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Systematic approaches for an industrially mature halophilic bioprocess to treat residual process brine

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Abstract

Residual process brines with high sodium chloride loads, generated during industrial production processes, can serve as valuable raw material, e.g. for membrane cell chlor-alkali electrolysis processes. However, residual process brines frequently contain organic or inorganic contamination, which often leads to challenges connected to their disposal or recycling. To utilize residual brines in chlor-alkali electrolysis, however, low levels of organic carbon (TOC: 1-10 ppm), nitrogen, and various ions are required to ensure an efficient process. Available physical and electrochemical approaches for the reduction of organic impurities in process brines, like sorption or oxidation, often come along with high energy consumption and operational expenditures, due to extended costs of chemicals and/or catalysts.

Therefore, the goal of this thesis was to provide an industrial mature bioprocess for the reduction of organic impurities in an industrial residual process brine. The potential of a biotechnological approach should be demonstrated to be integrated into chemical production chains. To do so, a continuous bioprocess using halophilic microorganisms should be characterized and optimized operating conditions should be provided. The industrial process brine used during this work was derived from the industrial production of 4,4'-methylenedianiline (MDA) and contained formate, aniline, phenol, and 4,4'-methylenedianiline as organic impurities.

To demonstrate an industrial mature bioprocess, a 1L-lab-scale bioreactor system was transferred to a 20-liter scale and implemented at an industrial production site. Continuous operation for >200 days showed robustness, efficiency, long-term feasibility, and stability of the biological treatment system. During unsterile pilot operation at the industrial site, the original halophilic archaeal culture was replaced by a halophilic mixed culture consisting of three bacterial genera (*Halomonas* sp., *Aliifodinibius* sp. and *Oceanobacillus* sp.), which showed excellent degradation properties for the organic contaminants. To increase the acceptance in the chemical industry to integrate bioprocesses, reliable analytical tools for process monitoring are required. Therefore, an integrated, non-invasive, and online biomass estimation was furthermore developed and successfully established, to serve as a control input for a feed-forward biomass control strategy. Moreover, a reliable HPLC method for the quantification of aromatic impurities in residual process brine feed and bioreactor samples was established. In that way, separation of peaks from bioreactor samples and aromatic target molecules according to the retention time was achieved, which enables a correct interpretation of the chromatograms. Also, the integration of a bioprocess into an industrial production chain requires a cost-efficient operation with minimal nutrient supplementation and optimized process control. Therefore, critical process parameters (specific glycerol uptake rate,

ammonium to glycerol yield, aniline feed concentration) for the degradation of organic impurities were identified, and optimized operating conditions for an efficient process were proposed.

All achievements were accomplished by the combined use of innovative process analytical tools as well as an intelligent and intensified process design. For the first time, long-term integration of a pilot-scale bioprocess treating MDA residual process brine into an industrial environment was successfully shown. Moreover, the potential to exploit natural microbial diversity for industrial purposes was underlined and a robust microbial system was found which could be operated during prolonged cultivation times, unsterile conditions, and changing residual process brine compositions. Finally, statistical experimental planning and evaluation enabled the definition and proposal of a cost-effective process operation space, ready for the transition into an industrial scale. Consequently, the work showed the successful integration of extremophilic bioprocesses into chemical production chains. In contrast to electro-chemical and physical approaches, the presented bioprocess solution offers a sustainable alternative for integration into industrial production chains with high degradation efficiencies. Future investigations could scope the potential of the novel halophilic mixed culture for treating different residual process brines. Moreover, the technology transfer to the production scale would be the final step of the process development.

Zusammenfassung

Prozessabwässer mit hohen Kochsalzkonzentrationen (Natriumchlorid) fallen bei vielen industriellen Produktionsprozessen an. Oft sind solche Prozessabwässer mit organischen und anorganischen Verunreinigungen kontaminiert, die häufig zu Problemen bei der Entsorgung oder dem Recycling der Abwässer führen. Auf der anderen Seite können solche Prozessabwässer aber auch einen wertvollen Rohstoff darstellen. So kann salzhaltiges Prozessabwasser zum Beispiel für die industrielle Erzeugung von Chlorgas oder Natriumhydroxid verwendet werden. Für einen solchen Chlor-alkali-Elektrolyseprozess wird meist das Membranzellverfahren eingesetzt. Bei diesem Prozess bestehen allerdings hohe Anforderungen an die Reinheit der verwendeten Sole. Um einen effizienten und sicheren Elektrolyseprozess gewährleisten zu können, sollten deswegen bestimmte Konzentrationen von organischem Kohlenstoff (TOC: 1-10 ppm), Stickstoff und anderen Ionen in der Sole nicht überschritten werden. Verfügbare elektrochemische und physikalische Ansätze zur Reduzierung organischer Verunreinigungen in Prozessabwässern sind oft mit einem hohen Betriebsaufwand und einem hohen Energieverbrauch verbunden.

Deshalb war das Ziel dieser Arbeit, einen biotechnologischen Reinigungsprozess für industrielles Prozessabwasser mit hohem Salzgehalt zu entwickeln und die Implementierung in einen industriellen Kontext zu demonstrieren. Das Prozessabwasser, welches in dieser Arbeit verwendet wurde, fällt bei der industriellen Herstellung von 4,4'-Methylendianilin an und enthält neben Formiat auch die aromatischen Verbindungen Anilin, Phenol und 4,4'-Methylendianilin.

Die vorliegende Arbeit hatte das Ziel einen kontinuierlich betriebenen Bioprozess für den Abbau organischer Verunreinigungen in einem industriellen Prozessabwasser zu entwickeln und zu charakterisieren, um diesen letztendlich in einen industriellen Prozesskreislauf zu integrieren. Um dieses Ziel zu erreichen wurde ein Bioprozess mit einem membran-basierten Zellrückhaltungssystem etabliert und eine Feed-Forward Kontrollstrategie für die Einstellung gewünschter Biomassekonzentrationen entwickelt. Für eine verbesserte Prozesskontrolle, wurde zudem eine nicht-invasive Echtzeit-Biomasseschätzung entwickelt und integriert. Dafür wurden Informationen aus Substratkonzentrationen im Feed und Konzentrationen von CO_2 und O_2 im Abgas verwendet.

Um einen industriell ausgereiften Bioprozess zu demonstrieren, wurde ein 1L-Bioreaktorsystem im Labormaßstab in einen 20-Liter Pilotmaßstab überführt und an einem industriellen Produktionsstandort implementiert. In einem Betrieb von mehr als 200 Tage

konnte die Robustheit, Effizienz, und Langzeitstabilität des biologischen Aufreinigungsprozesses gezeigt werden.

Während des Langzeitversuches wurde schließlich eine halophile Mischkultur, welche drei verschiedenen bakteriellen Gattungen (*Halomonas* sp., *Aliifodinibius* sp. und *Oceanobacillus* sp.) aufwies, entdeckt. Diese Mischkultur verdrängte die ursprüngliche Vorkultur aus dem extrem-halophilen Archaeon *Haloferax mediterranei*. Diese neue halophile Mischkultur zeigte sehr gute Wachstumseigenschaften im Prozessabwasser sowie Abbauleistung der vorhandenen organischen Verunreinigung.

Für die Integration eines Bioprozesses in eine industrielle Produktionskette sollte ein möglichst kosteneffizienter Betrieb mit minimaler Nährstoffzugabe umgesetzt werden. Daher wurden kritische Prozessparameter (spezifische Glycerinaufnahmerate, Ammonium-zu-Glycerin-Aufnahme, Anilinfeedkonzentration) für den Abbau organischer Verunreinigungen identifiziert und anschließend optimierte Betriebsbedingungen für einen effizienten Prozess untersucht und vorgeschlagen. Möglich wurde dies durch den kombinierten Einsatz innovativer prozessanalytischer Methoden sowie durch eine intelligente und intensivierte Prozessabwicklung.

Content

1 Introduction

1.1 Background

1.1.1 Industrial residual process brines as a raw material

In various industrial sectors, such as the petroleum industry, tannery, and textile industry, or during polyurethane and polycarbonate production, effluents with high salt content (>5% NaCl) are generated (Castillo-Carvajal et al., 2014; Le Borgne et al., 2008; Mainka et al., 2021; Muddemann et al., 2018; Silva et al., 2009). The share of saline residual process brines (RPB) is estimated to be 5% of worldwide generated wastewater and is likely to increase in the future (Le Borgne et al., 2008). Hence, the question arises of how to deal with industrial residual process brines, as they frequently contain hazardous organic or inorganic contamination, which often leads to challenges when disposed of or recycled (Mavukkandy et al., 2019; Moussa et al., 2006; Woolard & Irvine, 1995). Hence, reuse (or recycling) of RPB would not only relieve the natural water system from hazardous and saline waste streams but also saves cost in generating raw materials (like NaCl) and reduces water consumption (Blöcher et al., 2019). One possibility for the reuse of RPB for industrial purposes is the production of chlorine gas or caustic soda (NaOH) in the chlor-alkali industry, using the RPB as a raw material (Casas et al., 2012; Reig et al., 2014). The main applications for chlorine are the production of polyvinylchloride (PVC), isocyanate, and oxygenate, whereas caustic soda is mainly used for the production of organics (Euro Chlor, 2021). In 2020, membrane-based processes were the dominant technology for chlor-alkali electrolysis (CAE) processes. The installed capacity of membrane cell CAE processes accounted for 85% of the total capacity. Compared to other CAE processes (like mercury or diaphragm cells), membrane processes are seen as advantageous due to their reduced energy consumption, the production of high-quality caustic soda, and the avoidance of harmful chemicals during the process (Crook & Mousavi, 2016; Schmittinger et al., 2011). In a membrane cell CAE process, brine is fed to the anode compartment, which is separated from the cathode compartment by a cation-exchange membrane. Inside the membrane cell, three main reactions take place resulting in the production of chlorine and hydrogen gas, and a liquid sodium hydroxide solution (Crook & Mousavi, 2016; Schmittinger et al., 2011). On the cathode side chlorine is formed:

$$2\,Cl^- \rightarrow Cl_2 + 2\,e^- \quad (1)$$

From the anode compartment, hydrated sodium ions pass the membrane to the cathode side, where hydrogen and hydroxyl ions are formed (Equation 2). The latter react with sodium ions to form sodium hydroxide (Equation 3):

$$2\,H_2O + 2\,e^- \rightarrow H_2 + 2\,OH^- \quad (2)$$

$$Na^+ + OH^- \leftrightarrow NaOH \quad (3)$$

During the membrane cell CAE processes, harsh conditions (chlorine gas exposure, high salt loads in the anolyte, 90 °C reaction temperature, >30% NaOH solution) are applied to the membrane. To withstand such harsh conditions, perfluoro polymers are used as membrane materials, which are layered with sulfonate groups ($-SO_3^-$) on the anode side and carboxylate ($-COO^-$) groups on the cathode side (Schmittinger et al., 2011). The performance of the membrane cell mostly depends on four factors: i) concentration of anolyte/catholyte, ii) current density, iii) temperature, and iv) brine impurities (Schmittinger et al., 2011).

In brines, especially solid materials should be avoided, as they can damage the membrane materials. Thus, high concentrations of ions like magnesium and calcium have to be avoided in brines used in membrane cell CAE (Brinkmann et al., 2014; Schmittinger et al., 2011). Moreover, if nitrogen compounds are present in the membrane cells, explosive nitrogen trichloride (NCl_3) can be formed, thus nitrogen levels in brines have to be low (Brinkmann et al., 2014). Furthermore, organic impurities might lead to foaming and cause voltage increases, resulting in an inefficient process (Brinkmann et al., 2014; Casas et al., 2012; Keating & Behling, 1990; Schmittinger et al., 2011).

Thus, a pre-treatment of contaminated RPB before the membrane-based chlor-alkali process step is necessary. Physical and electro-chemical methods like sorption or oxidation can be used for brine treatment. However, reservations can arise due to extended costs of chemicals and/or catalysts, low organic removal efficiencies, or inappropriate handling of large brine streams (Bulan et al., 2019; Li et al., 2019b; Turkay et al., 2017; Zhou et al., 2011). Hence, biological systems are considered an economically more attractive alternative for residual process brine treatment (Bonfá et al., 2013; Jin et al., 2012; Le Borgne et al., 2008). In such a biological treatment strategy, microorganisms are used in a bioprocess unit operation to degrade the impurities present in the RPB (Figure 1) (Cao et al., 2016; Heckroth et al., 2019; Mainka et al., 2021; Tan et al., 2019).

After a biological pre-treatment step in which organic compounds should be removed, the brine has to be purified in further unit operations before potential reuse in industrial production processes. During the first purification step, especially magnesium and calcium ions should be

removed, as these ions can form large crystals near the cathode side, which leads to mechanical disruption of the membrane (Brinkmann et al., 2014; Schmittinger et al., 2011). Thus, magnesium and calcium are precipitated using sodium carbonate and sodium hydroxide before the CAE. In that way, calcium carbonate and magnesium hydroxide are formed (Brinkmann et al., 2014). Also, other ions and metals are precipitated by adding different precipitation salts. After precipitation, the solid particles are removed by either sedimentation, filtration, or a combined approach of both methods (Brinkmann et al., 2014). Nevertheless, for the membrane cell CAE process, brines have to be purified to a further extent. Thus, subsequential purification steps are applied to reduce magnesium and calcium levels. To do so, a polishing filtration step is applied to remove all solids sufficiently, in order to protect the resin used in the following ion-exchange chromatography step. The ion exchange is necessary to reduce magnesium and calcium levels below 20 ppb and achieve low current densities (<4 kA m^{-2}) (Brinkmann et al., 2014; Schmittinger et al., 2011).

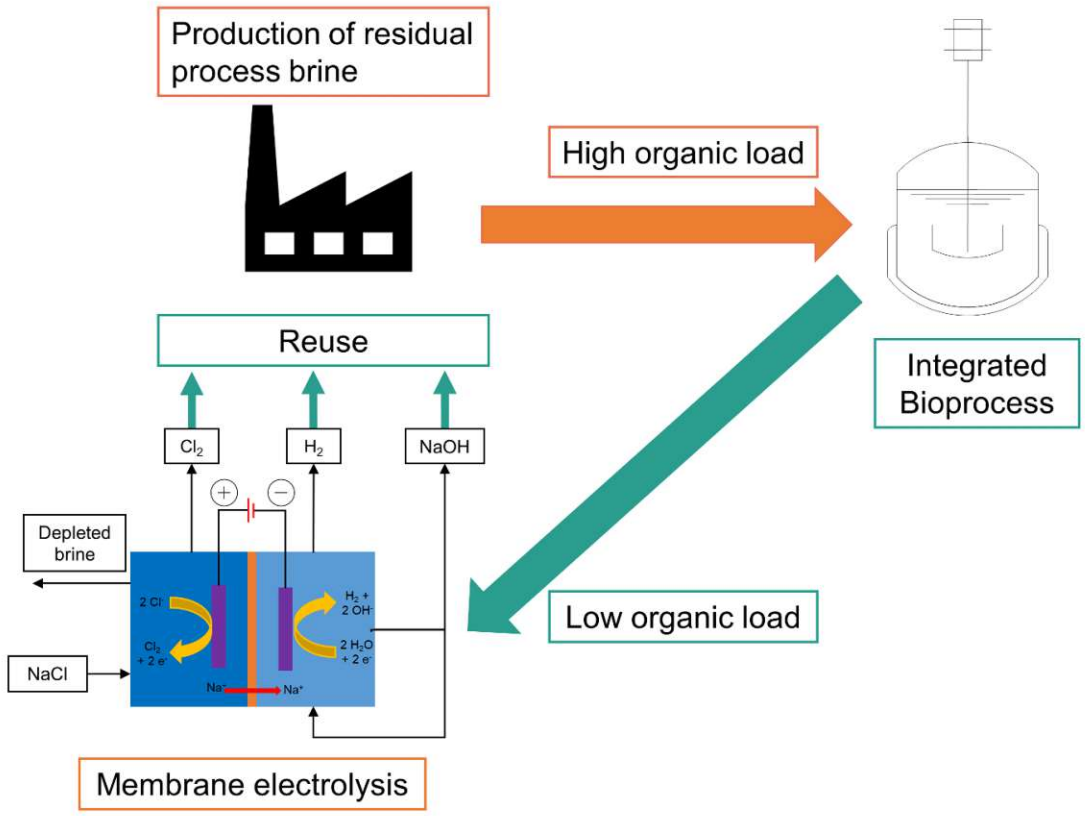

Figure 1. Schematic overview of a potential recycling strategy for industrial residual brine. From a production process, the residual process brine with a high organic load is transferred and treated in an integrated bioprocess using halophilic microorganisms. The treated brine with a low organic content can then be used in a chlor-alkali electrolysis step to produce H_2, Cl_2, and NaOH, which serve as raw materials for other industrial production steps (Bergner, 1982; Euro Chlor, 2021; Lakshmanan & Murugesan, 2014; Mainka et al., 2019; Schmittinger et al., 2011).

1.1.2 Residual process brine from polyurethane production

Polyurethanes (PU) are important polymers for the production of plastics, with a share of 7% in 2011 and 9% in 2016 among all plastic polymers in the European market (Kreye et al., 2013; Palm & Myrin, 2018). The main application forms of PU are soft or rigid foams (Gama et al., 2018). The industrial production of PU comprises the polyaddition reaction of a diisocyanate with a diol, in the presence of a tertiary amine, mostly triethylenediamine (TEDA) (Boros et al., 2018; Kreye et al., 2013). The most frequently used diisocyanate during PU production is methylene diphenyl diisocyanate (MDI), with a demand share of 61.3% (compared to 34.1% of toluene diisocyanate (TDI)) (Kreye et al., 2013; Schupp et al., 2018). The precursor of MDI is methylenedianiline (MDA), which is derived from the condensation reaction of aniline and formaldehyde (Figure 2A). Among the condensation products, 4,4'-methylenedianiline (4,4'-MDA) is the most abundant isomer (90-95%), compared to 2,4'-MDA (2-5%) and 2,2'-MDA (>1%) (Schupp et al., 2018). During the condensation reaction, an acid catalyst is used (mostly hydrochloride acid (HCl)). Thus, after the reaction, the mixture is neutralized using a base (mostly sodium hydroxide), and the organic phase containing the product (MDA) is separated from the aqueous phase by extraction and distillation steps (Bulan et al., 2019; Merenov et al., 2015; Thornton, 1968). Thus, the residual process brine derived from MDA production comprises an aqueous, alkaline (pH up to 13) solution containing sodium and chloride ions, residuals of the formate, and traces ($c < 20$ mg L^{-1}) of the aromatic compounds aniline and 4,4'-MDA (Figure 2A+C) (Heuser et al., 2005). In addition, also phenol ($c < 20$ mg L^{-1}) is frequently present in MDA residual process brine, as a residual from aniline production (Figure 2B) (Pohl et al., 2009). As organic impurities, formate and aromatic compounds should be removed if RPB from MDA production is used as raw material in a membrane cell CAE process. Moreover, the treatment of RPB comprising aniline and 4,4'-MDA is of interest when it should be disposed of, as both molecules were reported to potentially be carcinogenic (Bomhard & Herbold, 2005; McQueen & Williams, 1990; Schütze et al., 1995; Ward et al., 1991). Conventional treatment methods of RPB derived from MDA production are stripping out organic compounds by using steam and subsequent treatment with activated carbon (Bulan et al., 2019). However, activated carbon has a limited absorbance capacity and has to be renewed in intervals, resulting in high material costs. Also, ozonation can be used for the reduction of organic content in RPB is known. However, the generation of ozone (O_3) is energy-intensive, and the use of oxygen is costly (Bulan et al., 2019). In an electrolysis process using Boron-doped diamond anodes, the TOC levels of an MDA-derived RPB could be decreased with an efficiency of 95% (Muddemann et al., 2018). Although MDA and formate could be

reduced with an efficiency of >90%, aniline and phenol concentrations were only reduced with an efficiency of 73.1 and 74.9%, respectively.

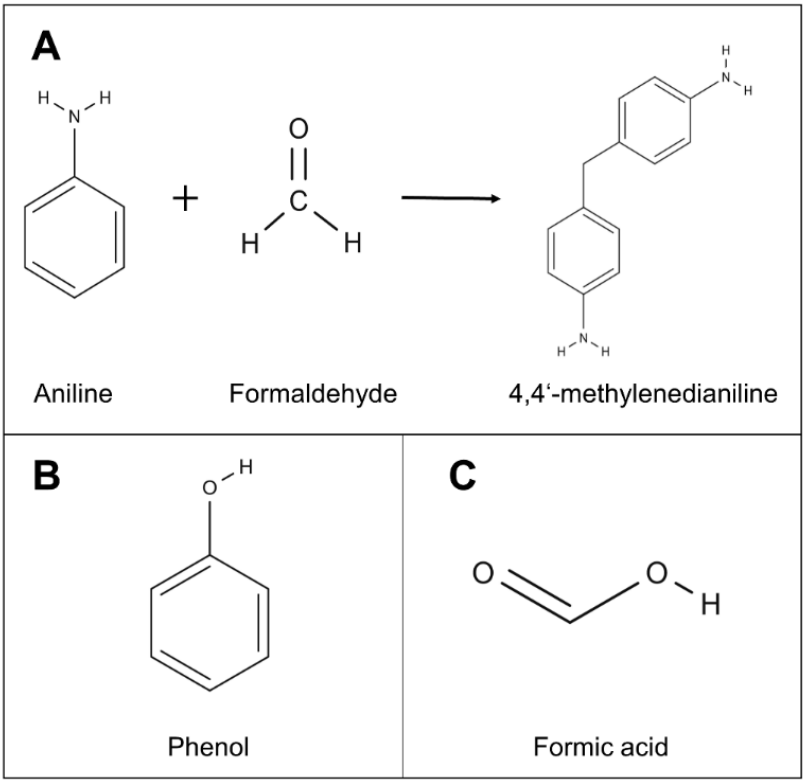

Figure 2. (A) Condensation reaction of aniline and formaldehyde to 4,4'-methylenedianline (Schupp et al., 2018). **(B)** Structure of phenol. **(C)** Structure of formic acid.

1.1.3 Halophilic microorganisms

For the biological treatment of saline RPB, the use of halophilic microorganisms is necessary, due to increased sodium chloride concentrations. Halophilic microorganisms are classified into four different groups, according to their level of salt tolerance: halotolerant, slight, moderate, and extreme halophilic microorganisms (Amoozegar et al., 2017). Halotolerant microorganisms are able to grow in saline environments but do not necessarily require high salt concentrations (Zhuang et al., 2010). In contrast, true halophiles are classified as slight (1–3% NaCl), moderate (3–15% NaCl), and extreme halophiles (15–30% NaCl) according to the salt concentration they require for growth (Fofonoff, 1985; Oren, 2002; Oren, 2008; Zhuang et al., 2010).

Generally, there are two different strategies for balancing osmotic pressure in high-salt environments, which are the salt-in and the compatible solute strategy (Amoozegar et al.,

2017; Gunde-Cimerman et al., 2018; Mainka et al., 2021; Oren, 2002; Oren, 2006; Oren, 2008). In the taxonomic group of halophilic archaea, the use of the salt-in strategy is widespread. Microorganisms using the salt-in strategy are accumulating inorganic ions (K^+ and Cl^-) intracellularly, in order to provide an osmotic balance (Margesin & Schinner, 2001; Oren, 2002; Oren, 2008). To do so, potassium ions can either be transported passively or actively into the cells. As the intracellular accumulation of negatively charged chloride ions would be repressed by the inside-negative membrane potential, an energy-dependent mechanism is required (Gunde-Cimerman et al., 2018; Oren, 1999).

In contrast, mostly halophilic bacteria produce or accumulate so-called compatible solutes. This group contains organic substances which are osmotically active and highly water-soluble. Prominent representatives of these molecules are glycerol or ectoine. Additionally, compatible solutes are reported to have stabilizing effects on DNA, enzymes, and whole cells against stress factors such as freezing, drying, and heating (Oren, 2002; Oren, 2006; Oren, 2008; Roberts, 2005; Shivanand & Mugeraya, 2011). Microorganisms accumulating compatible solutes, usually have high adaptability to changes in the extracellular salinity, compared to microorganisms using the salt-in strategy. In general, the accumulation of compatible solutes strategy is widespread among halophilic microorganisms, although it requires more energy than the intracellular accumulation of ions, because organic solutes have to be synthesized de novo (Gunde-Cimerman et al., 2018; Mainka et al., 2021; Oren, 2002).

Extremophilic microorganisms, like halophiles, offer a huge potential for industrial, medical, or cosmetical applications. They are a suitable source for producing highly valuable biomolecules like polyhydroxyalkanoates (PHA), pigments/carotenoids, or enzymes (Amoozegar et al., 2017; Corral et al., 2019; DasSarma et al., 2009; Delgado-García et al., 2018; Haque et al., 2020; Mitra et al., 2020). Moreover, extremophilic enzymes are very interesting for industrial purposes, as they are adapted to harsh conditions like low water activities, high or low pH, and high or low temperatures (Amoozegar et al., 2017; Delgado-García et al., 2018). For instance, a haloarchaeal alcohol dehydrogenase was reported to be stable in organic solvents (Alsafadi & Paradisi, 2013; Haque et al., 2020; Timpson et al., 2013).

1.1.4 Suitable bioprocess operation for integration into industrial process chains

To solve the problem of treating industrial residual brine with halophilic microorganisms and effectively degrade the organic contaminants, a suitable bioprocess is crucial. This bioprocess

should be designed in a way, that it should meet the requirements for implementation in an industrial environment. As during industrial production, residual process brines are generated in high volumes, and the bioprocess operation mode has to be chosen accurately. Out of the three typical operation modes (batch, fed-batch, and continuous), the continuous operation mode is seen as the most suitable for industrial integration, as it combines several advantages. For biological wastewater treatment processes, also sequencing batch reactors are frequently used, which work by sequential repetition of four phases (Amin et al., 2014; Deive et al., 2012; Golshan et al., 2019; Jiang et al., 2016b; Jiang et al., 2016c; Kayranli & Ugurlu, 2011; Martín-Hernández et al., 2009; Woolard & Irvine, 1995):

i) filling of the inoculated bioreactor with wastewater, ii) reaction and aeration time, iii) settlement phase where the cells sink to the bottom, and iv) removal of effluent with a specific volumetric exchange ratio.

However, the sequencing operation mode is also the major drawback of SBR, as high liquid throughputs of RPB require high reactor volumes. Thus, for treating industrial RPB, a continuous operation mode allows the application of higher volumetric rates. Additionally, as lower bioreactor volumes are necessary, higher space-time yields can be reached and lower energy inputs are needed (Konstantinov & Cooney, 2015; Schofield, 2018). A continuous operation mode moreover reduces potential cleaning times, compared to discrete batch or fed-batch processes (Croughan et al., 2015; Konstantinov & Cooney, 2015; Schofield, 2018; Vees et al., 2020; Zydney, 2015).

Potential drawbacks of continuous bioprocesses arise from higher technical requirements and complexities, as well as from a higher risk of contamination, due to a prolonged process time (Zydney, 2015). The latter problem can be overcome by the use of halophilic organisms which are adapted to the process conditions in saline RPB. However, cross-contamination might occur with other halophilic strains, which are also able to grow in the specific RPB.

During this work, a continuous bioprocess was used and extended with a membrane-based cell retention system. The general principle of a retentostat cultivation process is explained as follows (see Figure 3): a feed flow F_F is applied to the bioreactor which contains the growth medium with one or more substrates. Through an external loop line, cell broth from the bioreactor is circulated over a hollow-fiber membrane. A cell-free harvest flow F_H is withdrawn from the permeate side of the membrane. A smaller, cell containing bleed flow F_B is removed directly from the loop or the bioreactor. The system can be described by the liquid dilution rate D and the retention rate R with Equations 4 and 5, respectively:

$$D = {}^{F_F}/_{V_R} \tag{4}$$

$$R = {}^{F_H}/_{V_F} \tag{5}$$

Such a bioprocess system increases the potential throughput of RPB, as the specific growth rate μ of the microorganisms is decoupled from the liquid dilution rate D by the retention rate (or recycle ratio) R (Doran, 1995; Heckroth et al., 2019; Lorantfy et al., 2014a; Mainka et al., 2019):

$$\mu = (1 - R) * D \tag{6}$$

Thus, by applying high retention rates R, a lower substrate use is sufficient for maintaining the same biomass concentration as in chemostat cultivation. The addition of a co-substrate, which enables and/or enhances the growth of the used microorganisms depends on several factors. As the organic impurities can also serve as the sole carbon source for the microorganisms, the concentrations and the biomass formation yields have to be high enough to provide sufficient biomass concentrations during the process.

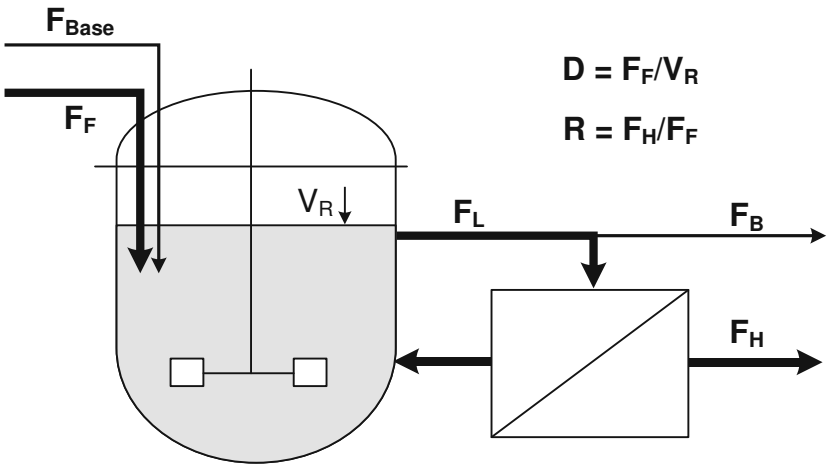

Figure 3. Scheme of the cell retention setup. A constant feed (F_F) supplies the cells with fresh substrate and media components. Base (F_{Base}) is added to hold the pH at a constant level. A pump continuously circulates the cell suspension as loop flow (F_L) through the membrane module to separate cell-free harvest (F_H). Bleed flow (F_B) is continuously removed to eliminate cells and sustain steady-state conditions. To guarantee a constant reactor volume (V_R) flow for Feed, Base, Harvest and Bleed have to meet the following equation: $F_F + F_{Base} = F_H + F_B$. Biomass is monitored using a turbidity probe and a soft sensor that is driven by measurements of off-gas composition (Mainka et al., 2019).

If concentrations of organic impurities are low or only a small part is incorporated to form biomass, an additional growth substrate has to be introduced in the bioprocess. As the supplement of a co-substrate is an additional cost factor, a sustainable substrate and an optimized feed strategy are crucial for the industrial integration of a treatment bioprocess.

Glycerol, acetate, and/or succinate could serve as sustainable co-substrates in a biological treatment process, as their production can be ensured by sustainable raw materials (Bucheli-Witschel et al., 2009; Khan et al., 2011; Luthfi et al., 2017; Mainka et al., 2022; Mainka et al., 2019; Novak & Pflügl, 2018; Pflügl et al., 2014; Rasool et al., 2015; Rüegg et al., 2007; Russmayer et al., 2019; Shan et al., 2009; Tan et al., 2016). Also, the feeding strategy of the co-substrate is important, as it was reported in retentostat cultures, that cells are prone to co-utilize substrates which are not co-utilized during carbon excess conditions, including organic compounds like benzoate (Marozava et al., 2014).

Moreover, the use of cell retention systems can prevent cell washout, compared to conventional CSTR processes, which might accelerate the adaptation of microbial systems to changes in wastewater compositions (Jang et al., 2013). Besides a membrane-based cell retention systems, also (i) the immobilization of cells magnetically inside the bioreactor (Jiang et al., 2016a; Wang et al., 2007a), (ii) biofilm cultivations (Gebara, 1999; Qureshi et al., 2004), or (iii) acoustic retention using ultrasound (Chisti, 2003; Gorenflo et al., 2002) are suitable technologies. However, in municipal and industrial wastewater treatment processes, membrane-based approaches are widely used (Henze et al., 2008; Marrot et al., 2004; Neoh et al., 2016; Santos et al., 2011). Thus, the application of membrane-based bioprocesses is widely accepted in the industry.

In addition to the process operation mode, the industrial integration of a biological treatment process also requires a suitable bioreactor design, which enables not only efficient degradation of impurities but also allows a cost-effective operation. In biotechnology, the most abundant bioreactor design is a stirred tank reactor (STR), where the cell suspension is mixed inside the reactor vessel mechanically. Continuous stirred tank reactors (CSTR) were shown to be suitable for the treatment of industrial residual process waters and brines (Gargouri et al., 2011; Papadimitriou et al., 2009). In contrast, bubble column reactor (BCR), the mixing of the liquid and gaseous phase is provided without mechanical stirring but by the sparging of the gas (Rollbusch et al., 2015; Sánchez Pérez et al., 2006). However, volumetric energy inputs are lower in BCR compared to STR, when the same oxygen transfer rates (OTR) should be reached (Mahler et al., 2018). Therefore, as BCR requires lower energy input compared to STR, operational expenditures can be lowered. In addition, BCRs are widely used in the chemical industry (Rollbusch et al., 2015). Thus, the acceptance of the chemical industry towards the integration of bioprocess unit operations into chemical production chains can be improved when BCRs are used. The feasibility of using a BCR with a membrane-based cell retention unit for the treatment of MDA residual process brine has been shown previously (Figure 4) (Mahler et al., 2018). During this study, a 21 L BCR (15 L working volume) was

operated continuously for 35 hours. Therefore, the presented BCR depicts a promising pilot-scale model for the implementation of an RPB treatment bioprocess in an industrial context.

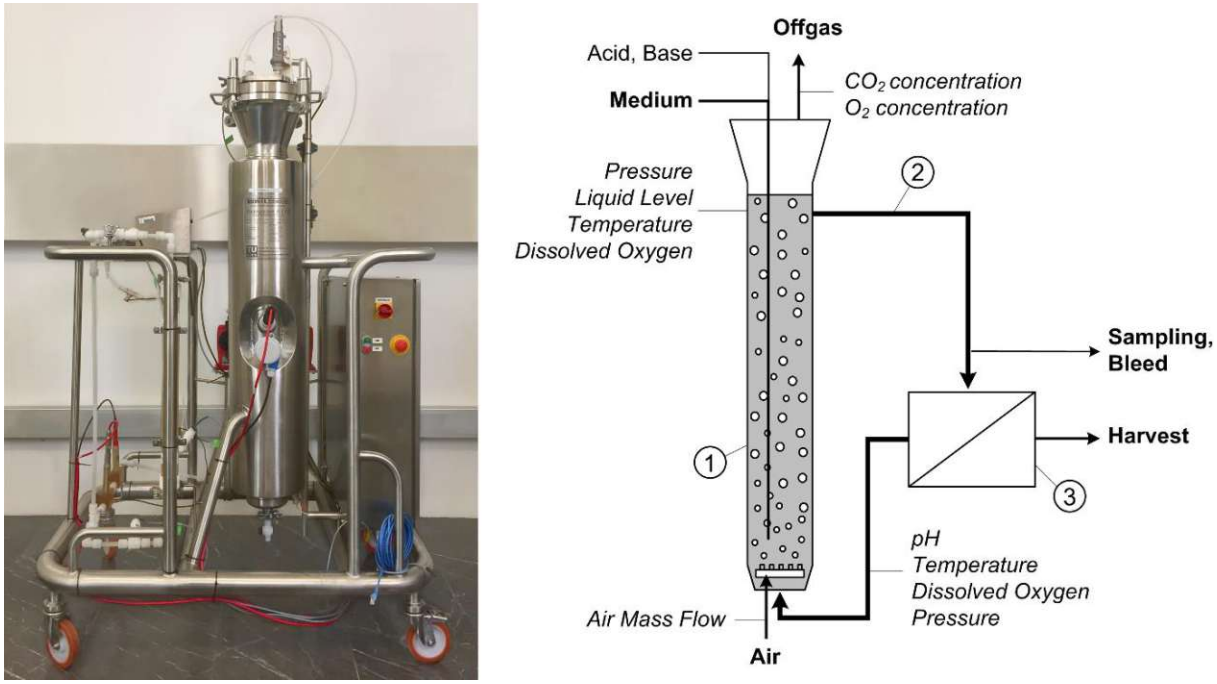

Figure 4. Schematic diagram of the BCR: (1) Pressure resistant bubble column vessel, (2) continuously circulating loop driven by a diaphragm pump, and (3) microfiltration unit for cell retention. Probes are indicated in italic, and continuous fluid flows are indicated in bold (Mahler et al., 2018).

1.1.5 Halophiles for the treatment of industrial residual process brine

For the treatment of process brines, a suitable halophilic microbial system has to be selected according to (1) the corresponding sodium chloride concentration in the brine, and (2) the present organic impurities to enable efficient degradation. Moreover, a suitable microbial system should be robust against changes in brine composition and/or salinities, which might occur in industrial RPB.

Various halophilic microorganisms have been reported to be able to degrade organic substances in process brines (Castillo-Carvajal et al., 2014; Fathepure, 2014; Le Borgne et al., 2008). As mentioned above, the industrial residual process brine used during this study contained four organic impurities: formate, aniline, phenol, and 4,4'-methylenedianiline. The main organic impurity, formate, is usually oxidized to CO_2 via enzymes, which are classified into two different groups (Niks & Hille, 2019). The first group consists of metal-independent,

but NAD^+-dependent formate dehydrogenases, which oxidize formate according to Equation 7 (Alpdağtaş et al., 2021; Tishkov & Popov, 2006):

$$HCOOH + NAD^+ \leftrightarrow CO_2 + NADH \quad (7)$$

The second group are metal-containing formate hydrogen lyases, which have molybdenum or tungsten containing components (Equation 8) (Maia et al., 2017; Niks & Hille, 2019; Yu et al., 2017):

$$HCOOH \leftrightarrow CO_2 + H^+ + 2e^- \quad (8)$$

Among halophiles, the halophilic bacterium *Halomonas* sp. MA-C was reported to degrade formate. However, only less than 5% of formate was incorporated into biomass, as shown by experiments using ^{14}C-labeled formate (Azachi et al., 1995; Heckroth et al., 2018b; Oren et al., 1992).

The degradation of aromatic compounds, like aniline or phenol, has frequently been studied for halophilic microorganisms (Arora, 2015; Bonete et al., 2015; Castillo-Carvajal et al., 2014; Fuchs et al., 2011; Krzmarzick et al., 2018; Li et al., 2019a; Nogales et al., 2017). For the aerobic and anaerobic degradation of small aromatic compounds, several pathways and the involved enzymes were described (Arora, 2015; Corti Monzon et al., 2018; Fuchs et al., 2011). For the aerobic degradation of small aromatics like aniline or phenol, two main pathways, the meta- and the ortho-cleavage pathway, exist (Figure 5). Both pathways start with an initial hydroxylation reaction, which leads to the formation of the intermediate catechol. While during the meta-cleavage pathway, 2-hydroxy muconic semialdehyde is formed as the next intermediate, during the ortho-cleavage pathway, muconic acid is formed. Ultimately, the intermediates of both pathways are incorporated into the TCA cycle (Corti Monzon et al., 2018; Fuchs et al., 2011; Li et al., 2019a). Halophilic microorganisms which are able to degrade aromatic compounds are mostly found in seawater, salt lakes, or soils, contaminated with industrial waste (Dehvari et al., 2020; Díaz et al., 2002; Feng et al., 2018; Lofthus et al., 2018; Tan et al., 2017b; Wang et al., 2007b). Thus, industrial sites were shown to be excellent places to exploit microbial diversity in the context of a residual process brine treatment.

Although the degradation of aromatic compounds present in industrial RPB was reported for various halophilic microorganisms, bioprocesses using real industrial process brines are scarce. Mostly synthetic brines are used in academic studies and organic impurities are added manually (Heckroth et al., 2018a; Praveen & Loh, 2016; Tan et al., 2017a; Wang et al., 2017). The slightly halophilic strain *Oceanomonas* sp. was reported to degrade phenol in saline wastewater (0-6.5% NaCl) (Tan et al., 2017a). The degradation process was carried out in a

cell retention system by immobilizing *Oceanomonas* cells to a polypropylene fiber in a 10L bioreactor. For the extremely halophilic archaeon *H. mediterranei*, continuous bioreactor cultivations showed the capability of degrading organic content from an MDA residual process brine (Heckroth et al., 2019; Mahler et al., 2018). Thus, *H. mediterranei* depicts a promising microbial system for the degradation of organic impurities in industrial RPB containing aromatic compounds.

