On-line Verification of Hemodialysis Water Quality

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Abstract

Renal diseases are an increasing problem worldwide and when kidney transplantation is not possible, dialysis treatment is the solution. An increasing number of patients choose to perform their dialysis treatment at home by implementing the dialysis machine and a water purification system in current electric and water supply, so called home hemodialysis. The quality of the water used for the preparation of dialysis fluid is essential due to the very close contact with the patient's blood. However, today's methods for verification of water quality are time consuming, non-frequently performed and costly.

The aims of this thesis were to investigate if an on-line, accurate and specific method for water quality verification is possible, without limitations as sample preparation, expensive and massive apparatus and professional skills needed for monitoring.

Attention has been paid to the requirements and expectations of such a device and an extensive literature study of potential techniques is presented. Some promising examples are biosensor applications for detection of bacteria and endotoxins, micromachined total organic carbon analyzers, amperometric chlorine analysers and flow cytometers.

Subsequent to this, an experimental investigation was performed to examine whether bacterial detection by their autofluorescence can be performed using a *FACSCanto II* flow cytometer. On the basis of the experimental results, no such autofluorescence could be detected. However, the high speed technique of flow cytometry could be further refined to replace the old fashioned conventional techniques for microbiological quantification.

Preface

This master thesis was accomplished as the final completion of the *M.Sc. programme in Engineering Nanoscience* at the Faculty of Engineering at Lund University. The work was performed at the Department of Biomedical Engineering in collaboration with the health care company Baxter in Lund. The practical part of the thesis was done at the Biomedical Center.

The project started in February 2016 and was finished in October the same year, covering 30 ECTS credits.

Acknowledgment

Firstly, we would like to express our sincere gratitude to our thesis supervisors Anders Wieslander at Baxter and Per Augustsson at the Department of Biomedical Engineering at Lund University, for their continuous support, encouragement and advisement throughout the thesis. Both their vast knowledge and their engagement have helped us whenever we encountered obstacles during the work and inspired us to new approaches.

Beside our supervisors we would like to thank the helpful staff at Baxter and especially Bo Olde, Staffan Bergström, Thomas Hertz and Helena Jeppsson for sharing their expertise and guiding us through many practicalities with motivating and always clever advices.

We would also like to thank Pelle Ohlsson, Maria Antfolk, Anke Urbansky and Cecilia Magnusson, since without their help and guidance through the flow cytometry measurements, the practical part of this thesis would have been nothing but a grope in the dark. We are very grateful for the time and effort they have dedicated to help and supervise us.

Lastly, we would like to thank all the other persons who have helped us during this thesis, such as the helpful and hospitable personnel at the Bulltofta Water Plant, the Division of Clinical Microbiology and the dialysis clinic at SUS in Lund for giving us useful insights and at last but not least, Daniel Baldor for an inspiring and touching introduction to the dialysis treatment.

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Contents

Chapter 1

Introduction

1.1 Disposition of the Report

The report is divided into four different chapters due to the diversity of the parts of this thesis. The initial part will introduce the reader to the project and motivate the defined objectives of the work. The second chapter presents the study of the demands and regulations needed to be fulfilled to accomplish the defined goals. This part merges into chapter three were a thorough literature review and investigation of possible techniques are presented. Finally, the last chapter of this report describes and demonstrates the experimental part of the project and concludes its results.

1.2 Background

This project was done on behalf of Baxter Sverige, aiming to investigate a device or devices for water quality verification for dialysis applications. This section will give the reader a brief background and basic knowledge of Baxter, the dialysis treatment and water quality.

1.2.1 Baxter

Baxter Sverige is a part of Baxter International, a multipurpose health care company with main focus in treatment of hemophilia, immune disorders and kidney diseases. The company has its headquarter in Deerfield, outside Chicago in USA. It is present in over 100 countries worldwide and have about 50 000 employees globally. In 2013, Baxter ac-

quired Gambro AB, a Swedish company with global innovation and manufacturing of in-center hemodialysis and acute renal care products. The facility located in Lund, where the work of this thesis has been carried out, is specialized in renal disease devices. This constitutes around a third of Baxter's market [1].

1.2.2 Kidney Functions and Renal Diseases

The kidneys are vital organs responsible for the regulation of electrolyte balance, regulation of the acid-base balance and the removal of excess fluid and body waste products like urea and creatinine. This process is achieved through the production of urine, including an advanced combination of excretion and re-absorption. Additionally, the kidneys are important producers of several necessary hormones and enzymes such as erythropoietin, EPO, and renin that regulate blood pressure and red blood cell production inter alia. A person with malfunctioning kidneys is not able to excrete urine. Fluid and poisonous waste products will accumulate within the body resulting in uremia. Since the kidneys regulate the uptake and elimination of several essential electrolytes like sodium, potassium, calcium and acetate ions, adjusted by the body's need, renal failure will cause electrolyte imbalance. This affects the heart and could cause arrythmia. Furthermore, the accumulation of electrolytes will retain fluid causing hypertension and an elevated risk of heart attacks and strokes. When the kidneys have lost 85 to 90 percent of their function the condition is life threatening and renal therapy is needed, either by kidney transplantation or dialysis [2].

1.2.3 Dialysis

The dialysis is an essential treatment for patients with renal diseases who are unable to undergo or are waiting for a renal transplantation. The treatment aims to replace the function of a normal kidney by removal of waste products and excess fluid and maintenance of acid-base balance and electrolyte levels. The general principle of dialysis is a fluid exchange between the blood and a dialysis fluid across a semipermeable membrane. The dialysis fluid, also called the dialysate, contains the electrolytes and nutrients wanted in blood while the blood contains waste products and excess fluid. This difference in solute content creates a concentration gradient and since solutes always migrate from

higher to lower concentration the result is a diffusion process. Electrolytes are accordingly taken up by the blood and excess fluid and waste products are removed from the blood, all controlled by the composition of the dialysis fluid. To regulate and amplify the fluid removal a pressure gradient is applied between the two fluid compartments. This can be conducted by applying a lower pressure in the dialysis fluid compartment which will force fluid from the blood to migrate over the membrane into the dialysis fluid by a hydrostatic pressure gradient. It can also be achieved by addition of an osmotic agent to the dialysis fluid, which draws fluid from the blood by the developed osmotic pressure gradient.

In general there are two types of dialysis treatment; *hemodialysis* and *peritoneal dialysis*. The main difference between these is the dialysis site. In hemodialysis the blood is diverted to an external machine where the filtration is performed, whereas in peritoneal dialysis the dialysis fluid is pumped into the peritoneal cavity in the abdomen and the peritoneum serves as the membrane for fluid exchange.

1.2.4 Hemodialysis

In hemodialysis blood is transferred in and out of the body through a previously created blood vessel, an arteriovenous fistula, which is a vein and an artery connected by surgery to increase the blood flow. The higher flow facilitates the dialysis process and forms a thick blood vessel with high resistance to withstand repeated needle penetrations. The principle of the fistula is illustrated in Figure 1.1.

When transferred into the dialysis apparatus the blood enters the dialyzer, the artificial kidney composed by multiple thin capillaries of semipermeable membrane, where it encounters the dialysis fluid. The dialysis fluid is conducted in opposite direction relative to the blood flow in order to enhance the solute exchange by the raised concentration gradient. The permeability and thickness of the membrane determines the flux across the dialyzer. The membrane porosity selectively permits water and small molecules to pass but retains bigger molecules like blood cells and proteins. The porosity of the capillary membranes is visible in the *scanning electron microscopy* images in Figure 1.2.

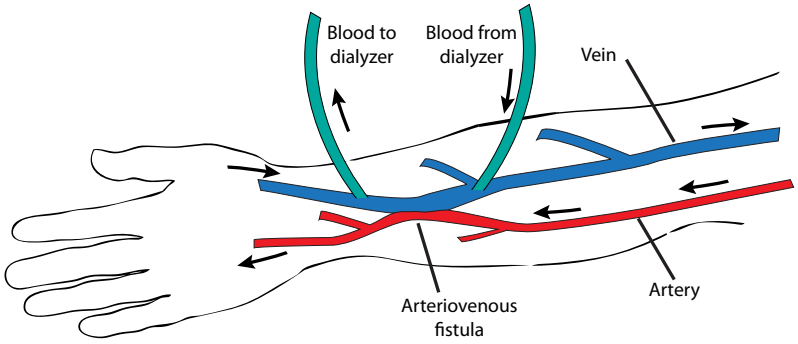


Figure 1.1: A sketch of a vein and an artery joint together forming an arteriovenous fistula with the in- and outlet of the dialysis connection marked.

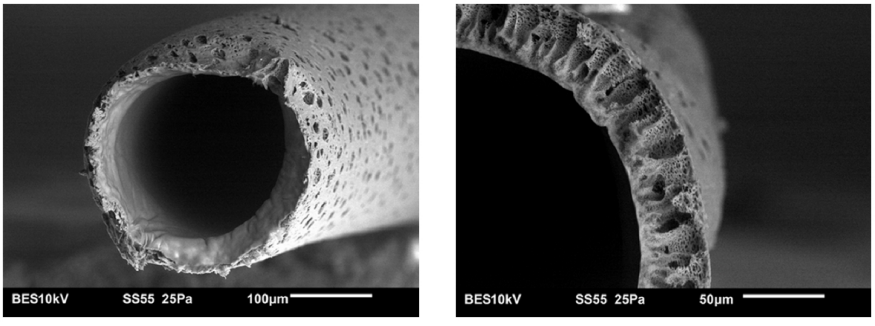


Figure 1.2: Scanning electron microscopy-images of cross-sections of a porous capillary in Baxter's dialyzer Polyflux 210 H. Blood flows inside the capillary that is surrounded with dialysate. Reproduced from *A. Hedayat et. al*[3] under the Creative Commons Attribution License.

When the exchange is accomplished the blood is led back to the body circulation and the contaminated dialysis fluid is sent to drain.

Since the blood is in very close contact with the dialysis fluid during the treatment, high fluid standard is essential. The composition should resemble blood plasma and not contain any contaminants to avoid immune system triggering. Furthermore, electrolytes and nutrients like sodium, calcium, potassium and glucose are vital. A comparison between typical concentration values of electrolytes and other substances in blood serum and dialysis fluid is shown in Table 1.1.

Substance	Concentration in blood serum	Gradient	Concentration in dialysate
Na	140 mEq/L	$\longleftrightarrow$	140 mEq/L
K	4.5 mEq/L	$\longrightarrow$	2 mEq/L
Cl	100 mEq/L	$\longleftrightarrow$	100 mEq/L
CO_2/HCO_3	24 mEq/L	$\longleftarrow$	35 mEq/L
Mg	2.0 mg/dL	$\longrightarrow$	1.2 mg/dL
Ca	2.4 mEq/L	$\longleftarrow$	2.5 mEq/L
Creatinine	5 mg/dL	$\longrightarrow$	–
Blood Urea N	30 mg/dL	$\longrightarrow$	–
Albumin	4 g/dL	$\longrightarrow$	–

Table 1.1: Typical concentrations of electrolytes and substances of interest in blood serum and dialysate together with the expected diffusion gradient. Concentrations in blood serum for dialysis patients can vary much and are often considerably higher than stated above [4].

Not only is the chemical composition of the dialysate of great importance for the performance and safety of the dialysis treatment. Several physical parameters need to be accurately controlled and monitored as well as chemical composition, wherefore both the blood circuit and the fluid circuit contain many sensing elements. For each sensor that is a part of the regulation process an additional monitoring sensor is necessary for safety reasons at all controlling parts of the dialysis system. An overview of the hemodialysis process, containing both the fluid circuit and the blood circuit, is illustrated in Figure 1.3.

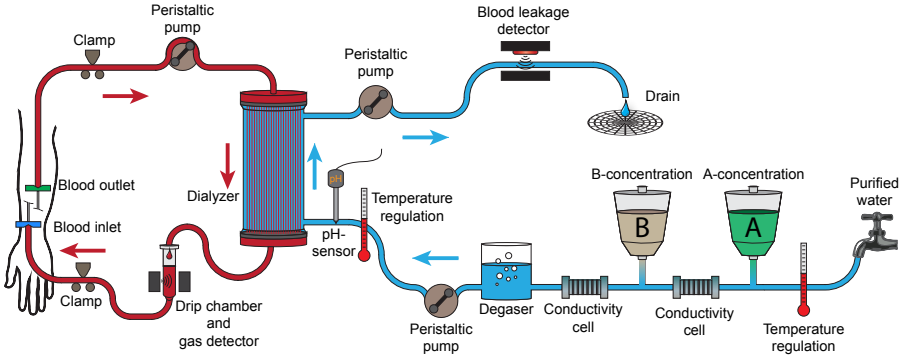


Figure 1.3: The general process of hemodialysis containing the blood circuit and the fluid circuit.

A typical flow velocity of the dialysate during hemodialysis treatment is around 500 ml/min, which means that over 100 liters of dialysate is used during a 4 hours treatment occasion. To avoid transport of those large amounts of dialysate to the treatment place, purified tap water is mixed with concentrated dialysate in the beginning of the fluid circuit. Prior to that, the water is heated to around body temperature, not too high so that proteins in the blood denature and not too low as it causes discomfort for the patient. During the dialysate mixing process two concentrates are pumped into the water; the first, the A-concentrate, contains all electrolytes except from bicarbonate and the second, the B-concentrate, contains just bicarbonate. The concentration of the mixture is measured with conductivity cells after both the addition of A- and B-concentrate, since the addition of a certain amount of electrolytes causes a known change in conductivity. Peristaltic pumps, sited before and after the dialyzer, control the flow velocity in the fluidic circuit, which together with pumps in the blood circuit regulates the pressure difference over the dialyzer, called the *transmembrane pressure* (TMP). The negative pressure produces a removal of excess water from the blood through ultrafiltration across the membrane.

