Universität für Bodenkultur Wien

# Economic and ecological assessment of clean water for biopharmaceuticals

Dissertation zur Erlangung des Doktorgrades an der Universität für Bodenkultur Wien

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Ich erkläre eidesstattlich, dass ich die Arbeit selbständig angefertigt habe. Es wurden keine anderen als die angegebenen Hilfsmittel benutzt. Die aus fremden Quellen direkt oder indirekt übernommenen Formulierungen und Gedanken sind als solche kenntlich gemacht. Diese schriftliche Arbeit wurde noch an keiner Stelle vorgelegt.

Ort, Datum

Unterschrift Alessandro Cataldo

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# Abstract

Water consumption in biopharmaceutical manufacturing is one of the most important parameters to assess the economic and ecological impact of a process. Currently, only total water consumption is considered but the production of different classes of clean water is neglected. In 2018 the process mass intensity (PMI) metric was introduced to biopharmaceutical production, which quantifies all consumed resources in order to produce 1 kg of product. More than 90 % of the PMI is dedicated to water.

Since the PMI mainly quantifies the amount of water used in a process without considering carbon footprint a novel metric was developed, which converts the water related PMI to its $CO_2$ and to the energy consumption, the WAter Related Impact of ENergy (WARIEN). The WARIEN metric quantifies how much water related $CO_2$ is emitted by analyzing the entire flow path of water from tap to waste. In this work, the electric energy consumption and thermal energy consumption for steam generation is quantified for the production of different classes of clean water.

Multiple process scenarios were evaluated and all water production related cost parameters were examined to enable a holistic overview of process design considering equipment occupancy, available floor space and scaling effects. In a further analysis we proved that the production of pure Protein A can also be optimized in order to reduce the overall footprint. However, this decrease cannot be displayed by the PMI of the antibody process. The use of the WARIEN metric further revealed that the costs of clean water have no significant impact on process costs. However, from an ecological perspective it can be used as design criterion for process development. The WARIEN and its new connection to energy consumption and $CO_2$ emission for pharmaceutical process water is the first step towards a full life cycle assessment of pharmaceutical products and need to be supplemented by data for other raw materials than water in future research.

# Zusammenfassung

Der Wasserverbrauch in der biopharmazeutischen Produktion ist einer der wichtigsten Parameter zur Beurteilung ökonomischer und ökologischer Auswirkungen eines Prozesses. Derzeit wird nur der Gesamtwasserverbrauch betrachtet, aber die Produktion der verschiedenen Klassen von sauberem Wasser wird vernachlässigt. Im Jahr 2018 wurde die „Process Mass Intensity“ (PMI)-Kennzahl für die biopharmazeutische Produktion eingeführt, die alle verbrauchten Ressourcen quantifiziert, um 1 kg Produkt herzustellen. Mehr als 90 % des PMIs entfallen auf Wasser.

Da der PMI hauptsächlich die in einem Prozess verbrauchte Wassermenge quantifiziert, ohne die $CO_2$ Emissionen zu berücksichtigen, wurde eine neuartige Kennzahl entwickelt, die den wasserbezogenen PMI in seinen $CO_2$- und Energieverbrauch umrechnet, der „WAter Related Impact of ENergy“ (WARIEN). Die WARIEN-Kennzahl stellt dar, wie viel wasserbezogenes $CO_2$ emittiert wird, indem der gesamte Fließweg des Wassers von der Quelle bis zum Ausguss analysiert wird. In dieser Arbeit wird der elektrische und der thermische Energieverbrauch für die Produktion von verschiedenen Reinwasserklassen quantifiziert.

Es wurden mehrere Prozessszenarien bewertet und alle wasserbezogenen Kostenparameter untersucht, um einen ganzheitlichen Überblick über das Prozessdesign zu ermöglichen. In einer weiteren Analyse konnten wir nachweisen, dass auch die Produktion von reinem Protein A optimiert werden kann, um den gesamten Fußabdruck zu reduzieren. Diese Verringerung kann jedoch nicht durch den PMI des Antikörperprozesses dargestellt werden. Die WARIEN-Evaluierung zeigte außerdem, dass die Reinwasserkosten keinen signifikanten Einfluss auf die Prozesskosten haben. Aus ökologischer Sicht kann sie jedoch als Designkriterium für die Prozessentwicklung verwendet werden. Der WARIEN ist der erste Schritt in Richtung einer vollständigen Ökobilanz pharmazeutischer Produkte und muss in zukünftigen Forschungen durch Daten für andere Rohstoffe als Wasser ergänzt werden.

# Table of Contents

# 1 Introduction

The evaluation of consumed water in production chains is a frequently applied method of ecological and cost modelling. For instance, producing 1 kg of beef meat consumes about 15,000 L of water including many parts of the production chain such as feed production, growing cattle with nutrients, meat processing and feed production [1]. For biopharmaceutical production the principle of water consumption connected to the final product can also be used as design criterion of potential process improvement. However, the actual manufacturing processes of such products are more complex and more sophisticated compared to meat production. The quality of water used in biopharmaceutical production is strictly regulated by the according Pharmacopoeia of each particular region worldwide [2]–[5]. In that manner, water which is used for parenteral application such as for pharmaceuticals must comply to specific quality attributes in order to be used as "Water for injection" (WFI) [3]. With the definition of WFI, hazardous contaminations such as bacterial endotoxins and total organic carbon (TOC) caused by inappropriate water quality must be reduced below strict thresholds [6].

Recycling process water is an integral part of the chemical production of pharmaceuticals or conventional food preparation to significantly reduce production costs since the recovery effort is less cost intensive than fresh water. Depending on the efficacy and the specification of the process, lower costs and environmental goals can be combined. Although buffer as well as media recycling concepts have been developed for biopharmaceutical production processes, those developments have not been applied yet [7]. The recovery of WFI requires not only additional space for storage, but its endotoxin and TOC levels no longer comply the regulatory standards after being in contact with biologic process material, because waste streams in biotech are only heat inactivated and no efficient buffer recycling is available [8]. Since buffer recycling currently is not profitable with the biopharmaceutical production setting, a different methodology needs to be developed in order to evaluate whether reduced water consumption and process costs can be combined for biopharmaceutical production processes.

In the following thesis potential challenges of water related evaluation were categorized into process related water reduction and overall evaluation of the WFI production train considering both economic along with ecological aspects. Therefore, industrial relevant data provided by several biomanufacturers, its suppliers and plant constructors were collected and evaluated. Furthermore, the impact of water consumption was linked to energy consumption, $CO_2$ emission and plastic waste. The gathered data can further assist future academic as well as industrial evaluation applications.

## 1.1 Economic modelling for industrial processes

Overall positive economic cash flows are essential for the design of a successful manufacturing process. Therefore, cost models need to be developed alongside of the process development of the process itself [9]. Performing an economic process evaluation two major models can be applied, namely a greenfield analysis or an evaluation of an existing facility. A greenfield analysis is typically applied used in early development stages assuming that the entire facility needs to be built on a green field. Therefore, capital expenses play a decisive role for the evaluation and the overall project lifetime or campaign length need to be considered as well as any potentially optimized process design. For greenfield analysis the process scale is based on the desired annual product output. In the case of economic evaluations of existing facilities the impact of capital expenses is reduced compared to a greenfield analysis, because the facility might be already depreciated or the facility is capable of manufacturing multiple different products in different campaigns [10]–[13].

At early stages of process development a significant number of assumptions needs to be made. By using the correct evaluation methods and models the most impactful parameters can be identified and the priorities during development can be specified. By using commercially available modelling software or approved in-house cost models the potential process can be simulated accurately. Besides sensitivity analysis tools, such as Monte-Carlo simulation the commercially available software BioSolve, developed by BioPharm Services, provides an industrial relevant database and several platform biomanufacturing processes [12], [14], [15]. With BioSolve a holistic evaluation of an entire manufacturing site can performed considering all upcoming cost parameters. For capital expenses construction, engineering, floor space, equipment and clean room associated costs are considered. For operational costs all relevant consumables, materials, labor, and several miscellaneous costs are considered as well. With the launch of BioSolve v7.5 the software enabled further sensitivity analysis, where several combinations of varying cost parameters can be evaluated individually.

For an economic modelling different approaches, such as developing neural networks or applying empiric models can be performed in order to evaluate a process. The present thesis focuses on empiric data generated in lab together with manufacturing scales. Therefore, a significant amount of assumptions is required. For each relevant cost parameter, the assumption can be made based on four different sources, which are sorted in the following list by their precision from most to least:

(1) Direct process data, (2) data available in the BioSolve database, (3) quote of an independent supplier, (4) predictions based on representative experiences.

At the beginning of a project the designed model is mainly filled with predictions based on representative experiences. With evolvement of the project, these assumptions are thoroughly exchanged by more precise data.

## 1.2 Ecological modelling for industrial processes

The ecological impact of a process can be assessed by several process characteristics, such as overall $CO_2$ emission, resource consumption or waste treatment. Thus, several metrics can be applied according to Figure 1.

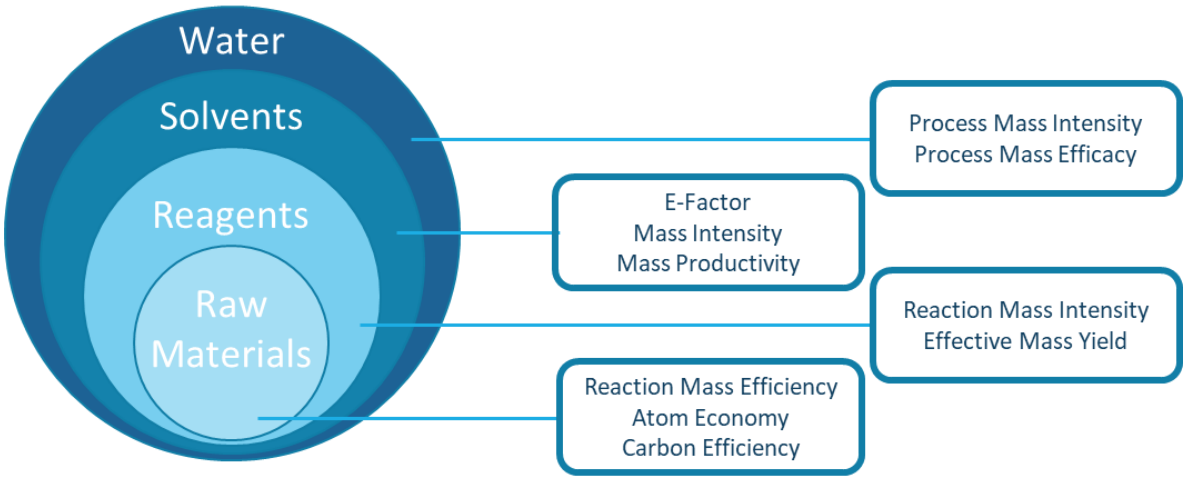


***Figure 1: Ecological metrics and their considered resources as proposed in "greenchemuoft" [16]***

All shown metrics evaluate different types of material for chemical production and visualize various perspectives of the environmental footprint, regardless of the product. Nevertheless, the implementation of those metrics has not yet been established for biopharmaceutical production processes. In general, operational and strategic decisions are not dependent on ecological parameters, because costs and environmental factors showed no proven correlative behavior so far [17]. In the following chapters a variety of parameters are described, which are relevant for ecological modeling of biopharmaceutical production processes.

### 1.2.1 Footprint and ventilation

High shares of overall carbon footprint of the biopharmaceutical production are not related directly to the process itself but to its environment. For instance, lots of energy is consumed by maintaining a clean environment in the manufacturing hall. In biomanufacturing different classes of clean room qualities are specified by norms. According to Table 1 the shown clean

room classes have different requirements regarding the amount of particles per $m^3$ internal volume of the room [18]–[20].

***Table 1: Cleanroom classifications according to ISO 14644-1 [21], [22]***

| **Clean Room Class** | | **Permitted Particles per $m^3$ in Operation (≥0.5 µm)** | **Air changes per hour** |
|---|---|---|---|
| Class | ISO | | |
| A | Class 5 | 3520 | 240-480 |
| B | Class 5 | 352,000 | 240-480 |
| C | Class 7 | 3,520,000 | 60-90 |
| D | Class 8 | Not defined | 5-48 |

These high standards are constantly controlled and require high efforts of air ventilation, proper room temperatures and pressure differences in order to enable a contained process. In the latest stages of production, such as product formulation the cleanest clean room classes B for personnel and A for the product containing isolators are necessary. For up- and downstream processing usually clean room classes C or D are applied [23]. Universal floor space, such as offices without air lock is classified as class U. The energy consumption per $m^2$ floor space in a clean room can be up to 25 times higher compared to a U-classified room [19]. The overall aeration of a clean room is organized by heating, ventilation and air-conditioning (HVAC) systems, which must comply to GMP requirements. Therefore, room temperature, humidity, pressure, air flow and air exchange rates need to be considered as displayed in Figure 2 [24]. In order to minimize the ecological footprint of a potential facility the entire clean room and HVAC system needs to be properly designed before it is constructed. Hence, an efficient process layout is essential for designing a more environmentally friendly facility at early stages. Reducing the floor space can significantly lower the energy consumption and hence $CO_2$ emission. In theory the required floor space for biomanufacturing can be minimized by switching from batch to continuous operation [25], [26]. Consequently, the equipment scales can be decreased while equipment occupancy of the entire facility can be increased for a more efficient scheduling [12]. The modelling software BioSolve takes clean room specific costs and environmental footprint into account by implementing reference values. Those numbers are regularly updated and based on industrial processes provided by the BioSolve community [27]. That type of process assessment demonstrates that an overall process evaluation is not only dependent on the process itself, but also on efficient utilization of its environment.

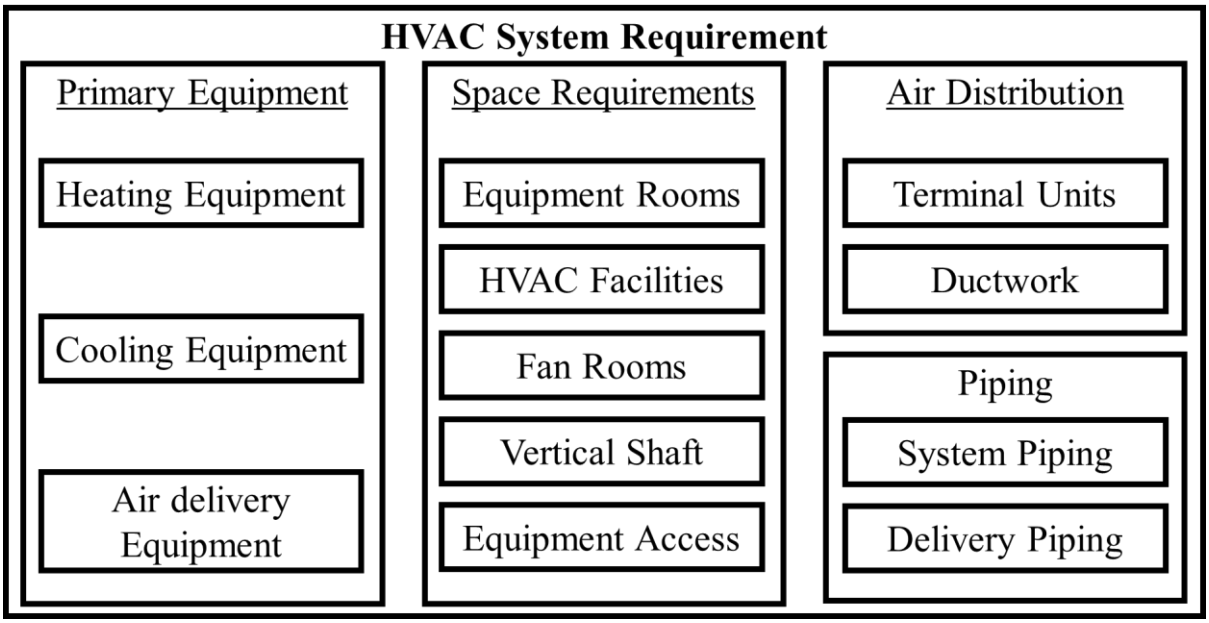


***Figure 2: Overview of HVAC system requirements in a clean room as proposed by Seyam [24]***

## 1.2.2 Water consumption

For biomanufacturing following challenges need to be considered in terms of water consumption evaluation: (1) water might not be recycled due to the contact of biological material and (2) water used in a process as well as for cleaning must comply with the regulatory standards. In this regard the prevention of contaminations has the highest priority. Thus, the water requires to be purified in a normalized process in order to be called “purified water” (PW) or “water for injection” (WFI). WFI and PW are regulated terms, which imply that specific unit operations have to be carried out for its production [2], [3].

The conventional production process can be seen in Figure 3. Until 2017 distillation of PW was mandatory for the production of WFI in Europe. In that year, the European Pharmacopoeia approved the utilization of membrane-based unit operations to convert PW into WFI [3]. For further discussion I will refer for distillation-based WFI production to “Hot WFI” and for membrane-based WFI to “Cold WFI”.

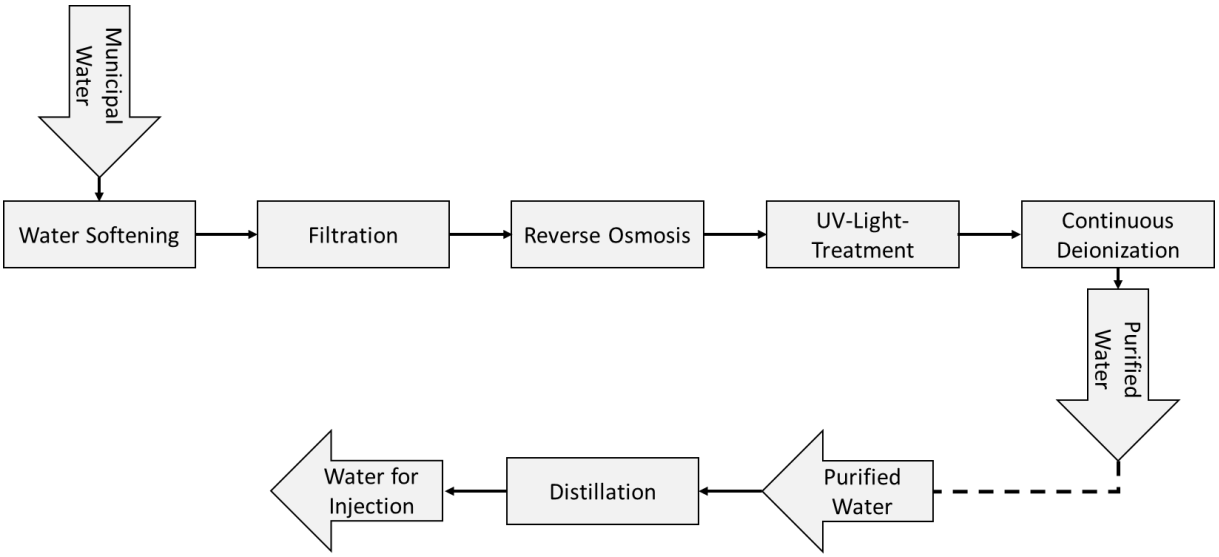


***Figure 3: Conventional WFI production train as proposed by Budzinski et al. [28]***

The production scheme of Figure 3 is originated in a manuscript titled "Introduction of a process mass intensity for biologics" developed and written by Budzinski et al. [28]. The authors are members of the ACS Green Chemistry Institute Pharmaceutical Roundtable (ACS GCI PR). The roundtable consists of a consortium of global acting biomanufacturers aiming on a more sustainable production of pharmaceuticals. In the stated manuscript the process mass intensity (PMI) metric, which is also outlined in Figure 1 was analyzed for the first time in a bioprocess production setting. The PMI quantifies the amount of resources consumed in order to produce 1 kg of product by taking into account the weight of each resource used in the production (See Eq. (1)).

$$Total\ PMI = \frac{total\ water, raw\ materials, consumables\ used\ in\ process\ (kg)}{active\ pharmaceutical\ ingredient(kg)} \quad \text{Eq. (1)}$$

In Figure 4 a detailed analysis of the PMI composition of a biopharmaceutical is visualized. Since only the weight of each resource is considered, 1 kg of a plastic or a powdered material has the same impact than a single kg of water towards the PMI determination. According to the calculation more than 90 % of the PMI is covered due to water consumption [28], [29]. Hence, the PMI enables a water related evaluation of a bioprocess while the footprint of all materials and consumables is not representative since only its weight is considered. Through the introduction of the PMI in biopharmaceutical production, ecological models can be based on an established metric applied in several other industries. However, the actual environmental impact of an increased water consumption is not defined by the PMI. When the PMI manuscript

by the ACS GCI PR was first initiated, the developers of the BioSolve software implemented the metric as a first approach of an ecological model, enabling further applications for the entire BioSolve community. It further empowered the combination of ecological as well as economic modeling in terms of water consumption.

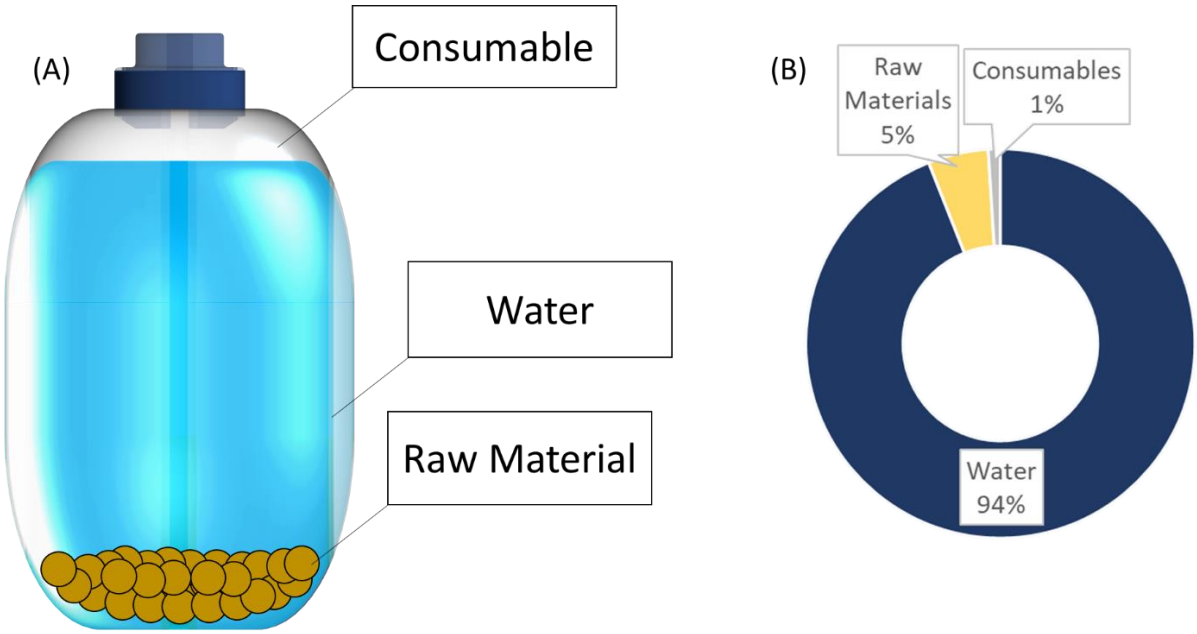


***Figure 4: (A) Considered parameters of the PMI; (B) Relative shares of PMI relevant parameters as proposed by Budzinski et al. [28]***

The PMI itself cannot show if reduced cost and water consumption are correlated, as cost of consumables and materials need to be considered for economic modelling as well. As previously described an entire process assessment is also dependent on its environment however several cost factors are related to water consumption. For instance, the scale of water production or media preparation shows a significant impact in terms of environmental reliance. Larger amounts of media can be operated either by increasing storage tank volume and hence increasing the overall footprint or by increasing the media preparation labor effort in order to produce more batches of media or buffer in the same devices. Both reactions lead to an increased effort. Additionally, several relevant cost parameters are linked to water consumption as well. Budzinski et al. [28] showed that high shares of required water for monoclonal antibody production are based on bind-and-elute chromatography steps, which are further explained in chapter 1.4 “Monoclonal antibody production”. However, the actual economic and ecological impact of focusing on water reduction for that specific step was not established by this work. The overall impact of water consumption also depends on the purity grade of the water. For PW and WFI production several WFI skid suppliers provided economic models of different WFI production methods [30]–[33]. To complicate matters further, accurate data is scarce, as

biomanufacturing companies can and do perform their own individual economic evaluation, but do not publicly disclose their findings. The same issues are present for the decontamination of the water [8]. Overall, within the production train of water not only process operators have to interact with the water, but also the responsible personnel for WFI production, buffer or media preparation, and decontamination [34]. For potential process optimization each department is able to contribute to reduced water consumption. Nevertheless, the exact impact of this reduction is not clearly defined and depends on the facility together with the process itself. Using single-use systems is a valid method of reducing total water consumption since less devices need to be cleaned. However, plastic waste increases as a tradeoff [35], [36].

### 1.2.3 Consumables

The importance of single-use systems is continuously rising for biopharmaceutical production due to the reduced contamination risk [37]. Single-use systems reduce the total effort of cleaning and hence water consumption as well as changeover time between two batches leading to higher productivity. Single-use bags, bioreactors, as well as filters or pre-packed chromatography columns are the most frequently used types of single-use systems [35], [36], [38], [39]. The suppliers of these systems are responsible for its validation and enables outsourcing of that part of the biomanufacturing process [40], [41]. Total effort of validation and the product specification of the single-use systems itself lead to an increased price of the consumable. In order to evaluate the environmental footprint of the consumables an overall life cycle assessment is required considering manufacturing, transport, disposal etc. [42]. Within the PMI only the weight of a consumable is considered however not its life cycle assessment. Thus, the PMI is not representative in regards of the environmental impact of consumables and novel methods need to be developed in order to evaluate the overall $CO_2$ emission of single-use systems.

In 2013 Pietrzykowski et al. [36] analyzed the environmental life cycle for a chosen monoclonal antibody production comparing specific single-use and stainless-steel scenarios. Within this analysis all consumables and skids were further inspected and their overall environmental impact considering their manufacturing, transport, handling and disposal. This manuscript impressively demonstrated that using single-use systems substantially outperform the utilization of stainless-steel devices in terms of ecological footprint. The environmental impact of cleaning and steaming all devices is significantly higher than gamma irradiating the plastics for sterilization and incinerating it for its disposal. Overall, the fermentation, Protein A chromatography and as already described the cleaning and steaming skid cover about 70 % of

the entire environmental footprint in this model. The findings of this manuscript serve as assistance for the performed ecological process evaluation and water assessment in this thesis.

## 1.3 Modelling challenges of industry and academia

For pharmaceuticals, the costs of a single product cannot be determined precisely due to clinical studies and the cost coverage of failed studies. The timeframe between the development of a pharmaceutical and the final drug approval takes multiple years and is split into different phases (see Figure 5). For each pharmaceutical the total costs of the entire study are in the range of billion $ within a timeframe of approximately 10 years from the development till registration With additional financial risk producing phase 3 material before phase 1 ends the time can be significantly shortened. However, this is only appropriate in exceptional circumstances such as a pandemic situation. In all other circumstances shortening the development with such measures can mean financial ruin for the company in case the drug fails [43]–[45].

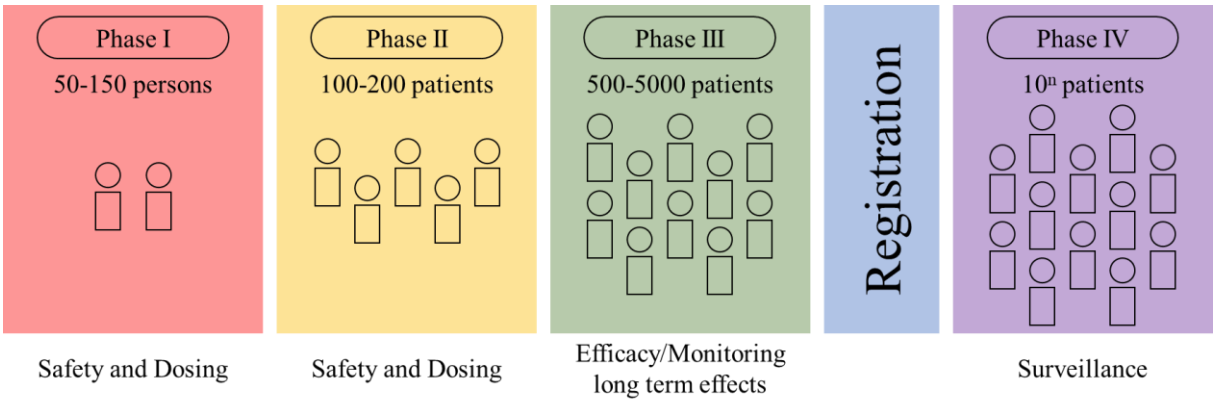


***Figure 5: Clinical trial phases - pharmaceutical approval [43], [46]***

Additionally, approximately 1 out of 10 pharmaceuticals is approved (i.e. it reaches Phase IV), while the rest fails throughout one of the trial phases [47]. In conclusion, one single pharmaceutical needs to cover the clinical trial costs of 10 pharmaceuticals on average in order to be profitable. Depending on the host cell of a biopharmaceutical, the final production scale, the individual preferences along with the manufacturing process are designed to cover the relevant demand of the market in the given facility, while the production costs are minimized [48]. During all clinical phases the required amount of product needs to be produced either in a small-scale facility or in cooperation with a contract manufacturer. By outsourcing of the process to a contract manufacturer no facility needs to be built for a potential product, which might fail in one of the clinical studies [49].

Once a biopharmaceutical is approved and the manufacturing facility fulfills the regulatory requirements, the designed process can be applied in order to produce the final product in large quantities. For the biomanufacturing industry an exact cost evaluation up to that point is challenging. For simulating all industrial relevant cost parameters, such as capital expenses for equipment and operational expenses for consumables, materials or labor need to be considered. The impact of each parameter can be evaluated during the production phase but due to the high complexity of the process the determination of the most impactful cost parameters represents an essential challenge for the industry.

Academic research institutions are struggling to perform industrial relevant process evaluation due to the lack of data [50]. Lots of experiments can only be performed in smaller scales. For instance, several bioprocess manuscripts describe a process, where cell disruption is performed via ultrasonication or similar methods. However, in larger scales mainly high pressure homogenization is applied in order to operate large scales of fermentation broth properly. Walther and Dürauer [51] compared the performance of each cell disruption method in microscale and concluded that using bead mills as small scale substitute for high pressure homogenization is the most suitable disruption technique for most cytoplasmic products expressed in *E. coli*. Performing an economic evaluation of a process as an academic institute without having information of an actual manufacturing process is even more complicated since the audience of this research topic is mainly from industrial companies. Though, publications written by industrial companies are often motivated by marketing strategies, some good and useful publications are available. As a company there is usually no intention to publish sensitive data in order to demonstrate how process design in a large scale can be optimized. In academic research projects together with industrial partners, economic models can be directly implemented into processes with significant industrial relevance. Publishing economic methods and findings in that setup have a more significant impact on the audience compared to manuscript purely written by an academic or industrial institution. Hence, those findings can be adapted and adjusted by others, even if no industrial process is directly involved. In 2019 an economic review provided by Yang et al. [52] listed several manuscripts dedicated on economic modelling for biopharmaceutical production between 2013 and 2018. Table 2 summarizes a list of those publications with slight modifications in order to reveal the usage of BioSolve for the particular evaluations.

In 2015 the EU funded project “NextBioPharm DSP” (http://nextbiopharmdsp.eu/) was launched. Project partners included renowned academic institutes, biomanufacturing companies and a supplier for single use material. During the project different methods of

monoclonal antibody production were compared, considering the implementation of single-use systems and continuous operation in relation to a conventional process at various production scales. The economic and ecological process assessment of the developed processes was also covered in a specific work package enabling the application of empiric PMI and cost models. The project was finished in 2019 and resulted in several publications [53]–[56]. After the fully integrated manufacturing platform suited for monoclonal antibodies was developed the available data of all working packages could be used for economic and ecological evaluation.

