD, RAMEB, QABCD, BCDPS), including also the native alpha- and beta-cyclodextrins, and their derivatives on the inhibition of biofilm formation. To identify if the effect of the tested cyclodextrin concentrations, the effect of contact time and the interaction between CD-concentration and time were significant, we performed Repeated Measures Analysis of Variance (RM ANOVA).

The results of the RM ANOVA demonstrated that both the CD-concentration and the time have influenced the evolution of biofilm for all CDs. However, the extent of this effect was statistically different for the various cyclodextrins. Additionally, Repeated-Measures ANOVA indicated highly significant time x CD-concentration interactions for all CDs.

According to the results, the ACD and its derivatives – the RAMEA and the ACDPS – had, in general, higher inhibitory effect than the BCD derivatives. This may be explained also with the cavity size of the cyclodextrins, since the diameter of the ACD cavity is smaller, thus it can more efficiently complex the acyl side chain. Meanwhile, in order to have a clear picture it would be advisable for the future to determine the stability coefficients between the cyclodextrins and the potential signal molecules, which would provide supplementary information on the complex formation and its stability. In addition, the extent of biofilm formation could be influenced also by many other parameters in the model system.

Our results presented that the random methylated derivatives (RAMEA, RAMEB) have a higher inhibitory effect on the long term at 2,5 mM and 12,5 mM concentrations, compared to the native CD-s. *Figure 9* and *Table 2.7* show the significant effect of RAMEA on biofilm formation.

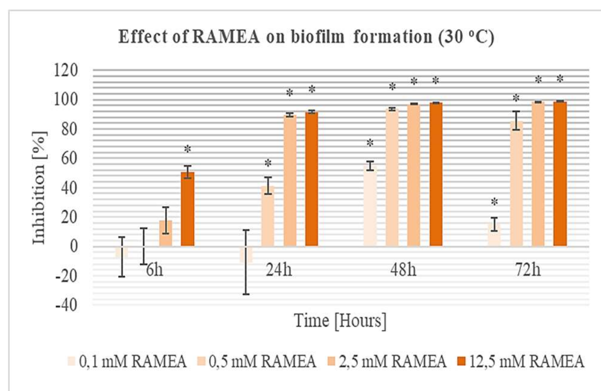


***Fig. 2.9** – Effect of RAMEA on biofilm formation (30 °C)*

Effect	Repeated Measures Analysis of Variance. Sigma-restricted parameterization - RAMEA 30 °C				
	SS	Degr. of	MS	F	p
Intercept	20.39	1.00	20.39	1882.99	0.000
CD	20.17	4.00	5.04	465.77	0.000
Error	0.15	14.00	0.01		
Time	3.77	3.00	1.26	113.51	0.000
Time*CD	8.58	12.00	0.71	64.50	0.000
Error	0.47	42.00	0.01		

***Table 2.7** – RM ANOVA results over time to evaluate effects of RAMEA on biofilm formation. Red numbers indicate significant differences at $p<0.05$ (30 °C)*

The inhibitory effect of ACDPS was also efficient at both temperatures, while the efficiency of BCDPS at 22 °C was higher. The QAACD and the QABCD had the lowest inhibitory effect both at 22 °C and 30 °C, while in many cases they had even stimulatory effect.

BCD inhibited biofilm formation at both temperatures, but the extent was much lower compared to ACD. Concerning the temperature of incubation, in all cases the inhibition was higher at 30 °C than at 22 °C, with the exception of BCDPS.

For comparative evaluation of the effect of cyclodextrins we determined by statistical means (with the Origin 8.5 software) the EC_{20} values, namely the effective concentrations resulting 20% inhibition. The concentration showing 20% inhibition is a good indicator of the cyclodextrin concentration which may be already effective for the inhibition of biofilm formation and a good tool for setting a priority order in the efficiency of cyclodextrins. The smaller the value, the higher is the efficiency.