Additional sensor elements in the fluid circuit are pH-sensors ahead of the dialyzer to give an extra assurance that the right dialysate mixture is used. Furthermore blood leakage detectors, placed after the dialyzer, use infrared light to detect if any hemoglobin has leaked out through the membrane, which if so happens requires an immediate stop of the dialysis process. On the purified blood's way back to the body it passes a drip chamber that evacuates any air introduced in the dialysis process. Also, an ultrasound gas detector is placed in connection to the drip chamber to ensure that no remaining gas enters the patients bloodstream. If that happens it can cause the fatal state of aeroembolism.

1.2.5 Home Hemodialysis

Hemodialysis is mostly preformed in-center at hospitals, but in some cases the treatment is also available in the patient's home. When done at home, the patients obtain higher freedom and control of their own treatment, since schedule and dialysis time can be adjusted to the pa-

tient's life and requests. Many patients experience higher life quality since there is no need for hospital visits several times a week and more time can be spent at home with family and friends. However, *home hemodialysis*, hereafter abbreviated HHD, puts higher demands on the patients and their relatives since they are in charge and responsible for the preparation, running and caring of the dialysis machine [5].

In HHD, the dialysis machine is connected to existing electricity and water supply. The water retrieved from the present water supplement is conducted to a purification system to achieve ultrapure water required for the dialysis treatment. As previously described, electrolytes and buffers are thereafter added to the purified water and transported to the dialyzer to purify the blood.

1.2.6 Water Quality

Water is essential in the dialysis treatment since it is the basis of the dialysis fluid. As a dialysis patient is exposed to approximately 40 times as much water through the dialyzer as a healthy person is orally, the demands on water control and monitoring are considerably higher [6]. Since elements in water may be small enough to pass the membrane into the patient's blood, the risk of provoking immune reactions or cause illness is higher than when water is ingested orally. Thus, the assertion of water and dialysate quality is vital for the protection of dialysis patients health.

In dialysis context, water quality is mainly based on three terms: chemical contaminants, presence of endotoxins and presence of microorganisms. Additionally, physical elements of the water such as temperature, pH and conductivity are indicators of its quality.

Common problem-causing contaminants for dialysis patients are those added deliberately to enhance public health, such as aluminum, chlorine/chloramines and fluorine [7]. These are added as coagulants to gather microorganisms, as disinfectants to kill bacteria and to reduce tooth decay respectively. However, they can cause severe reactions for a dialysis patient due to direct contact with the blood, which is not the case with drinking water, where they have to pass the intestinal barrier.

Presence of chemical substances within the dialysis fluid can cause reactions like motor difficulties, seizures, nausea, hypotension and diarrhea. Each chemical can provoke a specific reaction, for which reason numerous parameters need to be analyzed. If not removed properly from the dialysis fluid aluminum, a toxic metal, can enter the blood directly and accumulate within the body causing severe disorders like bone disease, microcytic anemia and encephalopathy [8]. Chlorine and chloramines have effects on cellular proteins, causing denaturation and haemolytic anaemia [9] and flouride can cause disturbance in bone mineralization leading to weak bones and bone pain.

Bacterial contamination of the dialysis fluid can provoke chronic inflammation and bacteremia, presence of bacteria in the blood, leading to sepsis. The small endotoxins may pass through the dialyzer membrane or appear in close contact with the white blood cells to elicit pyrogenic reactions [10].

Particular requirements and defined limits depend on the fluid application. The incoming water, called the *feed water* should obey the restrictions of *drinking water* quality and is controlled by the water suppliant. When treated and purified the water should fulfill the demands of *dialysis water* which is used for the preparation of the *dialysate/dialysis fluid* by adding electrolytes and buffer.

1.2.7 On-site Water Purification Systems

In general there are two types of water treatment systems: large *central water purification systems*, CWPs, used in-center at hospitals and small portable systems used for HHD or for a small number of patients. Even though they differ much in size and capacity the typical setup is the same since the same impurities and excess minerals have to be removed. A typical water purification process for HHD is shown in Figure 1.4.

In the process to achieve high water quality the incoming drinking water requires purification. The extent of the purification is dependent on the condition of incoming water and may differ due to the water source. Two commonly pretreatment devices are the carbon filter and the water softener. The carbon filter effectively adsorbs chlorine, chloramines

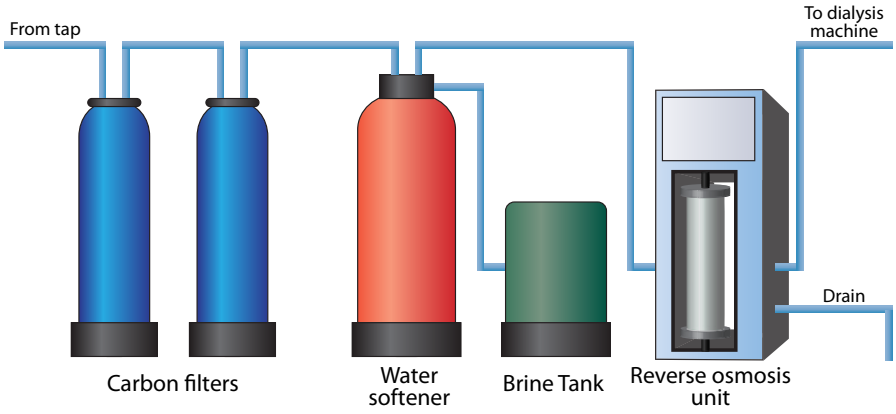


Figure 1.4: The production of dialysis water from feed water. On the way from the tap to the dialysis machine it passes one or several carbon filters, a water softener and a reverse osmosis unit.

and low molecular weight organics by its porous structure. Contaminants are trapped on the large surface area of the carbon filter and the trapping efficiency depends on flow rate and level of saturation [11].

The following water softener conducts an ion exchange by removing positively charged ions like calcium and magnesium and replacing them with sodium ions. By reducing the hard water content the forthcoming water purification system is protected and its life time is prolonged [12].

The main purification feature is the semipermeable *reverse osmosis* membrane that allows small water molecules to pass but hinders the majority of dissolved salts, bacteria and organic contaminants to follow the water. The method is considered very effective. For example, Baxter's RO-unit WRO 300 H removes more than 95% of dissolved salts and more than 99% of all particles, bacteria and pyrogens of the incoming water [13]. As described in Figure 1.5, a pressure greater than the natural osmotic pressure is needed to push the water through the membrane. The efficiency of the membrane relies on the flow, temperature and pressure, hence these parameters need to be kept constant upon validation [14].

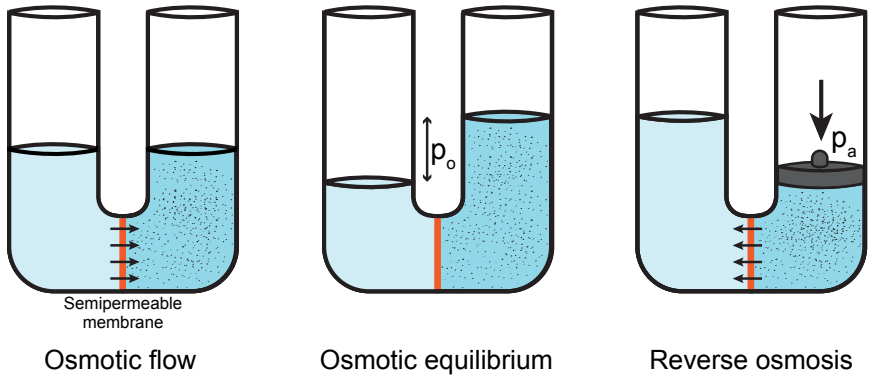


Figure 1.5: The principle of osmosis and reverse osmosis. When a semipermeable membrane separates two solutions with different concentrations of electrolytes, the solvent will spontaneously flow towards the higher concentrated region. When the two solutions are equally concentrated or when an outer pressure prevents any further flow, an equilibrium is reached. In this case the height difference between the fluid pillars during equilibrium constitutes the osmotic pressure, p_o. Reverse osmosis occurs when a pressure, p_a, higher than the osmotic pressure, is applied on the higher concentrated fluid. This will induce a flow in the opposite direction and increase the difference in concentrations which is the phenomenon that can be used for water purification purposes.

1.3 Aim and Motivation of the Thesis

As the background section indicates, the access to ultrapure water is extremely important for a successful dialysis treatment. The purification system helps to provide this but nevertheless the water quality has to be verified. This is an ongoing isssue, especially for the clinics offering home hemodialysis. Recurring sample-taking is necessary for each and every patient and not only for the CWP as at the clinic, which currently makes HHD a more time-consuming treatment method.

On behalf of Baxter this thesis aims at attacking the problem by finding on-line solutions for the water quality verification. Moving the extensive laboratory work to the patient's home could be a quicker, cheaper and safer way that would enable health care providers to give more kidney disease patients the care they need. This could also make HHD an available alternative for a larger group of renal patients.

The general objectives set up for this thesis in collaboration with Baxter were:

1. to investigate and identify the customer needs and market advantages with on-line verification of the water quality in home hemodialysis,
2. to survey the current available water quality verification methods from a microbial, endotoxic and chemical perspective,
3. to construct a hypothesis with possible techniques that are applicable and integrable on dialysis equipment,
4. to implement and evaluate at least one of these techniques experimentally.

1.4 Disposition of the Work

In order to accomplish the objectives stated in section 1.3 the work was divided into three parts (corresponding to chapter 2, 3 and 4):

The *requirements analysis* aims at fulfilling objective number 1 and to define what demands and regulations there are on an on-line method for water verification and what parameters that should be taken into account when searching for new water analysis techniques.

Consequently, the analysis forms the basis for the *literature review and investigation of possible techniques* that was the main research task of this project. It was performed with the intention of accomplishing objectives 2 and 3. The authors' background in the field of technical nanoscience with specializations in biomedicine should were helpful when investigating, inter alia, the feasibility in miniaturization of existing approaches.

Finally, the *experimental work* was performed as a proof of concept for one of the chosen techniques in accordance with objective number 4.

The two authors contributed equally in the planning, performance and presentation of this thesis and the work was mostly performed together.

Chapter 2

Requirements Analysis

This requirements analysis was performed as a first step towards understanding the demands and expectations on an on-line dialysis water quality verification system. The ambition was that it should give insight in what expectations not only the manufacturing company would have on such a medicine technical product but also clinics, nurses, technicians, patients, relatives and regulatory authorities.

2.1 Method

2.1.1 Investigation of Regulatory Standardizations for Dialysis Water Validation

With the intention to minimize the risk of contaminants in the dialysis water and the dialysis fluid, numerous organizations have set up standards were they define and motivate concentration limits of contaminants and with what methods this should be measured. During this step of the requirements analysis a review and a comparison of some important standards and regulative documents were made.

Since almost every country has its own regulations for dialysis water the focus was on the International Organization for Standardization's standard ISO:13959(2014); *Water for Haemodialysis and Related Therapies* [6], the Swedish Pharmaceuticals Standard, SLS:2016.0; *Tillverkning och hantering av hemodialysvätskor inom sjukvården* [14], the European Pharmacopoeia, Ph. Eur. 01/2009:1167; *Haemodialysis Solutions, Concentrated, Water for Diluting* [15] and the International Con-

ference on Harmonisation's guideline, ICH:Q2(R1); *Validation of Analytical Procedures* [16].

2.1.2 Investigation of Existing Methods for Dialysis Water Validation

In order to find improvement possibilities of water quality measurement, existing methods were investigated. These current methods, identified in previously mentioned standards, were studied and interpreted by literature searching and study visits. Information about current methods was also acquired by skillful personnel at Baxter.

2.1.3 Survey on Methods for Water Validation at Home-hemodialysis Clinics

In order to understand how the dialysis water quality verification is implemented and practiced by the HHD-clinics almost all of the Swedish dialysis clinics[1] were contacted and asked whether they offered their patients HHD-treatment. Those who answered that they did so were asked to fill in a formulary[2] regarding their routines for quality verification of their HHD-patients purified dialysis water.

Since the routines at the clinics were expected to be diverse, the formulary offered only free text answers, wherefore the analysis of the investigation were always meant to be qualitative rather than quantitative and not to be evaluated statistically.

2.1.4 Study Visits

To obtain a more empirical view on the prescribed circumstances with HHD, water purification and microbiological laboratory work, three study visits were carried out.

[1]The dialysis clinics contacted were those with contact information listed on the Swedish Renal Medicine Nurse Association's homepage [17].

[2]The structure and content of the formulary are described in Appendix A.

The Dialysis Clinic at Skåne University Hospital, Lund

At the clinics in the Alwall-building in Lund, patients can receive traditional hemodialysis treatment but also be trained for the self-treatment methods *peritoneal dialysis*, *home-hemodialysis* and *self-care hemodialysis* [18]. The study visit contained interviews with a HHD patient and a tour to the different dialysis facilities in the building.

Bulltofta Water Plant, Malmö

The study visit to Bulltofta Water Plant was performed to gain knowledge about how the water is pre-treated before it reaches a HHD-patient's house or a dialysis clinic and also to see what analysis methods that are used in the laboratory for the drinking water quality verification.

The water plant is operated by the water and sewerage supplier VA SYD and it treats and purifies water from the Grevie source, providing approximately 15 % of Malmö's drinking water [19].

The Division of Clinical Microbiology at the University Hospital of Skåne, Lund

For the purpose of getting a hands-on experience of the current techniques used for microbial and endotoxin analysis, a study visit was carried out at the clinical microbiology laboratory at the university hospital in Lund. The laboratory analyzes all of the water samples from the dialysis clinic.

2.1.5 International Aspects of HHD-possibilities and Regulations on Drinking Water Quality

Even though the survey and study visits were expected to give a better insight in the case of water for dialysis usage, they were limited to Sweden, which only has a minimal fraction of the world's patients under HHD treatment. Therefore, in order to get a more international perspective on the case, an investigation was made on how prevalent HHD is globally and what conditions that are necessary to enable HHD treatment at any location. Information about this was obtained from statistical databases of several international dialysis organizations and from organizations and magazines reporting about global aspects of dialysis.

