Innovating Quality Control Mechanisms in Aseptic Drug Manufacturing by Means of Isothermal Microcalorimetry and Tunable Diode Laser Absorption Spectroscopy

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Preamble

Transforming academic research into a practical use for industry is certainly challenging. Yet, within the following work a large pharmaceutical producer and an academic institution have collaborated to reach a common target: Implementing innovation into quality control mechanisms applied to aseptic drug manufacturing procedures. Due to such interaction, a trade-off between scientific aspects and economic topics has been made in the writing of this doctoral thesis. This explains why this doctoral thesis often discusses efficiency improvements, process modifications and overall cost reductions based on scientific insights gathered experimentally. Finally, such interaction between science and practice led to mutual benefits being reflected in the following work.

Table of Contents

Acknowledgements

I express at this point my deepest thank to all people who contributed to the successful development of this work performed in form of an industrial doctoral thesis.

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Summary

Aseptic manufacturing refers to bringing the sterile drug solution into decontaminated containers in a way that product sterility and therapeutic effectiveness is maintained. At this stage the produced drug has a significant value, reflecting relatively large financial risk in case of failure during manufacturing procedures. Therefore, environmental monitoring activities strictly control production surroundings to ensure that no accidental product contamination occurs.

Media fills are part of environmental monitoring activities and imitate the aseptic (free from pathogenic microorganisms) filling procedure with microbial growth medium instead of the liquid drug product. After filling, media fills are inspected visually on turbidity, which represents the control on filling line asepticity. Such inspection is time-consuming, manually performed and therefore considered for potential automation. A laser-based technology was used (called tunable diode laser absorption spectroscopy) abbreviated as TDLAS to determine CO_2 and O_2 variations in media fill headspaces as related to metabolic activity of growing microorganisms. The study results demonstrated that TDLAS can automate the visual media fill inspection reliably (inspection rate of 100 containers per minute) allowing a roughly 90% faster inspection than achieved by the manual visual inspection on turbidity.
TDLAS was further assessed on its potential in simplifying conventional measurement techniques in the field of calorespirometry. Calorespirometry deals with the simultaneous analysis of O_2 consumption, CO_2 production and heat emission by living systems such as tissues or organism cultures. TDLAS is a well-performing and convenient way to evaluate non-invasively the rates of O_2 consumption, CO_2 production during mentioned studies.

In aseptic manufacturing the sterility assessment is the last control of product sterility before an entire batch is released to the market. The assessment usually consists of a final visual inspection on turbidity 14 days after drug preparation. Isothermal microcalorimetry (IMC) is a methodology measuring small amounts of emitted heat and can thereby detect growing microorganisms. It is more sensitive than the visual inspection on turbidity and was therefore applied as alternative test for microbial growth to sterility assessments. IMC appears to have a large potential to improve the sterility assessment as all tested microorganisms were earlier detected by IMC as by the visual inspection.

Performed projects demonstrate that IMC and TDLAS can improve quality control mechanism by designing those more efficiently. Therefore, ongoing IMC and TDLAS based research is recommended to exploit the full potential of the aforementioned technologies.

Abbreviations

ATCC	American type culture collection
ATP	Adenosine triphosphate
BCN	Bacterial cell number
CCI	Container closure integrity
CFU	Colony forming units
CI	Confidence interval
CR_{CO2}	Calorespirometric ratio for CO_2 (emitted heat per CO_2)
CR_{O2}	Calorespirometric ratio for O_2 (emitted heat per O_2)
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EMT	Environmental monitoring
FDA	Food and Drug Administration
FN	False negatives
FP	False positives
FTM	Fluid thioglycollate
GMP	Good manufacturing practice
GPT	Growth promotion testing
IMC	Isothermal microcalorimetry
LHI	Lighthouse Instruments
M9	Minimal medium
M9N	Minimal medium plus nitrate
OD	Optical density
PhEur	European Pharmacopoeia
R	Statistics program
$R_{q,CO2,O2}$	Rate of heat emission, CO_2 production, or O_2 consumption
RMM	Rapid microbial methods
RQ	Respiratory quotient
$T_{H,CO2,O2}$	Threshold for heat emission, CO_2 production or O_2 consumption
TDLAS	Tunable diode laser absorption spectroscopy
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TtD	Time to detection
VI	Visual inspection (on turbidity)
WHO	World Health Organization

List of Figures, Tables and Equations

Introduction

Discussion

CHAPTER 1

General Introduction

1. General Introduction

1.1 Biologics and the Importance of Quality

Biologics (or biopharmaceuticals) are the most elegant and innovative achievements in modern drug science among existing medication. The active component of biologics is derived from living cell systems by means of recombinant DNA and controlled gene expression in bacteria or yeast. In contrast to conventional chemically synthesized drugs, biopharmaceuticals are large and complex structures typically based on polypeptides, (glyco-) proteins, and nucleic acids. The specific form of biologics enables interacting remarkably well with corresponding targets permitting the use of highly effective and selective therapies (Declerck 2011, Otto et al. 2014).

Product quality ought to be one of the superior assets delivered to patients when dealing with the manufacture of biologics (Haleem et al. 2015). All product contacting production steps are strictly regulated and controlled as already minor process variations could result in heterogenic, contaminated, not effective or even damaging medication (Declerck 2011). Biologics are administered through subcutaneous and intramuscular injections or intravenous infusions as an efficient drug uptake through the gastro-intestinal tract would be hindered by protein degradation in the stomach (Irvine et al. 2010). Therefore, drug sterility is indispensable and manufacturing procedures of rather complex nature. The slightest biological, chemical or physical contaminations may lead to exacerbated health conditions endangering the, in most cases, immunocompromised patients further (Figure 1).

Due to the protein based composition, which is sensitive to variation in physical conditions, biopharmaceuticals cannot be terminally sterilized before market release as contained proteins would be denatured and therapeutic drug efficacy be lost (Stockdale 2016). Thus, biopharmaceutical manufacturers are faced with designing production processes in a way that product asepticity (free of microbial contamination) is enabled during the entire drug product manufacturing process. Such premises are challenging, subject to constant improvement (Food and Drug Administration 2004) and dedicated to the wording “aseptic manufacturing” (Figure 1).

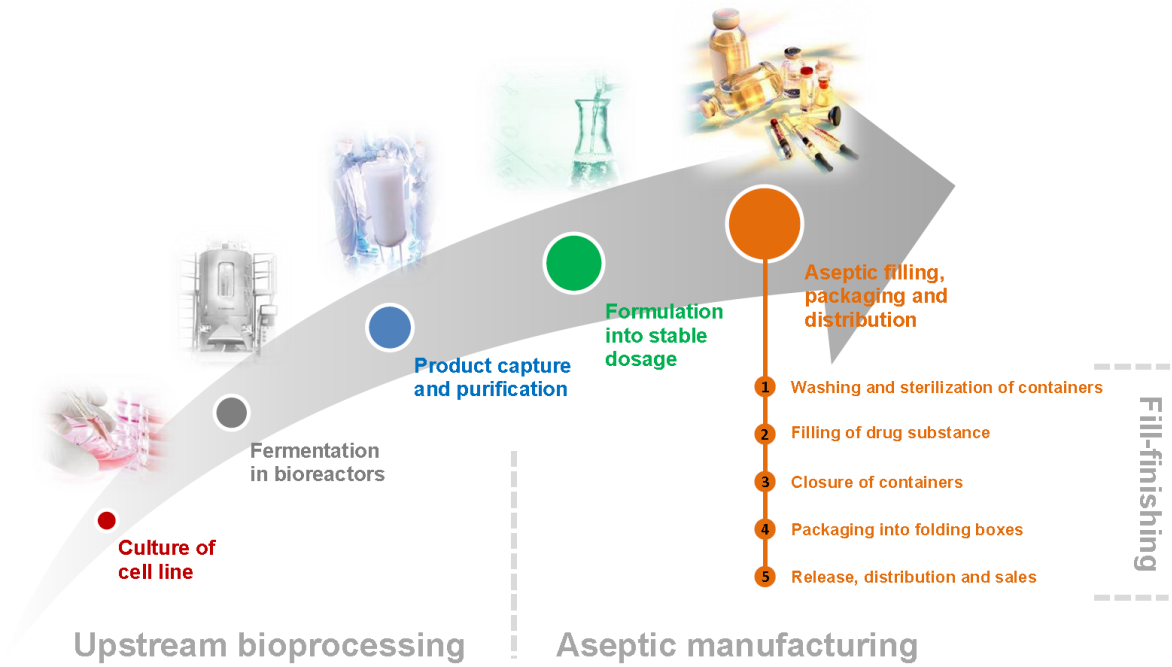

Figure 1: End-to-end production steps for biopharmaceuticals. During upstream bioprocessing modified cell-lines are cultivated in media and successively transferred to larger bioreactors for fermentation. By the addition of optimized and cell line specific nutrient media, production of the desired protein is enabled. The protein is separated from the biomass followed by centrifugation, purification, and concentration steps. During aseptic manufacturing the extracted pure drug substance is formulated into a stable dosage form and filled under aseptic conditions in sterile primary (such as vials, syringes, cartridges, etc.) and secondary (such as folding boxes, etc.) packaging (see also orange sub steps 1-5, indicating fill-finishing procedures). After quality approval of the final drug product, packaged biologics are shipped and sold to customers. (BioPharma Asia 2015, Ams Biotechnology 2013, Arcane Industries 2017, Bandageer 2013, BioRefinery 2009)

1.2 Aseptic Manufacturing and Drug Product Quality Control

Aseptic manufacturing is following aseptic upstream bioprocessing and refers to bringing the sterile drug substance into decontaminated containers in a way that sterility and effectiveness is maintained (Kovarcik 2016, Food and Drug Administration 2004). Such procedures include the formulation, mixture or general preparation of the sterile drug substance (for example in liquid or lyophilized form) as well as container filling and sealing (fill-finishing) (Figure 1). At the stage of aseptic manufacturing the final drug substance has significant value, reflecting noteworthy risk in case of failure during manufacturing activities (Scott et al. 2011). To minimize the risk of product contamination special measures are used to maintain and control surrounding cleanliness (Food and Drug Administration 2016, European Pharmacopoeia 2007, U.S. Federal Standard 2015b) and keep humans, the main source of pollution in aseptic manufacturing, away from the product (Figure 2). The concept of clean room areas is the basis for such risk minimization and separated into several zones (European commission 2008, U.S. Federal Standard 2015a). Zone A is the cleanliest area, in which products are filled. Zone F is the least cleanliest, where people are present in street clothes (no special measured applied); thus, zones B, C, and D, are in-between with altering specification on allowed bio burden (Stockdale 2016).

The zones are determined by the degree of allowed particulate contamination per cubic meter air and behave according the principle of onion skins: the closer the zone to the open product, the less particulate contamination is allowed implying additional measures needed to protect the product (see Figure 2, a & b). Therefore, operators entering zone D (the second least cleanliest zone after zone F) need to wear special clothing to keep contamination risk of bacteria colonizing their body and clothes as low as possible (Park et al. 2014). In addition, clean rooms are regulated in terms of humidity and temperature and are constantly ventilated by sterile filtered air streaming from ceiling to floor, using the principle of laminar flow (Figure 2, c). Thus, generating less favorable conditions for fungal growth and lowering bio burden in the air (U.S. Federal Standard 2015b). Furthermore, standard cleaning procedures are applied, such as: hand disinfection, use of antimicrobial cleaning media, and alcohol application at workbenches. Fill-finishing is the action with the close proximity to the product in aseptic manufacturing and is therefore performed in isolators replicating the environment of zone A (no human presence allowed), and separating the valuable product entirely from potential sources of pollution (Figure 2, d).

a)

Zone name	Recommended limits for microbial contamination			
	Air sample (1) cfu/m³	Settle plates (2) (*diameter 90mm*) cfu/4 h	Contact plates (3) (*diameter 55 mm*) cfu/plate	Glove print (2) (*5 fingers*) cfu/glove
A	< 1	< 1	< 1	< 1
B	10	5	5	5
C	100	50	25	-
D	200	100	50	-

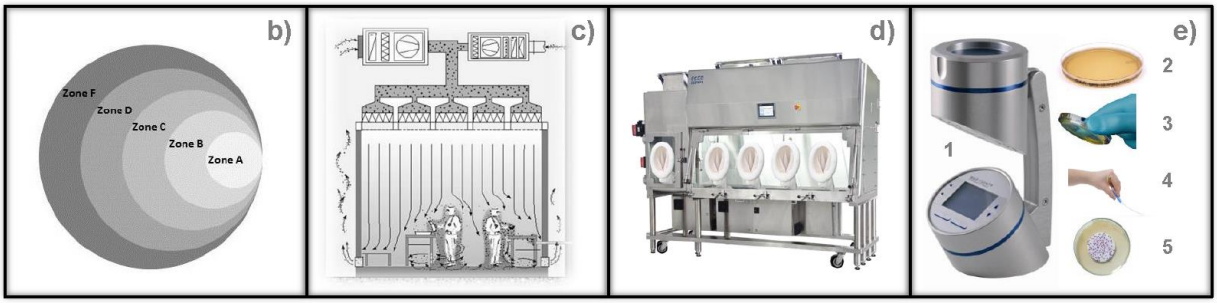

Figure 2: Methods to control and maintain cleanliness in aseptic manufacturing. Figure a) and b) describe the recommended limits for microbial contamination in relation to the clean room concept. Figure c) visualizes the principle of laminar flow applied in clean rooms and isolators. Illustration d) is an example of a standalone isolator. In large scale manufacturing such isolators are directly integrated into filling lines. Finally, e) shows applied techniques in environmental monitoring to determine and control bio burden in air (1 and 2), on surfaces (3 and 4) and in water (5). (Ingeniarg SA 2017, ESCO Pharma 2017, Pharmaceutical Online 2017, Biomérieux 2017, Xebios Diagnostics GmbH 2015, Sigma Aldrich 2017, European commission 2008, U.S. Federal Standard 2015b, Hygiena LLC 2017).

Isolators are closed chambers widely used in the pharmaceutical industry and guarantee near absolute sterility due to their conceptual design. Chamber sterility is achieved by a routinely performed and fully automated bio-decontamination utilizing hydrogen peroxide (H_2O_2), an oxidizing agent reacting with organic matter, killing microbes potentially remaining in the isolator (Bayliss et al. 1980). In case of complications in the isolator, access to the concerned objects is exclusively possible through installed gloves (or robotic arms), whereat a constant flow of filtered air from ceiling to bottom (high efficiency particulate air filters of ~0.3µm pore size are used) generates a steady air turnover enabling an air renewal in the isolator. Existing overpressure is additionally used to keep potential contaminants away from the sterile area. Consequently, the isolator technology allows proper performances of fill-finishing procedures under aseptic conditions. In addition, environmental monitoring (EMT) controls microbiological quality of surfaces (in isolators and surroundings), water and air (Figure 2, e 1-5). Such controls permits immediate reaction in case of action level excess (U.S. Pharmacopoeia 2013, European Pharmacopoeia 2007) therefore allowing the defined levels of zone specific bio burden to be maintained. In the context of EMT, fill-finishing lines are evaluated semi-annually on their degree of sterility.

Such evaluations include the filling of culture media, tryptic soy broth (TSB), instead of the drug substance solution, into product containers, known as “mediafills” (Figure 3). After filling, the media fill containers are inspected on integrity including the careful control of scratches, breaches, loose caps and further mistakes that may have occurred during the fill-finishing process. Subsequently inspected vials are incubated for a minimum of 7 days at 20- 25°C (22.5°C). Following the incubation process, operators visually inspect the media fills on the degree of turbidity (sometimes this intermediate inspection is skipped as not mandatory), which is an indicator for microbial growth relating to a contaminated filling line. The containers are once more incubated for a minimum of 7 days between 30-35°C (32.5°C) followed by a second mandatory visual inspection on turbidity (Food and Drug Administration 2004, Pastowski 2013). Finally, the number of contaminated units is counted (normally equal to zero) and the results documented in a manufacturing execution system.

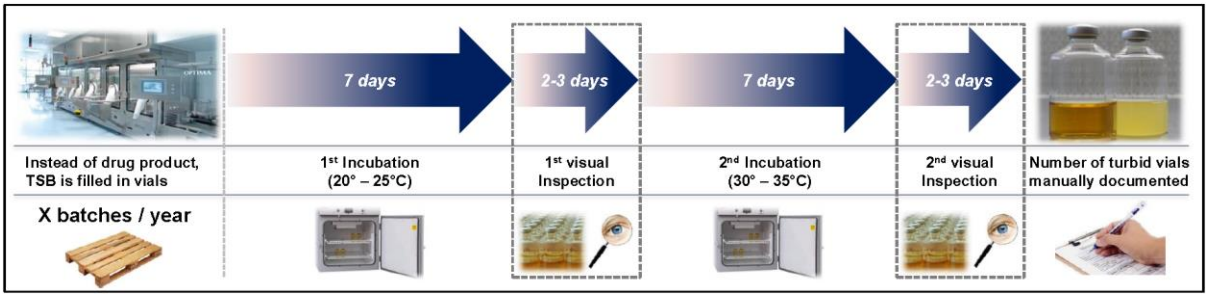

Figure 3: Media fill process description. First the vials are filled with TSB media at the filling line (inspection on integrity is not shown here). Subsequently vials are incubated for 7 days at 20-25°C and inspected on turbidity. Afterwards the process is repeated with an incubation temperature of 30-35°C, to provide besides fungi also bacteria optimal growth conditions. The entire media fill process from filling to final inspection takes a minimum of 18 days. Some manufacturers even prolong incubation periods to ensure that also very slow growing or dormant organisms become detectable. (Metall & Plastic GmbH 2011, Direct Industry 2017a, gesund.at 2017, Astra Nova Ltd. 2015).

The potential detection of contaminated units during the media fill inspection is linked with follow-up actions. The investigational extent of such actions is related to the number of units filled. For example if 5,000 to 10,000 media fill units are filled and one contaminated unit is detected, this should result in an investigation, including the consideration of a media fill repetition. In contrast, two contaminated units are considered cause for filling line revalidation, following diverse investigations (Stärk 2004, Food and Drug Administration 2004). For proper drug product release the result of a media fill is of concern as it represents drug product quality through the analysis of the filling line asepticity. However, of additional importance is the final sterility assessment (Figure 4). It uses filtration practices of meticulously chosen samples (20 – 30 units) combined with a visual inspection on turbidity, which is considered to be representative for the degree of sterility of an entirely filled product batch (20’000 – 100’000 units) (U.S. Pharmacopoeia 2011, European Pharmacopoeia 2005 , Food and Drug Administration 2009).

Consequently, by this approach chances are relatively low to detect single contaminated vials in a product batch, thus a successful sterility assessment is required to release products to the market (Moldenhauer et al. 2004, Sandle 2012). Again, the visual inspection of filtered samples on turbidity is used to assess the degree of batch contamination after an incubation of 14 days.

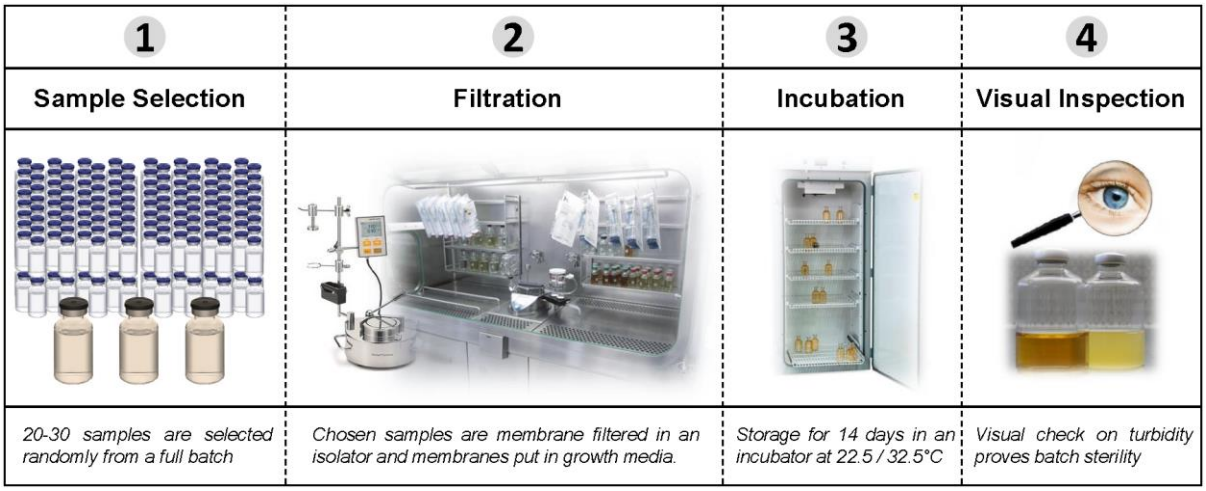

Figure 4: Sterility assessment procedure. From an entire batch ~30 samples are selected. In a H_2O_2 decontaminated isolator twice ~15 samples (the moiety) are filtered through two different filters. One filter is put into a tryptic soy broth (TSB) filled unit (aerobe cultivation), the other one into a fluid thioglycollate (FTM) filled unit (anaerobe cultivation). Subsequently the TSB unit is stored in a 20-25°C incubator and the FTM unit in a 30-35°C incubator. After an incubation of 14 days both units are inspected on turbidity. The product batch is released for commercialization if in both units no turbidity was detected. (BacteriostaticWater.com 2017, Hardy Diagnostics 2017, Franz Ziel GmbH 2017, Direct Industry 2017b, gesund.at 2017, Brueckner 2016b).

Despite the numerous quality control mechanisms utilized, approximately 207 injectable drugs (not only biologics) were recalled from the market within 2012 and 2016 (Table 1). Of the recalled cases, 38 were due to insufficient product sterility which could have led to infections in patients. Overall, it is hard to indicate a concrete number of potentially endangered humans if drug products were not recalled. However, the analysis illustrates that there is potential to design more secure and reliable quality control mechanisms, thus avoiding the release of products that are quality non-compliant. Technologies with the ability to facilitate the detection of drug quality defects and further allow a more efficient work performance are significantly supportive. Such methods could help to reduce recall rates as well as finally deliver manufacturers monetary benefits (in terms of lower recall rates or more efficient processes).

Table 1: Product quality related recalls. The analysis includes both recalls for injectable (207 cases) and non-injectable (223 cases) drug products. The values in brackets state the relative amount of injectable drugs from total recalls per category. There were 38 recalls observed for injectable drugs during 2012 and 2016 caused by deficient sterility control mechanisms. Accessed recall databases belonged to World Health Organization (A), government of the United Kingdom (B), drug office of Hong Kong (C), Swissmedic (D) and Food and Drug Administration (E). (World Health Organisation 2017, Gov.uk 2017, Drug Office Hong Kong 2017, Swissmedic 2017, Food and Drug Administration 2017)

Quality categories per recall database	A	B	C	D	E	Total
Packaging and labelling *(first and secondary packaging, leaflet, non-integer stoppers, wrong labelling, scratches, packaging with tendency for breakage)*	1	26	23	18	12	**80 (45%)**
Impurities and quality control failure *(Particles, contamination with drug, other non-biologic contamination, stability, crystallization, precipitation)*	4	24	46	31	38	**143 (58%)**
Patient safety *(Falsified drugs, wrong dosage, unexpected adverse events, general health hazards, treatment failures, organ failure)*	35	14	35	7	5	**96 (27%)**
General sterility problems *(deviations of EMT, linkage to packaging problems such as leakage or other risks of microbial contamination)*	1	8	9	2	22	**42 (76%)**
Sterility issue with isolates *(identified bacteria or fungi)*	0	1	0	0	6	**7 (86%)**
Others *(Clinical studies, market authorization, licensing, unexpected drug change)*	8	5	41	6	2	**62 (38%)**
Total	**49**	**78**	**154**	**64**	**85**	**430 (48%)**

1.3 Approaching the Improvement of Drug Quality Control Mechanisms

Since the use of modern isolator technologies in aseptic manufacturing, filling processes have become safer in terms of microbial contamination. As a result the need for media fills and sterility assessments are discussed (Glunz 2015). Still, regulatory authorities demand biologics manufacturers to perform media fills and sterility assessments as they are a fundamental part of the quality control concept in fill-finish processing. Therefore, the search for supportive and alternative approaches targeting a leaner and more efficient performance of the implied quality control processes is promoted.

The visual media fill inspection is time-consuming, costly and tedious, adding minor value to the product. Therefore, an automation of the visual inspection is of interest. Indeed, a need for alternative approaches with easy handling, non-invasive measurement of bacterial growth, comparable or faster time to result, providing operational savings and data integrity, exists. The same fulfillment requirement is demanded for a replacement of microbial growth detection within the sterility assessment of finished goods, where rapid time to result is of even more importance. Shortened growth detection could reduce requested incubation durations and allow an earlier release of finished goods to market.

Presently, various qualitative microbial methods exist, allowing faster detection of growing microorganisms compared to conventional approaches. Such methods are based on electro-optical, spectroscopic or biochemical reaction analysis having advantages and drawbacks (Miller 2016). However, among existing microbial methods it is relatively difficult to find one that fulfills demanded requirements such as non-invasive, continuous, and cost-efficient determination of microbial growth in closed systems. Therefore, within this study the search for alternative methods which enable microbial growth detection was widened beyond the boundary of classical techniques. Two technologies were identified and considered to be appropriate for the automation of the visual media fill inspection and the potential replacement of the sterility assessment. Due to the technologies generic applicability their usability in manufacturing related research was of further interest. Thus, the additional assessment of an alternative way to control microbial growth during drug substance manufacturing (tracking CO_2 and O_2 based respiratory and overall metabolic activities during for example fermentation) was targeted. Both methods used parameters resulting from microbial metabolic activity allowing growth detection in concerned test units.

1.4 Microbial Metabolisms

Microorganisms are subject to special attention in pharmaceutical and biotech industries. Although microbial cells are helpful for the production of biologics, their presence in aseptic manufacturing causes disturbances. The main threat of product contamination in aseptic manufacturing comes from organisms capable of surviving in aerobe surroundings; namely obligate aerobe, facultative-aerobe, or fermenting microbes (Table 2). Organisms such as *Staphylococcus sp., Micrococcus sp.* and *Corynebacterium sp.* are most frequently encountered in clean rooms as these organisms live on skin, lips, scalp, and within mouths and nostrils of humans (Park et al. 2014). Further, these species can easily access clean room areas through operators at work, yet are normally killed by means of standardized cleaning and sterilization procedures (Zuber 2014). In contrast, some microorganisms (e.g. *Bacillus sp., Clostridium sp.*) have spore-forming characteristics, meaning such microorganisms are capable of producing latent precursor units (spores) of fully developed cells, and are thus more robust against damaging environmental conditions. Therefore, they are hazardous to manufactured products.

Table 2: Typical microorganisms isolated from clean rooms. Aside from the microbial name and group name the table shows the relative appearance frequency (App. frequency) and metabolism types. As depicted, a minimal amount of appearing isolates is of anaerobe nature, as anaerobe conditions are less frequently encountered in aseptic manufacturing. (Park et al. 2014, Brueckner 2016a, Zuber 2014)

Microorganism	App. frequency	Metabolism type
*Staphylococcus group 1**	21.0%	facultative anaerobe
Micrococcus luteus	16.0%	aerobe
Corynebacterium sp.	9.4%	aerobe
*Staphylococcus group 2***	7.4%	facultative anaerobe
*Micrococcus group 2****	3.5%	mostly aerobe
Moraxella osloensis	3.5%	aerobe
Bacillus sp.	2.1%	aerobe
Acinetobacter sp.	2.0%	aerobe
Penicillium sp.	1.8%	fermentative
*Micrococcus group 1*****	1.6%	aerobe
Staphylococcus aureus	1.4%	facultative anaerobe
Aspergillus sp.	1.0%	aerobe
Cladosporium sp.	1.0%	anaerobe

* includes *S.capitis, S.caprae, S.epidermidis, S.haemolyticus, S.hominis, S.pasteuri, S.saccharolyticus, S.warneri*
** includes *Staphylococcus sp.* except *St. aureus*
*** includes *Micrococcus sp., Micrococcus lylae, Kocuria palustris*
**** includes *Kocuria carniphila, K.kristinae, K.rhizophila, K.varians,Kytococcus sp., Nesterenkonia sp.*

Nevertheless, for survival and replication microorganisms need nutrients enabling the production of chemical energy driving cell processes and biomass formation. They normally use organic compounds such as sugars, amino acids, fatty acids and alcohols as primary energy and carbon source. Overall microbial metabolisms can be separated into two sets of metabolic processes – catabolism and anabolism (Figure 5). Catabolism is the set of reactions breaking down organic compounds (nutrients) into smaller molecules. Those are either used to construct new compounds during anabolism, or are degraded further to simple waste products (e.g. CO_2, H_2O, lactic acid, urea, etc.). The production of cellular waste occurs normally through oxidation, releasing free energy, some of which is lost as heat (~40%), and some of which (~60%) enables the production of chemical energy, stored as adenosine triphosphate (ATP) (Fuchs et al. 2014, Madigan 2015).

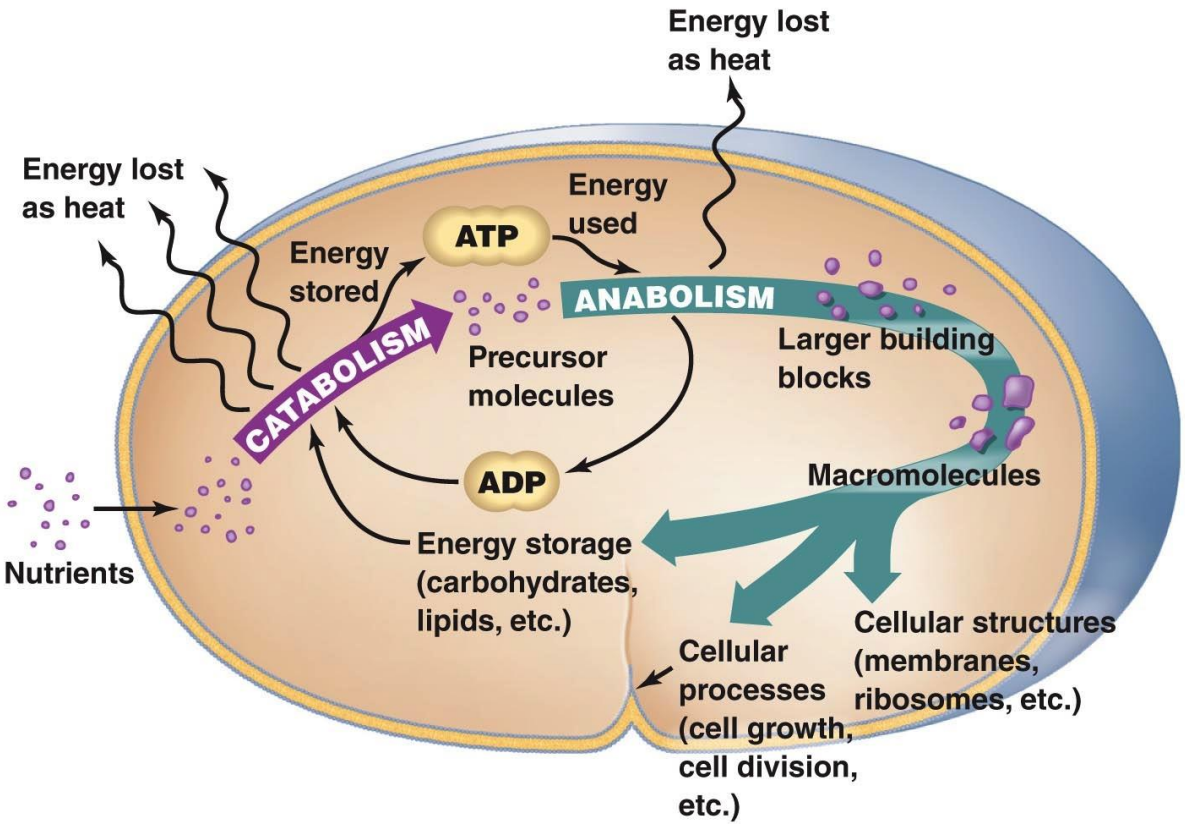

Figure 5: Simplified illustration of catabolic and anabolic pathways. Catabolism is used to break down large molecules into smaller molecules (sometimes precursor molecules) whereby energy is released (lost as heat or used for ATP synthesis). The energy in form of ATP enables the performance of anabolic reactions used by the cell to construct necessary macromolecules for survival, replication or other constructive processes. (WorldNews Network 2016)

Three main catabolic pathways used for ATP synthesis are encountered in cleanroom isolates. They are categorized into aerobe respiration, anaerobe respiration, and fermentation (Figure 6). Aerobe and anaerobe pathways are driven by electron accepting substances such as oxygen (O_2; aerobe) or e.g. sulfate and nitrate (SO_4^{2-} and NO_3^-; anaerobe), using Glycolysis and the Krebs cycle to synthesize ATP (Cowan 2016).

Fermentation is mostly used if access to electron acceptors other than organic compounds is not possible or if organisms synthesize ATP strictly by fermenting metabolisms. Normally microorganisms are capable to switch between several different metabolism types depending on their survival strategy (see also Table 3). If oxygen can be used as electron acceptor aerobe respiration is considered as primary pathway until its depletion. This happens as theoretical yield is highest for aerobe respiration (38 ATP per glucose) if compared to anaerobe respiration (2-36 ATP per glucose) or fermentation (2 ATP per glucose) (Cowan 2016).