The results are summarized in the tables (*Table 2.8–2.9*) below. The efficient treatments are coloured in red (and its shades), while the treatments of lower efficiency are coloured in green (and its shades).

Table 2.8 *Effective concentrations causing 20% inhibition of biofilm formation (22 °C)*

	Effective concentration- EC_{20} (22 °C) [mM]								
	ACD	**RAMEA**	**QAACD**	**ACDPS**		**BCD**	**RAMEB**	**QABCD**	**BCDPS**
6 h	0.11	0.06	n.g.	n.g.		5.84	n.g.	n.m.	2.48
24 h	1.03	0.14	n.g.	0.35		12.50	0.25	>12.5	0.17
48 h	2.48	0.52	11.90	0.17		2.40	0.44	n.m.	0.14
72 h	1.18	0.35	7.57	0.30		0.36	0.49	n.m.	0.09

(n.g.: no inhibition within the tested concentration range; n.m.: non-detectable)

Table 2.9 *Effective concentrations causing 20% inhibition of biofilm formation (30 °C)*

	Effective concentration - EC_{20} (30 °C) [mM]								
	ACD	**RAMEA**	**QAACD**	**ACDPS**		**BCD**	**RAMEB**	**QABCD**	**BCDPS**
6 h	3.75	2.48	1.63	0.17		n.g.	5.09	n.g.	0.98
24 h	0.57	0.47	2.42	0.35		0.17	0.06	0.17	10.90
48 h	0.30	0.09	0.73	0.42		0.11	0.09	n.m.	n.m.
72 h	0.19	0.08	0.70	0.09		0.54	0.47	0.03	0.16

n.g.: no inhibition within the tested concentration range; n.m.: non-detectable)

According to the incubation data at 22 °C, even the lowest RAMEA amounts have inhibitory effect at all time points, followed by BCDPS and ACD. RAMEB after 6 hours does not show yet any inhibition, but during the next time points, similarly to its alpha pair it could have efficient inhibitory effect even at very low concentrations compared to the other CDs studied. The efficiency of BCD may increase with time. However, the efficiency of the positively charged quaternary-amino-derivatives is the lowest as shown by the high value of the effective concentrations.

According to the incubation data at 30 °C altogether the efficiency of cyclodextrins may be higher. Based on the 24 and 48 hours data at 30 °C the RAMEB and RAMEA and the BCD were the most efficient. These results in terms of RAMEB and RAMEA are in accordance with the experiences of the *swarming* motility studies.

We measured both optical density (OD) and enzyme activity as additional endpoints of the biofilm formation experiments. For screening cytotoxicity, we monitored the reproduction phase of the cells and measured the optical density of both the entire well and of the planktonic supernatant.

The OD values did not decrease compared to control in case of any of the tested cyclodextrins, indicating that none of the CDs had any adverse effect on the cells. This fact had been confirmed in numerous scientific reports.

Nonetheless it is important to outline that the OD value is directly proportional with the total cell number which includes both the living and dead cells, therefore one should be very careful in or should refrain from drawing conclusions on the cytotoxic effect from these data.

In most experiments the enzyme activity was tested with resazurin, however, in case of QAACD and ACDPS besides the resazurin MTT tetrazolium salt was also applied. On the other hand, both indicators (MTT and resazurin) have been extensively used in viability tests, but due to their sensitivity their application has also disadvantages: Unfortunately, the standard deviation of both enzyme activity measurements was high, based on the results and, thus, they did not provide clear information on the potential cytotoxic effect of cyclodextrins.

According to both the scientific literature and our measurements, the behavior of CDs might have been influenced by several factors, such as the solubility of the formazan derivatives produced from MTT and the pH (especially in case of resazurin). In most cytotoxicity tests the CDs had no inhibitive effect. Therefore, mostly the quorum quenching effect of CDs was obvious. Meanwhile, in some instances, mainly at longer contact times and at the highest tested concentrations, especially the ACD, ACDPS and RAMEA had inhibitive effect. This may indicate the cytotoxic effect, for which we recommend application of further methods for determination of living cell concentration.