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*Table 2: List of previous economic publications for biopharmaceuticals according to Yang et al. [52]*

| Authors | Year | Authors affiliation | Content | BioSolve used? | Reference |
|---|---|---|---|---|---|
| Pollock et al. | 2013 | Academia + Industry | Fed-batch vs perfusion, cleaning | No | [57] |
| Pollock et al. | 2013 | Academia + Industry | Design Semi-continuous Periodic countercurrent chromatography (PCC) affinity for clinical and commercial manufacture | No | [58] |
| Liu et al. | 2013 | Academia | 5 days Cost-effective design (Batch) | No | [59] |
| Hammerschmidt et al. | 2014 | Academia + Industry | Batch vs continuous vs hybrid process (precipitation), cleaning | Yes | [60] |
| Xenopoulos | 2015 | Industry | Integrated batch vs continuous (multi-column chromatography) | Yes | [35] |
| Walther et al. | 2015 | Industry | Integrated batch vs continuous (multi-column chromatography) | Yes | [12] |
| Li and Venkatasu-bramanian | 2016 | Academia | Integrated batch process, focus on downstream | No | [61] |
| Klutz et al. | 2016 | Academia + Industry | Batch vs continuous (PCC) vs hybrid integrated process | No | [11] |
| Bunnak et al. | 2016 | Academia | Fed-Batch and perfusion | Yes | [62] |
| Torres-Acosta et al. | 2016 | Academia | Batch vs. batch with an aqueous two-phase system | Yes | [63] |
| Xu et al. | 2016 | Industry | Fed-batch vs perfusion vs concentrated fed-batch (media cost), cleaning | Yes | [64] |
| Liu et al. | 2016 | Academia | Integrated batch cost-effective optimization | No | [65] |
| Pollock et al. | 2017 | Academia + Industry | Batch vs semi-continuous vs hybrid integrated process | No | [26] |
| Grilo et al | 2017 | Academia | Batch process: Non-protein-A chromatographic platform vs protein A chromatographic platform | No | [66] |
| Arnold et al. | 2018 | Industry | Batch vs continuous integrated process (multi-column chromatography), cleaning | Yes | [67] |
| Hummel et. al. | 2018 | Industry | Stainless-steel batch vs single-use batch and single-use continuous downstream process, cleaning | Yes | [68] |

## 1.4 Monoclonal antibody production

The biotechnological platform process of monoclonal antibodies covers an essential share of the overall production of biopharmaceuticals. In 2020 about 50 % of all biopharmaceuticals approved in Germany have been monoclonal antibodies expressed in Chinese hamster ovary (CHO) cells and the market of monoclonal antibodies is constantly rising [69]–[71]. The production sequence of monoclonal antibodies can be seen in Figure 6. Additionally, the clean room classifications are shown according to chapter 1.2.1 “Footprint and ventilation” for each unit operation as well. In the present thesis not only the process sequence itself is analyzed but also all including process steps for GMP compliant water production. Furthermore, in Figure 6 the width of each unit operation box represents its water consumption in relation to the other unit operations [28], [29], [36].

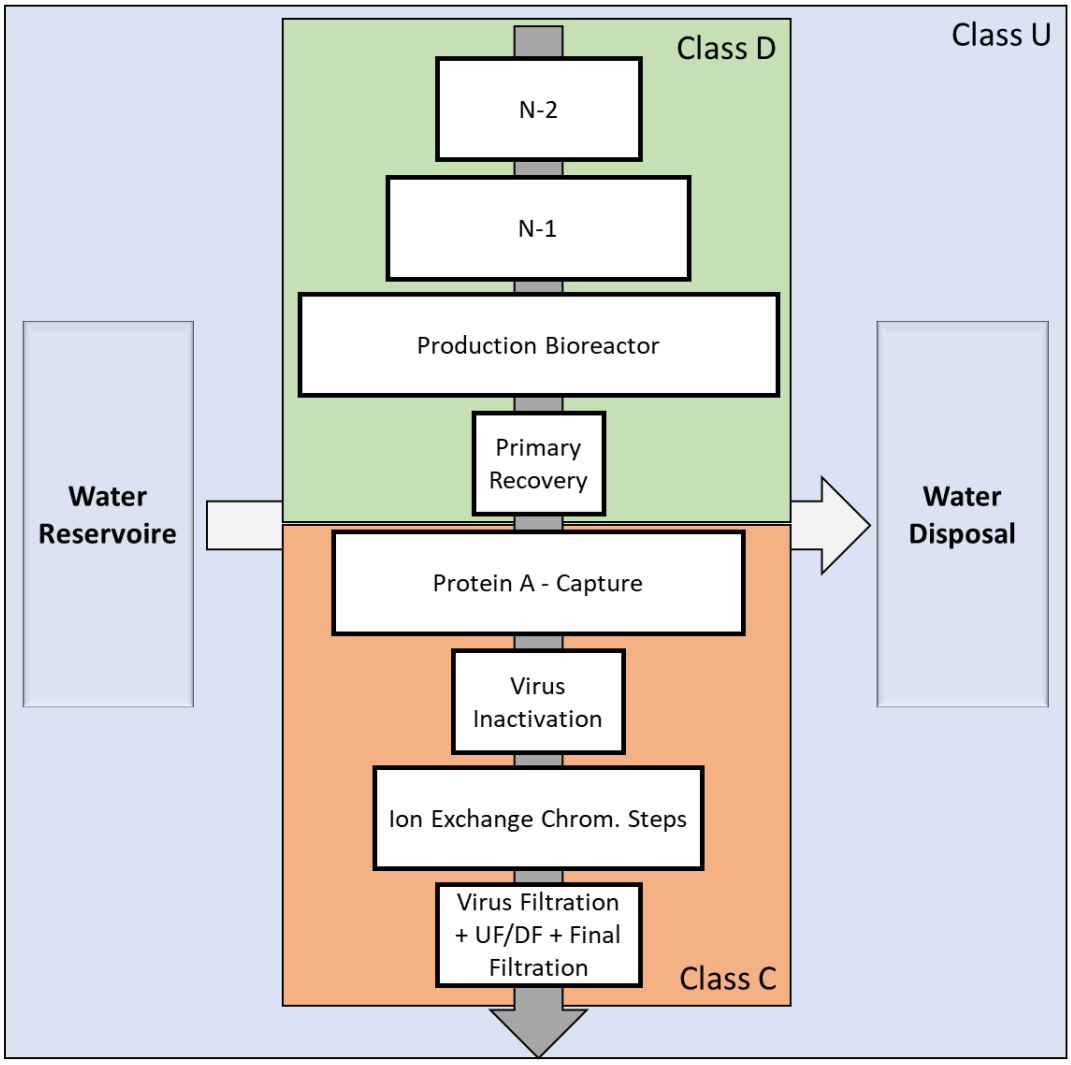


***Figure 6: Platform process for monoclonal antibody production and its clean room classes. The width of the white bars shows the amount of water consumed for each unit operation***

## 1.4.1 Characteristics of Protein A chromatography

The utilization of chromatography steps for capture and further purification of the final product is established in the majority of biopharmaceutical productions. The active pharmaceutical ingredient (API) can be purified by a variety of different chromatography columns based on its size, hydrophobicity, net charge or affinity. Protein A is originally appearing as surface protein of the bacteria *Staphylococcus aureus*. It is capable of binding specific IgG domains, which is essential for the purification of monoclonal antibodies [72]. In state-of-the-art commercially available protein A resins mostly the recombinant protein A produced in *Escherichia coli* or *Pichia pastoris* are used. Since the price of Protein A resins are significantly higher compared to other chromatography ligands, the production of Protein A plays a decisive role regarding the process economics of monoclonal antibodies. The production of Protein A itself is a biopharmaceutical process, as it is expressed in *E. coli* and conventionally secreted in the cytoplasm. For cytoplasmic proteins expressed in *E. coli* the cells need to be disrupted by homogenization in order to access the desired product. However, cell impurities, such as host cell proteins, host cell DNA, endotoxins or remaining cell debris are also released and must be removed accordingly [73], [74]. In 2020 Kastenhofer et al. [75] demonstrated that Protein A can be expressed extracellularly using a modified *E. coli* strain with an enhanced leakiness of the outer cell membrane. That strain can be utilized for a potential process innovation with significantly less impurities. By using the extracellular expression strategy, continuous perfusion fermentation can be applied and therefore simplifying the overall protein production compared to an intracellular product. Since homogenization becomes obsolete and thus leading to a reduced number of unit operations, the process economics will be positively impacted.

## 1.4.2 Challenges and bottlenecks

Using different product hosts for biomanufacturing leads to different production challenges. In earlier stages low product titers during fed-batch fermentation represented the bottleneck of the entire production. Within the last decades product titers of monoclonal antibodies expressed in CHO cells increased from a range of mg/L to multiple g/L nowadays [76]. State-of-the-art single-use bioreactors for monoclonal antibody production have a production volume of up to 2000 L. Within 14 days of fermentation, multiple 2000 L fermentations can be initiated every 2 days in staggered mode leading to similar fermentation output volumes than a single 15,000 L stainless-steel reactor. That strategy increases the occupancy of the downstream devices, while the overall footprint can be reduced compared to a single 15,000 L fed-batch fermentation

performed in a stainless-steel bioreactor. After reaching these milestones the economic bottleneck shifted towards the Protein A capture chromatography step [77].

Due to the performance of Protein A columns considering high binding capacity, efficacy and stability the number of unit operations is reduced. Hence, process complexity and process costs can be thoroughly reduced. However, bind-and-elute chromatography steps represent the biggest challenge among all downstream unit operations in terms of processing time, product yields and complexity. As mentioned before, Protein A is significantly more expensive than other ligands [78], [79]. Therefore, several strategies were developed within the last decade in order to optimize the Protein A step or substitute it entirely [80], [81]. For instance, Burgstaller et al. described a method using precipitation coupled with tangential flow filtration methods as potential substitute [54].

### 1.4.3 Disposables vs. reusables

In chapter 1.2.3 “Consumables” the benefits and challenges of using disposables were already described. For the production of monoclonal antibodies, the impact of the described bottlenecks described in chapter 1.4.2 “Challenges and bottlenecks” varies with different equipment utilized. Therefore, three essential unit operations were compared. In this work, for the fermentation process the economic and the ecological impact of multiple staggered single-use bioreactors are compared with a stainless-steel bioreactor, which is capable of producing the same amount of product. For primary recovery conventional stainless-steel centrifugation can be substituted by a two-stage filtration or a precipitation assisted depth filtration step, both performed in fully disposable filters [82]. For chromatography steps a sterile pre-packed column can be purchased compared to an in-house column packing and cleaning [83].

### 1.4.4 Batch vs. continuous operation

Besides utilizing single-use devices as described in chapter 1.4.3 “Disposables vs. reusables” continuous operation represents another substitution for conventional operation [37]. For antibodies expressed in CHO cells, mostly fed-batch is considered as upstream processing (USP) strategy by the industry. Within the last decade several approaches of using continuous perfusion fermentation have been evaluated based on its productivity and process economics as described in Table 2. By switching from batch to continuous fermentation the footprint of the facility is reduced, because smaller bioreactors running in continuous mode are capable of producing the same amount of product than a significantly larger bioreactor in fed-batch mode [84]–[86]. For downstream processing (DSP) unit operations the change from batch to

continuous is heavily dependent on the unit operations. As shown in Figure 6 the downstream process consists of several chromatography, filtration, virus inactivation and cell removal steps [87]. In order to specify a downstream process as fully continuous, the product needs to be processed into its final purity grade without interruption [88]. Therefore, breaks such as changing filters, regenerating the chromatography column or cleaning the disk stack centrifuge need to be avoided entirely. By using multiple alternated devices for each unit operation, the product flow remains constant, while one of each utilized devices is cleaned or exchanged. For conventional bind-and-elute chromatography no fully continuous operation can be performed [26], [89]. However, for ion exchange chromatography the product is purified based on its net charge the pH of the liquid. In that case the operation mode can be switched from bind-and-elute to flow-through mode by adjusting the pH of the liquid. Therefore, alternating columns used in flow-through mode approach enable a continuous product flow. However, Protein A chromatography must be operated in bind-and-elute mode according to chapter 1.4.1 "Characteristics of Protein A chromatography". Although no fully continuous flow can be achieved for bind-and-elute mode, a periodic system enables a significantly higher frequency of product output. By using periodic countercurrent chromatography (PCC) multiple columns in a smaller dimension are used simultaneously [90]. For instance, using three columns in the system enable a continuous load of column 1, while excessed product can be captured in column 2. In that period bound product in column 3 can be eluted and regenerated accordingly. After the binding capacity of column 1 is reached, the load is redirected to column 2 directly with column 3 for excessed product, while bound product is eluted from column 1. In that mode elution steps can be performed simultaneously with loading steps [90]–[93]. However, the product flow still is not fully continuous. In 2019 Burgstaller et al. [54] demonstrated a fully continuous substitute of Protein A chromatography as already mentioned in chapter 1.4.2 "Challenges and bottlenecks". In the manuscript the antibodies are precipitated by mixing the solution with polyethylene glycol 6000 coupled with zinc chloride. The precipitated product remains in the retentate of a total of two subsequent tangential flow filtration skids, while impurities are removed in the permeate.

For all of the described continuous and batch unit operations overall process costs coupled with maximum product output are the most relevant criteria in respect of the entire process design. However, all of the explained comparisons, single-use and stainless-steel, or batch and continuous operation also show differences in overall water consumption.

# 2 Objectives

The hypothesis of the thesis is that economics of a biopharmaceutical process are correlated with reduced water consumption. Improvement of the environmental footprint is not obtained on the expense of economics, in contrary it will add to a better economy.

Furthermore, it is hypothesized that the significance of the PMI metric can be enhanced by developing a novel metric, which includes the different types of clean water, energy consumption and $CO_2$ emission related to water production.

Therefore, the doctoral thesis had the following main objectives.

- *Holistic economic and ecological analysis of an industrial relevant monoclonal antibody biomanufacturing process and several process variants to get a deeper insight into the correlation between PMI and Cost of Goods*
- *Comparison of the PMI and Cost of Goods of conventional batch wise production of antibodies and continuous or hybrid processes.*
- *Development of a novel PMI based metric that includes the economic and environmental impact of production methods and use.*
- *Application of the extended metric on biotechnological case studies*
- *Effect of upstream processing on the PMI and Cost of Goods of a bacterial process*
- *Effect of failure rate on the process economics*

# 3 Results and discussion

The findings of this work are collected in this doctoral thesis and in total in four manuscripts. Publication 1: The manuscript with the title "*Economics and ecology: Modelling of continuous primary recovery and capture scenarios for recombinant antibody production"* is published in Journal of Biotechnology since December 2019 [94]. Contribution: First author - Conceptualization, methodology, writing - original draft.

Publication 2: The manuscript with the title "*Water related impact of energy: Cost and carbon footprint analysis of water for biopharmaceuticals from tap to waste"* is published in Chemical Engineering Science X since October 2020 [95]. Contribution: First author - Conceptualization, methodology, writing - original draft.

Publication 3: The manuscript with the title *"Impact of failure rates, lot definitions and scheduling on the productivity of continuous integrated bioprocesses"* is published in Journal of Chemical Technology & Biotechnology since December 2020 [96]. Contribution: Co-Author – Evaluation of economic impact for process failure.

Publication 4: The manuscript with the title *"Economic and ecological benefits of a leaky E. coli strain for downstream processing: a case study for Staphylococcal Protein"* is published in Journal of Chemical Technology & Biotechnology since February 2021 [97]. Contribution: Co-Author – Cost and PMI evaluation of Protein A process strategies.

## 3.1 PMI and process economics

To determine a correlation between reduced PMI and process costs all utilized data, methods and materials have to be evaluated individually. Therefore, all relevant process scenarios need to be defined properly and implemented into the BioSolve software. In this model the results, and the developed analysis methods are part of the overall outcome of the underlying thesis. BioSolve is a frequently utilized software throughout the biotech industry and its application fields are constantly evolving. Hence, the following chapters focus on further explanation as well as clarification how the results of Publication 1 were determined and further outline how BioSolve can enhance this evaluation.

### 3.1.1 Modelling with BioSolve

The generated data of the NextBioPharm DSP project were implemented into BioSolve considering different approaches based on sensitivity analysis. The advanced scenarios available in BioSolve 7.5 enable a dynamic process evaluation of varying scales, product titers and modes of operation. With the available data generated with a fermentation volume of

1000 L the scaling parameters of each device and consumable needed to be defined in order to perform the evaluations shown in Publication 1. In BioSolve the production scale can either be specified based on the annual product output or the fermentation volume. In case the process is scaled based on the product output the fermentation volume and the number of batches are adjusted automatically. For all devices listed in the BioSolve database costs, floor space and operation ranges are considered. The process scaling of BioSolve in order to reach the desired annual product output may lead to an inefficient equipment occupancy of the greenfield analysis according to chapter 1.1 "Economic modelling for industrial processes". For instance, a process with a given fermentation titer, number of batches and annual production scale required 2100 L fermentation volume according to BioSolve. The smallest bioreactor available in the database, which is capable of operating 2100 L has a capacity of 3000 L. The significant volume difference leads to an increased cleaning water demand and reduced occupancy of the reactor. In order to reduce the PMI and design the process more precisely, either the facility is scaled out, i.e. 2 x 2000 L are processed and hence the annual product output is increased or the given parameters (titer, batches, required product) has to be adjusted in order to fit in a 2000 L reactor. Simulating the process upscale also requires logistic adjustments, such as for choosing single-use or stainless-steel tanks for buffer or media hold and preparation tanks and its make-up basis. Cost data for all unit operations in the described scale was available considering the described evaluation of an existing facility. In order to enable a precise estimation according to the available cost data the cost scaling of each device, consumable and material needs to be defined individually. In Eq. (2) the cell manipulation of the Excel-based BioSolve model is exemplified with the daily costs of a pre-packed PCC column of varying scales. For continuous operation the perfusion runs with a net runtime of 15 days before it stays idle for 5 days during start-up of the next perfusion run. After each run the column is exchanged in order to guarantee a stable process. Within 15 days each column has performed about 360 cycles, which is almost double the number of the recommended maximum of 200 cycles per column. Therefore, columns have to be exchanged after 7.5 days of runtime. This exchange reduces the number of cycles per column to 180. Furthermore, it is predicted by experience that the total costs per column are increased by 50% with an increased column volume of 100 %. Pre-packed chromatography columns become cheaper per L column with increased column volumes, because the preparation process and validation of the column have no significant increase in costs for larger volumes. The available price reference as well as the empirically determined reference product input were implemented in the BioSolve database, while the current product input is calculated automatically by BioSolve for each scenario. With the formulated daily PCC costs, the 15 day

perfusion run can be compared to the three day fed-batch USP with continuous DSP. Within a three day operation the column has performed 72 cycles before it is exchanged for the next batch, leading to higher daily PCC costs with varying impact on different scales.

$$Daily\ costs\ PCC = 0.5 * \frac{1}{7.5d} * \left(1 + \frac{Reference\ product\ input}{Current\ product\ input}\right) * Price\ reference \qquad \text{Eq. (2)}$$

Materials, such as buffer or media ingredients either scale linearly or with a discount after reaching a threshold demand. Specific consumables, such as flex ware assemblies for the flow-through chromatography steps, have no significant price increase for different scales, because the effort of producing this consumable is equal for lab and pilot scale. All the mentioned parameters have a major impact on overall water consumption and the PMI but also on Cost of Goods (CoGs) resulting in a direct correlation.

### 3.1.2 Water related cost parameters

After implementing the NextBioPharm DSP process data into BioSolve the results of all 18 scenarios for all scales were analyzed to assess the PMI relevant input and output parameters. Therefore, the software splits the entire water consumption into each unit operation and its sub-units. For downstream processing, the chromatography steps are the most water consuming steps, caused by multiple cycles consisting of several washing, equilibration and elution steps. With a constant binding capacity of the column for each scenario the total input volume and product titer define the design and outline of the chromatography steps. For larger volumes with equal titers more material and a broader column are required in order to maximize the productivity and efficacy of the entire process. The PMI for varying scales remains constant, because the water consumption and the binding capacity of the product are correlating with the column volume. However, considering buffer preparation the amount of cleaning water per kg API for preparation and hold tanks are reduced as well as the product related effort of labor for buffer preparation.

The evaluation performed in Publication 1 showed that the biggest impact in increased costs and PMI is caused by product titer differences for perfusion and fed-batch fermentation. Fermentation titers of 1 g/L for perfusion and 4 g/L for fed-batch are applied referring to the process simulation of the NextBioPharm DSP project. According to the literature higher product titers are achievable for both strategies but within the project the source of real data had a higher priority than literature [27], [98], [99].

Assuming an equal annual product output of both fermentation strategies the fed-batch fermentation requires about 3 times increased reactor volume compared to the perfusion reactor. However, the reactor only needs to be filled once per 14 days, while the perfusion reactor has 1.5 volume exchanges per day leading to a significant increase in water consumption. Especially for cell culture media ingredients this represents a significant cost factor. With lower product titers the amount of media increases in relation of the product. Comparing a staggered fed-batch systems of six times 2000 L with a corresponding perfusion reactor volume of 4000 L the average amount of daily consumed media is 857 L per day for fed-batch and 4500 L per day for perfusion. Since each perfusion run has a non-productive period of 5 days for start-up the media demand per day for perfusion is higher than the expected 4-fold increase caused by the titer differences. Hence, the perfusion scenario requires more than 5 times more media than the fed-batch scenario. Furthermore, the 2000 L single-use threshold volume is exceeded for the perfusion reactor leading to an increased water demand caused by the cleaning of the stainless-steel reactor.

The entire downstream process requires smaller devices for the fed-batch scenario, because within every 2.3 days the broth of one of the staggered bioreactors is further processed. On average 8 kg of antibody enter the downstream train every 2.3 days and define the dimension of the Protein A column. For the 4000 L perfusion scenario about 14 kg of antibody enter the downstream train in the same time frame during the productive period. Hence, the PCC Protein A columns are about twice as large as for the fed-batch scenario. As already described in chapter 3.1.1 “Modelling with BioSolve” the columns need to be exchanged more often for the fed-batch scenarios and the price of the pre-packed column increases by 50 percent for a 100 % increase in column volume. Throughout the entire year unit operations performed in stainless-steel facilities can only be occupied for 80 % while single-use devices have a greater occupancy rate of 90 % due to reduced changeover times referring to process experience of industrial NextBioPharm DSP project partners. The lowered occupancy time of the perfusion reduces the annual costs for the column, while water consumption remains increased. In Figure 7 can be seen that the reduced costs of the chromatography steps in the perfusion scenario may outweigh the significantly higher PMI of the fermentation depending on the price of WFI. In discussion with the industrial project partners of the NextBioPharm DSP project further analysis of the water price was necessary in order to compare the described processes more specifically. These results are shown in chapter 3.2 “Economics of water consumption”.

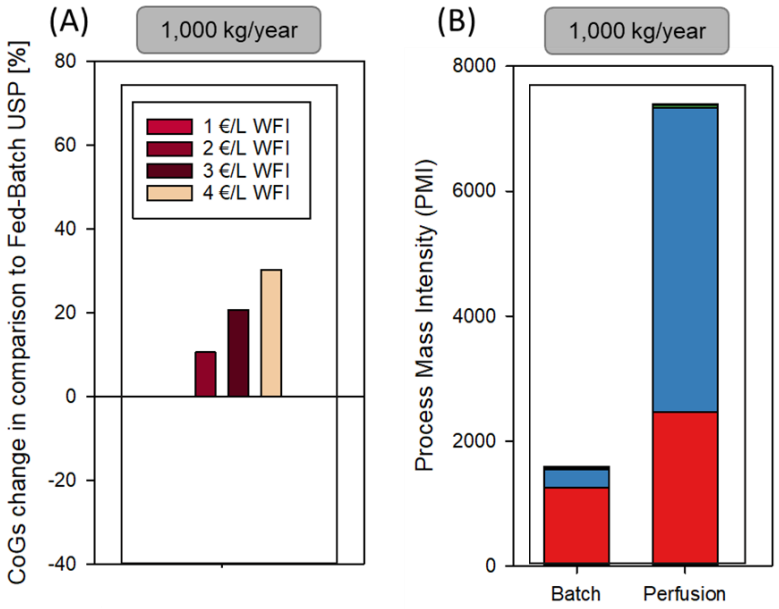


***Figure 7: (A) Cost comparison for 1000 kg/year USP; Fed-Batch is represented by the baseline; Perfusion by the bars; Bars below the line represent reduced costs for the perfusion USP and vice versa. (B) PMI comparison for 1000 kg/year USP [94]***

## 3.1.3 Consumables

In Publication 1 several unit operations regarding primary recovery for antibody production were assessed comparing a conventional centrifugation, a flocculation assisted depth filtration and a two-step membrane filtration. These unit operations were compared for 50 kg, 200 kg and 1000 kg antibody per year with varying fermentation titers. Figure 8 shows the performance of each solution, which considers data provided by industrial filter suppliers as well as biomanufacturing companies. The results of this comparison were already described and discussed in Publication 1. In short, the PMI cannot be applied for comparing single-use and stainless-steel operation since the price of consumables are not displayed in the PMI but in process costs. According to the generated process data, the number of required filters increases proportionally to the number of batches and fluid volume. Figure 8 is representative for the overall comparison of disposable and stainless-steel production trains in respect to the PMI evaluation, whereas the single-use systems are represented by bars and the conventional centrifugation as baseline. If a continuous centrifuge is used instead of filtration equipment costs represent the highest cost contribution throughout a depreciation period of 10 years. With increasing number of batches the costs per batch decrease due to higher equipment occupancy within the time frame. The PMI of the single-use systems remains relatively constant for all scales, whereas the PMI of the centrifugation based primary separation decreases for larger

scales. Taking this outcome into consideration the PMI is not representative for a cost comparison of sole single-use vs. conventional unit operations.

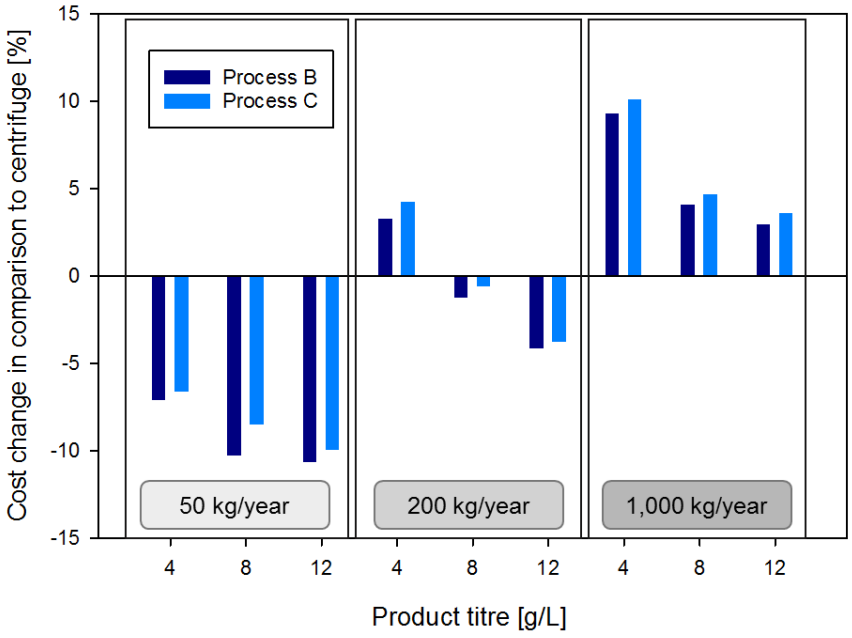


***Figure 8: Cost comparison - Primary recovery solutions [94]***

## 3.1.4 Cost and PMI evaluation of two Protein A process scenarios

In chapter 1.4.1 “Characteristics of Protein A chromatography” the characteristics and economic impact of Protein A was already described. In Publication 4 two Protein A manufacturing scenarios comparing different *E. coli* strains were designed and evaluated. Figure 9 provides a modified view compared to the results shown in Publication 4 in visualizing relative differences in (A) CoGs and (B) PMI for both processes. The performance of the conventional cytoplasmic process is represented by the baseline and the leaky process, where no cell disruption is required, is represented by the bars. It can be seen that for nearly each category the leaky strain reduces the total costs and water consumption caused by less unit operations and higher binding capacities for chromatography. For dilution the amount of water is increased compared to the cytoplasmic process, because centrifugation leads to a significant volume reduction directly after fermentation and less water is required for conductivity adjustment before the chromatography step. The results shown in Figure 9 express a total cost reduction of 24 % compared to the conventional process and a total PMI reduction of 36 % without considering the polishing steps. A price reduction in that dimension may affect the overall price of 1 L Protein A column for antibody capture depending on the share of the raw material Protein A in relation to the manufacturing of 1 L column. However, no statement can be made that the process cost reduction of 1 L Protein A column also reduces the retail price of

the column. Hence, the reduced footprint of the Protein A has no visible effect on process economics for the biomanufacturer, if the supplier does not adjust the price per L column according to the PMI reduction.

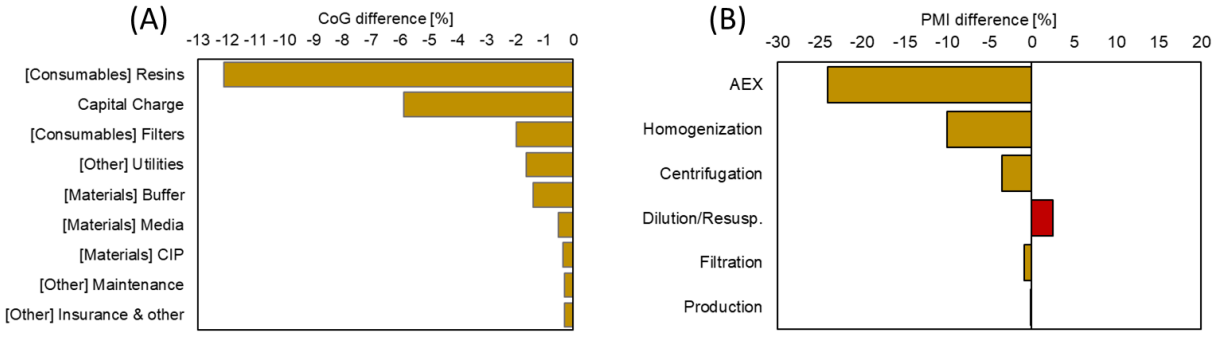


***Figure 9: (A) Cost and (B) PMI comparison Protein A production expressed in E. coli as explained in Publication 4; Conventional cytoplasmic process is represented by the baseline and production vith the leaky strain is represented by bars [97]***

## 3.1.5 Equipment occupancy and efficacy

In Publication 1 different process scenarios for monoclonal antibody production were compared in different scales. For the product output-based scales (50 kg/year and 1000 kg/year) fed-batch fermentation resulted in a lower PMI and lower CoGs. However, for the volume-based scale of the existing facility (1000 L fermentation) the perfusion with a titer of 1 g/L scenario had lower costs but an increased PMI compared to fed-batch with a titer of 4 g/L resulting in a non-correlative behavior (see Figure 10). For the volume-based scenarios the total amount of produced product varies significantly from 88 kg/year for fed-batch to 266 kg/year for perfusion. This significant difference leads to overall reduced CoGs for the perfusion process because the productivity of downstream operations is increased. For cell culture cultivation fed-batch fermentation represents the bottleneck of the entire process with about 14 days per batch. Antibody purification of the NextBioPharm DSP project was adjusted to process 1000 L of pooled fermentation broth within three days. In order to maximize the occupancy rate of the downstream devices multiple bioreactors can be installed in staggered mode. However, if multiple bioreactors or alternatively a 15 m³ bioreactor cannot be installed due to floor space limitations the efficiency of the available bioreactor needs to be increased by applying perfusion fermentation. With the inputs shown in Table 3 the average amount of product per L reactor volume per day increases from 0.29 to 1.13 g/Ld. With this setup the USP productivity can be increased by almost a factor of 4 while the DSP occupation can be increased to 75%. Due to the overall higher efficacy of available devices the overall costs can be decreased. By switching

from fed-batch to perfusion mode this process showed reduced process cost. However, the PMI is increased due to the increased amount of water per day for the perfusion (Figure 10).