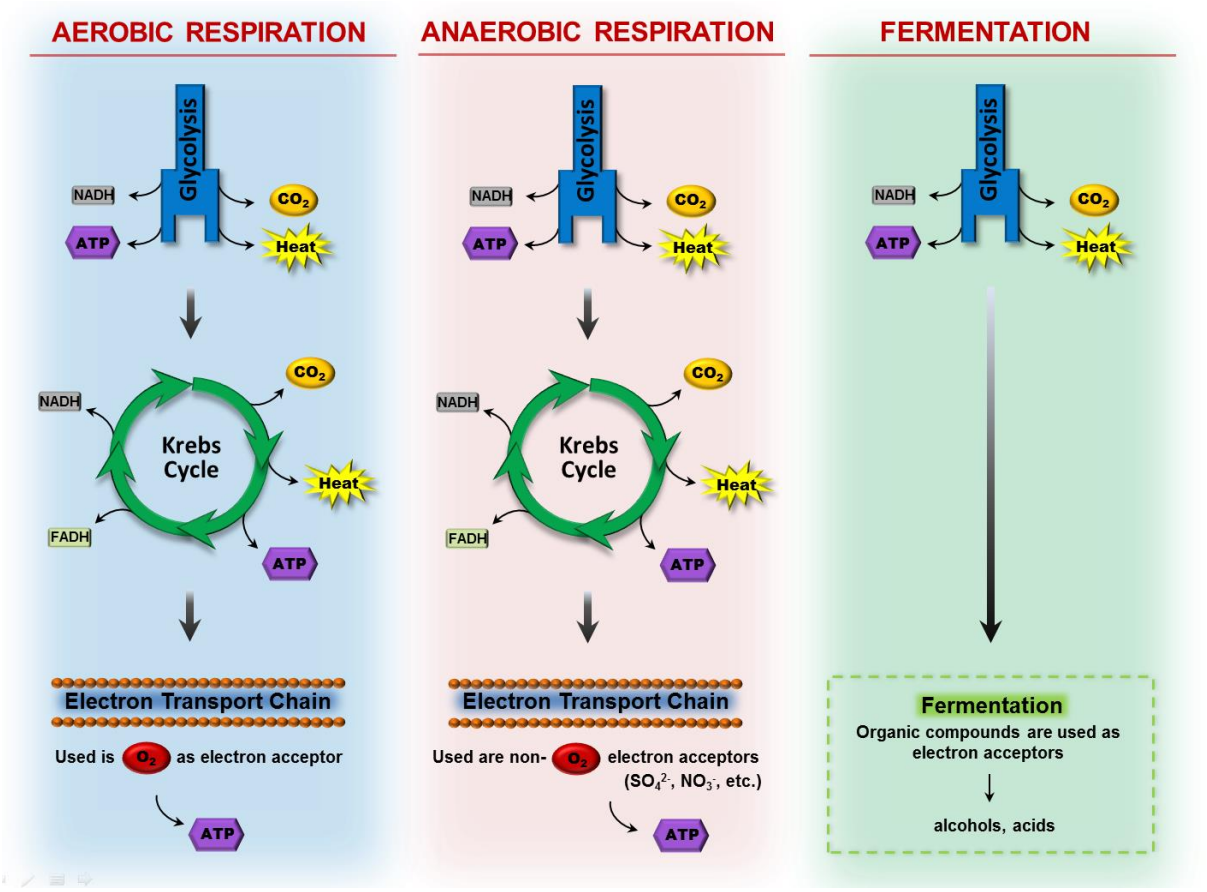

Figure 6: Catabolic main cycles frequently used by microorganisms. Aerobic and anaerobic respiration use Glycolysis and the Krebs cycle as pathways to gain energy out of catabolic processes. Either oxygen or other electron acceptors are used for substrate oxidation, releasing besides others CO_2 and heat as waste products. Fermentation occurs under exclusion of oxygen and uses organic compounds as electron acceptor. (Cowan 2016, modified)

ATP yield from catabolic reactions is driving anabolic pathways towards the construction of macromolecules out of precursor substances. More generally spoken, macromolecules allow the construction of cell structure (e.g. membranes, ribosomes, etc.), the performance of cell internal processes (e.g. maintenance, growth, cell division, etc.) or the assembly of energetic storages which can further be used for ATP regeneration. The sum of these constructive processes is dedicated to anabolism (Cowan 2016, Fuchs et al. 2014, Madigan 2015).

For environmental control in aseptic manufacturing the simple determination of microbial presence is of major relevance, much more than the understanding of cell internal processes. Methods applied perform a growth based assessment, treating microbial cells as black boxes that consume nutrients as well as electron acceptors (O_2, NO_3^-, etc.) and release waste such as CO_2 or heat, resulting under appropriate conditions in a detectable biomass increase (higher turbidity in liquid media or appearance of colony forming units on agar plates). However, besides the biomass increase also educts consumed and waste produced can be helpful in determining the presence of microorganisms (Table 3).

Table 3: Some common catabolic pathways encountered in clean rooms. Reactants are related to the initial substances used for energetic gain. Products relate to the result of the entire metabolic reaction also dedicated to metabolic waste. Heat, an additional product of metabolic performance, is not listed in this table. (Fuchs et al. 2014, Madigan 2015, van Maris et al. 2006)

Metabolism type	Reactants	Products	Sample organisms
Aerobe respiration	$C_6H_{12}O_6 + 6O_2$	$6CO_2 + 6H_2O$	*Staphylococci, Pseudomonas, etc.*
Alcoholic fermentation	$C_6H_{12}O_6$	$2C_2H_5OH + 2CO_2$	*Yeast, Zymomonas*
Homolactic fermentation	$C_6H_{12}O_6$	$2C_3H_6O_3$	*Streptococci, some Lactobacilli*
Heterolactic fermentation	$C_6H_{12}O_6$	$C_3H_6O_3 + C_2H_5OH + CO_2$	*Leuconostoc, some Lactobacilli*
Propionic acid fermentation	$3C_3H_6O_3$	$2C_3H_6O_2 + C_2H_4O_2 + CO_2 + H_2O$	*Propionibacterium, Clostridium propionicum*
Butyric acid fermentation	$C_6H_{12}O_6$	$C_4H_8O_2 + 2H_2 + 2CO_2$	*Clostridium butyricum*

Therefore, a basic metabolic understanding is required to identify techniques allowing uniform growth detection. As can be seen in Figure 5, Figure 6 and Table 3 heat emission, CO_2 exhaust and O_2 consumption are linked with most catabolic processes performed by microorganisms surviving in clean rooms. Therefore, the evaluation of such parameters by appropriate technologies could bring further innovation in the assessment of microbial presence.

1.5 Approaches Used for Non-invasive Microbial Growth Analysis

Metabolic Heat Determination by Isothermal Microcalorimetry

Virtually all biological and chemical processes emit (or consume) heat, thus thermal energy release is a suitable study parameter (Wadso et al. 2001). The analysis of heat dissipation can assist in evaluating presence of metabolically active cells, specifically in microbiology; where biological and chemical processes provide the cells necessary energy for survival (Trampuz et al. 2007, Braissant et al. 2015). In fact, metabolic heat dissipation can be utilized in the assessment of e.g. resistance to antibiotic drugs or optimal conditions for upstream bioprocessing (condition related yield maximization of a certain protein or other product) (Maskow et al. 2011, Braissant et al. 2010, Rohde et al. 2016b, Baldoni et al. 2009).

Each compound of a reaction contains energy, which is termed inner energy (U). During a reaction in a system inner energy has the potential to amass in form of thermal energy known as reaction enthalpy ΔH. Reaction enthalpy is the sum of the inner energy change ΔU (energy of sum of reaction products (U_2) – sum of reaction educts (U_1)) and work performed by a system (product of system pressure (p) and change in system volume (ΔV)). If the system is not performing, ΔH becomes equal to ΔU: in such cases, heat can be used as a reliable proxy for metabolic cell activity. A reaction releasing heat is of exothermal nature and thus, has a negative ΔH (Mortimer et al. 2007).

Metabolic reactions are by nature mostly exothermal (-ΔH) else the energetic gain needed for internal cell processes, could hardly be realized. The expected degree of heat dissipation from a metabolic reaction can be determined by calculations of enthalpy. Standard enthalpy values (ΔH^0_f) help realizing such calculations representing the enthalpy change during the formation of 1 mole substance from its initial compounds under standard conditions (25°C, 1 atm, pH7). To calculate ΔH^0 of an entire metabolic reaction (Equation 1), the difference between $\Sigma\Delta H^0_f$ (products) and $\Sigma\Delta H^0_f$ (reactants) is determined, which is based on the Hess' law (Figure 7).

$$\Delta H^0 = \Sigma \Delta H_f^0\ (products) - \Sigma \Delta H_f^0\ (reactants)$$

Equation 1: Formula for enthalpy calculation of a reaction based on standard enthalpy values. (Mortimer et al. 2007)

The law of Hess states that: the enthalpy change associated with the reaction depends only upon the identity of the reaction educts and products being completely independent of whether the reaction takes place in a series of steps or all at once (Mortimer et al. 2007).

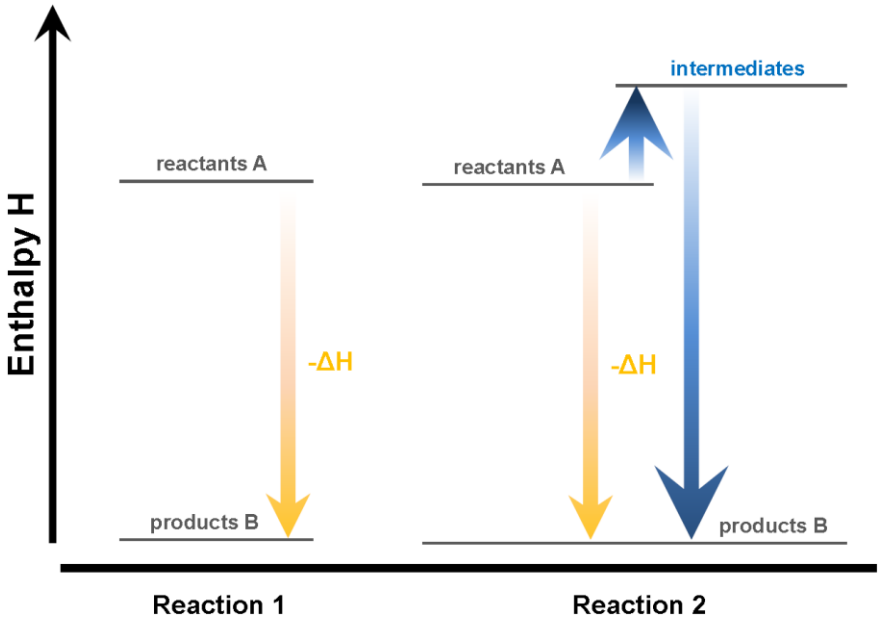

Figure 7: The principle of Hess' law is shown describing that the reaction enthalpy size is independent of whether the reaction takes place all at once (Reaction 1) or in a series of steps (Reaction 2), as long as reactants and educts remain the same. (Clark 2017)

Respecting the principles of Hess' law the calculated enthalpy change correlates directly with the amount of heat emitted (always considering that the system's work is negligible) which in turn depends on both, the kind of metabolism and the number of active cells in the system. Thus the meaning of heat dissipation originating from growing bacteria in a system becomes comprehensible (Table 4).

Table 4: Enthalpy calculations for frequent metabolism types. ΔH^0 is the enthalpy of the entire reaction. The respective sum of enthalpy of formation (ΔH^0_f) is shown for products and reactants. Standard enthalpies of $C_6H_{12}O_6$ (s) (-1271.0 kJ/mol), CO_2 (g) (-393.5 kJ/mol), O_2 (g) (0.0 kJ/mol), $C_3H_6O_3$ (s) (-688.3 kJ/mol), H_2O (l) (-285.8 kJ/mol), C_2H_5OH (l) (-277.6 kJ/mol), $C_3H_6O_2$ (l) (-510.0 kJ/mol), $C_2H_4O_2$ (l) (-484.5 kJ/mol), $C_4H_8O_2$ (l) (533.9 kJ/mol) and H_2 (g) (0.0kJ/mol) were taken from (Bigler 2017, NIST 2016, Earhart 2016, Crosby 2015, Li et al. 2016).

Metabolism type	ΔH^0	$\Sigma \Delta H^0_f$ (products)	$\Sigma \Delta H^0_f$ (reactants)
Aerobe respiration	-2805 kJ/mol	*-4076 kJ/mol*	*-1271 kJ/mol*
Alcoholic fermentation	-70 kJ/mol	*-1341 kJ/mol*	*-1271 kJ/mol*
Homolactic fermentation	-106 kJ/mol	*-1377 kJ/mol*	*-1271 kJ/mol*
Heterolactic fermentation	-88 kJ/mol	*-1395 kJ/mol*	*-1271 kJ/mol*
Propionic acid fermentation	-119 kJ/mol	*-2184 kJ/mol*	*-2065 kJ/mol*
Butyric acid fermentation	-50 kJ/mol	*-1321 kJ/mol*	*-1271 kJ/mol*

Isothermal microcalorimetry (IMC) allows for non-invasive and continuous monitoring of the rate of heat coming from the test sample, which is proportional to the rate of performed processes in the living system. Such heat is measured by means of heat-flow sensors (e.g. peltier elements) located between the sample and the heat sink (Figure 8). The sample, the sensor and the heat sink are placed in a thermally insulted bath with constant temperature to record minimal temperature variations in the reaction vessel. In case of heat variations in the sample, sensors test the sample against a reference and transform the corresponding heat flow into an electrical signal. This transformation is realized by the Seebeck effect where a voltage difference is generated across two different semiconductor materials if a temperature gradient is recognized through two junctions of the material (Kemp 1998, Wadso et al. 2001). The monitored voltage difference is normally converted into a Watts per second signal, which is the obtained output parameter of a calorimetric measurement enabling calculations in Joule.

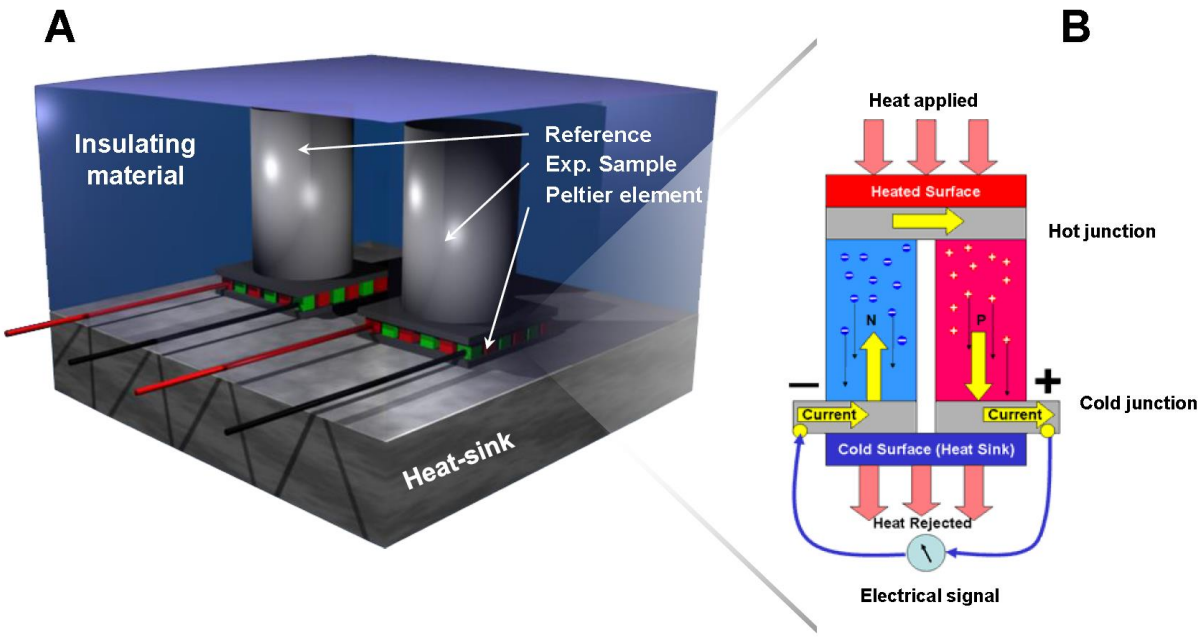

Figure 8: The concept of a calorimeter. On the left side (A) the inner life of a calorimeter is shown. Reference and sample unit are positioned each on a peltier element placed on a heat sink. The voltage signal originating from the temperature change in the sample is captured by the peltier elements, evaluated by a computer and transformed to a Watt signal. On the right side of the graph (B) the principle of a peltier element is shown transforming emitted heat into an electrical signal (Woodbank Communications Ltd 2005, Braissant 2016)

Merely 10^4 – 10^5 microbial cells are sufficient to reach the detection limit of an isothermal microcalorimeter (e.g. Tam III from TA Instruments) which corresponds to a sensitivity of 20nW – 200nW (Braissant et al. 2015). There are two different ways to analyze activity of microorganisms in an experimental sample, being either based on heat flow (raw data from a calorimeter) or on heat (integrated heat flow over time) assessments.

To determine the activity related time to detection of an organism or a culture, a threshold of heat or heat flow must be defined in order to determine the influence the media is having on the measurement over time (Figure 9, A & B). However, if going beyond simple activity assessments a better understanding of mentioned parameters is required to avoid misconceptions.

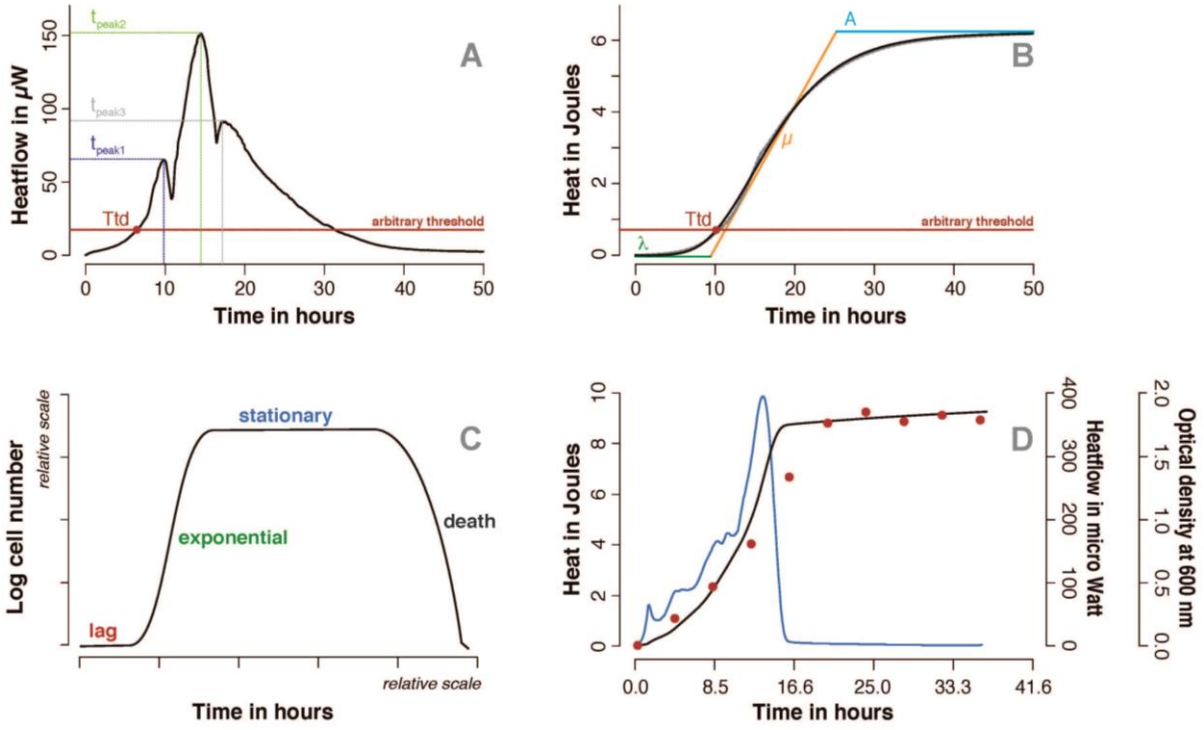

Figure 9: Activity and growth profiles. Figure A shows a typical heat flow profile (black curve) of *Escherichia coli* in basal medium eagle medium having three peaks that are potentially linked with the switch from one substrate to others. In figure B the corresponding heat profile (integrated heat flow over time shown as dotted grey line) and its approximation by the Gompertz growth model (black sigmoidal curve) are depicted together with the extracted microbiological parameters λ (lag phase), µ (growth factor) and A (maximal heat reached). In figure A and B the time point of microbial presence determination (Ttd = time to detection) is marked (red dot) considering that a heat flow and heat threshold was defined earlier. Figure C shows a typical microbial growth profile consisting out of 4 different phases. Figure D illustrates another typical heat (black curve) and heat flow (blue curve) profile of *Pseudomonas putida* indicating that optical density measurements (red dots - relating to the amount of produced cells), rather correlate with heat as with heat flow. (Braissant et al. 2015, Braissant et al. 2013)

Typical microbial growth goes through 4 main phases: the lag, the exponential growth, the stationary and the death phase (Figure 9, C). Similarly, a heat flow profile during a microbial growth experiment also follows rising and declining periods (Figure 9, A - black curve & D – blue curve); however, such curve comparability does not infer similarity between the involved processes. Microorganisms are often confronted with several carbon sources and might consume those successively by adjusting their metabolisms to the substrate.

During the transition state from one substrate to another the synthesis of new enzymes might be requested leading to a decrease in heat flow (Braissant et al. 2013).

In addition, heat flow must be used carefully as a proxy for microbial growth detection (or the cell number) as accuracy is only given as long as a correlation between heat flow and cell count (or optical density) exists. Such correlation typically exists, if at all, during the early exponential growth phase (Figure 9, D). Non-correlation is due to the heat production rate per cell, fluctuating as a result of the metabolism and the growth phase. However, the energetic cost of a cell should remain relatively constant. Thus, a constant amount of heat generated to produce a cell can be expected (Braissant et al. 2013, Maskow et al. 2006), making heat a more reliable proxy for microbial growth studies.

When an entire growth related heat flow pattern is collected in such a way that heat flow is allowed to rise and return to levels close to baseline, the heat-over-time curve typically reflects a sigmoidal profile. Sigmoidal profiles can be approximated by using the modified Gompertz growth model (Figure 9, B; Equation 2). Model based calculations are normally programmed in R by the “grofit” package (Kahm et al. 2010). Thereby, the extraction of microbial growth parameters, such growth rate (μ), lag phase duration (λ), and maximal heat reached (A), from a calorimetric dataset, is automated providing the basis for further assessment (Braissant et al. 2013).

$$y = A * e^{-e[\left(\mu * \frac{e}{A}\right) * (\lambda - 1) + 1]}$$

Equation 2: The modified Gompertz model allows an approximation of an entire sigmoidal growth curve, such as heat, resulting from the integration of the heat flow. A is the maximum value reached (maximum heat production), μ the maximum growth factor (growth speed or maximal steepness of the approximated growth profile) and λ the lag phase duration.

In contrast to the heat flow, heat profiles bear certain advantages in terms of assessing microbiological data, such as facilitated statistical analysis (extraction of parameters), easier correlation of growth data among organisms and estimation of microbial growth in relation to biomass production. Yet, the research purpose determines the suitability for the use of heat flow versus heat. In the case of simple analyses on microbial presence in test units, it is practical to employ both parameters.

Determining Metabolic O_2 Consumption and CO_2 Production by Tunable Diode Laser Absorption Spectroscopy

Carbon-dioxide and oxygen are additional parameters that can be used separately or in combination with isothermal microcalorimetry to detect microbial presence in test units, as directly related to active metabolisms and growth. In systems with closed boundaries (fermenters, vials, etc.) not allowing an exchange with the environment, growing microorganisms influence gas concentrations (such as CO_2 and O_2) strong enough to cause detectable changes in headspace compositions of used containers. Such an influence is proportional to the degree of metabolic activity and the rate of gas consumption or production, characteristic for the metabolism used by the microbial strain. Therefore, technologies, with the potential of detecting such and other gas changes non-invasively, can be a valuable tool for the analysis of microbial presence and growth (AS et al. 2017, Martinez-Cruz et al. 2012) (Figure 10).

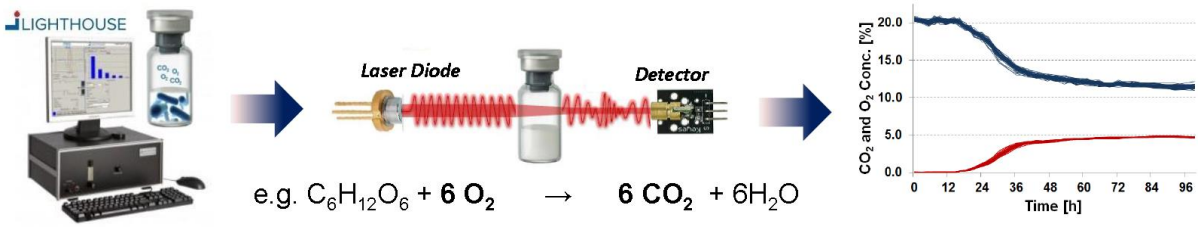

Figure 10: Description of the TDLAS measurement principle in closed containers contaminated with microbial cells. Headspaces of such containers are tested on changing CO_2 and O_2 concentrations being an indicator for active microbial metabolisms. Through several successive measurements growth profiles can be obtained. (Alibaba Group 2017, Lighthouse Instruments 2016)

Tunable diode laser absorption spectroscopy (TDLAS) can determine non-invasively and with a sensitivity ranging from ppm to ppb a large variety of gases (e.g. O_2, CO_2, N_2, NH_3, CH_4, etc. at T = 20-25°C) as such molecules absorb emitted laser light in the near and mid infrared spectral region (Lackner 2007). TDLAS is nowadays used for combustion, leakage, quality and emission control but found additional use in health care (i.e. the analysis of breath gas) and environmental (i.e. control of volcanic activities) industries (Giubileo et al. 2001, Webber et al. 2000, Pedone et al. 2014, Frish et al. 2005). Due to its measurement reliability TDLAS started to be increasingly applied in pharmaceutical process control enabling the assessment of lyophilisation processes (i.e. testing for residual vaporized water in product units) as well as the control of primary packaging material integrity (i.e. glass vials) (Schneid et al. 2011, Posset et al. 2015).

Although in the past TDLAS was only applied to a limited extent in detecting microbial presence, large potential remains for this technique in the field of applied microbiology. High sensitivity, user comfort and non-invasive nature make TDLAS attractive for process analytics and automation in the quality control sector. Especially in the pharmaceutical production where the detection of mainly aerobe and fermenting organisms is part of the daily business, a TDLAS based CO_2 and O_2 growth detection is reasonable (Figure 10).
Most TDLAS lasers applied measure in the near-infrared region. Thus, they can be operated at room temperature without need for cooling. The measurement principle is based on fundamental spectroscopic laws. Highly monochromatic laser light emitted from a distribution feedback electrode is passed through a sample of interest exciting molecules to rotational and vibrational movements. The resulting laser light attenuation is monitored with a detector which allows determining the gas concentration in the specimen headspace (Figure 10). Such determination is based on the Beer-Lambert law (Equation 3), stating a logarithmic relation between the transmission of the light through a gas, being equal to the product of the molar attenuation coefficient of the gas (ε), the distance (d) the light is travelling through the sample and the gas concentration (c) in the sample. (nanoplus 2015, Lackner 2007)

$$\log\left(\frac{I_0}{I}\right) = A = \varepsilon * d * c$$

Equation 3: Beer-Lambert law. It describes the relation among the laser light intensity (before (I_0) and after (I) passing through the sample), the absorption (A), the gas concentration (c), the sample path length (b) and the molar attenuation coefficient (ε). (Lackner 2007)

During the measurement TDLAS adjusts the emitted laser to the absorption wavelength of the target molecule and performs a tuning in its emission wavelength (λ) over the absorption band of the respective gas (Figure 11, A). This is done by altering the temperature or the injection current applied to the laser, targeting an exclusion of non-absorption regions into curve integration. Wavelength or frequency modulations are used to allow measurements with a better signal to noise ratio, raising the technique's result accuracy. As often the concentration of a single gas in a gas mixture is of interest, the selection of a suitable absorption line is of major importance, as otherwise interferences with prevailing gases might coexist and alter measurement results (Figure 11, B) (Zolo Technologies 2013, nanoplus 2015, Lackner 2007).

However, when using TDLAS as method for the analysis of microbial presence it needs to be considered that in more extreme fields than pharmaceutical production areas organisms often use other metabolic pathways, - sometimes even CO_2 and O_2 independent (Madigan 2015). This challenges a CO_2 and O_2 based measurement approach in terms of microbial growth detection. Therefore, TDLAS applied in microbiology shall always be linked with initially specified experimental purposes and microorganisms tested. An application in isolated systems is recommendable to keep external variable minimal. Such considerations can finally limit misinterpretation of measured parameters and allow users to identify TDLAS as a valuable tool for microbiologic assessments.

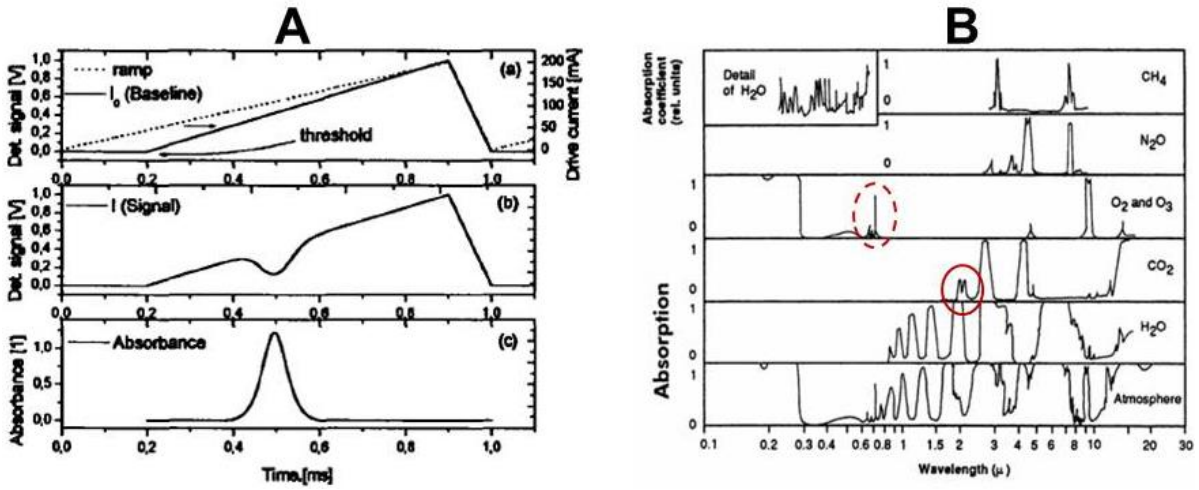

Figure 11: **Technical principles of TDLAS measurements.** The recording of an individual absorption line is shown in A. (a) gives an example for the current ramp applied to the laser and the baseline I_o; (b) shows the transmitted signal I and (c) provides the calculated absorbance according the Beer-Lambert law. In figure B absorption peaks of different gas types are illustrated. It becomes clear that choosing the right wavelength for TDLAS measurements of a certain gas is important, minimizing interferences with other prevailing gases. In our project the wavelength for O_2 was set to 760nm (red circle with dotted line) and for CO_2 to 2000nm (red circle) (Lackner 2007, Fleagle et al. 2006)

1.6 Dissertation Structure

The entire work was performed through research collaboration between industry (F. Hoffmann-La Roche Ltd., Novartis Ltd., Lighthouse Instruments Ltd., Wilco AG) and academia (University of Basel, Department of Biomedical Engineering, Center of Biomechanics and Biocalorimetry). F. Hoffmann-La Roche provided financial support and allowed use of facilities and materials for realizing planned work in the form of case studies. Other industrial partners participated by providing devices or access to data. Academia was the main contributor in terms of calorimetry and data modelling.

TDLAS Based Automation of the Visual Media Fill Inspection

Assessing the feasibility of automating the visual media fill inspection by TDLAS was the main project of the dissertation and included three subprojects. First, a proof of concept study was performed testing if TDLAS is generally capable to detect microbial growth in media fills. CO_2 and O_2 TDLAS benchtop devices (Lighthouse Instruments) were used to evaluate an obligate aerobe and a homo-fermenting (expected to have lower metabolic activity) microorganism on the potential in influencing O_2 and CO_2 media fill headspace concentrations (Brueckner et al. 2016). Second, growth detectability of a larger variety of microorganisms was targeted using again TDLAS benchtop devices (Brueckner et al. 2017). Novartis and F. Hoffmann-La Roche contributed equal amounts of data for a joint publication. Finally, an automated platform provided by Wilco AG, allowed assessing the automated media fill inspection on its reliability.

An Alternative Sterility Assessment

IMC was used to evaluate if a potential substitution of the sterility test's visual inspection on turbidity is possible. In addition, it was assessed if IMC allows a more sensitive growth analysis than the inspection approach in use, leading to a potential reduction of incubation and product holding times at aseptic manufacturing sites.

Combining TDLAS and IMC in Calorespirometric Analysis

Calorespirometry is the simultaneous analysis of the rate of heat emission, O_2 consumption, and CO_2 production by living systems such as tissues or organism cultures. The analysis provides useful knowledge about thermodynamic parameters relevant for e.g. biotechnology where parameter based yield maximization (fermentation) is highly relevant (Wadso et al. 2015). However, conventional techniques to determine CO_2 production and O_2 consumption rates are laborious. Therefore, tunable diode laser absorption spectroscopy (TDLAS) was assessed as an alternative approach for microbial respirometric analysis in order to facilitate the data collection procedure.