In summary, it can be concluded, that the results of the complex experimental series demonstrated the significant effect of cyclodextrins in the inhibition of biofilm formation. The cytotoxic effect of CDs was low, while their effect on the inhibition of biofilm formation was considerable, which demonstrates that the CDs act through quorum quenching. The QQ effect was significantly influenced by the applied concentration, contact time and the CD type (structure). These influencing factors need more detailed studies in the future.

The results clearly demonstrate the efficient and targeted applicability of cyclodextrins in the modulation of bacterial quorum sensing through formation of cyclodextrin traps, thus opening new opportunities in the fight against bacterial infections and controlling the efficiency of some environmental technologies.

2.2.6. Testing the cytotoxicity of cyclodextrins with Gram-negative and Gram-positive bacteria

The ACD at the highest tested concentrations (10–20 mM) proved to be very often significantly cytotoxic to the bacterial model systems applied in our quorum sensing studies. The cytotoxic effect was indicated by the results based on tetrazolium reduction. For detailed examination of this effect we initiated several experimental series in the last project activity period involving various bacteria.

The cytotoxic effect of cyclodextrins to human cells and cell lines had been confirmed in the scientific literature by several studies. This phenomenon is explained by the interaction between the methyl-β-cyclodextrins and the cell membrane lipids, primarily the cholesterol. For this reason, the β-cyclodextrins are applied in human cell biology research for the extraction of cholesterol from cell membrane and for studying its role in the cell functions. Meanwhile, detailed information about the cytotoxic effect of CDs on bacteria is not available.

Our aim was to study the cytotoxic effect of ACD in high-throughput, small volume, acute test systems. The potential toxic effect of α-cyclodextrin (ACD) was studied within the 0,625–20 mM concentration range, during 2h – 48 h residence time. The concentration- and time- dependent effect of cyclodextrin on *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* bacteria was monitored in 96-well microtiter plates applying the high-throughput screening method. The effect on reproduction was measured by optical density, while cytotoxicity by biological activities based on tetrazolium salts and resazurin reduction assays.

The effect of ACD on dehydrogenase enzyme activity was monitored by the following two indicators as artificial electron acceptors: INT, MMT. (INT: 2-(p-iodphenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium-chloride; MTT: 3-(4,5-dimetyltyazol-2-il)-2,5-diphenyltetrazolium-bromide). In case of undisturbed functioning of the respiratory chain the tetrazolium salts are reduced, the INT turns to red, the MTT to black-coloured formazan product, the light-absorption of which can be measured photometrically.

Additionally, for assessing the cytotoxic effect on *Pseudomonas aeruginosa* we applied a new indicator as well, namely the resazurin (7-hydroxi-3H-phenoxazine-3-on-10-oxide), which is a phenoxazine dye, similar to the tetrazolium salts. Resazurin is a fluorescent, nontoxic, cell-permeable substance, violet-bluish coloured at pH 6.5, but at neutral pH it turns to a pink-coloured and strongly fluorescent, irreversibly reducible rezorufin compound. The intensity of the fluorescent light emitted by rezorufin can be detected further to its excitation at a suitable wavelength. We compared the sensitivity of the three indicators in this bacterial test system.

According to our results and to the expectations, the tetrazolium and resazurin-reduction based methods were more sensitive for the characterisation of the cytotoxic effect compared to the optical density-based reproduction inhibition measurements. In most cases the optical density values were not significantly different from the control after 1 and 2 hours.

Furthermore, we found obvious ACD concentration-dependent effects after 1 and 2 hours, based on the enzyme activity results. Amongst the tested tetrazolium salts MTT proved to be more sensitive artificial electron acceptor than INT. Meanwhile, resazurin was the most sensitive indicator, but it can be applied only at pH higher than 6.5.