Especially, the report *Guidelines for drinking-water quality* from the *World Health Organization*, WHO [20] was taken into consideration.

2.2 Analysis and gained knowledge

2.2.1 Investigation of Regulatory Standardizations for Dialysis Water Validation

Standards for Manufacturing of Dialysis Water

The international standard ISO:13959(2014) influence the many local standards that regulates dialysis water when setting the limitations. According to Baxter it is the dialysis water standard that the manufacturers of equipment for dialysis firstly takes into consideration when designing for example water purification systems. The scope of the standard includes the specification of "...minimum requirements for water to be used in haemodialysis and related therapies." [6] and the types of liquids it regulates are "...water to be used in the preparation of concentrates, dialysis fluids for haemodialysis, haemodiafiltration and haemofiltration, and for the reprocessing of haemodialysers.". It contains an important section with definitions followed by the two main parts addressing dialysis water requirements and tests for compliance with them after which it ends with a rationale for its development.

The Swedish equivalent standard, SLS:2016.0 is built up in a slightly different way and contains two main parts. One part with guidelines to a GMP, *Good Manufacturing Practice*, for the production of water for dialysis and one part of a more advisory character, which aims at helping the manufacturer to establish the GMP. The guidelines include a table of maximum allowed concentrations of several contaminants together with a table of how often these levels shall be measured.

In addition to the large number of similarities between the two standards there are some minor and major differences. As for example, they have some differences in their requirement specification and in the ways they express them. Among them, the restriction of the concentrations of heavy metals, where ISO:13959(2014) has separate limits for individual heavy metals but SLS:2016.0 specifies a limit for heavy metals as a group. This can be considered as a bit unsuitable as the term

heavy metals often leads to ambiguity and thus, as a disadvantage with SLS:2016.0. Especially since it does not contain any rationale that motivates the selection of limits, which the ISO standard does contain. On the other hand, ISO:13959(2014) only states the limits and suitable detection methods for the respective analysis but does not carry any advice at which frequency the sample-taking and analysis should be performed, which is prescribed in SLS:2016.0.

Although both SLS:2016.0 and ISO:13959(2014) generally divides the quality control in terms of microbiological requirements and chemical contaminants, this report will hereafter separate the methods to fulfil the microbiological requirements into detection of *microbes* and *endotoxins* as the analysis methods are significantly different.

Pharmacopoeia

The monograph 01/2009:1167 *Haemodialysis solutions, concentrated, water for dialysis* of the European Pharmacopoeia consists of a set of conventional, validated test methods for many chemical parameters. The application of these methods is legally binding when determining the quality of dialysis water. If any other method is to be used it must be validated.

Validation of Analysis Methods

As previously mentioned, an analysis method that is not a pharmacopoeia method should be validated in accordance with the International Conference on Harmonization's guideline, ICH:Q2(R1), *Validation of Analytical Procedures*. It describes in its introduction that it rather "...serves as a collection of terms, and their definition, and is not intended to provide direction on how to accomplish validation." [16]. The characteristics normally necessary to evaluate for an analytical procedure that is meant to be testing for impurities, which is the most common case in water quality verification, are:

- Accuracy
    - Repeatability
    - Intermediate precision
- Specificity
- Detection limit

- Quantitation limit
- Linearity
- Range

In addition to these, the *robustness* – which means the method's "...capacity to remain unaffected by small, but deliberate variations in method parameters..." – should be evaluated.

2.2.2 Investigation of Existing Methods for Dialysis Water Validation

Microbial analysis

Routine microbial analysis is dominated by plate methods where the fluid to be analyzed is deposited in microbiological growth medium and incubated to enhance individual microorganism to grow to a colony forming unit. These methods are slow with several days waiting time to acquire data and can furthermore underestimate the real number of microorganisms since not all will grow to form a colony unit. The main steps in the method for determining bacteria concentration in water samples are illustrated in Figure 2.1.

According to ISO:13959(2014), as well as SLS:2016.0, the minimum requirement for water for dialysis is specified to 100 CFU/ml. The standards also recommend the agar plate counting as analysis method or other validated method with corresponding result [21] [6]. The outcome of the microbiological cultivation method is the result of a combination of nutrient media, incubation time and incubation temperature. Since poor cultivation conditions will underestimate the reality and favorable will overrate the microbial content, the standards also specify the type of agar plate accepted and the associated time and temperature. The SLS:2016.0 recommends the usage of either R_2A agar plates at 20°C for 5-7 days or TSA agar plates at 30-35°C for 3 days [14].

Endotoxin analysis

The *endotoxin analysis* is employed to detect and quantify the amount of endotoxins in the water sample. Bacterial endotoxins are lipopolysaccharides from the cell membrane in gramnegative bacteria, which in contact with the bloodstream they provoke intense pyrogenic immune

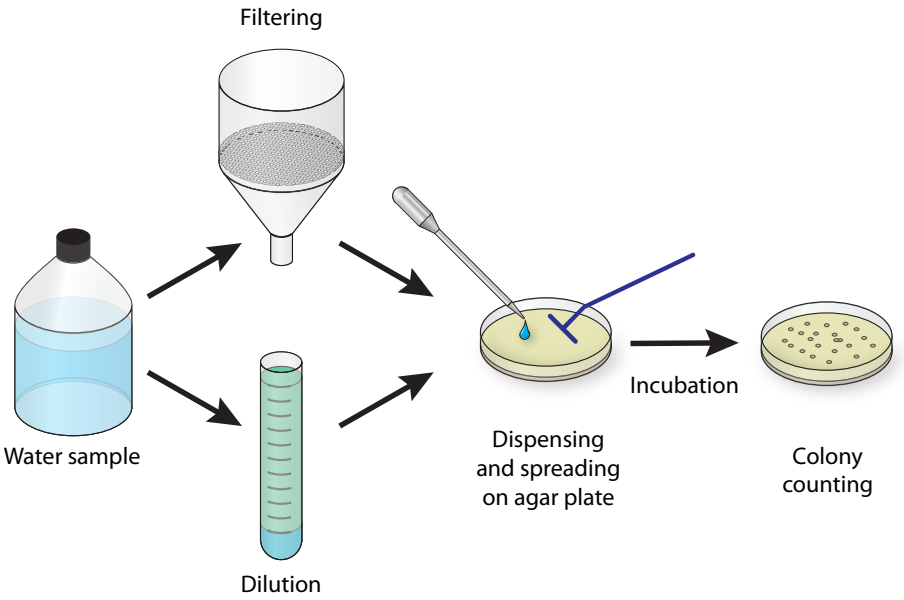


Figure 2.1: The general method to decide the bacteria concentration in water samples. First, the sample is either diluted or filtered to a concentration that is estimated to generate a countable number of colonies. The water is then dispensed on agar plates and evenly spread out until it has dried in to the agar. After incubation at a time and temperature dependent on the agar type and bacteria to detect, any present bacteria have formed colonies that can be counted.

responses. The most common approach to measure endotoxin content is the *limoulus amoebocyte lysate* (LAL)-test, recommended in both the American and European pharmacopoeia. The assay is accomplished by the extraction of the enzyme LAL from the horseshoe crab's blood which reacts with and inactivates the uncounted endotoxins. Three types of tests exist; *gel-clot* where the LAL enzyme coagulates in presence of endotoxins, *cromogenic* where cromogenic peptides release a chromophore upon reaction and the intensity is measured by spectrophotometry and thirdly *turbidimetric* where the turbidity of the sample serves as an indication of endotoxin presence. These can both be done at the end of the incubation time, so called *end-point-measurements*, or in close proximity after the mixing; *kinetic measurements* [22] [23].

The endotoxin content is measured and stated in units of IU (*International Unit*) or EU (*Endotoxin Unit*) which are equivalent. Both the SLS

2016.0 and the ISO:13959(2014) stipulate a maximal content of 0.25 IU/ml. Besides, SLS 2016.0 specifies quality controls twice a year.

Chemical analysis

For the chemical analysis it is harder to define one specific method since different analysis methods are available, depending on what component to analyze. For most analytes, different kinds of spectrometry or chromatography methods are recommended. For example, a commonly used method, that is recommended in the ISO:13959(2014) for several contaminants, is the ICP-MS, *Inductively Coupled Plasma Mass Spectrometry*. The method is performed by heating a carrier gas to induce plasma which in turn is used to ionize the sample. The created ions are then separated depending on the mass-to-charge ratio and the elemental components can be detected. The ICP-MS is rather advanced and the instrument is both big and expensive, although very precise with high resolution and capability to detect most elements.

The conductivity of the water should be monitored continuously with an integrated conductivity meter placed after the reverse osmosis-unit. A registered difference in conductivity is used as an indicator of contaminated water since higher conductivity is related to higher ion content within the fluid. The dissolved ions originates from dissolved salts and inorganic material. However, the monitoring is only a general indication of water quality and the method is unable to distinguish between different types of contamination [24].

2.2.3 Survey on Methods for Water Validation at Home-hemodialysis Clinics

Coverage

Out of 66 contacted dialysis clinics, 39 replied and answered the first question about whether they offered home hemodialysis or not to their patients. 16 clinics replied that they did so and out of them, 13 clinics filled in the formulary. Those 13 clinics reported to treat in total 121 patients which is equal to 88% of Sweden's 138 HHD-patients [25].

Sampling Frequency

The sampling frequency of the microbial detection and endotoxin testing varies between different clinics and ranges from once per month to once per year. Some of the clinics also reports that they are initially taking water samples more frequently until a good trend in water quality can be confirmed. However, a number of clinics do not satisfy the recommended sampling frequency for microbial control of once per month as stated in SLS:2016.0. All except one clinic followed the exhortation of two times per year as a minimum for endotoxin control. Even more nameworthy is the fact that only three out of thirteen clinics follow the recommendation of chemical control every second month. Without mentioning why, it becomes clear that the more or less mandatory document SLS:2016.0 is not complied to the letter regarding sampling frequency.

Sample-taking Routines

The answers upon the question *"Who performs the sample-taking?"* varies to be either a technician, a nurse or the patient. All clinics except one reports that they have a protocol describing the routines for the sample-taking but despite this, the fact that some patients tap the water themselves might be considered as an increased risk of contamination of the water.

After the water is tapped in the patients home, it is transported under well-regulated circumstances to the clinic or to the place where the analysis occurs. Most of the clinics send the water samples for analysis to internal hospital laboratories except from the samples for chemical control, that require more advanced equipment, where some clinics uses external analysis companies instead.

Costs

Since the microbial analysis mostly occurred at hospital laboratories, the cost for analysis is internally debited and therefore the actual price is not visible for the clinic. Neither the personnel cost for travelling to the patients and taking water samples is counted as a direct extra cost and is therefore hardly estimated. Anyway, one clinic reports that only the analysis costs up to 800 SEK but that the material cost is negligible.

According to Region Skåne's price list a bacterial cultivation from water costs 200 SEK while the external analysis company Eurofins takes 393 SEK [26][27]. Anyway, with the extensive sample-taking routines in mind the costs for microbial analysis are expected to be considerably higher than these sums.

Even in the case of endotoxin tests the analysis cost amounts to 800 SEK pursuant to the questionnaire answers while Region Skåne charges 283 SEK and Eurofins charges 1200 SEK. Again, it is reasonable to presume that the total cost will be substantially higher and that sample-taking for HHD is more expensive than for hemodialysis at clinic due to the expenditures that comes with housecalls.

As already mentioned, most clinics use external analysis companies for the chemical analysis since it includes several analytes and needs more expensive and advanced equipment. This made the costs more visible for the clinics and those who uses Eurofins reported a cost per sample of 1200 SEK excluding costs for sample-taking and shipping. Two clinics specifies chemical analysis costs of 35 respectively 39 SEK which appears to be unreasonably low prices. Either, all of the recommended parameters are not being measured or the actual costs are invisible for the clinic staff.

Response time

As with the costs, the time it takes for the clinics to get results from the different tests varies a lot. Most clinics states that the response time for the microbial tests and endotoxin tests is around one week while the response time for the chemical analysis ranges from a couple of hours for those who perform it internally up to 14 days for those who send it to an external analysis company.

The money and time consumed on all these controls together with the comprehensive routines are probably the main reasons to why the exhorted sample-taking frequency is not followed in many cases. This means that it is apparently both time and money to save on having an on-line method for the water quality verification if it can be sufficiently reliable and inexpensive.

2.2.4 Study Visits

As been mentioned before, the study visits were conducted to learn more about the different areas related to HHD treatment and rather than results, some of the gained knowledge is presented in this section.

The dialysis clinic at Skåne University Hospital, Lund

As there are big risks associated to an improper dialysis treatment, HHD-patients are trained at the clinic during eight weeks before they can start their treatment at home. The patients connected to the hospital in Lund operates some of the cleaning processes themselves and are also taught to perform the sample-taking for water quality analysis.

In Sweden, all parts of the HHD-treatment is free of charge for the patient, who is also given economic compensation for the increased consumption of water and electricity together with higher costs for transport and waste management. Each treatment occasion with HHD costs approximately 1200 SEK while the same cost for in-clinic treatment is around 3000 - 3500 SEK. This difference comes from reduced personnel and premises costs in HHD treatment.

An alternative method for the patients at the Alwall-clinic is night-time HHD, which is considered to be very effective. Anyhow, for some patients, the large impact on the body that dialysis causes together with noise from the machine results in disturbance of their night's rest.

There are shared opinions among patients and nurses whether automation of the dialysis treatment is good or not. For many patients it means better life quality to take control over their treatment and understand how the different settings affect their health, while for others it is convenient to not put so much effort in operating the dialysis machine that already is a big time-consumer in their lives [28].

Bulltofta Water Plant, Malmö

The water plant performs analyses of the outgoing drinking water in compliance with the regulations from the Swedish National Food Administration[21]. In common with the regulations for dialysis water they are looking for the presence of microorganisms in general but in

contrast they also have to ensure that there are absolutely no coliform bacteria, *Escherichia coli* or actinomyces present in the water. Besides that, they are not checking for endotoxins at all, as it is not considered as a risk if endotoxin containing water is consumed orally.