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CHAPTER 2

Comparison of Tunable Diode Laser Absorption Spectroscopy and Isothermal Microcalorimetry for Non-invasive Detection of Microbial Growth in Media Fills

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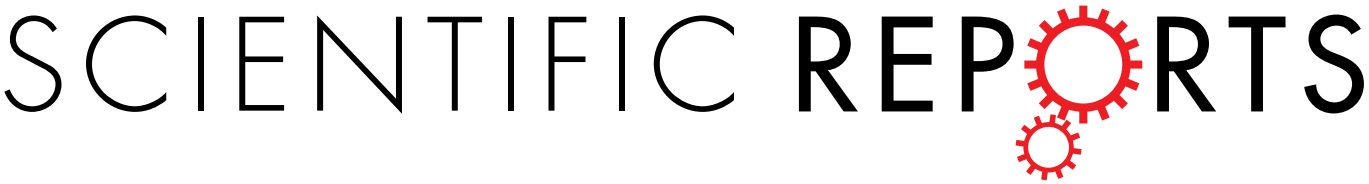

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Comparison of Tunable Diode Laser Absorption Spectroscopy and Isothermal Micro-calorimetry for Non-invasive Detection of Microbial Growth in Media Fills

David Brueckner[1,2], David Roesti[3], Ulrich Georg Zuber[2], Rainer Schmidt[2], Stefan Kraehenbuehl[4], Gernot Bonkat[1,5] & Olivier Braissant[1,5]

Two methods were investigated for non-invasive microbial growth-detection in intact glass vials as possible techniques for automated inspection of media-filled units. Tunable diode laser absorption spectroscopy (TDLAS) was used to determine microbially induced changes in O_2 and CO_2 concentrations within the vial headspaces. Isothermal microcalorimetry (IMC) allowed the detection of metabolic heat production. *Bacillus subtilis* and *Streptococcus salivarius* were chosen as test organisms. Parameters as robustness, sensitivity, comparability and time to detection (TtD) were evaluated to assess method adequacy. Both methods robustly detected growth of the tested microorganisms within less than 76 hours using an initial inoculum of <10CFU. $TDLAS_{O_2}$ turned out to be less sensitive than $TDLAS_{CO_2}$ and IMC, as some false negative results were observed. Compared to the visual media-fill examination of spiked samples, the investigated techniques were slightly slower regarding TtD. Although IMC showed shorter TtD than TDLAS the latter is proposed for automating the media-fill inspection, as larger throughput can be achieved. For routine use either $TDLAS_{CO_2}$ or a combination of $TDLAS_{CO_2}$ and $TDLAS_{O_2}$ should be considered. IMC may be helpful for replacing the sterility assessment of commercial drug products before release.

Aseptic processing is used to manufacture products that are intended to be sterile and ultimately injected to patients. The process is periodically simulated by filling microbial culture media (tryptic soy broth – TSB) instead of the drug product. This simulation is designated as a "media fill" and ensures the reliability and repeatability of aseptic processing[1,2] as even in pharmaceutical clean room environment a diverse microbial community can be found[3]. In conventional media fill procedures, intact media-filled vials are incubated for 7 days at 20–25 °C and another 7 days at 30–35 °C[4,5]. After 7 and 14 days, microbial contamination of media filled units is assessed by an operator through a visual inspection (VI), searching for an increased media turbidity as well as modifications in the aspect of media. However, VI is limited by several drawbacks including high and repetitive workload, weak data integrity, assumptions on media turbidity that is prone to human error, and inefficient data collection[6,7]. Although these drawbacks ensure patient safety and good manufacturing practices (GMP), they are associated with costs. Also the long turnover time required by incubation and examination of media fills leads to business risk regarding the sterility requirements if manufacturing has already resumed. Therefore, the establishment of new, non-invasive, more efficient, objective and faster methods replacing the conventional visual media fill inspection is needed. In this paper tunable diode laser absorption spectroscopy (TDLAS), measuring gas

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concentrations in vial headspaces, and isothermal micro-calorimetry (IMC), measuring metabolic heat production of microorganisms, are investigated as alternative methodologies.

The development of spectroscopic measurement techniques in the near- and mid-infrared spectral region[8–10] has led to their extensive use in the pharmaceutical industry for e.g., the determination of trapped gas inside vials, the analysis of porosity in tablets and the control of freeze-drying processes[11–13]. Considering parenteral drug production, contamination by microbes from the environment is a constant threat during aseptic filling[3]. These microbes are mostly aerobic and thus the standard media fill incubation is performed in aerobic conditions. Microorganisms using respiration as metabolic pathway consume oxygen and release carbon-dioxide (see equation 1). Non-invasive spectroscopic measurement techniques, such as TDLAS, can be used to detect growth related changes of O_2 and CO_2 concentrations in headspaces of sealed vials[14]. This provides insights on microbial contamination of media filled units. However, microbial fermentation can also occur and result in other end products such as lactic acid and alcohols (see equations 2 and 3). Nevertheless, many of these fermenters are not limited to their primary pathways and may therefore remain detectable by TDLAS. Still for some of those fermenters differences in cultivation medium composition (i.e., from different manufacturer, or different batches) might lead to use of metabolic pathway with little to no O_2 consumption and CO_2 production.

$$\text{Respiration} \qquad C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \qquad \Delta H = -2804,\ 90\,\text{kJ/mol} \tag{1}$$

$$\text{EtOH fermentation} \qquad C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \qquad \Delta H = -68.92\,\text{kJ/mol} \tag{2}$$

$$\text{Lactic acid fermentation} \qquad C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH \qquad \Delta H = -75.70\,\text{kJ/mol} \tag{3}$$

In addition, metabolic reactions resulting from microbial proliferation emit heat which can be quantified continuously and non-invasively by isothermal micro-calorimetry (IMC). IMC therefore detects enthalpy changes associated with the cell metabolism (see ΔH of equations 1–3) and has been used already to monitor microbial growth[15–19].

Here TDLAS and IMC are evaluated and compared as possible alternatives to current inspection procedures for parenteral drugs, aiming at a high-throughput substitution of the visual media fill inspection. Parameters such as robustness, sensitivity (i.e., the proportion of false negative), sensibility (i.e., the instrumental detection limit in our conditions), time to detection and comparability are investigated for each method, using the fast-growing, metabolically versatile *Bacillus subtilis* and the lactic acid fermenter *Streptococcus salivarius* as model contaminants. These organisms have been chosen as they represent extremes in terms of O_2 depletion and CO_2 production found in a preliminary screening. Growth measurements obtained using the different methods are compared using the Gompertz growth model[20].

Results

To determine microbial growth of *Streptococcus salivarius* and *Bacillus subtilis* by TDLAS and IMC a threshold was defined by a 4σ confidence interval defined on unicolutated samples. Repeated measurements of sterile TSB samples on heat production and headspace change in CO_2 and O_2 concentration provided data on the biochemical processes occurring in the medium, influencing heat flow and gas levels (Fig. 1 - section 2.1).

For inoculated samples, the intersection of fitted growth curves with the respective threshold defined the time to detection (TtD) (section 2.2). In turn, the distributions of TtD allowed making assumptions on method robustness, sensitivity, sensibility and comparability to visual inspection. In addition, an absolute TtD was determined for each organisms and method. Therefore worst-case fitting parameters of the Gompertz model (i.e., maximal λ (i.e., the longest lag phase) with smallest μ (lowest growth rate) and lowest X_{max} (minimum gas concentration change or heat produced)) were combined in this model to create a worst-case growth curve. The intersection of this worst-case growth curve with the respective threshold determined absolute TtD (Fig. 2). In parallel to each TDLAS and IMC measurement vials were inspected visually on the increase in turbidity. In addition, optical density was measured to allow a comparison of this commonly used measure with TDLAS and IMC profiles (Table 1, Fig. 3).

Thresholds for gases concentration (T_{CO_2}, T_{O_2}), heat production (T_H) and optical density (T_{OD}). In sterile TSB filled vials slight variations of oxygen and carbon-dioxide concentration were observed. Overall 82 vials out of 84 (2 contaminated samples were rejected) filled with two TSB lots differing in age were used to investigate these fluctuations in vial headspace. From the 14′645 TDLAS measurements obtained, 2′460 data points (16.8%) were omitted due to non-normal distribution of the data series, hindering the use of the 4σ approach. Another 2′025 (13.8%) points below detection limit were removed. Based on the remaining data (69.4%) T_{CO_2} and T_{O_2} were determined. To define the IMC threshold for growth detection 24 vials filled with three TSB lots differing in age were continuously measured. No vials or measurement points were rejected.

After 7 days T_{CO_2} was defined at 0.366% and T_{O_2} at 1.754%. T_H was defined at 0.416J and T_{OD} at 0.02 (Fig. 1).

Growth Profile and Parameter Analysis. The two microorganisms tested were detected by all methods used with robust and reproducible results across all the independent experimental runs (Fig. 3). However, TtD and number of false negative results varied slightly (Table 1). Lag phase duration, growth rate and maximal concentration/heat/biomass reached could be calculated for all organisms investigated and methods used. *Bacillus subtilis* showed higher reproducibility in λ and μ and reached higher changes in gas concentration and heat production compared to *S. salivarius* (Table 1). Differences in heat production and gas concentration patterns

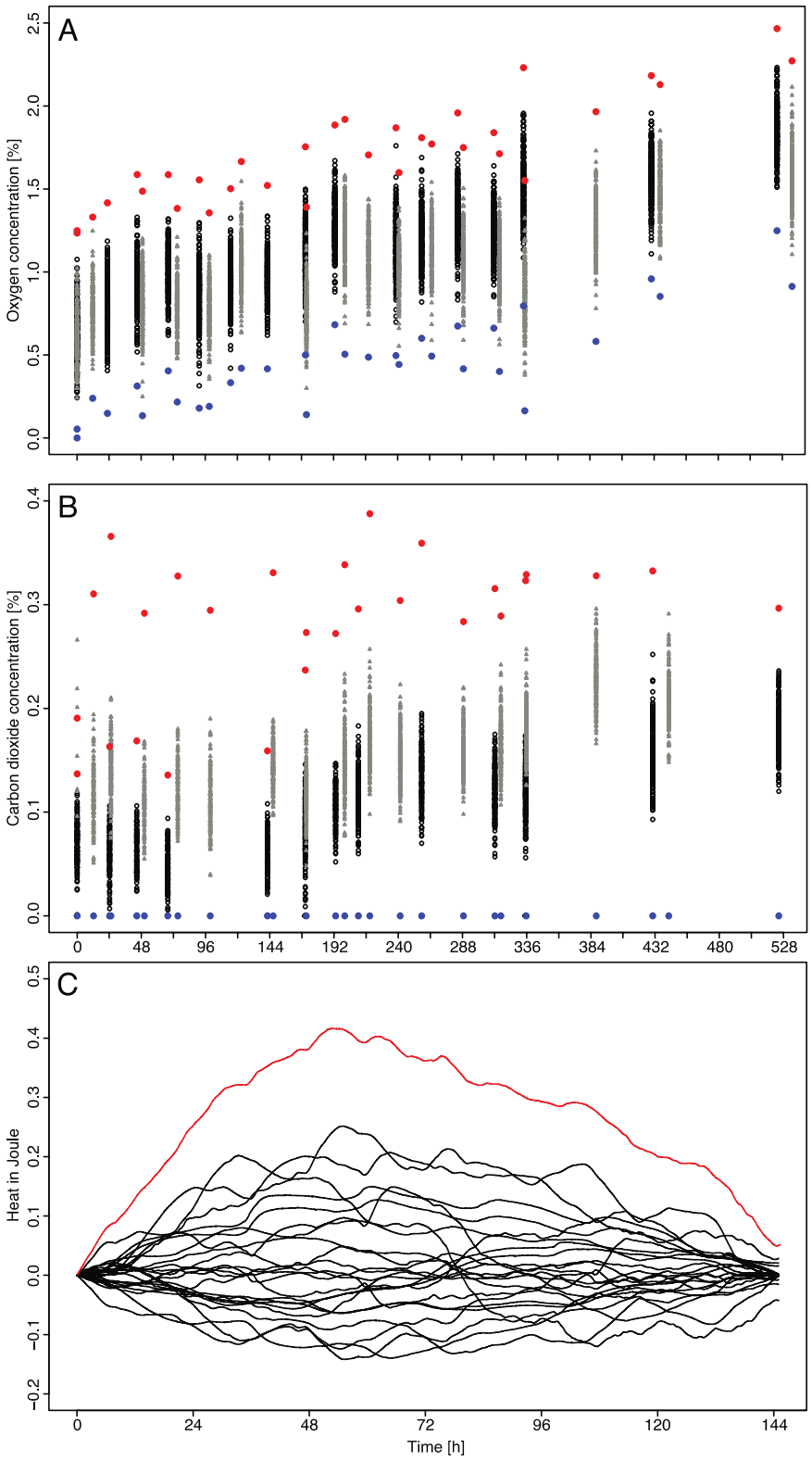

Figure 1. Plot (**A**) describes the inverted O_2 baseline development. It was determined by calculating the difference of $O_{2max} - O_2$ for all measurement series. The red and blue dots describe the upper and lower 4σ confidence intervals. Plot (**B**) describes the CO_2 baseline development. The red dots show the upper 4σ confidence intervals whereby the blue dots stand for the physical limits being equal to zero. The CO_2 and O_2 data is based on an old (grey) and a new (black) TSB lot being measured during at least 14 days. Plot (**C**) shows the heat emission during 145 hours of 24 blank TSB samples of three TSB lots differing in age. The superordinated red curve shows the 4σ confidence interval. Its maximum peak defines the threshold parameter for IMC.

	$TDLAS_{O_2}$ (min–max) (T_{O_2} = 1.754%)	$TDLAS_{CO_2}$ (min-max) (T_{CO_2} = 0.366%)	IMC_{HEAT} (min-max) (T_H = 0.416J)	OD_{595} [remaining values] (T_{OD} = 0.02)	Visual (all vials turbid)
Bacillus subtilis					
n	60	60	18	3	60
TtD (*time to detection*) [h] (intersection of B_{low} with threshold)	58.0 (36.7–57.0)	52.6 (35.7–54.4)	45.9 (34.4–47.2)	33.6 [28.3; 31.8]	45.0 (24.5–45.0)
λ (*lag phase*) [h]	39.85 (34.59–55.38)	45.65 (41.08–58.37)	40.58 (37.80–48.45)	36.40 (33.78–37.58)	n.a.
μ (*growth rate*) [% or J/h]	0.46 (0.35–0.61)	0.46 (0.38–0.58)	0.40 (0.30–0.63)	0.06 (0.05–0.06)	n.a.
$\mathbf{X_{max}}$ (C_{max}/*Heat*) [% or J]	19.89 (19.35–20.93)	17.93 (17.13–18.69)	12.79 (11.11–13.98)	1.55 (1.44–1.57)	n.a.
Streptococcus salivarius					
n	57	57	22	3	57
TtD (***time to detection***) **[h]** (intersection of B_{low} with threshold)	ND (51.0–ND)	76.0 (45.2–70.7)	71.2 (44.3–69.3)	51.9 [40.5; 50.0]	61.0 (37.3–61.0)
λ (*lag phase*) [h]	34.74 (17.75–57.20)	48.14 (40.12–68.96)	46.67 (42.20–65.74)	50.78 (41.55–52.40)	n.a.
μ (*growth rate*)[[% or J/h]	0.06 (0.017–0.1)	0.15 (0.05–0.33)	0.12 (0.08–0.20)	0.23 (0.08–0.57)	n.a.
$\mathbf{X_{max}}$ (C_{max}/*heat*) [% or J]	2.12 (1.41–3.4)	2.22 (1.13–3.14)	2.37 (1.79–2.90)	1.25 (0.78–1.75)	n.a.

Table 1. Time to detection, lag phase, growth factor and reached X_{max} for *B. subtilis* and *S. salivarius*, related to methods under investigation are presented. General TtD is expressed as number of hours where the lower growth boundary (B_{low}) crosses T_{o_2}, T_{co_2} or T_H. In brackets the TtD distribution of each single replicate. ND stands for not detected and is a direct measure for sensitivity as related to false negative results. Lag phase (λ), growth factor (μ) and maximal value reached (X_{max}) are demonstrated in form of median and range (in brackets). Unit for λ is hours, for X_{max} percentage, whereby μ goes without unit. Parameters were received from applying the Gompertz model in R ("grofit" package) on each individual growth profile. Measured OD_{595} values are of descriptive nature and used as reference method to show meaningfulness of results obtained for TDLAS and IMC. As a substantial number of OD_{595} samples was investigated it would lead to misconceptions in statistics if used differently. The single number is the maximal value of all three OD_{595} measurement runs. In brackets the remaining values.

between the 2 microorganisms is mainly attributed to the differences in *S. salivarius* and *B. subtilis* metabolism. Three TDLAS *S. salivarius* vials were not included due to secondary contamination.

TtD, the intersection between threshold and curve considered, for single curves of $TDLAS_{O_2}$, $TDLAS_{CO_2}$, OD_{595} and IMC were determined. TDLAS, OD_{595} and IMC measurements detected all *B. subtilis* inoculated replicates between 28.3 h and 57.0 h. *S. salivarius* samples became positive between 40.5 h and 70.7 h. However, 10% of the *S. salivarius* oxygen profiles did not reach the threshold, meaning that less than 1.754% O_2 was consumed after 7 days (Table 1). This emphasizes that the sensitivity of $TDLAS_{O_2}$ remained rather high as values were 100% and 90% for *B. subtilis* and *S. salivarius* respectively. Also, this supports the use of *S. salivarius* as worst-case scenario microbe.

Absolute TtD was modeled by using worst case parameters obtained for each organism with $TDLAS_{O_2}$, $TDLAS_{CO_2}$ and IMC data. This time point was defined by the intersection of B_{low} (computed using largest λ, minimal μ and smallest X_{max} – see section 5.4) with the respective threshold and was comprised between 45.9 and 76 hours (Table 1). The absolute TtD values showed that IMC outperformed TDLAS but that VI remained the fastest detection method. TtD for OD_{595} (used only for comparison purposes) was comparable to visual TtD and in most cases faster. However VI is performed only after day 7.

Discussion

TDLAS and IMC were evaluated for non-invasive microbial growth-detection in intact glass vials aiming at a rapid and automated inspection of media-filled units. Both methods proved to be technologies capable of detecting growth of *Bacillus subtilis* and *Streptococcus salivarius* in sealed vials. The results were robust and showed good reproducibility. In addition, TDLAS and IMC are comparable to the conventional visual media fill inspection performed after 7 days in terms of detection speed. However, 10% of all *S. salivarius* vials did not reach the set threshold (false negatives). This phenomenon was mainly linked with the auto-oxidative characteristic of TSB which affected the threshold chosen and resulted in a 4.8 times higher oxygen threshold compared to CO_2. Furthermore, the metabolism from *S. salivarius* led to relatively low O_2 consumption and CO_2 production, which might indicate the use of alternative pathways. As a consequence, oxygen depletion in vial headspaces was insufficient to reach the threshold. Considering reproducibility and robustness, different sources of variations exist. The origin of variations between runs of the same microorganism could be due to the different TSB lot used as this medium is not defined and variations in composition exist between batches and between manufacturers. The low CFU count used for the inoculation might be an additional source of errors as very little variation in the CFU number or the viability of the cells might have affected their growth. Finally the tendency of streptococci for self aggregation might also have influenced the reproducibility for this organism as inoculum size might have varied.

The sensibility for each method with respect to detection speed of microbial growth was clearly demonstrated (Fig. 3). With an inoculum <10 CFU per vial IMC outperformed TDLAS in terms of TtD. Still, it might be even optimized further. The calorimeter used in our experiment is an instrument of the mid-range performance class,

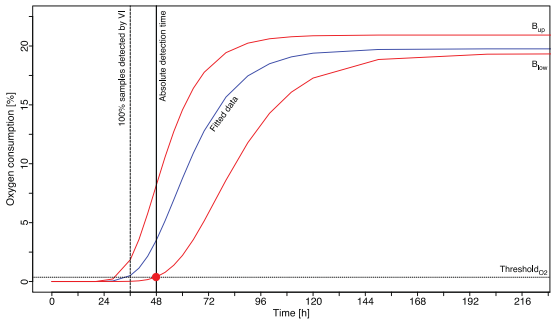

Figure 2. Example of an inverse O_2 growth profile of *Bacillus subtilis*. The inverted profile was determined by calculating the difference of $O_{2max} - O_2$ out of a collection of 60 inoculated vials. The red s-shaped curves illustrate upper (B_{up}) and lower (B_{low}) boundaries of growth associated oxygen consumption. The individual blue profile is the averaged fit of the entire sample collection. The dashed vertical line illustrates the time needed to detect visually 100% of all inoculated samples. The solid vertical line defines the absolute TtD for *Bacillus subtilis* based on oxygen measurements. The red dot marks the intersection of the lower boundary and the threshold T_{O_2}.

originally designed for studying cement curing[21]. The 2 ml vial format did not perfectly fit in the calorimeter's sample holder and results with even higher quality would have been obtained with samples of higher heat capacity and conductivity (i.e., 15–20 ml vials). The setup with a 2 ml vial inserted in a 20 ml plastic vial fitting into the IMC sample receiver probably resulted in additional measurement noise (i.e., increased limit of detection). Overall, there are many possibilities to further improve the IMC setup and to decrease the threshold thus resulting in lower TtD. The TDLAS setup had also some drawbacks. The short diameter of the 2 ml format results in a shorter measurement path than compared to containers of larger size. This leads to analytical fluctuations[12] and causes additional measurement errors. An improvement of measurement precision could be reached by using containers with larger diameters. However, the use of larger formats is also linked with bigger headspaces that might require more O_2 to be consumed or CO_2 to be produced to reach a similar gas concentration level. Thus, in contrast to IMC that measures heat produced in the whole vial independently of the vial format, TDLAS might be less appropriate for larger formats when fermenting microorganisms are considered as the larger filling volume is compensated by a larger headspace.

From an operational perspective, the visual inspection of a media fill assessment is comparable to the alternative methods investigated if growth detection occurs before the first inspection step. Indeed, first visual inspection activities are performed after 7 days (168 hours), meaning that despite variations in TtD all methods would have detected growth of *B. subtilis* or *S. salivarius* before the standard visual inspection.

However, it was observed that within the first 7 days OD_{595} delivered best performance in detection speed whereby IMC and TDLAS were slower than the visual inspection. Despite OD_{595}'s advantage in TtD its use in replacing the visual read-out of all vials (ca 10,000 per media fill) is not an option as sediments of growing microorganisms can fall to the vial bottom or form micro colonies. In this context, a shaking or vortexing step would be difficult to implement in an automated production line. Alternatively, in some cases such measurements might be impaired by autolysis as well (Fig. 3B,D). This would promote the risk for obtaining false negative results and complicate automated vial inspection. In addition, further applications of optical density measurements to poorly soluble drugs, provided as suspension or dispersion, is not possible due to the optical characteristic of such products.

Before a substitution of visual media fill read-out can be realized by either IMC or TDLAS, additional experiments are necessary to create a reliable decision base. Besides evaluating the impact on precision by using larger vial formats such studies should incorporate a specificity analysis by looking at a broader range of organisms with different metabolic pathways that are known contaminants in pharmaceutical drug manufacturing. Depending on the method and format the threshold will require a reassessment to provide an optimal specificity combined with a short TtD. Furthermore, microorganisms in drug manufacturing facilities are likely exposed to a lack of nutrition, heat shocks, hyperacidity, remnants of antibiotics, disinfectants, or other external influences. This results in cellular stress and in an extended lag phase, having a direct impact on TtD. Within this context it might be valuable to also include a growth development analysis of stressed organisms in future studies. Vial non-integrity is another issue that needs to be investigated as an exchange of gases between headspace and atmosphere could considerably impact the results. Indeed the false negative rate and TtD might be increased by impaired vial integrity.

When dealing with such technologies it is crucial to know what can be achieved, taking into account advantages and drawbacks of each method individually (Table 2).

Besides IMC's benefit of short TtD, the measurements are easy to handle and provide continuous real-time data[15]. Measuring heat-flow continuously can be an advantage as it allows getting some additional insights on metabolism of the potential contaminants, but also be a drawback since the throughput is limited to the number of measurement slots. In addition, metabolic heat emission is a temporary limited process, meaning that once growth terminates IMC cannot retrospectively assess bacterial contamination[22]. Growth of *B. subtilis* and *S. salivarius* stops after less than 150 hours (6.25 days), whereby the first inspection is planned at the 7th day of

Advantages/Disadvantages	TDLAS	IMC	VI
Objective inspection (quantitative results)	++	++	−−
High throughput rate	++	+	−−
Efficient and automatic data collection	++	++	−−
Measurement Sensibility/Speed in detection	+	+	++
Non-invasive inspection	++	++	++

Table 2. Describes and compares advantages and drawback of TDLAS, IMC and visual inspection. ++ fully applicable, + partly applicable, − partly not applicable, −− not applicable.

incubation. This calls for a method where metabolic activities remain detectable independently of the moment of inspection. O_2 and CO_2 changes in intact vial headspaces remain rather constant after bacterial growth took place. Because of this and the characteristic of enabling a high-throughput read-out, the individual $TDLAS_{CO_2}$ system (or in combination with $TDLAS_{O_2}$) is recommended here as the method of choice for automated inspection of media fills. Nevertheless, IMC remains of interest in areas where continuous measurements are demanded with lower throughput requirements, e.g. sterility control of commercial drug products before release. Finally the combination of IMC and TDLAS for CO_2 and O_2 measurement allows to perform metabolic studies focusing on different pathways or microorganisms in which this pathways would have been altered by mutagenesis. Such studies are referred to as calorespirometric studies and could be of much use oustide the field of sterility testing for example in assessing product formation[23–25].

Conclusions

The non-invasive application together with an accurate detection of bacterial growth render IMC and TDLAS as valuable tools for inspecting media-filled units and potentially commercial drug product as well. $TDLAS_{CO_2}$ and IMC were capable to detect growth of *B. subtilis* and *S. salivarius* reliably within much less than 7 days. False negative results were observed for $TDLAS_{O_2}$ which makes its single use without parallel $TDLAS_{CO_2}$ measurements questionable.

Although the verification of microbial growth by human visual inspection remained faster than TDLAS and IMC, its replacement stays a beneficial intention as augmented inspection objectivity, higher-throughput via automation, increased data integrity and efficient data collection can be realized. Due to the fact that automated vial handling for IMC is more difficult to achieve and less advanced than for TDLAS, the latter technique is suggested as substitution for visual media fill inspection despite IMC shorter TtD for growth detection. More potential is attributed to IMC in replacing the sterility assessment of commercial drug products before release. Nevertheless, further studies covering a wider range of microorganisms and formats are required to decide on whether or not IMC and/or TDLAS are appropriate method for a safer and more efficient sterility assessment procedures.

Materials and Methods

Microorganisms and culture conditions. *Bacillus* species are of obligate aerobe or facultative anaerobe, sporulating, gram-positive, rod-shaped nature and, appearing frequently in pharmaceutical environments[3]. They are often used in validation studies of microbial methods. In our study, *B. subtilis* (ATCC 6633) was obtained as bio balls® (Thermo Scientific) validated to a cell count of <100 CFU and was routinely maintained aerobically at 30 °C on tryptic soy agar (TSA).

Streptococcus salivarius (ATCC 7073; DSM 20560) is a facultative-anaerobe, gram-positive, catalase- and oxidase-negative bacteria that belongs to the group of lactic acid fermenters. Indeed it produces mostly lactate from glucose, therefore affecting minimally CO_2 and O_2 concentrations. The organism was provided by DSMZ as cryo-culture and maintained aerobically at 37 °C on TSA.

Preparation of experimental containers and bacterial suspensions. For all experiments 2 ml transparent, sterile tubed glass vials (Schott AG, Germany) were used. After manual filling of 1 ml TSB under aseptic conditions, containers were stoppered and capped with sterilized material and stored at 20–25 °C. As headspace gas concentrations are known to vary over time due to auto-oxidative processes in TSB[14], each container was flushed for six seconds with sterile air at a flow rate of ~0.25 l/min before inoculation. This "headspace flushing" was intended to simulate the conditions prevailing in newly produced units.

Inoculation through the self-sealing rubber stopper was performed using a syringe filled with 0.1 ml bacterial suspension containing <100 CFU/ml (i.e., <10 CFU per vial). Suspensions including *B. subtilis* were prepared by dissolving a bio ball® in Remel buffer, followed by diluting this suspension 1:10 times. *S. salivarius* was cultured twice for 48 h at 37 °C in TSB. The second culture was diluted by a factor of $1:10^{-6}$ in buffered sodium chloride-peptone bouillon and, with the addition of 15% glycerin, stored at −83 °C. The appropriate cell count for 0.1 ml suspension was determined by standard dilution series on agar plates.

Experimental Design. *Devices and Settings.* Single pass bench-top TDLA Spectrometers (Lighthouse Instruments, Charlottesville, VA) were used. O_2 and CO_2 concentrations were determined at 762 nm and 2000 nm respectively. Before each experiment the devices were preheated for at least 30 minutes. A two-point calibration with certified standards (0%, 20%) provided by the manufacturer was performed before each measurement series.

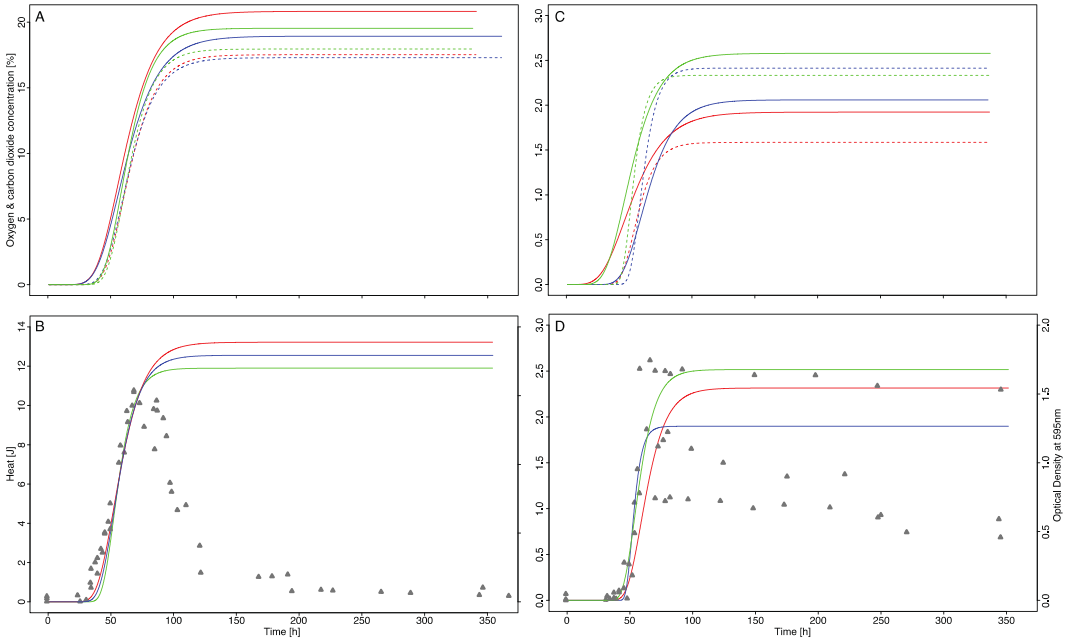

Figure 3. Fitted gas and thermogenic profiles together with raw data of OD_{595} measurements for *B. subtilis* and *S. salivarius*. Plots (**A**,**B**) illustrate plotted data for *Bacillus subtilis*, (**C**,**D**) plotted data for *Streptococcus salivarius*. The development of CO_2 (dotted) and O_2 (solid) profiles in fitted form are visualized in graph (**A**,**C**) for both organisms, whereby the color red describes the development of the first run, blue the second run and green the third run, including 20 samples each. Plot (**B**,**D**) visualize fitted heat (solid) of three runs with TSB differing in age and raw data of various OD_{595} measurements (Δ) over time. Considering the heat and gas profiles in more detail it becomes obvious that despite small deviations in profile development reproducibility and robustness is given for TDLAS and IMC measurements.

The measurement series were initiated after the measurement cap has been flooded with nitrogen (4 l/min) and the repeated analysis of standards with known concentration (4% and 8% respectively) was within specification limits (i.e., $\pm$0.25% deviation). Standards used were made of the same glass as the experimental containers to keep the signal to noise ratio minimal. In parallel to each TDLAS measurement (lasting 5 seconds), a spectrophotometer (Bio Photometer plus, Eppendorf) was used to measure the optical density at 595 nm (OD_{595}). OD_{595} data was gathered to allow comparison of this commonly used measure with TDLAS and IMC.

As initial experiments with TDLAS showed that the exponential growth phase of the organisms investigated had already stopped within less than hundred hours, it was decided to perform IMC experiments at 20°–25 °C only. For that, an eight-channel TamAir calorimeter (Waters/TA, Delaware, USA) was used. Once the eight samples were placed in the calorimeter, heat produced was recorded by the thermoelectric module placed between the samples and the heat-sink[15]. Reference vials used were identical to the experimental containers, but filled with sterile water thus providing an inert reference of similar heat capacity and conductivity. All 2 ml containers were additionally placed in a 20 ml plastic vial fitting the IMC sample receiver.

Baseline and Threshold Determination. To determine the threshold for microbial growth detection, sterile TSB samples were repeatedly measured for their heat production and headspace change in CO_2 and O_2 concentration. For TDLAS two sets of 42 vials were filled with new (0 days) and old (56 days) TSB. Their CO_2 and O_2 concentration was measured every 24 hours for at least 14 days. Each vial was analyzed 5 times to account for measurement variations[26]. The thresholds were defined after 7 and 14 days in order to be consistent with established inspection intervals.

To define the IMC threshold three sets of 8 vials containing TSB (3 up to 135 days in age) were measured continuously during 145 hours. This was considered as a sufficient time as the heat flow of all inoculated samples turned back to baseline after less than 100 hours.

For the definition of the OD_{595} threshold 100 measurements were performed with sterile TSB, filled in a cuvette.

Detection of bacterial growth by the different methods. For TDLAS measurements, three sets of TSB filled vials were prepared for both organisms. Each set included 20 inoculated vials and TSB lots varying from 1 day to 31 days in age. To reproduce the conditions of a real media fill, containers were incubated at 20–25 °C during 7 days and another 7 days at 30–35 °C. Before each measurement, vials were taken out of the incubator and were inspected visually by a qualified person for increased turbidity. After the 5 seconds lasting TDLAS measurement the containers were rapidly returned to the incubator. Measurements were taken every four hours during the growth phase.

Optical density (OD_{595}) measurements were performed in parallel to the TDLAS measurements with vials from an additional set prepared identically. Their content was transferred into transparent plastic cuvettes and then measured on absorption at 595 nm. Blank samples were determined in TSB.

IMC experiments used three sets of TSB filled vials for both organisms. Each set included at least 6 inoculated vials. Measurements were taken continuously and resampled to achieve an effective sampling rate of 1 data point per minute.

All evaluated samples showing differences from typical and expected growth profiles of the organism under investigation, were sent to the PCR identification lab and omitted in case of non-conformity.

Data Analysis. The data analysis described below was performed using the R statistical software[27] in combination with the grofit package[28].