The cyclodextrin concentration-dependent inhibition for the *B. subtilis* Gram-positive bacterium compared to the control ranged between 24–55%. Based on the first studies, ACD had no effect on *Escherichia coli* bacterium, even at the highest applied concentration (10 mM). At 10 mM ACD the inhibition compared to the control was 15–20% for the *Pseudomonas aeruginosa PAO1* strain with MTT as electron acceptor. However, this inhibition was almost 60% with resazurin. Based on our preliminary results the inhibitive effect of ACD to the Gram-positive *B. subtilis* bacterium was higher compared to the tested Gram-negative strains.

We plan to involve further bacteria (*Serrati sp., Proteus sp*. and *Staphylococcus sp.)* into our future experiments and to determine the cell concentrations by plate counting technique. Our additional primary targets are to detect the background and mechanism of the cytotoxic effect through the measurement of reactive oxygen species formation and the study of membrane integrity.

2.3. Summary and conclusions

According to our results, the QQ effect of cyclodextrins in all the tested bacterial model system was clearly demonstrated. This was the first complex research which used and evaluated comparatively the effects of native CDs and their derivatives for studying systematically on QS in various bacterial model system.

The autoinducer-dependent quorum sensing mechanism in *Aliivibrio fischeri* and *Pseudomonas aeruginosa* was markedly and significantly inhibited, the high quorum quenching effect of cyclodextrins was clearly demonstrated. The efficiency was influenced by several parameters; the size of the interior cavity, the structure and the concentration of the cyclodextrins, as well as the contact time with the cells. The application of a cyclodextrin-trap for complexation of signal molecules may be a novel, promising method for influencing QS interfering strategies, for example, to enhance the efficiency of various biotechnologies, as well as to find alternative approaches against bacterial proliferation and infections. Furthermore, our results could also serve as a basis for further research with bacterial or plant model systems, in which the same chemical signals may induce physiological responses.

One of our main future directions is to enhance further the CDs mediated QQ efficiency, adjusting the experimental parameters in a way to achieve nearly complete inhibition of the QS process (QS inactivation). According to our results, complete inactivation of the QS process would require a relatively high CD concentration, so we plan to find other parameters influencing the QQ effect.

3 SUPPORTING MATERIAL FOR THE FINAL REPORT OF THE PROJECT OTKA K_17 125093

3.1 Expected results

Expected results	Yes/No	Comment
AHL standards	✓	The AHL standards were purchased, not synthesized
6-alkylamino- and 6-alkylthio-derivatives of βCD	✓	Three deirvatives with alkyl chain length of C10, C12 and C16 were prepared using thiol coupling to ensure monosubstitution
A novel simple protocol applying *Aliivibrio fischeri* bioluminescence for characterizing the effect Quorum Sensing Inhibitors	✓	The methodology has been worked out and optimized for measuring the effect of CDs on QS
Mono-, di- and oligosubstituted 6-monoalkyl and 6-monoalkylthio α- and γCD derivatives with and without positive charge	✓	3-3 derivatives were synthesized with and without positive charge and characterized by NMR and HPLC
Characterization and comparative evaluation of inhibitory effects of CDs in different biological model systems.	✓	<10 various CD derivatives were tested in 4 bacterial model systems
Fluorescent-labeled CD monomer and polymer derivatives	✓	Fluorescent labelled RAMEB and BCDPS polymer crosslinked with epichlorohydrin were prepared for biological studies
Dissemination	✓	Conferences, Publications Teaching through research E-learning

3.2 Publications, oral and poster presentations related to our research project and with NKFIH acknowledgement

Molnár, M., Fenyvesi, E., Berkl, Zs., Németh, I., Fekete-Kertész , I., Márton, R., Vaszita, E., Varga., E., Ujj, D., Szente, L. (2021) Cyclodextrin-mediated quorum quenching in the *Aliivibrio fischeri* bioluminescence model system – Modulation of bacterial communication, *International Journal of Pharmaceutics*, 594, 120150. https://doi.org/10.1016/j.ijpharm.2020.120150