***Table 3: Chosen parameters of 1000 L fermentation scenarios***

| Parameter for 1000 L USP | Fed-Batch | Perfusion |
|---|---|---|
| Titer | 4 g/L | 1 g/L |
| Batch duration | 14 days | - |
| Perfusion Start-up | - | 5 days |
| Net perfusion runtime | - | 15 days |
| Volume exchanges per d | - | 1.5 |
| Average product per day | 285 g/d | 1125 g/d |
| Daily product per L volume | 0.29 g/Ld | 1.13 g/Ld |
| DSP occupancy | 21.4 % | 75 % |

Overall it can be concluded that switching the operation mode from fed-batch to perfusion respecting the data of the NextBioPharm DSP project leads to a non-correlative behavior of costs and the PMI, because different product output scales are compared.

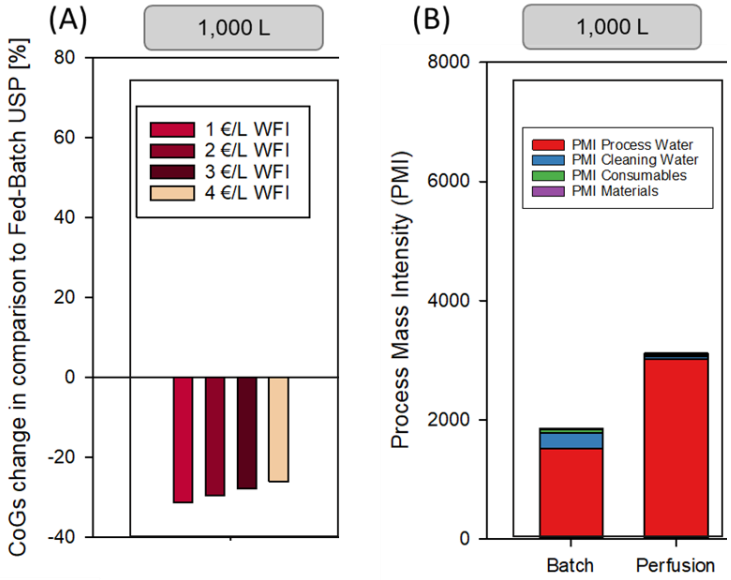


***Figure 10: (A) Cost comparison for 1000 L USP; Fed-Batch is represented by the baseline; Perfusion by the bars; Bars below the line represent reduced costs for the perfusion USP and vice versa. (B) PMI comparison for 1000 L USP [94]***

In Publication 3 process uptime and hence equipment occupancy of perfusion fermentation was evaluated considering failures and the flexibility after failure occurrence. By guaranteeing maximum flexibility to restart the N-1 fermentation run directly after the failure occurrence the N-1 reactor has a reduced occupancy rate compared to the other flexibility scenarios. However, no flexibility of starting the N-1 reactor will lead to a reduced occupancy of the perfusion reactor until the next perfusion run is scheduled. The strategies focusing on limited flexibility

according to Figure 11 will lead to an insignificant uptime reduction, while the correspondent N-1 reactor can be utilized for other procedures. In order to define the optimal frequency of the available time slot the entire facility considering all production lines and product variety needs to be considered.

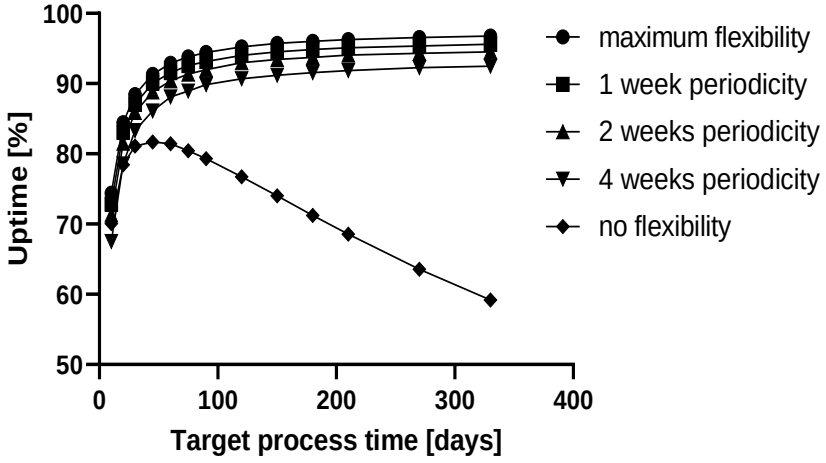


***Figure 11: Process uptime evaluation for different flexibility periods after perfusion failure occurrence [96]***

## 3.2 Economics of water production

The importance of water production economics was already exemplified in chapter 3.1.2 "Water related cost parameters". In Publication 2 the entire flow path of several clean water types was analyzed according to the flow scheme in Figure 12 in order to determine the price range of different water qualities [95]. In this flow path varying temperatures of PW, WFI and clean steam (CS) are indicated by different colors for the defined "hot" and "cold" WFI production methods described in chapter 1.2.2 "Water consumption". Several facility engineering companies provided economic calculations comparing different production methods [30], [32], [33]. However, the holistic approach considering different temperatures and decontamination efforts has not been developed yet. Biomanufacturing companies have their own department for water supply, where the costs of each facility can be calculated. But this information is not available for the public and academic research institutions in the field of biotechnology. Even though biomanufacturing operators and process planners performed several process evaluations considering clean water, such as process- or cleaning standard operating procedure (SOP) design, the overall impact of water is often ignored according to experiences of the ACS GCI PR. With the models created in Publication 2 the knowledge of different departments can be combined and water consumption can be used as design criterion

in order to define the process. The evaluation of different WFI production methods, which are shown in Figure 13 (B) visualize the cost saving potential from hot to cold WFI production depending on the relative share of utilized WFI compared to PW. For an increased amount of PW further process steps in order to produce WFI become obsolete and thus lead to better performance of hot WFI production, because the purity of PW is not specified for cold WFI production. However, for already existing facilities with a running hot WFI supply switching from hot to cold WFI production has no significant impact from a sole economic perspective. Additionally, the responsible personnel for the WFI supply may require additional effort, such as workshops in order to handle cold WFI production properly.

According to Publication 2 the price of WFI varies from about 35 € to 40 € per metric ton. Hence, the price of 1 L WFI is less than 0.04 €. Considering the results shown in chapter 3.1.2 "Water related cost parameters" and Figure 7 the economic performance of the perfusion fermentation is equalized due to the low costs of water. Furthermore, the supplementary files attached in Publication 2 enable an individual water calculation for academic institutions as well as industrial companies and further explain the calculation methods of each parameter.

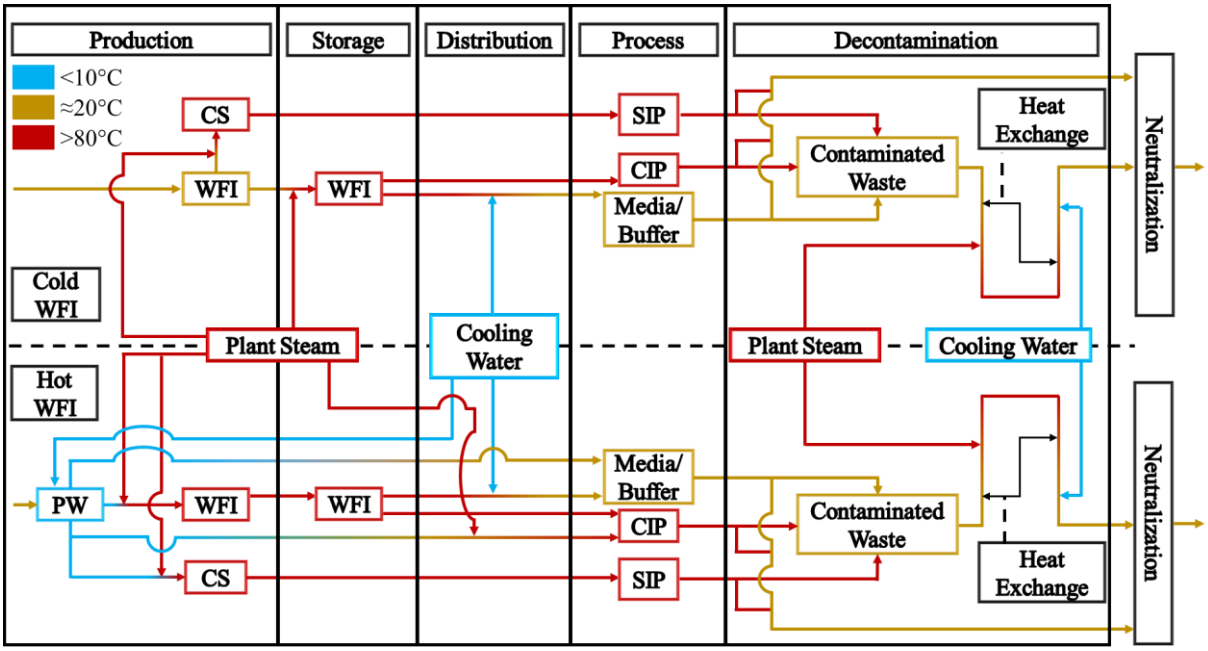


***Figure 12: Flow path of clean water from tap to waste [95]***

## 3.3 The WARIEN metric

In Publication 2 the WARIEN metric was introduced, which quantifies the amount of water related to consumed energy and emitted $CO_2$ per kg product. In order to calculate the WARIEN a PMI analysis of a process is necessary coupled with $CO_2$ emission intensity per kWh for the

specific region and the amount of required energy in order to produce 1 kg of WFI, PW or CS. The goal of the WARIEN metric is to further quantify the environmental impact of biopharmaceutical production. The economic analysis of the water production train explained in 1.2.2 “Water consumption” and Publication 2 was utilized in order to demonstrate how the WARIEN can be calculated. With the detailed calculation and the provided supplementary Excel sheet in Publication 2 the WARIEN can be calculated individually by other institutions and can therefore be used as design criterion for bioprocess development.

In Figure 13 the results of the (A) WARIEN and (B) water related process costs of a show case fermentation process are quantified. These results lead to the conclusion of the manuscript showing correlative behavior of water related costs and $CO_2$ emission. While the environmental footprint is proven to be higher compared to a described air ventilation scenario, the water related costs, considering energy related costs, overhead costs and $CO_2$ taxes have no significant impact on overall process costs, as already described in chapter 3.2 “Economics of water production”. Furthermore, reduced WARIEN can be observed for membrane-based WFI production compared to distillation-based and for utilizing single-use equipment instead of stainless-steel. Overall $CO_2$ evaluation of single-use systems require a life cycle assessment of each consumable considering its production, transport and disposal. However, comparing the overall WARIEN results with those of Pietrzykowski et al. [36] it can be concluded that the environmental damage caused by conventional stainless-steel devices is higher than for single-use systems.

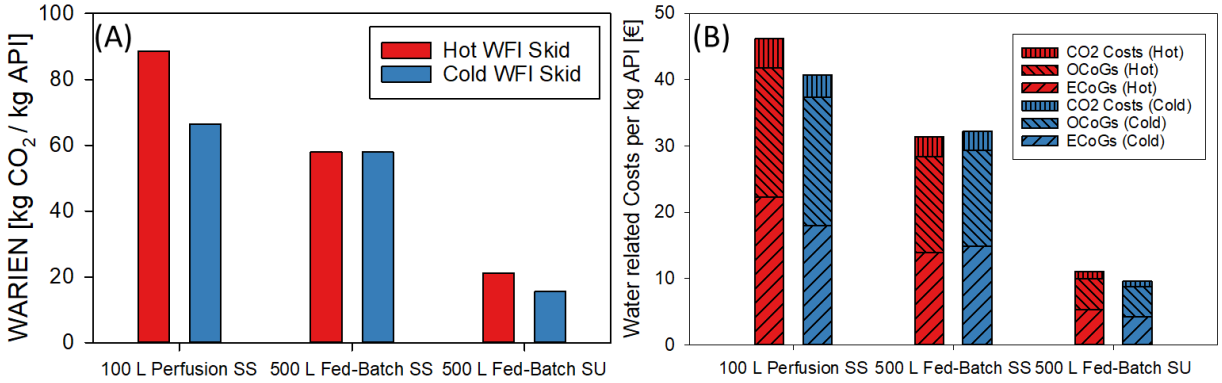


***Figure 13: (A) WARIEN calculation and (B) water related costs per kg API for the scenarios designed in Publication 2 [95]***

The application of the WARIEN requires utilization of clean water. The manufacturing process of other biotechnological products apart from biopharmaceuticals has lower standards regarding the water quality because the final product may not rely on strict regulatory. As an

example, cultivation of enzymes, which are capable of degrading plastics, can be performed with municipal water [100], [101]. For these bioprocesses alternative strategies need to be developed.

## 3.4 Formulation of future tasks

The results of Publication 1 and Publication 2 are based on biomanufacturing processes of monoclonal antibodies expressed in CHO cells. Even though monoclonal antibodies have the largest share of biopharmaceuticals approved in Germany the ecological impact of processes based on other host cells need to be considered as well. Recombinant proteins cultivated in *E. coli* also represent a significant number of biopharmaceuticals approved in Germany [69]. Process evaluation on an economic and ecological basis cannot be generalized for each *E. coli* expressed product, because the protein of interest can be either soluble or insoluble as an inclusion body located either in the cytoplasm, periplasm or extracellular.

Furthermore, no general affinity chromatography, such as Protein A for monoclonal antibodies can be performed. The methods of the PMI-Cost evaluation can be adapted for different *E. coli* production strategies aiming on the varying challenges. The same applies for the bioproduction of vaccines, enzymes or other recombinant proteins expressed in different hosts. For all listed bioproduction strategies the WARIEN metric can be applied according to the supplementary Excel-sheet in Publication 2. After several processes were evaluated the water related $CO_2$ emission can be coupled with the $CO_2$ emission from other sources after analyzing the life cycle assessments (LCA) of consumables, materials and equipment according to Pietrzykowski et al. [36]. Furthermore, the developers of the BioSolve software are continuously developing ecological calculation methods for its users. Hence, the impact of the WARIEN can be compared to overall $CO_2$ emission of a biomanufacturing facility.

# 4 Conclusion

In the present work economic and ecological aspects of biomanufacturing were assessed and novel methods of process evaluation have been developed based on the establishment of water related metrics. The introduction of the PMI in biotechnology by Budzinski et al. [28] clearly showed the impact of water in a process. However, its further economic and environmental consequences were not established in the work of Budzinski et al. Therefore, I have developed a process design criterion, which coupled the PMI with the production costs and energy of water and I called it "WAter Related Impact of ENergy (WARIEN)" metric. The generated process data of the NextBioPharm DSP project and the assistance of the ACS GCI PR enabled the extension of the PMI to the WARIEN metric with a broader meaning and significance and its application as a process design criterion.

By simulating a process with BioSolve or collecting empiric data of water consumption the PMI can be determined more easily. For the production of monoclonal antibodies, the demonstrated results showed under which conditions the PMI and process costs can be combined.

The introduction of the WARIEN metric enables further methods to apply the PMI within biotechnological process evaluation. Water related $CO_2$ emission for each type of clean water in respect of the energy source and the characteristic of the facility can be quantified with the newly developed WARIEN metric. It interconnects the knowledge of different company departments, such as process and cleaning experts, and the department responsible for water purification and disposal. Even though all methods were designed using antibody production processes as reference the described approaches are applicable for other biopharmaceutical manufacturing processes as well. The results of the WARIEN calculation also showed that the economic impact of the WFI price itself is in relation to the overall process costs lower. This indicates that costs of recycling water outweigh the economic benefits of saving resources, due to additional storage, purification and validation effort. The sole utilization of the PMI is not recommended for an ecological assessment of a process, but the elaboration or estimation of the PMI serves as basis for several conversion factors, such as for WARIEN and $CO_2$ quantification. However, not only the expertise of bioprocesses is necessary, but also chemical production of all single-use devices and raw materials.

To sum up:

- *Holistic economic and ecological analysis of an industrial relevant monoclonal antibody biomanufacturing process showed in which cases PMI and CoGs can be combined*
- *The WARIEN metric serves as a novel PMI based metric focusing on the production train of all clean water classifications*
- *The models are suitable for all biopharmaceutical production processes irrespective of the application of the product*
- *The WARIEN metric can be used to guide development of processes and materials to reduce the impacts on $CO_2$ emission.*
- *The type of upstream process has a significant impact in Cost of Goods and PMI of the product due to reduction of water and material consumption for the downstream processing.*

Beyond the original objectives I demonstrated that process optimization of *E. coli* derived Protein A production represents a complementary approach for antibody manufacturing evaluation. Reducing effort of Protein A as shown in Publication 4, has no effect on PMI for antibody production, however, on the overall environmental footprint.
In conclusion, a holistic ecological evaluation of biopharmaceuticals requires the inclusion of all life cycle assessments, but the demonstrated results guide how suitable models can be designed referring to the development of the WARIEN.

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# List of Figures

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# Abbreviations

| Abbreviation | Expression |
|---|---|
| ACS GCI PR | American Chemical Society Green Chemistry Institute Pharmaceutical Roundtable |
| API | Active pharmaceutical ingredient |
| CHO | Chinese hamster ovary |
| CS | Clean steam |
| CoGs | Cost of Goods |
| DSP | Downstream Processing |
| HVAC | Heating, ventilation and air conditioning |
| LCA | Life cycle assessment |
| PCC | Periodic countercurrent chromatpgraphy |
| PMI | Process Mass Intensity |
| PW | Purified Water |
| SOP | Standard operating procedure |
| TOC | Total organic carbon |
| USP | Upstream Processing |
| WARIEN | WAter Related Impact of ENergy |
| WFI | Water for Injection |

# Publications

Publication 1

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# Economics and ecology: Modelling of continuous primary recovery and capture scenarios for recombinant antibody production

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ABSTRACT

With the maturation of antibody production technologies, both economic optimization and ecological aspects have become important. Continuous downstream processing is a way to reduce the environmental footprint and improve process economics. We compared different primary recovery, capture, and fermentation methods for two output-based antibody production scales: 50 kg/year and 1000 kg/year. In addition, a fixed fermentation volume case of 1000 L was analysed in terms of total cost of goods and process mass intensity as a measure of the environmental footprint. In our scenario, a significant amount of water can be saved in downstream processing when single use equipment is utilized. The overall economic and ecological impact is governed by the product titre in our perfusion (1 g/L) and fed-batch (4 g/L). A low titre in fermentation with similar downstream purification leads to higher process mass intensity and cost of goods due to the higher media demand upstream. The economic perspective for continuous integrated biomanufacturing is very attractive, but environmental consequences should not be neglected. Here, we have shown that perfusion has a higher environmental footprint in the form of water consumption compared to fed-batch. As general guidance to improve process economics, we recommend reducing water consumption.

## 1. Introduction

Process development in the biopharmaceutical industry is not only guided by economic considerations, but also by ecological considerations. Traditionally, the upstream and downstream processing of a biopharmaceutical is batch-based, but in recent years several groups have demonstrated that upstream, as well as downstream, processing can successfully be performed continuously (Hammerschmidt et al., 2016; Shukla et al., 2017; Walther et al., 2015). However, how process economics and ecological factors are affected by a change from batch to continuous manufacturing is unclear. It is also reasonable to look at upstream and downstream processing separately and consider hybrid processes with continuous upstream and batch-based downstream or vice versa. By end-to-end continuous biomanufacturing, the cost of goods (CoG) can be reduced by 40 % for a 10-year scenario (Arnold et al., 2019; Walther et al., 2015).

Although the economic perspective for continuous integrated biomanufacturing is very attractive, the environmental consequences have mostly been neglected. In 2017, approximately 60 % of the market share of all biopharmaceuticals were antibodies and more thorough investigation of the consequences of continuous biomanufacturing on antibody processes is needed (Grand Review Search, 2017). Platform downstream processes have been established for recombinant antibodies (Biophorum Operations Group, 2014). Such a platform process consists of primary recovery, which is accomplished mainly by centrifugation with a disk stack centrifuge, followed by protein A capture chromatography, a virus inactivation step, at least two additional chromatography steps for intermediate purification and polishing, viral filtration, and an ultrafiltration/diafiltration step (Shukla et al., 2006). Although such processes are termed platform processes, the actual execution, design of primary recovery selection by chromatography, and selection of filters depends on the available facilities, manufacturing scale, product titre, and product characteristics (Farid, 2007; Gottschalk, 2017, 2008; Shukla et al., 2006). The implementation of unit operations that replace stainless steel devices with disposables, as well as continuous process development, create new possibilities for process optimization and further increase the variety in existing facilities. Due to its high cost, the substitution or improvement of protein A chromatography capture is a challenge for the purification of monoclonal antibodies (mAbs). Periodic counter-current chromatography

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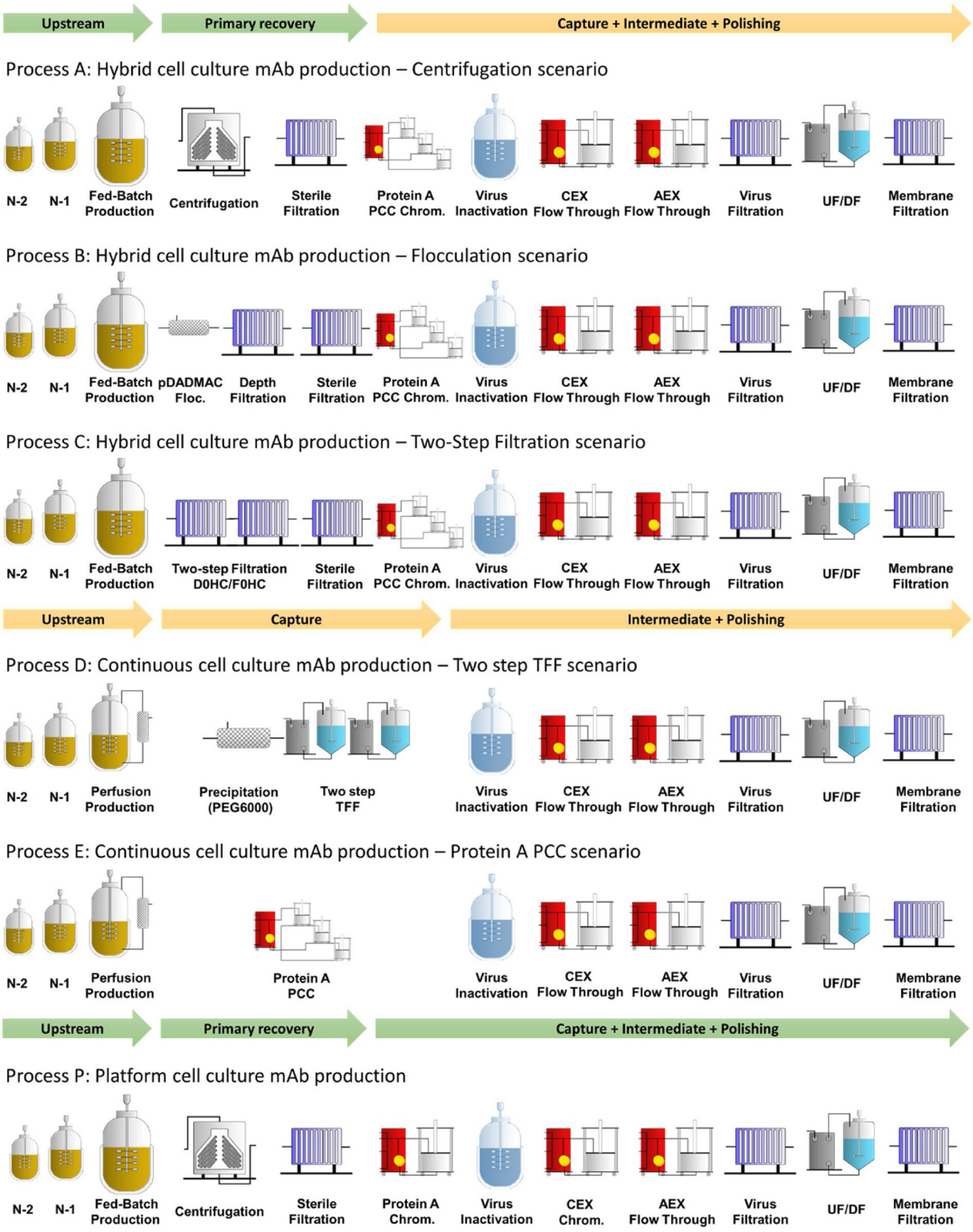


**Fig. 1.** Process scenarios for antibody production. The yellow arrows above the scenarios represent a continuous product flow, whereas the green arrows represent batch operations. Process A: Batch Upstream followed by conventional centrifugation and filtration for clarification. Process B: Batch Upstream followed by pDADMAC flocculation and two filtration steps. Process C: Batch Upstream followed by D0HC/F0HC filtration and sterile filtration. Process D: Perfusion Upstream followed by polyethylene glycol (PEG) precipitation in a tubular reactor and two diafiltration steps. Process E: Perfusion Upstream followed by a Protein-A PCC. Process P: Platform mAb batch production.

(PCC) is a semi-continuous method to reduce the column volume via higher column utilization and faster process operation. However, the process does become more complex because of additional valves, pumps, and multiple streams (Jungbauer, 2013; Steinebach et al., 2016). Affinity chromatography can be substituted by alternative capture methods, such as continuous precipitation using a tubular reactor followed by a two-step tangential flow filtration for precipitate harvest. This method allows fully continuous operation with a reduced footprint

without using protein A or columns (Burgstaller et al., 2019; Hammerschmidt et al., 2016).

For batch upstream processes, disk stack centrifugation is the most common unit operation to clarify the harvest from cells (Turner et al., 2018). Single-use substitutes for this unit operation can be cell flocculation combined with depth filtration or a sequence of various depth filters. With the polymer polydiallyldimethylammonium chloride (pDADMAC), the cells can be flocculated and removed by microfiltration. This process requires a smaller filter area than the removal of cells by microfiltration alone. The advantage of pDADMAC is the wide operating range and high yields (Burgstaller et al., 2018; EMD Millipore, 2016; McNerney et al., 2015; Singh and Peck, 2014; Tomic et al., 2014). Thus, it is obvious to compare flocculation-assisted cell removal to centrifugation.

The ecological impact is also a crucial element that needs to be considered in process evaluation. For other industry sectors, the process mass intensity (PMI) has been used to compare the environmental impact of different processes. It is a metric that describes the total resource consumption in mass per mass product (Eq. (1)) (Budzinski et al., 2018).

$$Total\ PMI = \frac{total\ water,\ raw\ materials,\ consumables\ usedinprocess\ (kg)}{active\ pharmaceuticalingredient\ (kg)} \quad (1)$$

Recently, the use of PMI for comparing different processes in biomanufacturing was suggested. In biopharmaceutical manufacturing, the highest contribution to the PMI is water consumption. Reducing the amount of water required has not only economic benefits due to less storage and water for injection (WFI) production effort, but is also environmentally aware (Budzinski et al., 2018; Jimenez-Gonzalez et al., 2011; Madabhushi et al., 2018).

For a meaningful comparison, it is important to define certain production scenarios, either greenfield or with an existing plant. High upfront investment may bias cost comparisons towards processes with disposable equipment, in which the CoG may actually dominate the overall costs. The cost structure will also depend on the phase of development and whether the product is produced for clinical trials or full-scale manufacturing over a long period of time. Nevertheless, the annual manufacturing amount will be crucial for selecting the most economic and environmentally friendly process. We used the computer program BioSolve, which allows custom calculations for different production scenarios of different scales. The models are based on existing data and data from the BioSolve database, which is regularly updated by the developers (Pollard et al., 2016).

In the present study, several batch and continuous manufacturing options for the production of monoclonal antibodies were compared. Possible process parameters and sequences were designed in discussion with our industry partners to operate a 1000 L fermentation broth within 3 days. For primary recovery, centrifugation and flocculation-assisted removal of the cells were compared. Next, capture and polishing steps in bind-elute and flow-through were investigated in batch and continuous operation. Two reference output scales were selected to demonstrate the impact of different scenarios for an orphan substance with an annual demand of 50 kg/year and a blockbuster substance with an annual demand of 1000 kg/year for a greenfield scenario in which a new facility is built (Farid, 2007; Gottschalk, 2017). In addition, the output and cost of the designed facility equipped with a 1000 L cultivation vessel is assessed using a conventional batch platform and the best options for each of the unit operations. We investigated whether the frequently used PMI and CoG positively correlate. If this proves to be true, a process with a small environmental footprint is also economically favourable.

## 2. Materials and methods

### 2.1. Process data

The economic evaluation is based on data generated in the EU-funded Horizon 2020 project NextBioPharm DSP (http://nextbiopharmdsp.eu/), in which academic institutions cooperate with research and manufacturing companies, having published certain process options and new unit operations (Burgstaller et al., 2019, 2018). All process scenarios were tested in lab-scale and applied in a pilot-scale facility. Based on the results of both scales and the BioSolve database, process parameters were realistically estimated in industrial-scale.

Process definitions of continuous capture via precipitation and pDADMAC-based flocculation scenarios are available in previous publications by Burgstaller et al. (Burgstaller et al., 2019, 2018). Fig. 1 shows all scenarios evaluated in this publication and the unit operations within the sequence. Each scenario consists of a hybrid process with fed-batch fermentation, followed by a continuous downstream train, or is run completely continuous, replacing the production fed-batch fermentation with a perfusion reactor.

For all hybrid scenarios, several primary recovery solutions were compared in a fermentation volume-based scenario of 1000 L, and in two output-based scenarios of 50 kg/year and 1000 kg/year for a direct unit operation comparison. The best performing solution was then applied in a simulated production of the corresponding scale. Capture and polishing were performed continuously and scaled based on the fermentation output to minimize the idle time of each unit operation. The evaluation of the hybrid processes focused on the comparison between different primary recovery solutions; thus, for all scenarios, a protein A capture PCC skid prototype was applied. The perfusion scenarios focussed on the comparison between the different capture solutions. Therefore, the stated PCC was compared to continuous precipitation, followed by two-step tangential flow filtration (TFF) (Burgstaller et al., 2019). Subsequently, continuous virus inactivation was performed, followed by flow-through CEX combined with an active carbon filter, pH exchange, flow-through AEX, viral filtration, diafiltration, and a final membrane filtration. The entire continuous purification train, except the two-step filtration in the precipitation scenario (Process D) via Äkta Flux®, was performed using continuously operating prototypes produced by Merck KGaA. Table 1 provides the specific process parameters and modelling inputs. For all scenarios performed in a single-use reactor, the equipment is assumed to be occupied for 90 % of the available time, 80 % for all stainless steel scenarios. For all perfusion scenarios, we assumed a total runtime of 20 days, including 5 days for the start-up phase. We also evaluated a default mAb process of BioSolve as a reference and compared it with our results (Process P).