At Bulltofta Water Plant, four parameters are continuously measured on-line; the water's hardness, acidity, conductivity and chlorine excess. All other parameters and contaminants are regularly analyzed at their own SWEDAC-accredited laboratory, where they also analyzes water samples for others.

The Division of Clinical Microbiology at the University Hospital of Skåne, Lund

When the water samples are transported from the patient's home and arrives at the laboratory, it is first filtered through a 0.2 μm pore filter, which is then placed on an agar plate. This step is performed since eventually present bacteria need to be enriched as the concentrations are expected to be very low in the purified water. The whole procedure of bacteria cultivation is done manually and so is even the analysis. The lab assistant looks on the plate after a certain incubation time and makes a subjective evaluation on the number of colony forming units if there is a visible microbial growth.

As well as microbial analysis, endotoxin tests are also performed in the same laboratory with the *Endosafe-MCS*^{TM} machine from *Charles River* company. The lab assistant gave a rough cost of 100 SEK per sample for the disposable cassette that is used.

2.2.5 International Aspects of HHD-possibilities and Regulations on Drinking Water Quality

International Distribution of Patients under HHD-treatment

The number of patients that are treated with hemodialysis in their homes varies strongly between different countries and continents. The possibilities to receive HHD-treatment in a less developed country is significantly lower since it demands high drinking-water quality. In addition to that, the cost-effectiveness is lower in these countries for HHD-treatment in comparison to hemodialysis at clinics since the personnel

cost is often lower. Two factors that are often thought to affect the ratio between dialysis at home or at clinic are urbanization and population density since clinic-visits are more circumstantial when a patient has a long traveling distance to the clinic. However, a study from 2006 shows no such correlation [5].

A selection of the HHD statistics for some countries are shown in Table 2.1.

Land	Patients w. HHD	p.m.i	% of pat.
USA [29]	8507	26.7	1.8
Sweden [25], [30]	138	14.9	1.6
Canada [31]	1042	29.6	2.5
Australia & New Zealand [32]	1629	59.0	11.0
Japan [33]	459	3.6	0.1

Table 2.1: The number of patients under HHD-treatment in total, per million inhabitants and as percent of treated patients.

When having a look at the European Union, there is no available summary for the union as a whole but the annual report [30] from ERA-EDTA (European Renal Association - European Dialysis and Transplant Association) contains two parts; one for countries that have provided individual patient data and one for countries and regions that has provided aggregated patient data. All of the countries in the European Union have not contributed with data, but of those who have, Wales (3.3%) and Denmark (3.0%) have the largest fractions of patients with HHD when considering the treatment methods in total. The country that treats the most patients with HHD is the United Kingdom (1168 patients) if considered as one country, while many of the countries do not provide HHD as a treatment method for renal patients.

Drinking-water quality

Access to high-quality drinking water is the most essential condition for home hemodialysis. But as is widely known, the access and quality of water is greatly different over the world and there is no globally accepted standardization made that regulates allowed levels of contaminants in drinking-water. Some countries have compiled their own recommendations while for example EU and USA have standards which specific directives must be abided by law [34][35]. For those countries

that lack standards, the *World Health Organization*, WHO has set up guidelines to support and be the basis of risk assessment strategies on the occasion of water contamination. The 500 pages long report also describes, in addition to requirements for maximum contaminant levels, procedures for monitoring and quality control [20].

Since the problem of drinking-water contamination is widespread and often noticed, the regulation of drinking-water quality around the world is more comprehensive and detailed than the one for water for dialysis and thus containing more measurement parameters. This despite the fact that the demands on the water quality is much higher for dialysis than for drinking purposes. As for example in Sweden, the feedwater for hemodialysis must at least achieve drinking-water quality [14].

Therefore, if HHD-treatment should be possible in countries with insufficient drinking-water quality an on-line system for verifying the quality of the purified water is almost necessary since the risk for complications is considerably higher.

2.3 Conclusions

The summarized conclusions of this requirements analysis with importance for the objectives of this thesis are:

- A potential on-line method for control of microbial presence must measure something else than Colony Forming Units since a bacterium needs incubation time to divide into a visible colony of many bacteria. By obvious reasons, the incubation is incompatible with an on-line method. Therefore the microbial concentration unit of an on-line method must either be able to translate to CFUs/ml or the set of regulations has to be changed.
- The conventional methods for determination of the endotoxin content in water requires an addition of the LAL reagent which might be possible but not desirable at on-line measurements since it implicates handling of waste water. In addition to that, the kinetic LAL-methods claim frequent calibration with known endotoxin levels.

- A measuring method for verification of the water quality which is not prescribed in the effective standard or a pharmacopoeia must be validated towards the conventional method in compliance with the ICH guidelines.

- Many costs for the sample-taking and analysis in the health care appear to be unknown or underestimated. Likewise, some of the approached clinics in the survey do not fulfill the demands of SLS:2016.0 in terms of sample-taking frequency. A continuous, or at least frequently performed, on-line method would be suitable to prevent under-sampling.

- There is both time and money to save with an adequate on-line method for water quality verification. Both transport and personnel cost will be avoided or at least depressed through on line measurements. Despite this, the grand advantage will come from safety aspects since a good and stable water quality can be guaranteed. It must not necessarily replace the conventional methods but could serve as a complement so that early warnings of high levels of contaminants can be communicated.

Chapter 3

Literature Review and Investigation of Possible Techniques

3.1 Method

In order to gain greater knowledge and comprehension to the previous defined problem and to investigate possible technical solutions, an extensive literature study was done. The study was divided into three parts; *Microbial Analysis Methods, Endotoxin Analysis Methods* and *Chemical Analysis Methods*. Published material from article databases, web pages and patent databases were investigated and gathered in a list with an abstract together with a short evaluation of their relevance, scientific rigor and quality.

When evaluating possible methods the following requirements from the *Requirement Analysis* were kept in mind:

1. On-line measurement - preferable without addition of reagents and with results in real time
2. Detection limits within the specification interval
3. Specificity for selected analyte
4. Possibilities for development within this project

3.2 Results

The most relevant and potential technical solutions are presented and described in each category.

3.2.1 Methods for Microbial Analysis

Biosensors

A biosensor is a type of detector used for quantification and identification of biological material. The sensor usually consist of two components; a biological affinity element and a complimentary detection method. The biological recognition can be performed for example by antibodies, boronic acids, lectins or other elements that bind specifically to particular segments of the intended microorganism.

For a biosensor to be relevant within this project, the biorecognition element must have the capability of non-specific affinity with all types of bacteria and not just one species. Besides, a very low limit of detection is necessary since the predefined bacteria concentration is considerably low. Furthermore, no sample processing nor addition of reagents are desired, hence the binding needs to be exogenous as no bacterial disruption or lysis are practicable. The binding component does often have too high limit of detection as well as an incubation time for the binding to occur. These problems make the methods complicated to use for sensitive on-line applications.

When the specific binding is conducted at least one physicochemical state is changed, such as conductivity, mass or refractive index, which consequently can be detected and quantified by an appropriate detection method. The sensitivity of the biosensor is due to both the affinity element and detection method [36].

The biosensor field is developing and becomes important in an increasing number of applications. Common detection techniques for bacteria are the optical and the impedimetric methods, including *surface plasmon resonance* and direct monitoring of impedance variations.

Biosensors - Recognition Element: Antibodies

Monoclonal antibodies are common recognition elements used in biosensors. The antibodies are immobilized on a substrate and when encountering the corresponding antigen the formation of an antibody-antigen-complex occur [37]. A study done with antibodies immobilized on screen printed electrodes of gold achieved a very low limit of detection of 3.3 CFU/ml. However one hour of incubation was needed for the antibody-bacteria binding to occur and the detection was selective for *E. coli* bacteria [38]. The main advantage of antibodies as recognition elements is their selectivity, however within this project not only one, but all types of bacteria should be detected.

Biosensors - Recognition Element: Boronic Acids

To expand the detection span boronic acids, ligands which selectively bind to diol-compounds, can be used. Since the bacteria cell wall contains diol groups attached to polysaccharides, the boronic acids can chemoselectively bind to these diols and hence detect and quantify the total amount of bacteria. Other saccharides, such as glucose or endotoxins, can also bind to the boronic acids indicating that the water is not purified enough, though from a different perspective than bacterial content. In a study where boronic acids were used in combination with a potentiostatic step method the detection limit of $1.0 \cdot 10^2$ CFU/m1 was obtained, though with an incubation time of 20 minutes [39].

Biosensors - Recognition Element: Lectins

Lectins are carbohydrate-binding proteins in plants and animals which selectively can bind to saccharide components of the bacteria cell wall. Like boronic acids these are more general in their affinity with bacteria and hence superior to antibodies in the detection of all bacteria types. Also, due to the small size of lectins, higher density of sensing elements can be obtained on the substrate surface resulting in higher sensitivity and less non-specific adsorption. A lectin-based impedance biosensor is reported to achieve detection with concentration range of 1.8 to $1.8 \cdot 10^7$ CFU/ml [37]. The carbohydrate recognition conducted by lectins or boronic acids enable identification and detection of unexpected or novel bacteria. Also, they are more stable than antibodies and do not denature upon temperature or pH variations [40].

Biosensors - Detection Method: Surface Plasmon Resonance

As illustrated in Figure 3.1 Surface plasmon resonance sensors measure changes in the refractive index and so the resonant frequency upon analyte binding. The substrate, covered by immobilized recognition elements, is irradiated by plane-polarized light which passes through a prism situated below the surface. When analyte binding occur, the result is a shift in the reflected light which cause a change in the angle of light exiting the prism. This change can be measured with great accuracy and is proportional to the mass increase, whereas the concentration of the target analyte can be calculated [41]. The main setup of a surface plasmon resonance system includes a light source, a prism, a substrate surface with biomolecules, a flow system and a detector [42].

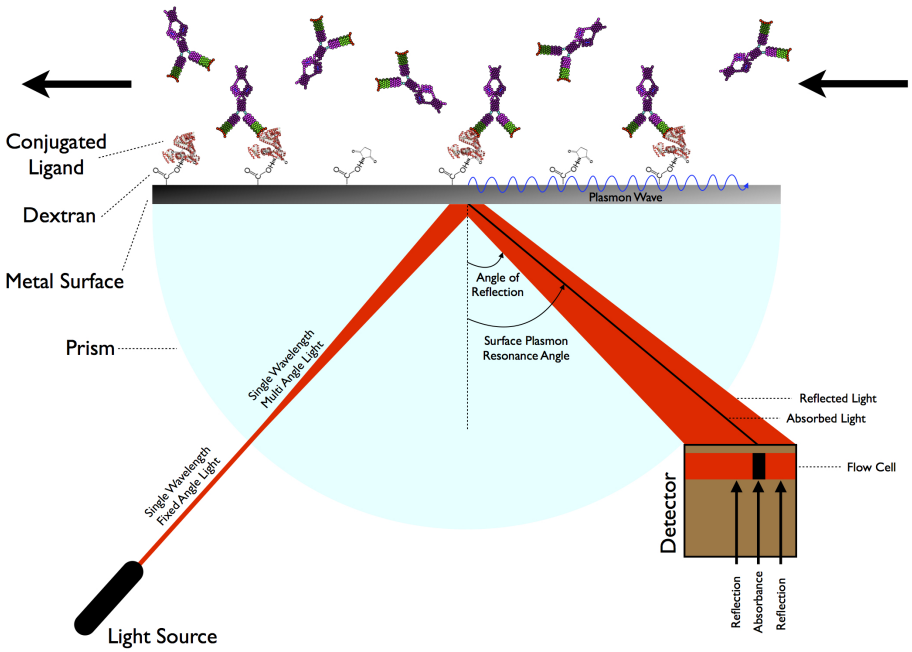


Figure 3.1: General setup for Surface Plasmon Resonance with a substrate surface covered with immobilized recognition elements, here Dextran, which selectively bind to analyte. Upon analyte binding, the resonance conditions of the surface plasmon waves and thereby the angle of reflection for the incoming light is changed. This small change is detected and can be associated with the amount bound. Reproduced under the Creative Commons Attribution-Share Alike 3.0 Unported license from source [43].

Biosensors - Detection Method: Impedance Spectroscopy

When for example a bacterium binds to the recognition molecule on the biosensor surface, the electrical properties of the surface changes. In (Electrochemical) Impedance Spectroscopy, this fact is used to determine the amount of bound bacteria by applying a sinusoidal voltage signal, $U(j\omega)$, with small amplitude to the electrode with immobilized affinity elements, while measuring the corresponding current, $I(j\omega)$. The ratio between the voltage and current, the complex impedance $Z(j\omega)$, consists of a real and an imaginary part that depends on the resistance and capacitance of the electrochemical cell. When alternating the frequency of the applied voltage, an impedance spectrum can be presented for example as a Nyquist plot with the resistance, $Re(Z)$, and the reactance, $Im(Z)$, on the axes [44].

Impedance spectroscopy can only be considered as a semi-continously detection method as an accurate characterization of the electrode surface requires recording of impedances over a broad range of frequencies. That often takes around 15-20 minutes.

Coulter Counter

In a *coulter counter*, a liquid is transferred from one reservoir to another through a micro channel that serves as an aperture. When the composition of the liquid changes in the channel, as when a bacterium passes, the electrical resistance changes due to movement of the conductive liquid. This change can be registered as an electric pulse by adding electrodes at the ends of the channel and measure impedance variations. Each pulse corresponds to the passage of a particle and the signal strength correlates to particle size and conductivity, wherefore individual particles can be quantified and sized. The coulter principle is illustrated in Figure 3.2.

The method has several advantages like low limit of detection, immediate detection results, portability and small size. However, the throughput is low and there is a high risk of clogging in the small channel. Another limitation is the identification sensitivity; the measurements only provide size information [45].