Analyzing Threshold Data for CO_2, O_2, Heat, OD_{595}, and VI. Each TDLAS measurement series (i.e., for each different time point) was checked on normality using the Shapiro-Wilk test. Non-normally distributed datasets, values equal to zero (i.e., below physical detection limit) and contaminated replicates were omitted from the analysis. The threshold parameters were estimated using a 4σ approach where the probability of receiving false positive results is around 0.006%. For the purpose of this study we choose to use 4σ instead of 3σ (commonly used in industry settings) to ensure safety of our results. The maximum value of mean plus four fold standard deviation was taken for the data gathered during the periods of 0 to 7 days and 7 to 14 days (see Fig. 1A,B) and considered as the threshold. The threshold parameter for emitted heat was defined by applying the 4σ approach to collected data and selecting the maximum of the 4σ confidence interval (Fig. 1C). The OD_{595} threshold was defined by the rounded 4σ confidence interval and the VI threshold by the perception of visible changes in turbidity of 100% of the inoculated samples.

Growth Profile Analysis and Data Transformation. Typical microbial growth appears in form of ascending sigmoidal curves (i.e., s-shaped curve). From a microbiology point of view, such curves can be described mathematically by 3 main parameters: lag phase duration (λ), exponential growth factor (μ) and maximal value reached (X_{max}). Those parameters can easily be estimated for TDLAS, OD_{595} and IMC curves by using the Gompertz model, Oxygen concentration profiles are inverted growth curves by nature as they reflect oxygen consumption. To allow the use of the Gompertz model the oxygen profiles were converted to conventional s-shaped curves. For this O_2 depletion was described as $O_{2max} - O_2$. Heat flow [in μW (μJ/s)] over time is usually composed of one single (or several) peak(s). Therefore, the Gompertz model was fitted on the integrated profile (i.e., the heat [J] over time curve), resulting in an s-shaped curve considered as a good approximation for microbial growth[15]. Additionally, OD_{595} curves are hill-shaped as some microorganisms might undergo lysis. To neglect this effect in the analysis the OD_{595} values were considered to be constant once they had reached the maximum, which allowed an adequate modelling of the OD_{595} curve.

All those transformations enabled to compare directly all the curves generated by IMC, TDLAS and OD_{595}.

Determination of Time to Detection (TtD). Once the thresholds for CO_2 (T_{CO_2}), O_2 (T_{O_2}), Heat (T_H) and OD_{595} (T_{OD}) were determined, each TDLAS, IMC and OD_{595} profile was described by λ, μ and X_{max} and the intersection with the respective threshold identified. For TDLAS and IMC a clear definition of lower (and upper) growth boundaries was needed to account for biological variation in growth and to determine a representative organism-related absolute TtD (see Fig. 2). Lower growth boundaries (B_{low}) were defined by combining maximal λ with smallest μ and lowest X_{max} in the Gompertz model. The intersection of B_{low} and the respective threshold determined the absolute TtD that CO_2, O_2 or heat measurements needed to detect either *S. salivarius* or *B. subtilis* (Fig. 3). Upper boundaries (B_{up}) were defined through combining minimal λ with highest μ and largest X_{max} in the Gompertz model. The definition of B_{low} and B_{up} imposes a measure for estimating total growth distribution. TtD for VI was defined as point where 100% of all inoculated samples turned turbid and is of qualitative nature.

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Author Contributions

D.B. and O.B. designed and performed the experimental work and wrote the main part of the manuscript. D.R. and S.K. contributed to the drafting of the article and to the data interpretation and analysis. They also critically reviewed the final version of the manuscript. U.G.Z., R.S. and G.B. critically reviewed the manuscript.

Additional Information

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CHAPTER 3

Tunable Diode Laser Absorption Spectroscopy as Method of Choice for Non-invasive and Automated Detection of Microbial Growth in Media Fills

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Tunable diode laser absorption spectroscopy as method of choice for non-invasive and automated detection of microbial growth in media fills

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ABSTRACT

Tunable diode laser absorption spectroscopy (TDLAS) was evaluated on its potential to detect bacterial growth of contaminated media fill vials. The target was a replacement/ automation of the traditional visual media fill inspection. TDLAS was used to determine non-invasively O_2 and/or CO_2 changes in headspaces of such vials being induced by metabolically active microorganisms. Four different vial formats, 34 microorganisms (inoculation volume < 10 cells) and two different media (TSB/FTM) were tested. Applying parallel CO_2 and O_2 headspace measurements all format-organism combinations were detected within < 11 days reliably with reproducible results. False negatives were exclusively observed for samples that were intentionally breached with syringes of 0.3 mm in diameter. Overall it was shown that TDLAS functionality for a replacement of the visual media fill inspection is given and that investing in further validation and implementation studies is valuable. Nevertheless, some small but vincible challenges remain to have this technology in practical use.

1. Introduction

Aseptic manufacturing is periodically evaluated by aseptic process simulations using microbiological culture media substituting the sterile product bulk-solution (i.e. typically tryptic soy broth [TSB] is used for aerobic micro-organisms, less frequently fluid Thioglycollate [FTM] for (facultative) anaerobic strains and finally vegetable peptone broth [VPB] where there is concern about prions). This simulation is designated as “media fill” and provides supportive data on the capability and reliability of aseptic processing activities. It is also used to qualify sterile working-techniques of aseptic cleanroom operators. In addition, media fills may identify potential weaknesses with regard to microbiological contamination during production line modification and qualification or during investigations of microbiological deviations (e.g. positive sterility test). The industrial standard in analyzing media filled units is the visual inspection (VI) performed by qualified human operators who check for turbidity increase and abnormality in the aspect of media [1,2]. Regulations and guidelines [3,4] specify that such inspections must be performed at least once after no less than 14 days with media filled units that have been incubated for minimally 7 days at 20–25 °C and another 7 days at 30–35 °C. Several manufacturers perform an intermediate VI after the first incubation period lasting 7 days since fast growing microorganisms would have grown and become visible by then. VI ensures good manufacturing practices (GMP) and contributes to a high standard of patient safety but is associated with high workload related to manual vial and data handling. Furthermore, the detection of medium turbidity by human eye may be prone to error, and requires regular qualification of operators. All this results in unnecessary cost.

Alternative non-invasive methods with the potential for media fill inspection automation could reduce cost, increase process efficiency, and decrease the risk of false negative media fill units (i.e. not detecting microbial growth) by eliminating the human factor. First advancements and proposals in this field were recently made [5,6]. Tunable Diode Laser Absorption Spectroscopy (TDLAS) was evaluated in determining non-invasively microbial growth related O_2 and CO_2 concentration changes in sealed (non-breached) media fill vial headspaces. Microorganisms in TSB were tested on their CO_2 emission and O_2 consumption. In addition, TDLAS data was compared to VI, optical density measurements and isothermal micro-calorimetry (IMC) to

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conclude on the sensibility of TDLAS and assure that gathered O_2/CO_2 data was growth related. Tested microorganisms were detected without observing false negatives and results were reproducible when using a combined O_2 and CO_2 TDLAS measurement. Method development studies for TDLAS including the analysis of a wider range of microorganisms in 2 different media, stressed microbial cells (having normally a prolonged lag phase), additional format sizes and breached containers (headspace equilibration with environment leading to false negative results) are outlined in this work. The findings shall provide the basis for method implementation and validation in sterile drug product manufacturing.

2. Materials and methods

2.1. Basic study design

Two production sites dealing daily with aseptic production and media fill inspection were involved in the study (site X: *F. Hoffmann-La Roche* (Wurmisweg, 4303 Kaiseraugst, Switzerland), and site Y: *Novartis* (Schaffhauserstrasse 101, 4332 Stein, Switzerland)). Media fill incubation times were set to 7 days at 20–25 °C and 7 days at 30–35 °C (overall incubation time > 336 h) and kept during the experiment to allow a comparison of growth detection by VI and TDLAS.

Next to the standard pharmacopoeia (PhEur) reference strains, several plant isolates (i.e., local isolates) from site X and Y (selection based on relative occurrence during routine monitoring in cleanrooms, unpublished internal sources), worst-case organisms (i.e., organisms with little metabolic activity, known long lag phase) and microorganisms with homofermenting characteristics (reduced O_2/CO_2 consumption/release) were included in this study (see Table 1 for strain details).

Both sites used benchtop TDLAS analyzers (Lighthouse Instruments, Charlottesville) with integrated single pass laser beams able to determine O_2 (λ=762 nm) and CO_2 (λ=2000 nm) concentrations respectively. Prior to use analyzers were warmed-up for at least 30 min and then calibrated with attested standards (20%, 0% of the respective format) supplied by the manufacturer. Standards were made of the same glass as experimental containers to keep measurement noise minimal. The experiments started as soon as the measurement chamber was flushed with nitrogen (4 l/min) and the analysis of O_2/CO_2 reference standards (4%, 8%) was inside limits of defined specification (± 0.25%).

3. Preparation of working suspensions

Despite small site specific differences in preparing the microbial working suspension, the main steps were identical. Both sites acquired the PhEur organisms from ThermoFisher Scientific in form of lyophilized cultures adjusted to an approximate cell count of < 100 colony forming units (CFU) per 0.1 ml. Fungi (yeast and mold), homofermenting, aerobic and anaerobic microorganisms were either obtained from the site internal microbial strain collection (plant isolates) or from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen). Plant isolates and DSMZ strains were cultured twice at 30–37 °C in tryptic soy broth (or blood agar) to obtain suspensions with unstressed and viable organisms. By using 0.2 ml of these suspensions their cell-density was determined on appropriate media (tryptic soy agar, angular agar or blood agar). After cultivation the suspensions were diluted in sodium chloride-peptone buffer and enriched with 15% glycerin to obtain an appropriate inoculum size (working suspension: site X < 10 CFU in 0.1 ml; site Y < 20 CFU in 0.1 ml) and stored at less than −80 °C. The working suspensions of the eight PhEur organisms were created by dissolving the lyophilisates in Remel buffer being followed by a dilution (1:10) in sterile water according the desired CFU count. In addition site Y created suspensions of heat stressed microorganisms (see Tables 1, 2). The suspensions were exposed to a water bath (T=50–70 °C) during maximally 1–2 min, depending on the test microorganism. An additional experiment for *Corynebacterium afermentans* was executed by adding 0.1% of the detergent Tween80 to TSB media, acting as growth stimulant by increasing the lipophilicity of TSB [7].

3.1. Sample preparation and testing

At *site X* experimental media filled containers were either prepared manually (manual filling, stoppering and capping under aseptic conditions), or directly taken from inspected media fill batches. As gas concentrations in vial headspaces were known to change over time due to media related oxidation processes [5], the headspace of each sample unit was flushed prior inoculation with sterile compressed air until CO_2/O_2 concentrations were comparable to those in atmospheric air. This “headspace flushing” was intended to simulate the conditions prevailing in newly produced media fill units. Experimental containers of site Y were prepared comparably. Filling volumes amounted for FTM (fluid thioglycollate media) filled units to 20% and for TSB filled units to 35–60% of the respective format capacity. Filling levels were defined according internal standards following the regulatory requirement that all inner vial surfaces get in contact with media when units are moved [8].

Samples were then inoculated by injecting 0.1 ml of bacterial suspension through the self-sealing rubber stopper using a syringe (Microlance BD, 0.8 mm in diameter). Site X preparations were incubated for 7 days at 20–25 °C and for another 7 days at 30–35 °C. CO_2 and O_2 measurements were performed every 12 ± 2 h until changes in headspace concentrations remained minimal. After this time-point the measurement interval was increased to 24–48 h. Each organism and format was evaluated by 10 inoculated replicates. At site Y samples were incubated for 7 days at 20–25 °C and 10 days at 30–35 °C and mainly tested on CO_2 production. Besides start- and end-point measurements at least 3 additional CO_2 measurements were performed per week. The sample size per organism consisted of a total of 2 independent test runs with minimally 3 independent replicates. At every time point of a TDLAS measurement the visual inspection was performed in parallel to check for an increase in media turbidity.

Detection thresholds for each format and media combination were needed to identify the time point of growth detection. Medium induces changes in vial headspaces which are related to TSB auto-oxidation and CO_2 degassing effects [5,9]. Site X evaluated 42 blank 2 ml, 20 ml and 2×50 ml (TSB media from different producers) TSB vials for CO_2 and O_2 concentration change in the vial headspace every day (see Fig. 1). Their filling volumes were identical to those mentioned before. At site Y, 50 blank 10 ml vials filled with 2 ml TSB and 20 blank 10 ml vials filled with 6 ml FTM were used to determine every 3–4 days the respective media induced variation in CO_2 concentration (see Fig. 2). The age of media was between 1 and 120 days and originated either from Merck Millipore (50 ml vials), Sifin (10 ml vials), Becton Dickinson & Company (2 ml and 20 ml vials) or Oxoid (10 ml FTM vials) (see Table 3).

4. Design of leakage study

CO_2 and O_2 headspace concentrations were analyzed in intentionally breached vials to evaluate if growth detection remained possible over 14 days. The aim was to assess if leakage related O_2 inflow or CO_2 escape altered headspace concentrations exposed to microbial activity strong enough to produce false negative results. Samples were prepared and tested as described above but punctured through the gum stopper using a stainless steel needle of ~0.25 mm inner diameter imitating a worst case leakage. Site X used ten TSB filled 2 ml, 20 ml and 50 ml vials inoculated with the respective organism and tested their CO_2 and O_2 concentrations over time. Site Y used per (stressed) organism at least 15 replicates and investigated their CO_2 profiles in 10 ml TSB/FTM filled vials (Table 2). Microorganisms in this study

Table 1
Microorganisms and formats tested on their respective CO_2/O_2 concentrations during 14 days at the respective site. Applied stress (exposure to heat) and media used are also indicated.

PhEur Organisms	**Site (X/Y)**	**2 ml (O_2/CO_2)**	**10 ml (O_2/CO_2)**	**20 ml (O_2/CO_2)**	**50 ml (O_2/CO_2)**	**Stress**	**Media**
Bacillus subtilis, (ATCC 6633)	X, Y	CO_2 O_2	CO_2	CO_2 O_2	CO_2 O_2	No	TSB
Staphylococcus aureus, (ATCC 6538)	X, Y	CO_2 O_2	CO_2	CO_2 O_2	CO_2 O_2	No	TSB
Salmonella typhimurium, (ATCC 14028)	X	CO_2 O_2	–	CO_2 O_2	CO_2 O_2	No	TSB
Escherichia coli, (ATCC 8739)	X, Y	CO_2 O_2	CO_2	CO_2 O_2	CO_2 O_2	No	TSB
Candida albicans, (ATCC 10231)	X, Y	CO_2 O_2	CO_2	CO_2 O_2	CO_2 O_2	No	TSB
Aspergillus brasiliensis, (ATCC 16404)	X, Y	CO_2 O_2	CO_2	CO_2 O_2	CO_2 O_2	No	TSB
Pseudomonas aeruginosa, (ATCC 9027)	X, Y	CO_2 O_2	CO_2	CO_2 O_2	CO_2 O_2	No	TSB
Clostridium sporogenes, (ATCC 11437)	Y	–	CO_2	–	–	No	FTM
Aerobic Isolates	**Site (X/Y)**	**2 ml (O_2/CO_2)**	**10 ml (O_2/CO_2)**	**20 ml (O_2/CO_2)**	**50 ml (O_2/CO_2)**	**Stress**	**Media**
Bacillus cereus (plant isolate)	X	CO_2 O_2	–	CO_2 O_2	CO_2 O_2	No	TSB
Bacillus clausii (plant isolate)	Y	–	CO_2	–	–	No	TSB
Micrococcus luteus (plant isolate)	X, Y	CO_2 O_2	CO_2	CO_2 O_2	CO_2 O_2	No, Yes	TSB
Staphylococcus warnerii (plant isolate)	Y	–	CO_2	–	–	No	TSB
Pseudomonas stutzeri (plant isolate)	Y	–	CO_2	–	–	No	TSB
Staphylococcus epi-dermidis (plant isolate)	Y	CO_2 O_2	CO_2	CO_2 O_2	CO_2 O_2	No	TSB
Bacillus iridiensis (plant isolate)	Y	–	CO_2	–	–	No	TSB
Staphylococcus capitis (plant isolate)	Y	–	CO_2	–	–	No	TSB
C. afermentans + 0.1% Tween80 (DSM 44280)	Y	–	CO_2 O_2	–	–	Yes	TSB
*Methylobacterium radio-tolerans (*DSM 1819)	X, Y	CO_2 O_2	CO_2	CO_2 O_2	CO_2 O_2	No, Yes	TSB
Kokuria palustris (plant isolate)	Y	–	CO_2	–	–	No	TSB
*Corynebacterium afer-mentans (*DSM 44280)	X, Y	CO_2 O_2	CO_2	CO_2 O_2	CO_2 O_2	No, Yes	TSB
Homofermenters	**Site (X/Y)**	**2 ml (O_2/CO_2)**	**10 ml (O_2/CO_2)**	**20 ml (O_2/CO_2)**	**50 ml (O_2/CO_2)**	**Stress**	**Media**
Enterococcus faecalis (DSM 20478)	X, Y	CO_2 O_2	CO_2	CO_2 O_2	CO_2 O_2	No, Yes	TSB, FTM
Lactococcus lactis (DSM 20481)	X	CO_2 O_2	–	CO_2 O_2	CO_2 O_2	No	TSB

(continued on next page)

Table 1 *(continued)*

PhEur Organisms	**Site (X/Y)**	**2 ml (O_2/CO_2)**	**10 ml (O_2/CO_2)**	**20 ml (O_2/CO_2)**	**50 ml (O_2/CO_2)**	**Stress**	**Media**
Streptococcus salivarius (DSM 20560)	X	CO_2 O_2	–	CO_2 O_2	CO_2 O_2	No	TSB
Streptococcus pyogenes (DSM 20565)	X	CO_2 O_2	–	CO_2 O_2	CO_2 O_2	No	TSB
Lactobacillus delbrueckii (DSM 20072)	X	CO_2 O_2	–	CO_2 O_2	CO_2 O_2	No	TSB
Anaerobic Isolates	**Site (X/Y)**	**2 ml (O_2/CO_2)**	**10 ml (O_2/CO_2)**	**20 ml (O_2/CO_2)**	**50 ml (O_2/CO_2)**	**Stress**	**Media**
Propionibacterium acnes (plant isolate)	X, Y	CO_2 O_2	CO_2	CO_2 O_2	CO_2 O_2	No, Yes	TSB, FTM
Bacteroides vulgaris (DSM 1447)	Y	–	CO_2	–	–	No	FTM
Fungi	**Site (X/Y)**	**2 ml (O_2/CO_2)**	**10 ml (O_2/CO_2)**	**20 ml (O_2/CO_2)**	**50 ml (O_2/CO_2)**	**Stress**	**Media**
Cladosporium sp. (plant isolate)	X	CO_2 O_2	–	CO_2 O_2	CO_2 O_2	No	TSB
Penicillium sp. (plant isolate)	X, Y	CO_2 O_2	CO_2	CO_2 O_2	CO_2 O_2	No, Yes	TSB
Penicillium chrysogenum (ATCC 10106)	Y	–	CO_2	–	–	Yes	TSB
Penicillium olsonii (plant isolate)	Y	–	CO_2	–	–	No	TSB
Aspergillus ustus (plant isolate)	Y	–	CO_2	–	–	No	TSB
Aspergillus sp. (plant isolate)	Y	–	CO_2	–	–	No	TSB
Leptospaerulina chartarum (plant isolate)	Y	–	CO_2	–	–	No	TSB

Table 2
Leaking media fill vial formats were prepared, inoculated with different microorganisms and tested on their respective CO_2/O_2 concentrations during 14 days at the respective site. Applied stress (exposure to heat) and media used are also indicated.

Organisms used in Leakage study	**Site (X/Y)**	**2 ml (O_2/CO_2)**	**10 ml (O_2/CO_2)**	**20 ml (O_2/CO_2)**	**50 ml (O_2/CO_2)**	**Stress**	**Media**
Bacillus subtilis (ATCC 6633)	X, Y	CO_2 O_2	–	CO_2 O_2	CO_2 O_2	No, Yes	TSB
Cladosporium sp. (plant isolate)	X	CO_2 O_2	–	CO_2 O_2	CO_2 O_2	No	TSB
Enterococcus faecalis (DSM 20478)	X, Y	CO_2 O_2	CO_2	CO_2 O_2	CO_2 O_2	No, Yes	TSB
Streptococcus salivarius (DSM 20560)	X	CO_2 O_2	–	CO_2 O_2	CO_2 O_2	No	TSB
Salmonella typhimurium (ATCC 14028)	X	CO_2 O_2	–	CO_2 O_2	CO_2 O_2	No	TSB
*Methylobacterium radio-tolerans (*DSM 1819)	Y	–	CO_2	–	–	Yes	TSB
Micrococcus luteus (plant isolate)	Y	–	CO_2	–	–	Yes	TSB
Penicillium sp. (plant isolate)	Y	–	CO_2	–	–	Yes	TSB
Penicillium chrysogenum (ATCC 10106)	Y	–	CO_2	–	–	Yes	TSB

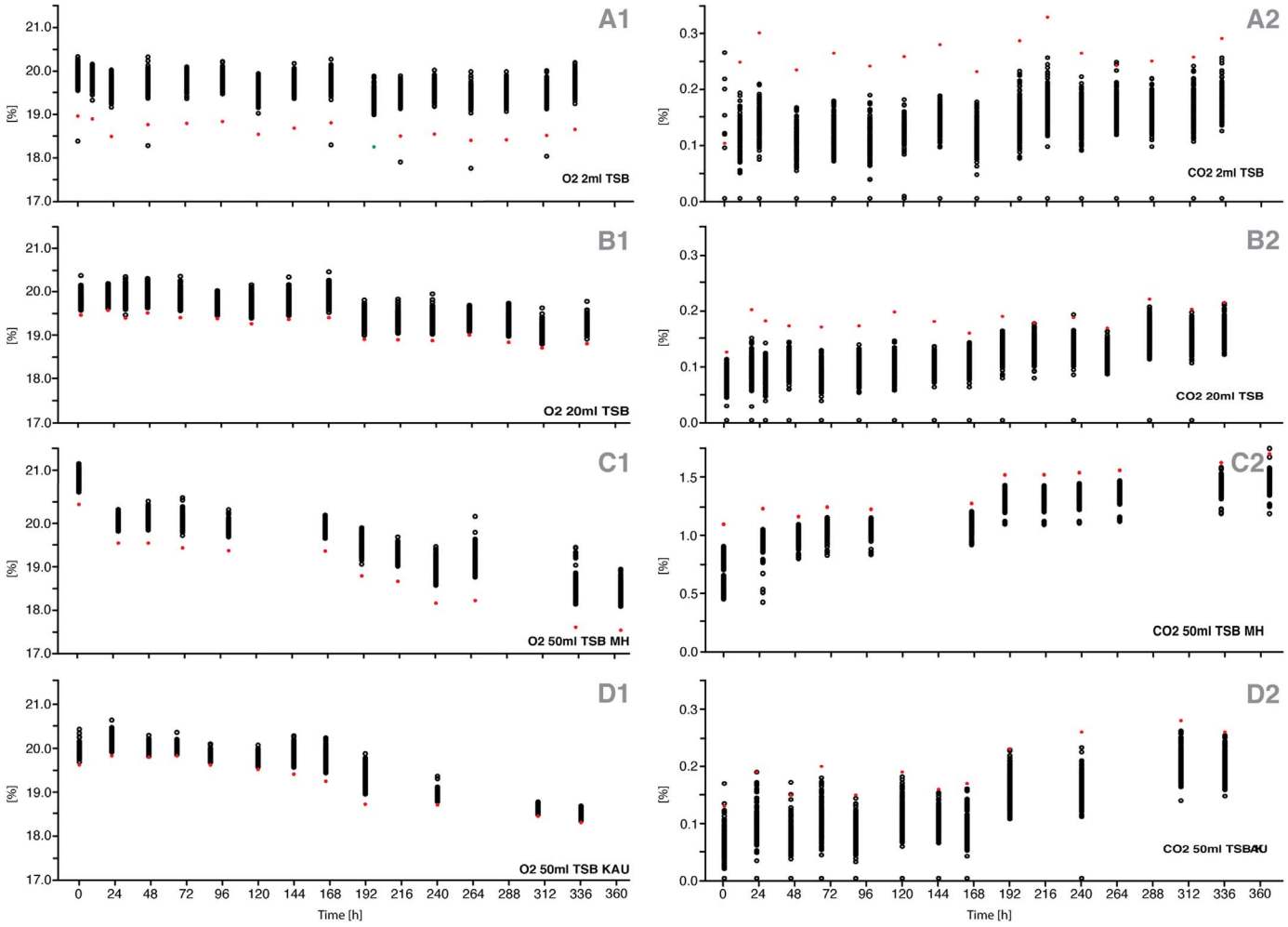

Fig. 1. Development of CO_2 and O_2 headspace concentrations of different formats with different TSB media from different producers evaluated at site X. A1-A2 (2 ml vials), B1-B2 (20 ml vials) and D1-D2 (50 ml vials) contained TSB from Becton Dickinson & Company. C1-C2 (50 ml vials) were filled with TSB from Merck Millipore. The red dots indicate the 3σ confidence intervals for each measurement point whereas the lowest (O_2) or highest (CO_2) 3σ level defined the threshold used for growth detection (see Table 3).

were selected due to their nature of consuming and exhausting O_2 and CO_2 and general phenotypical characteristics, targeting the analysis of fast/slow growing bacteria, fungi and homo-fermenting organisms (based on experiences made during subsequent studies). There was no difference detected among thresholds of leaking containers to those of sealed ones.

5. Data analysis and determination of time to detection (TtD)

With the collected data of blank samples a format specific three sigma (3σ) confidence interval (CI) was defined for O_2 and CO_2 headspace concentrations, specifying for each format the corresponding threshold for growth detection. In previous studies [6] it was shown that 10% of the $TDLAS_{O2}$ *S. salivarius* measurements remained undetected with a 4σ CI. To improve the detection rate, CI was reduced to 3σ which might however lead to an increased rate of false positives. For O_2 the minimal, and for CO_2 the maximal point of the 3σ CI after 14 days defined the threshold parameter of interest, which was taken as reference to determine the time point of growth detection also called time to detection (TtD) (see Figs. 1 and 2). TtD was reached when the gas concentration exceeded (CO_2) or passed below (O_2) the defined threshold. TtD of VI was reached as soon as 100% of all grown/inoculated samples turned turbid. All TtD points of O_2, CO_2 and VI were recorded and plotted in ranges (horizontal lines) to provide a condensed overview for all formats investigated per microorganism (Fig. 3). A false negative was defined as contaminated vial visually detectable but remaining undetectable for $TDLAS_{O2}$ and $TDLAS_{CO2}$. Vice versa false positives were defined as containers where media stayed clear but O_2 and CO_2 concentrations steadily reached the critical baseline level. Comparability among VI and TDLAS was given if microorganisms were detected by VI and TDLAS (O_2 and CO_2) within 14 days, as this is the requirement for a media fill demanded from regulatory agencies.

6. Results

6.1. Definition of format- and media-specific thresholds

It is important to set a format and media specific threshold prior study execution to allow the detection of microbial growth. Measured O_2 and CO_2 values, during the analysis of inoculated media fill units, that were below (for O_2) or above (for CO_2) these thresholds (3σ) were considered indicative of microbial growth and were defined accordingly (see Table 3, Figs. 1 and 2).

Considering medium providers and vial format only small differences for $TDLAS_{O2}$ (less than 1.2% difference on O_2 thresholds > 17.5%, see Table 3) were observed showing consistency in the results. In 50 ml vials TSB from BD versus TSB from Merck showed a rather small difference in the O_2 threshold (< 0.8%). A larger relative difference was observed for those two media when tested in the 50 ml vial on CO_2 that has a much lower initial concentration in vial headspace (> 1.4% difference). This indicates vendor or batch specific

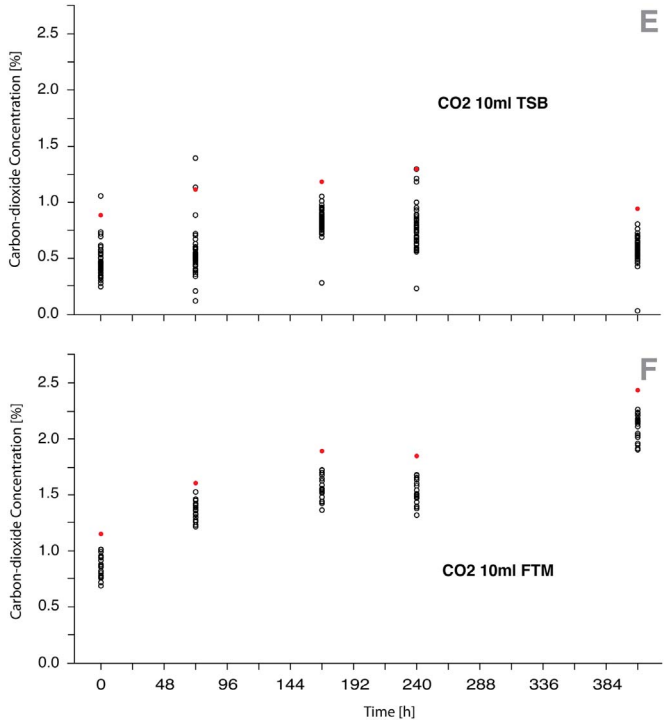

Fig. 2. Development of CO_2 headspace concentrations for TSB (E) and FTM (F) in blank 10 ml vials evaluated at site Y. The red dots indicate the 3σ confidence intervals for each measurement point whereas the highest 3σ level defined the threshold used for growth detection (see Table 3).

Table 3
Format specific O_2 and CO_2 thresholds (highest 3σ level) relating to the producers of the respective media. Data is based on repetitive measurements of blank media fill samples during minimally 14 days (see Fig. 1).

	2 ml	10 ml	20 ml	50 ml
O_2 Thresholds				
TSB from Becton, Dickinson and Company	18.3%	n.a.	18.7%	18.3%
TSB from Merck Millipore	n.a.	n.a.	n.a.	17.5%
CO_2 Thresholds				
TSB from Sifin	n.a.	1.3%	n.a.	n.a.
TSB from Becton, Dickinson and Company	0.3%	n.a.	0.2%	0.3%
TSB from Merck Millipore	n.a.	n.a.	n.a.	1.7%
FTM from Oxoid	n.a.	2.4%	n.a.	n.a.

differences and underlines the importance to set thresholds individually for a medium-format combination.

6.2. Analyzing the microbial spectrum (specificity analysis)

In total 34 different microorganisms were evaluated together with 4 different formats and two different media (TSB/FTM – Fig. 3A-F). All combinations of formats and microorganisms were detected by $TDLAS_{O2}$ and/or $TDLAS_{CO2}$ reliably. First the compendia reference strains from PhEur were investigated since they are routinely used in pharmaceutical industry for method suitability and growth promotion testing. As expected these strains were reliably detected between 24 h and 215 h by TDLAS (O_2 and CO_2) in all formats (see Fig. 3B). Most abundant strains from pharmaceutical clean rooms were also included in the study as they are highly relevant for media fill detection. Again TDLAS (O_2 and CO_2) was able to detect all plant isolates within less than 300 h (see Fig. 3C and F) proving its capability to detect relevant microorganisms. With regard to worst-case organisms such as *P. acnes* (slow grower), to stressed microorganisms and homofermenters (reduced O_2/CO_2 consumption/ exhaust) TDLAS was also able to detect their microbial proliferation (see Fig. 3D and E). Raw data (minimal, median, and maximum detection time) are presented in Tables S1-S5 for all formats and organisms tested.

Concerning the TtD some differences between VI, $TDLAS_{O2}$ and $TDLAS_{CO2}$ were observed. TtD using CO_2 was slightly faster for a majority of tested samples compared to TtD using O_2 (see Fig. 3A-F). Overall VI enabled the shortest TtD (range of 20.5–245.0 h). It is noteworthy that TDLAS and VI provided equivalent results for ca. 90% of all tested microorganisms after 7 days (i.e., intermediate inspection). Exceptions (ca. 10%) were samples inoculated with *A. brasiliensis* (50 ml), *Cladosporium* sp. (50 ml), *Penicillium* sp. (50 ml), *C. afermentans* (50 ml without Tween80 in TSB), *M. radiotolerans* (10 ml, 20 ml and 50 ml), *S. epidermidis* (2 ml), *K. palustris* (10 ml), *M. luteus* (50 ml) and *B. vulgaris* (10 ml). Some or all of those inoculated replicates remained for TDLAS undetectable within 7 days and needed up to maximally 11 days to reach the critical gas concentration. Overall, VI detected microbial growth slightly faster than TDLAS indicating that a bacterial cell mass of 10^7 CFU/ml (J. Mordenhauer et al., 2004) was in most cases not sufficient to allow a significant drop/increase of O_2/CO_2 in vial headspaces.

6.3. *The* Corynebacterium afermentans *case*

Corynebacterium sp. are present in the clean room flora [10] and grow slowly even on enriched media, whereas better growth is observed under aerobe than under anaerobe conditions [11]. *Corynebacterium afermentans* was selected in this study as model organism since it was recovered at site Y from monitoring in grade B area (in non-operating state max. 29 particles with size≥5.0 µm per m^3; see EC GMP Guideline Annex 1 for more details). Using 10 ml vials with TSB from Sifin allowed visual detection after 8 days. Small hardly detectable cell clumps appeared in the media around that time whereas an explicit turbidity increase was not detected. A significant change in CO_2 and O_2 headspace concentrations could also not be observed, even not after 14 days. This could be interpreted as a false negative result. At the same time, it is generally known that media with higher lipophilicity promotes growth of certain *Corynebacterium species* (Riegel et al., 1993). Adding 0.1% of the detergent Tween80 to TSB clearly improved the detection of *C. afermentans* within 7–8 days by VI as well as by $TDLAS_{O2}$ and $TDLAS_{CO2}$. This might reason future media adaptation for media fills in sterile drug product manufacturing. In TSB from Becton, Dickinson & Co. being filled in 2 ml, 20 ml and 50 ml formats no growth could be observed at all. However, the organism grew unexpectedly in 50 ml vials filled with TSB from Merck Millipore and could be detected visually (between 215.5 and 245.0 h) and by $TDLAS_{O2/CO2}$ (between 245.0 and 292.5 h). This is the longest organism specific detection time observed for TDLAS which positions *C. afermentans* as worst-case bacteria. The CO_2 measurement detected its growth slightly faster (max. 268 h) than compared to the O_2 (max. 292.5 h) measurement.