### 2.2. Cost data and modelling

Economic modelling requires a considerable amount of process and equipment data. The cost of equipment for each device and the operational costs for all utilized consumables, materials, personnel, and utilities need to be analysed. Therefore, it is important that certain assumptions be realistic to avoid misleading results. The modelling software BioSolve (v7.5) by BioPharm Services was utilized for data that could not be determined otherwise. This software provides a dataset based on yearly cost reports from the industry, which allows the user to minimize the number of errors based on non-comprehensible assumptions. We generated data on the 1000 L fermentation scale, and all relevant process costs could be determined directly from our experiments. All other production scenarios were scaled based on the data generated from 1000 L and complemented with BioSolve data when necessary. Consumables were scaled according to their use in the scenario (e.g., flexware assemblies were used according to the corresponding skid and lifetime, whereas the amount of protein A resin was scaled for the amount of product). For the entire process design we used

**Table 1**
Scenario parameters.

| | Scenario | Capture method | USP titre | DSP yield[a] | | | Annual output | USP volume |
|---|---|---|---|---|---|---|---|---|
| | | | | Total | Prim. | Cap. | | |
| A | **Fed-Batch Centrifugation** | PCC | 4 g/L | 81 % | 95 % | 95 % | 50 kg/year<br>1000 kg/year<br>81 kg/year | 650 L (SU)<br>2 × 8000 L (SS)6 × 2000 L (SU)<br>1000 L (SU) |
| B | **Fed-Batch pDADMAC-Flocculation** | PCC | 4 g/L | 81 % | 95 % | 95 % | 50 kg/year<br>1000 kg/year<br>81 kg/year | 650 L (SU)<br>8 × 2000 L (SU)<br>1000 L (SU) |
| C | **Fed-Batch<br>Two-step Filtration** | PCC | 4 g/L | 81 % | 95 % | 95 % | 50 kg/year<br>1000 kg/year<br>81 kg/year | 650 L (SU)<br>8 × 2000 L (SU)<br>1000 L (SU) |
| D | **Perfusion**[b]<br>**Precipitation + TFF** | Prec. + TFF | 1 g/L | 86 % | – | 95 % | 50 kg/year<br>1000 kg/year<br>266 kg/year | 200 L (SU)<br>4000 L (SS)<br>1000 L (SU) |
| E | **Perfusion**[b]<br>**PCC** | PCC | 1 g/L | 86 % | – | 95 % | 50 kg/year<br>1000 kg/year<br>266 kg/year | 200 L (SU)<br>4000 L (SS)<br>1000 L (SU) |
| P | **Platform mAb process** | Batch Prot. A | 4 g/L | 54 % | 85 % | 90 % | 50 kg/year<br>1000 kg/year<br>54 kg/year | 950 L (SU)<br>2 × 11,000 L (SS)<br>1000 L (SU) |

[a] All yields were determined in 1000 L scale production except for scenario P, in which a BioSolve process was utilized as reference.
[b] 1.5 vol exchanges per day.

the default values of the BioSolve "parameter" sheet. This sheet contains construction and manufacturing related parameters. Media and buffers were modelled to be prepared once per batch for fed-batch scenarios and once per production day for all perfusion scenarios. No buffer and media concentration was included in this model. For all 1000 kg/year scenarios we estimated a total personnel amount of 25. For all other scenarios we estimated a total personnel amount of 10. The unit operations were scaled according to the actual scenarios (e.g., primary separations were scaled to wet cell weight and not fermentation volume). In addition to the economic evaluation, modelling with BioSolve enabled analysis of the ecological impact, as well as the PMI, which allows a comparison of the total resource consumption of each scenario. Compared to the PMI of Eq. (1), BioSolve also includes cleaning water in the calculations. For all scenarios the PMI calculation of Eq. (2) is applied.

$$TotalPMI = \frac{processwater\,(kg) + cleaningwater\,(kg) + rawmaterials\,(kg) + consumables\,(kg)}{product\,(kg)} \quad (2)$$

The price of 1 L water can vary depending on different factors like purity grade, produced amount of clean water, municipal water quality or production handling. The comparison of purchased water to water produced within the facility needs to be considered as well. All of these uncertainty factors may cause a miscalculation of the water price.

To reduce the risk of this miscalculation, a sensitivity analysis was integrated into the modelling to evaluate the impact of the water price for each scenario. The price per litre WFI was compared within the range of 1 €/L and 4 €/L. These ranges were chosen to display concrete changes of the CoG in case the price per L WFI is 4 times higher. This kind of sensitivity analysis simplifies the understanding of cost dedication within BioSolve. Furthermore, it shows a different way apart from the WFI price calculation, which is classically generated by the software. With the collected data, all scenarios were compared based on the price impact per litre WFI, CoG, PMI, footprint, and cost split between Upstream Processing (USP) and Downstream Processing (DSP).

## 3. Results and discussion

### 3.1. Definition of scenarios

In our evaluation, we compared different manufacturing scenarios for a platform antibody process (Fig. 1, Process P) and compared this platform process to different primary recovery and capture alternatives (Fig. 1, Process A–C). These scenarios were then converted to a continuous downstream process. As such, we compared perfusion with fed-batch fermentation, a precipitation-based capture scenario with PCC Protein A capture, and single-use primary recovery methods using flocculation and depth filtration with a conventional centrifuge (Fig. 1, Table 1). The actual process was operated on a 1000 L fermentation scale, with which we then modelled different output-based scenarios of 50 kg/year and 1000 kg/year.

### 3.2. Floor space requirements

For each unit operation, we measured the required floor space for the 1000 L scale. Each unit operation has dedicated air classification varying from class A to D for production and U for non-classified air requirements. The required floor space for each scale was calculated using the physical dimensions of each device and allowing for free space within them, which was expressed as the total occupancy rate in the facility. For each scenario using single-use equipment, we assumed 50 % occupancy due to mobile accessible devices and fewer intermediate tanks. Thus, 50 % of the available floor space of the production hall is filled with equipment, whereas the rest of the floor space is required for personnel. For all stainless steel fermentations, an occupancy rate of 35 % was assumed for more intermediate tanks and equipment accessibility. Case studies, where integrated continuous biomanufacturing processes were evaluated, already showed design principles, where clean room class D can be applied for antibody purification. Ideally fully continuous unit operations do not require any storage tanks. PCC operations however have an interrupted eluate output. When precipation is used in a fully closed system, we consider reduced air quality requirements from C to D for this unit operation to demonstrate potential differences (Boedeker and Magnus, 2017). The floor space required for each continuous downstream scenario is shown in Fig. 2A. For each scale, continuous upstream processing with perfusion and continuous capture with PCC requires the smallest footprint.

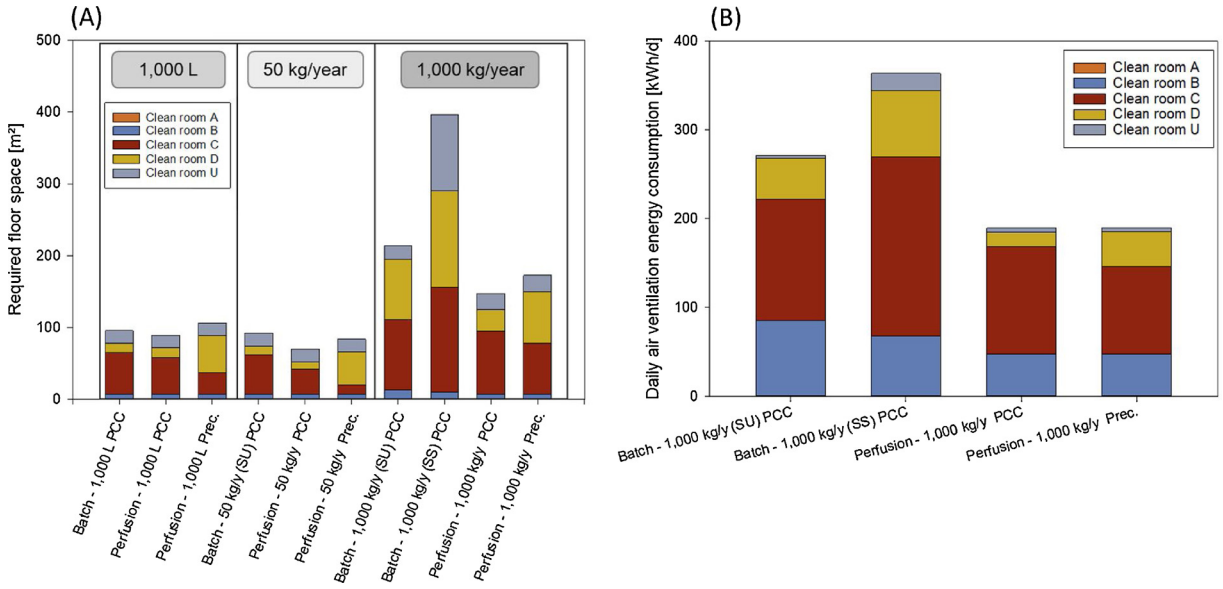


**Fig. 2.** A: Floor space dedication for each production scenario. All scenarios were calculated with single-use (SU) equipment for fermentation except the 1000 kg/year stainless steel (SS) scenario; B: Energy consumption caused by air ventilation for all 1000 kg/year scenarios.

The required stainless steel buffer and media tanks, as well as the piping and instrumentation for larger bioreactors, cause the difference between the 1000 kg/year stainless steel production and the other scenarios. By switching from a hybrid process of fed-batch upstream and continuous downstream towards fully continuous production, the footprint can be reduced by up to 63 % for stainless steel fed-batch compared to perfusion PCC and 31 % for single-use fed-batch compared to perfusion PCC (Fig. 2A).

According to Annex 6, 2002 WHO report clean room class D can also be classified as class 100,000 and clean room class C as class 10,000 (WHO Expert Committee, 2008). Jaisinghani showed how many air changes per hour are required for each clean room class. Based on these values we determined an average amount of 360/360/75/30/10 air changes per hour for clean room classes A/B/C/D/U (Aircuity guidelines, 2012; Jaisinghani, 2006). Based on the amount of air changes per hour, a room height of 4 m and a 24 h operation per day we calculated the energy consumption for all 1000 kg/year scenarios using a specific fan power of 0.192 Wh/m$^3$ (Pérez-Lombard et al., 2012). Fig. 2B shows that both perfusion scenarios consume less energy caused by air ventilation compared to the fed-batch scenarios.

Nearly all perfusion scenarios have reduced floor space demand compared to all fed-batch scenarios. Due to the water needed for cleaning, the stainless steel fed-batch scenario requires more floor space than the single-use scenario.

Precipitation or PCC have relatively similar floor space requirements, but the air quality requirement is lower for precipitation and will lead to reduced CoG and upfront investment.

### 3.3. Primary recovery scenarios

We compared three different primary separation setups (Fig. 1, Process A–C) in terms of cost in BioSolve and the ecological footprint on the basis of PMI analysis. The first scenario was a conventional centrifugation step using a disk stack centrifuge (Fig. 1, Process A), which has a high initial capital cost but low running cost. The second scenario was flocculation-assisted depth filtration using pDADMAC (Fig. 1, Process B). The third scenario was conventional depth filtration using a course and fine depth filter for primary separation (Fig. 1, Process C). Capture, intermediate, and polishing were not taken into account because they would not change the evaluation. The volume and product yield after primary recovery for each process option were the same. Both depth filtration-based separations were run in single-use mode in this scenario, replacing all tubing and filters after each batch, whereas the disk stack centrifuge is used for a lifetime of 10 years. We evaluated different scales of production (expressed in annual production of antibody) for all scenarios and compared the respective cost for each. Depending on the scales and facility, all scenarios had different benefits for certain production scales. Fig. 3 shows the cost difference of both single-use options compared to the disk stack centrifuge for the primary recovery steps. The flocculation-assisted scenario using pDADMAC saves up to 10 % for the CoG compared to the centrifugation for smaller scales of 50 kg/year. For larger scales, however, both single-use solutions increased costs up to 10 %.

The performance of the centrifugation scenario compared to the other scenarios can shift depending on the fermentation volume, titre,

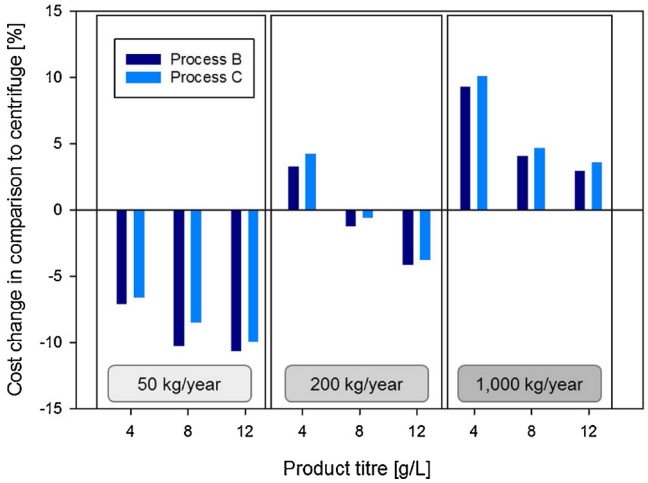


**Fig. 3.** Relative unit-operation related CoG comparison for the pDADMAC flocculation-assisted microfiltration (Process B) and two-step filtration (Process C) scenarios compared to conventional centrifugation (Process A). The single-use solutions are represented as bars. The conventional centrifugation is implemented as reference and represented by the baseline. Bars above the baseline represent higher CoG of the single-use scenarios compared to the centrifugation and vice versa. The titre varied from 4 *g*/L to 12 *g*/L, and the annual output represents an orphan, intermediate, and blockbuster antibody based on the output.

and annual product demand. Various scenarios with different titres and annual product output were compared to the disk stack centrifuge in terms of cost (Fig. 3). With lower annual output (50 kg/year), the improvement in product titre had a small impact on the CoG. The single-use options are more favourable than a disk stack centrifuge for smaller operations (i.e., 50 kg/year). A simulation was added for a production scale of 200 kg/year. Walther and Godawat (Walther et al., 2015) also stated that this is a common manufacturing scale. At low titre, the centrifugation (Process A) has better economic performance compared to single-use solutions (Process B and C). Higher process volumes must be handled at lower titres, increasing the required filter area. The flocculation-assisted depth filtration with pDADMAC consistently outperformed the conventional two-step filtration on all scales and with all titres. The capital costs for the disk stack centrifuge are included in the model by distributing the cost over the number of batches during the life of the centrifuge, which is typically assumed with a 10-year depreciation period. This means that, with higher production per year, the contribution of the capital costs reduces progressively.

The PMIs for the 50 kg and 1000 kg annual production were compared. For both scales, the water consumption for centrifugation was much higher compared to the single-use solutions (Fig. 4). We hypothesized that PMI correlates with the CoG. A correlation between the PMI and CoG was seen for smaller scales (compare Figs. 3 and 4A). The PMI of the primary recovery by filtration and CoG were lower compared to centrifugation. However, the PMI of the centrifugation was the highest but the CoG the lowest with the 1000 kg scale (compare Figs. 3 and 4B). This finding can be explained by the high consumable cost of both single-use solutions. On the large-scale, the centrifugation becomes more efficient and less water is consumed for cleaning the equipment. Total filter costs outweigh the costs of water consumption in centrifugation. In this case, a reduced PMI can be achieved by filtration, but the disadvantage of large expenses outweighs the benefit of the PMI.

### 3.4. Switching from conventional batch DSP to continuous

Batch platform antibody production was used as a reference (Fig. 1, Process P) and compared to different versions of batch USP and batch primary recovery followed by continuous DSP (Fig. 1, Process A and B). Process C (Fig. 1) was not considered because we have shown that the primary recovery was not better than with Process B. In Fig. 5, the CoG of continuous DSP is compared to the reference process. For each scale, continuous DSP had reduced CoG. Especially for smaller scales and higher titres (12 g/L), the potential cost reduction of 57 % can be achieved by applying the flocculation-assisted filtration. The product yield of the continuous DSP was 81 %, compared to 54 % for the platform reference process from BioSolve. This is a very conservative calculation because the state of the art antibody process may also have an overall product yield of more than 70 % (Walther et al., 2015). The difference in cost is reduced for higher scales in the course of the contribution of consumable costs. Though only SU bioreactors are used for the continuous scenarios, the platform process for larger scales was operated by stainless steel fermentation. We also took pre-packed columns into consideration, which were compared to acrylic columns for the conventional process. The change from single-use to stainless steel for the platform process can reduce costs for 1000 kg/year scale processes, but the PMI is increased 53 % when comparing 50 kg/year and 1000 kg/year.

### 3.5. Economics of alternative continuous capture steps

To quantify the influence of future non-chromatographic unit operations, such as precipitation, we used the data generated in our Horizon 2020 project (Burgstaller et al., 2019). Polyethylene glycol (PEG) precipitation was applied as a capture step and is suitable for coupling to an industrial perfusion reactor. We compared this non-chromatographic unit operation (Fig. 1, Process D) with PCC (Fig. 1, Process E) in a completely end-to-end continuous process on different scales: 50 kg/year, 1000 kg/year, and a 1000 L fermentation. The CoG and process water consumption were calculated. For the PCC scenario (Process E), the capture output volume was based on the eluate, whereas for precipitation, a 13-fold concentration was implemented based on lab-scale data. For our model, we assumed that a titre of 1 g/L is reached in the perfusion culture. This assumption is based on the perfusion culture of our Horizon 2020 project, which was run at pilot-scale. The parameters for economic evaluation and process water consumption were obtained from this end-to-end pilot-scale experiments. The CoG and PMI of both scenarios (Process D and E) at different scales are shown in Fig. 6A. For larger scales, the PMI and CoG were higher in the precipitation scenarios than the PCC scenarios. For the 1000 L scale, in which 266 kg/year can be produced, and the 1000 kg/year scale, the increased CoG can be correlated with the PMI. The volume reduction is lower for the precipitation than the chromatographic process (Process E). To keep the residence time constant for the subsequent chromatographic flow-through steps, we had to increase the size of the AEX and CEX columns to process the material at the same time. However, the volume stays constant in precipitation regardless of titre. Therefore, this method has advantages for high titre feedstocks. Budzinski et al. (Budzinski et al., 2018) stated that the PMI increases with the number of chromatography unit operations in a process. Based on this statement we can indicate that increased column volumes with standard bind-elute operation methods lead to a higher water consumption. Increasing the binding capacity of the column will lead to less column volumes and less buffer consumption. For protein A capture we tested MabSelect SuRe, with a dynamic binding capacity of 44 $g_{mAb}/L_{Resin}$ and Amsphere A3, which had a dynamic binding capacity of 65 $g_{mAb}/L_{Resin}$. The PMI

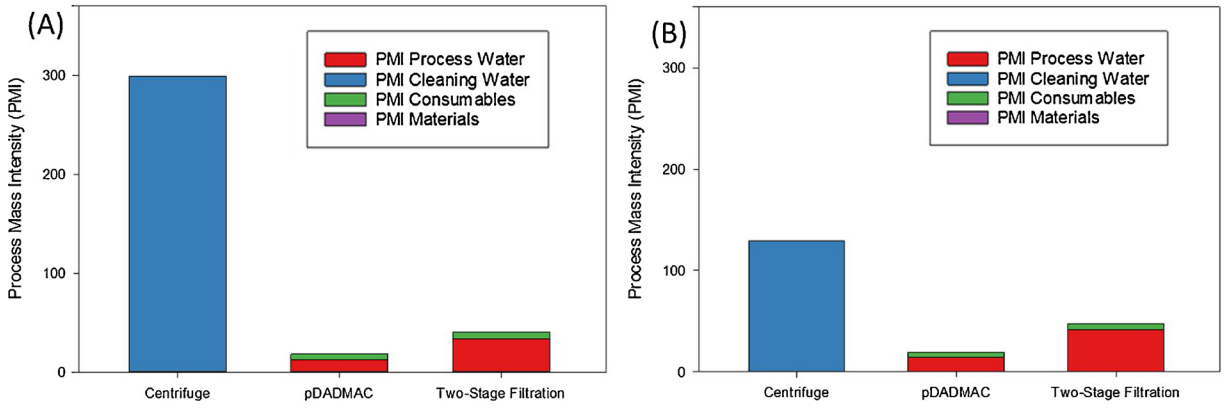


**Fig. 4.** PMI of primary recovery scenarios. A: 50 kg/year scale; B: 1000 kg/year scale. This figure considers the PMI of the specific primary recovery devices without taking other unit operations into account.

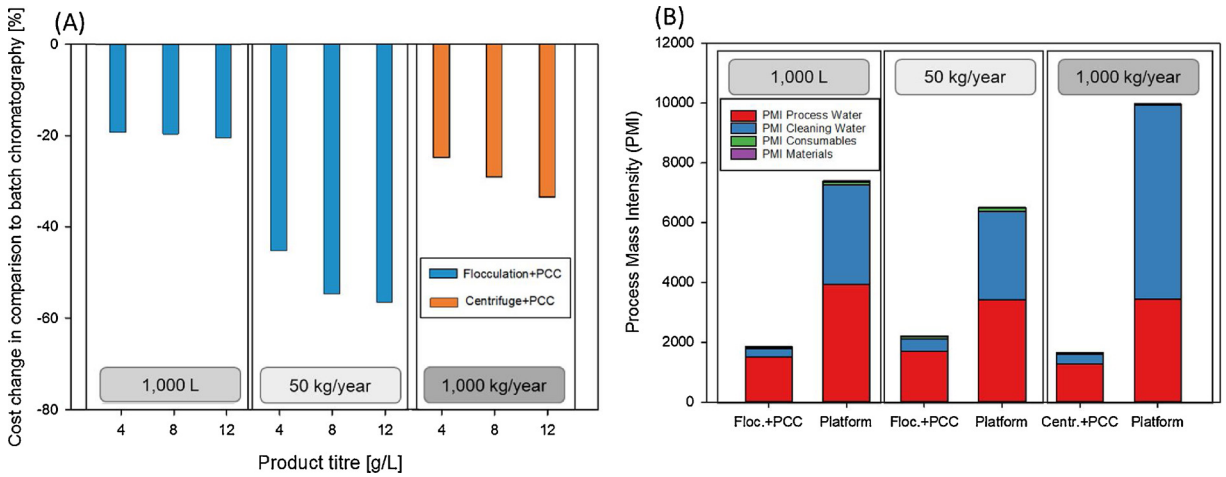


**Fig. 5.** A: Relative CoG evaluation of the entire production train. Continuous downstream prototype containing Protein A PCC (Process B for 1000 L and 50 kg/year, Process A for 1000 kg/year)) was compared to a conventional antibody process using batch protein A chromatography (Process P). The continuous operations are represented as bars. The conventional antibody process is implemented as reference and represented by the baseline. Bars above the baseline represent higher CoG of continuous downstream compared to Process P and vice versa B: PMI analysis of the platform process and continuous purification. This figure considers the PMI of the entire production train.

of MabSelect Sure was thereby higher than the PMI of Amsphere A3. This impact however is reduced for flow-through chromatography steps, where less column volumes for regeneration are required until the column can be reloaded.

For a yearly production of 50 kg, the lower volume reduction and larger polishing columns when using precipitation as a capture step are compensated for by the lower cost of unit operation. Protein A costs dominate the CoG for the total process for the 50 kg/year scale. For each scale, the PMI was significantly higher for the precipitation process than for the PCC (Fig. 6B). For higher scales, the CoG were higher for precipitation than the PCC. In addition, the precipitation performance varies based on the price of the WFI. These numbers do not take into account a potential difference in performance of the polishing unit when switching to alternative technologies, as there is no data available on our process in regards to polishing precipitated material. Therefore, these numbers have to be considered with caution and will need to be partially revised once more data is available in the literature for polishing of precipitated mAb material.

For the 1000 L scale, higher WFI prices had greater CoG differences, and the gap of the 1000 kg/year scale was reduced for higher WFI prices. In this scenario, a relative comparison of the CoG revealed a price difference of approximately 30 %, whereas the PMI was increased only 15 % for the precipitation. This means that, even though the absolute CoG for the precipitation is higher than for the PCC, the relative difference of both scenarios is reduced. For the 50 kg/year scale, the PMI benefit of the PCC scenario cannot achieve cost savings compared to precipitation because of the impact of the cost of the protein A column.

### 3.6. Hybrid and perfusion scenarios

We compared a hybrid process to fed-batch fermentation with a product titre of 4 g/L, followed by the best performing primary recovery and PCC for capture at each scale (Process A or Process B) with perfusion fermentation and a product titre of 1 g/L followed by PCC capture (Process E). Primary recovery was not required because the effluent of the perfusion culture could be processed directly by PCC. For the output-based scenarios, however, the fed-batch scenarios had reduced CoG. Fig. 7A shows the CoG comparison of a fully continuous process (Process E) to a hybrid process (Process A or B) and, for the 50

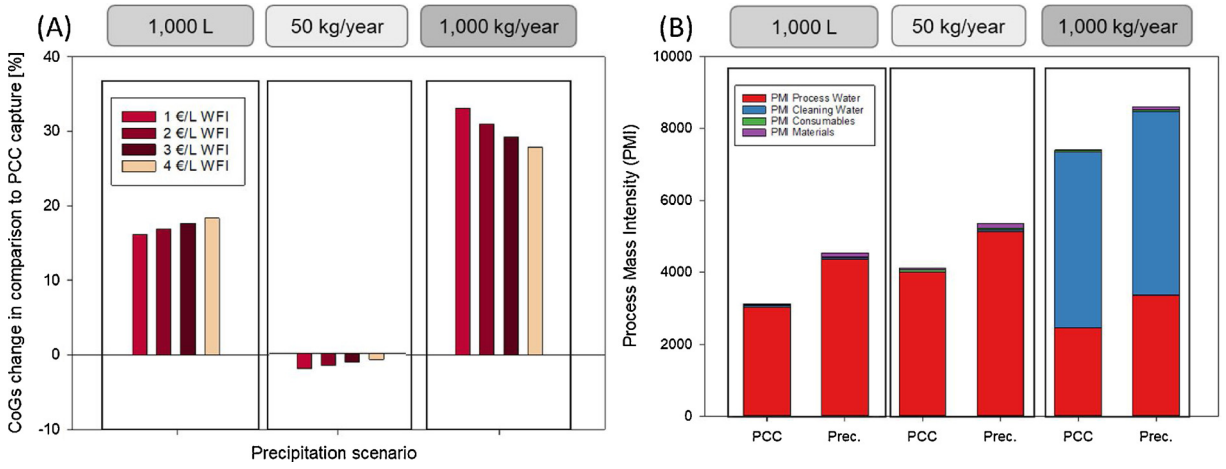


**Fig. 6.** A: CoG evaluation comparing the Perfusion Precipitation scenario (Process D) and Perfusion PCC scenario (Process E). Process D is represented with bars. Process B is implemented as reference and represented by the baseline. Different water prices were considered for both scenarios. Bars above the baseline represent higher CoG of Process D compared to Process E and vice versa B: PMI analysis of the Perfusion PCC and Perfusion Precipitation scenario. This figure considers the PMI of the entire production train.

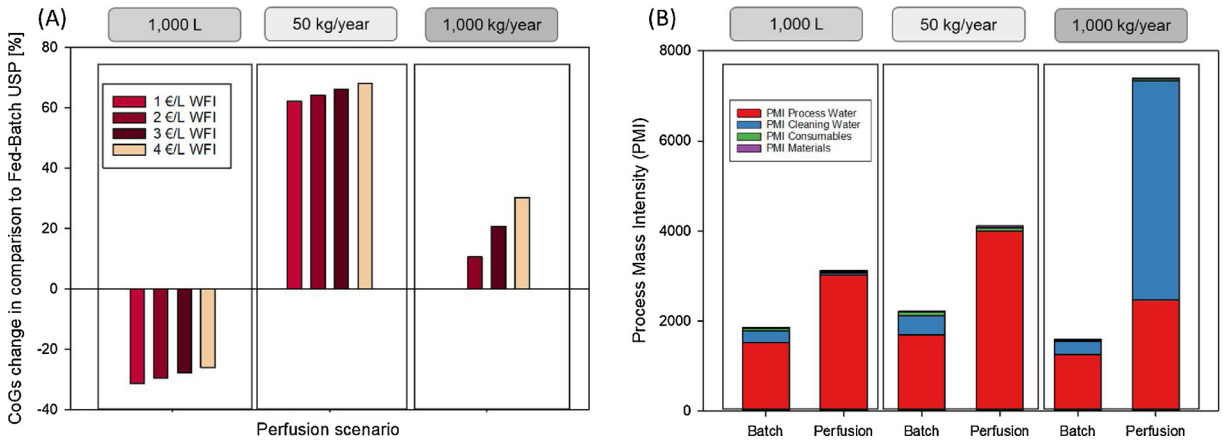


**Fig. 7.** A: CoG evaluation of the perfusion PCC scenarios (Process E) compared to the fed-batch PCC scenarios. Process E is represented with bars. The best performing fed-batch scenario (Process B for 50 kg/year and 1000 L, Process A for 1000 kg/year) is taken as reference and represented by the baseline. Different water prices were considered for both scenarios. Bars above the baseline represent higher CoG of Process E compared to fed-batch operation and vice versa. B: PMI analysis of the batch PCC and perfusion PCC scenarios. This figure considers the PMI of the entire production train.

kg/year scale, the fully continuous process costs are up to 68 % higher than fed-batch. For a 1000 kg/year scale, the performance of the perfusion scenario depends on the price per litre WFI. For this scale, the WFI price has a significant impact on the CoG. The reason for the sensitivity of perfusion-based processes on the cost of process water is the higher water demand of such processes (Fig. 7B). The relatively low titre of 1 g/L in perfusion processes compared to the 4 g/L for fed-batch leads to an increased demand for media to achieve the same product output as fed-batch and, therefore, higher water consumption. For the 1000 kg/year scale, we replaced all single-use storage bags in the models, which were larger than 2000 L with stainless steel vessels. The perfusion scenario on the 1000 kg/year scale had a significant increase in PMI due to cleaning the stainless steel vessels. By achieving a perfusion titre of 2 g/L, we calculated a reduced CoG for the perfusion on the 1000 kg/year scale of up to 24 % compared to a titre of 1 g/L and reduced PMI of 34 %.

Considering both the overall process water consumption and the CoG of these scenarios, batch processes have an advantage over perfusion processes.

Previous comparisons (Process A–C; Process A, B, P; Process D, E) showed potential water and cost savings for switching from batch-wise operation to continuous operation and from single-use to stainless steel devices. However, continuous upstream operations resulting in lower titres compared to fed-batch have a substantial impact on the economics of fully continuous antibody production (Hammerschmidt et al., 2016).

### *3.7. Water related process parameters*

The PMI displays the total water consumption of a process. However previous results have shown that not only the direct contribution of water is responsible for different CoG. Each unit operation with a relative high amount of consumed water have some high impact cost factors, which are connected to a higher water consumption. For USP a higher PMI is connected to higher media costs. Larger amounts of high cost cell culture media lead to higher CoG. For DSP especially chromatography operations have a strong impact on an increased PMI (see 3.5). Buffer ingredients are hereby the driving costs linked with the water consumption. Throughout the entire process, media and buffers need to be prepared and stored in bags or vessels. The equipment sizing is linked to the amount of required water. Bigger vessels require more floor space and have increased capital costs as well. Also other auxiliary costs such as piping and instrumentation are increased with a higher water consumption. Acquiring all of this information separately increases the quantity of assumptions for the economic evaluation. The risk of miscalculation caused by assumptions in a wrong dimension increases. Using the PMI as a key metric simplifies the overall amount of assumptions if the cost contribution of single-use consumables is taken into account.

## 4. Conclusion

From our economic evaluation and analysis of PMI, we conclude that Process A is the best for the large-scale scenario with 1000 kg/year and Process B for the small-scale scenario with 50 kg/year. Switching from fed-batch USP with PCC capture to perfusion with PCC capture reduces the floor space requirements in all of the evaluated scenarios. The fermentation volume-based 1000 L scale is not comparable to the other scales due to different annual product outputs for fed-batch and perfusion USP (81 kg/year and 266 kg/year, respectively). However, perfusion has higher equipment utilization for the DSP equipment, which is one of the biggest benefits of continuous manufacturing compared to batch operation. This leads to improved CoG despite a higher PMI. The hypothesis that the CoG correlates with the PMI is true in all scenarios except when the consumable costs outweigh the water costs. Our evaluation confirms that the PMI can be used to determine potential cost savings in terms of storage floor space, tanks, and labour. Implementation of the PMI can also lead to more sustainable production regarding buffer recycling, which is currently still a burden for the biopharmaceutical industry (Jungbauer and Walch, 2015).

An overall judgement regarding the best performing unit operations and process trains cannot be reached. It is very important to take the annual scale into consideration. The most striking example is primary recovery at small-scale, for which the flocculation-assisted filtration performs better than centrifugation, and at large-scale is the opposite. The economic perspective for continuous integrated biomanufacturing is very attractive, but environmental consequences should not be neglected. Here, we have shown that perfusion has a higher environmental footprint in the form of water consumption compared to fed-batch. As general guidance to improve process economics, we recommend reducing water consumption.

## Author contributions

Cataldo A.L: Conducted economic modelling and drafted the manuscript

Burgstaller D: Experiments for continuous antibody capture, basis for parameter estimation of modelling

Hribar G: Provided data for modelling of industrial scale and helped to define the scenario for manufacturing

Jungbauer A: Designed the research project and co-authored the manuscript, overlooked the research

Satzer P: Supervised the project and deliverables of economic modelling

## Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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# Water related impact of energy: Cost and carbon footprint analysis of water for biopharmaceuticals from tap to waste

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ABSTRACT

Manufacturing of biopharmaceuticals requires high quality water. Overall conditions for production of clean water vary across the globe due to availability and quality. We combined economic and ecological modeling to assess energy consumption of water production from tap to waste. We defined a metric, the WAter Related Impact of ENergy (WARIEN) to directly correlate the amount of $CO_2$ emitted per kg biopharmaceutical and included membrane- and distillation-based methods for clean water production. Three scenarios for production of antibodies with a 500 L fed-batch fermentation with stainless steel, or single-use or a 100 L perfusion were assessed. The WARIEN varied from 16 to 89 kg $CO_2$/kg antibody. Highest is the production with perfusion in stainless steel using distillation-based water and lowest the fed-batch with single-use using membrane-based water. The water related costs per kg product and the WARIEN correlate and therefore can be used as a design criterion.