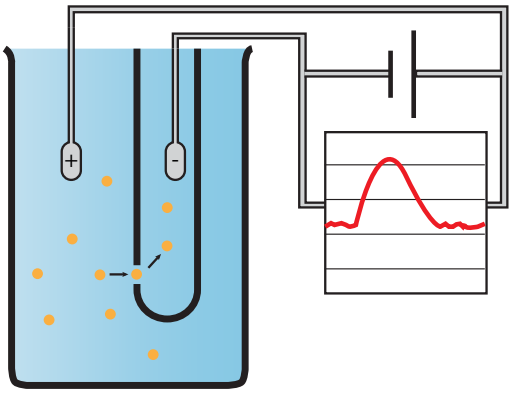


Figure 3.2: The general principle of a coulter counter. Conductive solution is separated in two containers with the exception of a small aperture. When a particle is passing the aperture, electrodes present in the liquid register the change in impedance and a pulse signal is generated.

Flow Cytometry Measurements of Bacterial Auto-fluorescence

Flow cytometry is an optical technology that uses lasers with specific wavelengths to, in real time, identify and quantify scattering events in a stream of liquid. The general principle of flow cytometry is illustrated in Figure 3.3 The method is often used for cell applications but can also detect objects as small as bacteria, then often labeled with fluorophores. However, it has been previously shown that intracellular components, e.g. flavins, can be fluorescent and emit detectable light between 500-560 nm when exposed to a laser beam with the wavelength 450-490 nm. The approach has been applied to eight different bacterial strains, all emitting autofluorescent signals but with a large variation in intensity. The intensity spectra of each strain can hence potentially be used to detect and identify the type of bacteria. Compared to the fluorescence of eukaryotic cells the bacterial autofluorescence is much lower and the cells are smaller. Consequently higher sensitivity of the flow cytometer is required.

This method achieved accurate bacterial detection at single-cell level with rapid and reliable results. The further refinement of the method is restricted by the apparatus sensitivity and background fluorescence causing disturbances in the measurements [46].

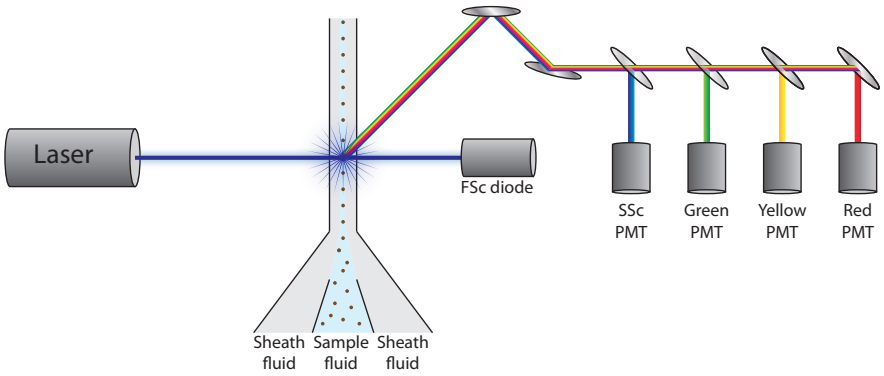


Figure 3.3: The main principle of flow cytometry. When light from a laser hits a particle in the focused flow the laser light is both side and forward scattered. From the side scattered light, eventual fluorescence with lower wavelengths are sorted out with dicroic mirrors and filters and thereafter amplified and detected by photomultiplier tubes.

3.2.2 Methods for Endotoxin Analysis

Biosensors - Recognition Element: Molecular Imprinted Polymers

Beside microbial detection, biosensors can be useful also for detection and quantification of endotoxin. One way to specifically adress endotoxins in the affinity at the sensor surface is to use *molecular imprinted polymers*, MIPs. Those are synthetic receptor molecules produced in a polymerization process where a set of different monomers can bind to a template covered by immobilized target molecules. The MIPs can then be extracted from the templates thus obtaining high affinity molecules which thereafter can bind to the target analyte during the analysis. Upon analyte binding to these artificial recognition sites the amount of bound analyte can be quantified by a correlated detection method. The MIPs can obtain similar binding characteristics as antibodies but with advantages such as lower cost, higher stability, easier fabrication and chemical modifications. Functional groups can be attached to the MIPs to enhance covalent binding and to act as a self assembled monolayer.

A study employed of the MIP method together with *Escherichia coli* endotoxins used as template, achieved a limit of detection at 15.6 ng/ml, which roughly corresponds to 75-150 EU/ml [47]. The endotoxin recognition by MIPs was combined with surface plasmon resonance for de-

tection. The accomplished limit of detection was however rather high to match the requirements of endotoxin content in dialysis water, but since it was the lowest concentration tested one can not exclude the possibility of an even lower limit of detection [48].

Biosensors - Recognition Element: Aptamers

Aptamers are short strands of peptides or oligonucleotides, such as RNA or single strand DNA, which can be produced synthetically to target practically any molecule. The aptamers can be designed to mimic the antibody binding to specific molecules with high affinity and selectivity. Like MIPs, aptamers may be linked to functional groups and can form a self assembled monolayer on a gold electrode. The aptamers can substitute the usage of antibodies due to their several advantages; higher stability, easier and more cost-effective production and non-immunogenic behavior. An aptamer-based biosensor with impedance detection reached a limit of detection of 1 pg/ml for endotoxin analysis. The method has high affinity for endotoxins, sufficiently low detection limit to be applied on RO-purified water, simple operation and low cost production. However, no further information of incubation time is available, thus it is unclear if implementable in an on-line monitoring system [49].

3.2.3 Methods for Chemical Analysis

Total Organic Carbon-analyzers

In terms of water analysis, *total organic carbon*, TOC, refers to the total amount of organic carbon in the water, which can be used as a non-specific estimation of water quality. It can be measured in laboratory by for instance consumption of the oxidizing agent permanganate, MnO_4^-, which is the method referred to in SLS:2016.0 [14]. However, there are instruments on the market that can measure the amount of TOC continuously in flowing water with very high precision. The principle they use is to detect a secondary parameter that is dependent on the amount of CO_2 before and after a step of total oxidization of the carbon in the water. One common technique to do this, used among others by the international company Merck Millipore in their MilliQ instruments is through conductivity measurements and oxidization by UV-light [50]. When irradiating the water with UV-light, water molecules are con-

verted into hydroxyl radicals. These will in turn oxidize the organic content in the water into carbon dioxide that is dissolved in the water as dihydrogen carbonate, which then split up to ions. The created ions will increase the conductivity of the liquid and by measuring the conductivity before and after the irradiation a value of the TOC content can be received [51]. An illustration of this is shown in Figure 3.4 below.

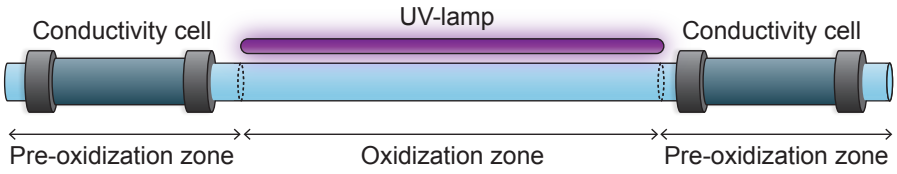


Figure 3.4: For a UV-TOC-analyzer, the conductivity of the water is measured before and after total oxidization of all organic carbon

The chemical equations are as follows:

$$\begin{aligned} C_xH_xO_x &\longrightarrow CO_2 + H_2O \\ CO_2 + H_2O &\longrightarrow H_2CO_3 \\ H_2CO_3 &\rightleftharpoons HCO_3^- + H^+ \end{aligned}$$

The method requires that all organic content is completely oxidized to not give false results, consequently the flux can not be too high nor too massive since it will shorten the time of UV-excitation and oxidization. The achieved limit of detection for this method can be really low e.g. the *5000TOCi* system from *Mettler-Toledo Inc.* has an LOD of 0.025 ppb carbon which corresponds to approximately 0.12 ppb permanganate consumption [52][53]. A value that is profoundly below 8 mg/l, which is the limit prescribed in SLS:2016.0.

Another common way to measure the amount of oxidized compound is through *non-dispersive infrared analysis*, NDIR, where the difference in IR absorption before and after oxidization is measured at a wavelength specific for carbon dioxide. This difference correlates to the concentration of CO_2 at the moment of detection.

Total Chlorine-analyzers

As with TOC analyzers, there are many chlorine analyzers for on-line use available on the market with different functions, designs and to

different costs. Among these, there are in general two types of chlorine monitors: *DPD colorimetric analyzers* and *amperometric analyzers*. The DPD colorimetric method requires titration of N,N-diethyl-p-phenylenediamine (DPD) that reacts with all free chlorine molecules in the water and creates a colored product and thus the chlorine concentration can be read as a color shift by photosensors. Amperometric methods are reagent free and expected to require less maintenance. In addition to that, they are also expected to be relatively free from interference from other compounds than chlorine in comparison to colorimetric sensors.

The principle of amperometric analyzers is to apply a voltage over an anode and a cathode and measure the passing current. The size of the current is dependent on the reduction of hypochlorous acid that takes place at the cathode. Since free chlorine appears as either hypochlorous acid (HOCl) or hypochlorite ions (OCl^-), with a pH-dependent distrubution, the concentration can be determined if the pH is known. The sensors can also be modified to register both free chlorine and combined chlorine, which results in total chlorine. As a commonly existing additive for drinking water is chloramines, which are a type of combined chlorine. Not only the free chlorine but the total chlorine amount needs to be detected in the case of water quality verification for dialysis [54].

One advantage with amperometric chlorine sensors is that the technique is rather easily miniaturized with moderately preserved detection limit. A micromachined free chlorine monitor with three electrode configuration showed a linear range of detection between 0.3 ppm to 1.6 ppm which indeed is three times higher than the dialysis water chlorine limit prescribed in SLS:2016.0 but which probably could be improved a lot if industrially produced [55]. Larger amperometric total chlorine analyzers can have much lower limits of detection and detect chlorine concentrations on the ppb level [56].

Flow-head for Miniaturized Chemical Sensors

To enable simultaneous monitoring of several chemical elements a design of a multicomponent chemical analysis detector has been developed. The device contains a set of selective chemical sensors which can detect a specific analyte on-line and without sample preparation. The

flow-head allows the implementation of ten different chemical sensors, hence up to ten distinctive elements can be measured simultaneously. The different sensor cells are placed in series with a common reference electrode in the center to maintain the same distance to each sensor. Therefore, results from potentiometric sensors are comparable due to the equal measuring conditions. The design of sensor mounting allows various types of sensors to be incorporated, e.g. ion selective electrodes, silicone solid state electrodes or optical sensors [57].

If placed in a flow configuration and incorporated with different sensors the device can be suitable for an on-line, real-time multiparameter measurement. *Ion selective electrodes*, ISEs, can be purchased and easy integrated within the flow-head system. The principle of an ISE is simple and consist of an ion-selective membrane which selectively allows the passage of a specific ion-type. The ion flux creates a potential difference across the membrane surface which can be converted into an electrical or optical signal. The strength of the signal is proportional to the ion content of the fluid and consequently, the ion concentration can be monitored. ISEs are commonly used both in industry and in laboratory environment and are available for numerous analytes including chlorine, fluoride, sodium, nitrate and ammonium [58].

3.3 Discussion and Conclusion

Biosensors

Biosensors have big potential due to their label free, fast, sensitive and specific measurements of bacterial and endotoxin content. Furthermore, several biorecognition elements and corresponding detection methods are available, with studies done where low limits of detection were obtained. The biggest challenge with biosensors is anyhow the obtainment of sufficiently low limit of detection to be used within dialysis applications. In order to achieve this, a low flow velocity is necessary to enhance the analysis sensitivity and enable binding of all bacteria or all endotoxins within the selected flow.

If it in the future is going to be possible to use biosensors in connection to the dialysis system, it is essential to ensure that the recognition biomolecules are fully immobilized on the sensor surface and that no

undesired substances leaks out in the water stream that goes to the patient. Consequently, it is essential to make routine controls of sensor performance and coverage.

Coulter Counter

The coulter counter offers a simple and sensitive method with real-time analysis results. Single bacteria can be detected and identified with great accuracy but since the medium within this application is or at least is supposed to be ultrapure, the conductivity is very low. Hence it can be difficult to measure electrical variations within the fluid. On the other hand due to the low concentration within the media other common problems for coulter counters can be avoided; the risk of coincidence and clogging. The probability of two particles passing the sensing zone of the detector simultaneously is rather low. Thus, false double amplitude signal and count loss are not likely.

Flow Cytometry

The available flow cytometers are often large and very expensive and therefore not possibly placed in the patient's home. However, even if the existing equipment often is very advanced with multi-purpose use, the principle is rather simple and does in theory not require too many components. Therefore, if flow cytometry should be used for dialysis water quality verification it definitely needs to be simplified and miniaturized. For example, existing flow cytometers use a buffert to hydrodynamically focus the sample fluid so that particles line up. But if it is known how big the excited volume is, the concentration could be calculated anyway.

Furthermore, as reported in the flow cytometry study concerning bacterial autofluorescence [46], the flow velocity needs to be very slow, thus requiring a stable control system.

TOC-Analyzers

As reported in section 3.2.3 the TOC-analyzers are already an existing high performance tool on the market for water quality measurements. However, organic carbon is not necessarily a dangerous compound in itself and more to consider as an indicator of the water quality and the

performance of the purification system. As the WRO 300 H-unit from Baxter already includes accurate conductivity monitoring, an integration of a TOC analyzing by UV-oxidization and conductivity measuring would not be a too large step to take.

Total Chlorine Analyzers

The chlorine content is almost a necessary parameter to measure online, or at least on-site, since it is a volatile and according to SLS:2016.0 the determination of the total chlorine amount should be made within one hour after the sample-taking. Therefore an amperometric total chlorine sensor would be suitable to prioritize if integrating sensor equipment for water quality analysis in home hemodialysis systems. Especially since chlorine historically has been associated to severe complications in hemodialysis [7]. As with TOC analyzers the technique is already on the market, it just has to be produced to a lower cost and custom-made to fit in dialysis context.