In addition, also the use of halophilic mixed cultures offers a high potential for the treatment of RPB, as the chance of accumulating highly adapted species, which are able to degrade the occurring impurities, is improved (Mainka et al., 2021). For instance, an activated sludge consisting of three halophilic bacterial genera (*Oceanomonas* sp., *Arthrobacter* sp., *Vibrio* sp.) was reported to degrade phenol in a synthetic wastewater (Tan et al., 2017b).

However, so far, most bioprocess studies for the treatment of RPB using halophilic microorganisms lack investigations regarding the feasibility of integration into an industrial scope, like long-term studies, monitoring, and control strategies, or definition of cost-effective and optimized operating conditions.

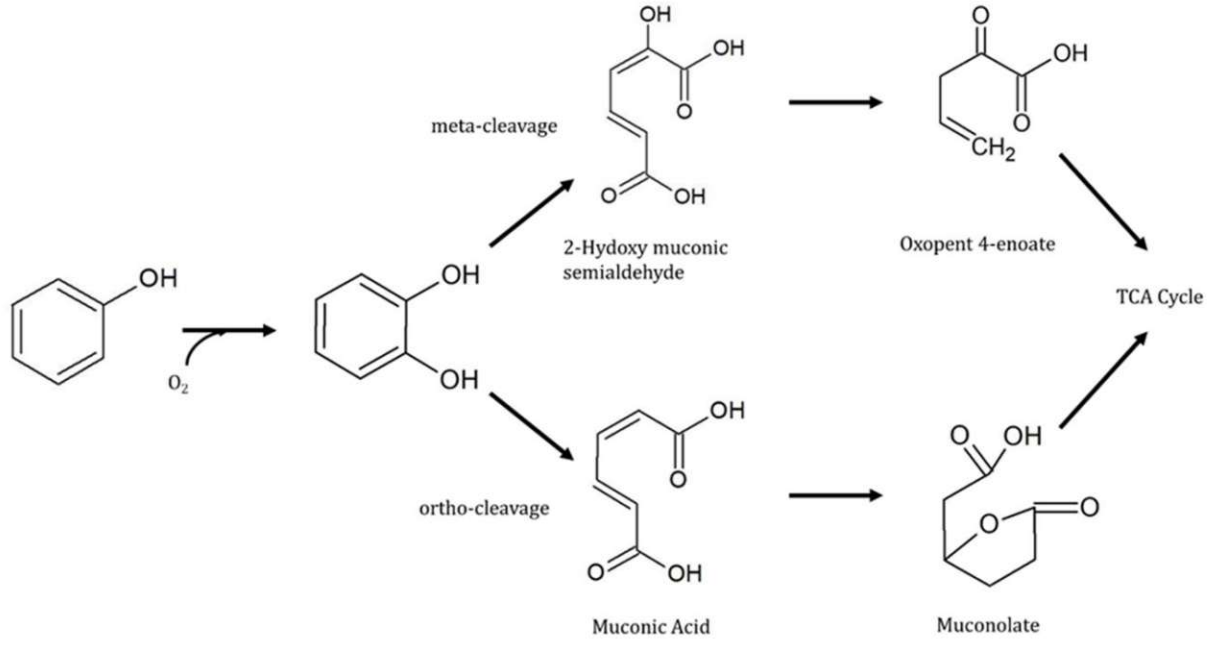

Figure 5. Aerobic pathway of phenolic degradation (meta- and ortho-cleavage) (Li et al., 2019a; Mainka et al., 2021).

1.2 Aim and structure of this thesis

This thesis aimed the development and characterization, of an industrial mature biological treatment process for the efficient reduction of organic impurities, present in an industrial residual process brine (RPB), by using halophilic microorganisms. To that end, the treated RPB, which is derived from the chemical production of 4,4'-methylenedianiline (MDA), should serve as a raw material for a membrane cell CAE process. However, brines used in membrane cell chlor-alkali electrolysis processes require a low organic content (TOC: 1-10 ppm). This thesis aims, therefore, (i) to propose a bioprocess, which is capable of degrading the organic impurities (formate, aniline, phenol, and MDA) in an industrial scope and (ii) to provide process operating conditions suitable for the implementation in an industrial production chain. Furthermore, (iii) reliable analytical tools to monitor, control and evaluate the bioprocess should be established.

Acceptance criteria for achieving these goals were, that (i) a robust microbial system, which achieves constantly high degradation efficiencies over a prolonged cultivation time under unsterile and changing environmental conditions, is provided, (ii) cost-efficient and optimized operating conditions by achieving nutrient-limitations for essential growth elements (C, N, and P) are applied while maintaining high degradation efficiencies, (iii) organic target molecules in RPB feed and bioreactor samples can be qualified and quantified reliably, and (iv) that specific growth rates and yields for halophiles can be estimated online and be used for a biomass monitoring and control strategy. Thus, this thesis is structured into four parts, which deal with individual steps of the process development for an industrial mature, biological treatment process to reduce organic content in a residual process brine (Figure 6).

The first part (chapter 2.1) of this work aimed at the implementation and long-term operation of a pilot-scale biological treatment process in an industrial environment. To do so, an established pilot-scale bioreactor system was put into operation at an industrial production site and was continuously operated for more than six months. During this work, the process robustness under real industrial conditions with changing RPB compositions was investigated. Therefore, the question should be answered if:

a stable and robust bioprocess be integrated into an industrial residual process brine production plant and be operated under changing raw material conditions for a prolonged cultivation time?

The experiments performed in the pilot-scale bioreactor at the industrial production site led to the discovery of a halophilic culture, which replaced the original inoculum culture of *H. mediterranei*. Therefore, this chapter also focuses on the identification of the found halophilic mixed culture as well as their process performance in a continuous RPB treatment process (Mainka et al., 2022).

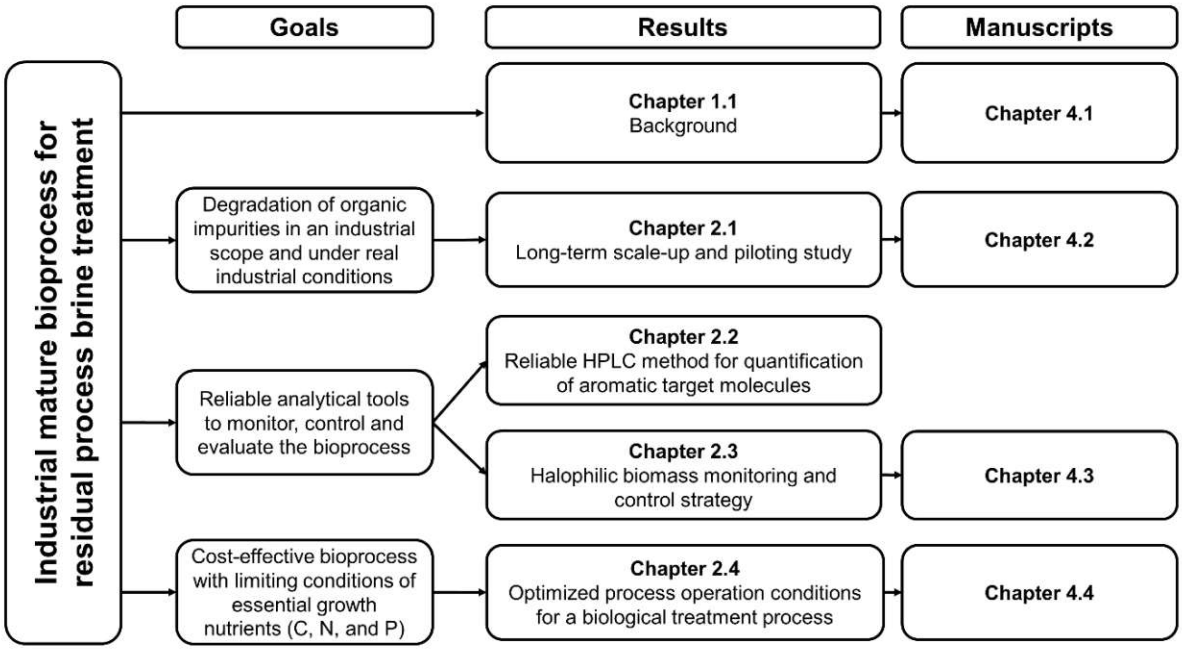

Figure 6. Structure of the thesis.

During the second part of this work (chapter 2.2), a reliable offline qualification and quantification approach of aromatic impurities in RPB feed and bioreactor samples should be developed, which is essential to evaluate the degradation efficiency of the proposed biological treatment process. Therefore, the following questions should be answered during this study:

How can peaks from bioreactor samples be distinguished from target molecules with similar retention times?

How can an HPLC method be improved to achieve reliable separation and identification of peaks occurring in bioreactor samples and the aromatic target molecules?

The developed method is able to robustly measure and distinguish aromatic impurities of the RPB feed from other peaks in bioreactor samples, based on efficient peak separation and identification tools.

The third part (chapter 2.3) aimed the development of an online, non-invasive biomass monitoring and control strategy for the cultivation of halophilic microorganisms. During this study, the following questions should be answered:

What are the requirements of a bioprocessing strategy when applied to the treatment of an industrial residual process brine?

How can a robust biomass monitoring strategy be established for use in an industrial-scale biological residual water treatment process?

Can a suitable biomass monitoring approach identify important physiological parameters of the microbial system?

Therefore, a feed-forward control strategy for a lab-scale bioprocess in a continuous operation mode, including a cell retention setup was developed. Combined with the soft sensor-based biomass estimation, a reliable monitoring, and control strategy for the biomass concentration was provided, which additionally delivers information about metabolic rates and physiological parameters like yield coefficients. The results of this work were published in a peer-reviewed journal paper (Mainka et al., 2019).

Finally, chapter 2.4 optimized process operating conditions should be proposed for the cost-effective operation of a biological treatment process at high degradation efficiencies. Therefore, additional media supplements should be minimized, and co-substrate feeding should be optimized to enhance process performance. Thus, the following questions arose and should be answered during this study:

Is it possible to establish a triple nutrient-limitation for the carbon, nitrogen, and phosphorus source and maintain an efficient degradation for organic impurities?

What is the influence of the co-substrate glycerol on the degradation efficiency of organic impurities?

Furthermore, other critical process parameters are investigated for their potential influence on process performance. Optimized process operation conditions are proposed to minimize operational expenditures and decrease efforts for preparation and polishing steps before the treated RPB is used for chlor-alkali electrolysis.

2 Results

2.1 Long-term scale-up and piloting study

Problem statement

To integrate a bioprocess for the treatment of industrial residual process brine into a chemical production chain, robustness and stability are required for the bioprocess and the used microbial system. Additionally, changing raw material conditions of the residual process might influence the degradation efficiency of the biosystem.

State-of-the-art

A bioprocess for the continuous cultivation of *Haloferax mediterranei* to treat a residual process brine derived from the production of 4,4'-methylenedianiline was successfully established (Mainka et al., 2019). The bioprocess was equipped with a membrane-based cell retention system, which allows the application of liquid dilution rates higher than the specific growth rates of microorganisms. The cultivation was performed for more than 54 days, however, under highly controllable laboratory conditions and only one residual process brine batch was used. Another lab-scale process was described for the removal of aromatic compounds in synthetic wastewater using an aerobic granular reactor (Franca et al., 2018; Ramos et al., 2016a; Ramos et al., 2015; Ramos et al., 2016b). There, long-term conditions were investigated, however, only synthetic, non-saline wastewater was used. Thus, simulating a real industrial environment (unsterile conditions) and changing residual process brine compositions were not addressed before. Additionally, a scale-able bioprocess platform for halophilic microorganisms suitable for pilot-scale studies has not yet been studied for its long-term applicability in industrial conditions (Mahler et al., 2018).

Goal of this study

This study aimed the implementation and long-term operation of a pilot-scale bioprocess into a chemical production chain for the continuous treatment of an industrial residual process brine.

Scientific question

Can a stable and robust bioprocess be integrated into an industrial residual process brine production plant and be operated under changing raw material conditions for a prolonged cultivation time?

Approach

We aimed to implement a pilot-scale bioprocess platform for the continuous treatment of MDA residual process brine into an industrial production chain. We hypothesized that by using a robust microbial culture, a long-term cultivation and successful degradation of organic impurities can be achieved. The combined use of a feed-forward control strategy and a membrane-based retentostats system should enhance the success of this study. In course of the study, a highly adapted and efficient degrading halophilic mixed culture was found as described to consist of mainly three halophilic genera. Using the discovered microbial system, the successful cultivation of more than 200 days was achieved at a high degradation efficiency for the present organic impurities.

Results and Discussion

Can a stable and robust bioprocess be integrated into an industrial residual process brine production plant and be operated under changing raw material conditions for a prolonged cultivation time?

One of the main reservations of integrating bioprocesses into industrial, chemical production chains is the long-term robustness and stability of a suitable microbial system, to ensure high degradation efficiencies during RPB treatment processes. To prove the applicability of a bioprocess for an industrial scope RPB treatment process, a pilot-plant bubble column bioreactor system was implemented at an industrial MDA production site and operated continuously for more than 210 days. Such a long continuous cultivation time under unsterile conditions faces a significant challenge regarding the stability of the microbial system. Hence, the microorganisms have to be highly adapted to the current environmental conditions.

During this study, a novel halophilic mixed culture, consisting of at least three different bacterial genera (*Halomonas* sp., *Aliifodinibius* sp., and *Oceanobacillus* sp.), was identified and characterized. So far, it is the first time that a halophilic mixed culture consisting of these three halophilic genera is described for use in a biological RPB treatment process. The described bacterial mixed culture was able to replace the original inoculation culture of the extremely halophilic archaeon *H. mediterranei* from the start of the cultivation. A possible reason for the replacement of *H. mediterranei* with the halophilic mixed culture could be an advanced adaptation to the RPB medium. This was proven, as the halophilic mixed culture showed higher growth rates in shake flask experiments than *H. mediterranei* when grown in the RPB medium. Although the original culture of *H. mediterranei* was replaced, the discovery and the use of novel mixed culture might be advantageous for the biological treatment process. Strains from the genera of *Halomonas* have already been described previously to efficiently degrade

aromatic compounds as well as formate (Alva & Peyton, 2003; Azachi et al., 1995; Govarthanan et al., 2020; Haddadi & Shavandi, 2013; Heckroth et al., 2018b; Oren et al., 1992; Piubeli et al., 2012; Tena-Garitaonaindia et al., 2019).

Moreover, the mixed culture showed a stable and robust culture throughout the entire process time and maintained equally high degradation efficiency. This is astonishing as the culture was not only robust against constant changes in the organic compositions of the RPB constantly changed, but also against potential, unmeasured variations in the RPB, which could influence the process performance.

During the piloting study, the successful integration of a pilot-scale bioprocess into an MDA residual process brine production plant was shown. The goal of establishing a stable and robust cultivation process as well as an efficient organic removal was not only achieved by using a novel and highly adapted mixed culture. Moreover, with the use of an intensified retentostats bioprocess, constantly high degradation efficiencies and sufficient biomass concentrations could be maintained throughout the entire cultivation time of more than 200 days. Using moderate biomass concentrations (OD_{600}) enabled the use of only one membrane unit for cell retention, without a necessary change or cleaning of the filter. Hence, combined with a low concentration of glycerol (1.5 g L^{-1} feed concentration) a cost-efficient process was provided.

One challenge for integrating a biological treatment process into the RPB production chain is the constantly changing concentrations of organic impurities between different RPB batches. Concentrations of aromatic compounds in the RPB feed varied significantly between the batches. Nevertheless, all aromatic impurities present originally in the RPB were degraded completely during the whole cultivation time of >200 days, underlying the efficiency of the found halophilic mixed culture. Besides, the results showed that aniline concentrations in the RPB feed are positively correlated to the accumulation of a potential, yet unknown intermediate, found in harvest samples. Also, the feed concentrations of the main organic impurity, formate, varied significantly between RPB batches. Degradation efficiencies of 90-98% were reached and did not decrease over a prolonged cultivation time. Residual concentrations of formate in harvest samples were low (10 and 20 mg L^{-1}), which corresponds to theoretical TOC levels of 3–6 ppm, which is below TOC specifications for chlor-alkali electrolysis processes. In addition, it was shown that limiting conditions for the co-substrate are required for a sufficient formate degradation. When the growth substrate glycerol was overfed and thus, accumulated in the bioreactor, also the degradation efficiency for formate was decreasing. The results of the present study further indicated a correlation between the glycerol feeding and the formate

degradation, as applying the lowest glycerol concentration combined with the highest dilution rate, the lowest residual formate concentrations were reached.

Outlook

The found novel halophilic mixed culture showed high degradation performance in a biological RPB treatment process. Although three halophilic genera could already be identified, the identification of single present strains should be performed. So far, further molecular biological tests indicated the presence of at least the halophilic strain of *H. organivorans*, a halophilic bacterium known to degrade organic pollutants in residual brines. Hence, further characterization of the mixed culture could investigate the specific roles of the single strains in the degradation of the organic impurities. Specifically, the investigation of *Aliifodinibius* strains and their ability to degrade organic impurities in residual brines could contribute to the diversity of halophilic bacteria. Additionally, the use of the novel found mixed culture for the treatment of other residual process brines, even with other salt concentration ranges, could be investigated.

Besides, the important role of glycerol in the degradation of formate should be further investigated to increase process knowledge and improve the cost-efficiency of the process. To do so, and to increase process flexibility, the RPB feed could be decoupled from the co-substrate feeding by applying an additional glycerol feed to the system. Furthermore, a potential correlation between glycerol feeding and formate degradation should be investigated to minimize the use of glycerol and optimize the formate degradation efficiency. Also, the identity of the unknown intermediate substance during aniline degradation, as well as its potential influence in further chlor-alkali electrolysis should be investigated. Ultimately, the present study underlines the potential of an alternative and sustainable bioprocess for treating residual process water, and once more emphasizes the possibilities, which natural microbial diversity offers for exploitation in an industrial context.

2.2 Reliable HPLC method for quantifying mono-aromatic compounds

Background and problem statement

Aromatic compounds, like aniline, phenol, or many vitamins, can be qualified and quantified using various offline analytical methods, like high performance liquid chromatography (HPLC) or gas chromatography (GC) (Cordin et al., 2021; Hofer & Herwig, 2017; Jen et al., 2001; Trost et al., 1997; Zhao et al., 2002). To detect aromatic compounds, several detection approaches are available, among the most used are flame ionization (FID), mass spectrometry (MS), or ultraviolet (UV) light (Brunmark et al., 1992; Dearth et al., 1992; Hofer & Herwig, 2017; Mainka et al., 2022; Tiljander & Skarping, 1990; Trost et al., 1997).

For the reduction or removal of aromatic compounds from industrial residual process brine (RPB) using biological treatment processes, online monitoring of aromatic target molecules could be helpful, especially in terms of real-time control strategies (Mainka et al., 2021). Therefore, several online monitoring approaches for the determination of aromatics were reported (Buerck et al., 2001; Gutés et al., 2005; Jen et al., 2001; Korkut et al., 2016; Mu, 2006; Rahemi et al., 2020; Zhang & Li, 2009). However, offline (re-)calibrations are still necessary to ensure reliable measurements of the online methods.

Samples from industrial RPB usually contain only the aromatic target molecules, which should ultimately be degraded in a biological treatment process. In contrast, samples derived from bioreactors after the treatment are frequently more complex, as potential intermediate substances might occur. Therefore, offline analytics for bioremediation processes are necessary which achieve a reliable peak separation of target molecules and other substances occurring in bioreactor samples.

The industrial RPB, used during this study, comprises three small aromatic compounds (aniline, phenol, and 4,4'-methylenedianiline), in concentrations below 20 mg L^{-1} (Mainka et al., 2022). Thus, as the concentrations of the target molecules are relatively low, a sensitive quantification method is crucial. Furthermore, the RPB contains a high NaCl load (8-15%), which has to be considered during the analytical procedure. Therefore, HPLC methods are considered to be the most suitable analytical approach to quantify aromatic impurities in the used industrial RPB.

An HPLC method for the quantification of aromatic compounds in an industrial RPB was previously described (Mainka et al., 2022). However, during the analysis of bioreactor samples from a biological piloting study for the continuous treatment of an industrial RPB (Mainka et

al., 2022), a peak shift was observed during the measurement of the same bioreactor samples at two different HPLC runs, with the same method. The peak had a similar retention time as aniline. As the analysis and quantification of aromatic impurities are essential for the interpretation of the biological treatment process efficiency, a reliable measurement of aromatics is crucial. Therefore, during this study the following questions should be answered:

- *How can peaks from bioreactor samples be distinguished from target molecules with similar retention times?*
- *How can an HPLC method be improved to achieve reliable separation and identification of peaks occurring in bioreactor samples and the aromatic target molecules?*

Thus, we hypothesized that using an adapted HPLC method and advanced peak detection methods (UV-spectrum and dual-wavelength absorption ratios) improves identifying and distinguishing aromatic target molecules from potential intermediate substances present in bioreactor samples.

Material and Methods

Used chemicals and residual process brine

Pure aromatic substances aniline, phenol, and catechol were purchased from Sigma-Aldrich. The project partner, Covestro AG, provided pure 4,4'-methylenedianiline and the residual process brine derived from the industrial production of MDA.

Sample and standard preparation

Prior to analysis, samples derived from RPB feeds and bioreactor cultivations were centrifuged for 10 minutes at 4 °C and 14,000 rpm. The supernatant was removed from the pellets and was used for analysis undiluted.

Standards of the aromatic target molecules were dissolved in water at a concentration of 20 mg L^{-1}. Afterwards a dilution series was prepared to reach concentrations of 0.1, 0.5, 1.0, 2.0, 5.0, and 10.0 mg L^{-1}.

HPLC analysis: method 1

For the quantification of aromatic compounds in feed and harvest samples, a reversed-phase HPLC measurement (U3000 UHPLC systems, Thermo-Fisher, USA) was carried out, using an Acclaim™ PolarAdvantage column (Thermo Scientific, USA, C16, 3 µm, 120 Å, 4.6x150 mm) at 30 °C (Mainka et al., 2022; Mainka et al., 2019). Aromatic compounds were detected using

a UV detector at 210 nm. The flow was 1 mL min^{-1} with a gradient system (see Table 1). The eluents were A) acetonitrile; B) 25 mM KH_2PO_4 (pH 3.5 with 1 M H_3PO_4); and C) MiliQ water. The injection volume of samples and standards was set to 10 µL.

Table 1. HPLC gradient program (method 1) for quantification of aromatic compounds in an industrial RPB.

t [min]	0	2.5	2.5	5	20	20	25.5	35	35	40
% A	5	5	25	25	70	25	5	5	5	5
% B	95	95	75	75	30	0	0	0	95	95
%C	0	0	0	0	0	75	95	95	0	0

HPLC analysis: method 2

Quantification of aromatic compounds in feed and harvest samples was performed using a reversed-phase HPLC measurement method (Vanquish UHPLC systems, Thermo-Fisher, USA) with an Acclaim™ PolarAdvantage column (Thermo Scientific, USA, C16, 3 µm, 120 Å, 4.6x150 mm) at 30 °C (Mainka et al., 2022; Mainka et al., 2019). Aromatic compounds were detected using a UV detector (diode array detector) at different wavelengths. The flow was set to 0.6 mL min^{-1} and a gradient system was used (see Table 2). Eluents were A) acetonitrile; B) Milli-Q water. The injection volume of samples and standards was set to 10 µL.

Table 2. HPLC gradient program (method 2) for quantification of aromatic compounds in an industrial RPB.

t [min]	0	5	5	10	33	35	40	45
% A	5	5	25	25	70	70	5	5
% B	95	95	75	75	30	30	95	95

Data analysis

Chromatograms were analyzed using Chromeleon™ 7 Chromatography Data System (Version 7.2, Thermo Scientific™, USA). Samples were derived from a continuous bioprocess treating an industrial RPB, which was published previously (Mainka et al., 2022).

Results and discussion

During a previously published study, aromatic compounds (aniline, phenol, and 4,4'-MDA) in an industrial RPB should be degraded using halophilic microorganisms (Mainka et al., 2022). In HPLC measurements of bioreactor samples taken during the process, shifts in a peak, which was previously identified as aniline, were detected. The shifts were observed as the same bioreactor samples were measured at two different HPLC runs, both using method 1 (see material and methods section). In addition, the aniline peaks in chromatograms derived from RPB feed samples did not shift and were retained at similar times as the aniline standards (Figures 7 and 8). However, in the chromatogram derived from HPLC run 2, the "aniline" peak of the bioreactor sample was shifted to the left, when compared to the chromatogram derived from HPLC run 1. Therefore, it was assumed that the "aniline peaks" in chromatograms of bioreactor samples are not actually aniline, but another, yet unidentified substance.

To confirm this hypothesis, dual-wavelength absorbance ratios (197 nm and 230 nm), were calculated for the "aniline" peak in a bioreactor sample and compared with the peaks from an aniline standard and an RPB feed sample (Figure 9) (Drouen et al., 1984; Marr et al., 1990; Tiljander & Skarping, 1990). Assuming the "aniline" peak of the bioreactor is aniline, the absorbance ratio should be equal to the absorbance ratios of the aniline standard. Similar absorbance ratios were indeed observed for the aniline standard and aniline from RPB feed samples at both HPLC runs 1 and 2. However, absorbance ratios calculated for the "aniline" peak of the bioreactor sample were less than half of the value, calculated for the aniline standard peak areas (Figure 9). Therefore, the results of the HPLC measurements indicate, that no aniline is present in the bioreactor sample and aniline from RPB feed was metabolized completely by the microorganisms. Thus, it is assumed, that another metabolite was formed during the process, which has a similar retention time as aniline when using the HPLC method 1. The metabolite measured at the retention time of aniline, however, might be an intermediate of the microbial aromatic degradation pathway.

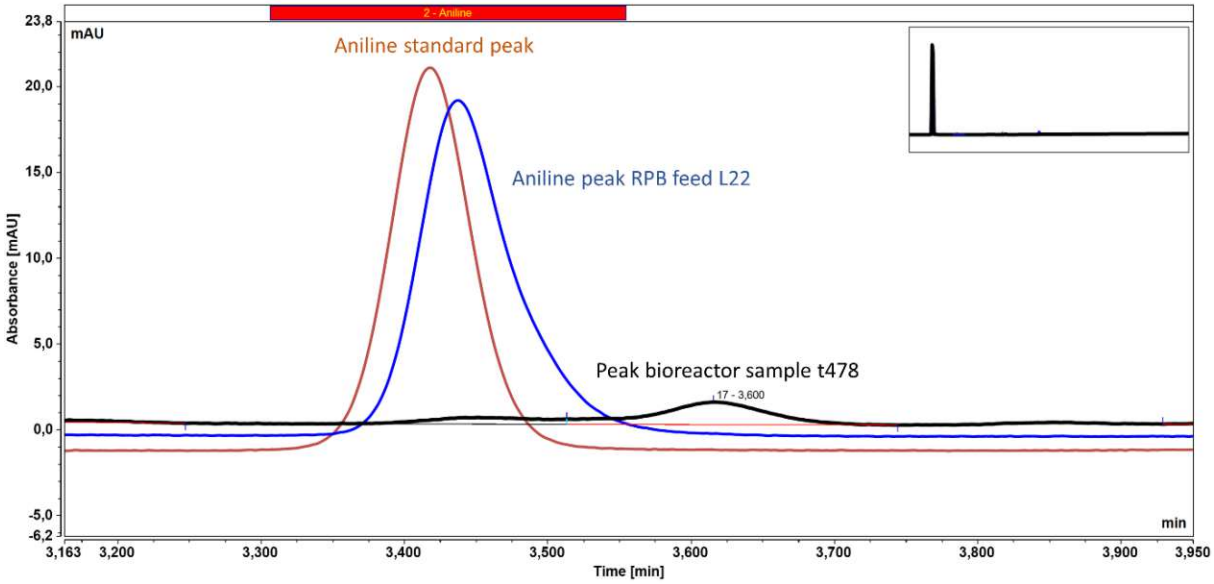

Figure 7. Aniline peaks in chromatograms of HPLC run 1. Chromatograms of bioreactor samples (t478), feed sample, and aniline standards (c = 1 mg L^{-1}). The HPLC runs were performed with the same method.

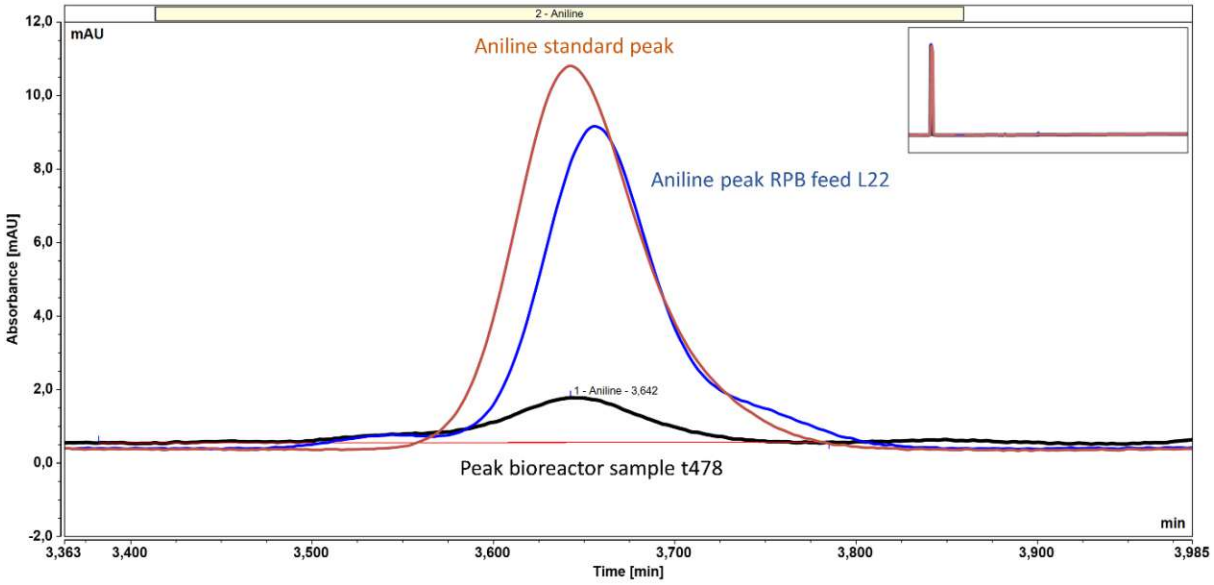

Figure 8. Aniline peaks in chromatograms of HPLC run 2. Chromatograms of bioreactor samples (t478), feed sample and aniline standards (c = 1 mg L^{-1}). The HPLC runs were performed with the same method.

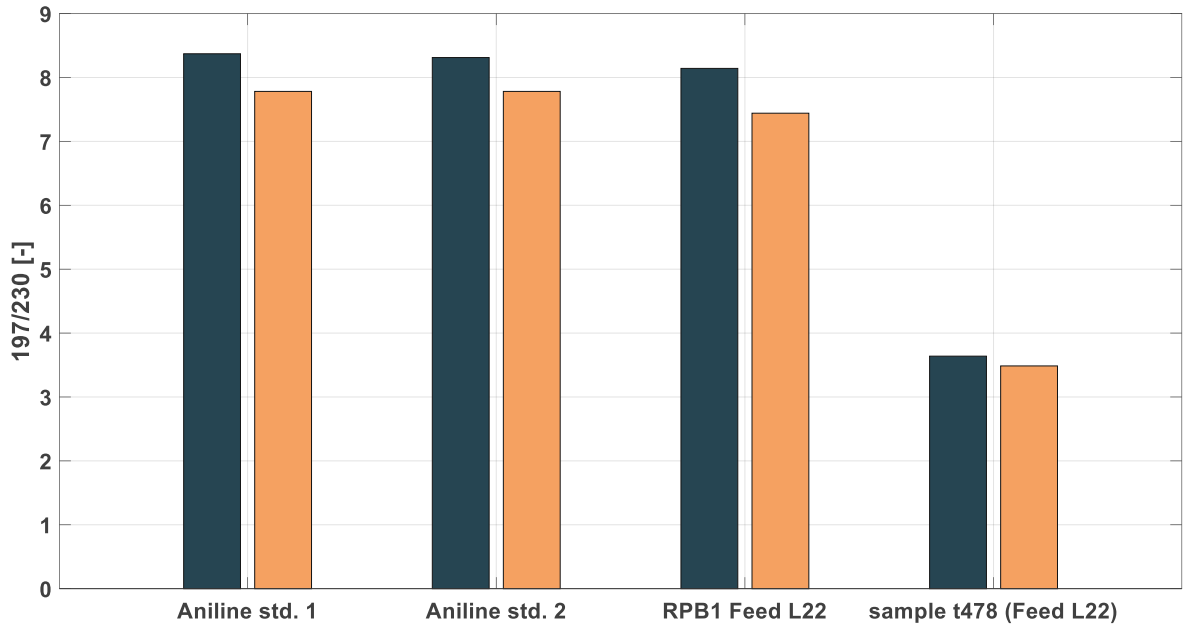

Figure 9. Dual-wavelength absorbance ratios of peak areas were integrated from peaks at $\lambda_{ab.}$ = 197 nm and $\lambda_{ab.}$ = 230 nm. Left bars (dark): HPLC run 1; right bars (light): HPLC run 2.

To further investigate aromatic compounds in bioreactor samples, HPLC method 1 was adapted in a way, that no buffers were used anymore, in order to avoid potential operator effects. In the new HPLC method (indicated as HPLC method 2), Mili-Q water and acetonitrile were used as eluents. Thus, it was possible, that peaks of potential intermediates in bioreactor samples were separated from peaks of the target aromatics. Furthermore, peak identification was improved by measuring the UV spectra of each peak. Thus, peaks could be compared with reference standards and target aromatics could be distinguished from potential intermediated eluted at similar retention times. Such a procedure was already reported for the confirmation of peaks with their corresponding reference standards (Wang et al., 2004). Aniline showed UV absorption maxima at three different wavelengths, 195 nm, 232 nm, and 283 nm (see Figure 10). Additionally, absorption maxima of three other small aromatic compounds (phenol, catechol, and 4,4-MDA) were determined (Table 3). Thus, the comparison of absorption maxima allows the specific identification during the HPLC measurement, as non-overlapping maxima were determined. Therefore, peak integration for the specific aromatic compounds should be performed at the specific absorption maxima wavelengths. By doing so, the sensitivity of the HPLC quantification method can be increased, as the limit of detection can be improved. It is suggested to analyze and integrate peaks for aniline at 230 nm, phenol at 212 or 270 nm, and 4,4'-MDA at 240 nm.

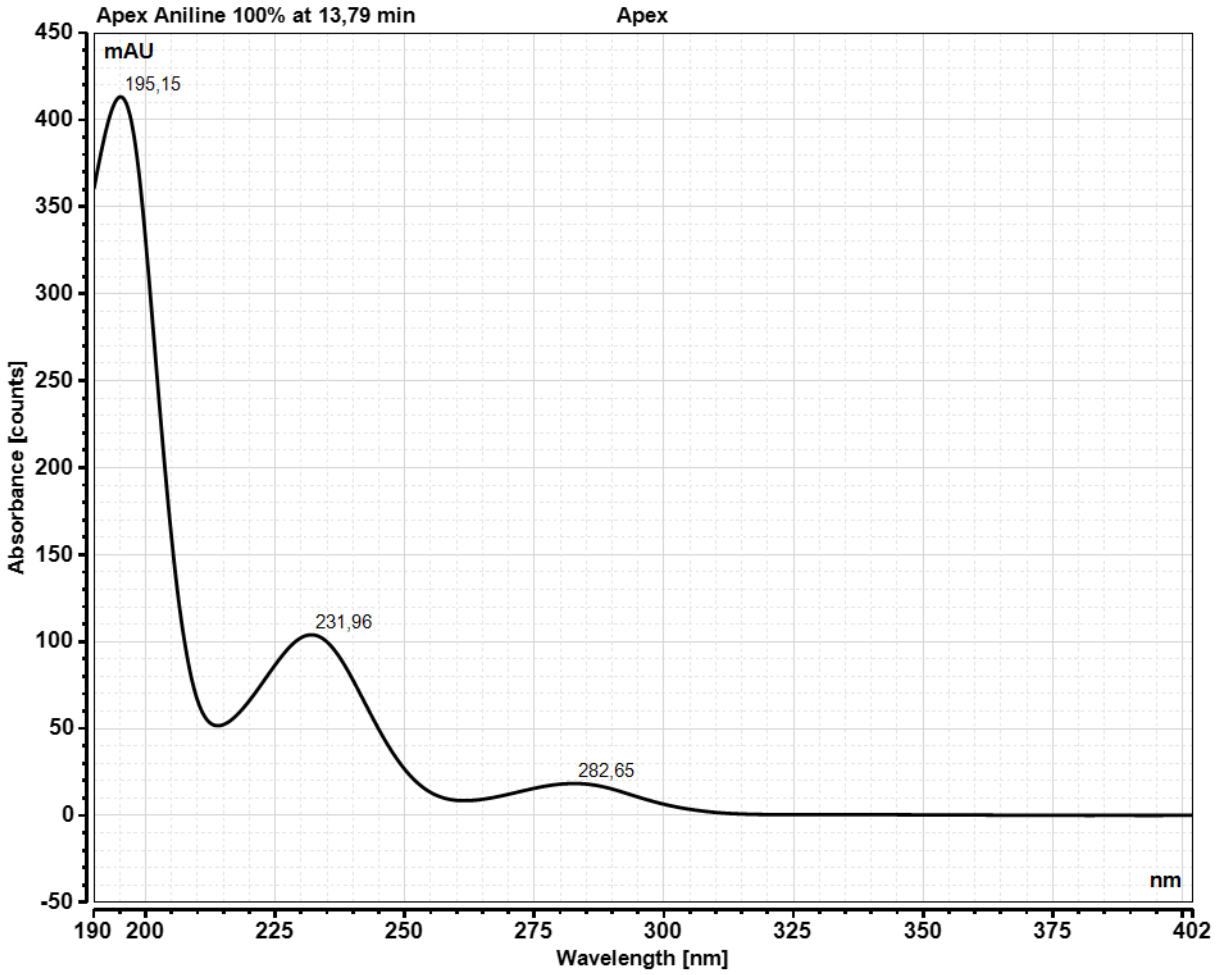

Figure 10. UV absorption spectrum of the aniline standard peak (retention time: 13.79 min).

The results of the aromatic absorption spectra are in line with literature values. For aniline, strong UV absorption was reported for ranges of 225-235 and 280-290 nm (King et al., 2010; Roberts et al., 2012; Thompson et al., 2015). For phenol, also previously published studies reported absorption maxima at two wavelength ranges (210 nm and 268-273 nm) (Dearden & Forbes, 1959). Absorption maxima were also reported for catechol to be 274 nm (Nikolic et al., 1998; Pillar-Little et al., 2015).

Table 3. UV absorption maxima of small aromatic compounds.

Aromatic compound	Absorption maximum λ_1 [nm]	Absorption maximum λ_2 [nm]	Absorption maximum λ_3 [nm]
Aniline	195-196	231-232	282-283
Phenol	211-212	270-271	
4,4-methylenedianiline	199-200	243-244	289-290
Catechol	193-194	276-277	

The developed HPLC method could furthermore successfully be applied for the quantification of aromatic compounds in RPB feed and bioreactor samples. The aromatic target molecules aniline, phenol, and 4,4'-MDA in RPB feed samples were effectively qualified and quantified (Figure 11). Moreover, after biological RPB treatment, aniline, phenol, and 4,4'-MDA could not be detected in bioreactor samples, and no overlapping of other peaks with the target molecule peaks was observed.