## 1. Introduction

### 1.1. Cold and hot WFI

The quality and production process of pharmaceutical water is regulated by several monographs. The production of water for injection (WFI) and purified water (PW) is clearly defined (Ph. Eur.0008, 2005; Ph.Eur.0169, 2016) in the European Pharmacopoeia, likewise in the US pharmacopeia and others (JP17 Rev., 2016; Ph.Eur.0008, 2005; Ph.Eur.0169, 2016; US Pharmacopoeia, 2006). Before 2017, distillation of feed water was the exclusive method for the production of WFI in Europe. Since then, membrane-based techniques are also listed as acceptable methods for the purification of water (Ph.Eur.0008, 2005; Ph.Eur.0169, 2016).

Membrane-based methods can run at a lower temperature and are therefore referred to as cold WFI production. The term hot WFI production is used for the distillation technique (Fig. 1).

In the European Pharmacopeia (Ph. Eur. Monograph 0008) water is specified as PW when a softening step via ion exchange chromatography or antiscalant followed by reverse osmosis (RO) and electro-deionization (EDI) is applied. The addition of antiscalants is beneficial for membrane-based methods since they reduce fouling, but they require additional monitoring. For WFI production, the PW is produced by distillation and called hot WFI or by ultrafiltration step called cold WFI according to the Ph. Eur. Monograph 0169. Depending on the needed quality, clean steam (CS) is either produced via vaporization of PW or WFI

*Abbreviations:* API, Active pharmaceutical ingredient; c, Specific heat capacity; C, Cooling; CEI, $CO_2$ emission intensity; CEQ, $CO_2$ equivalent; CIP, Cleaning in Place; CMI, Cleaning Mass Intensity; CoG, OCoG, Cost of Goods; CS, Clean Steam; CSC, Clean Steam (Cold); CSH, Clean Steam (Hot); DEC, Dedicated Energy Consumption; E, Electric; ECoG, Energy Cost of Goods; EDI, Electro-deionization; EER, Energy efficiency ration; M, Mass; P, Price; PMI, Process Mass Intensity; PS, Plant Steam; PW, Purified Water; Q, Heat; RO, Reverse Osmosis; S, Steam; SIP, Sterilization in Place; SOP, Standard operating procedure; T, Temperature; TOC, Total organic carbon; UC, Universal (Cold); UH, Universal (Hot); W, Water; WARIEN, WAter Related Impact of ENergy; WC, WFI (Cold); WFI, Water for Injection; WH, WFI (Hot); η, Efficiency.

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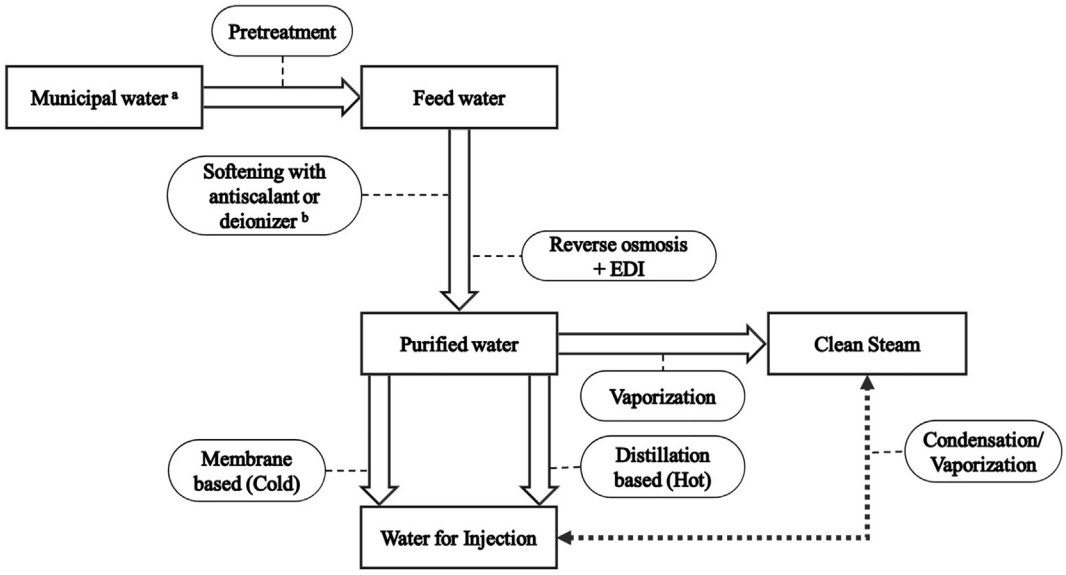


**Fig. 1.** Production flow diagram of all biopharmaceutical clean water classifications according to Ph. Eur. Monograph 0008 and 0169 (Ph.Eur.0008, 2005; Ph.Eur.0169, 2016).

(BWT, 2020; Letzner, 2016; MECO, 2019; Ph.Eur.0008, 2005; Ph. Eur.0169, 2016).

To assess the prevalence of hot or cold WFI production, we surveyed manufacturers and consultants from the biotech industry in Japan, US and Germany about the challenges of membrane-based water purification compared to distillation based. Survey participants included member companies of the American Chemical Society (ACS) Green Chemistry Institute Pharmaceutical Roundtable (GCIPR; https://www.acs.org/gcipharmaroundtable), as well as manufacturers and consultants of WFI plants.

In Japan, WFI is allowed to be produced via membrane-based methods according to the Japanese Pharmacopoeia (JP17 Rev., 2016). However, the precaution of avoiding microbial contamination has the highest priority for the purification process, thus, in reality WFI is produced only via distillation in Japanese biomanufacturing companies, regardless of the Pharmacopoeia. In Europe the majority of companies also produce WFI via distillation. However, since 2017 WFI skid manufacturers are keen to demonstrate that the purity of membrane-based WFI is equivalent compared to distillation based WFI, while costs can be reduced (BWT, 2020; Letzner, 2016; MECO, 2019). In the US WFI produced via membrane-based methods needs to be proven to have a quality that is "equivalent or superior to distillation" (US Pharmacopoeia, 2006).

We received data of 25 different WFI facilities within the ranges of 0.7 to 10.2 $m^3$/h. All of these facilities produce WFI via distillation.

Although the majority of companies use hot WFI production, applying membrane-based purification exhibits certain advantages. It reduces the steam demand significantly, which represents the biggest cost contribution of distillation based WFI (MECO, 2019). Furthermore, monitoring of PW quality becomes obsolete, because the skid is designed to produce WFI without intermediate storage of PW (Letzner, 2016).

However, for cold WFI the effort of avoiding microbial contamination represents a greater challenge than for hot WFI. After distillation WFI is stored with a temperature of 80 °C to keep the total organic carbon (TOC) and endotoxin levels low. For cold WFI ozonation coupled with UV-treatment can be applied in order to reduce all organic compounds in the water. The monitoring effort of cold ozonated WFI is increased and still represents a challenge. Alternatively, also WFI produced with membrane technology can be heated to 80 °C with steam to reduce the risk of microbial contamination (WHO, 2010).

### 1.2. Economic importance of WFI water supply

As we mentioned different production scenarios for WFI, the price of producing 1 $m^3$ WFI cannot be generalized, but heavily depends on the actual facility. Different attributes of the production site may facilitate or complicate the purification: Size of the water production facility, availability and purity grade of municipal water in different regions, changing water pretreatment for different seasons, required water temperature at the point of use and certainly the different methods of producing WFI. Also available rivers next to the site can be used to reduce energy costs for cooling (MECO, 2019; Röder, 2016).

For the conventional production of recombinant monoclonal antibody high impact cost parameters do correlate with increased water consumption. As an example, reduced product titers of a fermentation increase the total media demand to produce the same amount of product. The floor space demand for media storage is increased as well as the labor demand during media preparation and the use of WFI itself (Cataldo et al., 2020). The process mass intensity (PMI) is a valuable tool to depict the resource consumption for producing 1 kg of an active pharmaceutical ingredient (API) according to Eq. (1). In this metric, only the weight of each resource, which is used in the process, is considered (Budzinski et al., 2019; Cataldo et al., 2020; Madabhushi et al., 2018).

$$TotalPMI = \frac{totalwater, rawmaterials, consumablesusedinprocess(kg)}{activepharmaceuticalingredient(kg)} \quad (1)$$

Using this metric, the contribution of water represents more than 90% of the total PMI and is therefore more a metric of total water consumption and underrepresenting significantly other resource consumptions (Budzinski et al., 2019).

Performing an economic and ecological model for biomanufacturing represents a great challenge. The PMI can be applied more easily than performing exact cost of goods evaluation. The PMI is restricted to the kg of water used in the process, but does not relate to the different quality levels of water, and the respective energy needed to produce them. We expanded the PMI to include energy

contributions to produce the water with different quality. We introduced a novel metric, where we included the energy demand required to produce different stages of clean water: The WAter Related Impact of ENergy (WARIEN). The utilization of the WARIEN enhances the economic and the ecological evaluation of a bioprocess. The environmental footprint can be determined more specifically including the energy demand of the process as well as costs for $CO_2$ taxes for energy production.

In this manuscript, we consider different production methods to produce WFI, PW and CS. In contrast to previous economic water price calculations focusing on clean water production only, we follow a holistic approach and consider cost and carbon footprint for clean water production and decontamination. We also take into consideration varying quality requirements of water in the process. Furthermore, we evaluate three different cell culture cultivation methods regarding overall water consumption. Water consumption for process and cleaning procedures for a 100 L perfusion culture with a duration of 30 days is compared with two consecutive 500 L fed-batch cultures. For fed-batch culture we considered stainless steel and single-use.

We also show that the WARIEN metric quantifies how much water related $CO_2$ is emitted per kg API, in the actual case antibody. We also explore, if the WARIEN correlates with costs for water production and is suitable as design criterion.

## 2. Materials and methods

### 2.1. Definition of WARIEN

The WARIEN metric enables further utilization of the PMI and interconnects resources used by the bioprocesses with the production train of water. Furthermore, as the WARIEN yields energy consumption data, it enables a $CO_2$ evaluation using the $CO_2$ emission intensity (CEI) per kWh of the specific country where the facility is located (Table 1). The total $CO_2$ output then can be used to estimate process related costs of purchasing $CO_2$ certificates or indicate the environmental impact of water-related energy consumption of the facility.

The WARIEN for cold and hot WFI is derived via Eq. (2) for hot WFI and Eq. (3) for cold WFI. Besides the water related PMI values the correspondent $CO_2$ equivalent (CEQ) is required for the determination of the WARIEN. For Eq. (2) the sum CEQ values coupled with the PMI of hot WFI (WH), PW and CS of the hot skid (CSH) need to be considered and for Eq. (3) the values of cold WFI (WC) and CS of the cold skid (CSC).

$$WARIEN = PMI_{WFI} * CEQ_{WH} + PMI_{PW} * CEQ_{PW} + PMI_{CS} * CEQ_{CSH} \quad (2)$$

**Table 1**
$CEI_E$ of different countries.

| Country | $CEI_E$ [kg $CO_2$/kWh] | Year | Reference |
|---|---|---|---|
| EU average | 0.296 | 2016 | (EEA, 2018) |
| Austria | 0.085 | 2016 | (EEA, 2018) |
| Germany | 0.441 | 2016 | (EEA, 2018) |
| France | 0.059 | 2016 | (EEA, 2018) |
| United Kingdom | 0.281 | 2016 | (EEA, 2018) |
| Italy | 0.256 | 2016 | (EEA, 2018) |
| Spain | 0.265 | 2016 | (EEA, 2018) |
| Poland | 0.773 | 2016 | (EEA, 2018) |
| Japan | 0.516 | 2016 | (FEPC, 2018) |
| United States | 0.449 | 2018 | (EIA, 2020) |
| India | 0.680 | 2018 | (IEA, 2020) |
| China | 0.711 | 2013 | (Compare your country, 2014) |

$$WARIEN = (PMI_{WFI} + PMI_{PW}) * CEQ_{WC} + PMI_{CS} * CEQ_{CSC} \quad (3)$$

At first, the total PMI (Eq. (1)) can be adjusted to reveal the total water consumption per kg API using the water related PMI ($PMI_W$) in Eq. (4).

$$PMI_W = \frac{totalwaterusedinprocess(kg)}{activepharmaceuticalingredient(kg)} \quad (4)$$

The $PMI_W$ can be determined with economic modelling software, such as BioSolve Process 8 by BioPharm Services©. In this calculation the entire consumption of cleaning water is implemented in the PMI as well. Apart from the PMI Budzinski et al. (Budzinski et al., 2019) suggested to analyze cleaning processes of storage tanks or bioreactors individually using the Cleaning Mass Intensity (CMI) metric. With this metric water consumption of cleaning operations can be evaluated individually. Here we will use the BioSolve approach and include cleaning water in the PMI, thus $PMI_W$ can be further split into $PMI_{WFI}$, $PMI_{PW}$ and $PMI_{CS.}$ (Eq. (5)).

$$PMI_W = PMI_{PW} + PMI_{CS} + PMI_{WFI} \quad (5)$$

By splitting the PMI into its contributing components, the process related amount of required water for each component can be addressed individually.

For each quality grade of water we evaluated datasheets of different water facilities regarding the production capacity and the energy consumption. In this model the total energy consumption is split into three sections; electric energy, steam energy and cooling energy (Müller et al., 2014). We included compressed air as part of the electric energy, because its cost contribution is low compared to steam and cooling demand (Röder, 2016). Electric energy is required to run compressors, pumps, sensors etc. in order to run the facility. Plant Steam (PS) is required for distillation, clean steam generation, heating for storage and decontamination purposes. Steam generation is mainly based on oil or gas energy sources and has a different impact on costs and $CO_2$ emission compared to electric energy (Nieuwlaar et al., 2016). Cooling energy is generated via electric power consumption as well. However, different suppliers of recirculation coolers offer compressors with different energy efficiency ratios (EER) (Warwicker, 2010; "Water Re-Cooler,", 2020). For instance, compressors with an EER of 4 are able to produce up to 4 times more energy in a cooling circuit in relation to the electric power consumed, which reduces the costs per kWh cooling energy. For each clean water classification and each energy source the dedicated energy consumption (DEC) per metric ton water needs to be applied as a part of cost of goods (CoG) determination. The DEC reveals the amount of electric/steam/cooling energy required in order to produce 1 ton of WFI/PW/CS. For a detailed DEC calculation, a common energy demand required for all classifications of clean water is added up with each specific dedicated energy consumption after reaching the different branches of production. The dedicated universal energy consumption for hot WFI production ($DEC_{UH}$) explains the common energy consumption of all involved clean water classifications until the point of split shown in Eq. (6). For hot WFI production the point of split appears after the PW storage tank. For cold WFI production the $DEC_{UC}$ is applied considering the WFI storage tank as point of split.

$$DEC_{UHorUC} = \frac{Totalannual(electric)or(steam)or(cooling)energyuntilpointofsplit}{Annualnetwaterdemand} \quad (6)$$

The specific DEC values ($DEC_E$ for electric energy, $DEC_S$ for steam energy, $DEC_C$ for cooling energy) of CS and WFI consist of the universal DEC values and the additional energy required to produce and distribute the correspondent clean water classification

according to Eq. (7). As an example, the total DEC of purified water consists of the $DEC_{UH}$, the dedicated heating energy, which is required during distribution for the utilization of PW during a CIP process and the according decontamination energy demand. In this calculation in total 15 different DEC values are calculated considering 3 types of energy and 5 different clean water classifications. A detailed description of individual DEC calculations is shown in the supplementary sheet.

$$DEC = DEC_{UHorUC} + \frac{Totalannual(electric)or(steam)or(cooling)energyafterpointofsplit}{Annual(PW)or(WFI)or(CS)demand} \quad (7)$$

The $CO_2$ emission greatly varies depending on the power supply of different regions. Table 1 shows the CEI of several countries, exemplifying the large variety between countries. For PS generation mainly oil or gas are used, and their consumption can be directly related to the $CO_2$ output. These sources need to be treated separately, as well as cooling energy shown in Table 2, which is first adjusted for cooling efficiency and then treated as electric energy to be converted to $CO_2$ emission. The price per kWh may vary significantly depending on the market price of oil or gas.

For each stated source of consumed energy the CEQ needs to be calculated in order to reveal, how much $CO_2$ is emitted per metric ton clean water. Therefore, each calculated DEC needs to be multiplied with the correspondent CEI for each energy source (Eq. (8)).

$$CEQ = CEI_E * DEC_E + CEI_S * DEC_S + CEI_C * DEC_C \quad (8)$$

With the specific CEQ and PMI for each classification the WARIEN can be calculated in order to reveal the water related $CO_2$ emission per kg API.

### 2.2. Showcase fermentation

The WARIEN metric is applied in three mammalian cell culture showcase unit operations where an industrial relevant monoclonal antibody is expressed. The unit operation definition including antibody titers, sequence of operations, size of operation etc. is largely based on an industrial showcase published earlier (Burgstaller et al., 2019; Cataldo et al., 2020). We compare a fed-batch cultivation performed in a 500 L stainless steel tank, performed in a 500 L single-use tank and a 100 L perfusion culture operated in a stainless steel tank. For comparability of the fed-batch processes and the continuous process, both are designed to yield the same yearly amount of antibody as already described (Cataldo et al., 2020). In this comparison the different $PMI_W$ are going to be analyzed as well as two simulated water plants comparing distillation and membrane-based WFI production.

The WARIEN was determined for each scenario. In Table 3 the process parameters for a 30 day production scenario are shown. During this period a perfusion with a net runtime of 25 days or 2 consecutive fed-batch processes can be performed. For cleaning it is assumed that 2 reactor volumes of water are required respecting the correspondent cleaning standard operating procedure (SOP). The showcase is based on existing data.

In the showcase facility the water supply has a capacity of 2 $m^3$/h, whereas 1.5 $m^3$/h are required on average. Assuming a runtime of 22 h per day with 345 production days per year (7600 h per year) a total of 11 400 $m^3$ clean water can be produced annually. In Table 4 information of hypothetical hot and cold WFI production skids is shown. The utilized data is based on personal communication to WFI skid suppliers and previous WFI production cost evaluations. The capital expenses are depreciated within 10 years. Additionally, maintenance costs are considered on an annual base assuming 20 000 € for both scenarios. Overall monitoring and audit costs are covered in this calculation with a total lump of 100 000 € for both scenarios. This parameter may increase the costs per $m^3$ WFI by about 9 €, if the value is increased to 200 000 € instead of 100 000 €.

Furthermore, it has no impact on the WARIEN calculation. The process yields during water purification of each process step are most significant for the determination of the total gross water demand. For instance, in order to produce 1 $m^3$ WFI with a total yield of 70% in total 1.43 $m^3$ feed water are required (Budzinski et al., 2019; Steris, 2015). Process yields of each clean water classification are either listed in the datasheets of the devices or based on experience values of the water purification department. The total feed water price consist of the actual price per $m^3$ municipal water and the price per $m^3$ of waste water (MECO, 2019). The amount of required gross feed water can be determined respecting the yields of each process step during water purification. Also, for the total costs of salt for regenerating the deionization columns a

**Table 2**
Attributes of the utilized energy sources; data on oil and gas from (Carbonindipendent, 2020); electricity from own assumptions.

| Energy source | Gas | Oil | Electricity | Cooling |
|---|---|---|---|---|
| Costs per kWh | 0.05 €/kWh | 0.05 €/kWh | 0.15 €/kWh | 0.04 €/kWh* |
| $CO_2$ emission | 2.1 kg $CO_2/m^3$ | 3.0 kg-$CO_2$/L | – | – |
| Energy per unit | 11.2 kWh/$m^3$ | 10.28 kWh/L | 1 kWh/kWh | 4 kWh Cooling capacity/kWh |
| CEI | 0.185 kg $CO_2$/kWh | 0.288 kg $CO_2$/kWh | See Table 1 | See Table 1* |

* Value is determined by dividing the correspondent electricity value by the EER of 4 ("Water Re-Cooler," 2020).

**Table 3**
Process parameter – showcase fermentation.

| Parameters for 30 days operation | Perfusion 100 L | Fed-Batch 500 L |
|---|---|---|
| Volume exchanges per day | 1.6 VVD | – |
| Net production | 25 Days | 2 Batches |
| Titer | 1 g/L | 4 g/L |
| Media demand WFI (20 °C) | 4000 L | 1000 L |
| Cleaning water demand PW (80 °C) | 200 L | 2000 L (0 L for SU) |
| Produced product | 4 kg | 4 kg |
| Steam demand (SIP) | 15 kg | 150 kg |
| PMI WFI | 1000 kg/kg | 250 kg/kg |
| PMI PW | 50 kg/kg | 500 kg/kg (0 kg/kg for SU) |
| PMI Steam | 3.8 kg/kg | 37.5 kg/kg (0 kg/kg for SU) |

**Table 4**
General parameter – hot/cold WFI production and decontamination.

| Parameter | WFI/PW/steam supply (hot) | WFI/steam supply (cold) |
|---|---|---|
| Total feed water yield | 80% PW/70% WFI/75.5% CS | 70% WFI/66% CS |
| Total annual gross water demand | 15 761 $m^3$ | 16 384 $m^3$ |
| Share WFI/PW/CS | 75% WFI/ 20% PW/5% CS | 95% WFI/ 5% CS |
| Equipment - WFI production* | 500 000 € | 400 000 € |
| Equipment – decontamination | 200 000 € | 200 000 € |
| Depreciation period | 10 years | 10 years |
| Labor/monitoring costs per year | 100 000 € | 100 000 € |
| Maintenance costs per year | 20 000 € | 20 000 € |
| Feed water | 0.46 €/$m^3$ | 0.46 €/$m^3$ |
| Waste water | 0.69 €/$m^3$ | 0.69 €/$m^3$ |
| Regeneration salt demand | 0.75 kg/$m^3$ | 0.75 kg/$m^3$ |
| Regeneration salt price | 0.16 €/kg | 0.16 €/kg |
| Energy demand per ton PS | 814 kWh | 814 kWh |
| Number of distillation columns | 6 | – |
| Ratio PS demand per ton WFI | 1.4 | 1.4 |
| Ratio PS demand per ton CS | 1.15 | 1.15 |

*Equipment costs include all automation, sensors, spare parts and auxiliary devices.

**Table 5**
Electric energy consumption – hot/cold WFI production and decontamination.

| Device | Energy consumption |
|---|---|
| Production skid PW (hot) | 11 kW |
| WFI distillation skid (hot) | 2.5 kW |
| Membrane-based WFI skid (cold) | 13.5 kW |
| Clean steam generation | 1 kW |
| Storage temperature hold | 2.5 kW |
| 4 × UV-light | 4 × 55 W |
| Decontamination skid | 2.5 kW |

certain amount of gross feed water is necessary, because the deionization column is one of the first steps during the water purification.

For all devices of the showcase water facility, energy consumption is based on the values stated in Table 5. The references for values in this table are stated in the following chapter.

Increased energy demand of the PW production as well as for the entire cold WFI production are caused by high pressure pumps required for running the reverse osmosis.

### 2.3. Data acquisition

The price per L clean water has a significant variance depending on the required amount in a process. Capital cost contribution is higher for reduced water demand produced in the same water supply. The values used and calculations shown in this manuscript are either based on assumptions related to real facilities or datasheets of different suppliers. To verify the precision of the stated estimations and calculations, we cooperated with multiple companies in Europe, the United States and Asia using the network of the ACS Green Chemistry Institute Pharmaceutical Roundtable, experience from water supply facility engineers and expertise of GMP biopharmaceutical plant construction consultants. Water requirement during cleaning processes in smaller scales could also be delivered in an on-site pilot plant at BOKU with available stainless steel bioreactor scales up to 200 L.

Uninterrupted WFI availability in the final process has the highest priority. Producing PW is a continuous process in order to reduce accumulation of organic material on the RO-filter membranes. For reduced water demand in the process the total flowrate during the water purification is reduced. For long term cost savings it is recommended to modify the water supply plant in order to optimize equipment occupancy and floor space utilization. For energy consumption of multi-effect water stills and pure steam generators devices datasheets of Steris© Finn-Aqua® T-series were analyzed (Steris, 2015). For cold and hot WFI production as well as for the pretreatment of municipal water datasheets of Bilfinger SE© SWG and PWG purification skids were utilized. General parameters about WFI production were taken of information sheets by Bosch Packaging Technology GmbH©, Letzner GmbH©, MECO Incorporated© and BWT AG© (Bosch Packaging Tehnology, 2017; BWT, 2020; Letzner, 2016; MECO, 2019). For continuous decontamination datasheets of effluent decontamination systems by ©Actini Group were analyzed (Actini, 2018). The according references also contain energy consumption for sanitization (Daugelat et al., 2008; Gregoriades et al., 2003; Van Vaerenbergh et al., 2012). For cooling purposes, the datasheets of recirculation coolers by the Q series by WTG-Quantor GmbH© were utilized ("Water Re-Cooler," 2020).

### 2.4. Heating and cooling

Energy demand for heating and cooling for storage, distribution and decontamination were calculated with the heat equation according to Eq. (9), where c is 4.2 kJ/kgK for water.

$$Q = c * m * \Delta T * \frac{1}{\eta} \tag{9}$$

The overall efficiency ($\eta$) 87.5% is assumed. For more complex pipe paths between the energy source and the heat exchanger this value may be lower and vice versa.

In Fig. 2 the possible paths of all clean water classifications are shown. Different stream colors represent the temperature of the streams. Temperatures of up to 10 °C are indicated in blue, room temperature liquids in yellow and hot liquids above 80 °C as well as steam in red. PW is cooled to 10 °C before storage (Bosch Packaging Tehnology, 2017). Depending on the application in the process the PW needs to be heated during the distribution in case of a cleaning operation. For media requirement the temperature is slowly adjusted to room temperature with controlled pipe isolation, which means that no energy needs to be consumed. In this simulation cold WFI is heated to 80 °C with PS for storage. The temperature of the heated WFI is kept constant, similarly to the distillation based WFI production, using a flow through heater with electric power supply. If the WFI is used for media or buffers, it needs to be cooled to 20 °C during distribution. For CIP purposes no cooling is required. For decontamination we estimate that 50% of the used clean water in the process is stored in a decontamination tank. This estimation is based on personal communication with companies of the ACS GCIPR. The contaminated waste stream

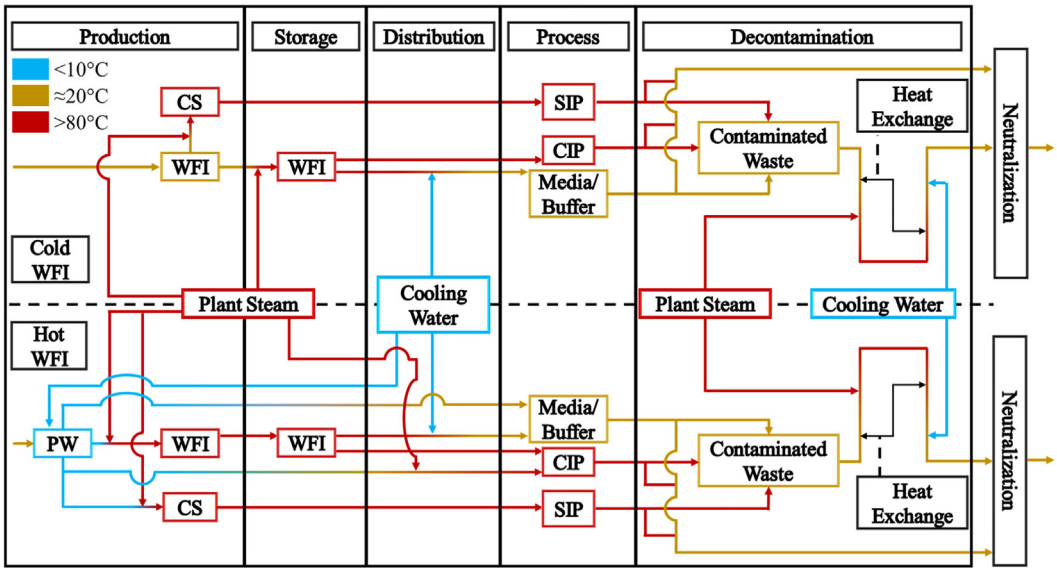


**Fig. 2.** Flow scheme of hot/cold WFI production train from tap to waste and its heating/cooling utilities. Different colors of the streams and tanks indicate the temperature.

is heated to 140 °C for sterilization and needs to be cooled to 20 °C before the stream is mixed with non-contaminated waste and a final pH adjustment step (Actini, 2018; Gregoriades et al., 2003). Liquids, which have no contact with biologic material flow directly into the final neutralization tank. During this heating and cooling process heat is recovered within the waste stream. The simulated waste stream is assumed to achieve 20 °C heating/cooling exchange with the heat recovery (Actini, 2018).

### 2.5. Water price determination

A generalized determination of the price per L WFI cannot be made because the variance between different facilities is too high and we therefore opted for a specific scenario. In this manuscript capital expenses are included in the price determination applying a 10 year depreciation. The annual depreciation rate can then be split by the amount of WFI generated in this period to calculate the capital cost contribution per $m^3$ WFI. For equipment it is assumed that the facility is built in an already existing room, where an old water plant is going to be replaced. As a consequence floor space demand is not considered a part of the cost calculation.

The corresponding values stated in Table 4 are based on personal communication with Bilfinger and a WFI price analysis by MECO (MECO, 2019). Capital expenses include the skid for water purification and decontamination, storage, piping, construction, automation, all probes and auxiliaries required to produce WFI GMP compliantly (Röder, 2016). Regular maintenance, requalification and spare part acquisition is included as a fixed value on an annual basis. For labor costs it is assumed that one person is full-time dedicated to the water plant for monitoring. Consumables, such as filter membranes, are also related to the skid and changed on a regular basis (Röder, 2016). Raw materials, such as salt for regenerating the ion exchange chromatography, as well as the costs of feed and waste water are calculated independently of the facility and based on the water throughput only (MECO, 2019). Energy related costs are split into electric, steam and cooling energy costs (Müller et al., 2014).

With the DEC of each energy source the energy cost of goods (ECoG) can be determined for each water classification according to Eq. (10) respecting the price per kWh (P) for each energy source.

$$ECoG = P_E * DEC_E + P_S * DEC_S + P_C * DEC_C \quad (10)$$

The overhead cost of goods (OCoG) is calculated by dividing all non-energy annual cost parameter by the net demand of required water per year (Eq. (11)).

$$OCoG = \frac{Equip. + Labor + Feed + Waste + Salt}{Annualnetwaterdemand} \quad (11)$$

The total CoG for each classification is determined by adding up the correspondent ECoG value with the general OCoG value of either the hot or cold production skid (Eq. (12)).

$$CoG = ECoG + OCoG \quad (12)$$

## 3. Results and discussion

### 3.1. Showcase fermentation – PMI and cleaning

In this study a production showcase was generated in order to analyze the overall impact of water consumption for the production of a biopharmaceutical, which was largely based on a real showcase of antibody production of our previous studies (Cataldo et al., 2020).

During 30 days of production a total of 2 fed-batch processes expressing Chinese hamster ovary cells can be performed based on the parameters given in Table 3. During that period in total 4 kg of monoclonal antibody can be produced in a 500 L reactor with a product titer of 4 g/L. The $PMI_W$ for this scenario is 787.5 kg water/kg API applying Eq. (5) using the data of Table 3. For fed-batch processes we are evaluating two scenarios, one single-use and one stainless steel. By using single-use technology all cleaning efforts, including validation and change over time, become obsolete. This reduces the dedicated $PMI_W$ with the data used in Table 3 to a total of 250 kg water/kg API. A perfusion process with 100 L production volume, a product titer of 1 g/L, net production time of 25 days and 1.6 volume exchanges per day is capable of producing the same amount of product in the same time period. However, the reduced product titer of this process shows a higher $PMI_W$ of 1053.8 kg water/kg API mainly caused by media demand.