6.4. False negative and false positive results

From the collection of inoculated samples (753 vials) including the 2 ml, 10 ml, 20 ml and 50 ml formats 93 samples showed no growth despite inoculation. Thereof 60 samples were inoculated with *Lactobacillus delbrueckii* (which is not a typical organism in clean room areas but an organism with homofermenting characteristics) and *Propionibacterium acnes*. Apparently both organisms did not grow due to their need for different growth conditions. The two strains normally prefer (slightly) anaerobic conditions and 37 °C. The most probable root cause for a lack of growth in the remaining 33 samples (ca. 4.3% from 753) may be the small inoculation volume. As working suspensions were prepared with less than 10 CFU (site X) and less than 20 CFU (site Y), it is statistically expected that some vials do not contain any/little CFU (less than 3) reducing chances for successful outgrow.

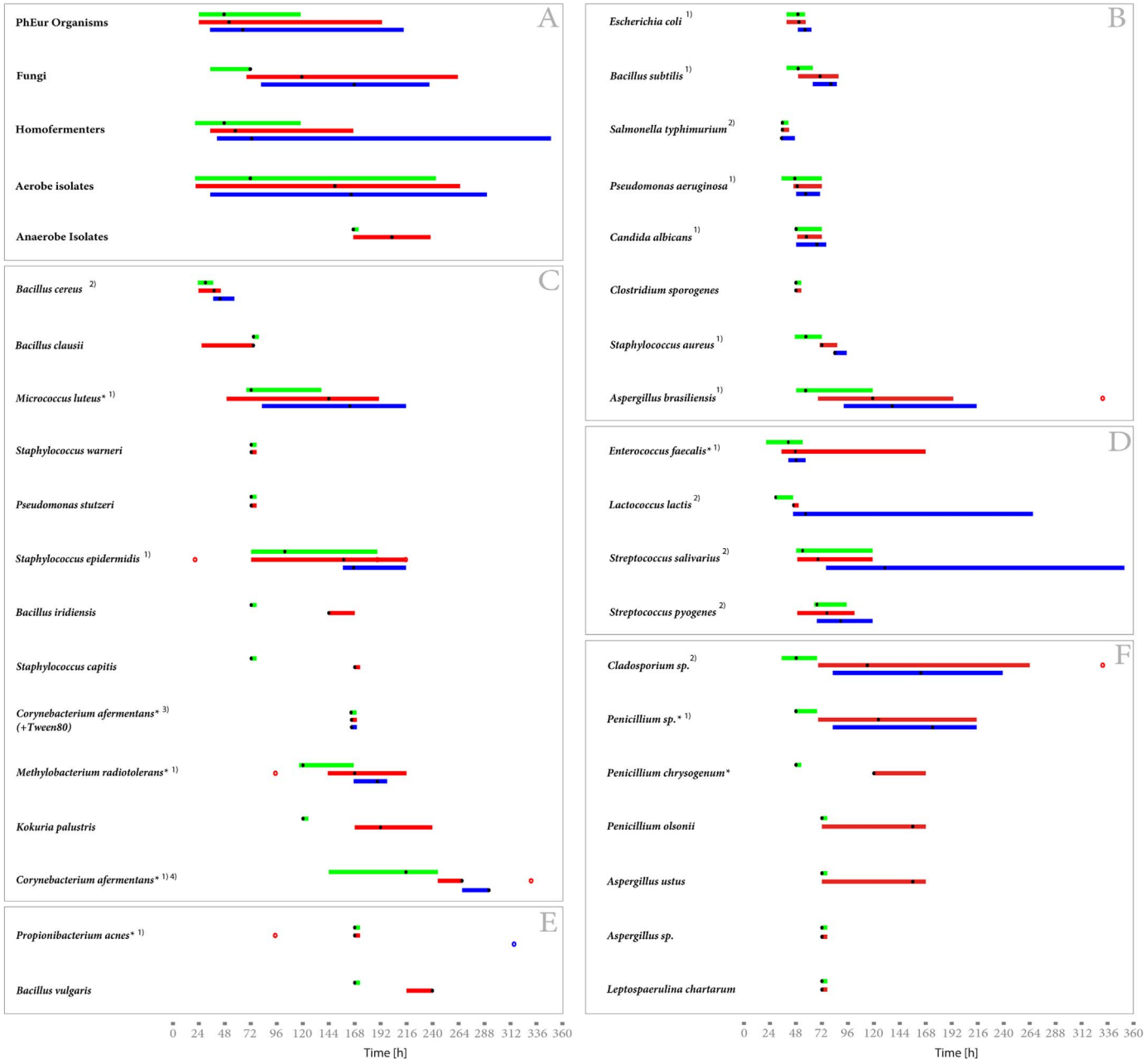

Fig. 3. Detection times in form of range (line) and median (black dot on line) are depicted per microorganism for the visual method (green), $TDLAS_{CO2}$ (red) and $TDLAS_{O2}$ (blue). Plot A illustrates an overall summary of the five organism groups in terms of detection speed per analyzed parameter (VI, O_2 and CO_2). Plot B represents detection times for PhEur organisms, Plot C for aerobic, plot D for homofermenting, plot E for anaerobic microorganisms and plot F for fungi including yeast and mold. The (*) refers to organisms that were heat stressed in 10 ml vials at site Y. The subscript 1) is indexing the microorganisms that were tested in 2, 10, 20 and 50 ml vials. 2) refers to organisms tested in 2, 20 and 50 ml vials only. 3) marks the 10 ml *C. afermentans* experiment being executed by site Y with TSB+0.1% Tween80. 4) relates to the TSB samples (no Twenn80) inoculated with *C. afermentans* being detected visually at site Y in 10 ml vials and at site X by TDLAS and VI in 50 ml vials (TSB from Merck Millipore). Organisms without subscript indexes were exclusively tested in 10 ml vials. Anaerobic organisms as well as *C. sporogenes* and part of *E. faecalis* were cultured in FTM instead of TSB. *Lactobacillus delbrueckii* was also part of the study but did not show any growth which is the reason for not being part of this table. Inconsistencies in TtD due to fluctuations around the baseline of either O_2 or CO_2 are shown as hollow dots in the respective color.

False positives were neither detected after 7 days nor after 14 days when applying parallel CO_2 and O_2 headspace concentration measurements (non-parallel measurements would have resulted in 4 false positives for CO_2 and 3 false positives for O_2 after 14 days in the 2 ml format).

6.5. Leakage study

Container closure integrity (CCI) testing is a standard procedure in the release of sterile drug products. CCI evaluates the goodness of container integrity as it directly relates to keeping the aseptically filled drug product sterile. Because leakages may occur also in media fills and could potentially influence CO_2/O_2 growth curves by headspace equilibration effects with the environment it is of high relevance to evaluate inoculated but leaking samples. Within the study a leakage representing worst case conditions was applied by inserting a needle into the stopper (inner needle diameter ~0.25 mm). As expected it was shown that non-integrity has an impact on growth related CO_2 and O_2 gas profiles leading in some few cases (3 out of 232 cases) to the appearance of false negatives. Still most samples were detected with at least the CO_2 measurement reliably. CO_2 and O_2 profiles were hill-(CO_2) or valley-shaped (O_2) (Fig. 4), whereas the impact of the gas

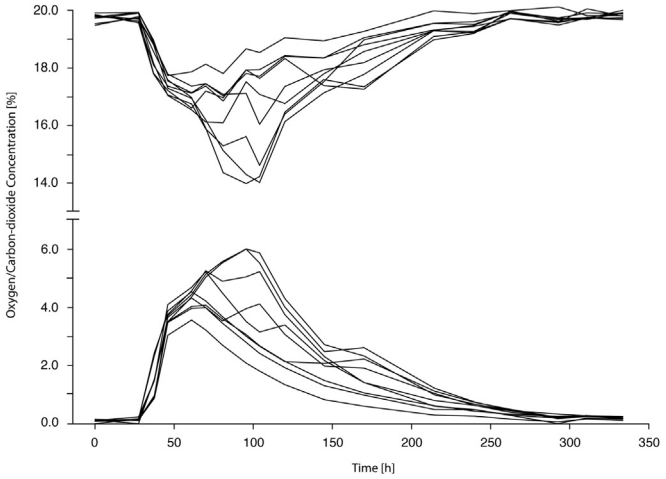

Fig. 4. Example for growth curve development of leaking vials inoculated with *Enterococcus faecalis*. Diverse leaking vials were tested on their O_2 and CO_2 related growth. On the bottom the CO_2 profile and on the top its corresponding O_2 profile. The hill- and valley shaped profiles are typical for leaking vials. In this experiment the equilibration was that strong that gas concentrations turned back to their initial level, leading to false negative results after 14 days.

concentration equilibration with the environment became more visible when the growth plateau phase was reached. At this point the respective gas concentration increased or decreased as O_2 inflow and CO_2 escape from the vial headspace became the leading process. Usually this happened between day 5 and 9. Indeed, compared to the remaining three evaluated organisms (see Table 2, Organisms used in leakage study), the majority of *Enterococcus faecalis* (2 ml) and *Streptococcus salivarius* (2 ml – CO_2 only) inoculated vials were detectable in the course of analysis (i.e. between 38.0 h and 263.0 h). However, after 14 days those samples were interpreted as false negatives as gas concentrations had equilibrated with the environment. It was not observed that the organism specific TtD was increased due to the leakage applied.

7. Discussion

To test an alternative microbial monitoring method several validation parameters need to be evaluated [12–14]. Within this study specificity (detectability of a wide organism spectrum as defined by [12]), robustness (reproducibility), sensitivity (ratio of false negative results) and comparability (to the conventional method VI) were tested for TDLAS. TDLAS was used to detected microbial growth in sealed glass vials by measuring non-invasively O_2 and CO_2 headspace concentrations aiming at a substitution of the visual media fill inspection (VI). The study included 34 different microorganisms in four glass vial formats (2 ml, 10 ml, 20 ml and 50 ml) filled with mainly TSB (and FTM) whereas growth of organisms was detected reproducibly without observation of false negative/positive results [5] during the period of 14 days. An increased rate of false positives can be expected when performing measurements after 14 days and needs further evaluation if applicable. Although the visual detection was in most cases faster than TDLAS, growth was determined within 14 days (11 days). This is in agreement with regulatory requirements for media fill inspection (specifying an incubation duration of not less than 14 days before the final visual inspection) and proof for method comparability [4]. Sites with isolator technology in use might therefore think about abolishing the intermediate inspection when using TDLAS since the intermediate inspection of a media fill after 7 days is not mandatory. This would save time and appears to be safe considering that 100% of the tested organisms used in this study were detected by TDLAS after 14 days.

An implementation and application of an automated TDLAS platform (machinery) would lead to diverse advantages. One of them is a ~17x faster read-out than what can be achieved today by the visual media fill inspection (TDLAS platform inspects at least 100 vials/min; visual inspection approx. 6 vials/min; average batch size ~15'000 units, two inspections), resulting in 17x higher cost effectiveness. In addition, TDLAS would increase data integrity, facilitate data handling including its transmission and help to efficiently realize technology transfers (e.g. implementation of a new format) as such transfers are often linked to three media fills in a row for validation purposes. In addition, higher safety could be expected when dealing with the determination of bacterial growth in contaminated vials that contain lysed bacteria as those would remain hardly visible for human eyes. In turn this would lead to false negative results. Such events are rare but tend to happen sometimes in units with scarce growth induced by autolysins [15]. Finally, false positive units that turned turbid due to chemical precipitation or haziness and not due to microbial growth would unlikely result in CO_2 or O_2 headspace concentration variations, thus avoiding unnecessary investigations and/or uncertainty.

High-throughput will be needed to deliver the expected efficiency increase, which is linked to short measurement times (results are based on 5 s measurements). Accuracy and precision are dependent on the measurement path length (vial diameter) and duration of the measurement becoming worse with decreasing vial size. It was experienced that a ~10 fold decrease in measurement time increases the measurement fluctuation by a factor of 3–4, independently of the format size. Therefore a trade-off between threshold definition and measurement speed will be needed to achieve a successful site-specific validation with zero false negative values.

To optimize and shorten detection times (i.e., < 11 days) of bacterial growth (TtD) filling levels could be increased thus lowering headspace volumes, resulting in higher gas concentration changes in such lower headspace volumes. Such adaptations are only recommended as long as an unhindered TDLAS headspace measurement is enabled, organisms are exposed to enough oxygen (dependent on the headspace size), and regulatory filling levels are respected [8]. Results also suggested that microbial stress had rather less influence on lag phase extension and TtD (data not shown). Much more impact on TtD was attributed to metabolic characteristics combined with unfavorable headspace volumes. Therefore, during validation studies we would recommend to focus rather on optimal fill-headspace volumes instead of applying stress to microorganism.

When thriving towards an implementation of the TDLAS methodology the case of *Corynebacterium afermentans* shall not be neglected. This bacterium was identified during first experiments as worst case organism because growth detection was possible by VI exclusively (false negative by TDLAS) in media from Sifin. The lack of detection was then resolved by adding 0.1% of Tween80 to filled TSB [7]. Furthermore, growth and TtD of *C. afermentans* was heavily dependent on the TSB media producer/composition and should be considered carefully when planning a substitution of the visual media fill inspection by TDLAS. Therefore growth of *C. afermentans* in commonly used TSB needs to be evaluated carefully as it could lead to relatively long TtD or even to false negative results, demanding for media adjustments (increase of lipophilicity). These findings are important since *Corynebacterium* strains may belong to the usual clean room flora. However, we are aware that this was a single experiment and that results cannot be generalized to all *Corynebacterium* species. Anyhow, media modification would only make sense if the risk of observing false negative results by using a certain media is existent.

It was also observed that non-integrity did have an impact on microbial growth profiles and could even lead in some cases to non-detectability. However, leakages applied here were of much bigger size than normally encountered in production as the absolute worst case was constituted. Therefore, it can be assumed that leaking contaminated vials passing the inspection on quality before the media fill

inspection will become detectable and should not result in false negative findings caused through headspace equilibration effects. Anyhow, container closure integrity testing is a state of the art analysis integrated in quality control processes reducing the risk of dealing with leaking units even further [16]. Alternative approaches inducing leakages close to more realistic size such as copper wires of the size 10–120 µm to simulate leakages [17] could be used during subsequent validation studies to further evaluate the degree of impact on microbial growth curves.

Hence, the discussed points illustrate that TDLAS is a technology with much potential in substituting the visual media fill inspection and may even lead to a reconsideration of incubation time and temperature for media fills [5].

8. Conclusion

TDLAS was evaluated as a potential method for automating the visual media fill inspection based on CO_2 and O_2 headspace measurements. Growth of a wide collection of (stressed) organism-format combinations without observing false negative/positive results was possible. Therefore, the method appears to be a promising alternative whereas the optimization of filling volumes might allow even faster growth detection. The risk of not-detecting contaminated vials due to leakages was estimated as minimal but needs further analysis.

An implementation should take into account that a high-throughput approach implies shorter measurement times, potentially affecting measurement precision negatively. Nevertheless, with the proposed filling modifications and a combined CO_2 and O_2 TDLAS headspace analysis it is expected that TDLAS satisfies regulatory requirements following an appropriate validation.

Compliance with ethical standards

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Conflict of interest

David Brueckner declares that has no conflict of interest.
David Roesti declares that he has no conflict of interest.
Ulrich Zuber declares that has no conflict of interest.
Meik Sacher declares that he has no conflict of interest.
Stephan Krähenbühl declares that has no conflict of interest.
Olivier Braissant declares that he has no conflict of interest.
Derek Duncan is employed at Lighthouse instruments that provided the TDLAS instruments.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2017.01.088.

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CHAPTER 4

A Case Study with a TDLAS Based Semi-automated Media Fill Inspection Platform

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Abstract

Media fills are process simulations imitating the aseptic filling procedure with microbial culture medium instead of the liquid drug product. The visual inspection on turbidity of fill-finished media fills represents the control on filling line asepticity and is a rather time-consuming activity. Former studies have discussed the feasibility of automating this visual media fill inspection by tunable diode laser absorption spectroscopy (TDLAS) benchtop devices. The idea of using TDLAS was based on determining non-invasively CO_2 and O_2 headspace concentration changes as indicator for microbial growth in test samples. Since the results from former studies were highly promising in terms of detecting contaminated media fill samples, a follow-up case study was performed with a semi-automated system provided by Wilco AG, enabling high-throughput detection (100 vials per minute). The study delivered very promising results in terms of media fill inspection automation and gives evidence for a more efficient design of the entire media fill process. Therefore, an implementation in aseptic drug product manufacturing is highly recommendable.

Keywords: Absorption spectroscopy, Automation, Microbial growth, Media fill, TDLAS

Introduction

Media fills are simulations imitating the aseptic filling procedure with medium (tryptic soy broth [TSB]) instead of the liquid drug product. The simulation's purpose is to identify deficiencies in the aseptic conditions during the filling process leading to microbial contamination. Several thousands of vials are filled, stoppered and crimped within one of such process simulation. The fill-finished media units are normally incubated for minimally 7 days at 20-25°C and for 7 days at 30-35°C, followed by a visual inspection (VI) through qualified human operators assessing the increase in media turbidity or abnormalities in medium appearance. Industrial regulations state that such inspections have to be performed after minimally 14 days [4,8,12,13]. However, a first VI after 7 days (intermediate inspection) is sometimes performed as fast growing microbes could already be detectable by then. A more efficient design of the media fill inspection procedure could be beneficial for manufacturers as VI is a qualitative assessment and therefore prone to subjectivity and errors. In addition manual vial and data handling during media fills are time-consuming resulting in unneeded costs for production facilities.

Previous studies [2,3,5] evaluated the potential of tunable diode laser absorption spectroscopy (TDLAS) as an alternative for VI. Benchtop TDLAS devices were used to detect microbial growth in media fills by measuring CO_2 and O_2 concentration changes in vial headspaces. Such changes were mainly resulting from metabolic activity of growing microorganisms and allowed non-invasive growth detection. The methodology detects growth of many different micro-organisms reliably and reproducibly, in various combinations of format sizes and media types as well as in vials subject to leakage. A transfer of the TDLAS technology to an automated platform leading potentially to a higher inspection rate (100 vials / min is targeted) can thus be a valuable investment. In turn, a more objective assessment, increased data integrity and monetary cost savings would result from such automation.

A first platform prototype allowing a high-throughput media fill inspection was assessed on its capability to detect microbial growth by a CO_2 and O_2 headspace analysis. In parallel, a comparison to previously published results [2,5] obtained from measurements with benchtop devices consolidated the experimental results. In this study, the media fill inspection automation is assessed based on sensitivity and specificity. The use of such an automated platform is deemed reasonable by the authors.

Materials and Methods

Automated Platform and Experimental Start-up

An automated tunable diode laser absorption spectroscopy (TDLAS) platform for high-throughput media fill inspection was provided by Wilco AG, Rigackerstrasse 11, 5610 Wohlen (see figure 1). It contains a slide-in unit (fig. 1 [a]) where operators can prepare up to 200 vials for the imminent analysis. A conveyor belt (fig. 1 [a]) transports the prepared samples towards a rotary plate (fig. 1 [b]) with format specific sample holders picking up the approaching vials. Subsequently the rotary plate passes the samples through the installed O_2 and CO_2 lasers (fig. 1 [c]) with a throughput speed of 100 vials per minute. The lasers measure each headspace of passing vials for 0.1 seconds. After the measurement corresponding vials are classified in two groups: non-sterile (fig. 1 [d]) and sterile units (fig. 1 [f]).

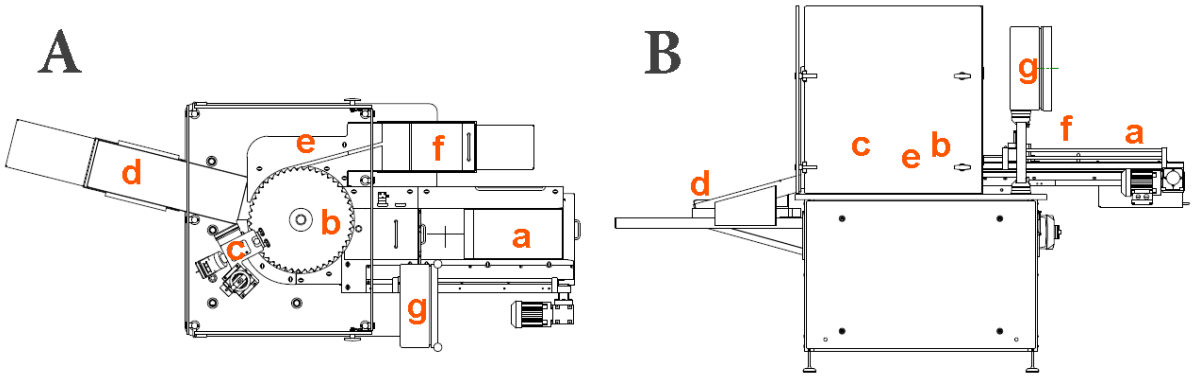

Figure 1: A) shows the top view and B) the side view of the inspection platform in use (Wilco AG, Rigackerstrasse 11, 5610 Wohlen). a) is attributable to the slide in unit as well as to the conveyor belt used for sample preparation and sample transportation towards the rotary plate marked as b). c) refers to the CO_2 and O_2 laser heads whereby d) is the sample exits for non-sterile units. e) is the connection between the rotary plate and the sterile sample exit f). g) refers to the human machinery interface allowing interaction with the device [1].

The classification is based on defined CO_2 and O_2 threshold values and allows operators to inspect samples (identified as non-sterile) visually on turbidity, controlling if the separation was justified or attributable to false positive measurements. Units that are determined as sterile are transported to the final sterile exit, taken off by an operator and put back for incubation. Fig. 1 [e] shows the transport channel being the connection between rotary plate and exit for sterile units. All interaction with the machinery, such as specification of baselines, batch initiation etc. occurred over a human machine interface (HMI, fig.1 [g]).

All experiments were done with 20ml vials having a diameter of 31.5mm, an overall height of 58.0mm and a filling volume of 8.0ml. The automated platform determined with two adjusted single pass TDLAS laser beams the corresponding O_2 (λ=760nm) and CO_2 (λ=2000nm) vial headspace concentrations. Prior its use, the system was preheated for minimally 10 minutes and measuring gaps were flushed with nitrogen (max. 4 l/min) enabling an unbiased measurement. Nitrogen flushing was constant during the experimental phase. The system validation was performed with certified standards before each measurement series (O_2: 0.1%, 1.9%, 19.9%; CO_2: 0.00%, 0.52%, 2.06% made and certified by Wilco AG). All standards were manufactured of the same glass as tested samples to keep background noise minimal.

Determination of Threshold Parameters

The definition of the CO_2 and O_2 thresholds was performed using 500 blank vials of a freshly produced media fill batch (B019) filled with TSB on an operative aseptic filling line. Vials were stored for 7 days in a 20-25°C incubator and subsequently transferred to a 30-35°C incubator and kept for another 7 days. O_2 and CO_2 concentrations in vial headspaces were tested 9 times during 14 days. After 14 days the upper (for CO_2) and lower (for O_2) 3σ confidence interval (CI) was defined for each measurement series whereas the highest and lowest values defined the threshold used as reference for microbial growth determination.

A confidence interval of 3σ was considered appropriate as minimal detection times of microbial growth were targeted by risking as few false negative results as possible but, instead, potentially accepting false positive samples.

Feasibility of Automated Growth Detection

The automated platform was first evaluated by performing a growth study for checking if the platform was generally capable to detect microbial growth. Fresh media fills (batch B019) manually inoculated with microorganisms were used for this purpose. Microorganisms used for vial contamination were chosen based on previous studies [2,3,11] targeting a well-balanced distribution of fast/slow growing organisms and the inclusion of worst case microorganisms (e.g. long lag-phase or reduced drop/increase of O_2/CO_2). Organism's availability, relative occurrence in cleanroom monitoring and their relevance in PhEur for validation studies [7] were also respected.

The selection involved *Bacillus subtilis (ATCC6633), Cladosporium sp. (site isolate), Micrococcus luteus (site isolate), Methylobacterium radiotolerans (DSM1819), Penicillium sp. (site isolate), Staphylococcus epidermidis (site isolate), Streptococcus salivarius (DSM20560) and Aspergillus brasiliensis (ATCC16404)*. Microbial suspensions in use were created according published procedures [2,3].

Ten vials were prepared per microorganism and each inoculated with <10 colony forming units (CFU). The prepared samples were tested once a day during two weeks using the automated platform so that a growth profile could be obtained. Between the measurements, vials were placed in an incubator at 20-25°C, for day 0 till day 7 and at 30-35°C for day 8 till day 14. The defined O_2 and CO_2 thresholds (3σ CI [10]) were used to determine detection points for each microorganism species by checking the time point when the growth profile passed these thresholds. This provided insight into microorganism specific time to detection determined through the automated platform and confirmed that the detection points were comparable with former study results.

Determining Detection Reliability of Contaminated Units

Determining whether the automated platform enables a reliable detection and separation of contaminated media fill vials randomly distributed within a batch of sterile samples is of major importance. Therefore a second assessment was performed also using B019 batch vials. However, the age of the batch amounted already 14 days ($T_{storage}$ = 20-25°C). As CO_2 and O_2 headspace gas concentrations are known to change over time induced due to media auto-oxidation [3,5], the previously defined baseline values were shifted accordingly. The values of the shifted CO_2 and O_2 threshold were defined by first assessing the lower (for O_2) and upper (for CO_2) 3σ CI of aged media fills before the experimental start. To this 3σ CI the variation in 3σ CI (rounded values) of fresh media fills after 14 days was added or subtracted and considered as the shifted baseline (fig. 2). This approach is justified as during former studies (D. Brueckner, 2017, unpublished data) the extent of media induced changes in headspace gas concentrations remained similar in 20ml vials independent of the medium age.

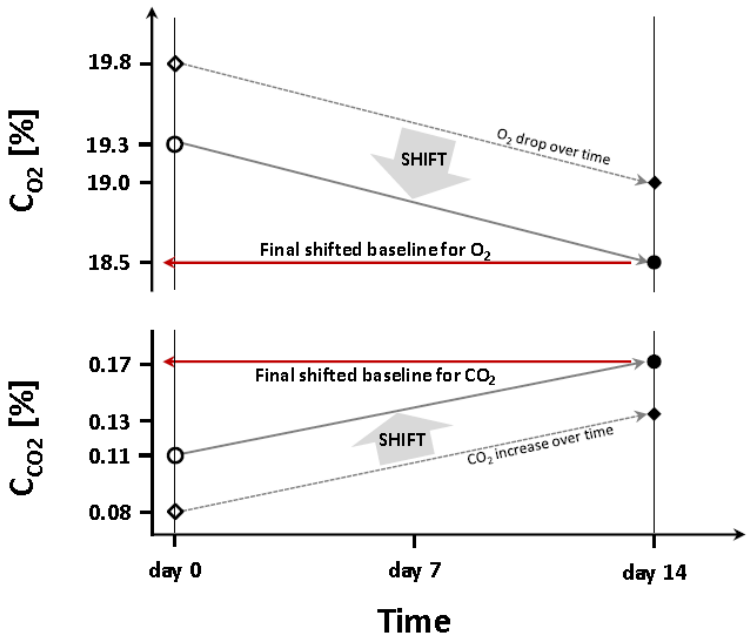

Figure 2: The illustration shows the approach of shifting the defined baseline for aged (14 days) media fills according the time-dependent CO_2 and O_2 headspace concentration variation induced by the media. The empty and filled rhombs define the 3σ confidence intervals (CI) of initial and changed headspace concentrations of fresh media fills. Empty circles depict the initial headspace concentration (3σ CI) of media fills after storage for 14 days at 20-25°C. The filled circles define finally the values for the shifted baselines which were used to determine the Wilco platform's detection reliability of contaminated media fill vials.

From a collection of 1208 sterile media fill samples 80 units were chosen at random, marked and contaminated with the same microorganisms used for the above described growth study. Always 10 units were inoculated with the same microorganism. Inoculation volume, suspension preparation, media fill incubation process, preheating, system validation and nitrogen purging remained identical. Inoculated samples were randomly mixed with sterile units whereas the entire collection was measured iteratively every ~24h during 14 days. In parallel to the automated inspection by the platform a visual inspection on turbidity was performed every day, assessing if and when growth of contaminated samples became detectable. Indeed, the visual inspection served as reference to determine the number of true positive (TP), true negative (TN), false positive (FP) and false negative (FN) measurements performed by the automated inspection platform. In addition, sensitivity (TP/(TP+FN)) and specificity (TN/(TN+FP)) were individually calculated for O_2 and CO_2 measurements.

Results

Threshold Parameters

All previous studies that investigated TDLAS as method for automated media fill inspection used media fills considerably older than encountered in production. Since CO_2 and O_2 headspace concentrations are known to change over time due to media auto-oxidation and CO_2 degassing, aged vials were flushed with sterile compressed air to neutralize that effect. Therefore, using fresh and unaffected media fills for this study is important to confirm that headspace concentrations are exposed to comparable variations as observed in previous studies. This is especially important for the threshold definition of growth determination done here by the analysis of 500 freshly produced media fills whose development over time is shown in figure 3 and table 1. The calculated lower (fig. 3 A) and upper (fig. 3 B) 3σ confidence interval (CI) after 14 days defined the final threshold for growth detection.

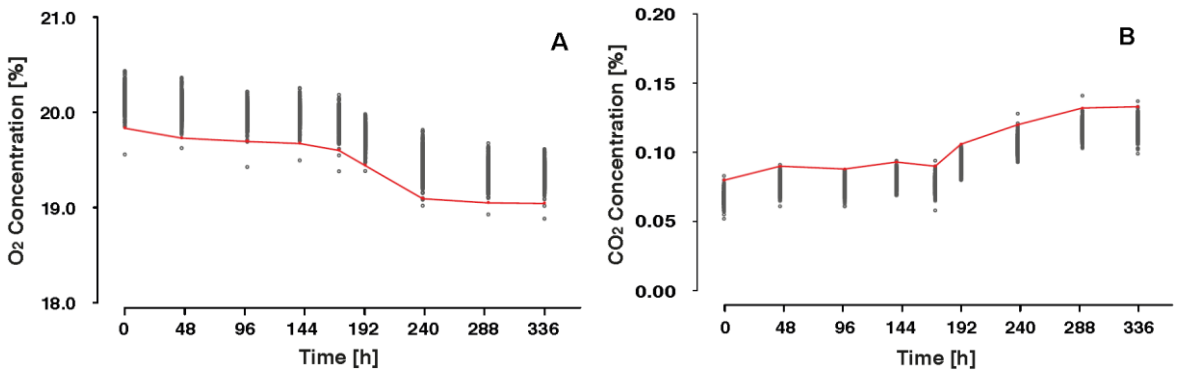

Figure 3: Development of O_2 and CO_2 headspace concentrations during 14 days. In red the continuous 3 sigma confidence interval is shown whereby the last value of the 3σ CI defines the baseline for O_2 or CO_2 based growth detection in fresh media fills.

The threshold for O_2 was set to 19.048% and the one for CO_2 to 0.133% (fig 3, tab. 1). Four O_2 values from the entire threshold study (4'500 measurements) were below and two CO_2 values above the defined threshold parameters, being theoretically attributable to six false positive results.

Table 1: Calculated continuous 3σ CI values. Grey shaded and bold values define the baseline for O_2 and CO_2 for freshly produced media fills.

Time [h]	**0.0**	**45.5**	**98.0**	**140.0**	**171.5**	**192.5**	**238.5**	**291.0**	**336.0**
3σ baseline O_2 [%]	19.837	19.732	19.698	19.676	19.604	19.445	19.097	19.053	**19.048**
3σ baseline CO_2 [%]	0.080	0.090	0.088	0.093	0.090	0.106	0.120	0.132	**0.133**

Automated Growth Detection

The automated platform was capable to readily detect microbial growth in media fills. Headspace concentrations from 75 out of 80 inoculated samples fell below or exceed the defined O_2 and/or CO_2 baseline(s) within 172h (figure 4). Only one sample inoculated with *Staphylococcus epidermidis* was detected after 172h (growth detection of that single sample occurred at 246h).