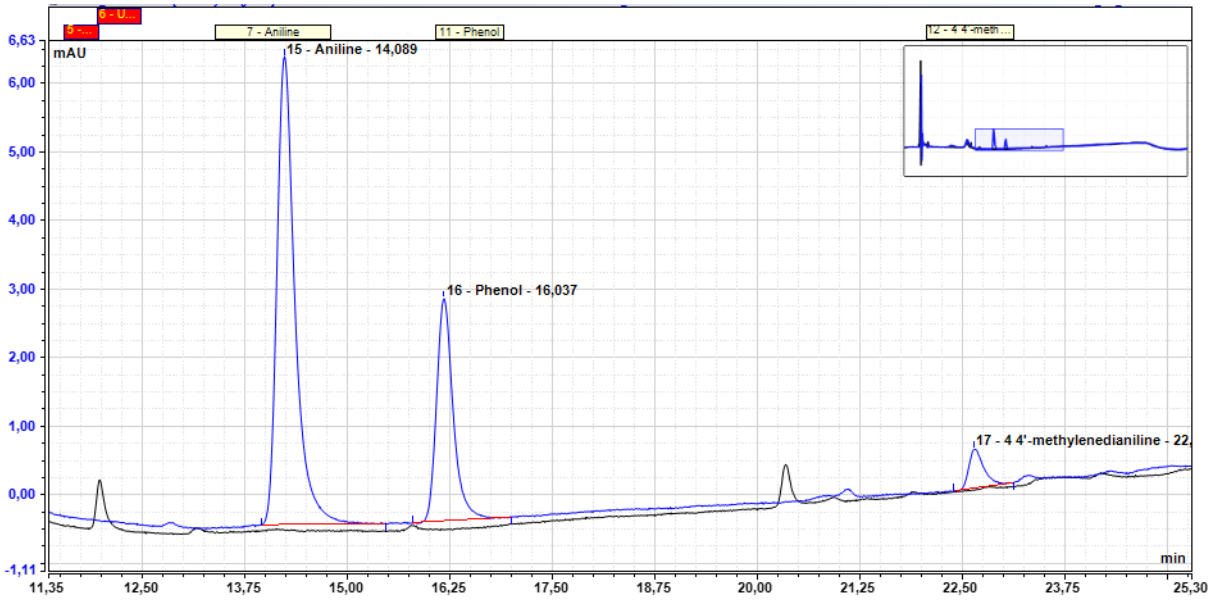

Figure 11. HPLC chromatogram for RPB feed (blue) and bioreactor (black) samples at λ=230 nm. As indicated in the chromatogram, the three aromatic target molecules (aniline, phenol, and 4,4'-MDA) are separated from any peaks in bioreactor samples.

Conclusion

During continuous bioreactor cultivations, an HPLC method for the quantification of aromatic compounds in an industrial residual brine was shown to be unsuitable, as a peak in bioreactor samples was eluting at a similar retention time as the aromatic target molecule aniline. Using dual-wavelength absorbance ratios, it was demonstrated that the peak in the bioreactor samples was not aniline, but a yet unidentified substance. Thus, to avoid potential interferences and wrong interpretations of HPLC chromatograms, the existing HPLC method was adapted, and it was demonstrated that the novel HPLC method was able to detect and quantify small aromatic compounds like aniline, phenol, catechol, and MDA. With the novel HPLC method interferences of the target molecules with potential intermediate substances were avoided. Moreover, by expanding the novel HPLC method with UV spectrum and dual-wavelength absorbance ratios analysis, intermediate substances can easily be distinguished

from the aromatic target molecules, present in the industrial RPB feed. Therefore, this study showed the importance of reliable measurement and detection methods for the correct interpretation of HPLC chromatograms of bioreactor samples derived from RPB treatment processes. Moreover, further investigations on improving process parameters and flow rates should be performed to reach faster elution times of the target molecules. Therefore, a potential real-time HPLC analysis can be applied during a biological treatment process of aromatic containing RPB. Furthermore, efforts for the identification of the unknown intermediates, which are detected in bioreactor samples should be performed. Therefore, mass spectrometry analysis could be used to determine the molecular masses of the present substances. However, special attention has to be paid to the salt content of bioreactor samples, which can lead to precipitates on ionization devices of the mass spectrometer.

2.3 Halophilic biomass monitoring and control strategy

Problem statement

The industrial integration of a bioprocess for the treatment of high salt RPB requires a robust and scalable process design and operation. To increase the acceptance of the chemical industry to integrate a bioprocess into chemical production chains, a simple and real-time control and monitoring strategy for the most important process parameters is crucial. For bioprocesses, biomass concentration is one of the most important process parameters, as biomass is the main catalyst in bioreactions. For halophiles, so far, no online biomass monitoring approach and control strategy was proposed in an industrial context. Therefore, in a rententostat bioprocess, the biomass concentration control should be established by regulating the parameters glycerol feed concentration, dilution rate D, and the retention rate R.

State-of-the-art

So far, retentostat processes have been used for the cultivation of slow-growing extremophiles (Lee et al., 1998; Schiraldi et al., 2001). However, mainly complete cell retention was used in order to increase the biomass concentration, instead of maintaining a stable steady-state. For *H. mediterranei*, a retentostat bioprocess system was applied to reach biomass concentrations of 5 g L^{-1}, however, not in the context of residual process brine treatment (Lorantfy et al., 2014a). For the determination of the biomass concentration, several offline and online concepts are known and established (cell dry weight, capacity, or optical density) (Sonnleitner, 2013). The most used method in the cultivation of halophilic microorganisms is the measurement of the optical density at 600 nm (Fang et al., 2010; Martinez-Espinosa et al., 2015). For a continuous bioprocess operated in an industrial environment, fast and reliable online measurements of the biomass are required. The application of online turbidity probes is often limited by a sensor saturation already at low concentrations, non-linear biomass to turbidity behavior, and disturbing effects of agitation and aeration rates (Gregory & Thornhill, 1997; Münzberg et al., 2017). Hence, the use of exhaust gas measurements and substrate feed concentrations are suitable for the estimation of online metabolic rates (Kager et al., 2017; Kager et al., 2018; Reichelt et al., 2016; Sagmeister et al., 2013; Ulonska et al., 2018; Wechselberger et al., 2013). Therefore, both, a biomass monitoring and control strategy have to be adapted for retentostat bioprocesses using halophilic microorganisms.

Goal of this study

This study aimed the development of an online monitoring and control strategy for the biomass concentration of a halophilic model organism (*Haloferax mediterranei*).

Scientific question

What are the requirements of a bioprocessing strategy when applied to the treatment of an industrial residual process brine?

How can a robust biomass monitoring strategy be established for use in an industrial-scale biological residual water treatment process?

Can a suitable biomass monitoring approach identify important physiological parameters of the microbial system?

Approach

We aimed to develop a robust and scalable bioprocess for the continuous treatment of a high-salt industrial residual water contaminated with organic impurities. To do so, a bioreactor setup was equipped with a membrane-based cell retention system. This bioprocess system allowed the simple and robust control of the biomass concentration by adjusting the feed concentration of the growth substrate and the retention rate R (ratio of harvest flow and feed flow). To complete the biomass control concept, an online monitoring approach was developed which uses concentrations of consumed substrates as well as exhaust gas measurements of O_2 and CO_2 to estimate a biomass turnover rate. Ultimately, this rate can be applied to calculate biomass concentrations at steady-state conditions, which can be used as input variables for the biomass control strategy.

Results and discussion

During this work, a feedforward control strategy for a membrane-based cell retention system was combined with a soft sensor-based monitoring tool, which allows the control and real-time estimation of biomass concentrations for the halophilic model organism *H. mediterranei*. The feedforward control strategy was depending on the retention rate R and the substrate concentration, which were investigated over a broad range of concentrations. This new approach was successfully applied in a high throughput industrial RPB treatment process. Moreover, removal of the four organic pollutants formate, phenol, aniline, and MDA at high productivity and degradation efficiency was demonstrated. The results in this work provide an excellent framework for future applications of halophiles in continuous bioprocesses.

What are the requirements of a bioprocessing strategy when applied to the treatment of an industrial residual process brine?

To integrate a bioprocess for the treatment of an industrial and high-salt residual water, the desired process has to be robust and scalable. An important parameter for any bioprocess is the biomass concentration. Thus, for improving the acceptance for the integration of a bioprocess into chemical production chains, a simple and effective control strategy for the biomass concentration in continuous bioprocesses is crucial. Hence, we hypothesized that a continuous bioprocessing strategy using cell retention allows the achievement of high space-time yields for slow-growing organisms such as halophiles.

The feed-forward strategy suggested in this study successfully achieved regulation of steady-state biomass concentrations. In contrast to a chemostat process, the presented rententostat bioprocess introduced an additional process parameter for the control of biomass concentration, namely the retention rate *R*. Hence, the control of the biomass concentration in the bioreactor was established by a regulation of the parameters glycerol feed concentration, dilution rate *D* and the retention rate *R,* which was independent of the microbial growth rate μ. Hence, decoupling the liquid dilution rate *D* from the specific growth rate μ allows higher residual water throughputs as with conventional chemostats. Additionally, the operational costs can be further optimized by using low substrate uptake rates r_S and retention rates *R*. By doing so, costs are reduced for i) the substrate supplementation in the growth medium, and ii) the disposal of residual biomass. It was also shown, that the cultivation of *H. mediterranei* was successful under unsterile conditions, using industrial RPB for more than 54 days. Therefore, the industrial applicability of the bioprocess strategy was demonstrated and integration into a chemical production chain is feasible.

How can a robust biomass monitoring strategy be established for use in an industrial-scale biological residual water treatment process?

Efficient methods for online monitoring of biomass concentration in continuous halophilic bioprocesses are rare. Hence, the aim was to establish an online monitoring strategy for biomass in continuous cultures, which is non-invasive, reliable, and easy to implement in an industrial treatment process. In this study, an online approach was introduced for the determination of biomass concentrations in a retentostats process using exhaust gas measurements and substrate feed concentration. To do so, elemental balances were used to calculate turnover rates and ultimately calculate the biomass formation rate. Also, the redundant measurement system can be used to determine gross errors of the measurements to ensure reliable data generation (Jobe et al., 2003). Hence, the proposed method only

needed minimal a-priori knowledge (substrate feed concentration and biomass elemental composition) and no calibrated multivariate models. Moreover, good comparability between online soft sensor values and offline biomass values, derived from OD measurements, was shown (Lorantfy et al., 2014b). As it is a non-invasive approach, human interferences are minimized and offline sampling times during the process monitoring can be reduced, which increases the acceptance of integration into industrial processes (Kager et al., 2018; Vojinović et al., 2006).

Can a suitable biomass monitoring approach identify important physiological parameters of the microbial system?

The estimation of physiological parameters is of utmost importance, not only for monitoring but also for real-time evaluation of biotechnological processes (Kager et al., 2018; Reichelt et al., 2016). Especially for continuous cultivation processes operated at low growth rates, the knowledge of yield coefficients can be interesting, as they might depend on the growth rate (Lipson, 2015; Pirt, 1965; Pirt, 1987). The suggested biomass estimation approach successfully calculated biomass formation yields and specific growth rates in real-time. The biomass formation yield was also demonstrated to depend on the specific growth rate, which showed the applicability of the soft sensor-based approach to be used as a real-time control input for controlling the biomass concentration in a continuously operated bioprocess. Consequently, with only simple process knowledge (substrate concentration, biomass elemental composition), the proposed approach can be helping to further intensify continuous bioprocess development, especially for extremophiles.

Outlook

The study showed the applicability of a feed-forward biomass control strategy and a soft sensor-based biomass monitoring approach for the extremely halophilic archaeon *H. mediterranei* in synthetic and industrial residual process brine. Consequently, transferability to larger scales and the implementation into an industrial environment could accelerate the integration of bioprocess into chemical production chains. So far, only carbon-limited, and steady-state conditions can be displayed with the presented biomass monitoring strategy. To adapt the soft sensor model for dynamic concentration changes of the substrate, online measurement of the substrate concentration could serve as an additional model input. Therefore, it becomes possible to calculate biomass concentrations online, using online exhaust gas and substrate measurements. Moreover, it should be investigated if changes in residual water compositions can be detected by changes in physiological parameters like yield

coefficients for biomass, O_2, or CO_2. These changes could be monitored by the proposed soft sensor and be linked to a process control strategy to ensure equally high process performance.

2.4 Optimized process operating conditions for a biological treatment process

Problem statement

For using industrial residual brines as raw material for the membrane-cell chlor-alkali electrolysis, high-quality requirements have to be met, like a low organic content. Besides, also inorganic substances, like nitrogen, might be critical to the membrane-cell process, in terms of performance and process security (Brinkmann et al., 2014). Thus, organic, and inorganic impurities must be removed before brines can be used in the membrane-cell process. A bioprocess can serve as a sustainable way of treating residual process brines, however, integration of bioprocess into an industrial production chain requires an efficient and cost-optimized process. Thus, the use of additional nutrients, like a carbon, nitrogen, and phosphorus source, should be optimized.

State-of-the-art

A continuous bioprocess was developed for the treatment of industrial residual brines using halophilic microorganisms (Mahler et al., 2018; Mainka et al., 2019). It was shown that a robust and long-term bioprocess, as well as efficient degradation of organic impurities was successfully achieved (Mainka et al., 2022). However, the efficiency of an industrial treatment process is not only measured in the degradation performance of impurities. Also, a bioprocess should be efficient in operational expenditures, among which the addition of nutrient supplements plays a major role. So far, double limitations with the carbon, nitrogen, and/or phosphorus source were achieved in continuous cultivations (Egli, 1991; Egli & Zinn, 2003; Frank, 1999; Zinn et al., 2004). However, the effect of multiple nutrient limitations on the degradation of organic impurities still has to be shown.

Goal of this study

During this study, optimized process operating conditions should be proposed, which allows the cost-effective operation of a biological treatment process at high degradation efficiencies.

Scientific questions

Is it possible to establish a triple nutrient-limitation for the carbon, nitrogen, and phosphorus source and maintain an efficient degradation for organic impurities?

What is the influence of the co-substrate glycerol on the degradation efficiency of organic impurities?

Approach

We aimed to identify the effect of certain critical process parameters on the process performance of a bioprocess for the continuous treatment of MDA residual process brine. We hypothesized that optimizing the addition of the growth nutrients glycerol, ammonium, and phosphate in a way that limiting conditions can be reached at high degradation efficiencies of organic impurities. It was found that the specific glycerol uptake rate and the consumption yield of ammonium to glycerol mainly influenced the residual formate concentration. In addition to a stable microbial system and an efficient and robust bioprocess, the operational costs are of utmost interest for industrial implementation and integration of the investigated bioprocess. Thus, the liquid throughput of the RPB should be desirably high, and additional media supplementation should be kept to a minimum.

Results and discussion

Is it possible to establish a triple nutrient-limitation for the carbon, nitrogen, and phosphorus source and maintain an efficient degradation for organic impurities?

When used as a raw material for membrane-cell chlor-alkali electrolysis, RBP requires to have low organic carbon levels and needs to be free of any ammonium or organic nitrogen species. In the case of nitrogen, a limited use is not only of interest for operational expenditures (OpEX), but also for security reasons, as explosive nitrogen trichloride (NCl_3) can be formed during electrolysis (Brinkmann et al., 2014). Thus, for OpEX reasons, the addition of nitrogen and phosphorus sources should be limited, in a way to avoid residuals of C, N, and P, but simultaneously ensure high organic degradation efficiency. In nature, especially in marine ecosystems, co-limitations of nutrients like N and P often occur (Bracken et al., 2015; Chen et al., 2019; Harpole et al., 2011; Mills et al., 2008). Furthermore, co-limitation for nutrients, like Zn/C, Ni/N, and Fe/light was frequently reported for cultivations of phytoplankton biomass (Maldonado et al., 1999; Morel et al., 1994; Price & Morel, 1991; Sunda & Huntsman, 1997).

So far, continuous cultivations are known with a dual-nutrient limitation of the carbon and nitrogen source (Egli, 1991; Egli & Quayle, 1986; Egli & Zinn, 2003; Frank, 1999; Poblete-Castro et al., 2012; Zinn et al., 2004). However, the effect of dual- or triple-nutrient limitations on degradation efficiencies in biological residual water treatment processes has not yet been investigated. We hypothesized that nitrogen and phosphorus consumption can be linked to the consumption of glycerol to ensure complete uptake of N and P and simultaneously maintain high degradation efficiency of organic impurities. Therefore, different feed concentrations of ammonium (N source) and phosphate (P source) were tested to achieve the following nutrient-limited states: C, C:N, C:P, and C:N:P. It was shown, that a dual- and triple-limitation state can

successfully be achieved. Degradation efficiencies for all present organic impurities were high. No effect of limitation states was shown on the degradation of aromatics, as all originally present aromatic compounds were fully degraded. Besides, residual formate concentrations at all different nutrient limitation states were below 10 mg L^{-1}, which corresponds to theoretical TOC values below 3 ppm. However, when lower consumption yields of ammonium to glycerol $Y_{NH4+/glycerol}$ were applied, a slight increase in the residual formate concentration was observed (from 6.5 to 8.2 mg L^{-1}). Therefore, $Y_{NH4+/glycerol}$ should be chosen to be between 0.2-0.4 mol mol^{-1}. Thus, the feed concentration of ammonium has to be set accordingly to the glycerol concentration, to avoid residual nitrogen in the treated RPB.

What is the influence of the co-substrate glycerol on the degradation efficiency of organic impurities?

The co-substrate glycerol plays an important role in the presented biological treatment process, as it works as the growth substrate. In contrast to glycerol, the main organic impurity formate only contributes little to biomass forming, as shown for *Halomonas* strain sp. MA-C (Azachi et al., 1995; Oren et al., 1992). Although glycerol can be produced sustainably, it is still the priciest part of the operational costs in the presented biological treatment process. Hence, an optimized supplementation of glycerol is crucial for the overall process efficiency. We thus hypothesized that an optimized feeding strategy for glycerol improves the degradation and, hence, the process efficiency for the treatment bioprocess of MDA residual process brine.

In the course of this work, it was shown that an excess of glycerol feeding and thus accumulation of glycerol in the bioreactor, led to a decrease in the formate degradation and ultimately to higher residual formate concentrations. It was shown already that carbon-limited conditions are beneficial for the simultaneous utilization of a growth substrate and other organic compounds (Duetz et al., 1994; Rüegg et al., 2007). Evidently, a glycerol feeding strategy resulting in residual glycerol and higher residual formate concentrations are not suitable for a process reducing organic content, both, economically and in terms of degradation efficiency. Therefore, only limiting conditions for the growth substrate should be applied to ensure high process performance. Also, it is crucial to minimize the addition of the co-substrate glycerol. However, to ensure high degradation efficiency, it was investigated if applying low co-substrate feeding rates influences the degradation of organic impurities. During this study, it was found that specific glycerol uptake rate $q_{S,glycerol}$ had an influence on the degradation of formate. If $q_{S,glycerol}$ was kept below 8.0 x 10^{-3} g L^{-1} h^{-1} OD_{600}^{-1}, the residual formate concentration was increased. In contrast, a $q_{S,glycerol}$ over 16.0 x 10^{-3} g L^{-1} h^{-1} OD_{600}^{-1} did not improve the formate degradation. Hence, specific glycerol uptake rates of 8.0 – 16.0 x 10^{-3} g L^{-1} h^{-1} OD_{600}^{-1} are shown to be beneficial for the degradation efficiency and the process operational costs.

Outlook

During this study, a process operation space for an effective bioprocess to continuously reduce organic impurities in an industrial RPB was proposed. It was shown that reduction of media components can be successfully applied, and a triple-nutrient limitation is possible. To further reduce operational costs, a further reduction or even limitation of other media supplements like magnesium, calcium, or potassium can be achieved and should be investigated. However, further investigations are required to elucidate the influence of the ammonium (or nitrogen) metabolism on the degradation of formate. Moreover, the degradation metabolism of aniline and strategies to avoid an accumulation of potential degradation intermediates should be investigated. To do so, the identification of the yet unknown intermediate should be prioritized. Additionally, enhancing liquid dilution rates should be investigated as a potential strategy to improve overall process productivity. Ultimately, the process and specifically the halophilic mixed culture could be investigated for the treatment of saline residual process brines contaminated with different organic impurities than formate, aniline, phenol, and MDA.

3 Conclusion and outlook

During this work, an efficient continuous, and scalable bioprocess for the treatment of an industrial residual process brine was successfully developed, characterized, and integrated into an industrial production chain. The main achievements of this thesis were (i) the successful, robust and stable long-term operation of a biological RPB treatment process integrated into an industrial production chain with high degradation efficiency, (ii) the discovery of a highly adapted halophilic microbial community, able to efficiently degrade organic impurities in an industrial residual process brine, (iii) the development of a reliable HPLC method to quantify aromatic compounds in a high-salt residual process brine, (iv) the development and application of a combined online monitoring and control strategy for biomass concentration using halophilic microorganisms, (v) optimized control range for the glycerol uptake rate to ensure efficient formate degradation and (vi) the successful application of a triple-nutrient (carbon, nitrogen, and phosphorus source) during the degradation of organic impurities (Figure 12).

Furthermore, the feasibility of a bioprocess to continuously reduce organic matter in an industrial RPB was demonstrated. Organic impurities (formate, aniline, phenol, and 4,4'-MDA) were found to be efficiently degraded by a novel found halophilic mixed culture. During the piloting study, it was shown that the developed retentostat bioprocess is suitable for the long-term cultivation of halophilic microorganisms under changing RPB compositions. Therefore, the acceptance criteria of a robust and long-term bioprocess were achieved and proven to be suitable for the integration into industrial production chains. Moreover, the presented retentostat bioreactor system was shown suitable for the long-term cultivation of slow-growing extremophilic microorganisms, such as halophiles, especially for their application in an industrial context.

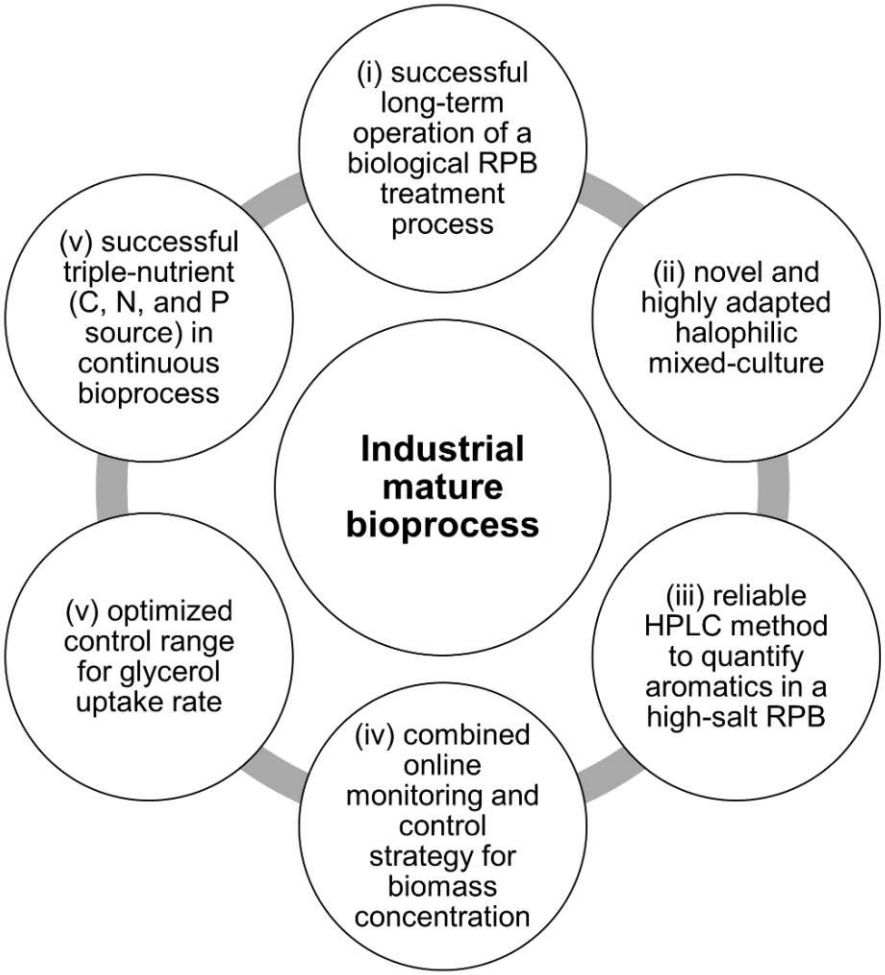

Figure 12. Achievements and findings of this thesis.

Moreover, a stable microbial system was demonstrated during the whole cultivation time. The halophilic mixed culture, consisting of three bacterial genera (*Halomonas* sp., *Aliifodinibius* sp., and *Oceanobacillus* sp.), was proven to be suitable for the reduction of organic content in industrial MDA residual process brine. A constant and efficient degradation of the four organic impurities (100% aromatic degradation, 90-98% formate degradation) was demonstrated. High degradation efficiencies were also shown under changing RPB feed compositions, as no decrease in process performance was observed. Thus, the found halophilic mixed culture showed promising attributes for the treatment of industrial residual brines. Therefore, the potential of the mixed culture for reducing organic content in other residual process brines should be exploited in future research.

As a crucial element for this work, an existing HPLC method for the quantification of aromatic compounds was adapted to avoid overlapping peaks with target molecules in bioreactor samples. The developed method was shown to be able to robustly measure and distinguish aromatic target molecules in the RPB feed from other peaks in bioreactor samples, based on

efficient peak separation and identification tools. It was demonstrated, that a reliable HPLC method is essential for the correct interpretation of chromatograms derived from bioreactor samples. Additionally, the importance of the combination of the detection methods and purity indices (dual-wavelength absorption ratios and UV spectra) was demonstrated to increase the reliability of HPLC measurements.

Moreover, as a key scientific success factor, a reliable online biomass monitoring strategy for halophilic microorganisms was developed, which is based on exhaust gas measurements and substrate concentrations. Therefore, it was possible to calculate metabolic rates and yield coefficients in real-time, with one or two carbon sources. The results also showed a dependency of the biomass yield coefficient on the specific microbial growth rate. Therefore, the proposed method is suitable to detect changes in microbial metabolism, which is especially important in the context of long-term continuous cultivations. Moreover, the biomass concentration calculated by the soft sensor can serve as a suitable real-time input variable for the feed-forward biomass control strategy, developed for a retentostat bioprocess.

Also, it was demonstrated that carbon-limiting conditions are essential for high degradation efficiencies of formate. Additionally, an optimized control range for the specific glycerol uptake rate was proposed, which ensures efficient formate degradation and resulted in the lowest residual formate concentrations. Ultimately, a cost-efficient operation space for an industrial integration of the bioprocess was proposed. Scientifically novel triple-nutrient limiting conditions were successfully applied which reduces not only operational costs but also potential downstream efforts to remove ions, disturbing the membrane cell electrolysis process. As shown with the nitrogen and phosphorus source, also a reduction of concentrations of other ions, like magnesium and calcium, and referring their consumption to the glycerol uptake seems reasonable. Thus, further research should be performed to optimize the medium and reduce both, the operational expenditures, and downstream efforts. According to a cost-effective process, also the throughput of the RPB and the disposal of residual biomass have to be considered. The results indicated that a process can be operated at least with a dilution rate of $D = 0.2\ h^{-1}$, which corresponds to a hydraulic residence time (HRT) of 5 h. Hence, during this study, a desirable high throughput of RPB was successfully applied. Still, the use of even higher dilution rates should be investigated in the future. Furthermore, high R values of up to 0.98 were successfully applied, which corresponds to only 2% of RPB feed streams that are generated as residual waste streams. Another beneficial impact of high retention rates is, that a cell wash-out can be prevented when high dilution rates and low glycerol concentrations in the feed are applied. Therefore, high throughputs of RPB combined

with minimal medium costs and a robust microbial system increase the potential of the developed process for industrial integration.

This thesis focused on the development and characterization of a bioprocess, ready to be used as a unit operation for the continuous treatment of an industrial residual process brine, integrated into an industrial production chain. Organic impurities could be effectively degraded in an industrial residual process brine using an efficient halophilic mixed culture. Moreover, analytical tools for monitoring, controlling, and evaluating the process performance were successfully developed and demonstrated in an industrial context. Finally, optimized process operation conditions for an efficient and industrial-mature bioprocess were proposed. In conclusion, the combined use of advanced retentostat bioprocessing, analytical approaches, and statistical experiment evaluations were key scientific success factors to gain knowledge about a biological treatment process for MDA residual process brine. Ultimately, this work demonstrated that the next steps towards the integration of bioprocesses into chemical production processes have been taken. To that end, the focus should be set on the technology transfer to an industrial scale, and further feasibility studies of this technology for the treatment of other brine effluents can be performed effectively.

4 Manuscripts

4.1 Potential applications of halophilic microorganisms for biological treatment of industrial process brines contaminated with aromatics

Title of manuscript

Potential applications of halophilic microorganisms for biological treatment of industrial process brines contaminated with aromatics

Citation

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Contribution

Conceptualization: Thomas Mainka, Stefan Pflügl; *Writing - original draft preparation*: Thomas Mainka, David Weirathmüller; *Writing - review and editing*: Stefan Pflügl, Christoph Herwig; *Visualization*: Thomas Mainka; *Funding acquisition*: Christoph Herwig; *Supervision*: Stefan Pflügl, Christoph Herwig

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Potential applications of halophilic microorganisms for biological treatment of industrial process brines contaminated with aromatics

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Abstract: Saline wastewater contaminated with aromatic compounds can be frequently found in various industrial sectors. Those compounds need to be degraded before reuse of wastewater in other process steps or release to the environment. Halophiles have been reported to efficiently degrade aromatics, but their application to treat industrial wastewater is rare. Halophilic processes for industrial wastewater treatment need to satisfy certain requirements: a continuous process mode, low operational expenditures, suitable reactor systems and a monitoring and control strategy. The aim of this review is to provide an overview of halophilic microorganisms, principles of aromatic biodegradation, and sources of saline wastewater containing aromatics and other contaminants. Finally, process examples for halophilic wastewater treatment and potential process monitoring strategies are discussed. To further illustrate the significant potential of halophiles for saline wastewater treatment and to facilitate development of ready-to-implement processes, future research should focus on scale-up and innovative process monitoring and control strategies.

Keywords: Bioremediation processes, Saline wastewater treatment, Industrial process brines

Introduction

Saline wastewater occur in various industrial sectors around the world, including the petroleum industry, tannery and textile industry, coal gasification and dye or polyurethane production (Castillo-Carvajal et al., 2014; Muddemann et al., 2018). In total, it is estimated that 5% of industrial wastewater contain high amount of salt and quantities are likely to increase in the future (Le Borgne et al., 2008). Due to the fact that saline wastewater mostly originates from heavy industry sectors, they are often contaminated with several toxic and hazardous substances (Woolard & Irvine, 1995; Moussa et al., 2006). Among them are various hydrocarbons, like mono- or polycyclic aromatic hydrocarbons (e.g., phenol, benzene, or anthracene) (Le Borgne et al., 2008). These substances pose a severe environmental and health threat, because they are toxic to most organisms and possess mutagenic and carcinogenic properties (Sun et al., 2019).

Often, saline wastewater is diluted with freshwater before they are released to the environment (Tan et al., 2019). However, on the basis of population- and economic growth as well as other global challenges like climate change, the availability of freshwater will become more challenging in the future (Du et al., 2018; Tong & Elimelech, 2016). Moreover, in some regions freshwater is produced by seawater desalination and brine is generated as a by-product, which has a negative environmental impact when disposed to the sea again, such as salinity gradients or reduction of the amount of flora (Casas et al., 2012). However, treatment of saline wastewater is not only of interest considering the release in the environment, but also its reuse for industrial applications. Brines from seawater or industrial processes can be used by the chlor-alkali industry to produce substances like chlorine, sodium- and potassium hydroxide. The most promising technique in the chlor-alkali industry is the membrane cell process (Brinkmann et al., 2014; Casas et al., 2012). In this process, however, organic and inorganic impurities in the saline wastewater can lead to a cell voltage increase. Therefore, a highly purified brine is needed to avoid a decrease in the membrane efficiency. Consequently, the degradation of pollutants in brines is gaining interest (Brinkmann et al., 2014; Casas et al., 2012; Le Borgne et al., 2008). In the past, it has been reported that physical and electro chemical methods like sorption or oxidation are expensive and energy consuming or inappropriate for large volumes of wastewater. Therefore, biological systems could represent an economically more attractive alternative for saline wastewater treatment (Bonfa et al., 2013; Jin et al., 2012). The biological treatment of saline wastewater with nonhalophilic microorganisms would require a pretreatment to decrease the salt concentration, either by dilution or desalination (Tan et al., 2019). However, these pretreatment strategies would increase operational expenditures significantly. Therefore, halophilic microorganisms are a better choice because direct operation with waste brine is possible (Bonfa et al., 2013; Díaz et al., 2002; Jin et al., 2012). Halophilic bacteria and archaea have already been the target in several studies dealing with the degradation of aromatic compounds, but reports for the application of halophiles in industrial bioremediation are scarce (Krzmarzick et al., 2018). Therefore, the aim of this review is to provide an overview of halophilic microorganisms, degradation of aromatic compounds, sources of saline wastewater polluted with aromatic compounds and other contaminants, and finally shows examples of application of halophilic organisms to treat industrial wastewater streams. Moreover, we address problems in process development which need to be solved for implementation to

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industrial production chains. Finally, future development potential and research goals are outlined.

Degradation of Aromatic Compounds in Halophilic Microorganisms

Halophilic Microorganisms

While nonhalophilic microorganisms show optimal growth at concentrations below 2% NaCl, halotolerant and halo-dependent microorganisms are able to grow in the presence of up to 30% NaCl (Castillo-Carvajal et al., 2014; Margesin & Schinner, 2001). The group of halophiles is highly diverse and present in all three domains of life (archaea, bacteria, eukarya) (Oren, 2002a, 2008; Roberts, 2005). Depending on the level of salt tolerance, halophiles are usually classified into four different groups: halotolerant, slight, moderate and extreme halophilic microorganisms. Halotolerant microorganisms can grow in saline environments, but do not necessarily require high salt concentrations (Zhuang et al., 2010). In contrast, true halophiles are classified as slight (1–3% NaCl), moderate (3–15% NaCl), and extreme halophiles (15–30% NaCl) according to the salt concentration they require for growth (compare sea water salinity: around 3.2%) (Fofonoff, 1985; Kushner, 1978; Oren, 2002a, 2008; Zhuang et al., 2010). In this review, halophilic physiology will not be discussed in detail. However, it is noted that two different strategies for balancing osmotic pressure in high-salt environments have evolved: the salt-in and the compatible solute strategy (Gunde-Cimerman et al., 2018; Oren, 2002a, 2002b, 2008).

The salt-in strategy is mainly used by halophilic archaea and is based on the intracellular accumulation of inorganic ions (K^+ and Cl^-) to provide an osmotic balance (Margesin & Schinner, 2001; Oren, 2002a, 2002b, 2008). The transport of potassium ions can be passive (through a K^+ channel) or active (through ATP-dependent transport systems). Therefore, an energy-dependent mechanism is required, because the intracellular accumulation of negatively charged chloride ions would be repressed by the inside-negative membrane potential (Gunde-Cimerman et al., 2018). Special membrane-bound retinal pigments such as bacteriorhodopsin and halorhodopsin are often found in halophilic archaea like *Halobacterium salinarium* and *Haloferax volcanii*, and enables these organisms to use energy from light directly to power bioenergetical processes (Gunde-Cimerman et al., 2018; Margesin & Schinner, 2001; Oren, 2002b, 2008; Roberts, 2005).

Within the domain of the archaea, the most widespread group are the *Halobacteria* and most of the members of this group require high salt concentrations above 15% (Gunde-Cimerman et al., 2018). To survive at such harsh conditions, the cellular structural components, the intracellular machinery and the intra- and extracellular enzymes are highly adapted (Le Borgne et al., 2008). To maintain enzyme activity and keep proteins soluble at such high intracellular ion concentrations, acidic amino acids are used more frequently on outside regions of proteins than hydrophobic amino acids (Margesin & Schinner, 2001; Gunde-Cimerman et al., 2018). To keep the level of the hydrophobic amino acids low, a high content of the "borderline hydrophobic amino acids" serine and threonine is required (Lanyi, 1974; Oren, 1999). Due to this unique amino acid composition, these microorganisms only have limited ability to adapt to changing conditions, because of protein denaturation at lower salt concentrations (Margesin & Schinner, 2001; Oren, 1999, 2008).

In contrast to accumulating inorganic ions for balancing osmotic potential, some halophilic microorganisms also employ the strategy of biosynthesis and/or accumulation of compatible solutes (Oren, 2008). Compatible solutes are organic, osmotically and highly water-soluble substances. There are a large number of different compatible solutes which can be classified into ionic, zwitterionic, or nonpolar molecules (Oren, 2002b, 2008; Roberts, 2005; Shivanand & Mugeraya, 2011). In addition to their task as salt antagonist, they stabilize DNA, enzymes and whole cells against stress factors such as freezing, drying and heating (Shivanand & Mugeraya, 2011). Thus, they have already been used as cryo-protectant (Roberts, 2005). Further, compatible solutes are often referred to as chemical chaperones, because they stabilize enzymes and assist during proper folding of polypeptide chains (Roberts, 2005; Shivanand & Mugeraya, 2011). This property leads to a high adaptability to changes of the extracellular salinity. The use of compatible solutes is more widespread among halophilic microorganisms, although it requires more energy than the intracellular accumulation of ions, because organic solutes have to be synthesized *de novo* (Gunde-Cimerman et al., 2018; Oren, 2002a).

Degradation Mechanisms of Aromatic Compounds in Halophilic Microorganisms

In the biosphere, aromatic compounds are the second most abundant family of organic constituents beside carbohydrates (Field et al., 1995). They occur frequently in saline waste streams in the petroleum industry (Alva & Peyton, 2003; Bonfa et al., 2011). Beside their frequent occurrence, some aromatic compounds like polycyclic aromatic hydrocarbons (PAH), phenol, toluene, or aromatic amines have toxic, mutagenic and carcinogenic properties (Bonfa et al., 2013; Castillo-Carvajal et al., 2014; González et al., 2012). Several aromatic compounds are considered as a widespread environmental pollutant and hazardous to ecosystems. Furthermore, they are highly persistent in the environment and may accumulate in natural systems (Alva & Peyton, 2003; Bonfa et al., 2011; Gomes et al., 2018; Seo et al., 2009).

The degradation of aromatic compounds has frequently been reported for halophilic microorganisms (Arora, 2015; Bonete et al., 2015; Castillo-Carvajal et al., 2014; Fuchs et al., 2011; Krzmarzick et al., 2018; Li et al., 2019; Nogales et al., 2017; Vaillancourt et al., 2006). For the aerobic and anaerobic degradation of small aromatic compounds, the pathways and involved enzymes are known and are shortly described in this review.

The first task of degrading aromatic compounds is to break the energetically stable aromatic ring shared by all aromatic compounds, which can be done either in the presence or absence of oxygen (Fuchs et al., 2011; Heider & Fuchs, 1997). Under aerobic conditions, the aromatic ring is cleaved at an intradiol bond, an extradiol bond or independently of a diol (Burroughs et al., 2019), whereas aromatic rings in anaerobic pathways are reduced to cyclohexane derivatives (Heider & Fuchs, 1997).

One of the most studied oxidative aromatic degradation pathways is the β-ketoadipate pathway, which starts with the conversion of aromatic compounds to catechol (e.g., phenol, benzene, or benzoate) or protocatechute (e.g., 4-hydroxybenzoate) (see Fig. 1) (Fuchs et al., 2011; Li et al., 2019; Vaillancourt et al., 2006; Wells & Ragauskas, 2012). Both molecules are then cleaved by oxygenases to *cis-cis*-muconate and 3-carboxy-*cis-cis*-muconate, respectively (ortho-cleavage pathway). The end products of this pathway (succinyl- and acetyl-CoA) finally enter the TCA cycle (Vaillancourt et al., 2006; Wells & Ragauskas, 2012). In contrast, when using the meta-cleavage pathway, catechol and protochatechute are finally transformed into pyruvate and acetaldehyde (Fuchs et al., 2011). Both pathways have been described for halophilic bacteria, like *Halomonas* or *Marinobacter*

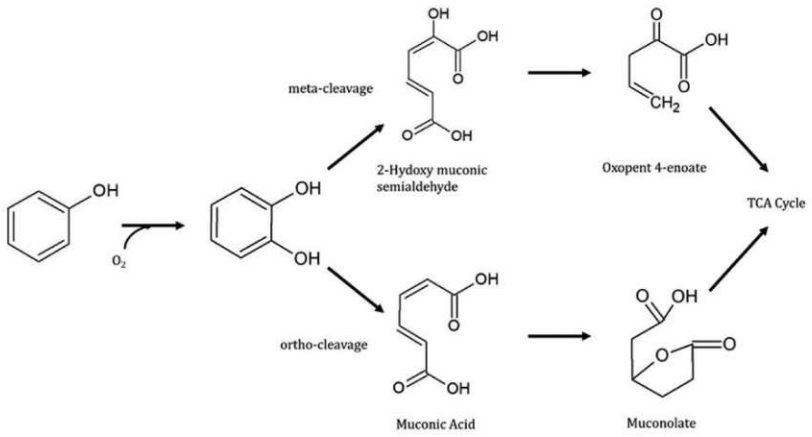

Fig. 1. Aerobic pathway of phenolic degradation (meta- and ortho-cleavage) (Li et al., 2019).

species, for various aromatic compounds like phenol, benzene or aniline (Li et al., 2019; Nicholson & Fathepure, 2004). For several *Pseudomonas* strains, which are halotolerant bacteria, it was reported that toluene is degraded, however, the strains used different pathways (Bordel et al., 2007; Duetz et al., 1994; Otenio et al., 2005; Yu et al., 2001). Reported pathways for toluene degradation were the conversion into 3-methylcatechol, *o*-cresol, *p*-cresol, or catechol (via benzoate), eventually resulting in the conversion to CO_2. Several studies showed also the degradation of toluene by various halophilic bacteria like *Marinobacter*, *Planococcus*, and *Arhodomonas* (Berlendis et al., 2010; Dalvi et al., 2014; Desouky et al., 2015). For the latter one, a degradation pathway of toluene via 3-methylcatechol and 4-hydroxy-2-oxovalerate into the TCA cycle was proposed, based on genomic and proteomic analyses (Dalvi et al., 2014). In 2002, Fairly et al. (Fairley et al., 2002) reported that only one ring cleavage oxygenase (gentisate 1,2-dioxygenase, Fu & Oriel, 1998) exists for archaea. Moreover, Wells et al. (Wells & Ragauskas, 2012) reported in 2012, that the β-ketoadipate pathway is not utilized by halophilic archaea and archaea in general. However, more recent studies reported the use of the β-ketoadipate pathway by halophilic archaea like *Haloferax*, *Haloarcula* of *Halobacterium* species, to degrade, for example, phenol or 4-hydroxy benzoate (Acikgoz & Ozcan, 2016; Erdoğmuş et al., 2013).