In the case of continuous operation, about 95% of the $PMI_W$ are media related. Several publications already showed that hybrid antibody processes using fed-batch fermentation and continuous downstream operation have a better economic performance than

a fully continuous or batch-wise process (Cataldo et al., 2020; Klutz et al., 2016). By doubling the titer to 2 g/L the $PMI_W$ will nearly halve to a total of 553.8 kg water/kg API, which is less than the stainless steel fed-batch scenario. Additionally, with a titer of 2 g/L the perfusion process is capable of producing the same amount of monoclonal antibody as a 1000 L fed-batch fermentation. Regarding the water consumption cleaning of the perfusion reactor is not significant. Thus, using a single-use reactor for perfusion is less beneficial compared to fed-batch fermentation. In our analysis for energy consumption, we also see differences between media and cleaning water. During media preparation the water requires room temperature, while cleaning water is processed with a higher temperature.

### 3.2. Showcase fermentation - price calculation of WFI plants

The price of clean water was calculated for hot and cold WFI production. All equations, which are shown in detail in the supplementary sheet, are dedicated to the different types of WFI production. The final results of the calculations can be seen in Fig. 3, Fig. 4, Fig. 5 and Table 6.

In Fig. 3 the results of the cost related calculations are shown. For the same amount of produced clean water it can be seen that the total annual costs show no significant difference between cold and hot water production. In the cold WFI production steam is used in order to minimize the contamination risk during storage. The difference between the distillation of the hot WFI and the storage heating for cold WFI only represent about 8% of the total annual costs. Additionally, distillation processes show a higher energetic efficiency, because the generated steam can be recovered. In this showcase the WFI distillation device with 6 distillation columns improves the efficacy of the distillation process by a factor of 6.This reduces the amount of required PS from 1.4 tons PS per ton WFI to 0.23 tons PS per ton WFI (Letzner, 2016; Steris, 2015). Labor and monitoring costs represent the highest share of process costs (40% for hot WFI and 46% for cold WFI). This value is determined by the amount of personnel costs dedicated to this WFI facility. If multiple facilities can be monitored by one person, the labor cost contribution for each facility is reduced. The monitoring effort for audits is independent of the amount of facilities. Comparing the total annual costs the utilization of the cold WFI is more cost effective. Furthermore, cold WFI production reduces the complexity of the heating and cooling loops shown in Fig. 2, because fewer streams are required. Annual costs for sanitization are below 100 €, because the amount of energy to heat the entire system to 80 °C is relatively low compared to the continuous steam demand for distillation or storage heating. In conclusion, total annual costs of the water plant can be reduced from 455 678 € (Hot) to 416 820 € (Cold).

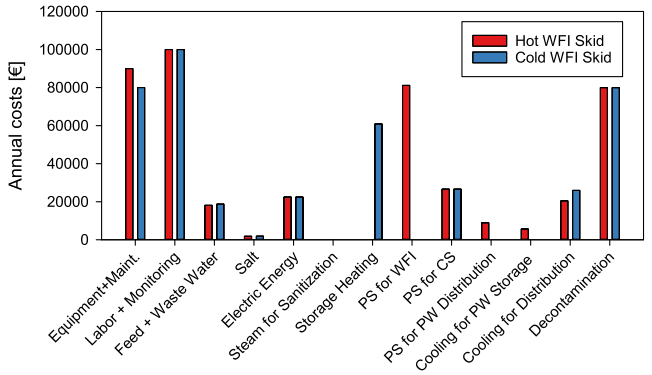


**Fig. 3.** Total annual cost split of a Hot/Cold WFI production train considering all energy related and overhead cost attributes.

### 3.3. Showcase fermentation – WARIEN and carbon footprint

The DEC values of each energy source for each clean water classification and the according CEQ are listed in Fig. 4 (A). For this calculation, Eq. (6) and Eq. (7) were applied individually for each energy source of each clean water classification to calculate the DEC. In total 15 different DEC values need to be calculated, represented by the stacked bars shown in Fig. 4 (A). For CS produced in the cold WFI setup the total steam energy covers about 92% of the total energy required. For cold WFI about 59% of the total energy demand is governed by steam energy required for storage heating and decontamination. DEC values for electric energy are relatively low for all classifications of water (4% of total energy for cold WFI). With the CEI of each energy source and the according DEC values the CEQ can be determined. The $CO_2$ emission of each type of clean water highly depends on the energy required to produce clean steam, with other contributions being almost negligible. For our calculation we used a showcase facility located in Austria, where the CEI is relatively low compared to other countries. In this simulation the application of a country with a high CEI has no significant impact on the WARIEN. The share of electric and cooling energy is relatively low compared to oil, which is used in this scenario in order to produce PS directly from combustion. In Fig. 4 (B) direct CEQ comparison of hot and cold water purification is shown. Total CEQ is reduced by 17% for the cold purification.

With the DEC values and the price per kWh of each energy source the ECoG for each classification can be calculated according to Eq. (10). The results of the calculation for each classification can be seen in Table 6. It can be seen that the ECoG highly correlates with the $DEC_S$ values, which is expected as the total energy needed is largely driven by energy needed for process steam. The overhead CoG for hot and cold WFI production were calculated according to Eq. (11). The total CoG for cold WFI are 14% lower compared to the hot WFI. It can be seen that the majority of the cost savings are based on the energy CoG rather than the overhead CoG. The energy CoG for WFI (Hot) is 67% more expensive compared to the energy CoG of the PW, which demonstrates that the large amount of steam required in this scenario leads to a significant increase of costs. CS (Hot) produced with PW is 89% more expensive than WFI (Hot) even though they both are vaporized at the same process stage. This significant price difference can be tracked on the $DEC_S$ values and is largely due to the increased efficacy of multiple distillation columns utilization for WFI production.

With the calculated CEQ values of each water type in Fig. 4 and the water related PMI values for each process scenario in Table 3 the WARIEN can be calculated according to Eq. (2) for hot WFI and Eq. (3) for cold WFI. The results can be seen in Fig. 5 (A). It is shown that the WARIEN values of the continuous production scenario is higher than for the 500 L SS scenario, because of the reduced titer and increased media demand. The WARIEN can be reduced by 26%, if the WFI is produced with membrane-based methods rather than distillation. Fig. 5 (B) shows the impact on water related costs respecting energy, overhead costs and costs of $CO_2$ tax. The $CO_2$ tax is estimated with 50 €/ton $CO_2$ based on average values in Europe ("Carbon Pricing Dashboard," 2020). The product related costs of $CO_2$ tax can be calculated with the WARIEN metric.

The correlating behavior between the WARIEN and the water related costs is explained by the DEC values, where WARIEN and energy cost calculation are based on.

The impact of the cleaning procedure is much bigger for the 500 L SS scenario in relation to the amount of produced API. For the fed-batch process the reactor needs to be cleaned twice during the given period of 30 days. The 100 L perfusion reactor only needs to be cleaned once in the same period and also has a reduced volume, which needs to be cleaned in order to produce the same

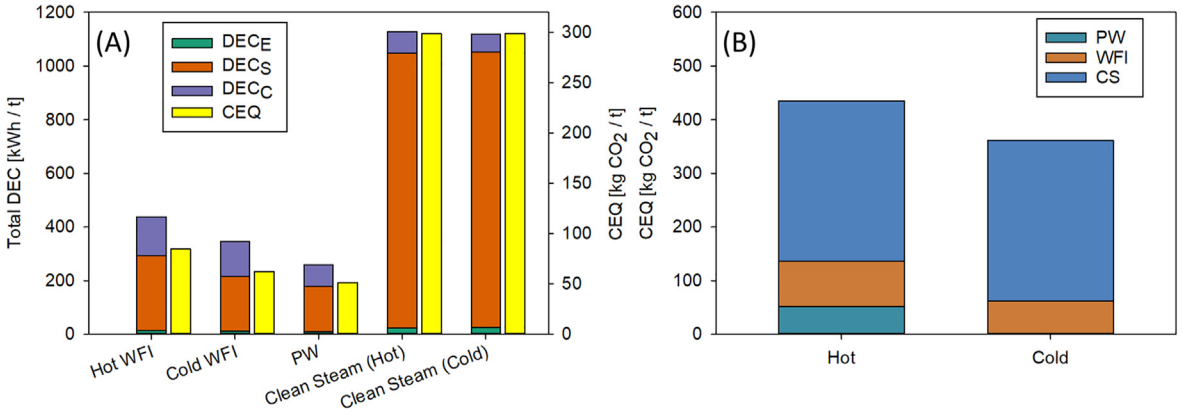


**Fig. 4.** (A): Results of the DEC calculation of each clean water classification and its corresponding CEQ. The stacked bars belong to the left Y-Axis, the yellow CEQ bar belongs to the right Y-Axis; (B): Accumulated CEQ values of hot and cold clean water classifications.

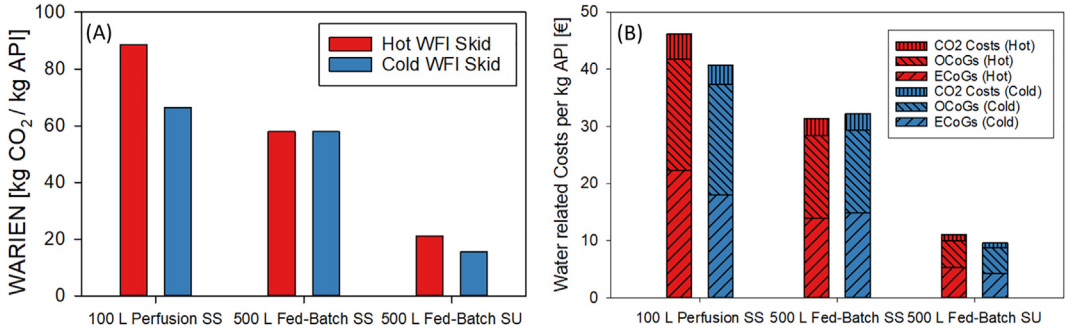


**Fig. 5.** (A) WARIEN of the showcase fermentation scenarios comparing hot and cold WFI production; (B) Water related costs of the Showcase Fermentation scenarios comparing hot and cold WFI production.

**Table 6**
Calculated CoG of each clean water classification.

| Parameter | ECoG per ton | OCoG per ton | Total costs per ton |
|---|---|---|---|
| WFI (hot) | 21.46 €/t | 18.43 €/t | 39.89 €/t |
| WFI (cold) | 16.91 €/t | 17.61 €/t | 34.52 €/t |
| PW | 12.83 €/t | 18.43 €/t | 31.26 €/t |
| CS (hot) | 57.70 €/t | 18.43 €/t | 76.13 €/t |
| CS (cold) | 57.70 €/t | 17.61 €/t | 75.31 €/t |

amount of product. The impact of the cleaning operation is even more significant, when the 500 L SS scenario is compared with the 500 L SU scenario, where no cleaning needs to be performed at all. In total the WARIEN is reduced by up to 72%, if single-use devices are applied in a fed-batch scenario. However, the $CO_2$ emission of the production of the single-use bags cannot be shown in the WARIEN model, which is tied to the water consumptions.

The WARIEN metric also shows that in the showcase facility the $CO_2$ consumption can be reduced for continuous operation and single-use fed-batch fermentation, if the WFI is produced via membrane-based methods.

Although the WARIEN has been exemplified for antibody production the metric is universal. It may serve as an indicator in biotechnology where excessive water is used and may be a rational guidance where to improve the environmental footprint of bioprocesses.

### *3.4. Impact of WARIEN*

Here we show how the WARIEN can be used for economic and ecological modeling of a bioprocess. In Fig. 5 (A) and (B), a correlation of WARIEN and water related CoG of the API can be observed. Previously we simulated a facility capable of producing 1,000 kg antibody per year with multiple 2,000 L reactors. In this simulation we showed that 272 kWh/d are consumed by air ventilation to achieve the respective cleanroom classification (Cataldo et al., 2020). With 345 production days per year and a CEIE of 0.085 kg CO2/kWh (Table 1; Austria) the respective CO2 emission is about 8 kg CO2/kg API. The lowest WARIEN calculated exclusively dedicated to fermentation with 16 kg CO2/kg API is higher compared to the air ventilation of an entire process including also downstream processing. The CoGs of antibody are in a range of 50 000–100 000 € per kg antibody according to Klutz et al. (Klutz et al., 2016). Considering this price range the contribution of the water related costs for fermentation would be below 1%. Typically this is not worth including it in economic optimization of a process. Overall, the DEC values are the basis of the WARIEN and the cost dedicated model. However WARIEN has a bigger significance on the ecological assessment of a process. The correlation seen in Fig. 5 clearly shows that reducing the carbon emission also leads to reduced costs. The WARIEN evaluation does not include the carbon footprint of single-use and stainless steel system manufacturing leading to a one-sided evaluation so far. The life cycle assessment of single-use systems and its total carbon footprint analysis require separate evaluation considering all emissions from raw materials to final disposal. Once such an analysis is available, overall emissions caused by single-use systems can be compared with the data generated with the WARIEN calculation.

## 4. Conclusion

We introduced a new metric called WARIEN to assess the water related $CO_2$ emission of a biopharmaceutical manufacturing processes and further analyzed the impact and correlation between

water related process costs and its carbon footprint. As an example we calculated the WARIEN of a conventional and single-use fed-batch and compared it with a continuous perfusion. We demonstrated the entire flow path of clean water in a facility from tap to waste and generated further utilization of the PMI metric. The calculations impressively showed that the $CO_2$ emission caused by water consumption parameters are correlating with water related process costs (see Fig. 5). While impact on carbon footprint is larger than air ventilation emission, the water related process costs are low compared to the overall biomanufacturing costs. The WARIEN may also be a decision aid to switch from hot to cold WFI production, depending on the manufacturing process. To calculate the WARIEN of an existing process may be also an incentive to redesign cleaning SOPs, because it may be an indicator for excessive water consumption. In our showcase the single-use fed-batch fermentation has the lowest water related $CO_2$ emission. However, additional research respecting the life cycle assessment of the plastics needs to be conducted in order to generate an equal carbon footprint comparison of stainless steel and single-use operation. Although the WARIEN has been exemplified for antibody production it can be used individually for future innovative bioprocess designs with the provided Excel-Sheet in the supplementary.

## CRediT authorship contribution statement

**Alessandro Luigi Cataldo:** Conceptualization, Methodology, Writing - original draft. **Bernhard Sissolak:** Project administration, Writing - review & editing, Funding acquisition. **Karl Metzger:** Investigation, Data curation. **Kristi Budzinski:** Validation, Investigation. **Osamu Shirokizawa:** Validation. **Markus Luchner:** Investigation, Validation. **Alois Jungbauer:** Supervision, Funding acquisition, Writing - review & editing. **Peter Satzer:** Conceptualization, Writing - review & editing, Supervision, Methodology.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data associated with this manuscript can be found in the online version: Supplementary File A; PDF-File: Calculation examples. Supplementary File B; Excel-File: Tool for individual WARIEN and WFI cost calculation. Supplementary data to this article can be found online at https://doi.org/10.1016/j.cesx.2020.100083.

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# Impact of failure rates, lot definitions and scheduling of upstream processes on the productivity of continuous integrated bioprocesses

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## Abstract

**BACKGROUND: The failure rates and the scheduling of bioprocesses have a substantial impact on process performance and economics but are often overlooked and neglected. Integrated continuous biomanufacturing is more flexible in respect to scheduling and the impact of failures can be reduced by appropriate scheduling and lot definitions.**

**RESULTS: In this work, we used a Monte Carlo approach on an integrated continuous biomanufacturing process with varying daily failure rates in the upstream and scheduling scenarios for seed fermentation (N-1 stage) to quantify the impact on the actual productive uptime of the integrated process. The optimum targeted production time in the continuous upstream ranges between 45 and 90 days depending on the daily failure rate and the lot definition used for the process. We showed that a minimal flexibility for planning of the seed fermentation is necessary to harvest the full potential of integrated continuous biomanufacturing. A comparison with batch manufacturing in the upstream processing showed a higher productive uptime for continuous biomanufacturing regardless of daily failure rates. Computation of productive uptime for different lot definitions showed that a daily lot definition only shows a loss of 3% to a maximum of 5% productivity, depending on the daily failure rate, compared to a real-time release approach.**

**CONCLUSIONS: With this study, we provide a decision-making tool for the scheduling of upstream processes and implementation of integrated continuous biomanufacturing taking failure into account, showing the extent to which planning of flexibility and batch definitions influence the productivity of continuous integrated bioprocesses.**


**Keywords:** productivity; lot; failure; continuous; bioprocessing

## INTRODUCTION

Continuous manufacturing is still in its infancy in the biotech sector mainly due to concerns for process stability, validation and quality, regulatory compliance and risk mitigation.[1–3] There is no rational understanding of how process failures may affect process economics and how they are affected by the scheduling of process trains. Continuous cell culture in perfusion mode is a crucial process intensification step for increased production capacities in a given volume.[4–6] In perfusion cultures a population of producing cells is maintained by constantly removing culture supernatant, bleeding off cells and constantly adding fresh media.[7] Efforts for integrated continuous manufacturing for the whole production train have to include downstream purification, where semi-continuous operations like periodic counter-current chromatography as well as truly continuous unit operations such as continuous precipitation for capture or polishing are employed.[5,8–10]

Despite clear evidence of the economic benefits, the flexibility for manufacturing and readily available upstream and downstream equipment for continuous processing, the transition to continuous biomanufacturing is in its early stages. Continuous integrated manufacturing comes with better economics and reduced facility footprint.[4,6,8] While continuous processing has

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been realized for small-molecule pharmaceutical production, the field of biotechnology is still reluctant. This is due to the high uncertainties connected with biological systems and risk assessment for continuous biomanufacturing is still an unknown territory for the biopharmaceutical industry.[11] In addition, the high investment costs for setting up new processes, and the already available facilities for batch-wise production of biopharmaceuticals hinder implementation further.[12] Nevertheless, the US Food and Drug Administration is taking steps to facilitate implementation of continuously produced drugs in the biopharmaceutical industry to improve product quality, reduce drug shortages, reduce costs for pharmaceuticals and implement automated monitoring for implementation of a quality-by-design approach[13,14] and has released draft guidance for continuous biomanufacturing.[15] Batch-wise and continuous manufacturing facilities must comply with the same quality standards, but a continuous process requires more sophisticated scheduling, monitoring tools, preferably on-line or in-line, and a higher degree of automation that can detect issues and react before a failure even occurs.[4,9]

Failure in a batch-defined setting is connected to one batch, whereas failure of a 'batch' in a continuous setting can mean a specific quantity or timeframe depending on the process definition.[13,16–18] Moreover, in an interconnected continuous manufacturing setup, a 'batch' failure can occur upstream, downstream or both. Hence, finding clear management strategies for handling failures and quantifying the potential impact is of particular importance.

A failure rate of production lots can be caused by equipment failure, human error or inability to meet specifications.[19] While preventative measures like equipment qualifications or personnel training can be applied to reduce them in a more general term, product-specific failures for not meeting certain specifications like aggregation levels or specific glycosylation have to be addressed case by case and are governed by the robustness of the product in terms of formulation, manufacturing procedure or analytical methodology.[20] While the failure rate definition for batch production is easily done with a statement of how many lots failed in comparison to how many succeeded, the definition for continuous manufacturing is more complicated as the failure may only apply to a certain quantity which can be isolated and discarded assuming proper monitoring and control strategies or can mean a catastrophic failure where the system needs to be restarted.[21,22] To ensure detection of failures, a more intense and accurate online monitoring and control instrumentation is required for increased product quality assurance in real time, which is amenable to real-time release testing approaches.[16,23–27] Real-time release ensures minimal product loss and an enhanced process reliability. One important parameter for lot description is the residence time distribution, dictating the spread of process disturbances and their traceability. A narrower residence time distribution results in less product lost in case of a failure.[13,28]

Failure rates and their impact in continuous manufacturing are hard to estimate and data are scarce especially for downstream operations, but for example Oyebolu *et al.* showed a continuous mAb process event, where ATF filter failure occurs with a probability of 2% causing a replacement of filters and perfusate discard of the consecutive 24 h of runtime.[29] However, occurrence rates and concrete impacts may vary significantly depending on the facility and expertise of the manufacturer, and whether the failure occurs upstream or downstream.[30] However, to the best of our knowledge, an investigation of how much product has to be discarded in the case of a failure and if this is significant enough in comparison to the effort of implementing real-time release has never been done.

Therefore, we evaluated the impact of different frequencies of batch failures at different points in biopharmaceutical production with a focus on upstream manufacturing including the seed fermentation stage (N-1) and scheduling of the N-1 stage. We further assumed a conservative approach in which a failure in the upstream process leads to a necessary restart of the perfusion process which takes several days. For downstream operations we assumed that a restart in the downstream process will lead to a temporary shutdown of 24 h. We used estimated failure rates[27] and utilized a Monte Carlo method to simulate the impact of different control strategies and lot definitions. This study quantifies the benefit of real-time release and the effects of scheduling of the N-1 stage as well as the facility flexibility on the loss of product and productive uptime of the entire facility.

## EXPERIMENTAL

### Process definition for Monte Carlo simulation

The process was defined as a day-by-day simulation of failure rates and a day-to-day allocation of possible failure events. The simulation was set up using a custom-built simulation in visual basic for applications run in Microsoft Excel and simulated a day-by-day run, accumulating 30 000 simulated runs for each data point and calculating a total uptime of the facility. The parameter uptime describes the ratio where the facility is actually productive and is defined by Eqn (1):

$$\text{Uptime} = \frac{t_{\text{total}} - t_{\text{start}} - t_{\text{flex}}}{t_{\text{total}}} \quad (1)$$

where $t_{\text{total}}$ represents the total time, $t_{\text{start}}$ the necessary start-up time for the upstream fermentation and $t_{\text{flex}}$ the time loss due to possible scheduling conflicts according to the scenario used for facility flexibility. The advantage of this key figure is that it is independent of scale and product, and can be translated to known product facilities. Additionally, it can be easily integrated into a future economic evaluation reducing the productive time of the respective process train.

The setup of the Monte Carlo simulation is depicted in Fig. 1, showing the start of each individual simulation, looping through a daily simulation and failure decision, adding the respective time penalty and saving it into the ensemble of individual runs for the specific daily failure rate and target runtime. From this ensemble in the database a total productive uptime is calculated, subtracting all non-productive days as described above.

Failure rates were estimated from the 15th Annual Report of Biopharmaceutical Manufacturing Capacity and Production.[27] A failure rate was attributed to each day individually and a range of failure rates was calculated as the estimation of failure rates from the literature is limited.

The process was defined as a seed fermentation (N-1), an upstream perfusion process with no interconnection to subsequent downstream operations, or one or more subsequent downstream operations. The seed fermentation was selected to be run in parallel with the upstream perfusion process if the next perfusion process starts on the planned day (and does not terminate early). Failure in the upstream leads to a complete process termination in our simulation as this simulation should inform the



  
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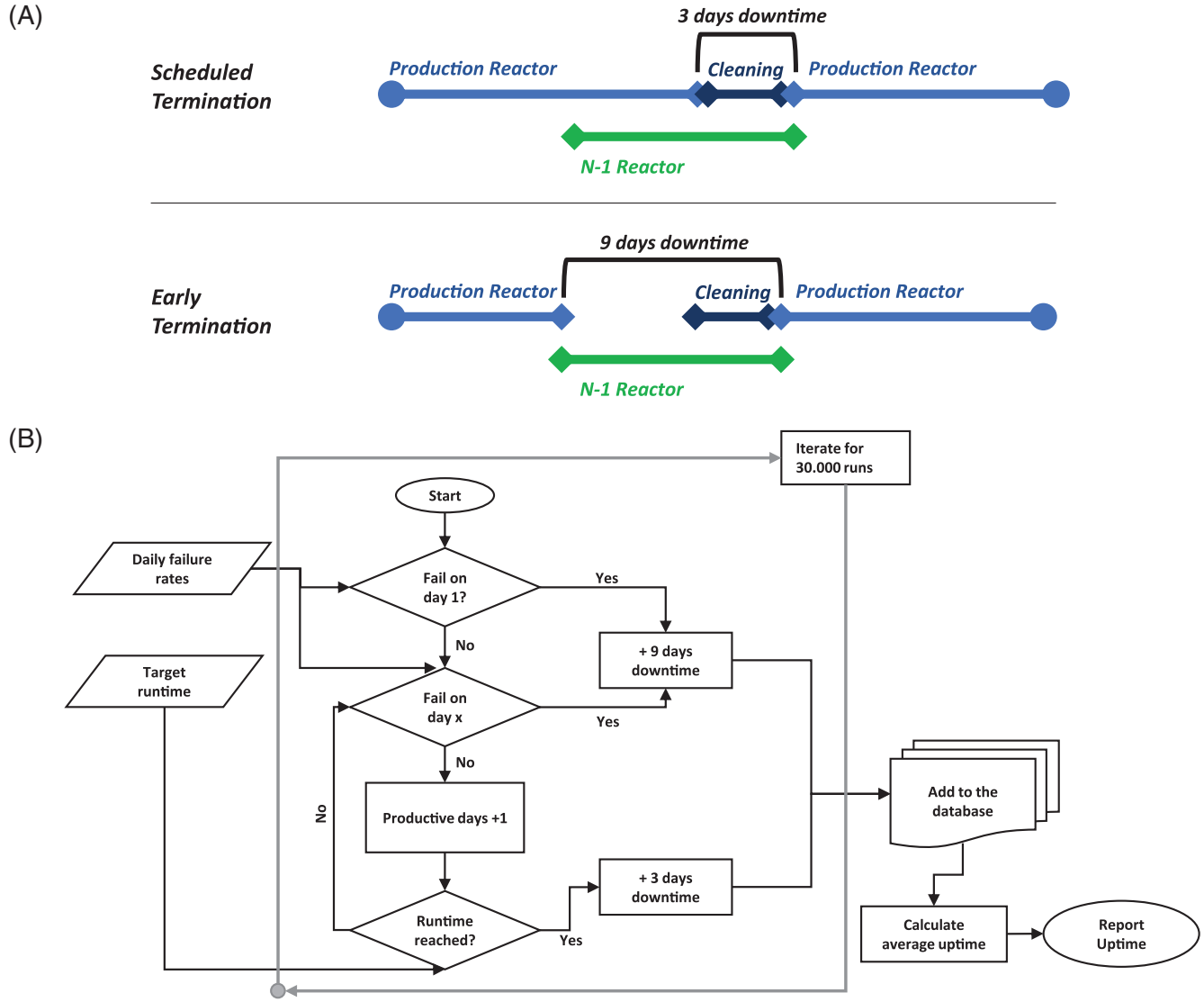


**Figure 1.** (A) Depiction of the downtime associated with a termination of the process on schedule with the N-1 started in advance of the process end, and an unscheduled termination of the process resulting in a larger downtime due to the start of the N-1 stage when the process terminates due to a failure. (B) Flow scheme of the Monte Carlo simulation depicting the simulation taking daily failure rates and target runtime as input, simulating individual runs with respective failure rates and reporting an average uptime across all simulated runs.

conservative stance on the impact on current implementation scenarios, and this reflects the current approach to batch production. Failure in the downstream does not lead to a complete termination in the model, but to a diversion of the production to waste for the time of the process interruption.

## RESULTS AND DISCUSSION

### Estimation of failure rates

Information on failure rates is limited in the literature, and only little information can be found on the cause of failures for batch production.[27] Information on failure rates for continuously operated manufacturing is not available at all and was estimated from batch failure rates. According to this survey, we can expect a batch failure rate due to contamination of 2.3%, and failures due to operator error, equipment failure or material failure of 1.5%, 1.4% and 0.6%, respectively. Additionally, there were failures due to failing to meet the specifications of 1.0%, cross-product contamination of 0.4% and other reasons of 0.4% (Fig. 2).

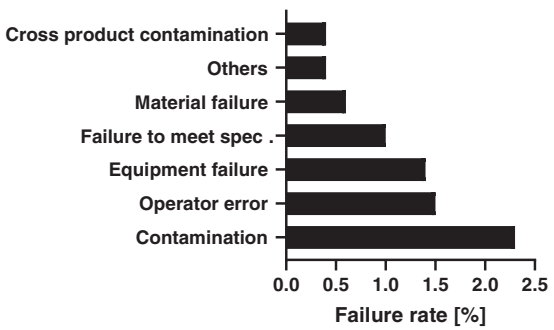


**Figure 2.** Reasons for batch failure for batches below a size of 1000 L. (Redrawn with data from the 15th Annual Report and Survey of Biopharmaceuticals Manufacturing Capacity and Production 2018.[27])

While the definition of failure rate as a ratio between successful and unsuccessful batches works for batch-mode operation, the definition of failure rate for continuous production must follow a



  
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different paradigm. In principle, continuous production is intended for an indefinite process. In reality, achieved process durations of 30 days or sometimes even shorter are already branded as continuous. However, the traditional batch definition can no longer be applied for continuous processes as material produced before a failure can still be used in the final product. This issue is also deeply connected with the definition of a 'lot' in regulatory terms for continuous products, as lots define the amount of material that needs to be discarded in the case of a failure. It is usually propagated that the ultimate goal for overcoming this issue in continuous manufacturing would be the application of real-time release, based on a sophisticated monitoring strategy and an advanced control regime.

The common redefinition of batches or lots for continuous operation is to treat a specific timeframe as lot, where the timeframe could be a minute, an hour or a day, depending on the monitoring capabilities implemented. In the case of a daily lot, the production material from the whole process train would be collected at the end for one day, and that collection can be tested for release as usual, or can be released automatically through a real-time release approach. This day-by-day definition will be used in this work to estimate the impact of failure rates on a daily basis on the whole-process productivity. In addition, we assume a conservative approach meaning that a failure of the culture leads to a termination of the process and a restart. This is a conservative approach that can be implemented today and we are aware that current and future developments might allow better process control.[9,31–37]

This continuous day-by-day definition and potential failures necessitate the handling of a dynamic schedule in the facility and the willingness to deal with early process terminations and restarting the continuous culture not only in a fixed schedule. In batch mode, the culture will run between 9 and 15 days in the production stage and will be discarded at the end when it is not suitable. In continuous mode, it has to be expected that some of the cultures, intended for 30 days or more, will not make it to the end. However, the material processed in the days before early termination will still go through purification, polishing and into a final product while the continuous culture will be restarted as soon as possible.

For transferring the batch failure rates into daily failure rates in a continuous setup, we decided to split the aforementioned risks mentioned in the survey into two groups. The first group consists of the contamination risk and the cross-product contamination risks which are more likely whenever there is a need for manipulation, for example during setup and inoculation at the start of the process, resulting in an estimated first day contamination risk of 2.7%. The second group consists of all other reasons, which can occur each day during cultivation, where equipment and materials can fail, and the operator can make errors. The accumulated total batch failure rate for these events can be estimated to be 4.9%. To transform this number to a daily failure risk we assume that the reported batches in this survey will have an average runtime of 12 days, and that these risks are evenly spread throughout the batch process. This translates then to a daily failure rate of 0.4% for all days during the production, and a failure rate of about 3% for the first day during setup of the process (including the contamination risk). We are aware that this translation of failure rates to the continuous system is prone to large systematic errors. The real failure rates could be substantially different for various reasons. Therefore, we chose to investigate a wide range of daily failure rates, ranging from 0.01% daily representing an exceptionally stable system, up to 2% daily, representing an exceptionally unstable system.

In addition, we assume in our models a completely uniform risk associated for the failure of different kinds for each day during production except the first day. Whereas this might be reasonable for operator errors and equipment failures, the chance for material failure or the failure to meet specifications might not be completely uniform. The culture might slowly drift out of specifications with time, and materials like plastics might age during the runtime and therefore have a higher associated risk later in the process than earlier. Unfortunately, there are no reliable data available in the literature to assess the nonlinearity of these risks, and our analysis might have to be revisited regarding these factors in the future when and if more data are available from industrial production plants running continuous manufacturing. For now, we intend to capture this variation sufficiently in our large range of daily failure rates investigated for this study, while more detailed and possible influence on specific process choices will have to be implemented in future studies when specific daily failure rate data are available. In this work we focus on catastrophic failures in production that lead to a restart of the system, as the data available are for catastrophic batch failures. Intermittent deviations where the process can be readjusted will be followed up in future publications as they require a detailed mechanistic or statistic process model of a specific process.