Benkovics, G., Bálint, M., Fenyvesi, É., Varga, E., Béni, S., Yannakopoulou, K., Malanga, M. (2019) Homo- and hetero-difunctionalized β-cyclodextrins: Short direct synthesis in gram scale and analysis of regiochemistry. *Beilstein Journal of Organic Chemistry* 15, 710-720. https://doi.org/10.3762/bjoc.15.66 (Imp. fact. 2.595)

Varnai, B., Grabarich, M., Szakács, Z. , Pagel, K. , Malanga, M., Sohajda, T., Beni, Sz. (2021) Structural characterization of fondaparinux interaction withper-6-amino-beta-cyclodextrin: An NMR and MS study, *Pharmaceutical and Biomedical Analysis* (JPBA-D-20-01312) 197, 113947. https://doi.org/10.1016/j.jpba.2021.113947 (Imp. fact. 3.209) (*This publication is not closely related to the project. We acknowledged for the OTKA grant as the applied per-amino-beta-cyclodextrin was prepared by applying also the synthetic methods developed in the project*.)

Berkl, Zs., Molnár, M., Bordohányi, Á., Fekete-Kertész, I., Németh, I., Fenyvesi, É., Szente, L., 2019. The effect of cyclodextrins on the biofilm formation of *Pseudomonas aeruginosa* – Modulation of quorum sensing. In: 6th European Conference on Cyclodextrins. Santiago de Compostela, Spain.

Molnár, M., Berkl, Zs., Németh, I., Fekete-Kertész, I., Márton, R., Timár, B., Fenyvesi, É., Szente, L., 2019. Cyclodextrin-mediated quorum quenching in the *Aliivibrio fischeri* bioluminescence model system – modulation of bacterial communication. In: 6th European Conference on Cyclodextrins. Santiago de Compostela, Spain. 2019.10.02–2019.10.04.

Fekete-Kertész, I., Berkl, Zs., Tóth, Zs., Fenyvesi, É., Szente, L., Molnár, M., 2019. Quorum quenching effect of cyclodextrins on the pyocyanin and pyoverdine production of *Pseudomonas aeruginosa*. In: 6th European Conference on Cyclodextrins. Santiago de Compostela, Spain.

Berkl, Zs., Molnár, M., Fenyvesi, É., Németh, I., Buda, K., Fekete-Kertész, I., Márton, R., Szente, L., 2020. Cyclodextrin-mediated quorum quenching in *Aliivibrio fischeri* model system. In: 4th National Conference of Young Biotechnologists, Debrecen, Hungary. 2020.12.19.

Berkl Zsófia: Mikroszkopikus rádiócsend – a bakteriális kommunikáció gátlása ciklodextrinekkel. *Élet és Tudomány* 2020, LXXV. évfolyam, 35. szám.

Buda Kata: Mozogjunk együtt! A baktériumok helyváltoztató mozgásformái. *Élet és Tudomány* 2021, LXXVI. évfolyam, 32. szám.

The project's research activities and achievements have been presented as invited talk at a *Scientific Symposium on the 145th anniversary of the BME's Faculty of Chemical Technology and Biotechnology foundation* (1 June 2018).

Manuscript accepted for publication

Fenyvesi. É., Sohajda, T. (2021) Cyclodextrin-enabled green environmental biotechnologies. *Environmental Science and Pollution Research* (ESPR-D-21-04125R1) accepted (Imp. fact. 3.056)

Manuscripts under submission

Fekete-Kertész, I., Berkl, Zs., Tóth, Zs., Vaszita, E., Márton, R., Fenyvesi, É., Szente, L., Molnár M. (2021) Quorum quenching effect of cyclodextrins on the pyocyanin and pyoverdine production of *Pseudomonas aeruginosa*. *Journal of Inclusion Phenomena and Macrocyclic Chemistry* (Imp. fact. 1.633)