The halophilic archaea *Haloferax* sp. D1227 was the first halophilic archaeon reported to grow solely on aromatic compounds, such as benzoate, cinnamate, and phenylpropanoate, using the enzyme gentisate 1,2-dioxygenase, which does not belong to either intradiol or extradiol oxygenases. Rather, gentisate is cleaved between the carboxyl- and the hydroxyl group to form maleylpyruvate (Emerson et al., 1994; Fu & Oriel, 1998). This pathway is also present in halophilic bacteria like *Martelella* strains (Huang et al., 2015). The gentisate pathway is active during the haloarchaeal degradation of aromatic compounds like 4-hydroxybenzoate, 3-hydroxybenzoate, anthranilate and salicylate (Fairley et al., 2002, 2006; Fu & Oriel, 1998; Vaillancourt et al., 2006). Nonhalophilic microorganisms use the same pathways for the degradation of aromatic compounds as halophiles, as the same degradation pathways via catechol, gentisate or protochatechute pathway have been reported (Ladino-Orjuela et al., 2016; Nair et al., 2008).

As reported in literature, anaerobic bacteria mostly metabolize aromatic compounds via the benzyl-CoA pathway, which is degraded to acetyl-CoA and finally CO_2 is released (Evans & Fuchs, 1988; Fuchs et al., 2011; Heider & Fuchs, 1997). Few hydroxylated aromatic compounds are degraded anaerobically via the polyphenolic intermediates phloroglucinol and resorcinol. These compounds are later also dearomatized and transformed to acetyl-CoA (Fuchs et al., 2011).

Fuchs et al. pointed out that the anaerobic degradation of the polycyclic aromatic naphthalene via benzyl-CoA appears to be not yet clear (Fuchs et al., 2011). Nevertheless, Nzila et al. recently summarized the anaerobic degradation of naphthalene, which happens via the nonaromatic molecule cis-2-carboxycyclohexylacetyl-CoA and later to CO_2 via acetyl-CoA (Meckenstock & Mouttaki, 2011; Nzila, 2018). Likewise, the degradation of other PAHs occurs via multiple step pathways resulting in the production of CO_2 (Nzila, 2018). The aerobic degradation of PAHs in halophilic bacteria and archaea has been reported for several halophilic strains like *Haloarcula* sp., *Haloferax* sp., or *Halomonas* sp. and has recently been reviewed (Bonete et al., 2015; Erdoğmuş et al., 2013; Ghosal et al., 2016; Govarthanan et al., 2020; Oren, 2019). Two aerobic degradation mechanisms of PAH's are described for nonhalophilic bacteria. The first one starts with the hydroxylation of an aromatic ring to a *cis*-dihydrodiol. This intermediate is metabolized via ortho- or meta-cleavage, resulting in the formation of catechol, which is finally degraded via the TCA cycle (Ghosal et al., 2016; Mallick et al., 2011). Additionally, the bacterial cythocrom P450-mediated pathway was reported for aerobic PAH degradation (Ghosal et al., 2016). This pathway initially forms *trans*-dihydrodiol molecules as shown for the degradation of benzo[*a*]pyrene by *Mycobacterium vanbaalenii* PYR-1 (Moody et al., 2004).

Industrial Saline Wastewater

Sources and Composition of Saline, Aromatic Compounds Containing Wastewater

Aromatic compounds are widespread among industrial saline wastewater (oil refineries, food-processing sites, tannery industry, etc.), which can contain concentrations from the mg l^{-1} to

even the g l^{-1} range, depending on the industry of origin, process conditions or dilution factors (Garcia et al., 2005; Le Borgne et al., 2008; Ramos et al., 2015; Woolard & Irvine, 1995). The efficient degradation of organics in industrial wastewater is therefore beneficial for environmental considerations as well as for potential reutilization of the purified brines in industrial processes. As the composition of industrial wastewater often changes, and companies rarely publish the exact species and amount of contaminants in their wastewater, most studies about bioremediation of saline wastewater use synthetic brines with model contaminants. Nevertheless, this chapter aims to summarize potential sources of saline wastewater and wants to give an overview of their aromatic contaminants and potential co-contaminants.

As oilfields are a widespread source of contamination for saline environments, plenty of studies deal with the treatment of wastewater contaminated with petroleum hydrocarbons (Al-Mailem et al., 2010; Dastgheib et al., 2012; Pugazhendi et al., 2017). During oil and gas extraction, so-called "produced water" is generated as a by-product in large volumes, because water is pumped from subsurface reservoirs, along with gas and oil (Fathepure, 2014; Veil et al., 2004a). The volume of produced water is equal to or even higher than the extracted volume of crude oil and in 2007, over 20 billion barrels of produced water have been generated alone in the United States (Clark & Veil, 2009; Piubeli et al., 2012b; Pugazhendi et al., 2017; Veil et al., 2004a). Produced waters are not only characterized by their high salt content, but it has also been reported that produced water contains toxic chemicals, heavy metals and organics (Al-Mailem et al., 2017; Díaz et al., 2002; Fathepure, 2014; Neff et al., 2011). Wastewater from petroleum industry is a complex mixture of organic substances like cycloalkanes, aliphatic, mono-, and polyaromatic compounds. Those wastewater mostly contains toluene, benzene, ethylbenzene, xylene, phenol, 2,4-dimethyl-phenol, or PAHs like naphthalene as aromatic contaminants (González et al., 2012; Piubeli et al., 2012a; Veil et al., 2004b). Information about the composition of produced water can also be found elsewhere (Neff et al., 2011). Due to the hazardous properties of wastewater originating from oil processing industries, the contamination of ecosystems like beaches, salt marshes, or salt lakes should be avoided. The disposal of contaminated wastewater into the environment could be realized by biological pretreatment of those saline wastewater with halophilic biological systems.

Another source of industrial wastewater originates from the coal chemical industry, where chemical products are generated from coal-based processes. Wastewater from this industrial sector might be reused and purified by membrane-based technologies, ultrafiltration and reversed osmosis, generating high saline liquid leftovers, contaminated with a complex mixture of organic compounds (Ge et al., 2019; Bian et al., 2020). The inorganic resources present in these wastewater (NaCl, $NaNO_3$, and Na_2SO_4) can be recovered by fractional crystallization. However, it is known that organic contaminants in the wastewater can negatively affect the crystallization reactions. For example, it was shown that phenol and quinolone affects the average crystal size remarkably, while phenol decreases the solubility of sodium sulfate during the crystallization. As the crystal size is important for the crystallization process itself and the further downstream processing, removal of the contaminants is beneficial for process efficiency (Bian et al., 2020; Cisternas & Rudd, 1993; Ge et al., 2019; Su et al., 2018).

During polyurethane production the generation of a high-saline process water (10–15% NaCl) is reported, which contains aromatic amines and phenol as well as formate in significant amounts. High pH values make this wastewater a difficult target for bioremediation, and require a pretreatment in order to lower the pH (Mainka et al., 2019; Muddemann et al., 2018). However, once the organic content is removed, this process water represents an ideal substrate, for example, for the chlor-alkali electrolysis in order to generate chlorine gas and sodium hydroxide.

The tannery industry is generating large saline waste streams, because salt is used to preserve the fresh skins (Lefebvre et al., 2005). The resulting soak liquor waste wastewater contains phenolic compounds (phenol, *p*-cresol, etc.) and aromatic carboxylic acids (benzoate, phthalate, phenylacetate, etc.) as organic contaminants (Lefebvre et al., 2005; Reemtsma & Jekel, 1997). Currently, biological treatment of tannery wastewater commonly relies on the use of activated sludge (Durai et al., 2011). However, it is noted that tannery wastewater composition can be highly complex and may negatively affect biological processes due to the presence of salts, tannins and sulfide (Munz et al., 2008).

In the textile and dye industry, saline wastewater containing aromatic compounds are generated. Dying wastewater contains high salt concentrations (4–10% NaCl), because NaCl is used to maximize the dye fixation on textiles (Liu et al., 2013). The release of dye-containing wastewater negatively affects the aquatic ecosystem because photosynthetic activities decrease which results in the depletion of dissolved oxygen in the water (Guadie et al., 2018). It has been demonstrated that azo dyes, a widely used group of dyes in textile industry, are transformed to aromatic amines by skin microbiota. As aromatic amines have mutagenic and potentially carcinogenic properties, the treatment of azo dyes is highly important to avoid release into the environment (Brüschweiler et al., 2014; Brüschweiler & Merlot, 2017; Dafale et al., 2008; Yurtsever et al., 2016). In literature, several azo dyes were used as model contaminants for biodegradation experiments as they are frequently used in the textile industry. Among them are, for example, Reactive Red 184 (Guadie et al., 2018), Reactive Black 5 (Işık & Sponza, 2008), or Acid Orange 7 (Liu et al., 2013).

The treatment of saline wastewater using microorganisms from conventional wastewater treatment plants or freshwater organisms will operate poorly in terms of degradation efficiency (He et al., 2017; Kargi & Dincer, 1996). Therefore, an adaptation of the microbial community to higher salinities is necessary. If higher salinities over 15% NaCl occur, even the use of halophilic or halotolerant is essential (Amin et al., 2014; Dinçer & Kargi, 2001; Kargi & Dinçer, 1998).

As most of the industrial saline wastewater do not only contain aromatic contaminants, but also aliphatic (e.g., alkenes in petroleum wastewater) or inorganic components (e.g., ammonium or heavy metals), the influence of the wastewater composition on the degradation efficiency is crucial and has to be investigated (Deng et al., 2014; Jiang et al., 2016a, 2016c; Lofthus et al., 2018; Pereira et al., 2019). For instance, it is reported, that phenol-containing wastewater is often contaminated with heavy metals, and found that phenol-degradation by the halotolerant fungi *Debaryomyces* sp. JS4 was lower, when Co(II) and Ni(II) were present in the medium (Jiang et al., 2016a).

In conclusion, various saline wastewater in different industrial sectors are generated, which contain aromatic and other contaminants. It is of high interest to treat these wastewater and degrade potential pollutants, because the release of toxic compounds into the environment could be prevented or organic free wastewater could be reutilized in other industrial approaches. Often the biological treatment of saline wastewater is inhibited by high salt concentrations, thus, efficient microbial systems for the

degradation of the present contaminations should be found and investigated.

Sources for Halophilic Microorganisms Able to Degrade Aromatics

For industrial wastewater treatment processes, it is highly important to find halophilic or halotolerant strains able to degrade the specific aromatic compounds present in the wastewater. Multiple studies reported the discovery of halophilic communities or halophilic strains around the world, suitable for bioremediation purposes (Bertrand et al., 1990; Oren et al., 1991, 1992; Rosenberg, 1983). Halophilic and halotolerant strains and communities were found at salt lakes, salterns, soda lakes, or coastal areas, as well as in seawater samples (Bertrand et al., 1990; Deng et al., 2014; Lofthus et al., 2018; Oren et al., 1991, 1992; Pereira et al., 2019; Todorova et al., 2014). Adapted microorganisms could be isolated from saline environments, which were contaminated with aromatic compounds, like from oil spills. An *Achromobacter* strain was found in oil-contaminated seawater and was able to degrade PAHs like phenanthrene with an efficiency of 50.6% (Deng et al., 2014). Similar results were shown for two other bacteria (*Bacillus methylotophicus* and *Pseudomonas sihuiensis*), isolated from sample spots at the Lagao do Peixe National Park in Brazil. These strains showed a degradation efficiency for phenanthrene (originated from petroleum contamination) of 33% under marine environment conditions. Additionally, microorganisms at natural, uncontaminated spots (e.g., from uncontaminated seawater samples) have also been the target of investigations to study their ability to degrade contaminants present in various industrial wastewater (Brakstad et al., 2015; Cuadros-Orellana et al., 2006, 2012; García et al., 2004; Lofthus et al., 2018). It was for example shown, that aromatic compounds (PAH's and phenols) from a produced water was degraded by marine microorganisms sampled from uncontaminated seawater (Lofthus et al., 2018). Another study investigated the composition of microbes in seawater contaminated with oil (Brakstad et al., 2015). The authors could show, that the abundance of specific degrading bacteria increased, when seawater was contaminated with oil. It was discovered, that Gammaproteobacteria are responsible for the oil compound degradation. Within this group, *Cycloclasticus* and *Marinobacter* were correlated with the biodegradation of aromatic hydrocarbons.

Thus, in Table 1 we want to give information about halophilic strains, found to degrade aromatic compounds, their place of origin, as well as the salinity they were grown in.

Existing and Potential Applications of Halophiles in Industrial Bioremediation Processes

This chapter focuses on halophilic bioremediation processes in bioreactor-scales, their potential to be transferred into industrial scale and future research subjects. Therefore, it will be discussed of which parts a biological process for saline wastewater treatment have to consist, and which requirements such processes have to meet.

A state-of-the-art saline wastewater treatment process should consist of a continuous process operation mode, a suitable halophilic microbial systems, and potential process monitoring approaches. Moreover, requirements of biological processes for saline wastewater treatment, such as a robust process behavior against disturbances, a continuous process mode, corrosion-resistant reactor systems and a simple monitoring and control strategy (see Fig. 2) are discussed. Moreover, certain main process characteristics are important to evaluate a process. Important criteria for an efficient process are the degradation efficiency, the hydraulic retention time (HRT), requirements for additional substrates, and the reactor volume (Fig. 2). The efficiency of a biological remediation process is usually evaluated by the removal efficiency (biodegradation) of organic pollutants (Tan et al., 2019). The hydraulic retention or residence time (HTR) is the ratio between the bioreactor volume and the feed flow rate (David et al., 2019; Deowan et al., 2015). It is an important process criteria, as it describes the duration for how long cells and/or substrates remain inside the bioreactor. When lower HRTs are used in processes, usually lower reactor volumes are required, as the organic loading rate (OLR) is increased. In contrast, higher HRTs are reported to achieve better removal efficiencies (Deowan et al., 2015). A summary of bioprocesses for the degradation of aromatic compounds in saline wastewater can be found in Table 2.

For the integration of bioremediation processes into industrial process chains, in general a continuous process mode is necessary, as high volumes of wastewater streams are continuously generated. Usually, liquid dilution rates of wastewater streams are larger than the specific growth rates of halophiles. To keep the liquid dilution rate in the range of growth rates, or even below, but to simultaneously apply high wastewater flows, large bioreactors have to be used. Alternatively, the use of cell retention systems offers the possibility to decouple growth rate and wastewater dilution rate. Therefore, lower bioreactor volumes are needed, which would reduce operational (OpEx) as well as investment (CaPex) costs (Mainka et al., 2019). The size of bioreactors is especially relevant for saline bioprocesses, because more expensive, highly corrosion-resistant materials have to be used. In comparison to conventional continuous stirred tank reactors (CSTRs), cell retention systems can prevent cell wash out, and thus, might accelerate the adaptation of microbial systems to changes in wastewater compositions (Jang et al., 2013). Several cell retention processes for the treatment of saline wastewater have already been reported. In general, two strategies have evolved regarding cell retention in the reactor: retaining cells in suspension (e.g., with membranes) or immobilizing cells to a carrier material. Alternatively, the cell suspension can be settled and cell-free medium withdrawn from the fermenter. The settling and withdrawal strategy is mostly used in conventional wastewater treatment process, but only allows a discontinuous process mode.

Additionally, to develop a successful bioremediation process, a suitable microbial systems has to be chosen. The microbial system has to be able to deal with the conditions present in the specific wastewater. In case of saline wastewater, the microbial system has to be selected according to (1) the potential contaminants to enable efficient degradation and (2) the salt concentration of the wastewater. The microbial system should also be robust against variations in composition and salinities of different wastewater batches. Salinities up to 15% NaCl can be handled by moderate halophilic or halotolerant microorganisms, whereas salinities above 15% NaCl usually require extremely halophilic microorganisms. However, the process development for extremely halophilic strains might be more complex as with moderately halophiles with respect to the cultivation, but also due to technical aspects (e.g., use of highly corrosion-resistant bioreactor setups). Also, at sudden drops in wastewater salinities, extreme halophiles might be contaminated with moderate halophiles, because growth rates at low saline environments are usually lower for extreme halophiles compared to moderate halophiles. In

Table 1. Overview of Halophilic or Halotolerant Microorganisms, Able to Degrade Aromatic Compounds and Their Place of Origin

Strain(s)/culture	Origin	Type of halophile	Salinity (%)	Aromatic compounds	Reference
Arthrobacter strains	Soils/bottom sediments with waste from chemical and salt mining industry (Verkhnekamskoe potash deposit, Permkrai, Russia)	Halotolerant bacteria	6%	Naphthalene, salicylate, gentisate, diesel fuel, tetradecane, octane, phenanthrene	Plotnikova et al. (2011)
Halophilic bacterial population (mostly related to *Halomonas* species)	Water and sediment of salterns and hypersaline soils in South Spain close to oil refineries and food-processing industries	Moderately halophilic bacteria	10%	Benzoate, *p*-hydroxybenzoate, cinnamate, salicylate, phenylacetate, phenol, *p*-coumarate, ferulate, *p*-aminosalicylate	García et al. (2005)
Halomonas organivorans	Saline soils from Isla Cristina (Spain)	Moderately halophilic bacterium	10%	Benzoate, *p*-hydroxybenzoate, cinnamate, salicylate, aminosalicylate, phenylpropionate, phenol, *p*-coumarate	García et al. (2004)
Geobacillus sp.	Production water (oil/water mixture) of the oil field TPS in Tunisia	Halotolerant (and thermophilic) bacterium	3%	Benzoate, *p*-hydroxybenzoate, protochatechute, vanilliate, phenol, *m*-cresol	Chamkha et al. (2008)
Halomonas sp. IMPC	Table-olive fermentation	Moderately halophilic bacterium	8%	*p*-Coumarate, benzoate, protochatechute, *p*-hydroxybenzoate, *p*-methoxy-benzoate, cinnamate, caffeate	Abdelkafi et al. (2006)
Halophilic archaeal strain (family of Halobacteriaceae)	Brine and sediment from Dead Sea (Jordan, December 2002)	Extremely halophilic archaea	34% (dead sea)	*p*-Hydroxybenzoate, benzoate	Cuadros-Orellana et al. (2012)
Halophilic consortium (*Halomonas* and *Marinobacter* strains	Saline soil sample contaminate with oil from industrial activity or accidents (Iran)	Moderately halophilic bacteria	1–17%	Phenanthrene	Dastgheib et al. (2012)
Halomonas sp. C2SS100	Production water from saline oilfield (Tunisia)	Moderately halophilic bacterium	5–8%	Crude oil [aliphatic hydrocarbons (C_{11}–C_{22})], carbazole (degradation activity was weak)	Mnif et al. (2009)
Haloferax sp., *Halobacterium* sp., *Halococcus* sp.	Soil and water samples from hypersaline coastal areas (supertidal "sabkha" from Kuwait and Abu Dhabi)	Extremely halophilic archaea	6–24%	*n*-alkanes (C_8–C_{40}), benzene, toluene, phentanthrene, biphenyl, naphthalene, *p*-hydroxybenzoic acid	Al-Mailem et al. (2010)
Debaromyces sp.	Activated sludge from a pharmaceutical plant (Wuhan, China)	Moderately halophilic fungus	5%	Phenol	Jiang et al. (2016a)
Haloferax sp. D1227	Top 5 cm of coarse, sandy soil surrounding an oil well (Grand Rapids, Michigan, USA)	Extremely halophilic archaeon	10–15%	Benzoate, cinnamate, phenylpropanoate	Emerson et al. (1994)
Haloferax sp., *Halobacterium piscisalsi*, *Halobacterium salinarium*, *Halorubrum ezzemoulense*, *Halorubrum* sp.	Brines samples from Çalmatı salterns (Turkey, 2007)	Extremely halophilic archaea	20%	*p*-Hydroxybenzoic acid, naphthalene, phenanthrene, pyrene	Erdoğmuş et al. (2013)

Table 1. Continued

Strain(s)/culture	Origin	Type of halophile	Salinity (%)	Aromatic compounds	Reference
Haloarcula sp. A235	Brine, salt, and saline soil samples from different (salt) lakes in Turkey in September 2000 and 2001 (see Ozcan et al., 2006)	Extremely halophilic archaeon	20%	Phenol	Acikgoz and Ozcan (2016)
Halomonas campisalis	Isolated near Soap Lake (WA, USA)	Moderately halophilic bacteria	0–15%	Phenol, catechol	Alva and Peyton (2003)
Haloarcula sp. EH4	Interface water sediment in a salt-marsh (Aigues-Mortes, France)	Extremely halophilic archaeon	15–30%	Acenaphthene, phenanthrene, anthracene, 9-methyl anthracene	Bertrand et al. (1990)
Haloferax alexandrinus st. KCTC 12962 BO3, BO6, AA31, AA35 *Haloferax* sp. CS1-9 BO7, MM17, PR13 *Haloferax* sp. HSC4 MM27 *Haloferax sulfurifontis* JCM 12327 CL47	Uyuni Salt Marsh, Bolivia, Cabo Rojo marine salterns, Puerto Rico, sabkhas (salt flats), Saudi Arabia, Dead Sea, Jordan, and Cahuil marine salterns, Chile	Extremely halophilic archaea	20%	Benzoate, *p*-hydroxybenzoate, salicylate, naphthalene, anthracene, phenanthrene, pyrene, benzo[*a*]anthracene	Bonfa et al. (2011)
Haloferax volcanii DSMZ 3757	Mud samples (1 m depth) in water close to the shore at the northern end of the Dead Sea	Extremely halophilic archaeon	20%	Anthracene, only with yeast extract: naphthalene, phenantrene, pyrene, benzo[*a*]anthracene	Bonfa et al. (2011), Mullakhanbhai and Larsen (1975)
Dietzia natronolimnaea JQ-AN	Industrial wastewater from Zhejiang Dragon Chemical Group Company (Hangzhou, China)	Moderately halophilic bacterium	0–6%	Aniline	Jin et al. (2012)
Unknown halophilc mixed culture	Soil samples from salterns at the Great Salt Lake Minerals Corporation (Utah, USA)	Moderately halophilic microorganisms	14%	Phenol	Woolard and Irvine (1995)
Halophilic enrichment culture	Two soil samples from the Great Salt Plains National Wildlife Refuge, OK, USA	Halophilic microorganisms	14.6%	Benzene and toluene	Nicholson and Fathepure (2005)
Bacillus methylotrophicus and *Pseudomonas sihuiensis*	Samples from National Park of Lagao do Peixe (sediment, sediment with seawater, seawater sample), Brazil	Halotolerant bacteria	3.5%	Aliphatic hydrocarbons (C8 to C33) and PAHs (anthracene, phenanthrene and pyrene)	Pereira et al. (2019)
Marine microorganisms from seawater samples	Seawater from Trondheimsfjord, Norway (depth: 80 m)	Halotolerant and/or halophilic microorganisms	3.4%	Produced water from Ula platform (North Sea): napthalenes, PAHs (2–3 rings and 4–6 rings), phenols	Lofthus et al. 2018)
Achromobacter sp. HZ01	Crude oil-contaminated seawater near the Mabianzhou Island, Daya Bay, Huizhou, China	Halotolerant bacterium	3%	Diesel oil, *n*-alkanes, anthracene, phenanthrene, and pyrene	Deng et al. (2014)
Arhodomonas sp. strain Seminole	Enriched microbial consortium (Nicholson & Fathepure, 2004)	Halophilic bacterium	14.6%	Benzene, toluene, phenol, 4-hydroxybenzoate, protochatechute, and phenylacetate	Dalvi et al. (2014)

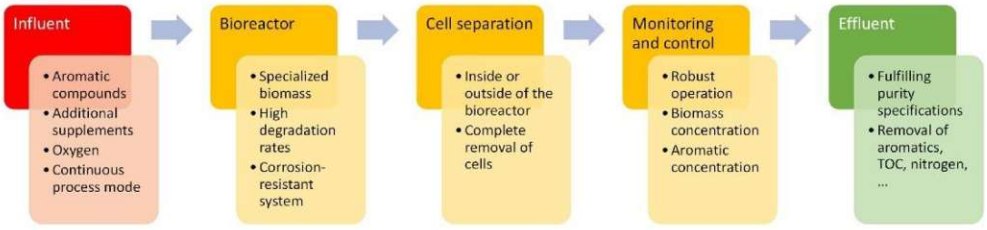

Fig. 2. Overview of process requirements for the implementation of halophilic bioremediation in industry. Red box: influent wastewater stream containing contaminants and additional supplements. Yellow boxes: bioreactor system containing biomass to degrade contaminants in the wastewater. Cell suspension is separated into cell-free effluent and broth remaining in the bioreactor. Cell separation can take place inside or outside the reactor. Control loop for a robust process control. Green box: treated wastewater stream within purity specifications.

general, the microbial system to be used in bioremediation processes can be chosen either from a microbial consortia originating from saline habitats or natural halophilic isolates from a strain collection [e.g., DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen)].

In addition to the operation mode of a bioremediation process and the used microbial system, also a process monitoring strategy contributes to a high process performance. Developing a monitoring strategy for wastewater treatment processes raises several questions. Those questions highly depend on the process and the process requirements. Therefore, a general statement for the need and the exact approach of process monitoring tools is impossible. Nevertheless, process monitoring is of utmost importance to guarantee the quality of the treated wastewater. Thus, it is important to first decide what parameters have to be measured. More precisely, it has to be decided if specific analyte concentrations (e.g., aniline concentration in the effluent, biomass concentration, etc.) are needed or if more unspecific parameters like the total organic carbon (TOC), the biological oxygen demand (BOD), or the chemical oxygen demand (COD) are sufficient (Bourgeois et al., 2001; Zhang & Li, 2009). The next questions would be how frequently data of the parameters are needed and what the most suitable measurement method is. Is it sufficient to take effluent samples once per day or is it necessary to obtain real-time data of the parameter? If only low numbers of data points per day are required, methods like HPLC, spectrometric methods or enzymatic essays might be used, as they offer high accuracy and well established protocols. However, if a permanent monitoring of the wastewater effluent is necessary, online hard or soft sensors offer data acquisition in real-time. Finally, the functionality of the monitoring of saline wastewater treatment processes is the applicability of the methods to high salt concentrations. Does the salt matrix disturb the method? Does dilution increase the measurement insecurity? Does the sensor corrode in the saline environments?

Wastewater Treatment with Halophilic Mixed Cultures

Using a microbial consortia from saline origins for treating saline wastewater increases the chances of accumulating species adapted to the environment and able to degrade the occurring contaminants. Such a strategy would finally allow to enrich special microorganisms able for the degradation of contaminants in the specific wastewater. In the following section, we summarized existing works using halophilic mixed cultures to treat aromatics-containing wastewater (e.g., phenol, etc.).

For instance, activated sludge from sea mud was used to degrade phenol containing wastewater mixed with seawater (Tan et al., 2017b). The cultivation was performed in an aerated 30 l bioreactor in a discontinuous way, as supernatant in the bioreactor was replaced by fresh medium once per day. The medium contained fresh nutrients like glucose (0.21 g l^{-1}), starch (0.1689 g l^{-1}) and in smaller amounts NH_4Cl and K_2PO_4. The salinity of the phenol-containing seawater–wastewater mixture was increased in the influent from 3.7% to 5.7%. The activated sludge was characterized, and three halophilic species (*Oceanomonas* sp., *Arthrobacter* sp., *Vibrio* sp.) could be identified in the activated sludge. All three species showed sufficient phenol removal capacities as over 80% of initial phenol concentration could be removed in batch experiments within 48 hr. The performance in the bioreactor experiments was similar as COD removal was always between 70% and 85%. Also, the phenol concentrations in the effluent never exceeded 40 mg l^{-1} (influent concentrations: 220–1 100 mg l^{-1}). Nevertheless, at the beginning of every new seawater–wastewater mixture, the performance of phenol and COD degradation decreased, probably due to an acclimatization phase. From this study we can learn, that activated sludge enriched with halophilic microorganisms is able to degrade aromatic compounds in slightly saline wastewater with an efficiency of over 70%. As a result, the process could be developed further by investigating the influence of the three halophilic strains found in the sea mud on the aromatic degradation. Furthermore, the operation mode should be changed into a continuous mode and an active cell retention system, like membrane-based technologies, should be used to reduce bioreactor volumes for following scale-up studies.

In addition to aerobic treatment processes also anaerobic treatment of saline wastewater containing aromatics has been described (Wang et al., 2017). Three upflow anaerobic sludge blanket (UASB) reactors (two saline reactors, one nonsaline reactor) with 3.5 l working volumes were inoculated with activated sludge from a municipal wastewater treatment plant in Hefei (China) and compared according to phenol degradation efficiency (Wang et al., 2017). It was shown, that phenol removal decreased at higher phenol (2 g l^{-1}) and Na^+ concentrations (20 gNa^+ l^{-1}). Synthetic wastewater with phenol, catechol, resorcinol and hydroquinone as aromatic contaminants was used at Na^+ concentrations of 10 and 20 g l^{-1}. The HRT was set to 48 hr, while the upflow velocity was 1.3 m h^{-1}. COD and phenol removal efficiencies always reached values above 95% and 98%, respectively, at influent concentrations of 100–500 mg l^{-1} phenol and 10 gNa^+ l^{-1}. However, the used microbial system suffered from a phenol shock when the influent concentration of phenol was increased to 1000 mg

Table 2. Bioprocesses for the Treatment of Saline Wastewater, Containing Aromatic Contaminants. Processes are Compared According to Their Process Parameters Like Reactor Volume, Operation Mode, HTR, and Removal Efficiency. Additionally, Information is Given About the Salt Content of the Wastewater, Additional Nutrients, and the Used Microorganisms

Wastewater source	Reactor volume	Operation mode	Salt concentration	Contaminants	Removal efficiency (%)	Nutrient supplementation	Microorganism	Reference
Synthetic wastewater	10 l	Continuous (HTR = 4.7–5.7 hr), immobilized cells	0–6.5%	Phenol	99	Salts, oxygen	*Oceanomonas* sp.	Tan et al. (2017a)
Synthetic wastewater	20 l	Continuous (HTR = 5 days), membrane-based cell retention	0–1.5%	Phenylphenol, acetanilide, bisphenol A, etc.	20–60	–	Activaled slduge (anaerobic)	Song et al. (2016)
Synthetic wastewater	625 l	Sequencing batch, immobilized cells	5%	Phenol	100	Salts, oxygen	*Camomonas* sp. JB	Jiang et al. (2016b)
Industrial wastewater	1 l	Continuous (HTR = 10 hr), membrane-based cell retention	15%	Formate, aniline, phenol, and MDA	100	Salts, glycerol, oxygen	*Haloferax mediterranei*	Mainka et al. (2019)
Industrial wastewater	30 l	Sequencing batch	3.7–5.7%	Phenol	80	Ammonium, phosphate, starch, glucose, oxygen	Activated sludge from sea mud	Tan et al. (2017b)
Synthetic wastewater	250 ml	Continuous (HTR = 3–14 hr), membrane based cell retention	Controlled to 50 mS (with NaCl)	Phenol	100	Salts and trace elements	*Pseudomonas putida* ATC 11172	Praveen and Loh (2016), Praveen et al. (2015)
Synthetic wastewater	1 l	Sequencing batch	14%	Phenol	99.5	Ammonia, phosphorus, iron, inorganic salts	Halophilic mixed culture	Woolard and Irvine (1995)
Synthetic wastewater	3.5 l	Continuous (HTR = 48 hr)	10–20 $gNa^+ l^{-1}$ = 2.4–4.8% NaCl	Phenol, catechol	95–98	Macro- and micronutrients	Activated sludge (anaerobic)	Wang et al. (2017)

l^{-1}. Thus, the phenol removal efficiencies were reduced to only 70–80%. After 10–20 days the removal efficiency was increased again to 95–97%. This study showed, that an anaerobic process for the removal of aromatics using activated sludge is possible. Nevertheless, this process could only be operated at lower salt concentrations. Poor process performance for anaerobic systems was also reported in other studies, which showed the inhibition of the anaerobic microbial community due to high salinities (Ng et al., 2014; Svojitka et al., 2017).

Similarly, another report showed comparable result by using activated sludge to treat saline wastewater in a 20 l anaerobic MBR (anMBR) system (Song et al., 2016). In this study, activated sludge from the Wollogong Wastewater Treatment Plant (Australia) was used to degrade 33 trace organic compounds (trOC) belonging to the four key groups of contaminants (i.e., pharmaceuticals, personal care products, industrial chemicals, and pesticides), among them are also aromatic compounds like phenylphenol and bisphenol A. The salinity in the reactor was increased from 0% to 1.5% with a rate of 0.1% per day. Only at NaCl concentrations below 1%, COD removal efficiency was 98%. When salt concentrations exceeded levels higher than 1%, COD removal efficiency decreased to 80%.

Wastewater Treatment with Halophilic Mono Cultures

Besides using halophilic microbial communities for the remediation of industrial waste streams, it is also possible to select halophilic strains according to their ability to degrade the present aromatic contaminants. Thus, a bioprocess for saline wastewater treatment can be tailored according to the existing conditions.

For example, an *Oceanomonas* sp. strain was used as inoculum for a biological contact oxidation reactor (BCOR), in order to degrade phenol in slightly saline wastewater [0–6.5% (wt/vol) NaCl] (Tan et al., 2017a). For cell retention, the cells were immobilized to polypropylene fibers in a 10 l reactor filled with seawater. The medium was additionally supplemented with ammonium, phosphate, calcium, magnesium and iron salts. Moreover, no additional carbon source was necessary. Because *Oceanomonas* is strictly aerobic, the reactor was aerated and the dissolved oxygen concentration was controlled to a minimum of 2.5 mg l^{-1} O_2 at a temperature of 25°C. The immobilization allowed liquid feeding rates of 1.33–2.13 l h^{-1}, which results in HRTs of 4.7–7.5 hr. As a result, saline wastewater containing phenol concentrations up to 1 500 mg l^{-1} and 6.5% (wt/vol) NaCl was successfully treated with removal efficiencies of 99%. Nevertheless, only synthetic wastewater under laboratory conditions was used in this study. Therefore, the investigation of long-term performance using real industrial wastewater would be interesting. Moreover, as salt concentrations were low in this study (below 6.5% NaCl), co-contaminations with other slightly halophilic microorganisms are possible. To test the robustness of the present approach against contaminations, unsterile industrial wastewater could be used as cultivation medium.

At a smaller scale of 625 ml, a system was developed which used magnetically immobilized *Comamonas* sp. JB for the degradation of phenol, *o*-cresol, *m*-cresol, and *p*-cresol in a synthetic wastewater containing up to 5% NaCl (Jiang et al., 2016b). In parallel, two other reactors were inoculated with *Comamonas* sp. JB, one without immobilized cells and the other with nonmagnetically immobilized cells. The wastewater was replaced in the reactors every 12 hr, which corresponds to a sequencing batch mode. Compared to nonimmobilized cells and nonmagnetically immobilized cells, the magnetically immobilized cells showed higher removal rates. In contrast to nonimmobilized cells, the magnetically immobilized cells even achieved high phenol removal rates of over 70% at salinities above 3% NaCl. The process performance could also be improved, by including electrodes into the bioreactor system for electrical stimulation of the cells. With this integrated system, phenol removal rates of 100% could be achieved, due to an increase of phenol hydroxylase activity from 0.135 to 0.31 U mg^{-1}. Besides high removal efficiencies, this systems lacks several factors, which are important for the implementation into industrial process chains. First, the volume of less than one liter is low, which makes scale-up studies necessary. Second, the process was not operated in a continuous mode. Therefore a strategy handling high volume waste streams has to be developed.

In contrast, using membranes is a common approach for retaining cells in the bioreactor. We have reported such a membrane-based retention system for the degradation of phenol, aniline, 4,4′-methylenedianiline (MDA) and formate in high-saline MDA process water using the extreme halophilic archaeon *Haloferax mediterranei* (process scheme, Fig. 3) (Mainka et al., 2019). The reported system allows not only higher hydraulic dilution rates D for the process water ($D = 0.1\ h^{-1}$, equals a HRT of 10 hr), but also monitoring and control of the biomass concentration in the reactor through a feedforward control strategy. The feedforward strategy controls the biomass concentration by adjusting the two parameters (1) glycerol concentration in the feed and (2) the ratio R of cell-free permeate flow F_H and the feed flow F_F (Fig. 3). The removal efficiencies for the aromatic compounds were up to 100% for all of the four organic contaminants. The additional carbon source glycerol was used as growth substrate in order to maintain high biomass concentrations and enable high degradation rates of the contaminants. Glycerol is a cheap and widely available carbon source. However, the utilization of other substrates such as acetate or lactate from waste sources is also possible (Erian et al., 2018; Pflügl et al., 2014; Russmayer et al., 2019). In terms of scale-up, we reported the construction and utilization of a 21 l corrosion-resistant bubble column bioreactor (BCR) equipped with a membrane-based cell retention setup (Mahler et al., 2018). While studying the BCR, we showed the successful cultivation of halophilic cultures in pilot-scale. Compared to the lab-scale bioreactor, the process parameters and yields were similar in the BCR, even though oxygen supply and mixing was provided only through bubbles and not by stirring. As a result, such a system reduces the operational costs at larger scales and makes the setup easier to maintain. As the experiments were performed under laboratory conditions, it would be interesting to investigate the effect of changing wastewater conditions. Therefore, a long-term cultivation would be interesting, where the composition of the wastewater is altered, in a way that simulates conditions found in a real industrial environment.

Another study used a forward osmotic hollow fiber filtration unit for cell retention in a continuous bioprocess for the degradation of phenol (influent concentration 0.6–2.5 g l^{-1}) with halotolerant *Pseudomonas putida* ATC 11172 (Praveen et al., 2015; Praveen & Loh, 2016). Additionally, an extractant impregnated membrane (EIM) was used as a partitioning phase to prevent inhibitory phenol concentrations in the aqueous phase by temporarily removal of phenol (Praveen & Loh, 2016). With this system, removal efficiencies of 100% were reached at HRT of 3–14 hr. However, the used volume of 250 ml was low and a scale-up to reactor-size should be carried out, in order to investigate potential scale-up effects. Moreover, problems with membrane fouling

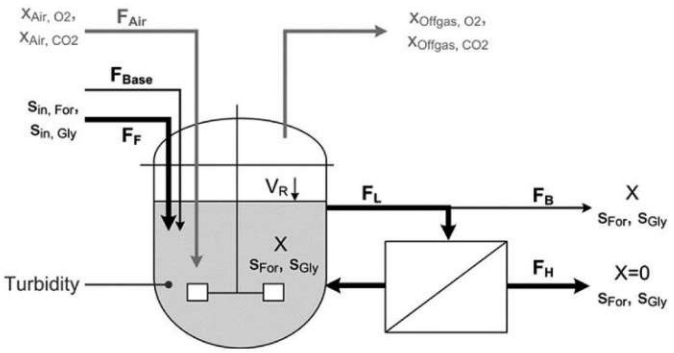

Fig. 3. Scheme of the cell retention setup. A constant feed (F_F) supplies the cells with fresh substrate and media components. Base (F_{Base}) is added to hold the pH on a constant level of 7.0. A pump continuously circulates the cell suspension as loop flow (F_L) through the membrane module to separate cell-free harvest (F_H). Bleed flow (F_B) is continuously removed to eliminate cells and sustain steady state conditions. To guarantee a constant reactor volume (V_R) flows for Feed, Base, Harvest, and Bleed have to meet the following equation: $F_F + F_{Base} = F_H + F_B$. Biomass is monitored using a turbidity probe and a soft sensor that is driven by measurements of off-gas composition (Mainka et al., 2019).

occurred, which were caused by proteins attached to the membrane (Praveen & Loh, 2016).