To enable easy translation of the results of this study to any production scenario or specific product, we calculate the actual uptime for the whole process train where the process train produces material. This uptime can be used regardless of facility size, product titre and yields in the process. Known parameters for a specific process can be easily adapted for a risk-including yield through the use of the planned uptime and the risk-reduced uptime reported in this study. This uptime can be further used to calculate the economics of a process including specific risk management strategies and expected failure rates. The expression of uptime is independent of cost, titre and scale and therefore more useful for a general investigation on the influence of risk and how to manage such risk successfully, while still keeping the possibility of integrating this key figure directly into any cost calculation.

### Influence of failure rate on scheduling and uptime in perfusion cultures and associated N-1 fermentations

To start the investigation, we modelled a perfusion culture and the connected pre-culture (N-1) which is needed for inoculation for the perfusion process. The perfusion process is planned for a certain maximum process runtime but might terminate early in the case of a failure. We modelled target process runtimes of between 7 and 320 days, where a shorter target runtime means that the process will more likely terminate as planned, while a longer target runtime naturally will lead to more early process terminations due to failure. In the case of a failure, the process is stopped, all material on the day of the failure goes to waste and all product produced before that will be further processed.

When the process reaches the planned maximum runtime, we assume that the N-1 stage is already finished as it can be planned to be run in parallel to the last days of the perfusion culture, whereas if the process terminates early, the perfusion reactor has to wait for the inoculum to be prepared. We assume that the N-1 stage will run for 9 days, which represents an additional downtime of the perfusion reactor in the case of early termination



  
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due to a failure. In addition, we assume that the cleaning, setup and start of the perfusion in the production reactor take 3 days which are not used for production, but can be accomplished in parallel to the N-1 stage. In summary in this first simulation, the time between consecutive batches is 3 days if the perfusion culture reached the target process runtime, whereas the time between batches is increased to 9 days in the case of an unexpected early termination due to a failure (Fig. 1(A)).

We simulated individual runs using Monte Carlo simulation, testing each day for a failure of the process with an associated daily failure rate and an increased failure rate for day one as described above. The simulation records each individual run and how long the productive time is and records the associated downtime for each run individually (either 3 days for a run completing on schedule or 9 days for a run terminating early due to a failure). The recorded runs are then used to calculate an average productive uptime across all runs. A process flow scheme for illustration of the simulation is presented in Fig. 1(B).

We simulated the uptime for different planned maximum process runtimes, from day one to the targeted end with daily failure rates between 0.01% and 2% (Fig. 3(A)). The uptime is improved with longer target runtimes with a markedly flattening curve for planned maximum process times longer than 90 days. When choosing 60 days planned maximum process time instead of 30 days we gain an additional 5–10% uptime depending on the daily failure rate. We gain almost nothing when changing from a 120 days planned process time to 150 days. As mentioned before, this is significant as some risk factors might not be linearly spread across the whole range, like genetic drift or the failure rate of plastics and other materials. From these curves, it is reasonable to argue for a planned runtime of 90 days regardless of daily failure rate. The gain in additional uptime is negligible for longer timeframes, and the uncertainty of the involved risks will increase with longer runtimes. Interestingly the curves in this simulation do not show an optimum, and a longer targeted runtime is always better. This is easily explained by the average time penalty for each day longer in the model. Each additional day of targeted runtime has an associated potential time penalty of 6 additional downtime days multiplied by the failure rate of that specific simulation (Eqn (2)). As long as the daily failure rate is below 16.7% (1 day additional productivity/6 days potential downtime) an additional day in the targeted runtime will always result in a higher average uptime (Eqn (3)).

$$[\text{average time penalty}] = [\text{time penalty for failure}] \times [\text{failure rate per day}] \quad (2)$$

$$[\text{breakeven failure rate}] = [\text{potentially gained productive time}] / [\text{time penalty for failure}] \quad (3)$$

Depending on the targeted process time and the daily risk, a larger or smaller portion of processes will be terminated early (Fig. 3(B)). Even for small failure rates of below 0.05% per day, the facility will have to accommodate for 10% of the runs

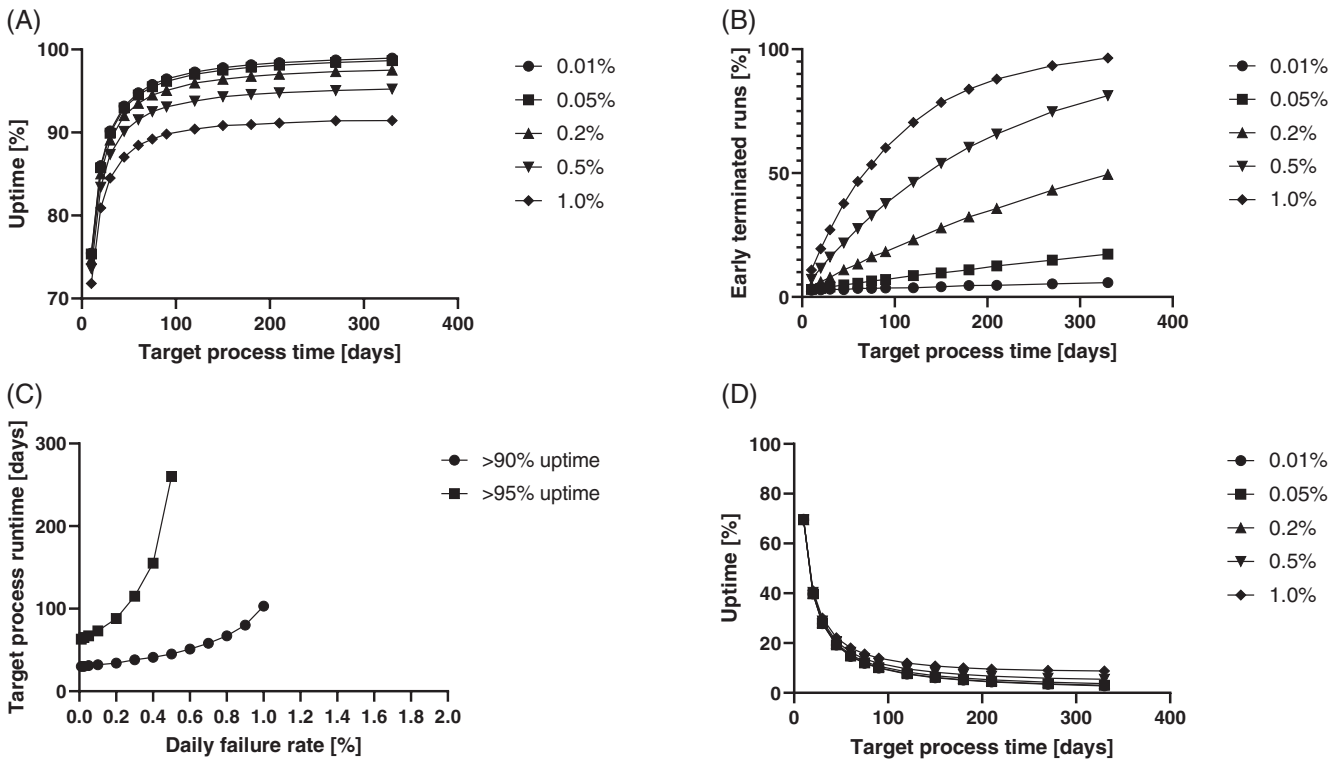


**Figure 3.** (A) Uptime for a perfusion culture with a planned process time and daily failure rates with a dedicated N-1 with instant availability. (B) Necessary target runtime for a specific goal of uptime of a perfusion reactor for a given daily failure rate of the process. (C) Utilization of the N-1 reactor for a given failure rate and targeted runtime. (D) Percentage of unexpected/unplanned N-1 runs in relation to the targeted anticipated runtime and daily failure rate. The data represent a total of *ca* 500 000 individual simulated runs.



  
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terminating early for a planned maximum production of 90 days. When we assume the transferability of batch failure rates as described above, resulting in a failure rate of about 0.3% per day we already have to accommodate for about 25% of the runs being terminated earlier than 90 days. Even under conservative failure assumptions of 0.1% or lower, a significant portion of perfusion processes will not make it to the targeted process end. Hence, early process termination has to be expected for continuous processes and needs to be planned for.

We think that this demonstrates the importance of a different viewpoint on failure rates when discussing continuous processes, as a significant portion of processes will terminate early, even if risks are reduced as much as possible.

These data can also be used for process design, as we can adjust the target runtime to a specific goal of uptime in a production facility with an associated cost in the organization due to the unscheduled starts of N-1 cultures. This is an organizational burden on management, as resources have to be reallocated quickly, both in equipment, as the N-1 stage has to be available, and in personnel. In many cases, running the N-1 equipment, or even having one dedicated instrument for the N-1 stage, is cheaper than postponing the start of the next cycle in the production reactor itself, but designing continuous processes will demand rethinking of the old batch schedule. For determining the necessary minimal target runtime for a given failure rate and a certain goal of uptime of the facility we can plot those factors and end up with an almost exponential curve (Fig. 3(C)), meaning that one can compensate a higher daily failure rate with a longer target runtime and get the same uptime for the process, but only to a certain point.

This analysis can also be used to determine the use of the N-1 stage connected to the perfusion culture, and to investigate the possibility of using one N-1 stage to produce the inoculum for multiple perfusion reactors. We determined the use of the N-1 stage, which is at around 10–15% for a targeted process runtime of 90 days almost regardless of the daily failure rate assumed for the perfusion culture (Fig. 3(D)). As expected, depending on the specific scheduling in the facility, it is feasible to use a single N-1 stage to feed multiple production lines, especially for long planned process runtimes and low failure rates.

### Influence of facility flexibility on uptime of perfusion cultures

In the model presented above, we assumed that the N-1 stage is always available as soon as the production process terminates resulting in a minimal delay. This represents a facility with a maximum in flexibility for both personnel as well as equipment availability. The need for rapid reallocation of resources can be reduced for the price of more downtime but the impact of such a strategy has to be quantified. We therefore calculated the loss in uptime for a production facility with three different scheduling scenarios for the N-1 stage: first, the scheduling as described above with maximum flexibility; second, a scheduling with an open slot for the N-1 culture every *x* days that can be used (called limited flexibility); and third, a completely restricted N-1 stage that is only available for the targeted process end (Fig. 4). Obviously, these strategies will lead to lower and lower uptimes for progressively more restricted scenarios, but the goal is to quantify the impact of each of these modes of scheduling. For implementation, the gained benefit for easier planning has to be evaluated for specific facilities individually, as for single-product facilities it might be feasible to implement a maximum flexibility approach, while multi-product facilities with no dedicated equipment might need stricter planning.

The limited flexibility approach can be designed with more or less flexibility, as a schedule can allow for the start of an N-1 culture either each week, each second week or with an even longer periodicity. We investigated schedules of a free slot every 7, 14, 21 and 28 days. Depending on the day of the failure, the next N-1 culture can either start immediately (if the process failed on the day before a slot is available) or has to wait up to the periodicity of the schedule (if the process fails right after the slot was available). We simulated the impact of three different daily failure rates of 0.05%, 0.3% and 1% and show the uptime for 7, 14, 21 and 28 days scheduling for the N-1 stage for a planned process time of 90 days (Fig. 5(A)). As before, the influence on the uptime is dependent on failure rate, which is expected as the introduced additional restriction only affects processes that are terminated early. We can also see that choosing a reasonable periodicity of 1 or 2 weeks instead of maximum flexibility in the facility will lower the uptime in production from 94.6% to 93.3% for a periodicity of 1 week and to 92.3% for a periodicity of 2 weeks for a process with a daily risk of 0.3%. The gain in uptime for a maximum flexibility approach is therefore minimal for such processes and most certainly not worth the effort. Scenarios of periodicities of 2 weeks for N-1 taking 7 days or less, or a cycle of 4 weeks for N-1 taking 14 days or less might be especially interesting, as this enables the N-1 to feed two production lines without the chance of any scheduling conflict due to processes terminating early and with only minimal losses in uptime of the production train.

In the next step, we wanted to simulate the combined influence of flexibility of the N-1 stage with various planned process times, as for longer planned process times the ratio of processes terminated early is substantially larger. We selected a maximum flexibility scenario, limited flexibility with 7, 14 and 28 days periodicity and the very inflexible approach for a daily failure rate of 0.3% and simulated planned runtimes of up to 320 days (Fig. 5(B)). This analysis shows that for very short processes of 30 days, the influence is minimal, as almost all processes that are run actually make it to the 30 days mark. For processes that yield high uptimes above 90%, meaning processes run for 60 days or longer, the impact of increased flexibility is visible, but only substantial for a periodicity of 28 days. As expected, the inflexible approach is very unsuitable for continuous manufacturing, as the uptime is markedly decreased for runtimes above 45 days.

While these results were expected in general terms, they have not been quantified before and we can see that there is no necessity for a daily reallocation of resources for potentially failing processes. Already a minimal flexibility of being able to start an N-1 every other week is already enough to come very close to the optimum achievable through maximal flexibility and rapid resource reallocation. And any achievable flexibility, even if it is monthly, is already vastly beneficial to no flexibility at all. A monthly or biweekly reallocation of a seed fermentation will be possible for any biopharmaceutical production facility, and this analysis shows that the pressure to restart the production stage as soon as possible is not warranted in a continuous perfusion production setup and a more conservative planning and scheduling approach will not lead to any significant losses. For all subsequent analyses we assume a weekly availability of the seed stage, as we think this is an approach that can be realized in almost any biopharmaceutical facility and avoids the rapid daily reallocation of resources necessary for maximum flexibility.



  
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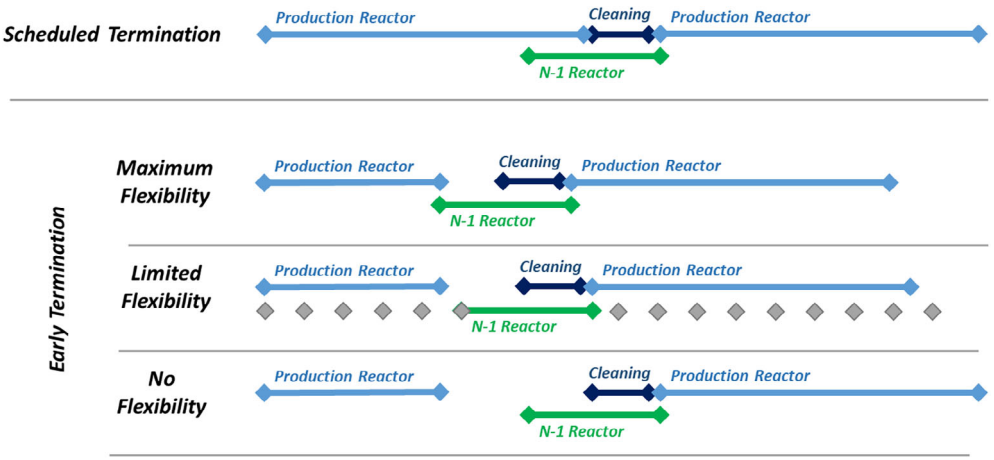


**Figure 4.** Graphical representation of the different modes of scheduling for the N-1 stage and handling of early termination of the production process. Maximum flexibility assumes the N-1 reactor to be usable as soon as the production reactor fails. Limited flexibility assumed allocated starting time slots (e.g. each week on Monday) and the start of the N-1 stage must wait for a free slot. No Flexibility assumes that the N-1 is only ready on the target planned time point.

### Influence of lot definition

How to define a lot in a continuously running system is a matter of ongoing debate. A hot topic is the implementation of real-time release based on online monitoring tools to maximize the product output and ensure full traceability of the product. This strategy allows the release of the product stream directly from the continuous production line and minimizing the output that has to be rejected in the case of a process disturbance by immediate detection of the deviation. No lot testing of the produced material after production is necessary. This maximizes the material that can be sold and minimizes cost in the analytical department, but current technology is not yet ready for implementing a complete real-time release. Approaches that are more conservative define a lot by a period of production time on an hourly, daily or weekly basis. While a longer period collected and defined as a lot reduces costs for testing of product quality, more material must be discarded in the case of any process failure. Efforts are being made in the scientific community to quantify the minimum that will have to be discarded through the modelling of residence time distributions through whole process trains showing a spread of minutes to hours, depending on the unit operations and the length of the process train.[28]

While real-time monitoring is a necessity for real-time release, the benefits in savings in the analytical department could also be realized without real-time release, enabling the combination with established analytical methods for lot-release testing. In this investigation we want to quantify the potential losses in product resulting from more conservative lot definitions that are possible to implement today. For this, we assume a continuous run with daily failure rates of 0.1%, 0.3% or 0.6%, in which the last lot before failure is discarded completely. We assume a lot definition of 1 h (representing real-time release), a lot definition of a day, or a week, progressively losing more material in the case of a failure. All scenarios assume an availability of the N-1 stage every week as discussed before, representing a medium flexibility in the production facility to handle failures.

Figure 6(A) shows nine curves of three different failure rates and three different lot definitions. For all definitions that do not include a real-time release, but daily or weekly definition and testing of lots, we see an optimum for the simulated uptime in terms of the selected planned process runtime, as the loss of material

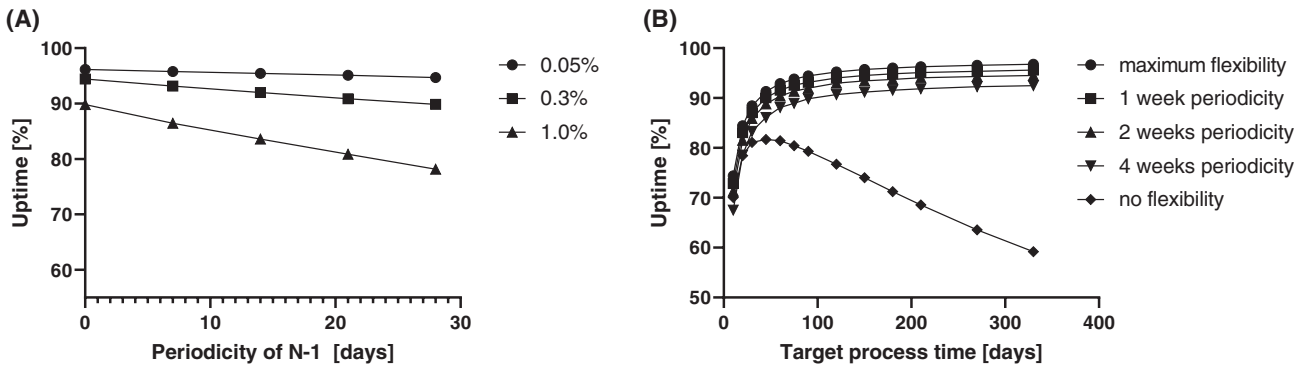


**Figure 5.** (A) Influence of reduced flexibility represented by a longer periodicity between available slots for the N-1 stage for 0.05%, 0.3% and 1% daily failure rates for a planned process time of 90 days. (B) Relationship between different planned process times and different flexibilities (periodicity of free slots) implemented for the N-1 stage.



  
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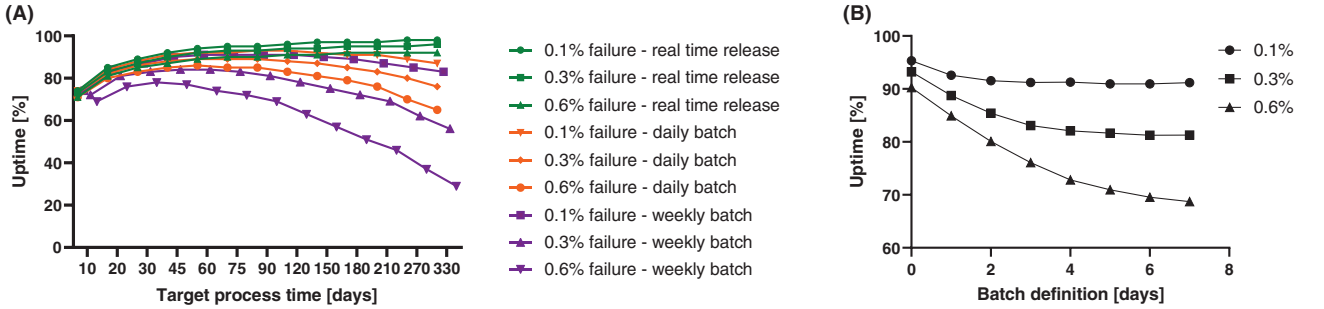


**Figure 6.** (A) Impact of different lot definitions (real-time release, daily or weekly) on the uptime of a continuous run planned for failure rates of 0.1%, 0.3% and 0.6% per day. (B) Impact of lot definition times between real-time release and 7 days in respect to daily failure rates of 0.1%, 0.3% and 0.6% for a planned process time of 90 days.

will become more and more significant if more and more lots fail. This optimum is at 60–120 days for a daily lot definition, and 45 to 90 days for a weekly lot definition. For a real-time release such an optimum does not exist, as longer target runtimes always mean higher uptime. Plotting the lot definition in days *versus* the uptime for a 90 days process, one can see a decrease in uptime for going to any daily lot definition from real-time release (Fig. 6(B)). While for a process with a daily failure rate of 0.1% the loss when implementing daily lots instead of real-time release is around 3%, and the loss for rates of 0.3% and 0.6% is already at 4% and 6%, respectively. Longer lot definition times are even worse, and a weekly lot definition will already reduce the uptime assuming a failure rate of 0.3% per day from 93% to 81%. From these data we can conclude that a continuous facility needs at least a day-to-day release of product to reduce losses to an acceptable minimum. A real-time release instead of a daily lot definition can gain an additional 3–5% of uptime, directly translating to additional revenue, but comes with the effort necessary for technology development and implementation as real-time release is hardly feasible with today's technology. For stable processes of 0.3% daily failure rate or below, the costs and additional challenges for real-time release might not be worthwhile and a day-to-day lot definition is sufficient. This analysis can also be used to estimate the potential impact of process analytical tools capable of determining a process deviation early or in real time as the loss of product is the delay time in detecting an error and/or the batch definition. From this we can also infer that a daily lot definition will need analytics suitably fast to detect failures in the process, as a daily lot definition, but analytics that take a week lead to an effective weekly lot definition as failure will be detected with a one-week delay leading to the production of unsuitable lots until the analytical data come in. To model the potential additional benefits of PAT, tools for advanced process control for the reduction of failures leading to process termination are not modelled here and would need the inclusion of a mechanistic or statistic process model.

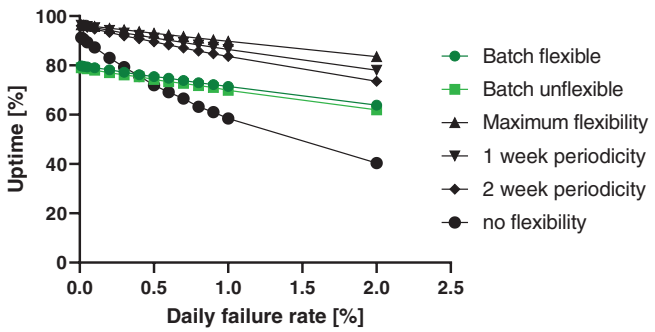


**Figure 7.** Comparison of uptime for batch and continuous processes, showing batch processes with high flexibility and low flexibility in the N-1 stage, and continuous processes with high flexibility, 1-week and 2-week cycle availability and no flexibility in the N-1 stage.

## Comparison to batch manufacturing scheduling

In the previous sections, we compared different scheduling approaches for continuous operation in handling failures during process time. In this section we compare these results to a standard traditional batch-based process. For a typical fed-batch fermentation, we assume a production time of 12 days in the production stage, and the same requirements for the seed and cleaning as for continuous production, meaning that the N-1 fermentation will take 9 days and can be started in parallel to the fed batch, and that cleaning between batches will take 3 days. We also use the same failure rates as for continuous manufacturing, as those data are based on batch failure rates anyway. We use the same flexibility scenarios for the N-1 stage for batch processes as for continuous processes, assuming either maximum flexibility or a rigid scheduling. For batch processes we omitted the limited flexibility cases, as the difference between a possible N-1 start every 7 days and no flexibility at all is very small because of the short target process time for the production reactor of 13 days. In comparison to a continuous process, all failed runs, regardless of when they failed, have to be discarded completely, as only material from a successful completed run can be transferred to further processing in a batch-operated facility.

This investigation is conducted for completely flexible and non-flexible batch scenarios, and for no flexibility, maximum flexibility and 1- or 2-week periodic availability for the N-1 stage for continuous processes with a target process time of 90 days (Fig. 7). We see that the design of flexibility in the N-1 stage is for both continuous and batch processes only important for higher daily failure rates. We also see that the batch process is significantly more insensitive to reduced flexibility in the N-1 planning as the process is anyway very short. In comparison, the no flexibility approach in the continuous process again markedly reduces the uptime of the unit. A large difference in this dataset can be seen between batch and continuous operation in the resulting uptime with a loss of uptime of between 10% and 20% for batch operation depending on the scenario. This is because of two factors, with the main contribution being the generally lower uptime even under best conditions (13 days production with 3 days cleaning and setup represent an uptime of only 81.25%). The second contribution is



  
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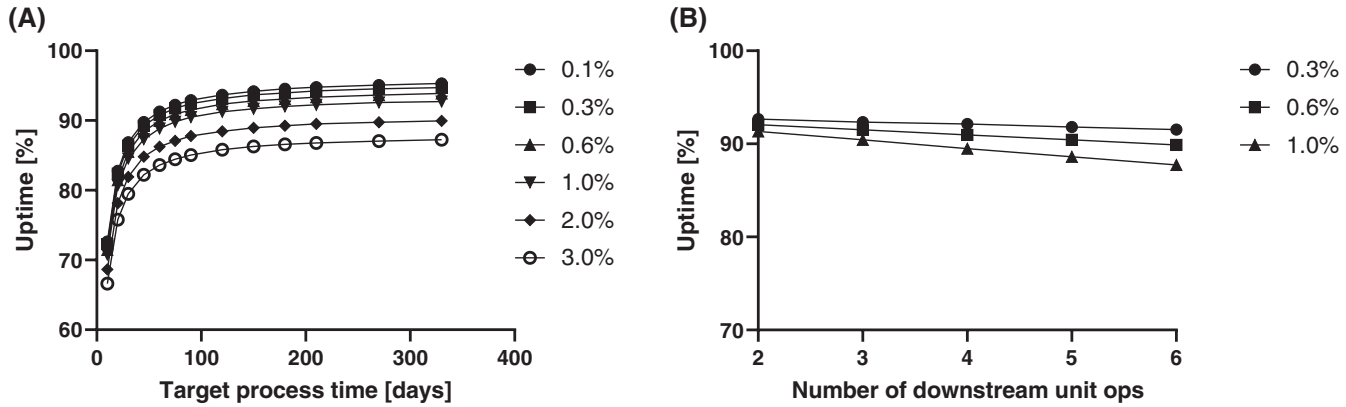


**Figure 8.** (A) Uptime for the entire process train at different downstream failure rates for a perfusion culture connected to three subsequent downstream unit operations and the uptime for the complete process train. (B) Influence of number of unit operations on the uptime of entire downstream train for a 90 day perfusion culture target.

from the difference between day 1 risk being significantly higher. This higher risk on day 1 also means that once the process is running, the continuous setup has significantly fewer 'day ones' to worry about, while the day-one event is a much more frequent occurrence in fed-batch production. From this comparison, we can see that a higher uptime for continuous production schemes is reached, even if only limited flexibility for continuous production is achievable in the facility. Only for high daily failure rates in combination with facilities than cannot implement any flexibility for the N-1 stage does the batch process option show a higher uptime.

### Failures in integrated upstream and downstream production trains

In addition to the assessment of failure and managing strategies for upstream production, it is important to simulate the uptime of a whole process train in the case of a directly interconnected downstream unit. This analysis helps to understand the importance (or non-importance) of catastrophic failures occurring in the downstream in comparison to catastrophic failures occurring in the upstream on a more general level. While we assumed total failure and restart for the upstream in the case of any failure, in downstream this is usually not the case for disturbances as unit operations can be reset quite quickly. We characterize such a failure as an intermittent unavailability of the unit which produces either nothing or product that is not within specifications. We assume that resolving an issue in the downstream will be possible within 24 h, as this gives enough time for chromatography columns to be exchanged, setting up replacement pumps or exchanging filters outside of normal schedule. Start-up phases of downstream units are usually within minutes or hours, so we think the 24 h definition is sufficient and conservative to capture all necessary steps to restart the downstream unit for production.

While defining the length of interruption is fairly straightforward with the assumption of a 24 h downtime regardless of the reason, defining the rate of occurrence of such events is much more complicated and this might also be dependent on the specific unit operation in question. While at least limited data were available for batch failures in the upstream, to our knowledge there is no report in the literature on failure rates for downstream equipment during continuous or batch production. Due to the lack of available data, and because a number of the aforementioned causes of failure for upstream are actually directly applicable to downstream as well (like material or equipment failure), we assumed the risk for a failure event will be of the same order of magnitude for the downstream as for the upstream. To determine the uncertainty associated with that risk estimation, we use for this analysis a failure rate range for downstream unit operations of 0.1% to 3% daily. We calculated the impact for a process with three subsequent downstream unit operations and a fixed upstream daily failure rate of 0.3% (Fig. 8(A)) with the entire process train not producing any product if any of the unit operation is either restarted (upstream) or on intermittent unavailability (downstream). From these data, we can see that the addition of unit operations and their failure rate reduces the uptime. However, the overall impact is much less pronounced than for the upstream process, even for very high daily failure rates of 3%. The same is true for the number of downstream operations, which have an impact, but changing from a four-step process to a three-step process only changes the uptime of the whole process train from 91% to 92% for a daily failure rate of 0.6% (Fig. 8(B)). As the number of unit operations in the downstream has a much more significant impact on the total product yield of the process as well as on the cost of the process, the influence on the uptime of the process can be safely neglected.

## CONCLUSIONS

In our simulations we were able to quantify the impact of different lot definitions and scheduling approaches for continuous manufacturing, especially the upstream production and scheduling of the N-1 seed train and compared this to the typical batch or fed-batch production for biopharmaceutical products. In the field of continuous biomanufacturing, much discussion is currently around meaningful lot definitions, real-time monitoring and release and scheduling and failure management. We were able to show that continuous biomanufacturing does not necessarily need real-time release or narrow lot definitions. A 24 h lot definition and a facility scheduled with limited flexibility, which can restart a failed process every 2 weeks, is only a few percent worse than a facility with maximum flexibility and real-time release. We showed that even when using such traditional and conservative approaches for the implementation of continuous biomanufacturing the productive uptime of the process is already



  
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significantly better than that of batch-based processes. Additionally, we were able to identify a planned maximum runtime of 45–90 days for continuous upstream as the most beneficial if failure rates are considered. In conclusion, we provided a rational basis for assessing if real-time release is feasible and worth the large investment for continuous manufacturing, especially for upstream production. A very flexible reallocation of resources in the seed fermentation is not necessary to be able to benefit from continuous manufacturing, and limited flexibility is already able to alleviate scheduling issues of continuous manufacturing facilities with the inevitable failures in mind.

## ACKNOWLEDGEMENTS

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# Economic and ecological benefits of a leaky *E. coli* strain for downstream processing: a case study for Staphylococcal Protein A

**Short title: Economic and ecological benefits of leaky *E. coli***

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## Abstract

BACKGROUND: Downstream processing of soluble recombinant proteins from *Escherichia coli* is complicated by the need to access the intracellular product by cell disruption and to separate the target protein from impurities, particularly host cell protein, DNA, endotoxins and lipids. We previously demonstrated the ability of the *E. coli* X-press strain to leak high amounts of product to the culture medium without sacrificing viability. In this case study, we assessed the economic and ecological benefit of this strain for downstream processing in direct comparison to the industrial standard *E. coli* BL21(DE3). Staphylococcal Protein A was used as a model protein. We performed recombinant protein production, primary recovery and capture by anion exchange chromatography in lab-scale and used the obtained data for estimating costs and resource consumption by economic modeling.

RESULTS: After primary recovery, the X-press process resulted in a 1.5-fold higher product purity, a 150-fold lower DNA, 3.5-fold lower endotoxin and 3.4-fold lower lipid load compared to BL21(DE3). Consequently, anion exchanger binding capacity was increased 2.7-fold and purity and concentration of the eluate was also increased. Extracellular protein production with X-press resulted in a 25% reduction of costs and a 36% reduction of both water usage and water-related $CO_2$ emissions compared to intracellular production with BL21(DE3).