Flow-head Multiparameter Sensor

The flow-head multiparameter setup enables monitoring of several parameters simultaneously but requires purchase of sensors and accordingly, implementation of these to the configuration. The method could be combined with easily produced screen printed electrodes but however, the problem with finding enduring and long functioning electrodes remain.

Sensor Placement

In order to verify the quality of the dialysis water and nothing else, any sensor with this purpose must be placed somewhere after the reverse osmosis membrane and somewhere before the first manipulative step in the dialysis circuit. This means that the sensors can be a part of either the purification unit, the dialysis machine or placed as separate units on the water pipe between these two. A benefit with an integration of the sensors in the dialysis machine is that it is a later step and any possible contamination on the water's way from the purification unit will be detected, while the advantage with adding it to the purification would be that these units are more often designed for HHD use specifically.

However, to obtain the low detection limits and high sensitivities needed, almost all of the analysis techniques described in the previous section requires low flow velocities, wherefore the sensors cannot be used to analyze the main flow directly. Figure 3.5 shows two different possibilities on how to perform this. The analyzed water can either be pumped back to the main flow or led away to drain. The first alternative means that no purified water is wasted and the second alternative enables the use of reagents, which makes it less devastating if the water is contaminated by the sensors. However, the second alternative results in an open circuit which implies an extra site for possible contamination of the main flow.

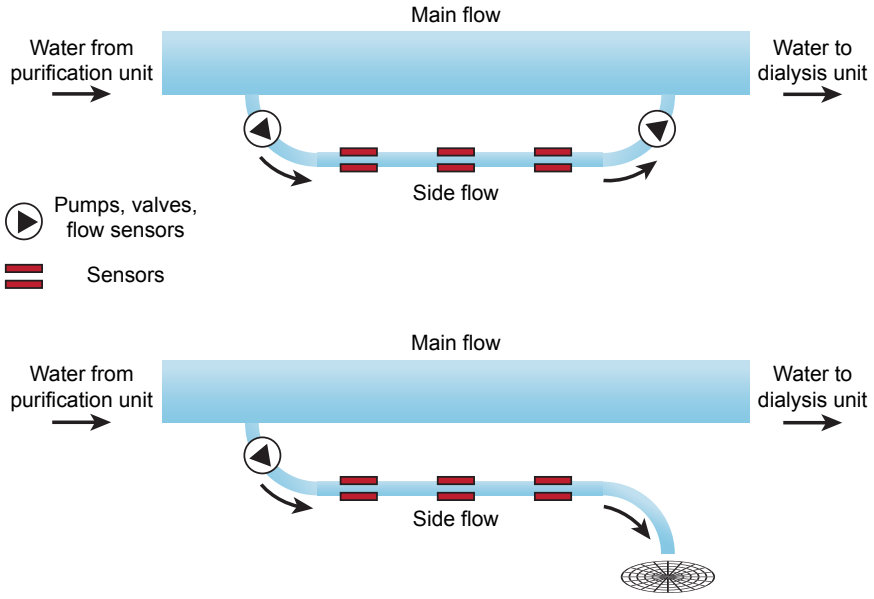


Figure 3.5: Two types of different sensor placements. In both of them the water from the main flow is diverted to a much smaller and preferably laminar flow with low flow velocity. This process is controlled with pumps, valves and flow sensors. In the upper type, water is pumped back to the main flow and in the lower type, analyzed water is wasted.

When leading away the water to the side flow, it is also important to verify that the composition of the removed water is identical with that of the main flow. If this is maintained and the flow velocity is known

with high accuracy, the concentrations in the sub-sampled side flow can easily be calculated and thought to represent the concentrations of the total flow.

Chapter 4

Experimental Work

4.1 Choice of Analytical Method

Several aspects were considered when making the decision of which technique to conduct in the following experimental work; fulfillment of the requirements stated in chapter two, possibilities for development within this project, the time perspective and the interests of the authors.

Biosensors seemed like a suitable solution with high accuracy, on-line monitoring capacity and relatively easy operation. However the time and equipment needed to fabricate, develop and implement these type of sensors together with the complexity of substrate attachment and sensitivity maximization made it improbable to fit within the boundaries of this thesis. One possibility would have been to use industrially manufactured sensors, but no suitable were found.

As a matter of fact, any fabrication of microstructures would have been possible but too time-consuming to have enough remaining time to evaluate what have been fabricated. Therefore, construction of a uniform micro channel without edges and roughness, needed for a sensitive coulter counter appeared unrealistic. Besides, the risk of clogging and entrapment of contamination particles during the fabrication process is high and the process require ultrapure environment. These device manufacturing complications were significant when choosing not to continue with the method due to the risk of not obtaining any results. However, the Department provided a regular size coulter counter, which could have been used for the tests but unfortunately it had been reported

to malfunction when used for bacteria counting. Ultimately, the coulter principle requires a conductive buffer for high sensitivity measurements and does consequently not go very well with ultrapure water.

A continuation of both the total organic carbon and the total chlorine analysis would involve the implementation and limitation testing of already existing monitors. These devices are, however, rather expensive and even if they could have been funded, the order times exceeded what was reasonable. However, it would be beneficial for Baxter to further investigate an implementation of these monitors to the existing WRO in a longer time perspective.

Finally, the choice fell on flow cytometry as a high quality cytometer was available for experiments at the Department. Furthermore, from the specialization in nanobiomedicine the authors had already gained some knowledge and practice within flow cytometry and the associated result analysis. Beside these practical factors the method had promising potential to be further investigated and adjusted to the dialysis application. The limit of detection were sufficiently low, no reagents were needed, the approach could be conducted on-line in real time and the specificity for bacteria could be investigated.

4.2 Hypothesis

As a result of the conclusions in chapter 3, a hypothesis was constructed in order to be the basis of the experimental work:

"...to continuously detect bacteria in the preparation water for dialysis in concentrations below 100 CFU/ml with help of flow cytometry measurements detecting the bacterial autofluorescence."

This hypothesis includes some requirements for the method. It should be selective for *bacteria*, including different species and excluding other types of fluorescent particles. The found bacteria should be found in *preparation water for dialysis* which means ultrapure water where they might not survive for a longer time due to a hypotonic environment and absence of nutrients. The method should also have a detection limit *below 100 CFU/ml* which makes it necessary to count only viable units. The bacteria should be discriminated by their *autofluorescence* as pri-

mary parameter.

4.3 Theory

4.3.1 Bacterial Autofluorescence

The principle of autofluorescence is the natural emission of light when a molecule, without any dyes or markers added, is excited with radiation of suitable wavelength. The energy from the radiating photons is absorbed and the molecule reaches an excited state with higher energy. When returning to ground state the decrease in energy generates an emission of photons with a specific wavelength, resulting in fluorescence. The released photons, the fluorescence, are always lower in energy and hence have longer wavelength. The difference between the excitation and emission wavelength depends upon the molecule.

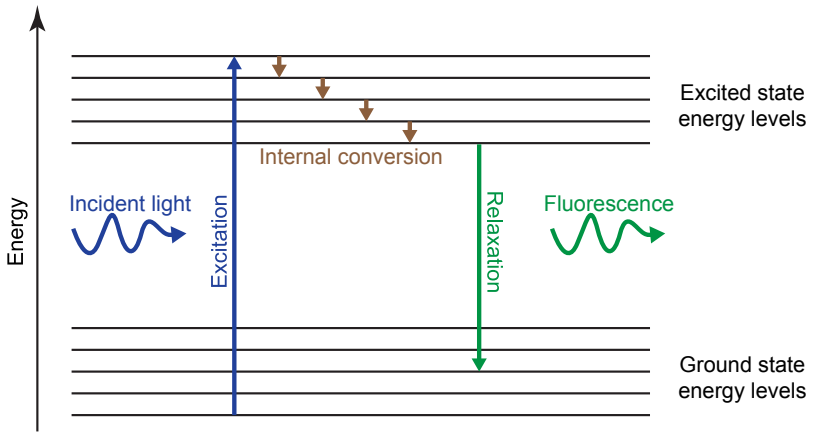


Figure 4.1: Excitation of a fluorophore to a higher energy state through radiation with a certain wavelength results in emission of fluorescent light during the relaxation to the ground state.

Most cells contain intracellular components with autofluorescent behavior, these include aromatic amino acids, NADPH and flavins. These endogenous molecules with specific excitation and emission wavelengths can be useful for biological detection and characterization. When illuminated by the lasers within a flow cytometer they absorb some of

the radiation energy and dispose it by releasing photons. Flavins that are important coenzymes in cellular metabolism have in their oxidized form excitation and emission spectra at 450-490 and 500-560 nm respectively. Advantageously, the flavin emission spectra overlaps with the commonly used fluorochrome FITC's spectrum and their autofluorescence can hence be detected in the same wavelength interval.

Nevertheless, in microscopy and flow cytometry assays the autofluorescence are often considered as a problem due to an interference with commonly used fluorescent labeled samples giving non-sensitive and blurry results. Consequently, the signals from autofluorescent molecules are usually sorted away [46].

4.3.2 Flow Cytometry

The flow cytometer is a diagnostic device for in vitro identification and enumeration of mainly biological material in suspension. Flow cytometers were originally designed for large eukaryotic cells, such as blood cells, for medical analysis, diagnosis and research. Mostly when used for bacterial detection different fluorescent dyes or markers are used [59]. The apparatus consist of three main parts; a fluidic system, an optical system and an electronic system to convert the signals.

The Fluidic System

Once placed into the cytometer the sample is pumped into the flow cell and the suspended particles are hydrodynamically focused into a single file with help of a buffer, called the sheath fluid. The dimension of the sample stream can be controlled by adjusting the pressure differences between the sheath fluid and the sample fluid. The particles in the sample fluid are then passing the laser beams detachedly so that sensitive scattering and fluorescence signals can be generated.

The Optical System

At the laser interrogation point, the light is scattered in all directions. Both signals from the forward- and side-scattering are detected. The forward-scattered light gives a measure of the particle size and the side-scattered light provides information of the granularity of the particle. Whereas the forward-scattered light move in the same direction as the

laser beam, the scattered light need to be directed by lenses through beam splitters and filters so that it reaches the photomultiplier tubes, PMTs, where it can be registered. Through division of the side-scattered light into wavelength intervals, any fluorescence of the particles can be detected.

Generally, two types of filters can be used for the spectral division; long pass filters and band pass filters. The long pass filter transmits all light with wavelength longer than a certain number and either attenuates or reflects light with shorter wavelength. The band pass filter does instead transmit light only within a certain wavelength interval and reflects all other incident light. Through a very subtle placement and combination of band pass and long pass filters, fluorescent light can be identified and separated for a number of common fluorophores in the visible light spectrum.

The Electronic System

As mentioned above, a signal is generated when incident photons hit the PMTs and by adjusting the voltage of the tubes, very low signals can be amplified.

The registered signal pulse is thereafter transported to the electronic board where the analog signal is converted to digital discrete data. The analog-digital-converter does this by sampling the signal in short time intervals and assign each interval with a measured value.

These values are then adjusted in time to correspond to the correct spatial displacement of the excitation laser beams. Only when a pulse exceed the preciously regulated threshold it is further processed by calculation of pulse height and area. The maximum digitized value correspond to the height whereas the pulse area is calculated by the sum of all measurements within a time interval.

This sample data can be analyzed and provide all the information about particle size, granularity and internal complexity. If several properties are detected simultaneously, information about every single particle can later be used to divide the total amount of particles into several populations and sub-populations with help of any proper software.

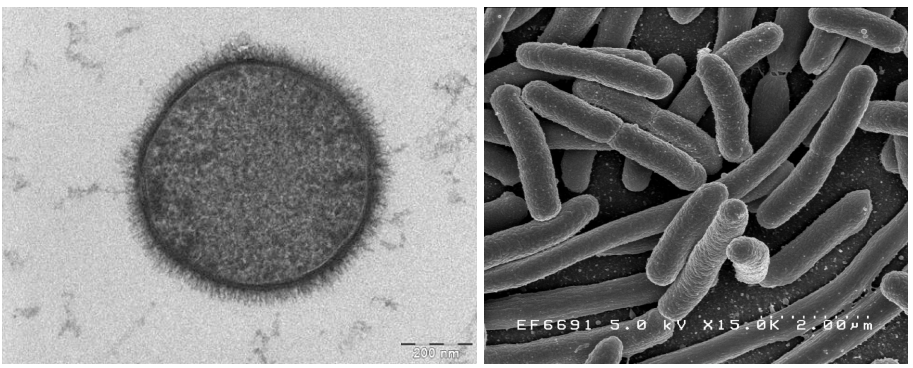
4.3.3 Bacterial Charachteristics

Escherichia coli

Escherichia coli bacteria are commonly used in research and biological lab experiments and are well-known organisms with establishment in many biological laboratories. The organism have several favorable characteristics; short doubling time, simple cultivation and do not sporulate, hence they are easily eradicated. The bacteria is gram-negative, rod-shaped and generally about one μm in size. Although generally harmless some strains can cause illness such as diarrhea [60].

Bacillus subtilis

Bacillus subtilis bacteria is naturally found in soil and vegetation and is broadly adapted to survive in several types of environment. Due to its normal harsh habitat with small amounts of nutrition the bacterium can endure extreme conditions of heat and desiccation by producing dormant endospores [61]. In contrast to the *E. coli* the *B. subtilis* is gram-positive and therefor have one thick cell membrane instead of two thinner [62]. The bacteria are non-pathogenic and are considered harmless for humans, animals and plants [63].



(a) *Bacillus subtilis* (b) *Escherichia coli*

Figure 4.2: Left: A cross-section image of *Bacillus subtilis* taken with a *Tecnai T-12* transmission electron microscope, by Allon Weiner, The Weizmann Institute of Science, Rehovot, Israel. 2006. Right: Scanning electron microscopy image of *Escherichia coli*. Taken by Rocky Mountain Laboratories, NIAID, NIH.