In summary, state-of-the-art bioprocesses for the treatment of saline wastewater, containing aromatic contaminants, deliver good performances according to aromatic degradation efficiencies. Various biological systems were used and different cell retention system could be applied successfully. A comparison of the presented processes can be found in Table 2.

Monitoring Approaches for Saline Wastewater Processes

The monitoring of critical process parameters depends on requirements of the wastewater treatment process. Parameters which can be monitored, are physical (temperature, pressure, etc.), chemical (e.g., analyte concentration, TOC or COD), or biological (e.g., biomass concentration, etc.) characteristics (Biechele et al., 2015; Bourgeois et al., 2001; Vanrolleghem & Lee, 2003). In general, a process monitoring strategy should help to obtain stable and reproducible bioprocesses which meet the desired quality criteria for the treated wastewater (Biechele et al., 2015). Although process monitoring is applied to saline and nonsaline processes, saline conditions and an industrial environment cause challenges to the design and usability of sensors and measurement methods. First of all, the high salt concentrations require materials to withstand the corrosive conditions. Moreover, measurement methods have to overcome interference problems with the salt matrix.

In the case of bioremediation of aromatics containing saline wastewater, the monitoring of biomass concentration, specific aromatic concentrations, and/or total organic concentrations (TOC, COD, or BOD) are potential approaches and are discussed in the following section.

Biomass monitoring

The determination of biomass related parameters, like absolute biomass concentration or specific growth rate, is an important topic for bioprocess development, as cells are the biocatalyst (Tamburini et al., 2003). For instance, in production bioprocesses often a correlation between specific productivity and specific growth rate can be found, thus monitoring the growth rate can help to maintain optimal production parameters (Looser et al., 2015, 2017; Zhang et al., 2005). Furthermore, the biomass concentration in wastewater treatment processes is a crucial parameter, not only important for degradation efficiency, but also as the disposal of waste biomass contributes to the operational costs of a wastewater treatment plant (Canales et al., 1994; Kayranli & Ugurlu, 2011; Wei et al., 2003). Methods published for the determination and estimation of biomass range from cell count methods on agar plats, to indirect gravimetric determination of cell dry weight to online methods like turbidity measurements and soft sensor estimations (Finn et al., 2006; Biechele et al., 2015; Kager et al., 2017; Mainka et al., 2019).

If a dependency between biomass concentration and process performance of bioremediation processes exists, the biomass concentration can be used as control parameter for process optimization. Control parameters need to be available in real-time, thus, data of the biomass concentration have to generated permanently. Thus, microbiological methods like cell count on agar plates or offline methods, such as gravimetric determinations or optical density (OD) measurements, are not suitable. Potential alternatives include online methods such as the measurement of turbidity with a turbidity probe, near infrared spectroscopy (NIRS), dielectric spectroscopy or mass balancing based on offgas data (Finn et al., 2006; Kiviharju et al., 2008; Mainka et al., 2019; Münzberg et al., 2017).

Turbidity probes are based on different optical measurement principles, such as transmission, reflection and transflection measurement (Münzberg et al., 2017). Probes based on reflection or backscattering are often preferred for in-line measurements, because these principles do not depend on the optical path length and are more resistant to probe fouling (Münzberg et al., 2017). Wavelengths for turbidity measurements in bioprocesses were reported at 600 nm or (860 ± 30) nm (Vojinović et al., 2006; Münzberg et al., 2017). However at 860 nm might be beneficial when used in colored systems, as the light absorbance is lower (Münzberg et al., 2017). For using turbidity probes to determine biomass concentration in bioreactors, an *a priori* calibration with offline biomass data, like the cell dry weight, is needed. Also other process parameters, like agitation or aeration, can influence the turbidity signal alter it in terms of turbidity-biomass-correlation (Gregory & Thornhill, 1997). Limitations using turbidity probes are occurring due to nonlinear correlations of turbidity and biomass

concentration (Gregory & Thornhill, 1997; Kiviharju et al., 2008; Münzberg et al., 2017). Those effects, however, can be avoided by special probe models where samples are passed into a degassed measurement chamber (Kiviharju et al., 2008; Olsson & Nielsen, 1997). Also, other suspended solids in the medium (e.g., dirt particles) can disturb the turbidity measurement and interfere with the biomass (Vojinović et al., 2006). Therefore, industrial wastewater should be particle free, if accurate turbidity data are needed.

A more sophisticated method for the determination of biomass concentration in cell broth would be the use of NIRS-probes. For biomass determination with NIRS-probes, a spectrometer scans wavelength ranges from 400 to 2 500 nm (Finn et al., 2006; Tamburini et al., 2003). Information about biomass concentration can, for example, be found in regions from 910 to 930 or 2260 to 2270 nm (Cervera et al., 2009; Finn et al., 2006). In order to extract biomass concentration data, it is common to pretreat the absorbance spectra, for example, calculating the second derivative, and build a chemometric model to estimate biomass concentrations (Cervera et al., 2009; Ge et al., 1994). Nevertheless, the NIRS measurements were found to be affected by the aeration rate, the agitation speed, and also the temperature, which has to be considered when building a biomass model (Ge et al., 1994; Scarff et al., 2006). One advantage of NIRS measurement for biomass estimation is the possibility of online *ex situ* measurements, realized by either flow-through cells or fiber optic process behind a glass wall (Cervera et al., 2009; Ge et al., 1994). Thus, no probes have to be inserted into saline medium containing bioreactors. Moreover, NIRS measurements were also reported for the determination of substrates and products in fermentation broths. Among those molecules, carbon sources such as glucose, ethanol, glycerol, or lactose were determined, but also ammonia or phosphate were reported to be measured (Cervera et al., 2009; Finn et al., 2006). The monitoring of such metabolites or by-products is important, when the total organic content in the wastewater should be removed. Also the accumulation of ammonia should be avoided in some cases, for example, for chlor-alkali electrolysis, thus online monitoring of ammonia could be beneficial for process efficiency and security (Brinkmann et al., 2014).

Alternatively, soft sensors offer the possibility to generate information about biomass concentration by using different process variables. Those variables are measured by several probes and are processed by a software-based algorithm to estimate biomass concentration (Biechele et al., 2015; Kiviharju et al., 2008). The underlying principles for soft sensors are either model-driven or data-driven. Whereas model-driven soft sensors are based on mass and energy balances, data-driven soft sensors use historical process data for online estimation (Biechele et al., 2015). Process variables which are commonly used for biomass soft sensors are offgas values (CO_2, O_2), base consumption or dissolved oxygen (pO_2) (Biechele et al., 2015; Kager et al., 2018; Kiviharju et al., 2008; Sundström & Enfors, 2008). In comparison to biomass estimation with hard sensors, also soft sensors need offline reference values of biomass concentration to develop and calibrate the underlying models. Nevertheless, soft sensors are able to extend the usability of probes and sensors commonly used for bioprocess and which are often already implemented in existing processes. Therefore, besides the software tools, no investments for additional devices and probes are necessary. Especially for the harsh conditions at treatment plants for industrial saline wastewater, avoiding sensitive probes is beneficial. Thus, soft sensors for the determination of biomass concentration in saline bioprocesses offer great potential for future process development. This potential could already be shown, as we have recently established a soft sensor for real-time estimation of biomass concentration based on offgas measurements and substrate concentrations in wastewater feeds (Mainka et al., 2019). The soft sensor was developed for the extremely halophilic archaeon *H. mediterranei*, used for continuously treating an industrial saline wastewater, containing various aromatic compounds.

Monitoring aromatic concentrations

The monitoring of concentrations of specific aromatic compounds (like aniline or phenol) can be useful, if, for example, thresholds of these compounds in the effluents have to be met. When the wastewater contains more than one aromatic compound, HPLC methods offer the possibility to measure several compounds at once and, depending on the method, with high accuracy. However, samples have to be taken, either manually or automatically, prepared and the HPLC measurement time considered (e.g., 30 min). HPLC measurements are time consuming and data are only generated discontinuously. Nevertheless, for purposes, which are uncritical in terms of time like for the comparison of concentrations of wastewater batches with environmental specifications, HPLC methods are suitable and sufficient. In contrast, if monitoring data are needed for control purposes, data have to be generated in real-time, which can, for example, be realized by sensors. In literature, several (bio)sensors and methods for the detection and quantification of aromatic compounds are described (Buerck et al., 2001; Gutés et al., 2005; Korkut et al., 2016; Mu, 2006; Rahemi et al., 2020; Zhang & Li, 2009).

Online measurement methods for aromatic compounds in wastewater treatment processes need to be highly sensitive and be able to detect also low concentrations. This is true, as the purpose of wastewater treatment plants is the removal of contaminations, and thus, contaminant concentrations should be close to zero. Therefore, the limit of detection of a potential sensor need to be even lower as the threshold, if the concentration of contaminants in a wastewater have to be decreased below a certain threshold. Otherwise, additional offline measurements are necessary to check the removal efficiency of the process.

As mentioned above (section "Monitoring Approaches for Saline Wastewater Processes"), NIRS measurements are able to determine carbon sources like glucose or glycerol, studies were also published using NIRS to measure aromatic compounds (Buerck et al., 2001; Mattioda et al., 2005). However, problems occur when aromatics are dissolved in aqueous solutions, as the OH-group of water absorbs strongly at 1450 and 1940 nm, and thus, overlaps with the CH peaks of aromatics (1600–1900 nm) (Buerck et al., 2001; Bürck et al., 1992). Therefore, an NIR–EFA sensor (EFA = evanescent field absorption) was developed, which enables the extraction and enrichment of apolar hydrocarbons into the cladding of quartz glass fiber sensor, according to the Nernst distribution law (Buerck et al., 2001). Prior to determining aromatic concentrations, spectra of pure aromatics were measured and calibration models were generated using partial least squares (PLS) method. As this sensor could be promising for monitoring aromatic compounds at wastewater treatment processes, still several things have to be taken into account. The first one is, that concentrations in a mixture of several different aromatics cannot be determined, but a cumulative concentration parameter could be calculated by including data from a filter photometer. Moreover, the response time of the sensor to concentration changes can range from 2 to more than 20 min, depending on the aromatic species. Also, the sensor has to be implemented inside the bioreactor or a

Table 3. Sensors for the Determination of Aromatic Compounds

Aromatic compound	Measurement principle	Probe	Linear conc. range	Reference
Catechol	Enzyme-based	Glassy carbon electrode	0.036–2.5 µM	Maleki et al. (2017)
Catechol	Electrochemical oxidation	Three-electrode system (working electrode: platinum or copolymer, counter electrode: platinum, reference electrode: calomel (SCE)	5–80 µM	Mu (2006)
Catechol (cat), hydroquinone (hyd)	Electrochemical detection	Glassy carbon electrode	cat: 0.5–40 µM hyd: 0.13–56.6 µM	Nazari et al. (2018)
Hydroquinone, 4-aminophenol	Enzyme-based	Electrode modified with TiO_2	4-Aminophenol: 0.05–2 µM	Rahemi et al. (2020)
3-Methoxyaniline	Electrochemical detection	Glassy carbon electrode	0.1 nM–0.1 mM	Rahman et al. (2019)
Hydroquinone, catechol, resorcinol (res)	Electrochemical oxidation	Glassy carbon electrode	hyd: 1–200 µM cat: 4–200 µM res: 3–400 µM	Wei et al. (2014)

bypass construction, as it has to be in contact with the medium. Therefore, stability and performance tests for saline wastewater are still necessary. Moreover, potential interferences with cells have to be investigated.

In contrast to measure cumulative concentration parameters of aromatics in general, as happened with NIRS measurements, sensors detecting only specific aromatics and being able to differentiate between these compounds have also been reported (Maleki et al., 2017; Mu, 2006; Nazari et al., 2018; Rahemi et al., 2020; Rahman et al., 2019; Wei et al., 2014; Zhang & Li, 2009). The principles used for the measurement range from optical based systems over enzymatic biosensors to electrochemical detectors. Aromatic compounds which were reported to be measured with sensors are, for example, aniline, catechol, hydroquinone, resorcinol, or 3-methoxyaniline (Maleki et al., 2017; Mu, 2006; Nazari et al., 2018; Rahemi et al., 2020; Rahman et al., 2019; Wei et al., 2014; Zhang & Li, 2009). Such systems offer the possibility of measuring specific compounds and are suitable for processes where only a small amount of different aromatics are present. However, at microbial degradation processes of aromatics or any other organic contamination, potential intermediates of the degradation pathways could accumulate. Thus, this accumulation could remain undetected if the sensors can measure the original contaminant but not possible intermediates. Nevertheless, sensors able to measure specific aromatic compounds could be used for quantifying contaminant concentrations in wastewater feeds. Those concentration data could consequently be used for feedforward control strategies, where the bioremediation process is controlled based on composition information of the influent. An overview of the aromatic (bio)sensors and their measurement principles is given in Table 3.

Monitoring TOC

Monitoring organics concentration in wastewater treatment plants is commonly carried out by measuring cumulative concentration parameters like TOC, COD, or BOD (Assmann et al., 2017; Bourgeois et al., 2001; Vanrolleghem & Lee, 2003). Those parameters are all measured in different ways. Therefore, the obtained results offer different possibilities to be interpreted. The standard BOD_5 method measures the consumption of dissolved oxygen of a microbial system during 5 days in a test sample (Vanrolleghem & Lee, 2003). The long timespan makes this measurement method obviously not suitable for real-time monitoring. However, BOD methods were developed which deliver results with 5–10 min (Bourgeois et al., 2001; Vanrolleghem & Lee, 2003). The COD analysis gives information about the oxidation ability of organic compounds in wastewater samples, by using strong oxidizing agents (e.g., dichromate, $Cr_2O_7^{2-}$) under acidic conditions (Bourgeois et al., 2001; Kayaalp et al., 2010; Vanrolleghem & Lee, 2003). Drawbacks of COD analysis are that biologically inert organic compounds cannot be differentiated from biodegradable content and the generation of hazardous waste (e.g., chromium) (Bourgeois et al., 2001; Guan et al., 2019). Simultaneously to organic substances, chloride ions in saline samples react with chromate ions and therefore increase measurement errors (Kayaalp et al., 2010). TOC analysis only measures the concentration of organic compounds, and is considered to be the most 'true' index of the total organic contamination in wastewater (Bourgeois et al., 2001). In general, the offline TOC analysis is based on the principle to convert organic matter into CO_2, either by using a catalyst at high temperatures (650–900°C) or by oxidizing organics with UV light and persulfate reagent, subsequently measuring the amount of formed CO_2 gas (Assmann et al., 2017; Bourgeois et al., 2001; Vanrolleghem & Lee, 2003). In both cases, inorganic carbon needs to be eliminated prior to measure TOC. Also, both methods have some drawbacks. The high temperature method is sensitive to salts, as salts could produce a melt on the catalytic surface. When using the persulfate method, incomplete oxidation could occur, if the pH is too low or the wastewater sample is too turbid (Vanrolleghem & Lee, 2003). Moreover, TOC measurements cannot give information about the biodegradability of wastewater samples (Vanrolleghem & Lee, 2003).

For the real-time measurement of TOC or COD-values, online spectrometers can be used. Those probes use the principle of many organic substances to absorb light at specific wavelengths (e.g., 254, 350, or 465 nm) (Guan et al., 2019; Mills & Fones, 2012). Online spectrometers can detect organic matter in concentration ranges down to ppb, and are widely used in wastewater treatment plants for monitoring and control purposes (Van Den Broeke et al., 2006). As the use of online spectrometers only delivers indirect measurement values, a calibration procedure with offline values is required (Langergraber et al., 2003). However, suspended solids influence spectroscopic measurements, due to light scattering and shading. Therefore,

compensations are necessary to obtain accurate measurements (Van Den Broeke et al., 2006).

Conclusion and Outlook

In this review, we showed the potential to use halophilic microorganisms for biological treatment of industrial saline wastewater contaminated with aromatic compounds. In that course, we highlighted the need for treating aromatic compounds-containing wastewater, suggested requirements for industrial wastewater processes, gave examples of halophilic bioremediation processes reported in the literature and discussed potential aspects of further research and development topics.

Studies dealing with the degradation of aromatics in saline wastewater showed the proof of principle of using halophiles, as high removal efficiencies for the tested contaminants were reached. Also, reports showed that continuous bioprocesses in lab-scale bioreactor systems including cell retention units are working successfully.

Nevertheless, for the implementation of halophilic bioremediation processes into industrial production environments, several problems still have to be solved or investigated. Most studies used lab scale systems with a volume well below 20 l. One reason might be the technical effort of large-scale experiments, but also investment costs play an important role. Therefore, experiments in larger scales are necessary to investigate potential scale-up effects. For that reason, collaborations with industrial project partners could intensify the research of large-scale bioremediation processes for saline wastewater. In particular, the usage of cell retention system in large scales should be examined considering long-term process performance. In addition, wastewater used in scientific studies were mostly synthetic wastewater with only little number of tested contaminants. For addressing the complexity of most industrial wastewater, more studies should be performed using real industrial waste streams. To do so, industrial collaborations would be again a suitable way to take the research a step further.

The industrial implementation of halophilic bioremediation processes also has to solve issues on the level of process technologies. For instance, corrosion-resistant bioreactor systems are needed when saline wastewater are used, in order to reduce technical problems like leakages in pipes, pumps, or valves. Furthermore, a complete halophilic bioremediation process necessitates a suitable process monitoring system for critical process parameters like biomass concentration, TOC levels or contaminant concentrations. Such a system could consist of both, hard and soft sensors. A complete monitoring and control strategy for a bioremediation process of aromatics containing wastewater should consist of different sensor systems. Online sensors for the measurement of aromatic concentrations in the feed, biomass estimation in the bioreactor based on soft sensors and the measurement of an organic sum parameter (e.g., TOC) in the effluent would enable monitoring the main process parameters. Those parameters could then be used as input parameters for a control strategy, which would help to improve and maintain process performance over longer operation periods. Although hard (e.g., TOC measurement) and soft sensors (e.g., for biomass estimation) are already established, more effort is required for further development of these systems. Information gathered by specialized sensors can help to improve process understanding, which could result in higher process efficiency.

Future research in the field of bioremediation of saline wastewater should also pay attention to environmental regulations concerning maximum levels of contaminants allowed to be released into the environment. As regulations differ between countries and also depend on the industrial sectors, information about maximum TOC or COD levels allowed for release should already be included during the process design phase.

In conclusion, halophilic organisms are promising catalysts for purification of saline, industrial wastewater. Combined with systematic bioprocess development which allows to establish efficient microbial degradation systems which meet industrial specifications, the time is ready for the application of halophilic cultures in large scale industrial systems.

Author Contributions

Conceptualization: Thomas Mainka, Stefan Pflügl; writing—original draft preparation: Thomas Mainka, David Weirathmüller; writing—review and editing: Stefan Pflügl, Christoph Herwig; visualization: Thomas Mainka; funding acquisition: Christoph Herwig; and supervision: Stefan Pflügl, Christoph Herwig.

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Conflict of Interest

The authors declare no conflict of interest.

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4.2 Reducing organic load from industrial MDA residual process brine with a novel halophilic mixed culture: scale-up and long-term piloting of an integrated bioprocess

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TM carried out the cultivation experiments. TM and SP conceived the study and analyzed the data. TM, CH, and SP wrote the manuscript. All authors have read and approved the final manuscript.

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Reducing Organic Load From Industrial Residual Process Brine With a Novel Halophilic Mixed Culture: Scale-Up and Long-Term Piloting of an Integrated Bioprocess

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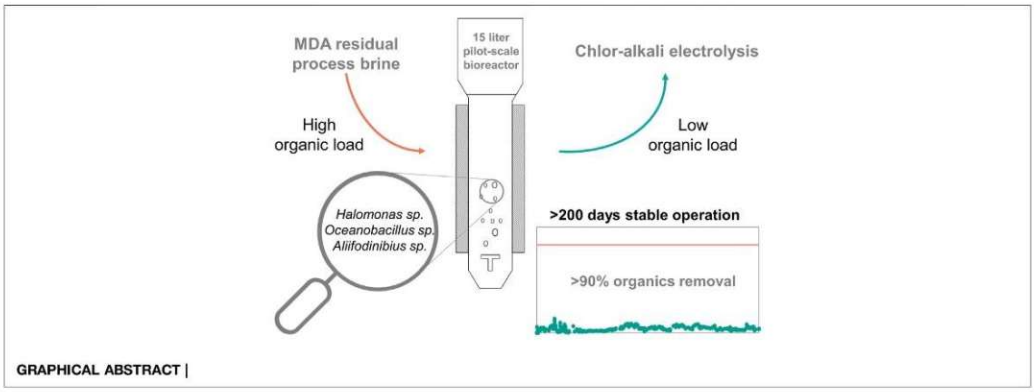
Integrating bioprocess solutions for treatment and subsequent reuse of saline residual process brine into industrial processes could increase the sustainability of production chains. However, such bioprocesses require large-scales and a robust operation over a prolonged period. Consequently, the aim of this study was to analyze scale-up equivalence as well as continuous and stable process performance of a previously established lab scale process for the degradation of organic contaminants (formate and aromatic compounds) in an industrial context. To that end, a pilot-scale bubble column bioreactor system equipped with a membrane-based cell retention system for process intensification was integrated at an industrial production site. The process was successfully scaled-up and continuously operated for more than 210 days. Overall, the process proved to be robust towards changing compositions of the residual process brine stream and degradation rates for organic contaminants were close to 100%. Interestingly, due to the unsterile process conditions, the original *Haloferax mediterranei* culture was replaced by a novel halophilic bacterial community consisting of three bacterial genera. To further improve process economics and productivity, an optimization of the co-substrate feeding strategy for glycerol is required, as results indicated a potential correlation between glycerol feeding and formate degradation rates. To that end, decoupling of the glycerol feeding from the residual process brine feed is a potential way to increase process control options and allow for easy adaptation of the process to changing residual process brine compositions. Ultimately, the process described here could be a promising alternative for chemical or physical methods of treating residual process brine and once more underlines the potential to exploit natural microbial diversity for industrial purposes.

Keywords: bioprocess scale-up, halophilic microbial community, industrial residual process brine treatment, aromatic degradation, long-term bioprocessing



GRAPHICAL ABSTRACT |

1 INTRODUCTION

In various industrial sectors, salt- and non-salt-containing residual process brine (RPB) are generated. Before RPB can be released to the environment, treatment is both necessary and challenging (Lefebvre and Moletta, 2006; Prasse et al., 2015; Crini and Lichtfouse, 2018). However, NaCl containing RPB is an excellent source for a more sustainable production of chlorine and sodium hydroxide (Du et al., 2018). Therefore, the use of RPB to produce basic chemicals offers a huge potential in making industrial production chains more sustainable and cost effective (Särkkä et al., 2015; Muddemann et al., 2018). Nevertheless, organic impurities in NaCl-containing RPB have a negative effect on membranes used in chlorine-alkali processes, since they can cause precipitates and foaming, resulting in potential voltage increases (Keating and Behling, 1990; Silva et al., 2009). Therefore, brine treatment is necessary to avoid an inefficient membrane process and even a damage to the membrane. Physical and electrochemical approaches for the reduction of organic impurities in chlorine-alkali brines have already been reported (Heuser et al., 2005; Muddemann et al., 2018; Bulan et al., 2019).

A promising alternative for the degradation of organic contaminants in RPB can be the treatment with halophilic microorganisms able to degrade the substances contaminating streams such as RPB (Bertrand et al., 1990; Alva and Peyton, 2003; Le Borgne et al., 2008; Tapilatu et al., 2010; Erdoğmuş et al., 2013; Castillo-Carvajal et al., 2014; Corti Monzon et al., 2018; Jamal and Pugazhendi, 2018; Aron et al., 2021; Mainka et al., 2021). However, the integration of biological unit operations into chemical production chains is often limited by the complexity of bioprocesses. Special nutrient supplementation, the risk of contamination and the widespread discontinuous process mode (mostly batch or fed-batch processes in biotechnology) are major concerns of the chemical industry. Processes developed with halophilic microorganisms have the potential to overcome several of the above-mentioned limitations. Due to high salt conditions, the risk of contamination is low (Schiraldi and De Rosa, 2002; Bonete and Martínez-Espinosa, 2011; Deive et al., 2012). Moreover, it has already been shown that nutrient supplementation can be kept to a minimum (use of minimal medium) and the use of easily accessible and renewably produced carbon sources such as glycerol is possible (Berrios and Skelton, 2008; Pflügl et al., 2014; Russmayer et al., 2019). In contrast to chemical processes, biological processes operated in continuous mode are perceived to bear an increased risk of instability, either genetically or through a decrease of specific productivity (Deive et al., 2012). Furthermore, continuous bioprocessing demands a more complex process setup. Nevertheless, it has already been shown for halophilic microorganisms, that continuous bioprocessing in lab-scale is successful and applicable for RPB treatment processes (Fallet et al., 2010; Mahler et al., 2018; Mainka et al., 2019).

However, academic studies frequently are time- and space-restricted. As a result, no studies are available which investigated long-term culturing effects on process performance of pilot-scale biological RPB treatment processes using halophilic microorganisms. The aim of this study was to establish a pilot-scale bubble-column fermentation system for the continuous biological treatment of RPB directly implemented at an industrial production site. The high-salt RPB contains the organic contaminants formate, aniline, phenol and 4,4'-methlyendianiline (MDA), which are substrates and products of the industrial MDA production. To provide RPB suitable for subsequent sodium chloride production, halophilic microorganisms are interesting biocatalysts. Degradation of the organic impurities by halophiles has already been demonstrated for several strains, including *Haloferax mediterranei*, *Halomonas* strain MA-C, *Haloarcula* sp. A235, *Oceanimonas* sp., or *Halomonas organivorans* (García et al., 2004; Li et al., 2010; de Lourdes Moreno et al., 2011; Acikgoz and Ozcan, 2016; Tan et al., 2017; Mahler et al., 2018; Heckroth et al., 2019; Mainka et al., 2019; Mainka et al., 2021). Consequently, this study compares process performance of a lab-scale and pilot-scale halophilic bioprocessing system to remove organic compounds from RPB. In order to extend the previously gained process knowledge from a lab-scale

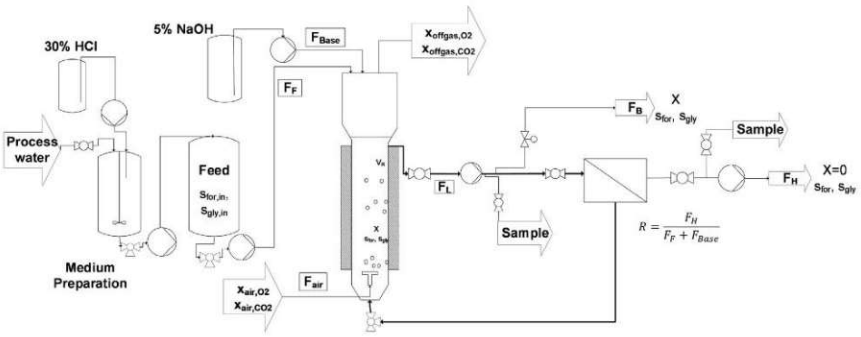


FIGURE 1 | Scheme of the fermentation setup. Residual process brine from the MDA production is prepared and supplemented with the required components in a first 200 L vessel. From there the medium is pumped into the feed vessel, from where a constant feed (FF) supplies the cells with fresh substrate and media components. 5 % NaOH (FBase) is added to hold the pH on a constant level of 7.0. A pump continuously circulates the cell suspension as loop flow (FL) through the membrane module to separate cell-free harvest (FH). The ratio of outgoing harvest flow to the total iput flow is called the retention rate R and is calculated according to the following equation: $R = \frac{F_H}{F_F + F_{Base}}$. Bleed flow (FB) is continuously removed to eliminate cells and sustain steady state conditions. To guarantee constant reactor volume (VR) flows for Feed, Base, Harvest and Bleed have to meet the following equation: FF + FBase = FH + FB. Biomass is monitored using a turbidity probe and a soft sensor that is driven by measurements of off-gas composition.

bioprocess using the extremely halophilic archaeon *H. mediterranei* was scaled-up to a bubble column bioreactor setup (Mahler et al., 2018; Mainka et al., 2019). Moreover, for the first time, such a pilot-scale bioreactor system was set up, implemented, and integrated in an industrial MDA-production site. To measure the success of the scale-up, process variables (dilution rate D, retention rate R, and substrate feeding) and process performance (degradation efficiency) of the lab-scale and pilot-scale process with *H. mediterranei* were compared.

In addition to comparing process scales, we investigated batch-to-batch variations of organic contaminant concentrations and their influence on the bioprocess. For industrial MDA-processes it is known that the RPB underlies variations in its composition as production conditions can change. However, in lab-scale experiments only a low number of RPB batches were used, so potential effects of changing feed conditions were not investigated. Thus, an advantage of the local integration of the bioprocess setup in the chemical production plant demonstrated in this study is the direct use of RPB without storage time between different RPB batches.

2 MATERIALS AND METHODS

2.1 Bioreactor Setup

The bioreactor setup, where the cultivations were performed, is a corrosion-resistant, custom-made bubble column reactor, with a total volume of 21 L (Diameter 13.4 cm; Height 1.1 m; Möstl Anlagenbau, Arzberg, Austria) (Mahler et al., 2018). The bioreactor consists of Hastelloy-C22 extended with a loop where all piping, valves, and screwing are made of PVDF or PTFE. The loop also contains a membrane-based cell retention unit (Hollow Fiber Cartridge, CFP-2-E-9A, 0.2 µm, 8400 cm2, GE, Westborough, United States) and a 4-piston diaphragm loop pump (Quattroflow, ALMATEC Maschinenbau, Germany) with a polypropylene pump head for circulation the cell suspension with a flow of around 100 L h^{-1}. A schematic diagram of the experimental setup is shown in **Figure 1**.

The bleed was released with a digitally operated valve (Bürkert, Germany) into the bleed vessel and worked as a control valve to keep the reactor volume constant. The filtrate was withdrawn through the membrane, using a peristaltic pump (ISMATEC Reglo Quick, Cole-Parmer, United States). The input flows to the bioreactor system were controlled with an analog pump (Magdos, Lutz-Jesco GmbH, Wedemark, Germany) to continuously add fresh medium and a digital pump (Magdos, Lutz-Jesco GmbH, Wedemark, Germany) to add base (5% NaOH) for adjusting the pH. The flow rates for feed and harvest were calculated based on gravimetric measurements with a resolution of 10 g (Mettler Toledo, United States). Flow rates of base and bleed were determined with a resolution of 0.1 g (Kern & Sohn GmbH, Balingen-Frommern, Germany).

The inlet airflow was kept constant at 3.2 L min^{-1} (0.2 vvm) *via* a mass flow controller (Brooks Instrument, United States). Dissolved oxygen was measured using an Oxyferm probe (Visiferm DO Arc 120, Hamilton, Bonaduz, Switzerland) placed in the loop piping. To increase oxygen transfer into the liquid phase, the pressure was kept constant at 2 bar with an electronic valve (Bürkert, Germany). The pH was measured using an Easyferm pH probes (InPro3250i, Mettler Toledo, Germany), which was placed inside the loopline. The temperature probe (Onigrad TR88, Endress + Hauser, Reinach, Switzerland) was placed on the reactor vessel wall to measure the temperature inside the reactor. In the bioreactor headspace, and in the feed, retentate and filtrate sides of the membrane, sensors were placed to measure the pressure (Signet

2450, Georg Fischer, United States). The composition of off-gas was determined using a BlueVary gas analyzer system (BlueSens, Herten, Germany). For preparing the RPB medium, a 200 L stirred vessel (Schwarzer Rührtechnik, Delmenhorst, Germany) made from polypropylene was used. The final feed was then transferred to second 200 L feed vessel, from which the fermenter was continuously fed.

2.2 Strain and Medium

Haloferax mediterranei DSM 1411 was obtained from DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkultur, Braunschweig, Germany). The preculture was cultivated in a 1 L lab-scale CSTR to a biomass concentration of around 8 g L^{-1} and cells were then transferred to the production site of the industrial partner. As 1 L of preculture was added to 15 L medium in the described pilot-scale bioreactor, the initial biomass concentration was 0.5 g L^{-1}. To increase the level of biomass, a batch cultivation was carried out followed by a continuous cultivation to reach a biomass concentration of 7–8 g L^{-1}. All cultivations were performed at a temperature of 30°C and at pH 7.0.

For the medium, RPB from an industrial partner was directly used from the RPB columns. The RPB contained 150 g L^{-1} NaCl and formate as an organic contaminant in a concentration range of 200 ± 20 mg L^{-1}. After adjustment of the pH from 13 to 4 with 30 % HCl, the RPB was supplemented with mineral media components in the following concentration (g L^{-1}): NH_4Cl 1; KH_2PO_4 0.15; $FeCl_3$ 0.005; $MgCl_2 \cdot 6\,H_2O$ 1.3; $MgSO_4 \cdot 7\,H_2O$ 1.1; $CaCl_2 \cdot 2\,H_2O$ 0.55; KCl 1.66; Trace elements solution 1 ml [(mg/100 ml): $FeSO_4 \cdot 7\,H_2O$ 139; $CuSO_4 \cdot 5\,H_2O$ 100; $CoCl_2 \cdot 2\,H_2O$ 44; $ZnSO_4 \cdot 7\,H_2O$ 86]; Manganese stock 1 ml [(mg/100 ml): $MnCl_2 \cdot 4\,H_2O$ 18]. Glycerol was added as a substrate in concentrations of 1.5-2.5 g L^{-1}. Agar plates consisted of the same media components, but agar-agar in a concentration of 15 g L^{-1} was added. All shake flask experiments were performed in 500 ml shake flaks without baffles and a liquid volume of 100 ml Samples from the shake flasks were taken under sterile conditions.

2.3 Calculations

2.3.1 Continuous Reactor Setup

The mathematical description of the continuous fermentation system used in this study has already been published (Mahler et al., 2018). The theory of chemostat cultures extended with cell retention was also described previously (Pirt and Kurowski, 1970).

Steady-state conditions was achieved by keeping the hydraulic dilution rate D and the retention (or recycle) rate R constant. D can be calculated according to **Eq. 1**. The retention rate R describes the ratio of cells retained within the bioreactor and were calculated using **Eq. 2**. As the reactor volume VR was kept constant during the process, volumetric input and output flows were equal (see **Eq. 3**). The volumetric flow rates for feed (F_F), harvest (F_H), bleed (F_B) and base (F_{Base}) were calculated based on online balance signals.

$$D = \frac{F_F + F_{Base}}{V_R} \tag{1}$$

TABLE 1 | Primers used for genetic analyses.

Primer	Sequence (5′–3′)
515f	GTG YCA GCM GCC GCG GTA A
806r	GGA CTA CNV GGG TWT CTA AT
27f	AGA GTT TGA TCC TGG CTC AG
HFX41f	CGA TTT AGC CAT GCT AGT TG
1494r	CTA CGG CTA CCT TGT TAC GA

$$R = \frac{F_H}{F_F + F_{Base}} = \frac{(F_F + F_{Base}) - F_B}{F_F + F_{Base}} \tag{2}$$

$$F_F + F_{Base} = F_B + F_H \tag{3}$$

2.3.2 Rate Calculations

The specific growth rate μ in shake flask experiments was calculated for the exponential growth phase using optical density measurements at a wavelength of 600 nm (OD_{600}).

In the case of a steady state, the biomass concentration is constant in a cell retention setup, thus, according to **Eq. 4**, the specific growth is dependent on D and R. Substrate uptake rates rS can be calculated using concentration values and liquid flow rates, as described in **Eq. 5**.

$$\mu = (1 - R) \cdot D \tag{4}$$

$$r_S = F_F \cdot (s_{in} - s) \tag{5}$$

2.4 Analytical Procedures

The samples were taken on the MDA production site and meanwhile stored at -20°C. After transport to the laboratories of the TU Wien, the samples were then further analyzed.

Substrate quantification in the feed and harvest samples was done as described previously (Erian et al., 2018), using an HPLC (Vanquish UHPLC systems, Thermo-Fisher, United States) with an Aminex HPX-87H column (Bio-Rad, United States) at 60°C, an isocratic eluent of 4 mM sulfuric acid in Milli-Q water with a flow of 0.6 ml min^{-1} followed by UV detection at 210 nm and RI detection (RefracoMax520, ERC, Germany). In brief, samples were centrifuged, and the supernatant was mixed with 40 mM sulfuric acid (9:1) before 10 µl was injected in the HPLC. The samples were analyzed for residual formate and glycerol, as well as the formation of organic acids. The standards, used for quantifications, were prepared the same way as the samples and mixed with 40 mM sulfuric acid (9:1).

For the quantification of aromatic compounds in feed and harvest samples, a reversed-phase HPLC measurement (Vanquish UHPLC systems, Thermo-Fisher, United States) was carried out, using an AcclaimTM PolarAdvantage column (Thermo Scientific, United States, C16, 3 µm, 120 Å, 4.6x150 mm) at 30 °C. Aromatic compounds were detected using a UV detector at 210 nm. The flow was 1 ml min-1 with a gradient system (0–2.5 min: 5 % A, 95 % B; 2.5–5 min: 25 % A, 75 % B; 5–20 min: linear decrease of B from 75 to 30 %, rest A). After each measurement, a washing step was carried out

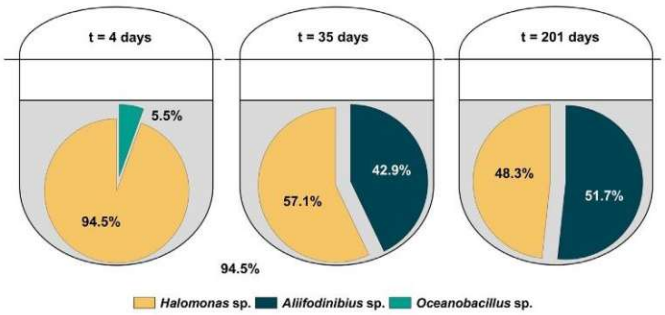


FIGURE 2 | Readout distribution from Amplicon Deep Sequencing of the halophilic microbial community after 4, 35 and 201 days of cultivation.

(0–5.5 min: linear increase of C from 75 to 95 %, rest A; 5.5–15 min: 5 % A, 95 % C; 15–20 min: 5 % A, 95 % B). The eluents were A) acetonitrile; B) 25 mM KH_2PO_4 (pH 3.5 with 1 M H_3PO_4); and C) MiliQ water. Samples were centrifuged prior to analysis and 10 µl undiluted supernatant was injected for HPLC analysis.

2.5 Genetic Analysis

2.5.1 Whole Genome and Amplicon Sequencing

For the analysis of whole genome sequences, samples were taken from the bioreactor and frozen to -20°C until the samples were transported to the lab in Vienna under dry ice conditions. Upon arriving in our lab, the samples were stored again at -20°C. For DNA isolation, 2 ml samples were centrifuged for 30 s, with 11,000 x g at room temperature and the pellet was treated with a DNA extraction kit (DNeasy® UltraClean® Microbial Kit, Qiagen, Netherlands) according to the manufacturer's recommendation. Whole genome sequencing was done by Microsynth AG (Switzerland) using purified genomic DNA samples obtained from biomass samples taken after 2 and 201 days of cultivation. For the Amplicon Deep Sequencing, purified genomic DNA from biomass samples was sent directly to the external laboratory (Microsynth AG, Switzerland). To that end, a Nextera two-step PCR was done using 515f and 806r as forward and reverse primers, respectively (**Table 1**). The products were sequenced with an Illumina MiSeq. Raw sequencing data were uploaded to the SRA database (accession number PRJNA813737).

2.5.2 16s rRNA Sequencing

For sequencing 16S rRNA, 2 ml samples were centrifuged for 10 min with 10,000 rpm at 4°C. the supernatant was discarded, and the pellet was frozen at -20°C until further use. Genomic DNA was isolated from frozen pellet samples using the DNeasy® Kit (UltraClean® Microbial Kit, Qiagen, Netherlands) according to the manufacturer's recommendation. Subsequently, 16s rDNA was amplified using primers 27f/HFX41f and 1494r (**Table 1**). Primers were purchased from Integrated DNA Technologies (IDT, Belgium). Sanger sequencing was done by Microsynth Austria GmbH (Austria).