CONCLUSIONS: This case study performed with Stapyholococcal Protein A demonstrated the potential of *E. coli* X-press to reduce costs for downstream processing and improve the environmental footprint by simplified primary recovery, lower impurity load and consequently higher chromatographic efficiency.

**Keywords:** capture chromatography; cost of goods sold; primary recovery; process mass intensity; protein secretion; soluble protein expression

## Introduction

The cost of recombinant protein production in *E. coli* is largely driven by the downstream process. Leaky *E. coli* strains may have economic and ecological benefits in form of reduced costs and resource consumption. The recombinant product produced in *E. coli* is usually located inside the cell, thus a disruption step is necessary and high amounts of contaminants, such as host cell protein (HCP), DNA, endotoxins and lipids, are released along with the product. Expressing the product as insoluble inclusion bodies mitigates purity issues and achieves high titers but requires a complex and costly inclusion body refolding process.[1,2] Extracellular production in *E. coli* might overcome these disadvantages, enable competition with other expression hosts for soluble proteins, such as yeasts or mammalian cells, and may facilitate the transition to continuous bioprocessing with *E. coli*.[3]

Apart from the costs, the ecological footprint is an increasingly important factor in the evaluation of recombinant protein production processes. Recently, the process mass intensity (PMI) was proposed as a metric to evaluate the resource efficiency of recombinant protein production.[4,5] The PMI relates the mass of consumed raw materials to the mass of produced protein. Water contributes over 90% to the PMI with an approximate magnitude of $10^3$-$10^4$ kg water per kg product.[6] Its production and storage are associated with significant energy consumption and consequent carbon emissions, which can be quantified by a recently introduced metric, the Water Related Impact of Energy (WARIEN).[7] Thus, using the WARIEN and PMI as metrics to assess the sustainability of bioprocesses can reveal potential savings, both in resource consumption and carbon footprint. Furthermore, reducing water utilization lowers costs by downscaling the equipment needed for water preparation and storage.[4,5,8]

Several leaky *E. coli* strains for extracellular protein production have been reported, but they often suffer from impaired growth or viability which limits their applicability.[9-12] The recently developed strain *E. coli* X-press was shown to enhance expression of various recombinant

proteins and we demonstrated that this strain leaks up to 90% of soluble recombinant product to the extracellular space while maintaining viability.[13,14] In this case study, we show the potential of *E. coli* X-press to reduce downstream costs and resource consumption in direct comparison to the industrial standard strain *E. coli* BL21(DE3). Recombinant staphylococcal Protein A (SpA) served as model protein and the up-stream production, primary recovery and chromatographic capture were performed and compared in lab-scale. Protein yield and purity as well as contaminant levels (HCP, DNA, endotoxins) were measured and by subsequent process simulation an estimate of the cost of goods sold (COGS) and the PMI were obtained.

# Experimental

### Strains

Two *E. coli* strains were used in this study: X-press, a proprietary strain developed by enGenes Biotech,[13,15] and the industrial standard BL21(DE3). Both strains produced the plasmid encoded, his-tagged IgG-binding domain of *Staphylococcus aureus* Protein A (SpA). The theoretical pI of SpA is 5.0, calculated with the ExPASy ProtParam tool (web.expasy.org/protparam). Details about the strains and the protein sequence are described elsewhere,[13,14] The processes using X-press for extracellular and BL21(DE3) for intracellular protein production are hereafter referred to as “process EX” and “process IN”, respectively.

### Upstream process

The upstream processes for production of SpA were conducted equally for both strains. The process parameters temperature and specific substrate uptake rate were screened in our preceding study and the parameters yielding the highest productivity of SpA in both strains were chosen for the present work.[14] The semi-synthetic media used for the preculture and the defined bioreactor medium were prepared as previously described.[14] The preparation of precultures and the 20 L bioreactor setup are described elsewhere.[13] Temperature was controlled at 37 °C during the uninduced phase and 30 °C during the induction phase. The pH

was maintained at 7.0 by addition of 12.5% ammonia. The overpressure was kept at maximally 1.0 bar and the dissolved oxygen level was kept at 30% by adjusting stirrer speed (800-1400 rpm) and aeration (up to 1.25 vvm). The batch was started by inoculating 8 L of defined medium with 200 mL preculture. After depletion of glucose, the fed-batch was started and the growth rate was set to 0.1 $h^{-1}$, assuming a biomass yield coefficient of 0.36 g $g^{-1}$. After 16 h, dry cell weight reached approximately 30 g $L^{-1}$ and SpA expression was induced by addition of 0.5 mM IPTG. The X-press strain was additionally induced with 100 mM L-arabinose. The process was stopped after 6 h of induction.

**Downstream process**

Figure 1 shows a flow scheme of the two downstream processes performed in lab-scale. The intended product location in process EX and process IN was extracellular and intracellular, respectively.

*Primary recovery and filtration*

After cultivation, the culture broth was harvested and centrifuged in 1 L bottles at 10,000 rcf and 4°C (Sorvall Lynx, Thermo Scientific, Waltham, MA). The supernatant and cell pellet were frozen and stored at -20 °C until further use.

For process IN, the cell pellet was resuspended in loading buffer (described below) to a concentration of 100 g $L^{-1}$ wet cell weight (approximately 25 g/L dry cell weight). Homogenization was done in 3 passages at 700 bar in a high pressure homogenizer (PandaPLUS 2000, GEA, Düsseldorf, Germany). Aliquots of the lysate were centrifuged in 50 mL tubes in a benchtop centrifuge (10,000 × g, 10 min, 4 °C).

After centrifugation in both processes, product solutions were sterile-filtered with a 0.2 µm polyethersulfone syringe filter (Sartoscale 25, Sartoguard PES, Sartorius, Goettingen, Germany).

*Capture by anion exchange chromatography*

In process EX, a dilution step was necessary to lower the conductivity for subsequent anion exchange chromatography (AEX). The culture supernatant was diluted 1:3.75 in loading buffer (see below) to reach a conductivity of 8.3 mS $cm^{-1}$. Preparative chromatography was performed with an Äkta pure system (Cytiva, Marlborough, MA). Conductivity as well as UV absorption (210/260/280 nm) were monitored. AEX was done with a 1 mL HiTrap CaptoQ column (Cytiva) at a flow rate of 1 column volume (CV) $min^{-1}$. The column was chosen based on the pI of the product and since strong anion exchangers have been shown to be applicable to SpA.[16] The column was equilibrated with 10 CVs buffer A (19 mM $NaH_2PO_4$, 31 mM $Na_2HPO_4$, pH 7.4). After loading, the column was washed with buffer A until the UV signal stabilized. Step elution was then performed with 30% buffer B (7.5 CVs; 19 mM $NaH_2PO_4$, 31 mM $Na_2HPO_4$, 1 M NaCl, pH 7.4) and a wash with 100% buffer B followed (8 CVs). Fractions of 1 mL were collected, frozen in liquid nitrogen and stored at -20°C until further analysis.

**Dynamic binding capacity**

Dynamic binding capacity at 10% breakthrough ($DBC_{10}$) of the AEX column was determined for both processes EX and IN. For intracellular SpA of process IN, the cell pellet was resuspended in loading buffer to a concentration of 60 g $L^{-1}$ wet cell weight. Homogenization and clarification of the lysate, preparation of the extracellular SpA of process EX and the AEX procedure were the same as described above. For intracellular SpA, 5 mL of the particle free lysate were loaded and fractions of 0.5 mL were collected. For extracellular SpA, 20 mL of the diluted culture supernatant were loaded and fractions of 1.3 mL were collected. The $DBC_{10}$ was calculated according to literature.[17]

**Analytics**

SpA concentrations were analyzed in triplicate by reversed phase HPLC (RP-HPLC).[18] Protein purity (i.e. the percentage of SpA with respect to total protein, comprising SpA and HCP) was determined by size exclusion HPLC (SEC-HPLC) using absorption at 214 nm.[19]

DNA was quantified in triplicate using the PicoGreen assay kit (Thermo Fisher Scientific, Waltham, MA). Samples were diluted with water to the standard range of 0.02 and 2 mg DNA $L^{-1}$. Limit of detection was 0.005 mg $L^{-1}$. Cell lysis in the cultivation was estimated with the DNA data according to a previous study.[14]

Endotoxins (ETs) were measured in triplicate with the EndoLISA assay kit (bioMérieux, Marcy l'Etoile, France). ET-free water was used for dilution of the samples to lie within the calibrated range (0.05-500 EU $mL^{-1}$).

Lipid extraction from the particle free culture supernatant and cell lysate of process EX and IN, respectively, was performed in triplicates according to the method by Bligh and Dyer.[20] The sample volume for extraction was 2 mL each. Lipids could not be extracted from subsequent process samples due to insufficient volumes. Lipids were quantified using 16:0-18:1-phosphoethanolamine (PE) as a proxy, since it is a major constituent of *E. coli* membranes[21,22] and a standard was commercially available (Avanti Polar Lipids, Alabaster, AL). Samples were analyzed on an Agilent 1100/1200 HPLC device (degassing unit, 1200SL binary gradient pump, column thermostat, and CTC Analytics HTC PAL autosampler) with an Agilent 6230 LC-TOF-MS equipped with an Agilent Dual AJS ESI source for detection. For separation of 16:0-18:1-PE from other lipophilic components, 2.5 µL of extracted samples were injected onto an Acclaim C30 column (Thermo Scientific, 2.1 x 100 mm, 3µm) kept at 40°C. The flow rate was 0.4 mL $min^{-1}$ with a mobile phase composed of 91% methanol, 5% MTBE, 3.9% water and 0.1% formic acid for A and 50% MTBE, 46% methanol, 3.9% water and 0.1 % formic acid for B. After equilibration at 5% (v/v) B for 5 min, analytes were eluted using a linear gradient starting at 5% (v/v) B to 95% (v/v) B for 20 min. After a 5 min washing step at 95 % (v/v) B

the column was re-equilibrated for 5 min at 5 % (v/v) B. Data was collected in positive ESI ionization mode and operated in the range from m/z 100 to 1700. Due to similar lipid profiles (Figures S1, S2), determining the amount of 16:0-18:1-PE allowed the comparison of overall lipid content between the processes.

### Economic and ecological calculations

The data obtained from the lab-scale experiments were used as a basis for economic and ecological calculations. The software BioSolve (v7.5; Biopharm, Chesham, UK) provided data that were not experimentally determined (including cost for equipment, consumables, materials, personnel and utilities) and was used to calculate COGS and the PMI. The WARIEN, expressed as kg $CO_2$ per kg product, was calculated using a previously published Excel-based tool.[7] The location of the production facility was assumed to be in Austria, resulting in a $CO_2$ emission intensity of 0.085 kg $kWh^{-1}$.[7]

## Results and Discussion

### Lab-scale experiments

*Upstream process*

The production of recombinant SpA was done in fed-batch cultivations, which were harvested 6 h after induction. Table 1 shows biomass concentration, SpA titer and localization. Process EX resulted in 30% lower biomass concentration due to induced growth repression in *E. coli* X-press.[13,14] The data for leakiness and lysis are in line with our previous study, where *E. coli* X-press and BL21(DE3) leaked approximately 80% and 30% of SpA, respectively, with low lysis under comparable process conditions.[14] Despite the lower cell density and slightly lower total titer in process EX, the product expression and secretion is much more efficient than expression in the intracellular process IN, thus leading to similar amounts of product in the respective target locations.

*Downstream process*

Primary recovery in *E. coli* processes usually requires cell disruption by high pressure homogenization to make the product available for purification. Homogenization is a critical unit operation that may have a large impact on further downstream steps, e.g. by introducing variance in pH or product concentration, and therefore requires optimization.[23-25] The major advantage of extracellular protein production is that primary recovery is simplified by omitting this cell disruption step.

The disadvantage in process EX was the need for a dilution step to reduce the salt concentration for AEX. Dilution is a simple step and therefore a common practice in industrial protein purification. A possible strategy to avoid dilution is ultra/diafiltration, which may also concentrate the product, but would add complexity to the process.

In case of process EX, clarified supernatant was obtained by centrifugation followed by microfiltration. This microfiltration step did not remove any impurities except solids and product losses were not observed. In case of process IN, the cells were harvested by centrifugation, lysed by high pressure homogenization and the homogenate was clarified by another centrifugation followed by microfiltration. The clarified broth of process EX and the clarified cell lysate of process IN were the starting material for the chromatographic capture step of the target protein, SpA. Table 2 summarizes the load conditions, product yield and titer. We expected not to exceed the binding capacity of the column, as possible $DBC_{10}$ values of over 100 g $L^{-1}$ are stated by the manufacturer of the column used in this study. While no product was found in the flowthrough of process EX, 14% of SpA were lost in the flowthrough of process IN. This is explained by the higher impurity of the starting material in process IN and the competition between impurities and product for binding sites. To enable comparison between both process outputs, the target step yield of the AEX capture was set to 70% and the

eluate fractions were collected accordingly. Process EX resulted in higher titer and less than half the volume after the capture step.

The impurity levels (HCP, DNA, endotoxins) are shown in Table 2. As expected, the load of process EX (the clarified culture supernatant) had a much higher purity compared to the load of process IN (the clarified cell lysate). The purification factor was similar for both capture steps, however the final purity in process EX was much higher with 88% purity compared to only 56% in process IN. Removal of DNA by AEX was efficient in both processes, which was expected due to the strong negative charge of nucleic acids. However, the amount of DNA in the load was approximately 150 times lower in process EX and therefore DNA could be removed below limit of detection. The endotoxin concentrations in the load of both processes were in the same order of magnitude (~$10^9$ EU $g_{SpA}^{-1}$). This is in contrast to the common perception that extracellular protein production reduces the endotoxin burden, but it can be explained by continuous release from the outer membrane to the medium during cultivation[26]. Increased outer membrane permeability, as encountered in process EX, may further accelerate endotoxin shedding into the culture medium. However, in the flowthrough and eluate of process EX much lower levels of endotoxins were found. This higher clearance efficacy for endotoxins is explained by lower concentrations of other impurities, such as *E. coli* HCP and DNA. The content of the lipid 16:0-18:1-PE in the load of processes EX and IN was 30 ± 3 and 103 ± 21 mg $g_{SpA}^{-1}$, respectively. This demonstrates significant reduction of lipid burden by extracellular protein production. Pollution of the recombinant product in process EX probably resulted from continuous release of membrane components from viable cells during cultivation. Impurities like HCP, DNA and lipids foul chromatography columns, which reduces the binding capacity for the product, increases the need for harsh cleaning procedures and, ultimately, shortens the column's life-time.[27,28] Apart from the benefit of lower contaminant levels in the purified product, extracellular recombinant protein production can therefore reduce resource and resin

consumption by lowering the burden on the column, as indicated by the present results with SpA.

The positive impact of the leaky X-press strain on the chromatographic binding capacity was corroborated in breakthrough experiments. The $DBC_{10}$ of the AEX column for SpA in the culture supernatant of process EX was 10.5 $g_{SpA}$ $L_{resin}^{-1}$. The corresponding value for the cell lysate of process IN was only 3.9 $g_{SpA}$ $L_{resin}^{-1}$. The breakthrough curve in Figure 2 shows, that extracellular SpA from process EX could bind more efficiently and was only found in the flowthrough after several CVs had been loaded. On the other hand, SpA from process IN was detected in the flowthrough immediately after loading, indicating that the high amounts of impurities in the load strongly compete with SpA for binding sites on the column.

**Economic and ecological calculations**

*Cost analysis*

The experimental purification steps and the data for $DBC_{10}$ as well as yield and volumes from the AEX capture served as a basis for estimating and comparing the economic performance as well as the ecological footprint of both processes for the production of SpA. The parameters obtained from the experimental results were used to model an upscaled process with a target product output of 500 kg per year. For process IN, the same centrifuge was used for harvesting the bacteria from the culture broth and for clarification of the cell lysate. Key parameters for the simulation are given in Table 4.

Normalized to the produced amount of product, COGS was 11.8 € $g^{-1}$ or 25% lower in process EX (36.6 € $g^{-1}$) compared to process IN (48.5 € $g^{-1}$). The contribution of each cost category (capital, material, consumables, labor, other) to the overall COGS is shown in Figure 3A. The highest contribution in both processes was the capital charge, although this was reduced by 2.8 € $g^{-1}$ in process EX (Figure 3B). These savings can be explained by avoiding a homogenization step, which is a capital-intensive unit operation. All other capital-intensive unit operations, such

as the bioreactor and centrifugation, are comparable. Reduced costs for consumables, specifically chromatography resins (reduced by 5.9 € $g^{-1}$), had the largest impact on overall reduction of COGS in the process using *E. coli* X-press (Figure 3). This is a consequence of higher purity of the AEX feed, the resulting higher $DBC_{10}$ (10.5 vs. 3.9 $g_{SpA}$ $L_{resin}^{-1}$) and, thus, lower resin consumption. The costs for chromatography are a major contributor to overall production costs[29,30] and the present results underline that they are a decisive factor in evaluating the economic benefit of extracellular over intracellular protein production. Utilizing the X-press strain may also result in reduced column fouling and therefore higher column utilization,[27,28] lowering chromatography costs even further. Moreover, pre-processing and conditioning steps, such as DNA precipitation with polyethyleneimine[31,32] or adsorptive flowthrough treatments of cell lysate,[33] may not be necessary in an extracellular production process due to lower impurity levels. Lastly, the AEX capture of extracellular SpA resulted in product of higher purity and concentration, which likely simplifies subsequent purification steps. This would offer even more saving potential than the currently estimated 25% reduction of COGS.

*Ecological impact*

The ecological footprint of the processes was assessed by estimating the PMI and WARIEN. Water contributed more than 99% to the PMI in both processes, thus other material contributions were neglected in further analysis. The PMI was reduced by 11.3 t $kg^{-1}$ or 36% in process EX (20.0 t $kg^{-1}$) compared to process IN (31.3 t $kg^{-1}$). The rather high values in comparison to other microbial recombinant protein production processes is explained by relatively low chromatographic efficiency, which inversely correlates with water usage[6]. The AEX step had the highest influence on the overall PMI, which is in agreement with previous findings (Figure 4A).[4,5] Furthermore, AEX had the highest fraction of process related water consumption, whereas the contribution of all other unit operations to the PMI was mainly due

to cleaning water. However, the more efficient AEX capture step in the extracellular process EX entailed the largest PMI savings compared to the intracellular process IN (Figure 4B). The absence of a homogenization step in process EX further contributed to a significant reduction in PMI. These results are similar to the COGS analysis, demonstrating the large saving potential of extracellular protein production in primary recovery and chromatography. This corroborates the previously stated hypothesis, that the PMI correlates with COGS and is therefore a metric of both economic and ecological meaning[8]. Finally, we determined the overall carbon footprint related to water preparation and storage. The WARIEN was estimated at 3.2 and 5.0 t $kg^{-1}$ in process EX and IN, respectively. Thus, a reduction of 36% was achieved by extracellular SpA production, which is equal to the PMI reduction.

**Concluding remarks**

Extracellular production of recombinant SpA with *E. coli* X-press resulted a 25% reduction of COGS and a 36% reduction of both PMI and WARIEN for the upstream process, primary recovery and chromatographic capture. This is due to a significant reduction of capital costs and water consumption by omitting homogenization. Furthermore, reduced levels of host cell impurities in the feed for the AEX capture step improved the binding capacity 2.7-fold compared to the intracellular production process, leading to major cost and resource savings. The chromatographic capture further yielded product of higher purity and concentration, which likely enhances efficiency of subsequent processing steps. We postulate that extracellular protein production enhances utilization of chromatography resins due to less fouling by host cell impurities, further reducing costs and resource consumption. Based on this case study relating to SpA, we conclude that *E. coli* X-press is a technology to reduce the economic and ecological impact of overall downstream processing. Further studies may investigate the purification of other recombinant proteins produced in *E. coli* X-press (particularly products

that tend to be insoluble), as well as the impact on further downstream purification steps and draw comparison to currently applied inclusion body processing.

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# Tables

**Table 1** Summary of the recombinant protein production processes.

| Process | Cell dry weight [g $L^{-1}$] | Total titer [g $L^{-1}$] | Intracellular titer [g $L^{-1}$] | Extracellular titer [g $L^{-1}$] | Leakiness [%] | Lysis [%] |
|---|---|---|---|---|---|---|
| EX | 30.3 ± 0.5 | 5.38 ± 0.12 | 0.93 ± 0.02 | 4.46 ± 0.12 | 83 ± 3 | 1 ± 0 |
| IN | 43.5 ± 1.0 | 5.88 ± 0.14 | 4.12 ± 0.13 | 1.76 ± 0.05 | 30 ± 1 | 1 ± 0 |

**Table 2** Summary of the experimental AEX capture in process EX and IN.

| | Load | | | Flowthr. | Eluate | | | |
|---|---|---|---|---|---|---|---|---|
| Process | Volume [CVs] | SpA [g $L_{resin}^{-1}$] | Protein* [g $L_{resin}^{-1}$] | SpA [g $L_{resin}^{-1}$] | Volume [CVs] | SpA [g $L_{resin}^{-1}$] | Yield [%] | Titer [g $L^{-1}$] |
| EX | 3 | 3.53 ± 0.07 | 7.8 | n.d.† | 2 | 2.53 ± 0.02 | 72 | 1.27 ± 0.01 |
| IN | 2 | 4.81 ± 0.07 | 16.0 | 0.67 ± 0.01 | 4.5 | 3.37 ± 0.03 | 70 | 0.75 ± 0.01 |

* Estimated via SEC-chromatography.
† Below detection limit.

**Table 3** Process related impurities and their reduction by AEX.

| Process | Load | Flowthrough | Eluate | Purification factor |
|---|---|---|---|---|
| | | Protein purity [%] | | |
| EX | 45 | n.a.* | 88 | 2.0 |
| IN | 30 | n.a.* | 56 | 1.9 |
| | | DNA [mg $g_{SpA}^{-1}$] | | |
| EX | 1.72 ± 0.07 | 0.48 ± 0.02 | n.d.† | - |
| IN | 263 ± 7 | 148 ± 3 | 0.37 ± 0.01 | 711 |
| | | Endotoxins [EU $g_{SpA}^{-1}$] | | |
| EX | 2.0 ± 0.2 $10^9$ | 1.0 ± 0.1 $10^6$ | 2.8 ± 0.2 $10^5$ | 7,142 |
| IN | 7.0 ± 0.9 $10^9$ | 1.4 ± 0.1 $10^9$ | 1.5 ± 0.1 $10^7$ | 466 |

* No data available
† Below detection limit

**Table 4** Key parameters for economic modeling.

| Process | USP titer [g $L^{-1}$] | Batches [$a^{-1}$] | DSP yield [%] | | $DBC_{10}$ [g $L_{resin}^{-1}$] | Output [kg $a^{-1}$] |
|---|---|---|---|---|---|---|
| | | | Prim. rec. | Capture | | |
| EX | 4.46 | 120 | 99 | 72 | 10.5 | 500 |
| IN | 4.12 | 120 | 99 | 70 | 3.8 | 500 |

# Figures

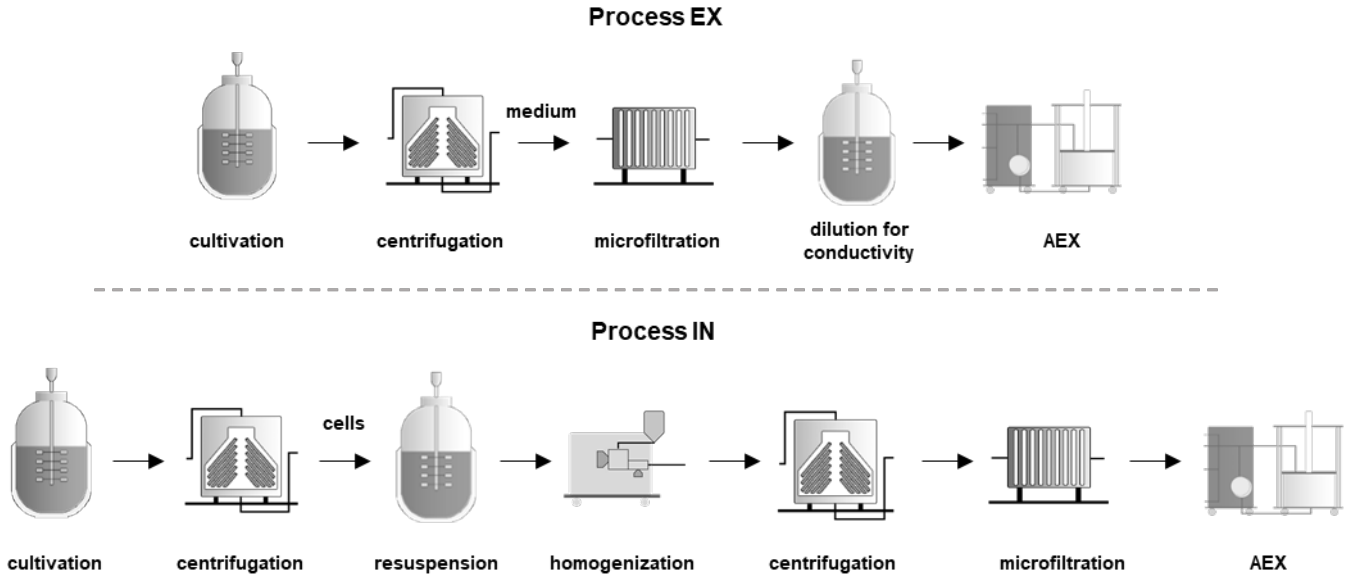


**Figure 1** Flow scheme of the processes EX (using *E. coli* X-press) and IN (using *E. coli* BL21(DE3)) from cultivation to capture using anion exchange chromatography (AEX).

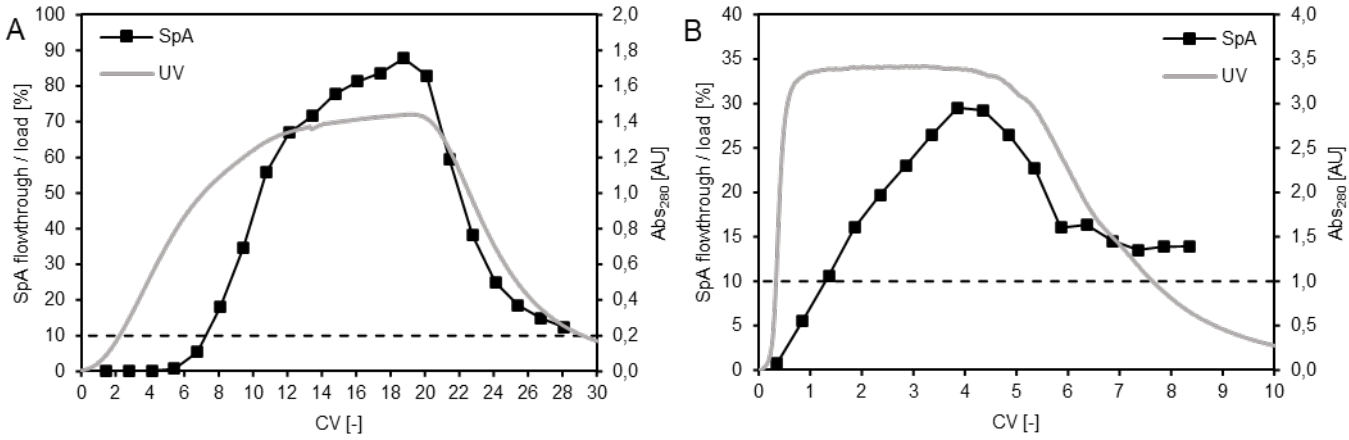


**Figure 2** Breakthrough curves for extracellular SpA from process EX (A) and intracellular SpA from process IN (B). Dashed lines mark the threshold at which the SpA concentration in the flowthrough equals 10% of the SpA concentration in the load.

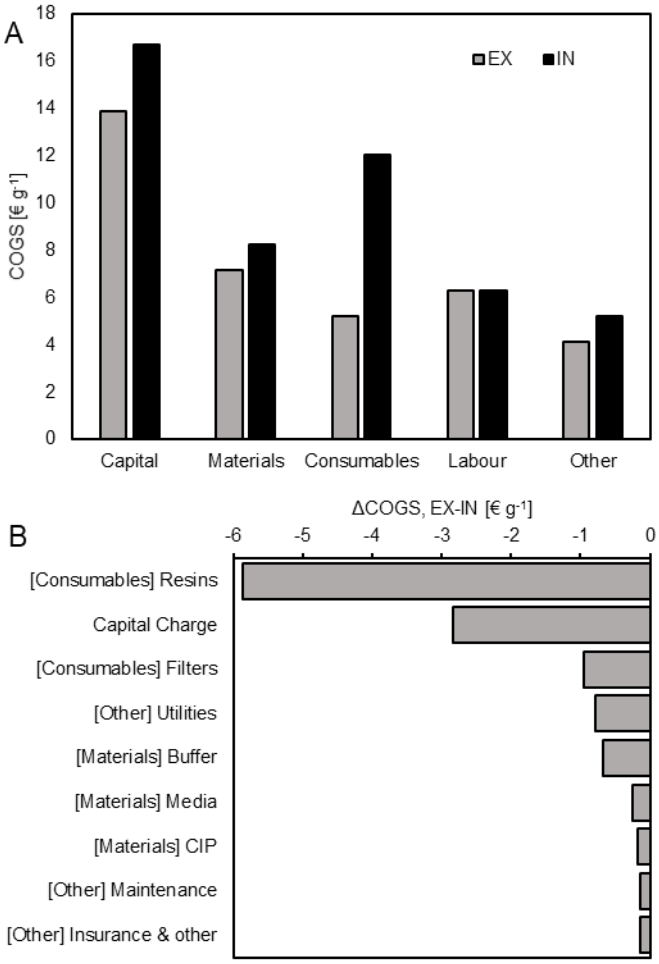


**Figure 3** Cost of goods sold (COGS) for production and capture of SpA in process EX and process IN, estimated with the BioSolve modeling software. A) Contribution of different COGS categories to total COGS. B) Difference in COGS between process EX and process IN, split into subcategories. The COGS of process IN was subtracted from the COGS of process EX, thus negative numbers indicate lower COGS in process EX. The total COGS difference was -11.8 € $g^{-1}$.

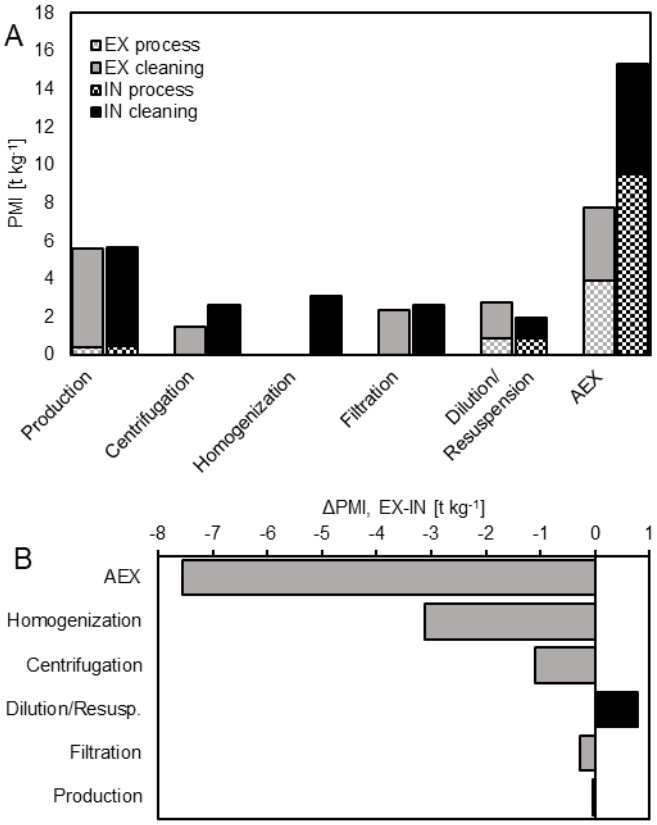


**Figure 4** Process mass intensity (PMI) of process EX and process IN for the production and capture of SpA, estimated with the BioSolve modeling software. A) Contribution of the different unit operations categories to total PMI. B) Difference in PMI between the unit operations of process EX and process IN. The PMI of process IN was subtracted from the PMI of process EX, thus negative numbers indicate a lower PMI in process EX and vice versa. The total PMI difference was -11.3 t $kg^{-1}$.