Molnár, M., Fenyvesi, E., Szente, L., Németh, I., Fekete-Kertész, I., Vaszita, E., Varga., E., Berkl, Zs. (2021) The effect of cyclodextrins (CD) and CD derivatives on the biofilm formation of *Pseudomonas aeruginosa. Science of the Total Environment* (Imp. fact. 7.963)

3.3 Thesis

- Antal Vivien (2020) Bakteriális kommunikáció befolyásolása alkil-szubsztituált ciklodextrinekkel – a biolumineszcencia vizsgálata *Aliivibrio fischeri* modellrendszerben (BME)
- Bajza Boglárka (2017) Bakteriális kommunikáció befoláysolása ciklodextrinekkel *Aliivibrio fischeri* biolumineszcencia modellrendszerben (Szakdolgozat, BME)
- Buda Kata (2019) Bakteriális kommunikáció befolyásolása ciklodextrinekkel *Aliivibrio fischeri* és Chromobacterium violaceum modellrendszerekben (Szakdolgozat, BME)
- Buda Kata (2021) Bakteriális kommunikáció befolyásolása ciklodextrinekkel Pseudomonas aeruginosa modellrendszerben – motilitás és biofilmképzés vizsgálata (TDK, BME)
- Göndöcs Ágnes (2020) Ciklodextrinek hatása az *Aliivibrio fischeri* biolumineszcenciájára – szignálmolekulák és a bakteriális kommunikáció vizsgálata növekedési kísérletekben (Szakdolgozat, BME)
- Gulyás Adrienn (2017) Ciklodextrinek hatásának tanulmányozása a bakteriális kommunikációra *Aliivibrio ficheri* tesztorganizmussal (Szakdolgozat, BME)
- Keller Nóra (2018) Kvórum érzékelés és csillapítás – bakteriális kommunikáció befolyásolása ciklodextrinekkel és triklozánnal *Aliivibrio fischeri* modellszervezetben. (Diplomamunka, BME)

- Lantosi Laura (2021) Ciklodextrinek hatása a bakteriális kommunikációra *Serratia marcescens* tesztorganizmussal – pigmenttermelés és biofilmképzés tanulmányozása (Szakdolgozat, BME)
- Ligethy Laura (2020) Ciklodextrin-limonén zárványkomplexek komplexképződési hatékonyságának és a bakteriális kommunikációra gyakorolt hatásának vizsgálata (Szakdolgozat, BME)
- Márton Rita (2018) Bakteriális kommunikáció befolyásolása ciklodextrinekkel. (Szakdolgozat, BME)
- Németh Imre (2019) Ciklodextrinek és triklozán hatása a bakteriális kommunikációra *Aliivibrio fischer*i, *Chromobacterium violaceum* és *Pseudomonas aeruginosa* tesztorganizmussal. (Diplomamunka, BME)
- Tar Alexandra (2017) A bakteriális kommunikációt befolyásoló antimikrobás anyagok ciklodextrinekkel szabályozott hatóanyag-leadása (Szakdolgozat, Cyclolab – SZIE, BME)
- Timár Blanka (2018) Bakteriális kommunikáció befolyásolása ciklodextrinekkel *Aliivibrio fischeri* biolumineszcencia modellrendszerben (Szakdolgozat, BME)
- Takács Dorottya (2021) *Pseudomonas aeruginosa* biofilmképzésének vizsgálata és befolyásolása ciklodextrinekkel (Diplomamunka, BME)
- Tar Alexandra (2018) Triklozán/CD komplexek hatása a quorum érzékelésre *Aliivibrio fischeri* biolumineszcencia modellrendszerben (Szt. István Egyetem, szakdolgozat)
- Ujj Dóra Viktória (2018) HPLC módszer kidolgozása homoszerinlaktonok elválasztására (szakdolgozat, ELTE)

3.4 E-learning

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https://www.enfo.hu/node/13363, https://www.enfo.hu/node/13364,

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