4.4 Method

In order to investigate the possibilities to register and detect non-labeled bacteria by their autofluorescence, a testplan with several sub-experiments was set up. Firstly, fluorescence labeled beads were analyzed to estimate the approximate size and find appropriate flow cytometer settings. Thereafter bacteria were stained and run in the flow cytometer to enable a comparison with unstained bacteria of the same strain. Two types of bacteria were studied to confirm the hypothesis for more than one bacteria type. Different concentrations of the bacteria were analyzed aiming to detect very small amounts of bacteria as stated in the requirement analysis. Also, a comparison of bacteria media was made to investigate the difference between nutritionless ultrapure water and nutrient broth where the bacteria flourish. Additionally it was tested if it was possible to discriminate live bacteria from dead with use of two separate fluorochromes.

4.4.1 Equipment

The flow cytometer used within the experiments was the BD FACSCanto II. The apparatus can be configured with two or three lasers with the capacity to detect eight different colors together with forward- and side-scattering, giving detailed information about size, granularity and fluorescence of the sample.

The associated software, BD FACSDiva, counts the events in the flow and presents the results in graphical plots with eligible parameters on the axis. With gating tools the user can find and identify different populations and compare the fluorescence strength. For small particles, as bacteria, the instrument settings are especially critical and the threshold level should be sufficient high to exclude background noise but low enough to include the size of the bacteria. The signal amplification settings permit adjustment of bacteria population placement within the plot and to facilitate the threshold settings the population should be situated in the center.

To analyze and gather the flow cytometer data the software package *FlowJo v.10* (from FlowJo LLC, Ashland, OR, USA) was used. The program enables an easy procedure to collect and organize statistics by

visualization in graphs, tables and histograms. This facilitate the comparison of different groups of samples, experiments or even multiple experiments at once. Gates can be drawn and applied to different plots to sort out data of interest.

4.4.2 Chemicals and Bacterial Cultures

In some of the cytometry measurements when the bacteria had to be stained, the *LIVE/DEAD BacLight Bacterial Viability and Counting Kit* from Molecular Probes/Invitrogen was used. The kit contains two nucleic acid stains; green-fluorescent SYTO 9 dye and red-fluorescent propidium iodide. The SYTO 9 binds to nucleic acids in all cells whereas the propidium iodide only stains bacteria with damaged cell membranes, hence live bacteria with intact membranes can only be stained with SYTO 9 whereas dead bacteria can be stained with both of the fluorescent dyes. However, the membrane penetration of propidium iodide cause a reduction in SYTO 9 fluorescence intensity and dead bacteria will therefor emit a weaker green fluorescent signal. A mixture of the two colors can be used to distinguish and quantify the amount of live and dead bacteria within a sample. The SYTO 9 have excitation/emission maxima at 480/500 nm whereas the same values for propidium iodide are 490/635 nm. These spectra are illustrated in Figure 4.3.

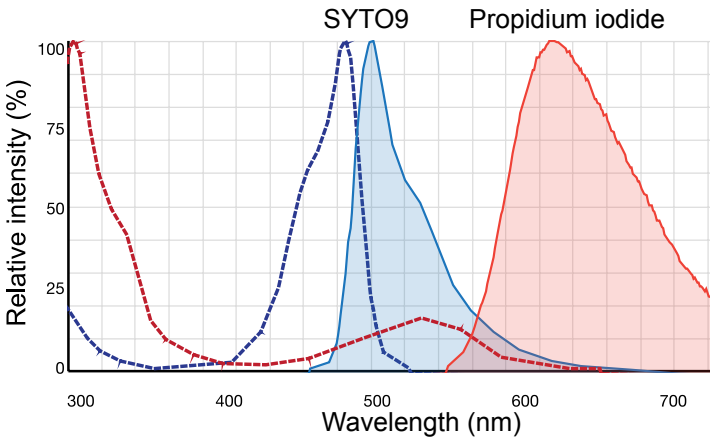


Figure 4.3: Emission spectra (dashed line) and excitation spectra (filled) for the the two fluorescent dyes SYTO9 (blue) and propidium iodide (red).

Two different bacteria strains were provided and used to evaluate and validate the method. The Escherichia coli, E.coli DH5alfa, acquired from the Department of Biomedical Engineering at Lund University were genetically modified with a constructed plasmid to achieve ampicillin resistance. Due to its antibiotic resistance contamination by other bacteria strains can be avoided by adding antibiotic to the samples. The Bacillus subtilis strain, CCUG 10779, from Culture Collection at University of Göteborg was not genetically modified to resist antibiotics, wherefore several risk assessments were necessary prior to use. Both bacteria strains can be considered non-pathogenic thus categorized in risk class 1 [64].

4.4.3 Experimental Processes

Bacterial Growth

To cultivate bacteria in a laboratory, a growth medium containing proteins, salts and growth enhancers that will support the bacteria are needed. In this project *lysogeny broth*, LB, was prepared, followed by the bacteria cultivation in the following steps:

1. LB-medium was prepared by mixing tryptone, yeast extract and NaCl in analytical grade milliQ water and later autoclaved.
2. Frozen bacteria were thawed and added to falcon tubes with LB-medium. Sterile pipette tips with filters were used during all experiments to avoid any contamination.
3. The tubes were incubated at 37°C in oven for approximately 24h. The tube lids were not tightened of to enable air circulation and oxygen supply.

Successfully grown bacteria culture was seen as an increase in turbidity. Since both bacteria strains are facultative anaerobes they gathered to a certain extent at the surface, forming a thin layer. The *B. subtilis* due to its endosporic nature formed a considerably more dense layer of bacteria, making the suspension harder to dissolve and less homogeneous.

Washing and Staining of Bacteria

The bacteria staining processes followed two different protocols. The first was provided by staff at BME and further refined to fit the measurements in this project. The general steps are listed below though varying in recurrence and performance time:

1. Washing by centrifugation, removal of supernatant and resuspension in filtered NaCl (9 g/l),
2. addition of dyes; SYTO9 and/or propidium iodide,
3. incubation of samples at room temperature in a dark space,
4. repetition of the washing procedure,
5. dilution to the desired level with either MilliQ-water, NaCl (9 g/l) or PBS+BSA (phosphate buffered saline plus bovine serum albumin),
6. dark and cool storage of the samples in refrigerator until analyzed in the flow cytometer to prevent bacterial growth or bleaching of the fluorophores.

The second protocol was the standard procedure for flow cytometry from the LIVE/DEAD BacLight staining kit manual and was used when accomplish the viability tests [65].

Flow Cytometry Measurements - General Settings

In the present study the settings of the FACSCanto II were alternated and analyzed to find the most appropriate ones. However, some settings were fixed for all measurements unless anything else stated.

The flow speed was set to the lowest possible, $\sim 10\ \mu$l/min, to maximize event detection and measurement sensitivity. The actual flow speed is somewhat uncertain since the flow is pressure regulated which results in varying flow rates for varying densities. Since many measurements were to be performed, the recording time was limited to 30 seconds.

Threshold limits were set on both scattering measurements, FSC (Forward-Scattering) and SSC (Side-scattering), to 200 to reduce signal noise since data below this boundary will not be acquired. During the experiments the solutions were stored in dark and mostly in refrigerator, and sonication was performed immediately prior to analysis.

Flow Cytometry Measurements - Autofluorescence

In order to investigate if bacterial detection by their autofluorescence is possible, unstained and stained bacteria of the same strain were compared. Firstly *E. coli* were stained with the previously described nucleic dye SYTO 9. To detect the fluorescence the FITC detector with a 530/30

band pass filter was used. The same detector was used for the unstained bacteria since the autofluorescence of the flavins is more or less in the same spectrum. Thereafter the same procedure was preformed with the *B. subtilis*. The population gate in forward- and side-scattering intensities for the bacteria was set after the stained bacteria since it could easily be detected through its fluorescence. The same gate were then used for the subsequent experiments with unstained bacteria to study whether any autofluorescence could be detected.

Flow Cytometry Measurements - Concentration Determination

With the purpose to investigate how low concentrations the FACSCanto II could detect, an assay was set up to count the number of bacteria through forward- and side-scattering events.

Firstly, high concentrated bacteria suspensions of unstained *B. subtilis* and *E. coli* with visible turbidity were prepared and diluted in steps of 1:10, until diluted $1{:}10^4$. The high concentrations were used to simplify characterization of the registered events as bacteria through gating. If the total count of a sample was not high enough to exceed the least-event-limit of the flow cytometer, no results were recorded. If so happened, the recording time was increased to 60 or 120 seconds to enable gathering of data of these samples.

Concentration Determination of CFUs/ml on Agar Plates

A method of counting colony forming units grown on agar plates was carried out to control the concentration of the analyzed samples as a comparison to the flow cytometry results. The TGEA Agar Plates were acquired from Baxter together with instructions for bacteria handling and the plate count procedure. The process was conducted in the following steps:

1. Dilution of the bacteria solution until weak turbidity is reached, indicating a concentration of 10^5 - 10^6 CFU/ml,
2. repetition of the dilution in steps of 1:10 until reaching concentration of approximately 10^0 CFU/ml,
3. distribution of each dilution sample, ranging from 10^0 to 10^5 - 10^6 CFU/ml, on the agar plates until the surface of the plates are dry,

4. incubation at 37°C for about 48 hours,
5. counting of appeared colonies. The appearance and morphology of the colonies should be similar for the same type of bacteria, hence a deviation in morphology indicates of a contamination.

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Flow Cytometry Measurements - Comparison Between Bacteria in Ultrapure Water and Isotonic Buffer

To investigate whether the solution media impacts the measurements or not in terms of background fluorescence and impact of bacterial size, a comparison of nutritionless ultrapure water and isotonic buffer was conducted. The ultrapure water was conceived from a Milli-Q water system while the isotonic buffer was prepared by filtration of NaCl in Milli-Q water together with bovine serumalbumin. The same concentration of bacteria was used for two different suspensions and later on run in the FACSCanto II.

Flow Cytometry Measurements - Viability

To quantitatively discriminate live and dead bacteria within a sample a viability assay was conducted. The instructions from the *LIVE/DEAD BacLight Bacterial Viability Kit* with some modifications were followed in the subsequent steps for *B. subtilis* and *E. coli* independently:

1. The bacteria suspensions were washed as in the previous experiments and divided into to equal amounts in two separate tubes. Thereafter, 95 % analytical grade ethanol was added to one of the tubes and incubated for one hour, with the intention to kill the bacteria. The same volume of NaCl solution was added to the second tube to keep the bacteria alive.
2. Live and dead bacteria suspensions were mixed with the following ratios; 0:100, 20:80, 40:60, 60:40, 80:20 and 100:0, with a total volume of 1 mL.
3. The two fluorescence dyes; SYTO 9 and propidium iodide, were mixed and added to all of the bacteria samples. The samples were then incubated in dark for 15 minutes.

Since no 70% isopropyl alcohol (recommended in the manual) was available, 95% ethanol was used instead.

During the flow cytometry, FITC (530/30 BP filter) and Per-CP (670 LP filter) detectors were used to register the fluorescence from the two dyes, SYTO 9 and propidium iodide, respectively.

4.5 Results

The results from the flow cytometry measurements are presented in graphic dot-plots. The acquired plots represent two parameters where each axis corresponds to the intensity of the selected parameter and each dot in the plot represents one registered event, with scattered intensities high enough to exceed the threshold values. In most cases logarithmic axes are used to allow all dots to be visualized in the window.

4.5.1 Flow Cytometry Measurements - Autofluorescence

One example of many performed flow cytometry measurements, that should prove the relation between the fluorescence from stained bacteria and the intrinsic fluorescence from unstained bacteria, is presented in Figure 4.4.

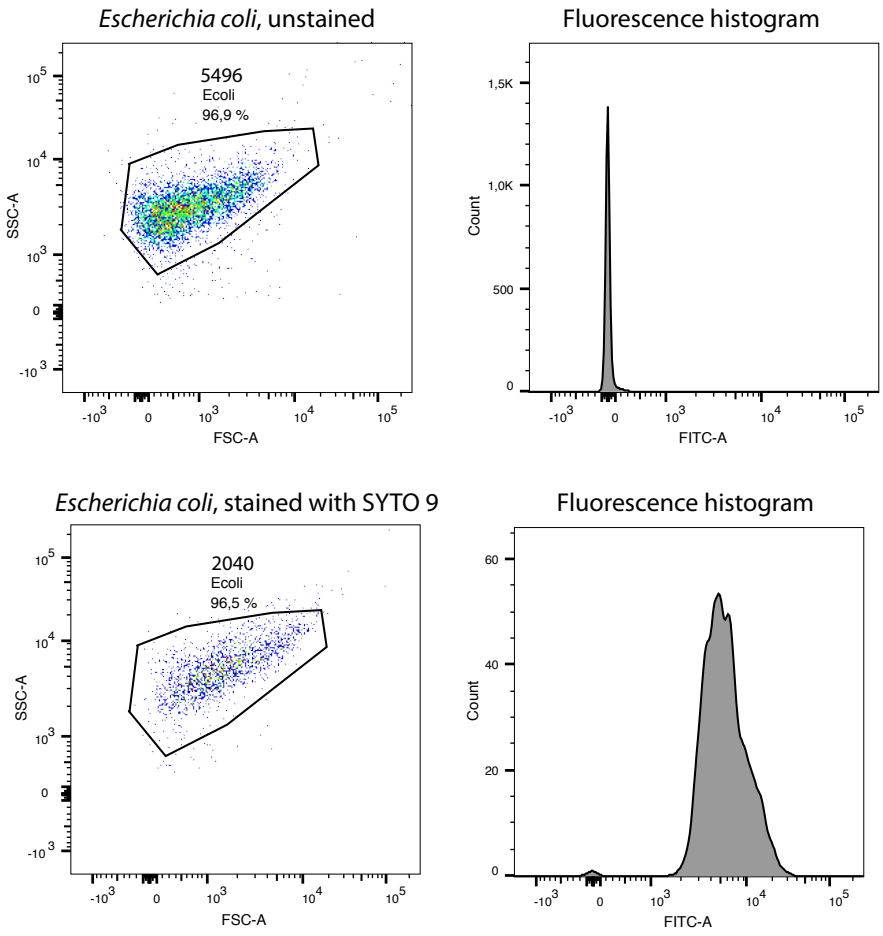

Figure 4.4: Flow cytometry measurements of two approximately same concentrations of *Escherichia coli*. The upper row shows unstained bacteria and the lower row shows the results for bacteria dyed with the nucleic acid stain SYTO 9. The left dot plots shows the FSC- and SSC-gates for the bacteria and histograms of the gated populations respectively green fluorescence are shown to the right. A shift from blue towards green and red on the event dots indicates a higher density of events at this position than what could be resolved in the image.