3 RESULTS AND DISCUSSION

3.1 Identification and Characterization of a Halophilic Consortium Found at an Industrial Production Site

This study investigated the effect of scale-up, changing RPB composition and long-term cultivation effects on the process behavior and degradation efficiency of a biological RPB treatment process. To that end, the pilot-scale bioreactor filled with 15 L RPB from the MDA production was inoculated with 1 L of a *H. mediterranei* preculture grown in a lab-scale bioreactor (Mainka et al., 2019). The process started with a batch phase followed by a continuous process phase with cell retention for biomass propagation and to establish a stable growing continuous culture. The experimental phase included the use of weekly changing RPB batches, applying different dilution rates (D) and substrate concentrations for glycerol. In total, the cultivation process was operated for more than 200 days (>4800 h). In addition to process performance, genetic stability was investigated by taking samples for subsequent genome analysis throughout the cultivation.

Specifically, genomic DNA from two biomass samples (days 2 and 201) were sequenced. Surprisingly, the analyzed biomass samples did not show the presence of *H. mediterranei*. Instead, several different bacterial strains mostly from the halophilic bacterial genera of *Halomonas* were identified. This finding was unexpected, as the process performance (organic contaminants were degraded in the measured samples) and growth behavior did not show unusual behavior indicative for a potential contamination. The pre-culture could be excluded as the root cause of the contamination as 16S rRNA analysis showed a pure culture of *H. mediterranei*. To further investigate and verify these results, Amplicon Deep Sequencing was performed, using biomass samples from three different cultivation time

points, representing the early (day 4), intermediate (day 35) and final (day 201) stage of the cultivation. To avoid any biased results, no additional cultivation step was performed between the sampling and the DNA analysis. For the Amplicon Deep Sequencing, 16S rRNA (primers 515f and 806r, see **Table 1**) is only partially amplified. Hence, only genera rather than single species could be identified. **Figure 2** shows the results of the Amplicon Deep Sequencing, indicating the percentage of readouts for the identified taxonomic genera. At day 4, the main part of readouts belonged to the bacterial genus *Halomonas* (>94%) while only a minor portion of readouts was identified as either *Oceanobacillus* sp. or *Aliifodinibius* sp. However, the composition of the novel halophilic mixed culture changed over time, as the main percentage of total readouts are allocated to *Halomonas* sp. and *Aliifodinibius* sp. after 35 and 201 days of cultivation. In contrast, the genus of *Oceanobacillus* sp., made up only 0.03 and 0.01% of total readouts at days 35 and 201, respectively. Therefore, it appears that the environment in the bioreactor favors the growth of *Aliifodinibius* and *Halomonas* strains over that of *Oceanobacillus* strains. All the identified genera belong to halophilic (*Halomonas* sp. and *Aliifodinibius* sp.) or halotolerant (*Oceanobacillus* sp.) bacteria (Lu et al., 2001; Mata et al., 2002; Takami et al., 2002; García et al., 2004; Kaye et al., 2004; Wang et al., 2013; Yin et al., 2015; Xia et al., 2016; Cho et al., 2017). To the best of the authors knowledge, this is the first time that a halophilic mixed culture consisting of these three halophilic genera (*Oceanobacillus* sp., *Halomonas* sp. and *Aliifodinibius* sp.) is described for the use in a biological RPB treatment process. The discovery and usage of novel isolated halophilic microorganisms and their exploitation in a biotechnological and industrial context was already successfully demonstrated (Guven et al., 2018; Rezaei Somee et al., 2018; Zare et al., 2019; Hasanzadeh et al., 2020; Villanova et al., 2021). Besides, the usage of a co-culture might be even beneficial for the present bioprocess. Moreover, it was reported for co-cultivations of microorganisms, that productivities and process efficiencies can be improved, compared to monocultures (Angell et al., 2006; Ishika et al., 2017; Anh et al., 2021). Moreover, the potential of halophilic consortia for the bioremediation of saline wastewater contaminated with organic impurities like aromatics was already proven in several cases (Jamal and Pugazhendi, 2018; Al-Shaikh and Jamal, 2020; Jamal and Pugazhendi, 2021; Jin et al., 2021).

To further investigate and characterize the mixed culture found in bioreactor samples, 16S rRNA sequencing experiments were performed, using genomic DNA purified from single colonies. These single colonies were obtained, as a culture sample from the end of the cultivation was grown on agar plates with undefined preculture medium. In total, nine single colonies were re-streaked on a fresh agar plate. From this plate, shake flasks were inoculated, genomic DNA was extracted and used as template for PCR amplification of the 16S rDNA. The results of the 16S rRNA sequencing are summarized in **Supplementary File S1** (see **Supplementary Table S1**).

According to 16S rRNA sequences, all nine single colonies were identified as *Halomonas organivorans* strain G-16.1 which is in line with the results from Amplicon Deep Sequencing where mainly *Halomonas* sp. and *Aliifodinibius* sp. were present in the reactor. Additionally, the identification of strain *H. organivorans* strain G16.1 in a salty environment, contaminated with aromatic substances, is not surprising, as this strain was reported to degrade aromatics, such as phenol (García et al., 2004; de Lourdes Moreno et al., 2011). Additionally, other *Halomonas* strains, such as *H. campisalis* or *H. anticariensis* FP35, are known for their ability to degrade various aromatic compounds, such as catechol or polycyclic aromatic hydrocarbons (PAH) (Alva and Peyton, 2003; Piubeli et al., 2012; Haddadi and Shavandi, 2013; Tena-Garitaonaindia et al., 2019; Govarthanan et al., 2020). Moreover, another *Halomonas* strain, namely *Halomonas* sp. strain MA-C, was previously reported to be able to degrade formate (Oren et al., 1992; Azachi et al., 1995; Heckroth et al., 2018).

The number of readouts for *Aliifodinibius* surpassed that of *Halomonas* sp. at day 201 of the cultivation. Generally, the discovery of *Aliifodinibius* species in the mixed culture and the fact that it become a major part of this culture is surprising as there are only a limited number of publications describe the genus *Aliifodinibius* (Wang et al., 2013; Hahnke et al., 2016; Xia et al., 2016; Cho et al., 2017; Cho and Whang, 2020; Zhao et al., 2020). Members of the genus *Aliifodinibius* include *Aliifodinibius roseus*, *A. sediminis* and *A. halophila* (Wang et al., 2013; Xia et al., 2016). To the best of our knowledge, so far none of the reported strains have been described to use aromatic compounds for growth or the ability to degrade aromatic compounds. The identification of this genus as part of the halophilic mixed culture, however, indicates the ability of *Aliifodinibius* sp. to adapt to the salt concentrations present in the RPB stream and its potential ability to grow on either glycerol or formate. Nevertheless, it is unclear which role this genus has played regarding the degradation of aromatic compounds, or if its presence is beneficial for the use of aromatic compounds by the other genera of *Halomonas* and *Oceanobacillus*.

The reason why the microbial composition in the bioreactor changed between sampling points could be, that a new substrate (glycerol) was introduced into the environment of the bacteria, resulting in a wash-out of cells growing slower with glycerol. Therefore, a halophilic strain could possibly replace all other strains in a bioreactor if its affinity to the growth substrate is the highest for process conditions and the media composition used here.

As *H. organivorans* was identified in the halophilic mixed culture, its ability to grow in the presence of RPB was further investigated. Therefore, shake flask experiments with industrial RPB and glycerol as a co-substrate were performed (results not shown). *H. organivorans* was able to degrade all present organic contaminants (aniline, phenol, MDA, and formate). Compared to the mixed culture, a pure culture of *H. organivorans* grew slower and to a lower maximum optical density. Moreover, during growth of *H. organivorans*, the formation of precipitates could be observed (**Supplementary Figure S1A**) and were investigated with light microscopy (**Supplementary Figure S1B**).

Furthermore, the growth rates, based on OD_{600} measurements, of the mixed culture and pure cultures of *H. organivorans* and *H. mediterranei* in the RPB were compared.

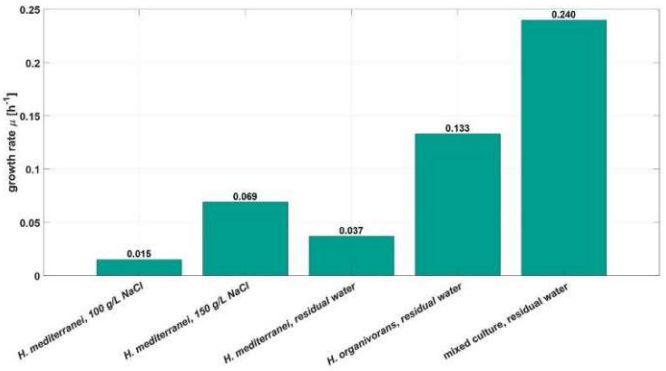


FIGURE 3 | Comparison of specific growth rates between *H. mediterranei*, *H. organivorans*, and the novel mixed culture in the industrial residual process brine. Additionally, *H. mediterranei* was grown in synthetic medium with 100 and 150 g L-1 NaCl.

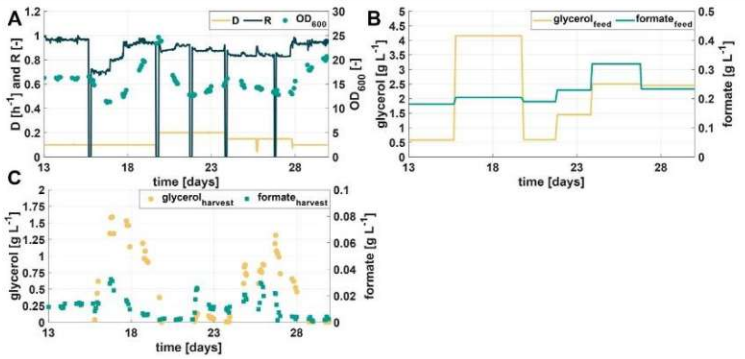


FIGURE 4 | Process performance during the second experimental phase **(A)** Optical density (OD_{600}) during the cultivation time of the second phase (17 days). Process parameters (dilution rate D and retention rate R) were changed to maintain a stable biomass concentration **(B)** Feed concentrations of the additional substrate glycerol and formate **(C)** Concentration of glycerol and formate in harvest samples.

Additionally, *H. mediterranei* was grown in synthetic RPB containing two different NaCl concentrations (100 and 150 g L^{-1}). Overall, *H. mediterranei* showed the lowest specific growth rate of the three microbial systems tested, independently of the growth medium (**Figure 3**). In contrast, the novel mixed culture showed the highest growth rate of 0.24 h^{-1}. Consequently, it appears likely that the initial growth conditions together with its general growth advantage favoured the growth of the bacterial mixed culture identified in this study which led to the replacement of *H. mediterranei*. The origin of the contamination could not be clearly identified. However, one plausible explanation might be that the members of the mixed culture found in the reactor grew at different locations of the MDA production site and were introduced to the bioreactor system and its periphery by the operator. As the system periphery could not be heat sterilized, the bacterial contamination was present in the bioreactor at the time of the inoculation and due to a higher level of adaptation to the process conditions, the mixed culture became dominant in the bioreactor.

3.2 Process Performance of Halophilic Mixed Culture for the Degradation of Organic Contaminants in an Industrial Residual Process Brine

To evaluate the process performance of the pilot-scale cultivation presented in this study, performance parameters and their acceptance criteria need to be defined. Moreover, the results

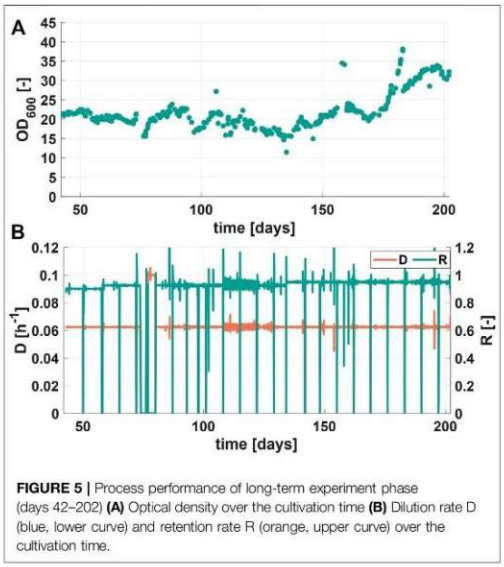


FIGURE 5 | Process performance of long-term experiment phase (days 42–202) **(A)** Optical density over the cultivation time **(B)** Dilution rate D (blue, lower curve) and retention rate R (orange, upper curve) over the cultivation time.

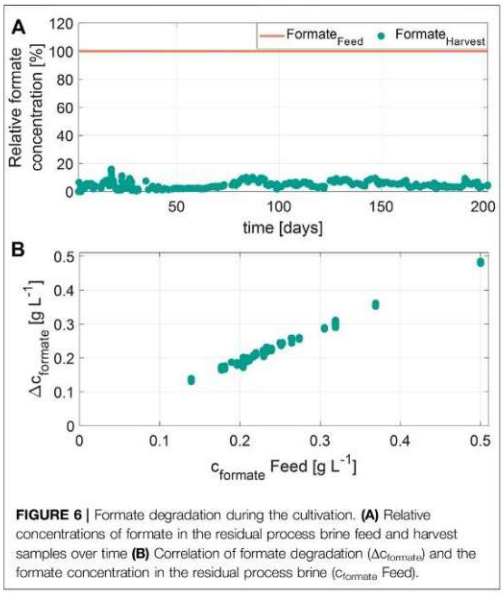


FIGURE 6 | Formate degradation during the cultivation. **(A)** Relative concentrations of formate in the residual process brine feed and harvest samples over time **(B)** Correlation of formate degradation ($\Delta c_{formate}$) and the formate concentration in the residual process brine ($c_{formate}$ Feed).

should be compared with an already established process, treating the same RPB (Mainka et al., 2019). As process variables the dilution rate D, retention rate R and the substrate feeding r_S were used. For evaluation of process performance, the efficiency of organic substance degradation was selected. Key process parameters were kept constant during the whole cultivation time: reactor temperature T (= 30 °C), pH (= 7), reactor volume V_R (= 16 L) and aeration rate F_{air} (= 0.2 vvm).

As mentioned before, the present study was divided into several process phases. During the first phase (day 1 to 13) the goal was to establish a stable operation of the cultivation setup, including the feed preparation devices. To continue, during a second phase of around 17 days, different dilution rates D (0.1–0.2 h^{-1}) and glycerol concentrations (0.5–4 g L^{-1}) in the RPB feed were applied to the system. The goal was to maintain a constant biomass level by adjusting the retention rate R accordingly. In the third phase of the study, long-term performance of the process was evaluated by applying in total 23 different RPB batches. In this phase, the dilution rate D was decreased to 0.0625 h^{-1}, to apply a weekly change of the RPB feed batch. In this phase, the glycerol concentration was maintained between 1.5 and 2 g L^{-1}, and R was adjusted between 0.925 and 0.95 to maintain a biomass concentration constant.

Figure 4A shows OD_{600} values, the dilution rate D, and the retention rate R of the second experimental phase (days 13–30) over a course of 17 days of continuous fermentations and **Figure 4B** shows the variations in the glycerol concentration in the feed. Formate concentrations of six different RPB batches varied between 0.2 and 0.32 g L^{-1} (**Figure 4B**). Interestingly, during this process phase glycerol accumulation in harvest samples together with decreasing biomass concentrations in the bioreactor was observed when the glycerol concentration in the feed was 4 g L^{-1}, operating at D of 0.1 h^{-1} (days three to six, **Figure 4C**). Simultaneously, the formate degradation decreased, indicated by higher formate concentrations in the harvest. The apparent substrate overfeeding could be linked to the specific growth rate of the culture. Decreasing the specific growth rate by increasing the retention rate R resulted in higher OD_{600} values (days three to six, **Figure 4A**) and lower glycerol and formate concentrations in the harvest samples (**Figure 4C**). The results further showed that integrated control of the process variables D, R and r_S results in a stable operation of a continuous bioprocess. Moreover, the highest tested dilution rate D (= 0.2 h^{-1}) combined with the lowest glycerol feed concentration (= 0.5 g L^{-1}) showed the best formate degradation results (>98% of efficiency) while no glycerol was accumulated (days six to eight, **Figure 4C**). Consequently, further investigations for identification of an optimal glycerol feeding strategy for obtaining high formate degradation would be beneficial for the process efficiency.

During the third phase (days 42–202), process variables (r_S, R, D) were chosen in a way that no accumulation of glycerol could be observed. Over the period of these 160 days, the OD_{600} values showed stable values between 15 and 25 (**Figure 5A**). However, process operation was disturbed due to operational failure of the process control system during two periods (days 73–75 and 182–182). A reason for the increase of OD_{600} to values over 30 during the last 25 days (days 177-202) could not be clearly identified but was probably a result of a failure of the temperature control system (caused by high environmental temperatures).

This failure could have either led to higher growth rates or to a change in the morphology of the cells, resulting in potentially larger cells. The dilution rate D, as can be seen in **Figure 5B**, shows a phase of higher values (0.1 h^{-1}). The value was set to 0.1 h^{-1} to consume all the RPB feed left, in order to prepare the new RPB batch according to the time schedule.

As one goal of this study was to investigate the long-term stability of the halophilic bioprocess, particular attention was paid to the influence of the changing contaminant concentrations on process performance. Among the present organic contaminants, phenol and MDA showed stable concentration values between all measured RPB batches (phenol: 3–6 mg L^{-1}, MDA: 0.12–0.30 mg L^{-1}). In contrast, aniline and formate could vary significantly (aniline: 0.5–15 mg L^{-1}, formate: 200–500 mg L^{-1}) in their initial feed concentrations.

Nonetheless, HPLC analysis during the long-term experiment showed no detection of residuals of the aromatic compounds aniline, phenol, and MDA in harvest samples. It is assumed that aniline, phenol, and MDA are finally metabolized to CO_2 through the TCA cycle, as described for halophilic bacteria (Wells and Ragauskas, 2012; Arora, 2015). However, during the metabolism of aromatic substances, intermediates might have occurred, as an unidentified peak was detected in the HPLC chromatograms. In contrast, in previous studies using MDA residual process brine in biological treatment processes, no intermediate accumulation was described (Mahler et al., 2018; Mainka et al., 2019). The peak detected during HPLC analysis with a retention time of 6.8–6.9 min could not yet be identified, thus it is referred to as *unknown substance*. The peak area of the *unknown substance*, however, showed a correlation with the concentration of aniline in the RPB feed (see **Supplementary Figure S2**). Thus, it is assumed that the *unknown substance* is formed during the degradation of aniline and might be an intermediate of the degradation pathway of aniline.

Besides, formate was degraded with an efficiency of 90-98%, independently of the concentration in the RPB feed (**Figure 6A**). However, the absolute amount of formate degraded (Δformate) showed a linear correlation to the concentration of formate in the feed (**Figure 6B**). No correlation between the formate degradation and RPB feed concentrations of glycerol, aniline, phenol, or MDA were found. Moreover, the residual concentrations of formate in the harvest were constantly between 10 and 20 mg L^{-1} over the entire cultivation time. It is assumed that formate is metabolized to CO_2, catalyzed by a formate dehydrogenase (Maia et al., 2017; Yu et al., 2017). Previously, the halophilic bacterium *Halomonas* strain sp. MA-C was shown to degrade formate (Oren et al., 1992; Azachi et al., 1995; Heckroth et al., 2018). Similar to the present study, it was reported, that the total amount of formate degraded by *Halomonas* strain sp. MA-C is dependent on the initial formate concentration, and the NaCl concentration (Heckroth et al., 2018).

In addition to formate oxidation, formate dehydrogenases are able to catalyze the reduction reaction from CO_2 to formate. The results shown in the present study might indicate that formate is not removed completely because an equilibrium with no further degradation is reached at values of 10–20 mg L^{-1} formate in the bioreactor (Maia et al., 2017; Yu et al., 2017). Furthermore, previous reports for *Halomonas* strain sp. MA-C showed that less than 5% of C^{14}-labeled formate was incorporated into cells. Therefore, it appears likely that formate plays only a minor role in biomass formation (Oren et al., 1992; Azachi et al., 1995).

TABLE 2 | Comparison of scale-up performance.

Parameter	Lab-scale	Pilot-scale
References	(Mainka et al., 2019)	(this study)
Reactor volume [L]	1	16
Microorganisms/strain	*H. mediterranei*	Halophilic mixed culture
No. of RPB batches	1	32
Temperature [°C]	37	30
pH	7.00	7.00
Dilution rate D [h−1]	0.1	0.0625-0.2
Retention rate R [-]	0.74-0.87	0.7-0.95
Glycerol concentration [g L−1]	2-10	0.5-2.5
Glycerol feeding rate [g L−1 h−1]	0.2-1.0	0.05-0.4
Formate removal [%]	100%	90–98%
Aromatics removal [%]	100%	100%

3.3 Comparison of Scale-Up Process to Existing Bioprocesses for MDA Residual Process Brine Treatment

In two previously published studies, RPB from MDA-production was biologically treated using the extremely halophilic archaeon *H. mediterranei* in a 1L lab-scale stirred tank reactor and a 16L bubble column reactor (Mahler et al., 2018; Mainka et al., 2019). There, the goal was also to reduce the organic load in the RPB for later reuse in base chemical production. The previous lab-scale study showed that 100% reduction of the present organic contaminants was possible for a cultivation time of over 54 days (Mainka et al., 2019). However, only one RPB batch was used, as the study focused on the influence of different process variables like D, R, and the glycerol feed concentration. In contrast to previous results, the present study investigated the influence of batch-to-batch variability of RPB on process performance. Moreover, higher values for the dilution rate D and the retention rate R, and lower glycerol feed concentrations were tested, key parameters to optimize overall process economy. High values for D and R increase the process productivity by increasing the capacity of the RPB reduces costs as less unused bleed stream is generated. Furthermore, decreased glycerol consumption improves overall process economics.

The previously published pilot-scale study showed the technical feasibility of using a bubble column reactor for the growth of *H. mediterranei* in salty RPB. However, the cultivation time of 35 h was short compared to the cultivation time in the present study. The process in the present study, therefore, combined and extended the previously gained knowledge, by scaling-up the previous process to a higher volume of 16L and an extended cultivation time of over 210 days. Moreover, for the first time it was achieved to directly integrate the process in the RPB production on the MDA production site. **Table 2** compares the process parameters and variables of this study with the previously published lab-scale process.

Different from the previous study, in this work the process temperature could be lowered to 30°C (from 37°C). Lower process temperatures are not only less energy consuming but also water evaporation during the process can be reduced. Furthermore, the present study showed that compared to the lab-scale study, simultaneous application of low glycerol feeding with high dilution and retention rates resulted in similar degradation rates for organic impurities. As a result, lower consumption of the additional carbon glycerol was observed, which could help to lower operational costs. Additionally, a reduced use of the additional carbon source necessitates a higher retention rate R to maintain the same biomass level. As already mentioned before, high R values in turn result in a reduced bleed stream volume. Therefore, less waste stream is generated as the bleed stream is currently not further used in the process setup. However, a potential future use of the bleed stream, e.g., by burning the biomass for heat generation, could further increase the process sustainability.

In addition to the bioreactor size and the energy input to the system, the bioprocess presented in this study differs mainly from previous studies in the microbial system used for degradation of organic pollutants. During this study, a halophilic community was identified which proved to be able to degrade all organic impurities in the RPB with high efficiency. Compared to *H. mediterranei*, the mixed culture has a higher growth rate when grown in the same RPB stream. The mixed culture found in this study consisted of halophilic bacteria which might be more flexible towards changing NaCl concentrations compared to *H. mediterranei*, especially at lower NaCl levels. In detail, salt tolerance in bacteria is based on the production of compatible solutes which could potentially provide an advantage to bacteria over archaea (relying on the "salting-in" strategy) to deal with changing salt concentrations (Oren, 2002; Mainka et al., 2021). Nevertheless, there is most likely an optimal range of the NaCl concentration, where the halophilic bacterial mixed culture most efficiently degrades formate and aromatic compounds.

4 CONCLUSION AND OUTLOOK

For the first time, a pilot-plant bubble column bioreactor system was successfully implemented at an industrial MDA production site and operated continuously for more than 210 days. Process parameters were compared to a small-scale process using RPB from the same production site. The results achieved with in the pilot-scale bioreactor showed degradation efficiencies as high as in the lab-scale bioreactor process. In this study, the bioreactor initially inoculated with *H. mediterranei*, was contaminated with a halophilic mixed culture, consisting of at least three different bacterial genera (*Halomonas* sp., *Aliifodinibius* sp. and *Oceanobacillus* sp.). The bacterial mixed culture could replace the original *H. mediterranei* culture possibly due to a better adaptation to the RPB stream. The identified microbial community was highly adapted to the environment of the industrial production site and showed high degradation efficiencies for the contaminants present in the RPB. Compared to *H. mediterranei*, the halophilic community also showed higher growth rates when grown in RPB. Nevertheless, additional investigations of the optimal salt concentration ranges for the degradation of organic contaminants in RPB treatment processes are required for an improved process understanding. Concentrations of aromatics in the RPB feed were changing significantly between the batches. The results showed that aniline concentrations in the RPB feed are positively correlated to the accumulation of a potential, yet unknown intermediate. So far it is not known if this intermediate is affecting the process efficiency of a chlor-alkali electrolysis process. Nevertheless, future investigations should be done to identify the substance and its effect on the efficiency of the membrane-cell chlor-alkali electrolysis process. Formate degradation showed a dependency on the formate concentration in the RPB feed. However, residual concentrations of formate were always between 10 and 20 mg L^{-1}. Moreover, if the additional substrate glycerol was overfed and thus accumulating in the bioreactor, also the degradation efficiency for formate was decreasing. The results of the present study further indicated a correlation between the glycerol feeding and the formate degradation, as at the lowest glycerol concentration combined with the highest dilution rate resulted in the lowest residual formate concentration. To increase process flexibility, the RPB feed could be decoupled from the co-substrate feeding by adding an additional glycerol feed to the system. Consequently, biomass concentration could be adapted based on fluctuating aromatic concentrations independently from the retention rate R solely by changes in the glycerol feed. Furthermore, a potential correlation of the glycerol feeding, and the formate degradation should be investigated to minimize the use of glycerol and to optimize the formate degradation efficiency. In conclusion, the present study underlines the potential of an alternative and sustainable bioprocess for treating residual process brine, and once more emphasizes the possibilities natural microbial diversity offers for exploitation in an industrial context.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA813737.

AUTHOR CONTRIBUTIONS

TM carried out the cultivation experiments. TM and SP conceived the study and analyzed the data. TM, CH, and SP wrote the manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.896576/full#supplementary-material

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4.3 Soft Sensor-Based Monitoring and Efficient Control Strategies of Biomass Concentration for Continuous Cultures of Haloferax mediterranei and Their Application to an Industrial Production Chain

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Contribution

Thomas Mainka: data analysis and evaluation; revision, structuring, and writing of the manuscript.
Nicole Mahler: planning and execution of experiments, initial data evaluation, first manuscript draft.
Stefan Pflügl: conceiving the study and writing the manuscript.
Christoph Herwig: conceiving the study and writing the manuscript.

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Soft Sensor-Based Monitoring and Efficient Control Strategies of Biomass Concentration for Continuous Cultures of *Haloferax mediterranei* and Their Application to an Industrial Production Chain

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Abstract: Continuous bioprocessing using cell retention allows the achievement of high space-time yields for slow-growing organisms such as halophiles. However, the lack of efficient methods for monitoring and control limits the application of biotechnological processes in the industry. The aim of this study was to implement a control and online monitoring strategy for biomass in continuous cultures. For the first time, a feedforward cultivation strategy in a membrane-based cell retention system allowed to control the biomass concentration of the extreme halophilic *Haloferax mediterranei* at defined levels. Moreover, soft sensor-based biomass estimation allowed reliable monitoring of biomass online. Application of the combined monitoring and control strategy using industrial process water containing formate, phenol, aniline and 4,4'-methylenedianiline could for the first time demonstrate high throughput degradation in this extremophilic bioremediation process, obtaining degradation efficiencies of up to 100%. This process demonstrates the usefulness of continuous halophilic cultures in a circular economy application.

Keywords: continuous bioprocessing with cell retention; soft sensor-based process monitoring; halophiles; bioremediation; *Haloferax mediterranei*

1. Introduction

Many areas of industrial production result in the continuous generation of large quantities of complex process water streams. Often, they require (pre)treatment, either chemically or biologically, before they can be released to the environment, which is necessary if the process water contains toxic, hazardous, or inhibitory contaminants with detrimental effects on the environment and public health [1–5]. There are several industrial sectors producing saline process water streams, for which halotolerant microorganisms offer a sustainable alternative for their treatment [6,7].

Halophilic microorganisms have huge potential in terms of waste water treatment since they can be cultivated under non-sterile conditions and are able to use a broad variety of carbon sources [8–10]. Furthermore, they are also able to degrade a large variety of contaminants in process waters, e.g., aromatic compounds like phenol or aniline [11,12].

On the other side, drawbacks of halophilic processes are low specific growth rates, rendering them unsuitable for high throughput processes as required for industrial process water treatment applications. In addition, biomass as the catalyst is the most critical process parameter in a biological remediation process and process performance (i.e., degradation efficiency) is directly proportional to the number of biocatalysts in the reactor. Therefore, a process for treatment of large quantities of industrial process water would have to meet three major criteria: (1) Simple control of biomass concentration, (2) accurate online monitoring of biomass concentration, and (3) high productivity to achieve high liquid throughput.

To establish a robust bioprocess that meets the defined requirements, continuous bioprocessing is necessary. However, due to low specific growth rates, a conventional chemostat process with halophiles can only be operated at low dilution rates. A chemostat fermentation system can be extended with a cell retention system, retaining a controlled number of cells inside the reactor [13,14]. This allows decoupling the liquid dilution rate from the specific growth rate of the culture (i.e., the biomass dilution rate) according to the retention rate and as a result higher dilution rates can be reached. The retention rate, or recycle ratio, describes the ratio of cell-free filtrate-flow from the reactor to the input feed-flow [14–16]. In conclusion, a cell retention set-up allows to adjust biomass concentrations independent from the specific growth rates and therefore allows to establish a process with high biomass concentrations and high liquid dilution rates.

Nevertheless, only suitable online monitoring of biomass concentration allows its accurate control. There are numerous concepts that work on the online determination of the process parameters like biomass concentration—among them, optical, capacitance, and calorimetric methods [17]. An overview of the advantages and disadvantages is given elsewhere [18]. Moreover, mathematical models or soft sensors could be an appropriate solution for describing and monitoring complex bioprocess variables (i.e., biomass) and help to control process states [19–21]. Soft sensors are a combination of a "software" and a "sensor", meaning signals are provided online by a sensor and are evaluated with mathematical models [20,22]. For calculating the biomass concentration in a bioreactor, soft sensors using balances for carbon and the degree of reduction have already been investigated previously [23,24]. However, for the approach in this study, existing models were extended for continuous systems with cell retention.

The aim of this study was the first implementation of a feedforward control strategy combined with an online monitoring concept for the biomass concentration for cultivations with the extreme halophilic *Haloferax mediterranei*. To achieve this, a lab-scale bioreactor was extended with a membrane to perform cell retention bioprocessing. For the control of biomass concentration, the parameters retention rate R and substrate concentration in feed s_{in} were used. The feedforward control loop was closed with a soft sensor-based tool for the online state estimation of biomass concentration. Therefore, existing principles of online biomass estimations using off-gas measurements and elemental balances were adapted to a continuous cell retention process and the performance compared to offline measurements.

Using either synthetic process water or real industrial process water with the organic contaminants formate, aniline, phenol, and 4,4′-methylenedianiline (MDA), the feasibility of both the control and monitoring concept could be demonstrated. Based on these results, the extreme halophilic archaeon *Haloferax mediterranei* was used for the first time in a high throughput treatment process to remove four organic contaminants from an industrial process water. This shows successful use of halophilic continuous cultures in a circular economy application, where an industrial process water after biological purification can be reused for further purposes (e.g., NaCl-containing process water for chlorine production), thus combining chemical and biological processing.

2. Material and Methods

2.1. Bioreactor Setup

A schematic diagram of the experimental setup is shown in Figure 1. Continuous cultures with and without cell retention were performed in a corrosion-resistant stirred tank reactor with a working

volume of 2.3 L (PEEK Labfors bioreactor, Infors, Switzerland), equipped with a 420 cm² microfiltration unit (model: CFP-2-E-4A, polysulfone membrane, 0.2 µm pore size, GE Healthcare, Germany). Peristaltic pumps were used for loop (Ecoline, Ismatec, Germany), feed, bleed, and harvest (Lambda Preciflow, Lambda Instruments, Switzerland). The dilution rate was kept constant at 0.1 h^{-1} for all experiments. The pH was measured using an Easyferm probe (Hamilton, Switzerland) and adjusted using 0.5 M NaOH via the integrated dosing system of the Labfors system. Reactor volume was kept constant at 1 ± 0.05 L. The reactor volume, feed, base, and acid consumption are continuously monitored by laboratory scales with 0.1 g resolution (Mettler Toledo, USA). The inlet airflow was kept constant at 100 mL min^{-1} via a mass flow controller (Brooks Instrument, USA). Dissolved oxygen was measured using an Oxyferm probe (Hamilton, Switzerland) and kept above 20% to guarantee aerobic conditions in the reactor. Oxygen transfer was adjusted by variation of stirrer speed between 400 and 560 rpm. Composition of off-gas was determined using a BlueSens gas analyzer system (BCP O_2 and CO_2, BlueSens, Germany). To reduce its water content the off-gas passed a countercurrent condenser before entering the gas analyzer system.

A corrosion-resistant turbidity probe (InPro8050, Mettler Toledo, USA) was used for online measurement of biomass concentration. The probe was calibrated to Nephelometric Turbidity Units (NTU) using Formazin calibration standards (Sigma Aldrich, USA). The probe is equipped with an infrared-LED to beam light at 880 nm via a fiber optic cable into the liquid medium. Backscattered light is captured and led back to the transmitter via a fiber optic cable where it is processed as turbidity signal.

The online data monitoring and process control were executed with a process information management system (Lucullus, SecureCell, Switzerland).

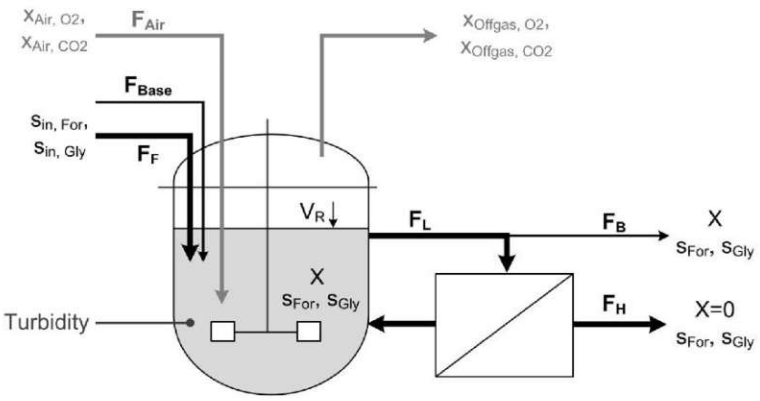

Figure 1. Scheme of the cell retention setup. A constant feed (F_F) supplies the cells with fresh substrate and media components. Base (F_{Base}) is added to hold the pH on a constant level of 7.0. A pump continuously circulates the cell suspension as loop flow (F_L) through the membrane module to separate cell-free harvest (F_H). Bleed flow (F_B) is continuously removed to eliminate cells and sustain steady state conditions. To guarantee a constant reactor volume (V_R) flows for Feed, Base, Harvest and Bleed have to meet the following equation: $F_F + F_{Base} = F_H + F_B$. Biomass is monitored using a turbidity probe and a soft sensor that is driven by measurements of off-gas composition.

2.2. Strain and Medium

Haloferax mediterranei DSM 1411 was obtained from DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkultur, Braunschweig, Germany). Prior to continuous culture, batch cultivation was carried out in the bioreactor to reach an initial biomass concentration of 3 g L^{-1}. All cultivations were performed at a temperature of 37 °C and at pH 7.0. Synthetic medium contained (g

L^{-1}): NaCl 150; NH_4Cl 1.5; KH_2PO_4 0.15; $FeCl_3$ 0.005; $MgCl_2 \cdot 6\ H_2O$ 1.3; $MgSO_4 \cdot 7\ H_2O$ 1.1; $CaCl_2 \cdot 2\ H_2O$ 0.55; KCl 1.66; Trace elements solution 1 mL [(mg/100 mL): $FeSO_4 \cdot 7\ H_2O$ 139; $CuSO_4 \cdot 5\ H_2O$ 100; $MnCl_2 \cdot 7\ H_2O$ 120; $CoCl_2 \cdot 2\ H_2O$ 44; $ZnSO_4 \cdot 7\ H_2O$ 86]. Glycerol was added as a substrate in a concentration of 1–4 g L^{-1}.

For the industrial medium, process water from polyurethane production of an industrial partner was used. It contained 150 g L^{-1} NaCl and formate as an organic contaminant in a concentration range of 200 ± 20 mg L^{-1}. For the industrial medium, the process water was supplemented with mineral media components (except NaCl) equivalent to the synthetic medium and glycerol to final concentrations of 2–10 g L^{-1} as indicated. The elemental composition of *H. mediterranei* grown in continuous cultivation on glycerol was determined by Universität Wien, Institute of Physical Chemistry [25]. The elemental composition of the *H. mediterranei* biomass on the substrate glycerol was $CH_{1.57}O_{0.63}N_{0.13}P_{0.02}S_{0.01}$ with a molar mass M_X of 26.4 g mol^{-1} [26]. The cell concentration was estimated by the measurement of the optical density at 600 nm (OD_{600}). In case absorption exceeded OD_{600} 0.5 the samples were diluted with a saline solution (150 g L^{-1} NaCl) to prevent lysis of the cells. Correlation of OD_{600} with biomass concentration was calculated as described in the literature [26]: Biomass (g L^{-1}) = 0.48 · OD_{600}.

2.3. Continuous Culture and Feedforward Control Concept

The steady state condition in biological systems with cell retention is determined by the dilution rate D and the retention rate R. The parameters are calculated as follows from the feed flow F_F, the base flow F_{Base}, the harvest flow F_H, the bleed Flow F_B and the reactor volume V_R:

$$D = \frac{F_F + F_{Base}}{V_R} \tag{1}$$

$$R = \frac{F_H}{F_F + F_{Base}} = \frac{(F_F + F_{Base}) - F_B}{F_F + F_{Base}} \tag{2}$$

$$F_F + F_{Base} = F_B + F_H \tag{3}$$

Calculation of the volumetric flow rates F_F, F_B, F_H, and F_{Base} was based on the change of balance signals over time. In a steady state system, the biomass concentration for cell retention reactors depends on the biomass yield $Y_{X/S}$, the substrate consumption (s_{in}-s) and the retention rate R (see Equation 4). Therefore, the set point for the biomass concentration X_{Set} can be calculated as stated in Equation 5. The dilution factor f_D, thereby, includes the dilution caused by base addition (see Equation 5). In the case of carbon-limited conditions, Equation 5 further can be simplified as s is set to zero. In a setup with cell retention, the specific growth rate μ is depending on the retention rate R and the dilution rate D (see Equation 6).

$$X_{Steady} = \frac{Y_{X/S} \cdot (s_{in} - s) * f_D}{(1 - R)} \tag{4}$$

$$f_D = \frac{F_F}{F_F + F_{Base}} \tag{5}$$

$$\mu_{Set} = (1 - R) \cdot D \tag{6}$$

2.4. Soft Sensor Rate Calculation and Statistical Test for Consistency

Soft sensors for physiological rate calculations are already described in the literature [23,24,27]. The biomass formation rate r_X is calculated by means of a redundant equation system (degree of redundancy of 1) comprising the Carbon balance (C balance) and Degree of Reduction balance (DoR balance) (see Equations 7 and 8):

- Carbon Balance: (7)

$$r_{S,For} + r_{S,Gly} = CER + r_X$$

- Degree of Reduction Balance:

$$\gamma_{For} \cdot r_{S,For} + \gamma_{Gly} \cdot r_{S,Gly} + \gamma_{O2} \cdot OUR = \gamma_X \cdot r_X \quad (8)$$

where r_S is the volumetric substrate uptake rate for formate and glycerol, calculated according to Equation 9; CER is the carbon dioxide evolution rate, calculated from off-gas composition according to Equation 10; OUR is the oxygen uptake rate, calculated from the off-gas composition according to Equation 11; and γ is the degree of reduction of the component indicated in the index. F_{Air} is the airflow into the reactor as determined by the mass flow controller, the different x are the molar fractions of CO_2, O_2, or H_2O, and V_{mol} is the molar standard volume. The term r_{inert} compensates the proportion of gaseous water x_{H2O} in the off-gas (Equation 12). All rates are referred to the number of carbon molecules with the objective of obtaining rates with a unit of C-mol h^{-1}. The aromatic compounds aniline, phenol, and MDA were not taken into account for the balances since they only contribute a maximum of 2% of the total carbon in the system.

$$r_S = F_F \cdot (s_{in} - s) \quad (9)$$

$$CER = \frac{F_{Air} \cdot r_{inert} \cdot (x_{CO2_{Offgas}} - x_{CO2_{Air,}})}{V_{mol}} \quad (10)$$

$$OUR = \frac{F_{Air} \cdot r_{inert} \cdot (x_{O2_{Air}} - x_{O2_{Offgas}})}{V_{mol}} \quad (11)$$

$$r_{inert} = \frac{1 - x_{O2_{Air}} - x_{CO2_{Air}}}{1 - x_{O2} - x_{CO2} - x_{H2O}} \quad (12)$$

The biomass turnover rate r_X is calculated by data reconciliation of elemental balances. For this purpose, Equations 8 and 9 are transformed in matrix form, which described elsewhere in detail [27].