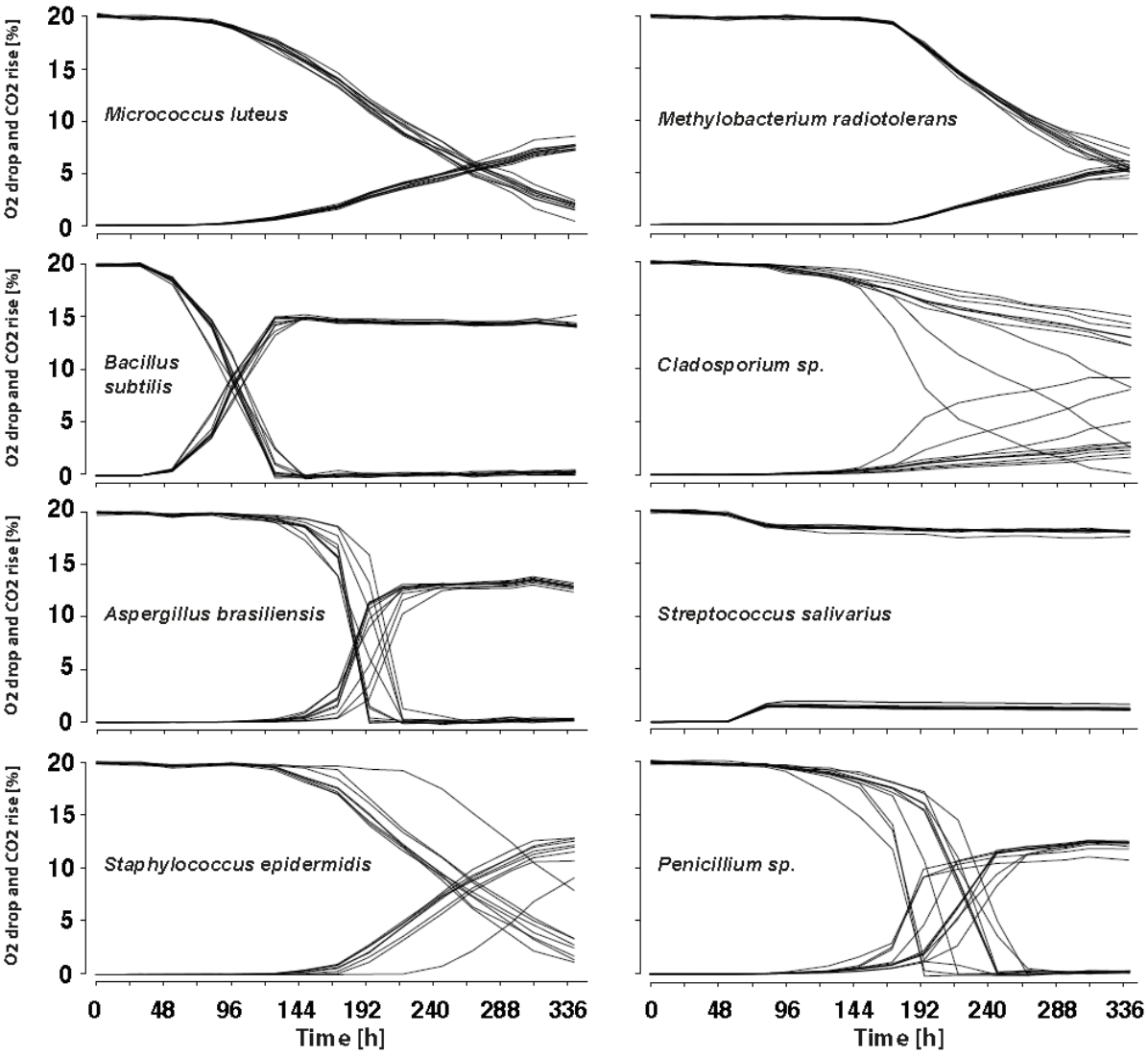

Figure 4: CO_2 and O_2 vial headspace developments (raw data) of eight selected microorganisms. Each illustration included 10 inoculated sampled which were tested by the Wilco inspection platform every ~24h during 14 days on their CO_2 and O_2 headspace concentration.

Four inoculated samples remained clear neither turning turbid nor experiencing any relevant change in CO_2 or O_2 headspace concentration. The automated platform detected growth in 35 units at the same time as the visual inspection on turbidity (applicable for all samples inoculated with *Methylobacterium radiotolerans*, *Micrococcus luteus* and *Bacillus subtilis* as well as for samples inoculated with of *Streptococcus salivarius* (1), *Staphylococcus epidermidis* (3) and *Aspergillus brasiliensis* (1)). Slightly lagged growth detection by the automated platform was observed for the remaining 41 samples. 53 samples were detected ~24h earlier by the CO_2 than by the O_2 measurement.

Detection Reliability of Contaminated Units

Results from the growth study demonstrated that microorganisms could be detected within <14 days by the automated platform. Those results were confirmed by letting the system randomly find 80 inoculated samples among a batch of 1'128 sterile media fill samples. As the media fill vials were aged (14 days) and TSB had slightly impacted CO_2 and O_2 headspace concentrations, for this study the previously defined baselines were shifted to values of 0.17% CO_2 and 18.50% O_2 (over the considered time period the changes of the headspace concentrations induced by TSB were assumed to be linear [2]), defining the new growth detection limits (fig. 2). Visual inspection on turbidity (VI) was used as reference for growth detection by the automated platform. Again, VI detected microbial growth in media fills slightly faster than measurements on CO_2 and O_2 headspace concentrations. A temporary appearance of false negative results was observed for CO_2 measurements between 45h - 137h and for O_2 measurements between 45h - 161h (fig. 5, A and B). Nevertheless, after 161 hours sensitivity for CO_2 reached 1 (100%) (fig. 5 C). There were no false positive results observed (neither for CO_2 nor for O_2 measurements) leading to an overall specificity of 1 (100%) during the entire test period.

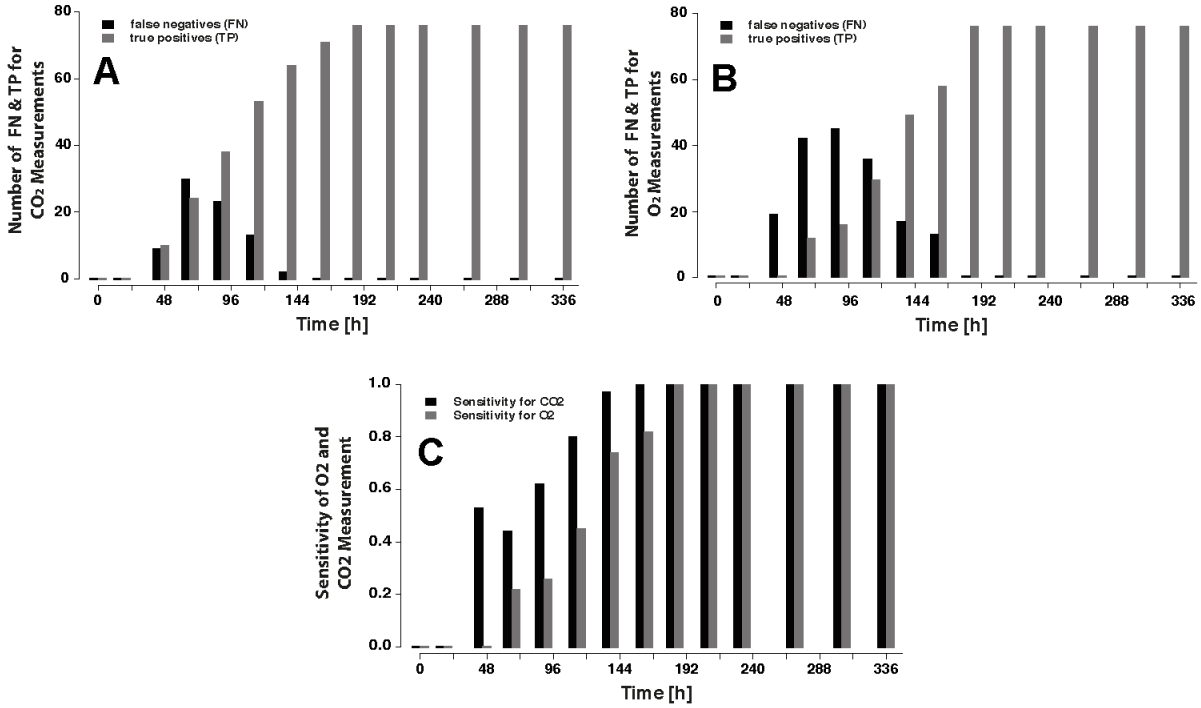

Figure 5: The number of false negatives and true positives is shown for CO_2 in plot A and for O_2 in plot B. Plot C shows the increasing sensitivity of the CO_2 and O_2 related headspace analysis during 14 days. The reference for determining false negatives was the visual inspection on turbidity. Values equal to zero are also indicated.

Discussion & Conclusion

It was clearly demonstrated that an automated and TDLAS-based media fill inspection, controlling 100 vials per minute, allows a reliable detection of contaminated 20ml TSB filled vials. In fact, besides one single vial (it contained *Staphylococcus epidermidis* and needed 246h until growth detection), all inoculated samples that were visually detected were also identified by the automated platform as non-sterile after ~7 days without observing any false negative or false positive results (fig. 5A-C).

As experienced during previous studies [2] performed with benchtop devices (manufactured for single measurements only) from another supplier (Lighthouse Instruments (LHI)), again the CO_2 measurement was more sensitive (fig. 5C, fig. 6A and B) and detected two third of contaminated units one day earlier than the O_2 analysis. Indeed, the O_2 headspace concentration is subjected to much stronger changes than for CO_2, because of auto-oxidation. Thus, the delta between O_2 threshold level and initial O_2 headspace concentration is much larger as compared to CO_2. As a consequence more time is needed until O_2 growth profiles reach the O_2 threshold, which explains the different periods of time needed for growth detection using CO_2 and O_2 measurements.

In addition the CO_2 measurement was more precise. Indeed the relative standard deviation (stdev) of a sample collection of 500 units was 0.0045% for CO_2 and higher for the O_2 analysis (stdev = 0.120%). In comparison to studies performed with older TSB no difference of the degree of media induced headspace variation of CO_2 and O_2 was detected between fresh (unaffected) and aged (regased) media fills.

A comparison to the mentioned previous studies that evaluated a visual media fill inspection substitution by TDLAS benchtop devices was of further interest. Such result comparability is valuable as knowledge is generated about what can be expected from the automated platform assessment if testing other formats (e.g. 2ml, 10ml, 15ml, and 50ml vials). Fresh media fill samples used in this work for baseline and growth analysis, were tested in parallel with an older generation of benchtop devices from LHI. The devices were set to the shortest measurement duration of 0.5s to achieve the closest possible comparability with the automated platform in use. The automated platform from Wilco AG achieved slightly shorter times to detection (fig. 6) as the benchtop devices. Indeed the slightly larger measurement fluctuations of the benchtop lasers imposed higher standard deviations for blank measurements (0.077% for CO_2 and 0.390% for O_2) and led to higher shifts in baseline values (17.856% O_2 and 0.365% CO_2) delaying time to detection. It is important to mention at this place that such benchtop devices are designed for optimum performance in a laboratory environment. They are not customized for assessments with short measurement durations as the internal data acquisition system and scanning rate are not fully designed for such measurement speed. Therefore, the comparison of benchtop devices and automated platform cannot be seen as an “apple-to-apple” comparison but shall be treated as additional experience being helpful for a future method transfer to an automated system. Furthermore, such comparison shall not be interpreted as a rating of the LHI benchtop device performance or their corresponding competences.

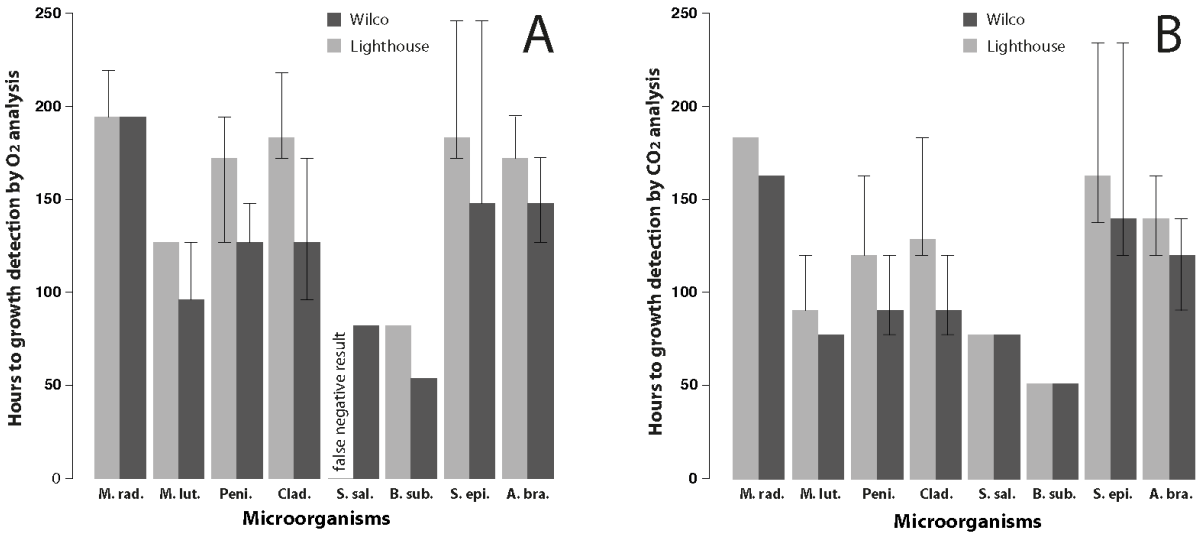

Figure 6: Comparison of detection times per microorganism obtained through measurements by the Wilco platform and the benchtop devices from LHI. It depicts clearly that devices developed for high-throughput inspection achieve even better results than laboratory equipment. 6A shows the results of O_2 measurements and 6B the results of CO_2 measurements. The end of each bar is attributable to the median of the detection time resulting from the analysis of 10 samples per microorganism inoculated with <10 colony forming units. The error bars define the maximal and minimal value for the detection time of microbial growth. (Please refere to fig. 4 or the materials and methods chapter to see the full microorganism names assignable to the abbreviations used in this figure).

Technical and methodological feasibility of automating the media fill inspection could be shown within this work. During media fills diverse process interventions are performed which need to be assignable to individual trays (a collection of multiple vials) filled during the intervention of concern. This is important as in the case of microbial growth detection a connection needs to be established between intervention and tray containing the contaminated unit. Such a connection allows finding and eliminating the source of filling line contamination. However, the actual design of the automated platform hardly allows separating one tray from the other. By letting trays pass the device individually the very last several vials of each tray remain in the transport channel (fig. 1, e) once left the rotary plate. Additional vials from the subsequent tray would need to undergo the measurement and transportation procedure pushing then the remaining vials in the exit for sterile units (fig.1 f). This makes a tray by tray separation challenging. The problem can easily be solved by tagging each vial with a specific barcode allocating it to the tray units or by customizing a baton, allowing operators to empty the transport channel manually (manual tray separation).
Besides this technical challenge the analysis of smaller formats (e.g. 2ml vials being filled with 1 ml TSB) and intentionally breached samples could additionally be performed as sealed 20ml vials are the most convenient format to be tested. In addition the technique provides a base for discussions in simplifying the inspection and incubation procedure.

It was shown in this work that worst case organisms defined in previous studies were reliably detected within ~7 days. Therefore, a single inspection after e.g. 8 days might be reasonable, but of course, demanding additional investigation in form of several experiments. However, if realized further monetary benefit can be expected for media fill processes applying two visual inspections as the second one would be eliminated.

Based on former studies [9], an alternative incubation approach using incubation temperatures of 28°C during the entire 14 days could be of further investigational interest influencing (shortening or prolonging) times to detection of bacteria and molds/fungi accordingly. By such temperature normalization a steadier baseline development could be expected as the transfer to 30-35°C is eliminated which normally slightly worsens CO_2 and O_2 headspace variation (caused through oxidation and degassing effects). If such a process adaptation was targeted more investigational effort would be required.

An easy, fast and safe electronic documentation of individual tray units or concerned vials attributed to process interventions must be allowed by the platform to fulfill efficiency expectations and requirements existing in a GMP environment. Therefore the impact of the documentation procedure on the overall inspection duration is subject to further clarifications involving exchanges of experience with subject matter experts. Depending on how the automated documentation approach is solved and how many media fills are produced per year a company could face acceptable savings after acquisition and implementation of a media fill inspection platform. Normally operators inspect ~6 media fill vials per minute during the visual inspection on turbidity. Considering that e.g. 16 batches are filled per year, one batch contains 15’000 units, and inspections are done after 7 and 14 days, operators would invest yearly ~1’300 hours in inspecting media fills. Using the automated platform would allow a throughput speed of 100 vials / minute (higher throughput speed is not recommended with this set up as pressure on vials might increase risk for breakage [6]). However, if higher throughput rates are requested machineries exist with a different vial handling system enabling such intention) and reduce the yearly inspection time to ~80 hours (6.2% of the time needed for the visual inspection). Such fact allows a strong efficiency increase in terms of visual media fill inspection and would shorten reaction times in case of a sterility breach at the filling line. However, such benefit can only work if documentation does not generate extra workload and is easily performed besides the automated inspection in a convenient, safe and user-friendly way.

Overall the automated platform is an excellent tool enabling an automated and secure media fill inspection.

Although some minor challenges have to be overcome for the inspection platform investigated here, such a platform can release employees from a rather repetitive task and allow manufacturing sites to save costs in case of method implementation.

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CHAPTER 5

An Alternative Sterility Assessment for Parenteral Drug Products Using Isothermal Microcalorimetry

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ORIGINAL ARTICLE

An alternative sterility assessment for parenteral drug products using isothermal microcalorimetry

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bacteria, contamination, drug safety, isothermal microcalorimetry, public health.

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Abstract

Aims: Production and release of injectable drug solutions are highly regulated since the administration of injectables bypasses natural body barriers. The sterility test is the last opportunity of product quality assessment. However, sterility is currently assessed by visual inspection (VI) that is time consuming and somewhat subjective. Therefore, we assessed isothermal microcalorimetry (IMC) as a replacement for the VI of the filtration based state-of-the-art sterility control.

Methods and Results: We used ATCC strains and house isolates to artificially contaminate frequently produced monoclonal antibodies (Avastin, Mabthera, Herceptin). After filtration, growth was assessed with IMC. Growth of all micro-organisms was reliably and reproducibly detected 4 days after inoculation, which was significantly faster than with VI.

Conclusions: The reliability and the sensitivity of IMC have a large potential to improve sterility controls. Further evaluation of this alternative method is therefore highly recommended.

Significance and Impact of the Study: Drug safety is of great concern for public health. Faster and safer drug production could be achieved using the technique described here. All the tests were performed with real manufactured drugs and complied with pharmaceutical standards. This suggests that drug sterility testing can be improved with potentially increased safety and cost reduction.

Introduction

Public health is dealing primarily with the protection of people's health, including the promotion of healthcare quality and accessibility and the limitation of healthcare disparities. Considering healthcare quality, fostering safe environments and developing sound health policies remain public health systems' key responsibilities (Centre of Disease Control 2016). That is why drug producers are required to strictly comply with regulations and guidelines targeting the highest possible level of drug product quality (Wilder and Kerrigan 2012). The design and control of manufacturing processes according to quality-compliant specifications is therefore crucial. Indeed, the execution of product-related quality assessments during the production process by analytical methods is important to achieve these requirements (Rathore and Winkle 2009). Injectable drugs have the highest quality requirements (including sterility of the solution) as parenteral applications bypass natural body barriers and could severely affect patient health in case of microbial contamination. Despite strict adherence to guidelines by producers, several drug recalls (approximately 430 out of which ~200 were related to injectables) occurred within the last 3 years (Fig. 1). A major reason was unmet specifications in the sterility assurance processes (Drug Office Hong Kong 2016; European Pharmaceutical

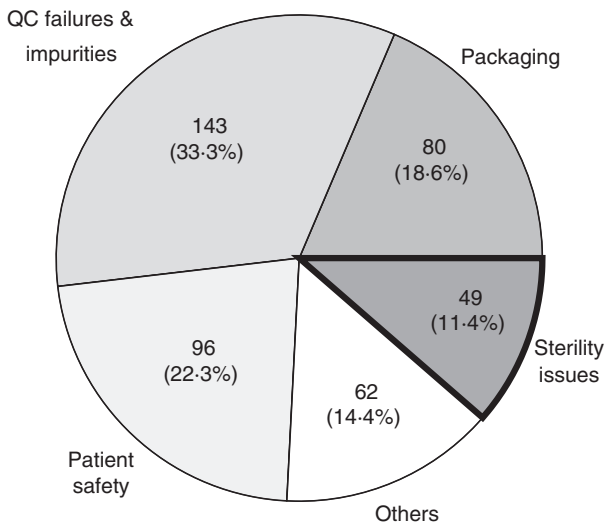

Figure 1 Approximation of the absolute recall number from January 2014 to October 2016 for US, JP, UK, CH (and few other countries) assigned to five categories. Various drug formulations (parenteral, oral, topic, etc.) were included in the study. However, the majority of the 'Sterility issues' section is assignable to injections and related to unmet specifications in sterility assurance processes such as identified micro-organisms in products, deviations from environmental monitoring, failed sterility tests and failed media fills. 'Packaging' refers to mistakes on leaflet, packaging damages, stopper issues, wrong labelling and scratches. 'QC failures and impurities' include particles (glass, rubber, etc.) in the product, cross-contamination with drugs, stability problems, precipitation and crystallization. 'Patient safety' is linked with falsified drugs, wrong dosage, unexpected adverse reactions, general health hazards and treatment failure, whereas 'Others' cover issues in clinical studies, market authorization, licensing and indication change.

Reviews 2016; Food and Drug Administration 2016; Medicines and Healthcare Products Regulatory Agency 2016; Swissmedic 2016; World Health Organization 2016) and the sample selection within a batch. Manufacturers can attempt to improve the sample selection by taking samples from the 'near start', 'middle' and 'near end' of a batch as contamination is unlikely to be evenly distributed (Sandle 2012). In addition an inclusion of samples close to any production process intervention performed is recommended. Still the probability remains low to find single contaminated units in one entire lot (Sandle 2012). Sterility tests are the last opportunity for product quality assessment. Sterility tests are, however, limited in their ability to detect microbial contamination in large production batches (up to 100 000 units) due to small sampling sizes (20–30 commercial units) (Food and Drug Administration 2004). Such tests include filtration of the sample through cellulose membranes under sterile conditions. Filters are then incubated during 14 days in tryptic soy broth (TSB) at 20–25°C and fluid Thioglycollate media (FTM) at 30–35°C enabling growth of aerobe and anaerobe micro-organisms. A final visual inspection (VI) on media turbidity assesses the cumulative sample sterility and allows approval for batch release (Food and Drug Administration 2009; Japanese Pharmacopoeia 2016; Ph Eur 8.0th edition 2014; US Pharmacopoeia-National Formulary 2016). Because of its low sensitivity (small sample size), positive results are not expected but, if occurring, are considered as a serious GMP issue that must be thoroughly investigated (Food and Drug Administration 2004). Therefore, use of quantitative methods detecting microbial growth faster than by VI on media turbidity (the human eye needs 10^7 cells per ml to detect a biomass increase in liquid medium (Moldenhauer and Sutton 2004)) would be advantageous. Reduced incubation duration (<14 days through a higher detection speed of microbial growth) decreasing product holding times until market release (direct monetary savings), higher growth detection objectivity, less inspection duration, shortened reaction time for GMP investigations as well as an optimized release duration of high-quality drug products delivered to patients could be achieved.

Different rapid microbial methods (RMM) such as, for example, Milliflex® Rapid Microbiology Detection and Enumeration System, BacT/ALERT® 3D, BD BACTEC™, Celsis® Advance II™, ScanRDI®, Growth Direct™, etc. are available. Growth detection of the RMM are based on electro-optical, spectroscopic or biochemical reaction analysis which all have advantages and drawbacks (Thorpe *et al.* 1990; Chollet *et al.* 2008; London *et al.* 2010; Parveen *et al.* 2011; Jimenez *et al.* 2012; Celsis 2017), and are often invasive by nature (Sandle 2016; rapidmicromethods 2017). Isothermal microcalorimetry (IMC) is a method capable to detect heat produced by biochemically active cells such as growing bacteria or fungi (Tan *et al.* 2012; Braissant *et al.* 2013). In theory, with the most sensitive instrument available, already 20 nW (10^4 cells)—200 nW (10^5 cells) are sufficient to allow noninvasive detection of microbial activity by IMC (Braissant *et al.* 2010). However, one needs to be aware that these value are only theoretical and that they vary a lot depending on micro-organism type, metabolic state (i.e. actively growing or dormant) and metabolisms type (i.e. respiration, fermentation, nitrate or sulphate reduction) (Braissant *et al.* 2010; Maskow *et al.* 2012). In this work, we will evaluate IMC as a possible alternative for the above described sterility control and highlight its advantages, drawbacks and potential impact on existing procedures.

Materials and methods

The experimental set-up targeted close simulation of the prevailing conditions during the sterility test of liquid drug products. For this, we used three commercial parenteral drug products (the monoclonal antibodies

Avastin, Herceptin and Mabthera) easily available from the production site. All other material used was purchased from Merck Millipore (Schaffhausen, Switzerland). For practical reasons (to avoid disturbances in the daily operations) product filtration was done by a multistation filtration device under laminar flow (instead of a certified and qualified isolator). Filtration units with installed filters (Microfil V, pore size 0·45 μm, 47-mm diameter) were filled with 10 ml fluid A (0·1% peptone, pH 7) and 2·0–6·0 ml of drug product were added. The entire solution was inoculated with 0·1 ml of micro-organism suspension (<10 colony forming units per 0·1 ml) imitating biologic product contamination. The inoculated solution was filtered by applying vacuum (EZ-Stream Pump; Merck Millipore) to the filtration unit. Subsequently, the filters were washed twice with 20 ml fluid A to get rid of excessive product protein and conservatives. Using sterile forceps, filters were folded and carefully introduced into sterile 4-ml glass vials fitting the measurement channels of an isothermal microcalorimeter (Tam48; TA instruments, New Castle, DE, USA). After having filled all glass vials with 3 ml of liquid media (either TSB or FTM (fluid thioglycollate)), they were sealed and introduced into the calorimeter for heat flow analysis following the two step thermal equilibration procedure recommended by the manufacturer. Anaerobe experiments were started as soon as the change in colour of the FTM medium was detected.

For each combination of medium—organism—filtered product, 5–10 replicates were prepared and investigated. Aerobic micro-organisms included *Candida albicans* (ATCC 10231), *Staphylococcus aureus* (ATCC 6538), *Penicillium* sp. (site isolate), *Bacillus subtilis* (ATCC 6633), *Aspergillus brasiliensis* (ATCC 16404) and *Pseudomonas aeruginosa* (ATCC 9027) and were incubated in the Tam48 at 25 ± 0·1°C in TSB. *Clostridium sporogenes* (ATCC 19404) was selected as model organism for anaerobe studies and cultured in FTM at 32·5 ± 0·1°C in the Tam48 calorimeter (for details about creation of microbial suspensions see (Brueckner *et al.* 2017)). The rationale for selecting the micro-organisms intended to include one house isolate with high growth variability (and long lag phase) and to cover the pharmacopoeia strains in use excluding fast growing species.

In addition, noninoculated samples (eight per product and media) were tested during ~100 h and went through the same preparation process as described above. This allowed creating a reference threshold for each medium. The threshold was defined by the fitted upper six sigma confidence interval of the respective sample collection (Fig. 2) as a high level threshold was targeted (decrease in false positive number). Since calorimetric baseline data are not flat by nature due to the insertion of the sample, producing a thermal perturbation, the baseline fitting was done using the R statistical package assuming a second-order exponential decay (allowed an approximation of such asymptotic curves). Growth detection was considered positive once the heat flow curve from inoculated samples crossed this threshold. Visual detection times for *C. sporogenes* were determined by the read-out of 38 individually inoculated (<10 organisms) FTM samples. The remaining visual data was taken from previous internal measurements using the same procedures. The organism-specific visual detection point was reached once all inoculated samples became turbid. In both cases the VI was performed by a qualified human operator (inspection results might vary among

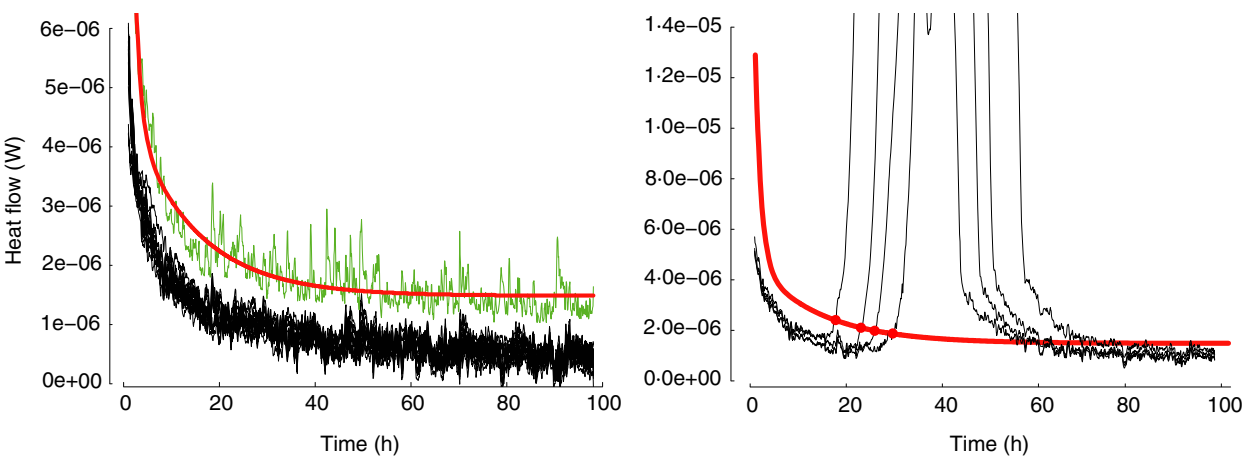

Figure 2 The left figure shows the upper six sigma confidence interval and its fit (red curve) based on the function of second order exponential decay. The fitted curve defines the growth threshold (noise from blank samples). The right figure describes the concept of determining the time point of growth, based on the intersection of growth curve and threshold (using *Pseudomonas aeruginosa*).

different human operators). The difference between the two detection methods (visual detection and IMC detection) was assessed using the Wilcox Test as values were non-normally distributed according to the Shapiro–Wilk test. The influence of the filtered drug product on microbial growth was analysed by the Kruskal–Wallis test. In addition, collected heat flow curves were integrated and fitted using the Gompertz growth model (Braissant *et al.* 2013, 2015). Thereby, additional descriptive parameters such as total heat, growth rate and lag phase duration could be extracted (Table 1).

Results

Several blank TSB and FTM samples were tested on their heat emission which determined the thresholds (a fitted six sigma confidence interval) for bacterial growth detection (Fig. 2). Due to equilibration processes between samples and measurement channels the thresholds showed a decline within the first 20 h and equilibrated at approximately $1{\cdot}57 \times 10^{-06}$ W (TSB) and $4{\cdot}73 \times 10^{-06}$ W (FTM). Detection times for IMC were extracted once the defined threshold was crossed by the growth curves and then compared to the detection time of VI.

All inoculated samples were detected by VI and IMC within 100 h. IMC identified microbial growth in 92% of the prepared filter samples ($n = 138$) earlier than VI. In addition, IMC was significantly faster ($P < 0{\cdot}05$) for six of the seven microbes when compared to VI (Fig. 3). In comparison, VI detected growth in few samples inoculated with *Penicillium* sp. (35%) and *C. sporogenes* (11%) sooner than IMC.

Highest fluctuations in total heat and lag phase were observed for *Penicillium* sp. and *A. brasiliensis*. Their maximal emitted heat (44·82 J and 24·95 J) reached high values. It is likely that the formation of biofilms on the incubated filter was responsible for the higher heat production rates (Donlan and Costerton 2002; Astasov-Frauenhoffer *et al.* 2012). The remaining organism samples showed uniform growth curves, whereas *B. subtilis* and *P. aeruginosa* grew with highest reproducibility. A significant ($P < 0{\cdot}05$—sample size in Table 1) influence of the filtered product on the time to detection (TtD) of growing micro-organisms was observed. Nevertheless, distributions of TtD values for the tested organisms were rather small (Fig. 3). Although statistically significant, such differences remain negligible for practical use.

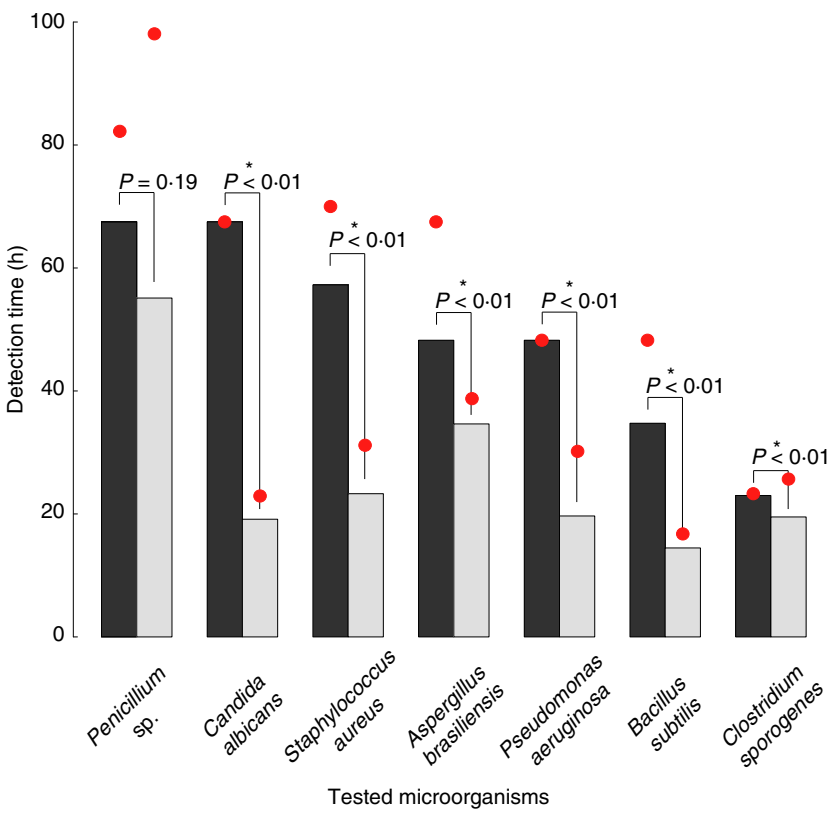

Figure 3 Comparison of detection times for IMC (light grey) and VI on turbidity (dark grey). Bar height represents the median of all detection points per organism. Dots represent maximal values of the respective sample collection. The samples size for IMC data was between 15 and 38, for VI between 9 and 38. Stars mark if the two groups (detection with VI or IMC) are significantly different ($P < 0{\cdot}05$; Wilcox test) for one micro-organism. The visual inspection was performed by a single human qualified operator.

Table 1 Extracted parameters from the Gompertz fitting analysis. Total heat, growth rate and lag phase duration are listed by their median. In brackets the min and max values are shown. Number of samples used per product is entitled as *n* and referred to Avastin (A), Mabthera (M) or Herceptin (H)

	Total heat (J)	Growth rate (h^{-1})	lag phase (h)	*n*
Bacillus subtilis (ATCC 6633)	8·88 (7·44–9·95)	0·34 (0·27–0·45)	20·47 (16·73–25·45)	5 (A), 5 (M), 5 (H)
Staphylococcus aureus (ATCC 6538)	9·14 (5·48–10·68)	0·18 (0·09–0·37)	27·72 (15·41–49·77)	10 (A), 5 (M), 5 (H)
Pseudomonas aeruginosa (ATCC 9027)	4·77 (3·99–5·76)	0·22 (0·08–0·37)	24·52 (12·00–44·01)	5 (A), 5 (M), 5 (H)
Penicillium sp. (site isolate)	4·63 (3·50–44·82)	0·04 (0·01–0·22)	115·29 (66·47–249·95)	5 (A), 10 (M), 5 (H)
Candida albicans (ATCC 10231)	6·00 (4·81–7·79)	0·12 (0·04–0·20)	21·82 (0–33·74)	5 (A), 5 (M), 5 (H)
Aspergillus brasiliensis (ATCC 16404)	5·34 (4·79–24·95)	0·07 (0·05–0·24)	74·66 (45·05–114·90)	5 (A), 5 (M), 5 (H)
Clostridium sporogenes (ATCC 19404)	16·84 (11·57–19·97)	0·70 (0·13–0·95)	28·78 (20·41–43·49)	13 (A), 15 (M), 10 (H)

Discussion

In this publication, the potential of IMC as a substitution for the VI of the sterility test for parenteral drug products (filtration, incubation and VI on turbidity) was assessed. IMC detected microbial growth in all inoculated samples and in a large majority of them (92%), microbial growth was detected significantly faster than with VI (Fig. 3).

A previous study (Tan *et al.* 2012) has suggested that IMC could be used for replacing the sterility assessment. However, this study used conditions that were neither relevant for sterility testing nor compliant with the respective guidelines (Food and Drug Administration 2009; Ph Eur 8.0th edition 2014; US Pharmacopoeia-National Formulary 2016). In addition, only few micro-organisms were investigated and no mention of replication was stated, rendering the results of this study doubtful. In the current study, growth profiles showed a high degree of reproducibility and reliability. This advocates for further assessment of IMC using additional micro-organisms (e.g. slow growers) and allowing the investigation of the applicability of this method in practice.

The real-time applicability of this technology is advantageous compared to VI, supporting the use of IMC in a GMP environment. As a consequence, the subjective visual analysis of sterility could be replaced by an electronically documented, objective assessment of microbial growth over time. Importantly, implementation of IMC in practice would require only minor changes in the existing procedures, since sample preparation and handling processes during sterility assessments would remain unchanged. Method validation would have to focus only on replacing visual growth detection by heat flow analysis because IMC measurements and VI are both noninvasive.

Interestingly, one sample of *A. brasiliensis* and *Penicillium* sp. each produced rather high and steady energy emissions of 24·95 J/44·82 J during the measurement period, being most probably related to biofilm formation (Said *et al.* 2014, 2015). Therefore, IMC could be of additional use in finding efficient ways to detect the presence of biofilm-forming micro-organisms in, for example, water pipes or air cooling systems of pharmaceutical production facilities—especially as biofilms might be tolerant to some of the applied sanitizing procedures.

Importantly, in the current study we used smaller volumes than in conventional sterility tests. This might have slightly influenced the time of visual growth detection, as initial turbidity is harder to see in smaller formats. During subsequent validation studies this is an aspect to be considered.

The analytical capacity of the instrument could easily be adapted, depending on the needs of different drug manufacturers, as commercially available instruments have a sample capacity ranging from 1 up to 48 measuring slots (Braissant *et al.* 2010, 2015). It must be noted, however, that some isothermal calorimeters used in research were built with up to 100 channels (Wadsö 2002). Such systems could additionally be used for other mandatory microbiological assessments, for example, the determination of the degree of water contamination (Maskow *et al.* 2012).

Our findings illustrate that IMC is a methodology with a high potential for replacing the VI of sterile solutions (for parenteral and potentially also other drug products). The main advantages of this method are not only its noninvasive nature and user friendliness but also its reliability and sensitivity, allowing earlier microbial growth detection than with VI. This could allow shorter product holding times before release, resulting in monetary savings that may reduce drug prices. IMC should not only be compared with VI, however, but also with other methods currently in use or planned for future applications such as 'Growth Direct' (Rapid Micro Biosystems 2011) which is also used for bio burden testing. Such comparisons could provide further insights on the performance of IMC and would therefore be valuable.

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Conflict of Interest

D.B. and U.Z. are currently employees of F. Hoffmann—La Roche AG. O.B., S.K. and G.B. have no conflict of interest to be declared.

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CHAPTER 6

A Combined Application of Tunable Diode Laser Absorption Spectroscopy and Isothermal Microcalorimetry for Calorespirometric Analysis

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A combined application of tunable diode laser absorption spectroscopy and isothermal micro-calorimetry for calorespirometric analysis

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ABSTRACT

Calorespirometry is the simultaneous analysis of the rate of heat emission (R_q), O_2 consumption (R_{O2}) and CO_2 production (R_{CO2}) by living systems such as tissues or organism cultures. The analysis provides useful knowledge about thermodynamic parameters relevant for e.g. biotechnology where parameter based yield maximization (fermentation) is relevant. The determination of metabolism related heat emission is easy and normally done by a calorimeter. However, measuring the amount of consumed O_2 and produced CO_2 can be more challenging, as additional preparation or instrumentation might be needed. Therefore, tunable diode laser absorption spectroscopy (TDLAS) was investigated as an alternative approach for respirometric analysis in order to facilitate the data collection procedure. The method determines by a spectroscopic laser non-invasively CO_2 and O_2 gas concentration changes in the respective vial headspaces. The gathered growth data from *Pseudomonas aeruginosa* cultured in two different scarce media was used to compute respiratory quotient (RQ) and calorespirometric ratios (CR_{CO2} [R_q/R_{CO2}], CR_{O2} [R_q/R_{O2}]). A comparison of the computed (experimental) values (for RQ, CR_{CO2} and CR_{O2}) with values reported in the literature confirmed the appropriateness of TDLAS in calorespirometric studies. Thus, it could be demonstrated that TDLAS is a well-performing and convenient way to evaluate non-invasively respiratory rates during calorespirometric studies. Therefore, the technique is definitively worth to be investigated further for its potential use in research and in diverse productive environments.

1. Introduction

Calorespirometry is the simultaneous analysis of the rates of heat emission (R_q), O_2 consumption (R_{O2}) and CO_2 production (R_{CO2}) by living systems (single cells, tissues, or organism cultures from culture or environmental samples). Calorespirometry provides useful information about efficiency in metabolic turnover and potentially on corresponding metabolic pathways (aerobe or anaerobe). Especially in biotechnology where yield maximization of biomass or alternatively of a certain product is of interest, calorespirometry helps to understand system thermodynamics, enabling an optimization of culturing conditions (Hansen et al., 2004; Schuler and Marison, 2012; Schuler et al., 2012). R_q is normally determined using an isothermal heat conduction or power compensation calorimeter (Wadsö and Hansen, 2015). A thermoelectric module located between the sample and the calorimeter heat sink measures the emitted heat and transforms it into an electronic signal. An accurate determination of R_{CO2} (and R_{O2}) is more difficult but has been described (Criddle et al., 1991; Criddle et al., 1990). The approach was based on the determination of the heat produced by the reaction of CO_2 with a 0.4 M NaOH solution (absorbent) contained in the reaction container as described by $CO_{2\,(g)} + 2OH^-_{(aq)} \rightarrow CO_3^{2-}{}_{(aq)} + H_2O_{(aq)}$ with $\Delta H_{abs} = -108.5\ kJ{\cdot}mol^{-1}$ (Wadsö and Hansen, 2015).

By subtracting heat rate values of samples lacking the absorbent from those including the absorbent, the amount of produced CO_2 can be determined and conclusions on metabolic efficiencies drawn. The described approach is used as a gold standard in works of calorespirometric analysis such as feeding control of biopolymer production, evaluations of soil organic matter decomposition, and metabolic assessments of microorganisms, plants, animals, fruits, and other living systems (Barros et al., 2015; Criddle et al., 1990; Hansen et al., 2002; Nogales et al., 2013; Regan et al., 2013; Rohde et al., 2016; Wadsö, 2002). The technique is limited by its invasive nature, leading to the absorption of the entire CO_2 in the system. The effect of CO_2 degassing in combination with the absorbent removes the entire CO_2 from the medium, generating a shift in the carbonate equilibrium ($HCO_3^- \rightarrow CO_2 + OH^-$). Hydroxide ions ($OH^-$) arise therefrom resulting in a pH increase potentially influencing the cell growth potential

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in weakly buffered systems. A more convenient approach to perform such experiments is the use of optrodes. Several calorespirometric studies used modified calorimeters with optrodes included in the measurement vessel to determine oxygen consumption. For example, moth metabolism or oxygen consumption by Maple tree leaf buds were studied using such modified instruments (Itoga and Hansen, 2009; Neven et al., 2014). Tunable diode laser absorption spectroscopy (TDLAS) is discussed in this work as an alternative approach capable to determine CO_2 production and O_2 consumption rates non-invasively and easily. The technology uses a laser to measure relative CO_2 (λ = 2000 nm) and O_2 (λ = 760 nm) concentrations in the sample headspace. Of major interest is the investigation of a combined use of IMC and TDLAS for calorespirometric studies based on one single model organism (*Pseudomonas aeruginosa*). Measurements were run in parallel and in different media (M9 and M9 + nitrate) to compare calorespirometric ratios under different metabolic conditions (respiration and denitrification [$NO_3^- \rightarrow NO_2^- \rightarrow NO + N_2O \rightarrow N_2$ (g)]). Biomass (cell count per ml medium, optical density, and protein concentration), substrate consumption (glucose concentration), gas concentrations and metabolic byproducts (nitrite concentration) were used to interpret the obtained respiratory quotient R_{CO2}/R_{O2} (RQ) and calorespirometric ratios R_q/R_{CO2} (CR_{CO2}) and R_q/R_{O2} (CR_{O2}). Those results were compared to values found in the literature to gain insight into reliability and applicability of TDLAS in the field of calorespirometry.

2. Materials and methods

Pseudomonas aeruginosa was chosen as a model organism for calorespirometric studies due to its aerobe and denitrifying metabolic characteristics. The microorganism was purchased from ThermoScientific™ (Quanti-Cult Plus, ATCC 9027, < 100 colony forming units/0.1 ml) and maintained at 37 °C in minimal salt medium (M9, Glucose concentration ~3.9 millimolar [mM]) enriched with 1% BME vitamins. In addition the same medium was supplemented with 100 mM nitrate (M9N) (Williams et al., 1978), which allowed a calorespirometric assessment of denitrification pathways.

Two sample sets (4 ml glass vials, TA Instruments) were prepared for the study filled with either 0.9 ml M9 (50 samples) or 0.9 ml M9N (80 samples). Samples were inoculated each with 0.1 ml media containing 1,000–2,000 CFUs (used microbial units were cultured in their respective media). Inoculated vials were capped under laminar flow and transferred into an isothermal micro-calorimeter or an incubator set at 37 °C. The M9 and M9N sample sets were further split in two groups for calorimetry and TDLAS measurements, made in parallel (i.e., 1 M9 + 1 M9N set for calorimetry and 1 M9 + 1 M9N set for TDLAS).

The metabolic heat emission rate was monitored by a 48 channel isothermal micro-calorimeter (TAM III, TA Instruments, Delaware, USA) set to an incubation temperature of 37 °C. Two sets of 10 inoculated vials and non-inoculated control samples (2 ×) filled with either M9 or M9N were tested. At the same time point, tunable diode laser absorption spectroscopy (TDLAS) analyzed the respiratory activity of growing bacteria by measuring O_2 (λ = 760 nm) and CO_2 (λ = 2000 nm) concentration changes in the headspaces of vials kept outside the calorimeter but within a thermostatic oven at the same temperature (i.e., 37 °C). Experiments were started with preheating the TDLAS analyzers (Lighthouse Instruments, Charlottesville) for minimally 30 min followed by a calibration with certified standards of known concentrations (0%, 4%, 8% and 20%). Flushing the TDLAS measurement channels with nitrogen (4 l/min) allowed a headspace analysis with improved signal to noise ratio. For both sample sets incubated in parallel in the oven (M9 and M9N) CO_2 and O_2 concentrations of 15 inoculated and 2 reference vials were tested three times every 2 to 3 h during ~100 h. Care was taken that the measurement time per sample was as short as possible to keep the sample temperature as close as possible to 37 °C. In addition, optical density (Bio Photometer plus, Eppendorf, λ = 595 nm) and CFU counts (per 0.1 ml media) were performed on tryptic soy agar (TSA) after each TDLAS measurement, with samples taken from an additionally prepared sample stock. To monitor nitrate reduction, glucose consumption, and protein aggregation, every 2 to 4 h one inoculated sample filled with M9N was taken from the sample stock and frozen at − 82 °C. At the end of the experiment the respective glucose content was determined for all thawed samples with an Accu-Chek Aviva diabetes device (detection range 0.6 mM–33.3 mM) from Roche Diagnostics. The device was calibrated with glucose standard dilutions of 1, 5, 10 and 25 mM prepared in M9 and M9N. Nitrite concentrations of the same samples were determined by colorimetric analysis using the Griess reagent (Sigma Aldrich). Assay calibration was performed with 0, 15, 40, 60 and 80 nmol/ml nitrite and absorption measured at a wavelength of 500 nm. Protein concentration changes in the medium were identified by the Bradford assay. Dependent on the protein concentration a respective colorimetric change occurred measurable at a wavelength of 586 nm. A standard curve for the assay was performed with 0, 20, 40, 100, 500 and 1000 µg/ml of bovine serum albumin (BSA) per assay.

The data analysis included integration of the measured heat flow data resulting in sigmoidal heat profiles. The respective medians of CO_2 production, O_2 consumption (inverse values) and heat were fitted using the Gompertz function in R ("grofit" package). Molar amounts of CO_2 and O_2 were then determined, respecting the size of the vial headspace (3.31 ml) and assuming that CO_2 and O_2 behaved as ideal gases. Using the ideal gas law, we estimated that in our condition (vials closed at 22.5 °C and 1.029 bar, followed by an increase of temperature to 37 °C) 1 mol of gas had a volume of 23.89 l. Calculations relating to the respiratory quotient (RQ: R_{CO2}/R_{O2}) and calorespirometric ratios for CO_2 (CR_{CO2}: R_q/R_{CO2} [kJ·mol^{-1}]) and O_2 (CR_{O2}: R_q/R_{O2} [kJ·mol^{-1}]) were performed for each data point between 0 and ~100 h by using the fitted values. Finally, standard deviations were determined for all relevant values.

3. Results

Pseudomonas aeruginosa cultured in M9 produced after ~100 h and in vials with a headspace volume of 3.31 ml, at the maximum (fitted data) 4.68 ± 0.08% CO_2 (6.49 ± 0.11 µmol), consumed 8.70 ± 0.22% O_2 (12.06 ± 0.31 µmol) and emitted 5.48 ± 0.19 J (Fig. 1A–B). Optical density (OD) and bacterial cell number (BCN/ml) reached their respective maxima after 47 h (absorption = 0.476) and 43 h ($2.1 \cdot 10^9$ cells) respectively (Fig. 1C–D, consistent with the development of the CO_2, O_2 and heat profiles. Growth of *P. aeruginosa* in nitrate supplemented M9 medium (M9N) was, in comparison, marginally lagged and reached after 100 h CO_2, O_2 and heat values of maximally 5.43 ± 0.07% (7.53 ± 0.10 µmol), 9.06 ± 0.22% (12.56 ± 0.31 µmol) and 6.38 ± 0.06 J (Fig. 1A–B) respectively. The related OD and BCN/ml values attained the top after 61 h (absorption = 0.295) and 69 h ($3.8 \cdot 10^9$ cells). A slight but steady drop in biomass after reaching the maximum was observed in both media (Fig. 1C–D).

A first decrease of the glucose concentration in the medium (~3.9 mM) could be observed after 17 h whereas after 26 h the carbon source was entirely depleted (Fig. 1E). The protein concentration started to increase after 17 h reaching a maximum amount of 59 µg/ml after 40 h incubation (Fig. 1E). Nitrite concentrations (resulting from denitrification) increased intensively after 20 h, reached a maximum of 201 nmol/ml within 26 h and equilibrated 20 h later at a level of approximately 19 nmol/ml (Fig. 1 F, dashed triangles). In addition, the main peak of the heat flow profile of samples containing M9N (Fig. 1 F brown curve) was observed at 16 h (65.2 ± 1.2 µW) whereas a second smaller peak was detected after 25 h (39.6 ± 0.9 µW). For growth in M9, two heat flow peaks were detected after 15 h (35.5 ± 5.6 µW) and 40 h (43.8 ± 1.7 µW) (Fig. 1F, orange curve). Flat heat flow profiles at baseline level (0 ± 0.2 µW) were observed for all tested references. Similarly, gas profiles remained flat varying very little around their

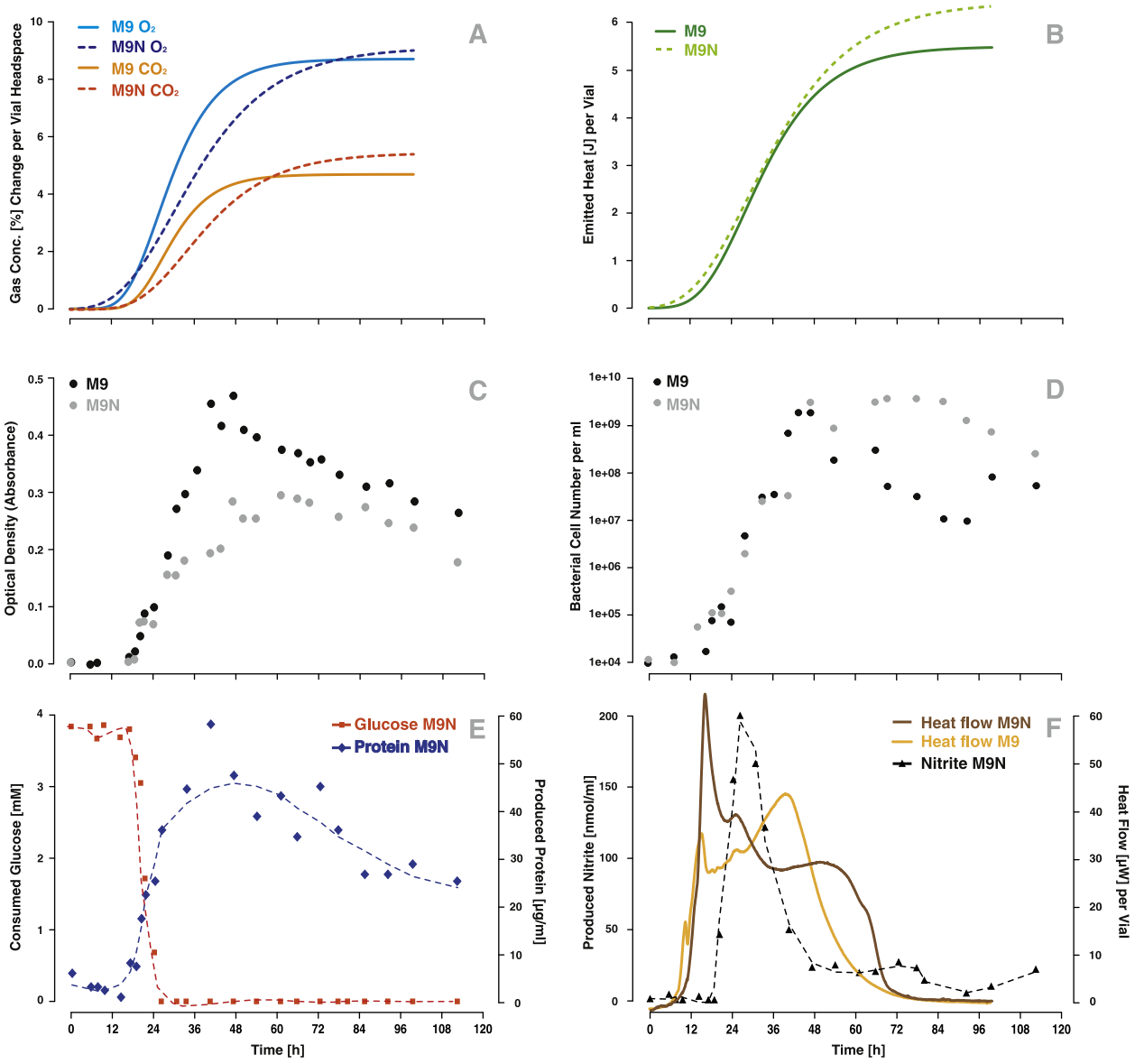

Fig. 1. Fitted profiles of O_2 consumption (R_{O2}), CO_2 production (R_{CO2}) and heat emission (R_q) in minimal salt (M9) and nitrite supplemented M9 medium (M9N) are illustrated in plot A and B. The curve development is based on the fitted median values of the respective measurements. Optical density (absorbance) and the number of counted cells per vial and media are shown in C and D. Determined concentrations of glucose (red squares [mM]) and protein (blue rhombs [µg/ml]) during the experiment are depicted in plot E. Plot F demonstrates the averaged heat flow profiles [µW] for each medium (orange for M9, brown for M9N) whereas the black triangles represent changes in nitrite concentration [nmol/ml]. Approximations for Glucose, Nitrite and Protein measurements were done by spline interpolation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

initial value for all tested references.

Based on the fitted CO_2 (R_{CO2}), O_2 (R_{O2}) and heat (R_q) data, respiratory quotient (RQ [R_{CO2}/R_{O2}]) and calorespirometric ratios (CR_{CO2} [R_q/R_{CO2}] and CR_{O2} [R_q/R_{O2}]) were calculated with respect to each medium (Fig. 2A–C). In M9 medium, RQ amounted to 0.54, CR_{CO2} to 845 kJ·mol^{-1} and CR_{O2} to 455 kJ·mol^{-1}. In M9N, values were slightly higher. RQ was equal to 0.60, CR_{CO2} reached 848 kJ·mol^{-1} and CR_{O2} equilibrated at 509 kJ·mol^{-1}.

4. Discussion

Calorespirometric studies deal with the simultaneous analysis of the rate of heat emission (R_q), CO_2 production (R_{CO2}) and O_2 consumption (R_{O2}) by living systems, enabling an association of observed effects with metabolic parameters. *Pseudomonas aeruginosa*, a facultative aerobe microorganism capable of using nitrate as terminal electron acceptor (denitrification), was used to evaluate whether TDLAS is of potential use in calorespirometric studies. A laser spectrometer measured CO_2 and O_2 concentration changes in vial headspaces, leaving the investigated biological system completely unaffected. Our results demonstrate that TDLAS is a well-performing and convenient way to evaluate O_2 consumption and CO_2 production rates non-invasively. This is an important advantage compared to invasive standard practices using CO_2 absorbing substances (NaOH), O_2 electrodes, or optrodes, that are less practical in such experimental settings and might require instrument modification (Itoga and Hansen, 2009; Neven et al., 2014). With respect to TDLAS reliability, parameters such as respiratory quotient (RQ) and calorespirometric ratios for O_2 (CR_{O2}) and CO_2 (CR_{CO2}) were calculated. They were mainly used to determine if TDLAS is a useful technique in calorespirometry by comparing them to literature values. Comparable results were indeed observed (Hansen et al., 2004; Kemp, 2000) but minor deviations were found (Table 1).

In this study, we chose to use two different media, allowing *Pseudomonas aeruginosa* to express different metabolic activities. Our

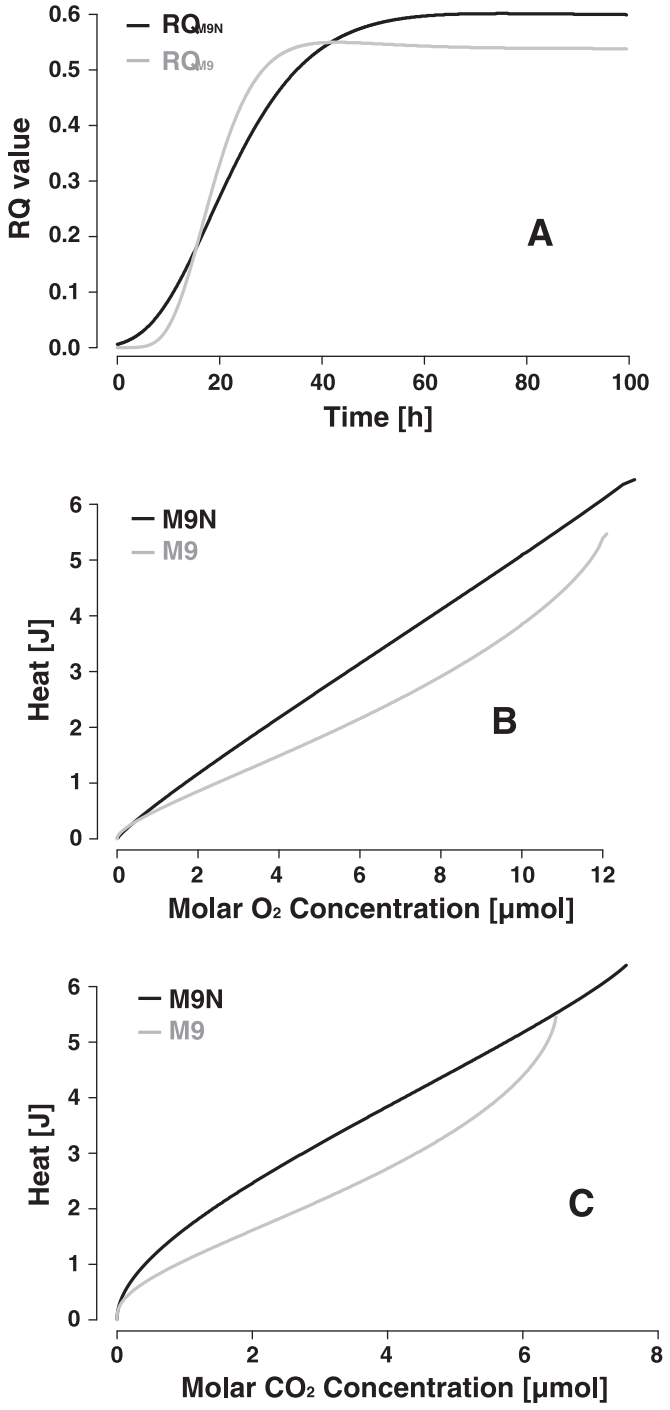

Fig. 2. The media dependent development of the respiratory quotient (RQ [R_{CO2}/R_{O2}]) is depicted in Plot A. Emitted heat per consumed O_2 and produced CO_2 is shown in plot B and C, being base for calculating the calorespirometric ratios CR_{O2} and CR_{CO2}.

Table 1
Calculated values of RQ, CR_{O2} and CR_{CO2} in M9 and M9N medium. Literature values used for comparison are listed at the table bottom (italic) (Hansen et al., 2004; Kemp, 2000).

	RQ	CR_{O2} (kJ·mol^{-1})	CR_{CO2} (kJ·mol^{-1})
Calculated values for:			
M9	0.54	455	845
M9N	0.60	509	848
Literature values:	(< or =) 1.00	450–480	0–600

objective was to determine whether TDLAS is able to detect subtle changes in metabolism (i.e., only respiration vs. respiration + denitrification). The calorespirometric growth study involved M9 and M9N (nitrate supplemented M9) media, whereby M9N was expected to lead to more growth and higher CO_2 production due to the presence of an additional terminal electron acceptor (Chen et al., 2003). Indeed, in M9N the maximal amount of growth related heat and CO_2 production surpassed the corresponding production rates in M9 medium (Fig. 1A–B). In contrast, the reached maximum of O_2 consumption remained comparable in both media as denitrifying pathways are of anoxic nature. Further evidence of active denitrification was provided by the measurement of nitrite concentrations (c_{max} = 201 nmol/ml after 26 h). The nitrite concentration-time profile correlated with the second heat flow peak of M9N samples whereas the subsequent nitrite decay suggested that nitrite was involved in further reduction to NO, N_2O and N_2. Here, an analysis of NO and N_2O headspace concentrations by a wavelength adapted TDLA spectrometer would have been interesting to evaluate denitrification efficiency but, was out of the study scope. The additional use of nitrate as terminal electron acceptor occurred after 20 h and was probably linked with the exhaustion of media dissolved O_2.

Further differences in medium could be seen when the respiratory quotient (RQ) and the calorespirometric ratios for O_2 (CR_{O2}) and CO_2 (CR_{CO2}) were calculated (Fig. 2, Table 1). In M9N RQ was slightly higher (0.60) compared to M9 (0.54), as the availability of another electron acceptor (i.e., NO_3^-) asides from O_2 may have caused a release of more CO_2. In theory, for pure glucose combustion ($C_6H_{12}O_6$ (s) + 6O_2 (g) → 6CO_2 (g) + 6H_2O (l) + heat, ΔH = − 2813.25 kJ·mol^{-1}) (Hammes and Hammes-Schiffer, 2007), literature refers to an RQ of 1.0 (Kemp, 2000) as same amounts of O_2 molecules are consumed as CO_2 molecules released. However, microbial growth leads to an uptake of carbon into biomass and thus lowers the CO_2 production, resulting in a decreased RQ value (RQ < 1.0). In addition, the RQ ratios for M9 and M9N differed only minimally (table 1) and roughly similar numbers of cells were found in the medium at the end of the culture (Fig. 1C–D). An additional protein assay at the end of the experiment (data not shown) confirmed a similar biomass as well. Thus, it is likely that the biomass formed is partly limited by the amount of glucose available in the medium, explaining the little differences. In both media glucose was entirely depleted after 26 h. Nevertheless, microbial growth still continued as illustrated by the ongoing rise in protein concentration, heat production, cell count, optical density as well as CO_2 and O_2 concentrations. Such growth continuation is explained by the microorganism's consumption of metabolic byproducts (such as low molecular weight organic acids, and/or polyhydroxybutyrate which is often produced for pseudomonads) as they are the only remaining nutrients in minimal media such as M9 and M9N (Ackermann et al., 1995; Singh Saharan et al., 2014).

With respect to the oxycaloric parameter (CR_{O2}, which can be defined as the theoretical value for the enthalpy change of the catabolic part of metabolism (Kemp, 2000)) for M9N samples, CR_{O2} was 53.82 kJ·mol^{-1} higher than the one obtained for M9 (Table 1), although comparable amounts of oxygen were consumed in both media. In M9N medium denitrification took place besides respiration. Therefore, the heat emission after ~100 h was slightly increased (by 0.91 J) and resulted in this higher CR_{O2} (R_q/R_{O2}) ratio. Comparable CR_{CO2} ratios (R_q/R_{CO2}) were observed for M9 and M9N (Table 1). The high and very similar CR_{CO2} values observed support the hypothesis that carbon was integrated into roughly the same amount of biomass.

In conclusion this study shows that TDLAS is a technique capable to fulfill the requirements needed for direct calorespirometric analysis. Therefore, additional TDLAS based evaluations in this field would be of interest as well as the construction of a TDLAS containing calorimeter prototype since TDLAS and microcalorimetry are complementary. The potential impact of the laser on heat measurements might be one of the biggest challenges that needs to be investigated when designing such a

prototype. In addition, our comparison of two closely related media (M9 and M9N) shows that a TDLAS based calorespirometric analysis might be beneficial for environmental research (e.g. soil analysis (Barros et al., 2015)) where many different electron acceptors such as sulfate are involved (Traore et al., 1981; Traore et al., 1982). Furthermore, in biotech, oil and food industries (Cocola, 2016; Muyzer and Stams, 2008; Regestein et al., 2013; Schuler et al., 2012), the non-invasive nature of TDLAS, as well as its efficiency and reliability are desired attributes for investigating metabolic activities, product durability or other processes happening in reaction vessels. Therefore, an ongoing investigation of TDLAS' role in calorespirometry is recommended. Still, previous studies have reported that the TDLAS O_2 measurement was less precise than the corresponding CO_2 measurement (Brueckner et al., 2017; Brueckner et al., 2016; Duncan et al., 2016) which might be an additional source of error, that should be further investigated. However, we believe that the technique remains of interest in calorespirometry. Thus, TDLAS allows a non-invasive, reliable and convenient determination of CO_2 and O_2 rates, with comparable results to conventional techniques.

Declaration of interest

The authors whose names are listed certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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CHAPTER 7

General Discussion & Outlook

7. General Discussion and Outlook

7.1 The Need for Process Innovation in a Changing Market Environment

Biologics allowed remarkable advances in the treatment of chronic diseases (such as cancer, psoriasis or rheumatoid arthritis) being linked with successes in terms of live prolongation and improvement of the overall quality of life. This promoted the demand for biologics and allowed manufacturers to charge high product prices. Perceived prospects of large profit generation caused a reorientation of corporate strategies towards the biopharmaceutical market segment (Figure A). Nowadays, biopharmaceuticals generate approximately $163 billion of global revenues being comparable to 20% of the entire pharmaceutical market (Otto et al. 2014). However, such favorable market conditions attract competition (Figure B). In 2006 Omnitrope from Sandoz, one of the first biosimilar of Genotropin (Somatotropin) was approved for market release (Evaluate Ltd. 2017) by the European Medicines Agency (Figure C).