To check the consistency of the model, a test function (χ^2 distribution) is used. Degree of freedom of χ^2 distribution equals the degree of redundancy of the equation system (here: 1) and the confidence level is set to 95%. For these parameters, the threshold for the statistical test value h is 3.84, i.e., with a probability of 95% the test value h is to be expected in the range of 0 to 3.84. For h values >3.84, consistency of the equation system is statistically rejected. For these time points, an outlier in soft sensor calculations might be indicated. A step-by-step explanation for calculation of r_X, as well as a more in-depth description of this consistency check can be found elsewhere [24,27–29]. However, the used formulas can be found the supplementary file. All calculations were performed with the software MATLAB (2018b, MathWorks, Natick, MA, USA).

2.5. Soft Sensor Biomass Estimation

Biomass concentration $X_{SteadyState}$ during steady state conditions is calculated according to Equation 4, by using the estimated biomass turnover rate r_X obtained from data reconciliation.

Online estimation of the biomass conversion rate r_X enables online estimation of parameters that are crucial for the assessment of the metabolic state. In Figure 2, a schematic overview of the soft sensor workflow to calculate the biomass concentration is shown. For the data reconciliation in order to calculate r_X, the balances described in Equations 8 and 9 are used.

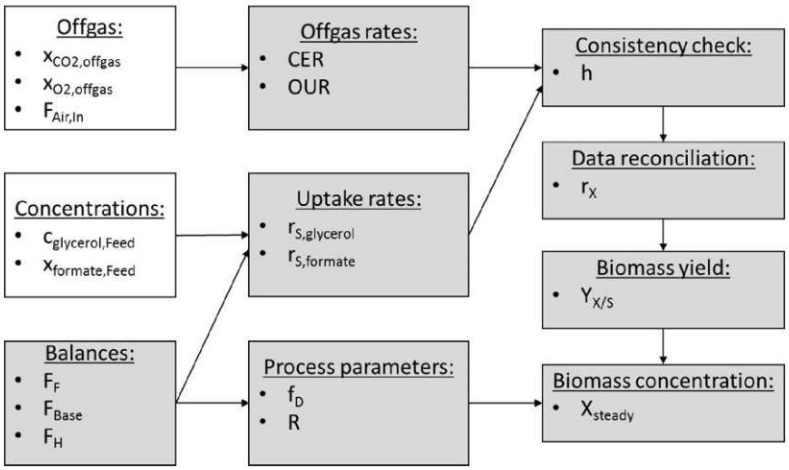

Figure 2. Soft sensor workflow. The calculation of the biomass concentration at steady state conditions in a bioreactor with a cell retention system is carried out according to this workflow. White boxes represent measured values and grey boxes represent calculated variables.

2.6. Analytical Procedures

Substrate quantification in the feed and harvest samples was done as described previously [25], using HPLC (Vanquish UHPLC systems, Thermo-Fisher, USA) with an Aminex HPX-87H column (Bio-Rad, USA) at 60 °C, an isocratic eluent of 4 mM sulfuric acid in Milli-Q water with a flow of 0.6 mL min^{-1}, followed by UV detection at 210 nm and RI detection (RefracoMax520, ERC, Germany). In brief, samples were centrifuged and the supernatant was diluted (1:10) with 40 mM sulfuric acid before 10 µL was injected in the HPLC. The samples were analyzed for residual formate and glycerol, as well as the formation of organic acids. The standards, used for quantifications, were prepared the same way as the samples and diluted with 40 mM sulfuric acid.

For the quantification of aromatic compounds in feed and harvest samples, a reversed-phase HPLC measurement (Vanquish UHPLC systems, Thermo-Fisher, USA) was carried out, using an Acclaim™ PolarAdvantage column (Thermo Scientific, USA, C16, 3 µm, 120 Å, 4.6x150 mm) at 30 °C. Aromatic compounds were detected using a UV detector at 210 nm. The flow was 1 mL min^{-1} with a gradient system (0–2.5 min: 5% A, 95% B; 2.5–5 min: 25% A, 75% B; 5–20 min: Linear decrease of B from 75% to 30%, rest A). After each measurement, a washing step was carried out (0–5.5 min: Linear increase of C from 75% to 95%, rest A; 5.5–15 min: 5% A, 95% C; 15–20 min: 5% A, 95% B). Eluents were: A) acetonitrile; B) 25 mM KH_2PO_4 (pH 3.5 with 1 M H_3PO_4); and C) MiliQ water. Samples were centrifuged prior to analysis and 10 µL undiluted supernatant was injected for HPLC analysis.

3. Results and Discussion

3.1. Development of a Biomass Control and Monitoring Concept on Synthetic Medium

In order to develop a control strategy that enables the control of biomass concentration of extremely halophilic *H. mediterranei*, a continuous membrane-based cell retention system was used. A feedforward biomass control strategy was successfully developed on synthetic medium using glycerol as a cheap and widely available carbon source [30–32]. Furthermore, a biomass online monitoring tool was established to calculate biomass concentrations in steady state experiments.

Moreover, formate as additional carbon source was also tested as the control concept strategy should later be transferred to real industrial process water contaminated with formate. Previous

experiments showed that *H. mediterranei* could not grow when cultivated on formate as sole carbon source (data not shown), but is degraded in the presence of glycerol.

The feedforward control concept was established by varying the parameters R and s_{gly} to obtain different biomass concentrations (Table 1). Doubling the substrate concentration in the feed leads to a two-fold increase of the steady state biomass concentration in the reactor. This can clearly be seen when comparing experiments 1 and 2, where the glycerol concentration in the feed was decreased from 4 g L^{-1} to 2 g L^{-1} resulting in a decrease of biomass concentration from 8.4 g L^{-1} to 4.1 g L^{-1}. This correlation is known from chemostat cultivations without cell retention. Additionally, by varying the retention rate R, the Bleed:Feed ratio (1-R) is also changed. An increase in R results in a lower ratio, meaning that less biomass is removed from the reactor via the bleed stream. As a direct result, decreasing the Bleed:Feed ratio by 50 % while also decreasing s_{gly} by 50 % the same overall biomass concentration reactor should be obtained. This can be seen when comparing experiments 2 and 3. Both aim for the same biomass concentration of 4.1 g L^{-1}, but the Bleed:Feed ratio for experiment 3 was only half compared to experiment 2. By additionally decreasing the glycerol concentration by 50%, the same biomass concentration was achieved (Table 1). For estimation of the variables r_X, r_S, $X_{soft\ sensor}$, and $Y_{X/S}$ in Table 1, data reconciliation and steady state equations as described in Material & Methods was used.

Table 1. Continuous states on the synthetic medium were controlled by different levels of R and s_{in}. Addition of formate is indicated with *. For the calculated values r_X, r_S, $X_{soft\ sensor}$, and $Y_{X/S}$, an error of 10% was assumed.

Experiment	R	S_{gly} [g L^{-1}]	S_{for} [g L^{-1}]	μ_{Set} [h^{-1}]	r_X [C-mmol $L^{-1}h^{-1}$]	r_S [C-mmol $L^{-1}h^{-1}$]	$X_{soft\ sensor}$ [g L^{-1}]	$Y_{X/S}$ [Cmol $Cmol^{-1}$]
1	0.74	4	0	0.026	8.01	12.35	8.4	0.65
2	0.74	2	0	0.026	3.83	5.99	4.1	0.64
2*	0.74	2	0.3	0.026	4.18	6.87	4.2	0.61
3	0.87	1	0	0.013	1.84	3.06	4.1	0.60
3*	0.87	1	0.3	0.013	2.14	3.79	4.7	0.56
4	0.74	2.6	0	0.026	4.89	7.98	5.2	0.61
5	0.74	1.4	0	0.026	2.66	4.19	3.0	0.63

To check for potential effects on biomass estimations by the soft sensor formate was added in a concentration of 300 mg L^{-1} in experiments 2* and 3* of the experimental design (Table 1). Formate was completely degraded in all experiments (data not shown) and the functionality of the soft sensor-based method was not influenced by additional formate in the medium.

An overview of the elements of the process control concept and their high interactivity is shown in Figure 3. Using a cell retention system allows to obtain high biomass concentrations while maintaining low substrate concentrations in the feed. This has a beneficial effect on the operational expenditures of the process (Opex), whereas high values of R result in low bleed streams and lower costs for disposal of redundant biomass. Hence, high retention rates are usually preferred in cell retention systems. The control concept uses the self-regulation mechanisms in continuous culture. Disturbing factors, e.g., temporary change in feed composition, lead to disturbance of biomass growth. However, in case the biomass concentration has not been decreased below a critical threshold, the culture will reach a steady state again, according to the settings of D and R.

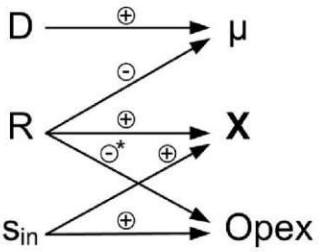

Figure 3. Elements and interactions of the process control concept. Control variables (dilution rate D, retention rate R and substrate concentration s_{in}) are used to vary process variables (specific growth rate μ, biomass concentration X) and operational expenditures (Opex). Positive correlation indicated with (+), negative correlation indicated with (−). * Influence of R on Opex is highly dependent on the process. A negative correlation can be seen when biomass has to be disposed of (e.g., for industrial process water treatment or extracellular products). When a product is intracellular, a high bleed stream is desirable.

3.2. *Application of the Feedforward Control Concept on Industrial Medium*

The process knowledge gained in the experiments on the synthetic medium was applied for continuous remediation of industrial brine containing 200 mg L^{-1} formate by *H. mediterranei*. In a 1300 h continuous process, biomass concentration was controlled stepwise to reach four different levels in the range of 5 to 20 g L^{-1}, while the dilution rate D was held constant for the entire duration of the experiment. The phases were held constant for a minimum of 96 h to guarantee that the culture reaches steady state conditions (equivalent to 9.6 volume changes). Analysis of harvest samples showed C-limited conditions at all time points. Each step was performed at two different specific growth rate levels (0.013 and 0.026 h^{-1}) to investigate the influence of μ on the yields (O2, CO2, and biomass). The variation of R, Bleed:Feed ratio (1-R), and rS over time can be seen in Figure 4A and B. Carbon dioxide evolution rate (CER) and oxygen uptake rate (OUR) as inputs for the monitoring tool are shown in Figure 4C. Using the soft sensor, the biomass formation rate rX was calculated (see Figure 4D). In Figure 4D, also the statistical test value h can be seen. It indicates the consistency of the soft sensor calculations. In almost all time points it was below the threshold of 3.84, where gross errors larger than 10% are not occurring. However, between day 32 and 35 of the continuous experiment, h exceeded the threshold value, indicating that the rates cannot be calculated within a 10% error interval. This might be caused by measurement errors in the gas rates or the substrate concentrations and leads to incorrect calculations. Nevertheless, calculated values for biomass concentration in steady states XS Steady using Equation 4 show good conformity when compared with offline OD measurements as well as with the applied set point for the biomass concentration (see Figure 4E).

Furthermore, the results show that biomass concentration could be held constant on the four different biomass concentration levels despite changing the specific growth rate μ on two levels (see Figure 4F).

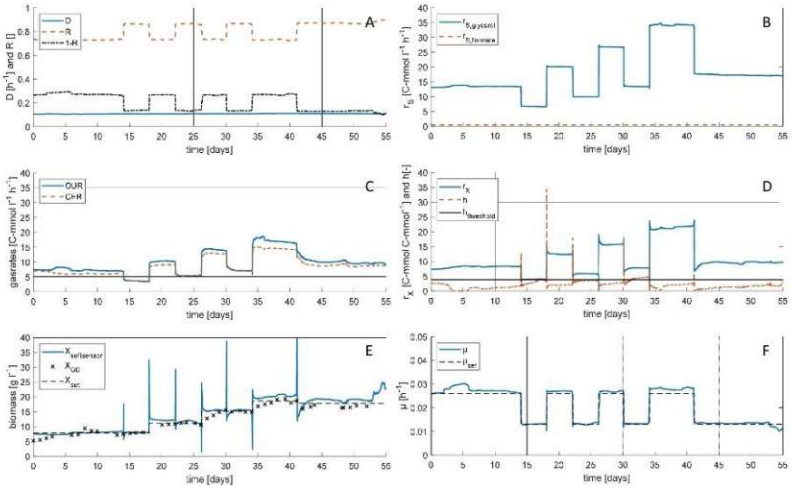

Figure 4. Rate calculations during continuous cultures of *H. mediterranei* with cell retention using real industrial brine. (**A**) Dilution rate *D*, retention rate *R*, and Bleed:Feed ratio (1-R) calculated from online balance data. (**B**) Substrate consumption rate *rs* calculated from feed rate and substrate concentrations. (**C**) Oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) calculated from online off-gas data. (**D**) Biomass turnover rate *rx* calculated by the soft sensor from reconciled data and the statistical test value h for soft sensor calculation. Additionally the threshold for h of 3.84 is marked as black line. (**E**) Biomass concentration calculated from the soft sensor and from OD values. Root-mean-square deviation (RMSD) of soft sensor measurements in comparison to biomass set points was determined to be 2.0 g L^{-1}. (**F**) Specific growth rate μ calculated with biomass turnover rate and biomass concentration from soft sensor.

Physiological rates, that are determined for the soft sensor, offer important additional information in terms of metabolic activities of the cells. Figure 5 shows the correlation of CER, OUR, and *rx* with substrate turnover rate *rs* in the industrial medium. The results show a linear correlation of all three rates. The slope of the graphs are the yields $Y_{CO2/S}$, $Y_{O2/S}$, and $Y_{X/S}$ (see Table 2). Yields show significant differences according to the specific growth rate μ. A higher μ (0.026 h^{-1}) leads to higher biomass yields $Y_{X/S}$, lower CO_2 yields $Y_{CO2/S}$, and lower O_2 yields $Y_{O2/S}$ compared to lower μ (0.013 h^{-1}).

A comparison of yields for O_2, CO_2, and biomass for synthetic and industrial medium did not show significant differences. Nevertheless, the results show the same effect of μ on the yields on both media, synthetic, and industrial. Therefore, it is important to use online determined yields for soft sensor-based biomass calculation instead of predetermined yields, since physiological states can change during a process, according to process parameters and medium.

Table 2. Yield coefficients reconciled along C and DoR balance with industrial medium.

	$Y_{O2/S}$ [mol Cmol^{-1}]	$Y_{CO2/S}$ [Cmol Cmol^{-1}]	$Y_{X/S}$ [Cmol Cmol^{-1}]
μ = 0.013 h^{-1}	0.60 ± 0.01	0.46 ± 0.01	0.54 ± 0.01
μ = 0.026 h^{-1}	0.55 ± 0.01	0.40 ± 0.01	0.60 ± 0.01

Since the proposed soft sensor is a direct measurement of metabolic activity, it is independent of changes for process parameters like aeration rate, stirrer speed, or change of flow rates for liquid

substrates, and only requires steady state conditions of continuous bioprocesses together with consistent offgas measurements and information on the substrate concentration in the feed. Additionally, undefined amounts of carbon introduced when complex media components are used have to be considered in the calculations for the biomass estimations. In conclusion, the developed monitoring strategy allows estimation of biomass concentration even in the presence of other particles, in colored or turbid media or under circumstances where cells form aggregates [33]. Furthermore, it does not require corrosion sensitive hard sensors. This is in stark contrast to standard procedures for measuring biomass concentration, requiring either corrosion sensitive hard sensors or the need for human interference. The tool uses a redundant measurement system (based on gas analysis), which can be used to check the measurements for gross error [28]. Once established, it is easy to handle and can be applied for a wide range of process parameters (different μ and R).

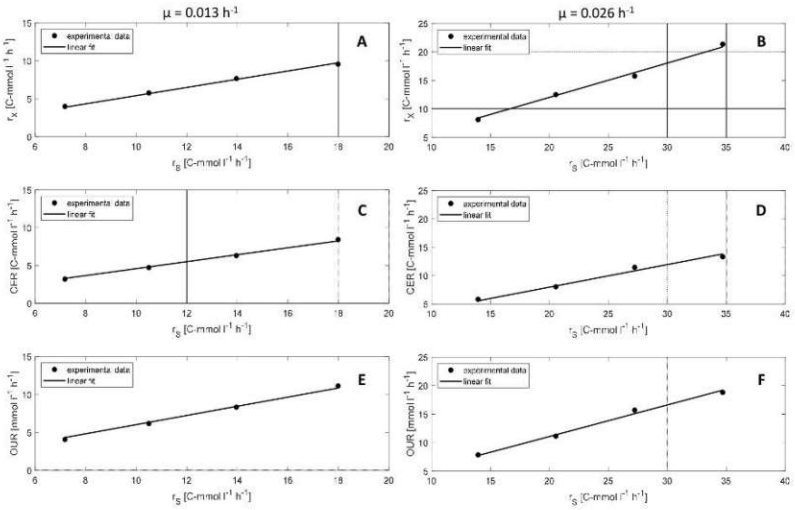

Figure 5. Metabolic yields determined by linear regression. **(A–F)** Biomass turnover rate (r_X), carbon dioxide emission rate (CER) and oxygen uptake rate (OUR) over substrate uptake rate (r_S) at a two different specific growth rates of (left: 0.013 h^{-1}, right: 0.026 h^{-1}) (• experimental data, - linear fit).

3.3. Degradation of Aromatic Compounds in Industrial Brine

After establishing a method for biomass control and monitoring in the membrane-based cell retention system, the ability of such a system to degrade organic pollutants from real industrial process water was evaluated. To that end, different biomass concentrations, specific growth rates and substrate uptake rate were used to study the impact on degradation efficiency of formate and the aromatic compounds phenol, aniline, and MDA contained in different concentrations in the industrial brine. Concentrations of the respective components in the harvest were compared to feed concentrations to determine the degradation efficiency (%, Figure 6A). The individual components were contained in the feed at fixed concentrations and degradation was studied for a period of 1300 h using a continuous process with a dilution rate of 0.1 h^{-1} and different settings for R (and therefore μ) and s_{Gly}.

Full formate and phenol degradation was observed throughout the entire process irrespective of the process parameters and biomass concentrations. This was a very interesting finding as it suggests that the degradation efficiency under the conditions tested does not seem to be dependent on a certain biomass concentration or concentration of the individual components. Therefore, high activity of the degradation pathways facilitating degradation of formate and phenol is assumed.

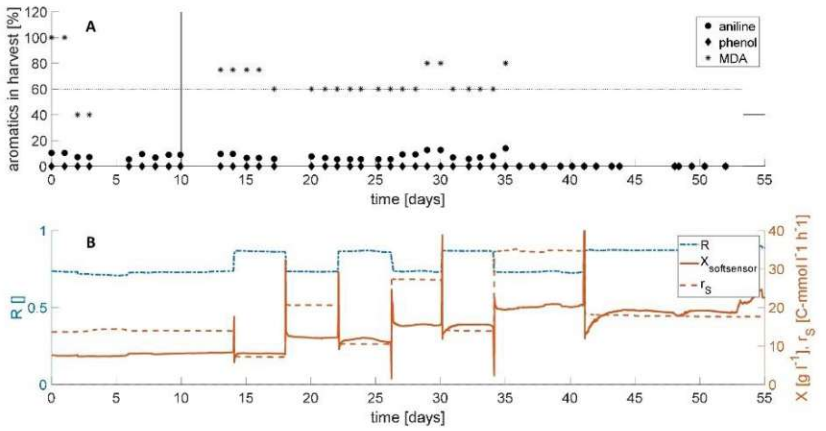

Figure 6. Aromatics in harvest compared with process parameters. (**A**) Share of aromatics left in harvest after treated in bioreactor. Values are calculated according to: Share = aromatics in harvest/aromatics in feed. (**B**) Process parameters X, rs, and μ over the process time. Increase of biomass concentration led to complete degradation of aromatics.

Degradation of aniline in the industrial process water was slightly less efficient compared to phenol, reaching 80–90% degradation for the time period of 0–35 days. It seems the capacity of the metabolism of *H. mediterranei* for the degradation of aniline is lower than for phenol. Comparing the degradation of aniline with biomass concentration showed that a simple increase of biomass concentration using the feedforward strategy (after day 35) led to complete degradation of aniline. The degradation of aniline in the real brine could therefore be successfully shown if a suitable bioprocessing strategy (in this case high biomass) is utilized. A similar behavior could be observed for the degradation of MDA. At low and intermediate biomass concentrations (<15 g L^{-1}) degradation was incomplete at around 20–40% efficiency. Like for aniline, by influencing the biomass concentration as process parameter, also the degradation efficiency of MDA could be increased from 40% to 100%. This indicates a biomass concentration-depending degradation of aniline and MDA, whereas the degradation of phenol seems to be independent from the biomass concentration. Furthermore, it could also be shown that the degradation does not depend on the specific growth rate, since no MDA and aniline were detected at two different settings for R and s_{in} (compare Figure 6B).

In literature, the pathway of phenol and aniline degradation in halophilic bacteria is described via meta- or ortho-cleavage of catechol [6,34,35]. On the other hand, MDA was reported to be degraded by activated sludge, also in saline waste water [36,37]. However, no degradation pathway for MDA has been reported so far, but it appears likely that the degradation pathway is similar to aniline degradation as MDA can be converted into aniline via cleavage of the methyl bridge between the two aromatic rings [37].

The results show, that the developed feedforward control strategy for biomass concentration works successfully and can be used for degradation of organic contaminants from real industrial brine at high efficiency and productivity.

Although membrane-based cell retention in bioreactors for continuous cultivation of extremophilic cells has already been reported [38–41], the current work nicely expands these approaches by using a simple but nevertheless efficient bioprocessing control and monitoring strategy to demonstrate the applicability of extreme halophilic organisms for a circular economy process that requires high throughput degradation of organic compounds, in order to reuse industrial process in further production steps.

Bioprocesses that are easy to handle and have a robust control strategy are more likely to be integrated into existing production facilities (e.g., in the chemical industry). Therefore, it is envisioned that the current work can contribute to overcome typical reservations against including biotechnological processes in non-biotechnological industries like high effort for sterile handling, time-consuming training of staff and low space-time yields. The feedforward control concept developed in this study provides the process transparency needed for an operation that is safe, robust and easily implementable in an industrial environment.

4. Conclusions

To conclude, a feedforward control strategy for a membrane-based cell retention system was combined with a soft sensor-based monitoring tool to control and real-time monitor biomass concentrations of *H. mediterranei*, depending on the retention rate R and the substrate concentration over a broad range of concentrations. This new approach was successfully applied in a high throughput industrial process water treatment process. Moreover, removal of the four organic pollutants formate, phenol, aniline, and MDA at high productivity and degradation efficiency was demonstrated.

The results presented here provide an excellent framework for future applications of halophiles in continuous bioprocesses.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1.

Author Contributions: N.M. carried out the cultivation experiments. N.M., T.M., C.H., and S.P. conceived the study and analyzed the data. T.M. and S.P. wrote the manuscript. All authors have read and approved the final manuscript.

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Conflicts of Interest: The authors declare that they have no competing interests.

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4.4 Optimized operating conditions for a biological treatment process of an industrial residual process brine using a halophilic mixed culture

Title of manuscript

Optimized operating conditions for a biological treatment process of an industrial residual process brine using a halophilic mixed culture

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Contribution

TM carried out the cultivation experiments. TM, CH, and SP conceived the study and analyzed the data. TM and SP wrote the manuscript. All authors have read and approved the final manuscript.

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Article

Optimized operating conditions for a biological treatment process of industrial residual process brine using a halophilic mixed culture

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Abstract: Residual process brine is a sustainable raw material for chlor-alkali electrolysis processes. This study investigated the influence of critical process parameters on the process performance of a continuous treatment process for residual process brine using halophilic microorganisms. The goal of the bioprocess is an efficient degradation of the organic impurities formate, aniline, phenol, and 4,4'-methylenedianline from this residual stream. It was shown that formate could be degraded with high efficiencies (89-98%) during the treatment process. It was observed that formate degradation was influenced by the co-substrate glycerol. The lowest residual formate concentrations were achieved with specific glycerol uptake rates between 8.0-16.0 x 10^{-3} g L^{-1} h^{-1} OD_{600}^{-1}. Moreover, a triple nutrient-limitation for glycerol, ammonium, and phosphate was successfully applied for continuous cultivations. It was furthermore shown that all aromatic impurities were degraded with an efficiency of 100%. Ultimately, this study proposed optimized operating conditions allowing efficient degradation of organics in the residual process brine under various process conditions. Future optimization steps will require a strategy to prevent accumulation of potential intermediate degradation products formed at high aniline feed concentrations and increasing liquid dilution rates of the system to achieve a higher throughput of brines.

Keywords: Residual Process Brine, Triple Nutrient Limitation, Formate and Aromatics Degradation, Continuous Bioprocessing, Halophilic Bioprocess

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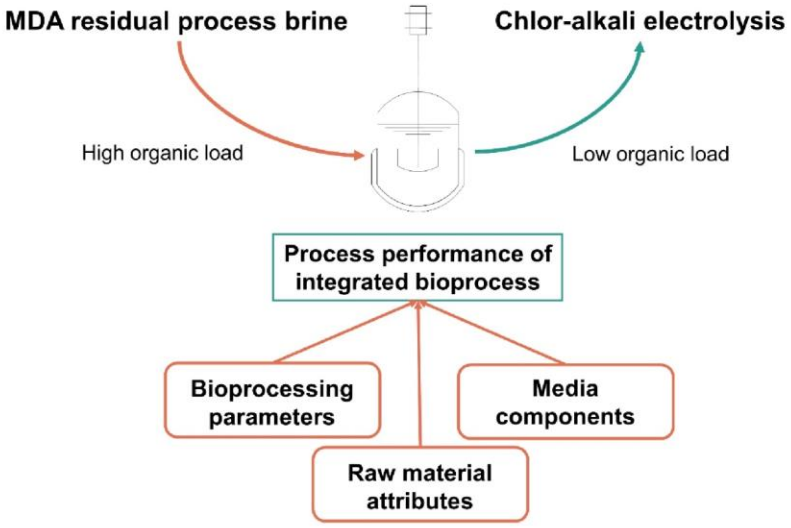
Graphical abstract



1. Introduction

The chlor-alkali industry uses brines for the electrolytic production of chlorine gas, sodium hydroxide, and hydrogen [1-3]. Especially the products sodium hydroxide and chlorine gas are widely used in various industries [1, 4]. The membrane cell technology is seen as the best available technology among chlor-alkali electrolysis processes [2, 4]. Brines comprise the raw material for the membrane cell process, and can be derived from seawater desalination plants or industrial production chains [5, 6]. However, there are high quality requirements for brines used as a raw material in membrane cell processes to ensure an efficient process [4]. For instance, organic contaminations in brines negatively affect the chlor-alkali membrane process performance [7-10]. Moreover, also inorganic impurities, like Mg^{2+}, Ca^{2+}, or nitrogen can decrease the membrane cell process [7, 10, 11]. Thus, a pre-treatment of industrial result process brines (RPB) is necessary. So far, biological treatment processes are seen as a cost-effective alternative for residual water treatment, compared to physical or chemical treatment approaches [12]. Moreover, several studies already have shown the applicability of halophilic bioprocesses for saline residual water treatment [13-19].

In this study, a biological process for the treatment of an RPB should be characterized for its integration into an industrial production chain. The RPB is derived from the industrial production process of 4,4'-methylenedianiline (MDA), which serves as a substrate in the production of methylene diphenyl diisocyanate (MDI) and ultimately of polyurethanes [20-22]. After the separation of the organic phase containing the product MDA and the aqueous phase, the RPB comprises four organic impurities (formate, aniline, phenol, and MDA) and high concentrations of sodium chloride (10-15%) [23-25]. Thus, the organic matter present in the RPB has to be removed, if the RPB is reused as raw material for a

membrane cell CAE process. As previously reported, RPB from industrial MDA-production can be successfully treated with a continuous retentostat bioprocess using halophilic microorganisms [13, 16].

During the production of MDA, process conditions might be varied, which ultimately leads to changes in organic impurity concentrations between different RPB batches. However, the effects of such changes in the raw material attributes (RMA) like the organic impurity concentrations on the degradation efficiency of the investigated biological treatment process are not known. Still, the degradation efficiency of organic contaminants is one major process performance variable, if the RPB shall serve as raw material for a chlor-alkali-electrolysis step. Besides RMA, also other critical process parameters like bioprocessing parameters (dilution rate, retention rate, or biomass concentration) and media components (different nutrient concentrations) might influence the process performance.

Therefore, the goal of this study is to determine the effect of critical process parameters on the process performance of a halophilic biological process for the treatment of an industrial RPB. This process understanding shall be used to propose optimized operating conditions where process parameters should be controlled in a way, that high process performance is achieved. Moreover, the industrial integration of bioprocesses requires cost-effective processing. Thus, this study aimed the reduction of operational costs, achieved by reducing media supplementation but maintaining high degradation efficiencies at the same time.

To do so, critical process parameters were identified and their influence on the process performance was investigated. In that way, also the question should be answered if process performance variables need to be known before the bioprocess step, or if it is sufficient to set process parameters within the control space, to obtain sufficient degradation performance. To that end, a total of three continuous cultivation experiments were performed and analyzed. It was shown for the first time in a biological RPB treatment process, that the degradation of the main organic contaminant formate is depending on the specific uptake rate of the co-substrate glycerol and the consumption yield of ammonium to glycerol. Thus, pre-defined settings for the glycerol uptake rate keep formate degradation high, even at large concentration changes in the RPB. Moreover, for the first time, this study showed the successful application of a triple nutrient limitation (carbon, nitrogen, and phosphorus source) in a continuous bioprocess.

2. Materials and Methods

2.1. Strain and medium

The microbial culture used in this study was a novel halophilic mixed culture first found in a biological MDA residual water treatment process [16]. The culture mainly consists of strains from the three halophilic genera *Halomonas* and *Aliifodinibius*, and to a small part of *Oceanobacillus* strains.

The RPB was supplemented with mineral salts and contained (g L^{-1}): $FeCl_3$ 0.005; $MgCl_2 \cdot 6\,H_2O$ 1.3; $MgSO_4 \cdot 7\,H_2O$ 1.1; $CaCl_2 \cdot 2\,H_2O$ 0.55; KCl 1.66; Trace elements solution 1 mL [(g L^{-1}): $FeSO_4 \cdot 7\,H_2O$ 1.39; $CuSO_4 \cdot 5\,H_2O$ 1.0; $CoCl_2 \cdot 2\,H_2O$ 0.62; $ZnSO_4 \cdot 7\,H_2O$ 0.86; Manganese stock 1 mL [(g L^{-1}): $MnCl_2 \cdot 4\,H_2O$ 0.18]. However, concentrations of $MgCl_2 \cdot 6\,H_2O$, $MgSO_4 \cdot 7\,H_2O$, and $CaCl_2 \cdot 2\,H_2O$ were altered as indicated. The pH value of the RPB medium was adjusted to pH 4, using 37% HCl.

The substrate glycerol was added in concentrations of 0.3-4.0 g L^{-1}, as indicated. As a nitrogen source, ammonium chloride (NH_4Cl) was added in concentrations of 0.1-1 g L^{-1}. As phosphorus source, potassium dihydrogen phosphate (KH_2PO_4) was used in concentrations of 0.05-0.15 g L^{-1}. The macronutrients, consisting of carbon, nitrogen, and phosphorus sources were either added to the RPB or combined in a separate supplement feed, as indicated. To the supplement feed, NaCl in a concentration of 100 g L^{-1} (10%) was added and the pH was adjusted to 7. Shake flask experiments were performed in 500 mL shake flasks filled with 100 mL medium additionally supplemented with 20 g L^{-1} MOPS, 5 g L^{-1} glycerol, 1 g L^{-1} NH_4Cl, and 0.15 g L^{-1} KH_2PO_4.

2.2. Bioreactor setup – continuous stirred-tank reactor

A schematic diagram of the experimental setup is shown in Figure 1. Continuous cultivations with cell retention were performed in a corrosion-resistant stirred tank reactor with a working volume of 2.3 L (PEEK Labfors bioreactor, Infors, Switzerland), equipped with a 420 cm^2 microfiltration unit (model: CFP-2-E-4A, polysulfone membrane, 0.2 µm pore size, GE Healthcare, Germany), as described previously [13]. Peristaltic pumps were used for circulating cell broth in the loop line (Ecoline, Ismatec, Germany), for the addition of feed (industrial residual brine) and base (peristaltic pump installed at the Infors bioreactor tower), as well as for bleed and harvest withdrawal (Lambda Preciflow, Lambda Instruments, Switzerland. The dilution rate *D* was kept constant at 0.1 h^{-1} for all experiments. The pH was measured using an Easyferm probe (Hamilton, Switzerland) and adjusted using 0.5 M NaOH via the integrated dosing system of the Labfors system. Reactor volume was kept constant at 1 ± 0.05 L. The reactor volume, feed (industrial residual brine), and base consumption were continuously monitored by laboratory scales with 0.1 g resolution (Mettler Toledo, USA).

This setup was altered for one continuous fermentation experiment, two separated feeds were used. One feed contained industrial residual brine containing organic contamination and micronutrients. The flow for the residual brine was set to 90 mL min^{-1}. The pH of the residual water feed was adjusted to values around 4, using hydrochloric acid. The second feed contained the carbon, nitrogen, and phosphorus source. The salt content of the second feed was adjusted to 10% NaCl and the pH was 7. The second feed was pumped by the feed pump installed at the Infors bioreactor tower (peristaltic pump) and the flow was set to 10 mL min^{-1}. This resulted in a combined feed flow of 100 mL min^{-1}, leading to a *D* of 0.1 h^{-1}.

The inlet airflow was kept constant at 100 mL min^{-1} using a mass flow controller (Brooks Instrument, USA). Dissolved oxygen was measured using an Oxyferm probe (Hamilton, Switzerland) and kept above 20% to maintain aerobic conditions in the reactor. Oxygen transfer was adjusted by variation of stirrer speed between 400 and 560 rpm. The off-gas composition was determined using a BlueSens gas analyzer system (BCP O_2 and CO_2, BlueSens, Germany). To reduce the water content, the off-gas passed a countercurrent condenser before entering the gas analyzer system. Online data monitoring and process control were executed using a process information management system (Lucullus, SecureCell, Switzerland).

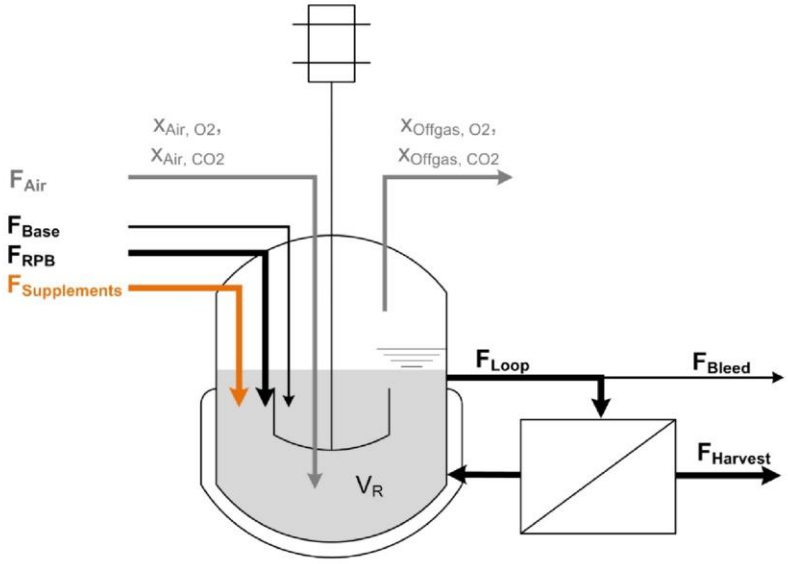


Figure 1. Scheme of the cell retention setup. In the first bioreactor setup, a constant feed (F_{RPB}) is adding fresh residual brine to the bioreactor broth and supplies the cells with substrate and media components. In a second setup the feed flow is split up into a residual brine feed (F_{RPB}) containing additional micronutrients and a supplement feed ($F_{Supplements}$) containing the carbon, nitrogen, and phosphorus source. Base (F_{Base}) is added to hold the pH on a constant level of 7.0. A pump continuously circulates the cell suspension as loop flow (F_{Loop}) through the membrane module to separate cell-free harvest ($F_{Harvest}$). Bleed flow (F_{Bleed}) is continuously removed to eliminate cells and sustain steady state conditions. To guarantee a constant reactor volume (V_R), flows for Feed, Base, Harvest and Bleed have to meet the following equation: $F_{RPB} + F_{Base} (+F_{Supplements}) = F_{Harvest} + F_{Bleed}$. Biomass is monitored using a turbidity probe and a soft sensor that is driven by measurements of off-gas composition. Oxygen is supplied with a constant flow of pressurized air F_{Air}. The off-gas composition of oxygen $x_{Offgas,O2}$ and carbon dioxide $x_{Offgas,CO2}$ was measured.

2.3. Calculations

All calculations in this study were performed using Matlab R2019b (Mathworks, USA).

2.3.1. Steady-state cultivation

Continuous cultivation experiments were performed, and steady-state conditions were assumed, as each experimental setpoint was performed for at least 96 h. During the experiments, the substrate glycerol was completely consumed, thus changes in concentrations in the bioreactor are zero over time according to:

$$\frac{dc_i}{dt} = 0, \quad (1)$$

Hence, the specific substrate uptake rate for glycerol under steady-state conditions was calculated with Equation 2:

$$q_S = D * (c_{i,Feed} - c_{i,Harvest})/OD_{600}, \quad (2)$$

where i indicates substrate components. D denotes for the liquid dilution rate and is calculated based on Equation 3:

$$D = \frac{F_{RPB}+F_{Supplements}+F_{Base}}{V_R} = \frac{F_{Feed}}{V_R} = \frac{1}{HRT'} \quad (3)$$

$$R = \frac{F_{Harvest}}{F_{Feed}}, \quad (4)$$

$$\mu = (1 - R) * D, \quad (5)$$

where F_{Feed} is the sum of input flows F_{RPB}, $F_{Supplements}$, and F_{Base}. F_{RPB} is the RPB feed flow rate, $F_{Supplements}$ the supplement feed flow, F_{Base} the flow of the base, and V_R the bioreactor volume. The dilution rate D can also be denoted as the reciprocal value of The Hydraulic Residence Time (HRT). The retention rate R is calculated based on flows of the RPB feed F_{Feed} and the cell-free harvest $F_{Harvest}$. Once steady-state conditions are reached, the growth rate μ of the microbial system can be calculated based on the retention rate R and the dilution rate D (Equation 5).

2.3.2. Multiple linear regression

For the investigation of effects of one or more predictor variables on one outcome (target variable) a multiple linear regression (MLR) model can be used [26, 27]. The relation between the outcome Y and the predictor variables X_k (k=1,2,…,p-1) is described as in Equation 6:

$$y_i = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_{p-1} x_{p-1} + \varepsilon_i, \quad (6)$$

where β represent the regression coefficients to the representing predictor variables. To estimate the coefficients the least squares criterion is applied where β is chosen to minimize the sum of squared errors of observed Y and the fitted model $\hat{y}$:

$$\sum_{i=1}^{n} (y_i - (\beta_0 + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_{p-1} x_{p-1} + \varepsilon_i))^2, \quad (7)$$

To test if regression coefficients and the regression model are significant, several statistical tests can be evaluated. The hypothesis H_0, that the regression coefficients $\beta_{1\text{-}k}$ are equal to zero is verified if the corresponding p-value is above 0.05 and the F-value is larger than the reference statistic:

$$p > 0.05, \quad (8)$$

$$F > F_{1-\alpha}(k, n - p), \quad (9)$$

where α is the significance interval (in this case 0.05), p is the number of regression parameters, and n is the number of samples. During this study, two different predictor variables were tested, which are the consumption yield of ammonium and phosphate per consumed glycerol ($Y_{NH4+/glycerol}$ and $Y_{PO43-/glycerol}$) in mmol mmol^{-1}. The chosen predictor variables were not correlated. As response variables, the residual formate concentration, and the residual amount of US2 were used. Linear regression was performed using the Matlab function *regress*.