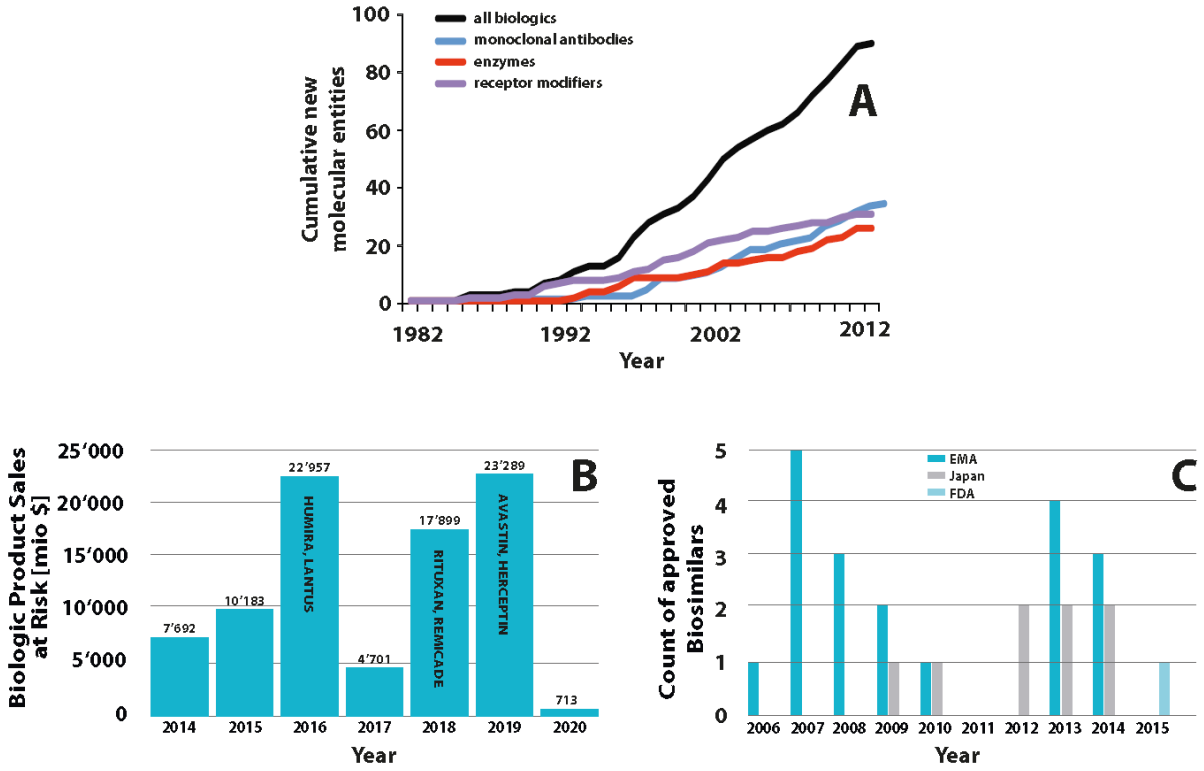


Figure 1: **Evolution of the biologics and biosimilar market.** Plot A describes the development of cumulative (black curve) and specific (violet, blue and red curve) new biological drug entities marketed between 1982 and 2012. However, biosimilars are expected to increasingly challenge branded biologicals in coming years. An estimated total of $87.4 billion of branded biological sales are at patent risk between 2014 and 2020 (B). The count of expected biosimilar approvals between 2006 and 2015 makes this obvious (C). (Evaluate Ltd. 2017, Kinch 2015)

Since then the situation became increasingly challenging for original biologic manufacturers as for a growing part of available biologics patent protection (normally 10-14 years from market approval) is expiring (Figure and Figure). Between 2014 and 2020 it is expected that globally up to ~$87.4 billion of revenues (Evaluate Ltd. 2017) coming from sales of biologics are endangered by the launch of biosimilars.

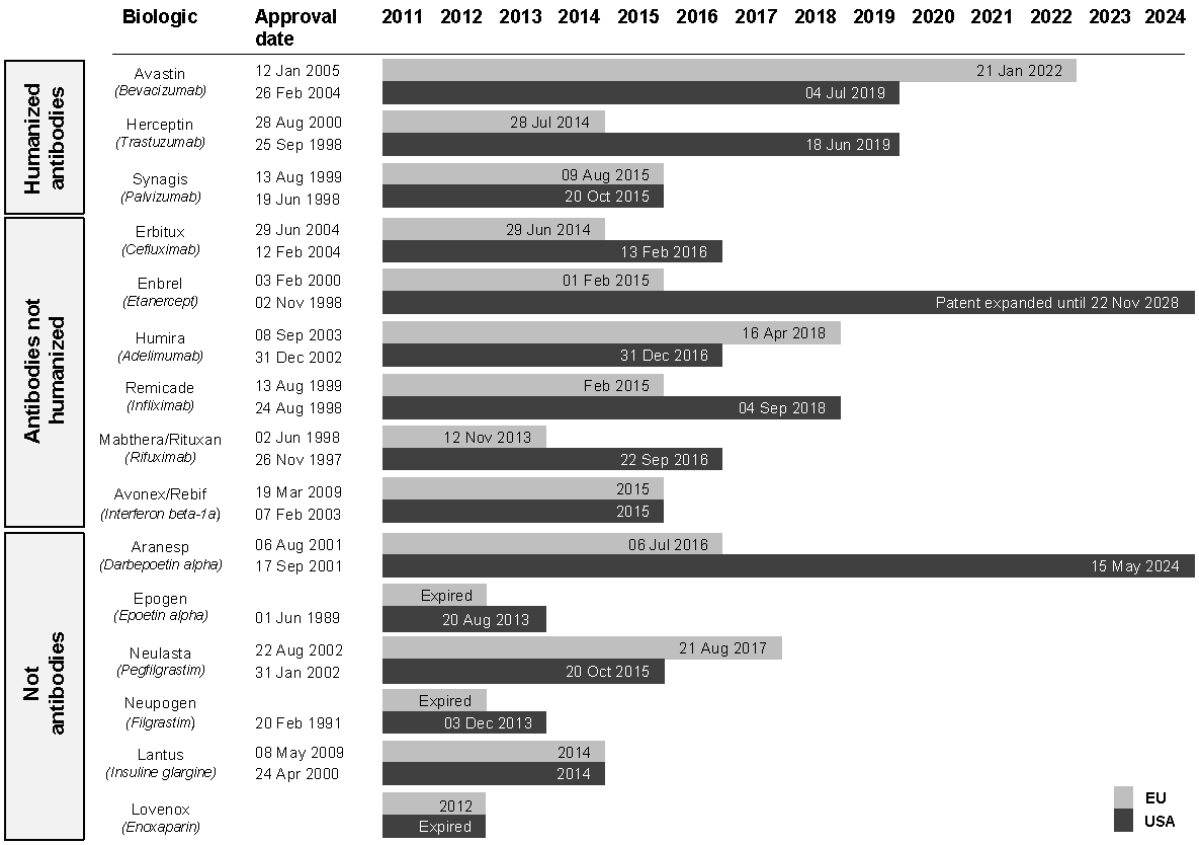


Figure 2: Patent approval and expiration dates for diverse biological products allocated to European Union and United States of America (GaBI 2011).

The overall pressure originating from patent expiration and biosimilar market intrusion redirects innovators to search for alternative strategies decreasing risks on their potential turnover. Thus, biologics manufacturers are forced increasingly to face equal challenges as other businesses, ensuring affordability, product quality, and delivery performance (Otto et al. 2014). Maximal process efficiency is thereby of major relevance being achieved by means of lean management as well as the use of innovative technologies (Chowdary et al. 2011, Gebauer et al. 2009). Therefore, the constant search, assessment, and implementation of technical and methodological innovations are important.

Aseptic manufacturing environments are strictly monitored on their microbial burden as already slightest product contaminations can endanger patients. Methodologies used nowadays for such control have not changed much since Robert Koch established microbiological techniques in 1881 (Miller 2010a). However, during the last years new technologies have appeared (Moldenhauer 2008, Miller 2016b, Thorpe et al. 1990, Jimenez 2001, Miller et al. 2009b, Miller et al. 2009a). Rapid microbial methods (RMM) allow the user to get test results for the analysis of microbial growth and presence faster as compared to traditional techniques (Figure). RMM techniques use principles of immunology, nucleic acid amplification, mass spectrometry, flow cytometry, as well as biochemistry.

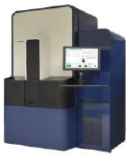
Product	Description of Principle
Milliflex® Rapid 	Detects ATP from a micro-colony based on a bioluminescence reaction before the colony becomes visible to unaided eyes.
BacT/ALERT® 3D 	Samples contain a sensor which respond to the concentration of CO_2 generated from metabolic activity of live microorganisms. The sensor is monitored by the instruments every ten minutes for color change.
Growth Direct™ 	The system automatically detects natural auto-fluorescence from living microorganisms using advanced digital imaging technology (can cut off the two-tiered incubation to 7 days).
Celsis Advance II™ 	Utilizes a combination of amplified-ATP (adenosine triphosphate) and advanced bioluminescence allowing the system to detect live microorganisms in a sample containing as low as 100 cells per milliliter.
Scan RDI® 	The system is utilizing solid-phase cytometry technology and can perform real-time microbial detection and enumeration.

Figure 3: Examples of rapid microbial methods that exist in the market using different principles to detect microbial presence and growth (Merck 2017, Chollet et al. 2008, Biomérieux 2017a, Rapid micro biosystems 2016, Celsis 2017, Biomérieux 2017c)

The demand for RMMs is expected to rise in the future from an estimated turnover of ~3.4 bn $ in 2015 up to ~6.2 bn$ in 2022, showing that the shift from conventional microbiological methods towards alternative ones is occurring (Credence Research 2016). Diverse documentation is depicting how to go for a structured and secure RMM evaluation allowing that such techniques can be successfully implemented at the site of interest (Kielpinski et al. 2005, Miller 2010b, Jasson et al. 2010, Parenteral Drug Association 2013, European

Pharmacopoeia 2009, U.S. Pharmacopoeia 2014). This obviously illustrates that despite high regulatory requirements for quality related process adaptations, willingness to improve exists, outgoing from manufacturers but also from regulatory agencies. Therefore, the time is right to move forward in this field and find more efficient ways to perform microbiology related quality control mechanisms without compromising drug and patient safety.

Therefore, tunable diode laser absorption spectroscopy (TDLAS) and isothermal microcalorimetry (IMC) were assessed on their innovation potential for mechanisms in quality control applied to aseptic manufacturing procedures. Both technologies showed high potential in enabling a leaner design of analytical processes related to microbial growth detection, being congruent with the nowadays discussed principles of RMM implementation and lean manufacturing.

7.2 The Role of TDLAS in Aseptic Manufacturing

Based on our research TDLAS was identified as well-suited for automating the visual media fill inspection. Time to detection comparability among TDLAS and conventional visual method was shown for diverse microorganism-format combinations (Brueckner et al. 2017, Brueckner et al. 2016). However, a convenient, fast and automated documentation is still needed and of major interest. Indeed, all containers in a media fill need to be identified and linked to a specific process-dependent intervention. In turn, this allows to rapidly spotting the breach in the sterility of the filling line which is highly relevant for evaluating a media fill, yet makes an automated documentation more complex. An individual vial tagging with 2D barcodes or RFID chips containing the relevant information (batch, tray unit and process intervention number) would probably fit best to allow such container specific allocation. Alternatively, a manual scanning of the tray barcodes being allocated digitally to the collection of tested tray units could be another option to automate the documentation. Considering GMP conformity (Food and Drug Administration 2016a) and pharmaceutical guideline documents, an automated documentation is linked to certain complexity. It therefore demands end-users to plan and specify the documentation concept in detail, being entirely aligned with the performed media fill procedure, before automation is achievable. This has certainly implications on project costs but is necessary before the transition to a well-working automated media fill inspection can be completed. Though, the potential of TDLAS is not constrained to media fill inspection automation reasoning the evaluation of its use in innovating calorespirometric assessments.

Calorespirometry is the combined study of heat, CO_2 and O_2 of metabolically active cells (Criddle et al. 1990). TDLAS can assist by an entirely non-invasive investigation of a living system being of potential benefit for e.g. biotech production process control (i.e. control of fermentation procedures) or environmental research (Barros et al. 2015, Rohde et al. 2016a, Schuler et al. 2012). However, when talking about the potential of TDLAS for microbiologic inquiries it is important to understand that not all metabolisms are based on the principles of O_2 consumption and CO_2 production. For example, most methanogens produce CH_4 and simultaneously consume CO_2, potentially leading to misinterpreted CO_2 data ($CO_2 + 4\ H_2 \rightarrow CH_4 + 2H_2O$) or even to non-detectability of microbial growth (Thauer et al. 1993). In addition, metabolisms of exotic fermenters or chemolithotroph species such as e.g. sulfate reducing bacteria (Table 1) can be independent from O_2 and/or CO_2 (Beijerinck 1895, Madigan 2015).

Of course exotic metabolisms are for clean rooms of minor relevance but can be encountered by environmental researchers willing to use the techniques applied here. In such case a combined analysis of TDLAS and IMC is highly recommended as IMC measures metabolic activities in a more generic way, as most of the active metabolism types emit heat. Still, life based on entropy has been shown and theorized (Liu et al. 2001, von Stockar et al. 1999). To approach unambiguous data collection further, TDLAS could be adapted in its wavelength to assess, besides CO_2 and O_2, additional gas types, such as e.g. CH_4 or N_2O/NO (from methanogens or denitrifying microorganisms) accumulating in the sample headspaces (Lackner 2007). Such data could be used as complementary parameter(s) to study and detect growth of exotic cultures.

Table 1: Description of CO_2 and O_2 independent metabolisms. Listed are metabolisms for sulfate reduction and exotic fermentation. All listed metabolism types are of minor relevance for cleanrooms but can be encountered by for example environmental researchers (Beijerinck 1895, Madigan 2015).

Metabolism type	Reaction
Sulfate reduction	SO_4^{2-} + acetic acid + 2 H^+ → HS^- + 2bicarbonate + 3 H^+
Acetylene fermentation	$2C_2H_2 + 3H_2O$ → ethanol + $acetate^-$ + H^+
Glycerol fermentation	$4Glycerol + 2HCO_3^-$ → $7acetate^- + 5H^+ + 4H_2O$
Resorcinol fermentation	$2C_6H_4(OH)_2 + 6H_2O$ → $4acetate^- + butyrate^- + 5H^+$
Phloroglucinol fermentation	$C_6H_6O_3 + 3H_2O$ → $3acetate^- + 3H^+$
Citrate fermentation	$Citrate^{3-} + 2H_2O$ → $formate^- + 2acetate^- + HCO_3^- + H^+$

Besides the use in microbiology, TDLAS can surely add value to general process control and supervision of pharmaceutical production procedures. Leakage testing is the standard procedure of assessing if primary packaging materials conserve the quality of manufactured and fill-finished products. It can be done by the blue dye ingress test where commercial units are placed for a certain time in a methylene blue bath (European Pharmacopoeia 2005b, U.S. Pharmacopoeia 2009). This allows the dye to penetrate potential leakages of the test unit. Such procedure demands hand protection as the dye is hard to be cleaned away from the fingers and could be substituted by TDLAS based measurements on CO_2. The basic test design could follow the principle of positioning a unit under investigation into a pressure chamber filled with CO_2, being followed by a TDLAS measurement on any increase in CO_2 headspace concentration.

During fill-finishing, product headspaces are either gassed with process air (contains ~0.041% CO_2) or N_2 (it avoids growth of potentially embedded aerobe microbes). Thus, any changes in CO_2 headspace concentration would indicate to a leakage of the primary packaging material. To show comparability to the blue dye ingress test experimental variables such as CO_2 pressure, leakage diameter and duration of pressure applied, including their correlation would need to be investigated. Once assessed in a laboratory setting with benchtop devices, a transfer towards a 100% in-line leakage inspection of commercial units after filling might be conceivable. This would minimize production mistakes and impact product quality directly as every leakage is spotted straight after filling and capping of product units.

TDLAS could add further value in validating the degree of isolator sterility after decontamination. Nowadays, isolator decontamination is performed by H_2O_2 killing prevailing microorganisms. The subsequent distribution of agar plates directly in the isolator checks if isolator sterility is given. Such conventional procedure could become obsolete if the H_2O_2 distribution during isolator decontamination could be monitored through wavelength adjusted TDLAS sensors. Of course, such sensors would need to be placed in isolators at spots less exposed to H_2O_2 and the entire approach be assessed on reliability.

Such examples for further research of TDLAS based measurements illustrate the technologies versatility. Due to its unique characteristics enabling non-invasive measurements, simple application and installation, high speed result reception and sample analysis without intermediate preparation steps, the technique is dedicated to in process control. In process control is a relevant part in lean manufacturing and helps, besides others, to keep quality related mechanisms and process interactions as efficient as possible (Figure). Furthermore, it targets the realization of quality by design, aiming at integrating quality into products through continuous monitoring activities during production. In conclusion, TDLAS can be part in performing such principles if applied adequately.

However, these are only some of the many helpful applications TDLAS could be used for bringing manufacturing sites one step closer to a fully optimized operational unit.

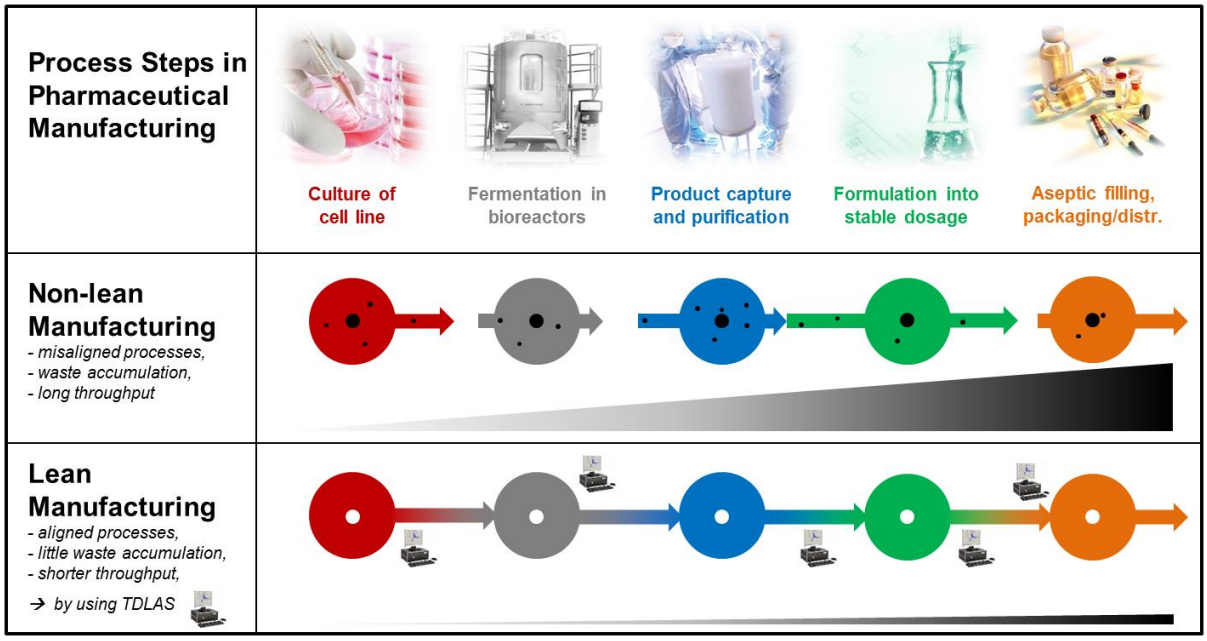


Figure 4: **Comparison of non-lean and lean production procedures.** The most important process steps in pharmaceutical manufacturing are depicted Non-lean processes generate and accumulate waste (black dots and ramp) - or in other words, activities are performed that do not add value to the product and customer. Often the performance of unnecessary steps leads to non-aligned processes extending the end-to-end throughput time of a product. In contrast, lean manufacturing focuses on the most relevant processes allowing a close process-process interaction and a fast product throughput time. TDLAS could help in realizing (in certain areas) a lean in process control. (Ams Biotechnology 2013, Arcane Industries 2017, Bandageer 2013, BioPharma Asia 2015, BioRefinery 2009, Gebauer et al. 2009)

7.3 General Implications for IMC

IMC is another technology having comparable advantages as TDLAS. IMC can test samples non-invasively and without additional preparation whereas data collection is relatively easy. However, differences in terms of usability exist as IMC measurements are more prone to variations coming from the environment depending on the instrument used. This demands a strictly controlled room temperature that allows the instrument to be efficient. Indeed, the surrounding of samples that are measured, needs to remain very stable in terms of temperature (0.1mK over 24 hours (TA Instruments 2012)). In optimal (or suboptimal) conditions, one of the methods main strengths is the very high sensitivity, detecting minimal amounts of heat emitted from biological or chemical reactions in real time. As such heat emission is a temporary phenomenon and cannot be detected anymore once over, the sample is demanded to stay in the device and on the measurement element during that time. This depicts clearly that the technique is suitable for an operational environment where samples are evaluated with rather low throughput rates (Wadso 2002). The low throughput as well as the unspecific measurement nature (several heat emitting processes cannot be differentiated from each other – i.e., only the cumulative heat signal is monitored) make IMC ideally for optimizing quality control mechanisms such as the control for microbial presence in test samples. Yet, targeting a heat based high-throughput analysis of experimental samples infrared thermal imaging might be the method of choice allowing such intention (Salaimeh et al. 2011, 2012).

In this work, studies performed with IMC targeted a substitution of the conventional sterility assessment. The sterility assessment tests quality into products which is not in alignment with nowadays targeted principles of quality by design. Still, the performance of sterility tests is demanded by regulatory agencies as final release assessment of fill-finished products and cannot be omitted. Therefore, appropriate measures must be taken to design a more efficient test. The IMC analysis of samples prepared similarly as during a sterility test delivered highly promising results and inoculated microorganisms could be detected earlier by IMC compared to the visual inspection on turbidity. This and the real-time monitoring character of IMC could potentially result in a reduction of the incubation procedure and the replacement of the sterility test.

Many companies which are targeting a faster performance of the sterility test are evaluating rapid microbial method (RMM) as potential test replacement (e.g. see Figure). Although many RMMs exist it is difficult to find an appropriate one, as the measurement must fulfill certain requirements to be considered valuable for sterility assessments.

The method must detect microbial growth faster than the visual detection on turbidity or colony counts and allow a non-invasive and non-destructive measurement so that cross contamination can be avoided and identification of grown microorganism by polymerase chain reaction is possible. Within this context the potential of IMC is still underestimated (shown by our study results) although fulfilling all the requirements to be categorized as useful RMM. The sensitivity of IMC as well as its non-invasive and non-destructive measurement positions it as well-suited sterility test replacement.

In addition to the optimization of the sterility test, microcalorimetry could be directly used for an assessment of potential microbial contaminants in the fill-finished product containers. Aqueous products (proteins such as anti-bodies) theoretically provide to microorganisms sufficient nutrition to enable growth until IMC detection is possible (possible with 10K – 100K cells per vial). The major benefit of such an approach would be the entire elimination of the filtration step of chosen product samples, thus designing a more efficient sterility test. However, such a potential process modification would require further investigations as the approach was not sufficiently studied in this work. Furthermore, process modifications of such kind are related to extensive product and country specific regulatory questions, endangering that expected efficiency gains (in case that good study results are obtained) are outperformed by regulatory costs.

In addition, growth promotion testing is the procedure that evaluates growth characteristics of microorganisms in media used for aseptic manufacturing control (European Pharmacopoeia 2007b). Nowadays, such tests are simply done by inoculating concerned media with defined microorganisms, followed by a visual check on colony forming units or turbidity (liquid media). Upon positive detection of all tested microorganisms (i.e., according to specifications) tested media are released for further use in aseptic manufacturing. By using IMC also growth promotion testing could be improved.

Nevertheless, some obstacles remain for IMC to become more established in the pharmaceutical and biotech market. Commercialized isothermal microcalorimeters capable to measure with the sensitivity needed for our and potential future studies are relatively expensive (500K CHF) and are therefore too costly to replace an analysis with low throughput and less burdensome performance such as the sterility test. High costs originate from device specifications which are above the demand for targeted applications in environmental monitoring. Commercial calorimeters allow for example to perform experiments in a relatively wide temperature range (i.e. from 15°C to 150°C (TA Instruments 2012)) whereas for applications related to microbial growth temperatures between 20°C and 40°C are already sufficient.

In addition, the applicability of such existing calorimeters is not yet fully aligned with a highly regulated drug production environment, making a sudden implementation rather difficult. Normally, diverse documentation is needed to perform the demanded validation and qualification (design qualification, installation qualification, operational qualification, and performance qualification) procedures, being prerequisite for device usage in a pharmaceutical and biotech environment. To further promote the use of microcalorimetry a simpler and more compact instrument with equal sensitivity and higher channel number (larger throughput) could be built. A modular design enabling besides the analysis of different vial and syringe formats also the assessment of agar plates would be highly convincing for usage in environmental monitoring and GPT. However, the realization from a technical point of view was not approached so far especially as the device's sensitivity is expected to diminish with reduced size (i.e. a smaller heat sink will be more sensitive to potential environmental temperature fluctuations).

Due to the versatility of IMC its potential is not limited to pharmaceutical quality control but can be of further use in clinics as well as research & development. Some studies have been performed about the speed in detecting antibiotic susceptibility of microorganisms causing urinary tract infections. Microcalorimetry achieved considerably faster times-to-detection (approximately 10 hours) as compared to conventional methods used to determine drug susceptibilities (Howell et al. 2012, Braissant et al. 2014). This is a considerable advantage and again shows the strength of microcalorimetry: its sensitivity. It is therefore only a matter of time until the technical evolution of IMC, that took place during the last 20 years, is realized promoting the technology's application for the diverse purposes it is and will be dedicated to.

7.4 Potential Influence of Innovation on Established Procedures

The performance of media fills, sterility assessments, and the related inspections on turbidity and growth are an integral part of the microbiological quality control system of aseptic facilities. Such quality control processes are based on a sequential (media-fills) or parallel (sterility assessment) two-tiered incubation of 20-25°C and/or 30-35°C. However, besides media fills and sterility assessments many other actions are performed to control bio burden in clean rooms, which are mostly exposed to the same incubation conditions. Despite disparity in guidance documents giving advice for applied incubation time and incubation temperature, many drug producers decided for a two-tiered incubation strategy and support their decisions with experimental data. The two-tiered incubation is preferred, as high yield of fungal growth is obtained at 20-25°C and bacteria grow considerably at 30-35°C.

However, recent studies have shown that incubation at 25°-30°C also yielded reasonable recovery of bacteria and fungi (Gordon et al. 2014). Such finding is highly promising as it theoretically allows facilitating the two-tiered incubation procedure for the entire set of microbiologic activities, which is in alignment with discussed lean manufacturing principles. All activities relating to transferring the incubated samples to another temperature could be omitted. Furthermore, a switch to one single incubation temperature would have implications for the media fill inspection automation. During threshold studies performed with TDLAS a slightly increased media induced CO_2 and O_2 variation of headspace concentrations was observed after the transfer from 20-25°C to 30-35°C (Brueckner et al. 2017, Brueckner et al. 2016). By normalizing applied incubation temperatures to 25-30°C such an effect could be neutralized which might result in lower thresholds being associated with a reduction of detection times. However, to allow such process alteration further experiments would be needed with a larger microbial spectrum.

Nowadays incubation times of the sterility test are set to 14 days at either 20-25°C (aerobe organisms) or 30-35°C (anaerobe organisms). Since IMC is more sensitive than the visual inspection on turbidity the use of IMC would allow a reduction of the established incubation durations. The release of fill-finished products is linked with the availability and quality of the sterility test results. Therefore, a reduction of incubation durations could permit an earlier availability of sterility test results and allow in parallel a faster market release of stored drug batches, being related with a more efficient market supply.

7.5 Acceptance of Innovation and Change

Altering established processes as well as implementing innovative technologies and methodologies is often costly. New equipment and research effort are necessary for such transformation, impacting frequently regulatory specifications. In addition, skepticism as against the proper functioning of planned changes exists as unexpected incidents could disturb the daily business, potentially endangering the unobstructed market supply. Therefore, sponsors are difficult to win for financing change related undertakings, if the expected transformation risk is not minimal and profitability not given within less than 2.5 – 3 years (Figure, Project C – Scenario 1). However, the success rate of projects weak in profitability can sometimes be increased through descriptions of qualitative benefits as well as risk minimization activities (Figure, Project C – Scenario 2). For the TDLAS based media fill inspection automation such challenges had to be overcome to push the technology forward. Therefore, the initially designed user requirement specifications for a platform allowing inspection automation were downsized, taking small efficiency reductions into account.

This resulted in a large cost reduction (costs could be reduced from 3'000K to 500K CHF) fitting the rather small efficiency gains (expected were ~200K CHF of yearly savings based on a process with two inspections) expected after realizing inspection automation. The additional description of qualitative benefits (such as data integrity, etc.), and the robust experimental data collected with an automated platform provided for free, further decreased the risk in acquisition. Chances for project realization increased and finally allowed to bring the project close to local and global implementation. Therefore, to successfully transform innovation projects with weak profitability, one needs to think about 1) existing qualitative benefits (not related to monetary savings), 2) actions helping to minimize the risk of failure and 3) possible cost reductions and 4) how to deal with the regulatory impact. This will certainly increase chances for project realization, yet there is no guarantee for success.

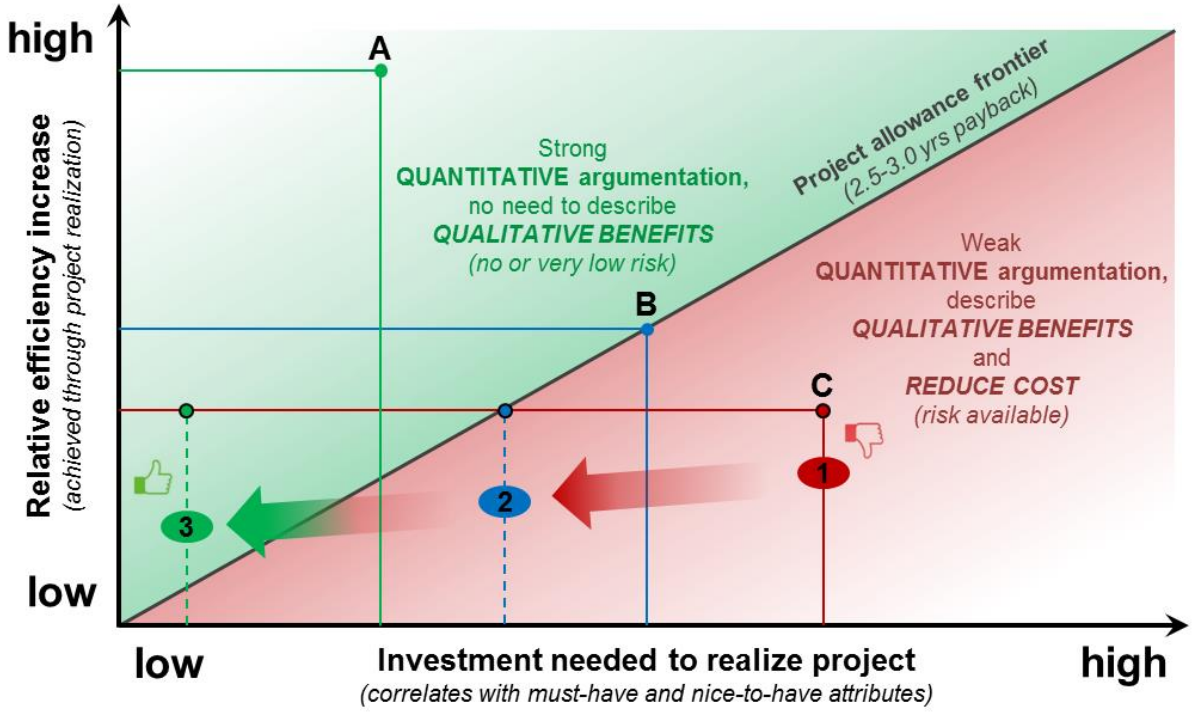


Figure 5: **Project allowance frontier.** Two hypothetic project scenarios A and B are shown whereas C represents the scenario applicable to the media fill inspection automation. All projects show a different impact on efficiency and have an altering financial demand for realization. The grey line illustrates the frontier of accepting a project to be financed. Project A is above this line and therefore likely to be realized (low risk of failure, high efficiency increase and low investment, correlate with high profitability). Project C is the exact contrary and comparable to the situation faced at the beginning of the project when targeting an automation of the visual media fill inspection. Therefore nice-to-have attributes needed to be omitted so that a cost reduction could be initiated allowing a move from situation C1 to situation C2. Situation C3 is of hypothetical nature showing that even more cost reduction would be necessary to assure financing. Generally, if the project is unprofitable, qualitative argumentation can help to ameliorate the situation, if the risk for failure is not too high.

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CHAPTER 8

General Conclusion

8. General Conclusion

Due to changes originating from existing and imminent transitions in the biotech and pharmaceutical market (patent expiration of blockbusters, release of biosimilars) aseptic manufacturers are increasingly forced to reorient their economic strategies. Tunable diode laser absorption spectroscopy (TDLAS) and isothermal micro-calorimetry (IMC) can ideally contribute to such reorientation through their potential in designing quality control mechanism more efficiently.

In this work TDLAS was used to determine microbial growth through an analysis of CO_2 and O_2 concentrations in the headspaces of media fill vials, targeting a redesign of the visual media fill inspection process. TDLAS is suited for automating the visual media fill inspection and capable to increase efficiency of the related process by minimally 90%. Neither false negative nor false positive results were obtained within the entire study. Therefore, a platform implementation is highly recommended although it was observed that a TDLAS based detection of microbial growth is slightly slower than the visual inspection on turbidity. Aside from many other possible applications (for example leakage detection of primary packaging material and assessment of the H_2O_2 distribution in isolators) TDLAS showed large potential in substituting invasive methodologies used nowadays in calorespirometry for the determination of CO_2 production and O_2 consumption of living systems. TDLAS can easily and non-invasively quantify CO_2 and O_2 concentrations whereby comparability to conventional techniques is given. Further experimentation is encouraged as the use of TDLAS in calorespirometry can be beneficial for more efficient control of fermentation conditions, needed to maximize yield of a specific biological products.

Although IMC is less suited for process automation, it can contribute differently to more efficient quality control mechanisms. Its high sensitivity allows a much faster detection of microbial growth than realizable by methods used usually (visual inspection on media turbidity or colony formation) in aseptic production facilities. IMC can replace the inspection procedure of the final sterility assessment of fill-finished products and, with its fast time to result, even shorten the duration of sample incubation. A technically simplified, modular, miniaturized, but equally sensitive calorimeter allowing the parallel assessment of diverse container forms (vials, syringes, agar plates, etc.) could probably promote the application of IMC in the pharmaceutical and biotech industry. Unfortunately such a device has not been commercialized yet.

The feasibility of innovating quality control mechanisms in aseptic manufacturing by the use of TDLAS and IMC was demonstrated. The mentioned technologies contribute to increase process efficiency in practice. However, their potential goes far beyond the value exploited in this study being the reason for further recommended research with TDLAS and IMC.