2.4. Analytical procedures

2.4.1. HPLC analysis

Substrate quantification for glycerol and formate in the feed and harvest samples was done as described previously [28], using HPLC (Vanquish UHPLC systems, Thermo-Fisher, USA) with an Aminex HPX-87H column (Bio-Rad, USA) at 60 °C, an isocratic eluent of 4 mM sulfuric acid in Milli-Q water with a flow of 0.6 ml min^{-1} followed by UV detection at 210 nm and RI detection (RefracoMax520, ERC, Germany). In brief, Samples and calibration standards were prepared by mixing 450 µl cell-free supernatant with 50 µl of 40 mM H_2SO_4. For analysis, 10 µl sample were injected to the column and 5-point calibration curves were used for quantification. The samples were analyzed for residual formate and glycerol, as well as the formation of organic acids. The standards, used for quantifications, were prepared the same way as the samples and diluted with 40 mM sulfuric acid [16].

Quantification of aromatic compounds in feed and harvest samples was performed using a reversed-phase HPLC measurement method (Vanquish UHPLC systems, Thermo-Fisher, USA) with an Acclaim™ PolarAdvantage column (Thermo Scientific, USA, C16, 3 µm, 120 Å, 4.6x150 mm) at 30 °C [13,16]. Aromatic compounds were detected using a UV detector at 210 nm. The flow was 0.6 mL min^{-1} and a gradient system was used: 0-5 min: 5% A, 95% B; 5-10 min: 25% A; 75% B; 10-33 min: linear increase of A from 25% to 70%, rest B; 33-35 min: 70%A, 30%B; 35-40 min: linear decrease of A from 70% to 5%, rest B; 40-45 min: 5% A, 95% B. Eluents were: A) acetonitrile; B) Milli-Q water. Samples were centrifuged before analysis and 10 µL undiluted supernatant was injected for HPLC analysis. Quantification of aromatic compounds in feed and harvest samples for cultivation experiment 1 was performed as described previously [16].

2.4.2. Media composition analysis

The determination of ammonium and phosphate was conducted in a Cedex Bio HT Analyzer (Roche, Germany), where enzymatic assays are used and combined with photometric measurements. For these measurements, the limits of detection (LOD) of the analyzer are 0.238 mmol L^{-1} for ammonium and 0.1 mmol L^{-1} for phosphate.

2.4.3. Determination of the chloride ion concentration

The determination of the chloride ion concentration in residual process brine samples was performed using the titration method as described previously (Fajans). The method is based on titration with $AgNO_3$ and dichlorfluorescein (2%) as indicator [29]. Together with chloride ions, silver ions form a poorly soluble precipitation of silver chloride:

$$Ag^+ + Cl^- \rightarrow AgCl$$

Before starting the titration, an $AgNO_3$ solution was prepared and standardized with a NaCl solution of known concentration. According to the titration of this known NaCl solution, the $AgNO_3$ solution had a concentration of 163.9 mmol L^{-1}. The pH of the samples was adjusted to pH 7, either by adding hydrochloric acid or sodium hydroxide. To avoid precipitation of the silver

chloride colloidal solution, 10 mL of chlorine-free dextrin solution (1%) were added to 2 mL of sample, resulting in a yellow color. Titration of the prepared sample with an $AgNO_3$ solution, the color of the liquid sharply turns to pink at the equivalence point, indicating the end of the titration.

3. Results and discussion

3.1. Definition of process performance variables and critical process parameters

The goal of this study was to propose optimized operating conditions, used for the control strategy of a biological treatment process to reduce organic impurities in an industrial RPB. To do so, potential factors which influence the degradation efficiency of bioprocess have to be identified (see Figure 2). To measure the degradation efficiency of the four organic impurities in the biological treatment process, the following process performance variables were defined:

- the **residual aromatic concentration** in the harvest $c(aromates_{Harvest})$ [mg L^{-1}];
- the **residual formate concentration** in the harvest $c(formate_{Harvest})$ [g L^{-1}];

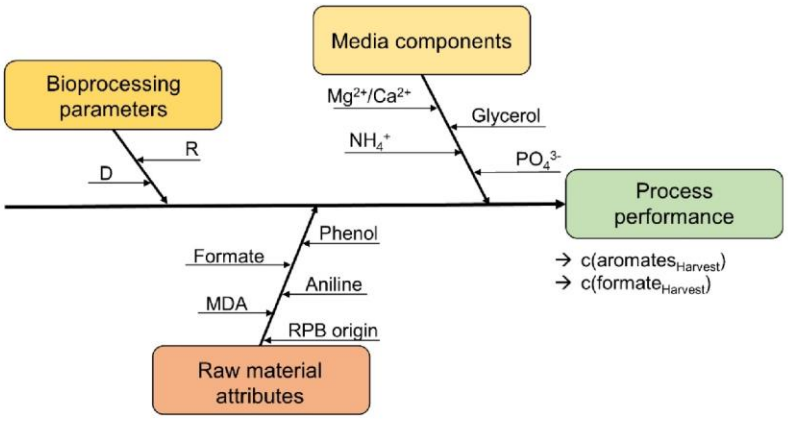


Figure 2. Potential parameters influencing a biological treatment process to reduce organic content in an MDA production residual brine. Bioprocess parameters, media components, and raw material attributes are summarized as critical process parameters. Parameter D refers to the dilution rate, and R to the retention rate R. The residual aromatic ($c(aromates_{Harvest})$) and formate concentrations ($c(formate_{Harvest})$) were defined as process performance variables.

As other process parameters might also influence the process efficiency, hence, critical process parameters were defined and analyzed during this study (see Table 1):

- **Bioprocess parameters** (dilution rate, retention rate, and the biomass concentration (indicated by the optical density));
- **Media components** (glycerol, ammonium, phosphate, magnesium, and calcium feed concentrations);
- **Raw material attributes** (RPB origin, formate, aniline, phenol, and 4,4'-MDA feed concentrations);

Table 1. Overview of critical process parameters investigated for their influence on the process performance.

Critical process parameter	Unit	Critical process parameter	Unit
Glycerol	g L^{-1}	Formate	mg L^{-1}
Ammonium (NH_4^+)	mmol L^{-1}	Aniline	mg L^{-1}
Phosphate (PO_4^{3-})	mmol L^{-1}	Phenol	mg L^{-1}
Magnesium (Mg^{2+})	g L^{-1}	4,4'-MDA	mg L^{-1}
Calcium (Ca^{2+})	g L^{-1}	Optical Density OD_{600}	-
Dilution rate D	h^{-1}	Retention rate R	-

To investigate the influence of the critical process parameters on the process performance variables, three different continuous cultivation experiments were performed. In each cultivation experiment, different settings of critical process parameters were tested. For simplification the cultivation experiments are indicated with cultivation 1, 2 and 3 (Table 2).

During this study, continuous cultivation experiments were performed in a 16L pilot-scale bioreactor system (cultivation 1) and in a 1L lab-scale bioreactor system (cultivation 2 and 3). For the experiments, RPBs from three different MDA production sites were used and compared for their impact on the process performance of the biological treatment process. The RPB origins are numbered with RPB1, RPB2, and RPB3. Cultivation experiments 1 and 2 only used RBP1. For cultivation 3, RPBs from three different MDA production sites were used and compared (RPB1-3). Moreover, for cultivation 3, a two feed-system was applied, where feed 1 consisted of the RPB and with feed 2, glycerol was supplied to decouple glycerol feeding from RPB feeding.

Table 2. Overview of cultivation experiments.

Parameter	Cultivation 1	Cultivation 2	Cultivation 3
Reference	[16]	This study	This study
Feed system	Feed: RPB and glycerol	Feed: RPB and glycerol	Feed 1: RPB Feed 2: glycerol
Reactor volume	16 L	1 L	1 L
Range of D	0.06 – 0.20 h^{-1}	0.09 h^{-1}	0.10 h^{-1}
Range of R	0.75 – 0.95	0.92 – 0.98	0.8 – 0.91
RPB origins	1	1	1, 2, 3
Cultivation time [days]	>200	39	35

3.2. Influence of critical raw material attributes on process performance

3.2.1. RPB origin

In previously performed shake flask experiments potential effects of the RPB origin on the microbial growth rate and the degradation efficiency of organic impurities were investigated. Shake flask experiments showed no differences in the specific growth rates and organic degradation efficiency for medium prepared from RPB1, 2, and 3 (data not shown). As previously reported, potential changes in the NaCl concentration in the RPB might occur and influence microbial growth rates [16]. Therefore, specific growth rates of the novel halophilic mixed culture

at different NaCl concentrations were determined in shake flasks (Figure 3). Growth rates stayed constant at NaCl concentrations between 50 and 100 g L^{-1}, whereas a decline of the growth rates was observed at NaCl contractions at 150 and 200 g L^{-1}. Hence, NaCl concentrations for the different RPB origins were determined (RPB1: 100.1±0.6 g L^{-1}, RPB2: 83.0±0.6 g L^{-1}, RPB3: 82.9±0.7 g L^{-1}). In conclusion, the determined growth rates indicated that the halophilic mixed culture is suitable for growth in the RPB as the NaCl concentration was measured between 80 and 100 g L^{-1}. NaCl concentrations higher than 100 g L^{-1} lower the growth rate to 0.04 h^{-1} at 200 g L^{-1}. Furthermore, results of bioreactor cultivation experiments indicated no influence of the RPB origin on the degradation efficiency of formate or aromatic compounds.

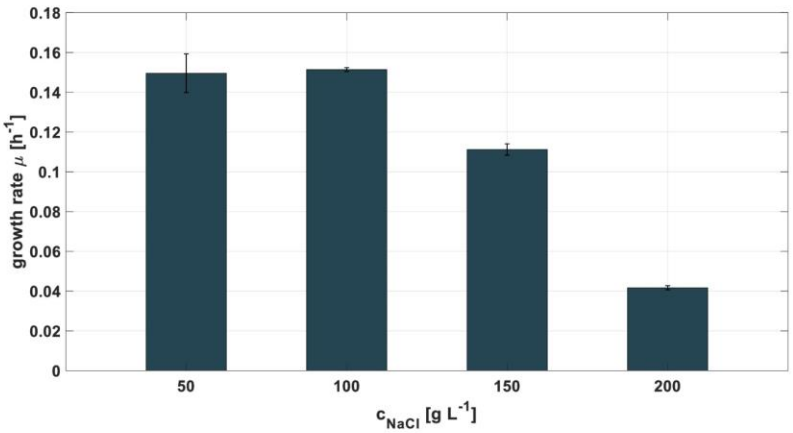


Figure 3. Determination of the specific growth rates of the novel halophilic mixed culture, depending on the NaCl concentration in the medium.

3.2.2. Aromatic compounds

The concentrations of aromatic contaminants might change between different RPB batches, caused by variations in the production process of MDA. The concentrations of aromatic compounds in the used RPB batches varied from 0.02 – 15.5 mg L^{-1} (Table 3). Effects of changing concentrations of aromatics in the RPB feeds were analyzed during this study. High degradation efficiencies of aromatic impurities (100%) were achieved during all continuous cultivation experiments.

Table 3. Concentration ranges of aromatic contaminants in residual process brine batches. For RPB 1 several batches were used and the range for each aromatic contaminant is shown. RPB 2 and 3 derived from one batch each.

Contaminant	RPB 1 [mg L^{-1}]*	RPB 2 [mg L^{-1}]	RPB 3 [mg L^{-1}]
Aniline	0.04 – 15.50	3.91	0.04
Phenol	1.37 – 7.71	15.00	2.99
4,4'-MDA	0.02 – 0.74	0.21	0.06

Although all aromatic compounds from the RPB feeds were degraded with a 100% efficiency during the bioprocess, potential intermediates in the aromatic degradation pathways were discovered during HPLC analyses of bioreactor samples. In bioreactor samples of all continuous cultivation experiments, yet unidentified peaks were observed in HPLC chromatograms. However, such unidentified peaks were not present in RPB feed samples. In cultivation 1 a different HPLC method for the detection of aromatic compounds was used than for cultivation 2 and 3. Therefore, the unidentified substance found during the cultivation 1 was indicated as *unknown substance 1* (US1), while the substance found during cultivations 2 and 3 was indicated as *unknown substance 2* (US2).

During microbial degradation of aromatic compounds like aniline and phenol, catechol is one of the first intermediate substances [30-33]. Afterwards, catechol is degraded via two main pathways (meta- and ortho- cleavage pathway) and ultimately transformed to CO_2 via the TCA cycle [31, 32].

However, US1 and US2 could not be identified as catechol (data not shown). Still, the peak areas of the two unidentified, potential intermediates US1 and US2 increased with an increasing aniline concentration in the RPB feed (Figure 4). In contrast, accumulation of these unidentified compounds did not correlate with phenol and 4,4'-MDA concentrations. In addition to the aniline feed concentration, no other critical process parameters were found to influence the accumulation of the unidentified substances.

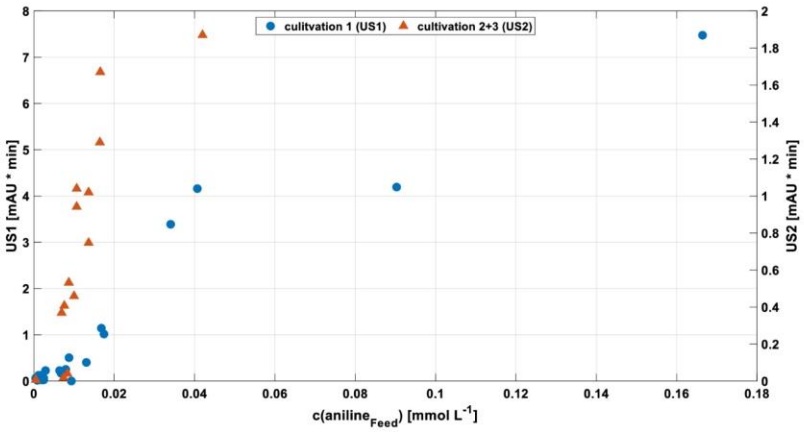


Figure 4. Accumulation of US1+2 depending on RPB feed concentrations of aniline. (●: peak area of unknown substance 1, found during cultivation experiment 1; ▲: peak area of unknown substance 2, found during cultivation experiments 2+3).

3.2.3. Formate

Generally, in RPB derived from MDA production, formate is the main organic impurity and showed a concentration range of 0.17 – 0.50 g L^{-1} in the used RPB batches. During the cultivation experiments, formate was degraded with an efficiency of 89 – 98%. Formate is usually oxidized to CO_2 via enzymes, which are classified into two different groups [34]. The first group consists of metal-independent, but NAD^+-dependent formate dehydrogenases, which oxidize formate according to Equation 7 [34, 35]:

$$HCOOH + NAD^+ \leftrightarrow CO_2 + NADH \quad (10)$$

The second group are metal-containing formate hydrogen lyases, which have mo-lybdenum or tungsten containing components (Equation 8) [36-38]:

$$HCOOH \leftrightarrow CO_2 + H^+ + 2e^- \quad (11)$$

Among halophiles, the halophilic bacterium *Halomonas* sp. MA-C was reported to degrade formate. However, only less than 5% of formate was incorporated into biomass, as shown by experiments using ^{14}C-labeled formate.

It was shown that the total amount of degraded formate and the formate concentration in the RPB feed were linearly correlated (Figure 5). This linear trend was consistent within all three cultivation experiments. Nevertheless, formate was not completely degraded, as residual formate was still measured in harvest samples (2-11% of original formate concentration). However, residual formate concentrations could not be linked to the corresponding formate concentration in the RPB feed. Therefore, further investigations of influential factors on formate degradation were carried out (see section 3.3.2).

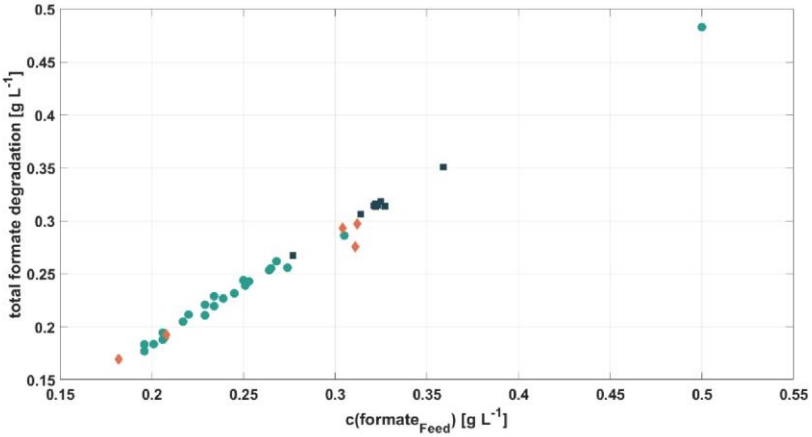


Figure 5. Linear correlation of the total amount of degraded formate and the formate concentration in RPB feed. (●: cultivation 1; ■: cultivation 2; ♦: cultivation 3).

3.3. Influence of media components on process performance

3.3.1. Ammonium and phosphate

In addition to low organic levels, RPB - used as a raw material in a membrane-cell chlor-alkali electrolysis - has to be free of any ammonium or organic nitrogen species, as explosive nitrogen trichloride (NCl_3) can be formed [7]. For microorganisms require nitrogen for growth and protein biosynthesis, ammonium was supplied in the cultivation medium as nitrogen source. However, to meet the raw material specifications for the chlor-alkali electrolysis, the ammonium concentration in the feed medium must be low enough to allow for

complete degradation by the microbial culture, but still high enough to ensure sufficient process performance.

Apart from ammonium, also phosphate plays a crucial role for microbial growth, as it is a major part in several cellular processes like DNA-synthesis and is a part of the energy molecule ATP. In contrast to ammonium species, it is not known to the authors, that phosphate could negatively affect the chlor-alkali process when it is present in brines. However, the reduction of the used phosphate source is of interest in terms of the process operational cost. Thus, the addition of phosphate should be limited to a minimum, that successful degradation of organic compounds is still ensured, but no residual phosphate was measured in harvest samples.

Therefore, during cultivation 2, the influence of ammonium and phosphate consumption on the process performance variables was investigated. To do so, the ammonium and phosphate concentrations in the RPB feeds were varied to compare degradation efficiency of organic compounds at limiting and non-limiting conditions for either ammonium, phosphate, or both (see Table 4). In all experiments, glycerol-limiting conditions were applied. All used RPB feeds were originated from RPB1. A successful application of a triple-nutrient limitation (glycerol, ammonium, and phosphate) could be achieved, and a sufficient degradation of organic impurities could be maintained. The results also showed that the lowest $c(formate_{Harvest})$ were reached with phosphate-limiting, but not nitrogen-limiting conditions (see Table 4, experiment 2.5-2.7). Besides, ammonium and phosphate consumption did not show an effect of the accumulation of US2.

Table 4. Experimental conditions and results of ammonium and phosphate limitation experiments. Concentrations of ammonium, phosphate, and formate in feed and harvest samples are denoted as $c((NH_4^+{}_{Feed})$, $c(NH_4^+{}_{Harv.})$, $c(PO_4^{3-}{}_{Feed})$, $c(PO_4^{3-}{}_{Harv.})$, $c(formate,_{Feed})$, and $c(formate,_{Harvest})$. Consumption yields of ammonium and phosphate referred to glycerol consumption are denoted as $Y_{NH4+/Glycerol}$, and $Y_{PO43-/Glycerol}$

Exp.	$c(NH_4^+{}_{Feed})$ [mM]	$c(NH_4^+{}_{Harv.})$ [mM]	$c(PO_4^{3-}{}_{Feed})$ [mM]	$c(PO_4^{3-}{}_{Harv.})$ [mM]	$Y_{NH4+/Glycerol}$ [$mol_N\ mol_{gly.}^{-1}$]	$Y_{PO43-/Glycerol}$ [$mol_P\ mol_{gly}$]	$c(formate,_{Feed})$ [mg L^{-1}]	$c(formate,_{Harvest})$ [mg L^{-1}]
2.1	19.75	10.87	1.11	0.76	0.426	0.017	314	7.44
2.2	19.90	12.86	0.07	0	0.340	0.003	323	8.15
2.3	4.63	0	1.02	0.30	0.199	0.031	359	8.11
2.4	4.69	0	0.07	0	0.219	0.004	322	8.52
2.5	9.65	1.28	0.34	0	0.412	0.017	322	5.89
2.6	8.72	0.92	0.37	0	0.390	0.019	325	6.40
2.7	9.75	0.52	0.35	0	0.434	0.009	321	6.66

Based on the results, the hypothesis of a significant influence of the consumed ammonium and phosphate on $c(formate_{Harvest})$ was tested by performing a multiple linear regression (MLR). To do so, the predictor variables $Y_{NH4+/glycerol}$ and $Y_{PO43-/glycerol}$ were applied for the target variable $c(formate_{Harvest})$. Among the tested predictor variables, only $Y_{NH4+/glycerol}$ showed a significant effect in the model for the $c(formate_{Harvest})$. Therefore, a second linear regression with $Y_{NH4+/glycerol}$ as the only predictor variable and $c(formate_{Harvest})$ as target variable was performed.

The results indicated a linear correlation between $Y_{NH4+/glycerol}$ and c(formate$_{Harvest}$) (Supplementary Figure S1), as the p-value was below 0.05 (p-value = 0.044). However, the results also showed that residual formate concentrations for all experiments were below 10 mg L^{-1}, which equals a theoretical TOC of less than 3 mg L^{-1}, which is below the specification level of 10 ppm required for chlor-alkali processes [7]:

$$TOC_{theor.} = \frac{M_{C-atom}}{M_{formate}} * c_{formate} = \frac{12\ g\ mol^{-1}}{46.03\ g\ mol^{-1}} * 10\ mg\ L^{-1} = 2.61\ mg\ L^{-1}$$

Hence, a feeding strategy for ammonium and phosphate was successfully applied in a way that both substrates are consumed completely to ensure less downstream efforts before a chlor-alkali-electrolysis step. Such a strategy could e.g., be linked to the glycerol consumption rate and thus to the glycerol feeding strategy. It should be mentioned that ammonium concentrations in the feed should not exceed the ratio of 0.4 mol_N mol_{gly}^{-1}, in order to avoid residual ammonium. A preferable ratio for ammonium to glycerol would be in the range of 0.20-0.22 mol_N mol_{gly}^{-1}, which ensures nitrogen-limiting conditions and reduces cost for ammonium supplementation. As no effect for the consumption of phosphate on the formate degradation could be observed, a ratio of the phosphate addition based on the glycerol consumption was proposed to be 0.003 – 0.004 mol_P mol_{gly}^{-1}.

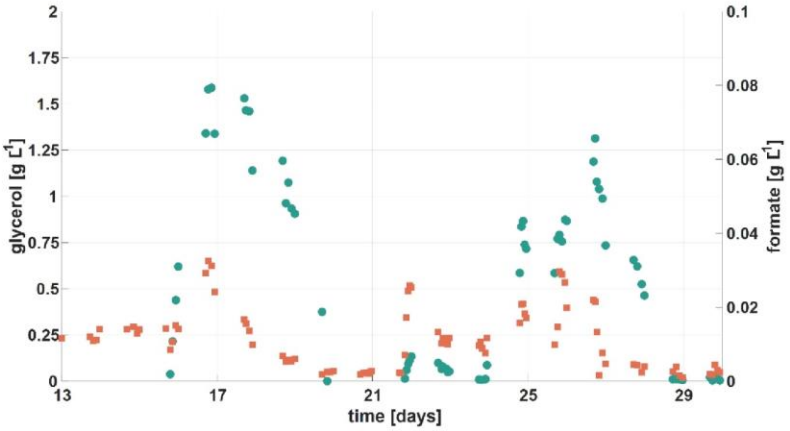
Nutrient-limiting conditions frequently occur in natural habits, like marine ecosystems and include co-limitations of N and P [39, 40]. For instance, co-limitations of Zn/C, Ni/N, or Fe/light were reported in bioprocesses with phytoplankton biomass [41-44]. So far, dual-nutrient limitations of the carbon, nitrogen, and/or phosphate source are known for fed-batch and continuous cultivations [45-51]. Nutrient limitations are mostly applied for biological production processes of secondary metabolites like polyhydroxybutyrate or polyhydroxyalkanoates [51-53]. However, an effect of dual- or triple-nutrient limitations on degradation efficiencies in biological residual water treatment processes has not yet been investigated.

The results show that a robust continuous cultivation with a halophilic microbial system and an efficient degradation of organic contaminants in a RPB is successful at triple limiting conditions for the nutrients glycerol, ammonium, and phosphate. The limitation of these compounds did not decrease the growth rate or significantly reduce the degradation efficiency. This allows not only the reduction of operational costs, but also ensures to meet specifications of additional substances in brines used for membrane-based chlor-alkali-electrolysis.

3.2.2. Glycerol

In addition to ammonium and phosphate, glycerol was added to each RPB feed as the main growth substrate. During cultivation 1, it was shown that a suitable feeding strategy for glycerol is crucial. Overfeeding of glycerol resulted in decreased degrada-tion of formate, indicated by an increased c(formateHarvest) and residual glycerol in the harvest (see Figure 6). Furthermore, other studies showed that carbon-limited condi-tions are beneficial

for the simultaneous utilization of a growth substrate and other organic compounds [54, 55].



Days	Glycerol Feed [g L^{-1}]	Formate Feed [g L^{-1}]
13-17	0.58	0.18
17-21	4.16	0.20
21-23	0.58	0.19
23-25	1.44	0.23
25-28	2.50	0.32
28-30	2.45	0.23

Figure 6. Process performance for cultivation 1 during the second experimental phase. Concentration of glycerol (●) and formate (■) in harvest samples; the table give information about the corresponding feed concentrations [16].

Therefore, data from cultivation experiments 2 and 3 were analyzed for a potential influence of glycerol feeding on formate degradation. Indeed, the results of the cultivation experiments indicated a correlation between the addition of glycerol and the formate degradation efficiency as well as c(formate$_{Harvest}$) (see Table 5).

Table 5. Overview of parameters used for glycerol feeding experiments. RPB denotes for the origin of the used residual process brine and $q_{S,gly}$ for the specific glycerol uptake rate. Feed concentrations of glycerol, ammonium and phosphate are denoted $c(Gly._{Feed})$, $c(NH_4^{+}{}_{Feed})$, and $c(PO_4^{3-}{}_{Feed})$.

Exp.	RPB	D [h^{-1}]	R [-]	$q_{S,gly}$ [g L^{-1} h^{-1} OD_{600}^{-1}]	$c(Gly._{Feed})$ [g L^{-1}]	$c(NH_4^{+}{}_{Feed})$ [mmol L^{-1}]	$c(PO_4^{3-}{}_{Feed})$ [mmol L^{-1}]
2.8	1	0.095	0.800	17.0 x 10^{-3}	3.74	5.10	0.36
2.9	1	0.095	0.800	15.9 x 10^{-3}	1.79	4.90	0.29
3.1	1	0.088	0.917	8.7 x 10^{-3}	2.07	5.23	0.33
3.2	1	0.088	0.980	2.1 x 10^{-3}	0.30	0.73	0.05
3.3	1	0.088	0.965	5.2 x 10^{-3}	0.57	1.52	0.10
3.4	2	0.088	0.965	7.7 x 10^{-3}	0.58	1.41	0.10
3.5	3	0.088	0.965	5.4 x 10^{-3}	0.59	1.54	0.10

An optimal control space for the specific glycerol uptake rate could therefore be located at 8.0-16.0 x 10^{-3} g L^{-1} h^{-1} OD_{600}^{-1}, where the lowest $c(formate_{Harvest})$ (~10 mg L^{-1}) was reached (see Figure 7). Similar values for the specific glycerol uptake rates were tested in other studies (10.2 x 10^{-3} g L^{-1} h^{-1} OD_{600}^{-1} [13] and 11.6 x 10^{-3} g L^{-1} h^{-1} OD_{600}^{-1} [56]), using *H. mediterranei*. However, in these studies, no effect on the influence of the specific glycerol uptake rate on $c(formate_{Harvest})$ was reported. Specific glycerol uptake rates below 8.0 x 10^{-3} g L^{-1} h^{-1} OD_{600}^{-1} led to an increase of $c(formate_{Harvest})$. This is also in line with other studies, which investigated low growth rates of bacteria in retentostats and observed co-utilization of substrates which are not co-utilized during carbon excess [54-57]. In contrast, higher specific glycerol uptake rates could not increase the formate degradation but would result in higher process costs due to a higher glycerol consumption. Moreover, it was shown in cultivation 1, that an excess of the carbon source glycerol led to decreased formate degradation.

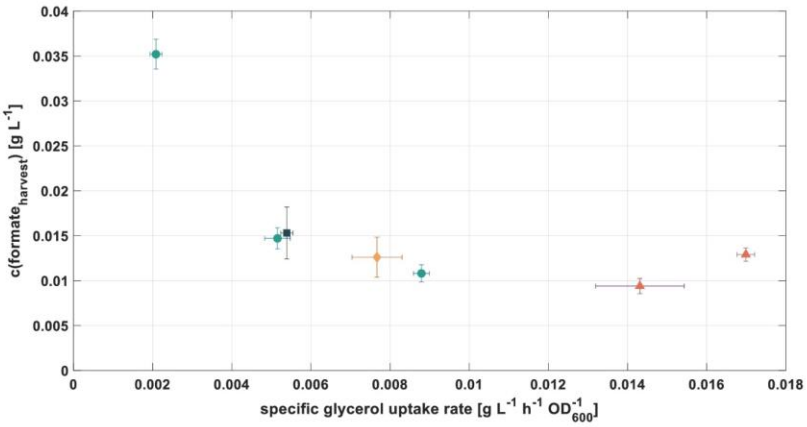


Figure 7. Influence of glycerol on process performance. Correlation of the specific glycerol uptake rate and the $c(formate_{Harvest})$. (●: cultivation 3, RPB 1; ♦: cultivation 3, RPB 2; ■: cultivation 3, RPB 3; ▲: cultivation 2, RPB 1).

3.4. Defining optimized operating conditions

To define optimized operating conditions for a biological treatment process of RPB from MDA production, critical process parameters, which influence the process performance were identified. Among those variables, the specific glycerol uptake rate $q_{S,gly}$ and the consumption yield of ammonium to glycerol $Y_{NH4+/glycerol}$ influenced the residual formate concentration c(formate$_{Harvest}$), whereas the aniline feed concentration was shown to have an influence on the intermediate accumulation.

Besides, several other process variables were tested and found to have no influence on any process performance variable. The dilution rate *D* (Equation 3) was varied from 0.036 to 0.2 h^{-1} in cultivation experiment 1. No influence of the dilution rate on the process performance could be observed. A process can therefore be operated at least with a dilution rate of $\boldsymbol{D = 0.2\ h^{-1}}$, which corresponds to a hydraulic residence time (HRT) of 5 h. In other biological processes for the reduction of organic content in wastewater, HRT values of 3-24 h were applied [13, 33, 58-60]. Hence, during this study, a desirable high throughput of RPB was successfully applied. However, in another study, a dilution rate of D = 0.37 h^{-1} (HRT = 2.7 h) was already shown to be successful for treating RPB from MDA production with *H. mediterranei* [61]. Thus, the usage of even higher dilution rates should be investigated in future. Nevertheless, the dilution rate also impacts the glycerol feeding, which was found to be influential for the formate degradation efficiency. It was shown, that with a specific glycerol uptake rate of 8.0-16.0 x 10^{-3} g L^{-1} h^{-1} OD_{600}^{-1} the highest formate degradation was achieved.

Furthermore, the retention rate *R* (Equation 4) was found to have no influence on the process performance variables and was tested at ranges from 0.70-0.98. A high value of $\boldsymbol{R = 0.98}$ is beneficial for the process operation costs, as less, yet unused, waste streams (bleed) are generated. Another beneficial impact of high retention rates is, that a cell wash-out can be prevented at high dilution rates and low glycerol concentrations in the feed. In general, the usage of a retention system for the degradation of organic compounds in RPBs can be seen beneficial, as it allows the application of higher dilution rates D for bioprocesses using slow growing microorganisms like extremophiles [62, 63]. Moreover, other studies showed, that at low growth rates achieved in carbon-limited retentostat cultures, bacteria prone to co-utilize substrates which are not co-utilized during carbon excess conditions including aromatic compounds like benzoate [56, 57].

Moreover, it was shown that the limitation of ammonium and phosphate due to reduced addition on the RPB feed did not negatively affect the process performance. Also, no negative influence on the process performance was observed when the concentration of Mg^{2+} and Ca^{2+} ions (in form of $MgCl_2$ x 6 H_2O, $MgSO_4$ x 7 H_2O, and $CaCl_2$ x 2 H_2O) was reduced to 10% of the original level (data not shown). Usually, magnesium and calcium ions can be removed by using ion-exchange membranes [10]. Nevertheless, a reduction of added magnesium and calcium salts to the growth medium would not only lower operational costs of the bioprocess, but also reduce the efforts for brine pre-treatment for the chlor-alkali electrolysis. During this study it was also achieved to remove $MgCl_2$ completely and supply magnesium only by addition of $MgSO_4$. Based on the results of this study, potential process parameter ranges for optimized process operating conditions were proposed (Table 6).

Table 6. Optimized operating conditions of process parameters for a control strategy.

Parameter	Control parameter	Proposed parameter values
Specific glycerol uptake rate [g L^{-1} h^{-1} OD_{600}^{-1}]	-	8.0 – 16.0 x 10^{-3}
Ammonium (NH_4^+) conc. feed	Ratio to glycerol [mol_N mol_{gly}^{-1}]	0.20 – 0.22
Phosphate (PO_4^{3-}) conc. feed	Ratio to glycerol [mol_P mol_{gly}^{-1}]	0.003 – 0.004
Magnesium (Mg^{2+}) conc. feed	Feed concentration for: $MgSO_4$ x 7 H_2O [g L^{-1}]	0.26 (equals 2.16 mmol L^{-1})
Calcium (Ca^{2+}) conc. feed	Feed concentration for: $CaCl_2$ x 2 H_2O [g L^{-1}]	0.055 (equals 0.51 mmol L^{-1})
Dilution rate D [h^{-1}]	-	0.2
Retention rate R [-]	-	0.98
NaCl [g L^{-1}]	-	50 – 100

4. Conclusion

In this study the influence of critical process parameters (bioprocessing parameters, media components, and raw material attributes) on the degradation efficiency of organic impurities in a biological treatment process was investigated. For the industrial integration of the biological treatment process, optimized operating conditions of process parameters was proposed, in which a cost-effective process control at high degradation efficiencies is enabled. It could be demonstrated that the aromatic impurities of the RPB (aniline, phenol, and MDA) were degraded with high efficiencies of 100%. However, during the cultivation experiments it was observed that high aniline concentrations in residual process brine feeds, resulted in an increased accumulation of potential intermediate substances. Therefore, in a next step, the intermediate substances and thus, the potential degradation pathway should be identified. Additionally, the main organic impurity formate was degraded with an efficiency of 89 – 98 %. Moreover, it was shown for the first time, that a triple limitation of the carbon, nitrogen, and phosphorus source could successfully be implemented while, at the same time, maintaining high levels of degradation for organic contaminants. The successful triple limitation does not only lower operational costs but also reduces further downstream efforts, especially in the case of nitrogen species. In course of this study, furthermore, an influence of the ammonium consumption yield and the co-substrate glycerol on the degradation of formate was discovered. In this course, it was shown that higher consumption yields of ammonium to glycerol $Y_{NH4+/glycerol}$ led to a slight increase of residual formate concentrations. Therefore, further research on the influence of ammonium on the formate degradation pathway could be beneficial for an increased process performance. Likewise, an excess of glycerol led to a decrease of the formate degradation. Additionally, it was found that specific glycerol uptake rate outside the range of 8.0-16.0 x 10^{-3} g L^{-1} h^{-1} OD_{600}^{-1} increased the residual formate concentration. In conclusion, this study shows the robustness of a biological system to continuously treat MDA residual process

brine under various process conditions. Optimized operating conditions were proposed, which support the cost-effective control of the biological treatment process, while maintaining high degradation efficiencies. Ultimately, the use of triple nutrient-limiting conditions shows the potential to improve the cost-effectiveness biological treatment or production processes towards an industrial scope. Further investigations should be performed regarding higher liquid dilution rates to increase productivity. Moreover, researching further influences on aromatic degradation and the nitrogen uptake would be beneficial for the process knowledge.

5. Patents

Covestro AG has filed patent applications Wo 2018/130510 A1, Wo 2018/037081 A1, EP18160929, WO 2019/121199 A1, 2020P30051WO comprising results of this study.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Results of linear regression with $Y_{NH4+/glycerol}$ as predictor variable and c(formate$_{Harvest}$) as target variable.

Author Contributions: Conceptualization, T.M, and S.P.; methodology, T.M, and S.P.; validation, T.M, and S.P.; formal analysis, T.M.; writing—original draft preparation, T.M, S.P.; writing—review and editing, T.M, S.P, and C.H.; visualization, T.M.; supervision, S.P. C.H.; project administration, C.H.; funding acquisition, C.H. All authors have read and agreed to the published version of the manuscript.

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Supplementary file

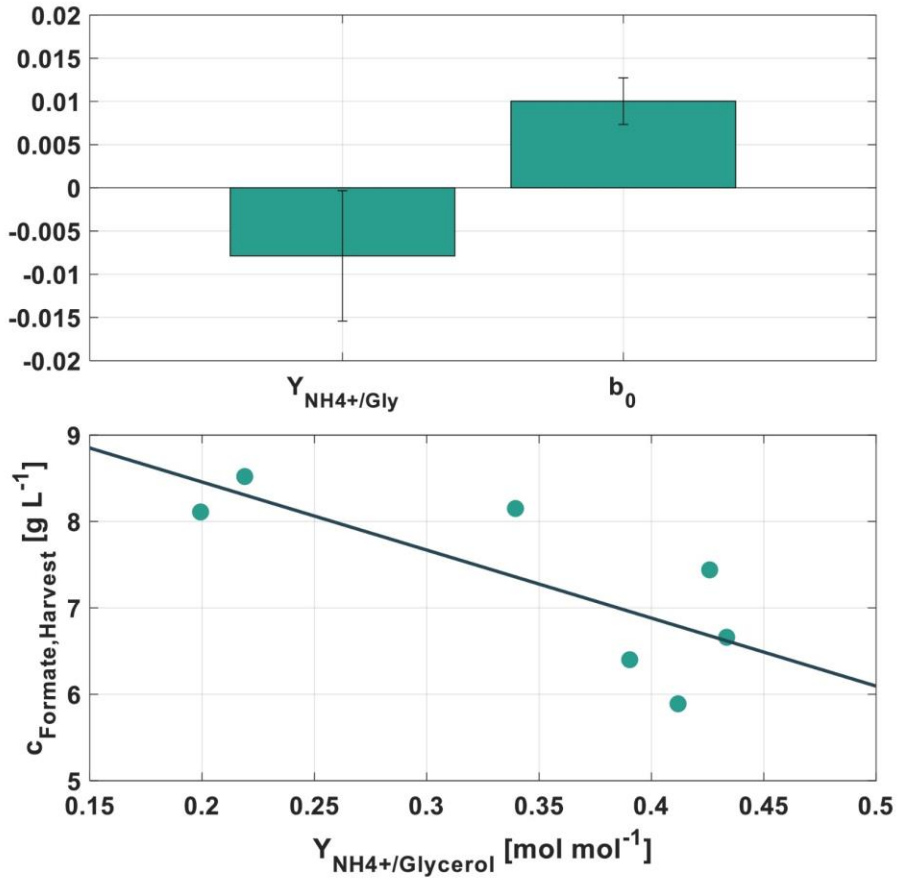

Figure S2. Results of linear regression with $Y_{NH4+/glycerol}$ as predictor variable and c(formate$_{Harvest}$) as target variable. A) Regression coefficients for and β_0 with error bars indicating the 95% significance interval. B) •: $Y_{NH4+/glycerol}$; ─: fitted linear regression model.

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6 Appendix

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