The registered number of events appear to be much lower for the stained bacteria even though roughly the same concentration were used before staining. With these settings, an obvious fluorescence is seen for the stained bacteria but no traces of the autofluorescence were found since all events in the upper right diagram of Figure 4.4 lies around zero in intensity.

4.5.2 Flow Cytometry Measurements - Concentration Determination

As described in the method section, four unstained samples of both bacteria types were examined in the flow cytometer. Dot plots for each sample is presented in Figure 4.5.

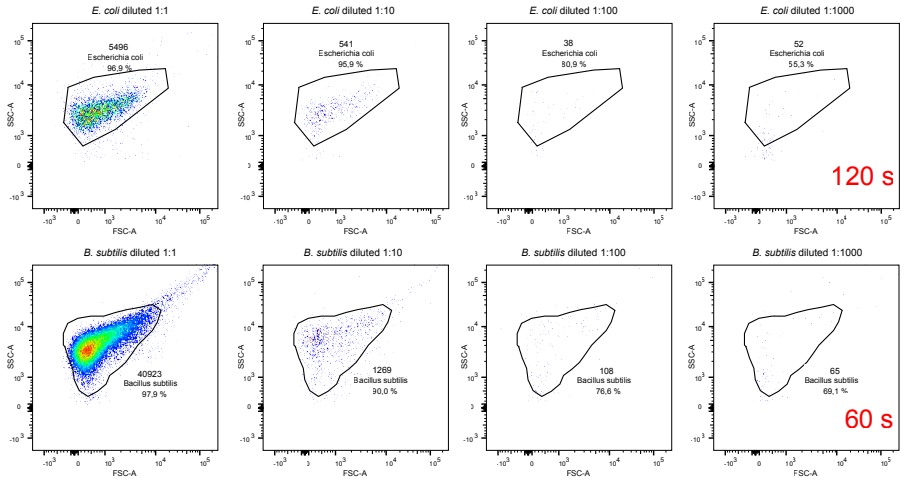

Figure 4.5: Flow cytometry measurements used to calculate the concentration of the bacteria suspensions at different levels of dilution. Suspensions with unstained *B. subtilis* and *E. coli* were run in dilutions from 1:1 - 1:1000 and plotted as a function of their forward- and side-scattered intensities. The acquisition time had to be increased from 30 to 60 respectively 120 s for the two samples diluted 1:1000 to obtain any data.

The gates were set in reference to the highest concentrations and should surround at least 95 % of the bacteria. For all samples, the numbers of events in the population were used to calculate the bacteria concentrations, c, of the undiluted samples with equation 4.1.

$$\frac{n}{Q \cdot t} \cdot f \tag{4.1}$$

where n is the number of events, Q is the flow rate ($\sim 10\mu$l/min), t is the acquisition time and f is the dilution factor. The calculated concentrations are presented in Figure 4.6.

The bacteria concentration of the samples in Figure 4.5 were also deter-

mined through standard plate counting. The number of colonies visible on each plate is reported in table 4.1

E.coli (unstained)	**CFU/plate**	**B.subtilis (unstained)**	**CFU/plate**
1:1	>1000	1:1	>1000
1:10	>1000	1:10	>1000
1:100	>200	1:100	>200
1:1000	54	1:1000	41

Table 4.1: The results from the concentration determination through plate counting for the same suspensions as used in Figure 4.5. The number of colony forming units grown on agar plates were counted for unstained bacteria, *Escherichia coli* and *Bacillius subtilis*, with four different dilutions.

From these counts the concentration of the undiluted sample (1:1) could easily be determined by division with the used volume and multiplication with the dilution factor. Concentrations should be calculated from plates with 30-300 CFUs and the best suited for this were the the ones diluted 1:1000 for both bacteria types.

$$c_{E.c.} = \frac{54}{0.100 \text{ ml}} \cdot 1000 = 5.4 \cdot 10^5 \text{ CFU/ml}$$

$$c_{B.s.} = \frac{41}{0.100 \text{ ml}} \cdot 1000 = 4.1 \cdot 10^5 \text{ CFU/ml}$$

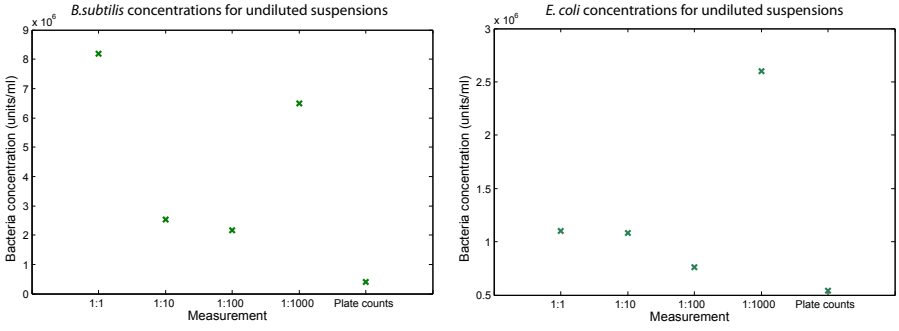

Figure 4.6: A plot over the calculated concentrations of both bacteria through both flow cytometry and standard plate counting. Which measurement that was regarded for which concentration is marked on the x-axis.

4.5.3 Flow Cytometry Measurements - Comparison Between Bacteria in Ultrapure Water and Isotonic Buffer

To analyze whether the type of medium used for the bacteria suspensions has any influence on the flow cytometry process or bacteria behavior, a comparison was made, with results presented in Figure 4.7.

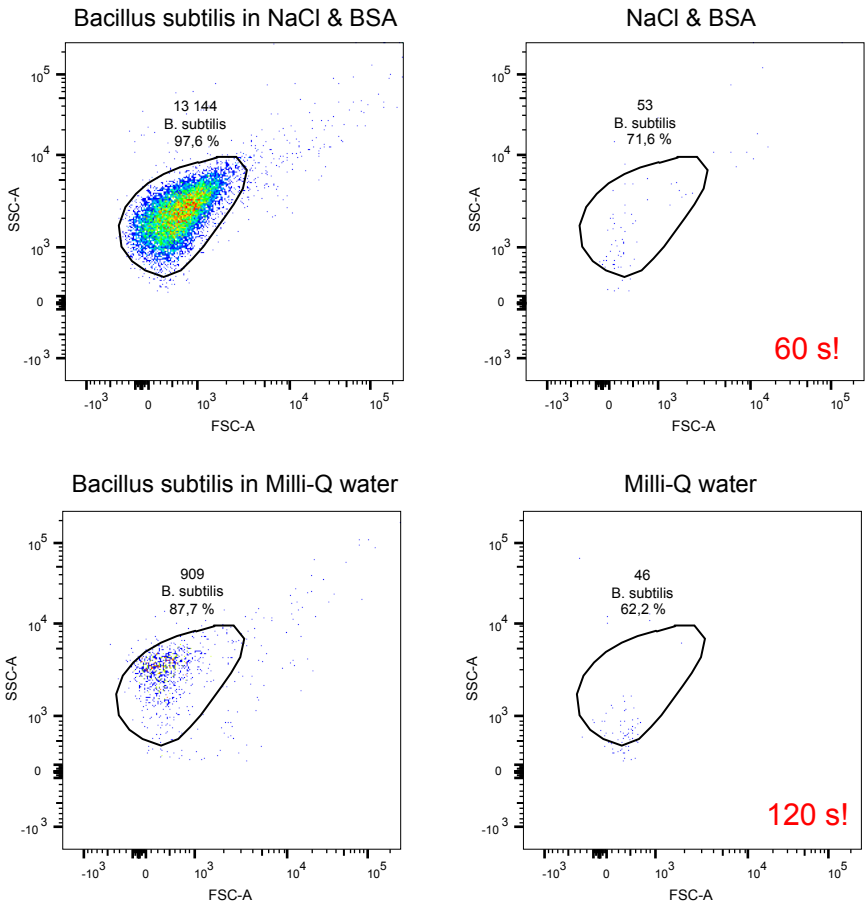

Figure 4.7: A comparison between similar amounts of *B. subtilis* bacteria suspended in either sodium chloride and bovine serum albumin or ultrapure Milli-Q water. The two right plots show the registered events of blank samples of both media, without any addition of bacteria. A population gate was set to define how big the false contribution from the medium is to the bacteria counts. Since the particle content of the blank samples were low, the acquisition time had to be longer than 30 s.

Something noteworthy is that the registered concentration is lower for bacteria suspended in Milli-Q water than in NaCl and PBS. However, Milli-Q water produced less undesired scattering events than the filtered buffer solution.

4.5.4 Flow Cytometry Measurements - Viability

To discriminate the amount of viable bacteria within a sample, viability tests were conducted for the two bacteria strains. Dot plots for three out of five examined mixtures of live and dead bacteria are presented in Figure 4.8 for both bacteria types.

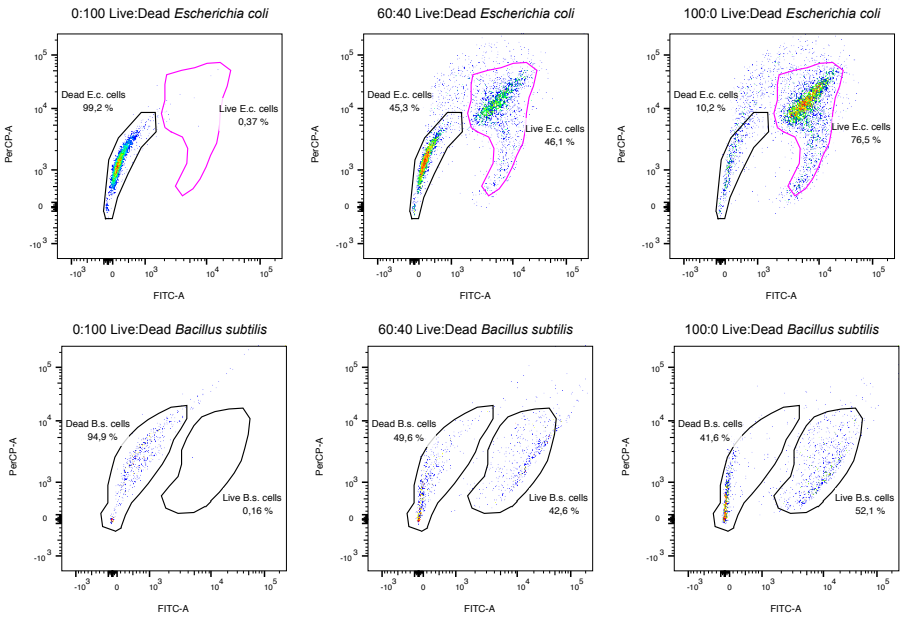

Figure 4.8: Flow cytometry discriminations between live and dead bacteria to decide the viability of a mixture. The samples in the upper row contained *E. coli* and the samples in the lower row contained *B. subtilis*. The samples in the left column had only content from the tube with killed bacteria, those in the left column came from the tube with live bacteria and the two in the middle consisted of a mixture between live and killed bacteria with the ratio 60:40. The two parameters measured are the green fluorescent intensity, FITC, on the x-axes and the red fluorescent intensity, PerCP, on the y-axes. The percentages annotated beside the populations are the fractions of the total counts belonging to each populations.

The number of registered events in each population was then used to decide the proportion of live bacteria by dividing the numbers of events in the live population with the sum of events in the both gates. All events that lie outside the two gates are considered to be uncharacterized and are not included in the viability estimation.

The viability estimation is made through a comparison of the live proportions measured with flow cytometry and the intended fractions of live cells of the mixing process. A graphical presentation of this comparison can be viewed in Figure 4.9

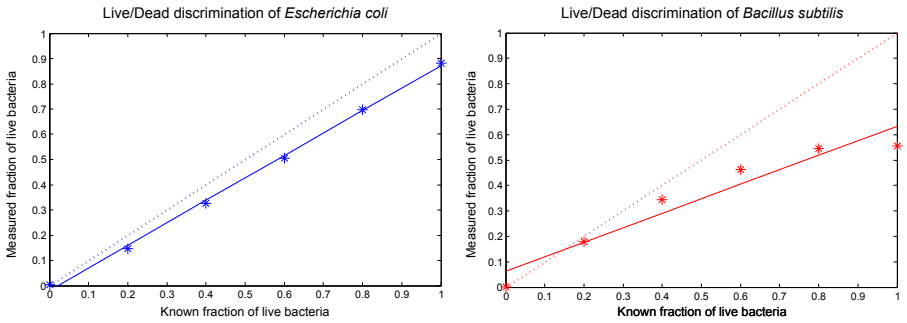

Figure 4.9: An estimation of the measured viabilities for *E. coli* respectively *B. subtilis* in relation to the known viability plotted as stars for the five different fractions. ”Known” viability is in this case defined as the ratio between added amount of live bacteria suspension and killed bacteria suspension. The filled line shows the least square fit of the points and when extrapolating it to the expected values (dashed line) an estimation of the amount of dead bacteria in the live suspension is obtained. If evaluating the graphs above, these values are 11 % for *E. coli* and 43 % for *B. subtilis*.

With this data in mind, it is clear that the *E. coli* mixtures generated a more linear viability distribution curve than the *B. subtilis* mixtures. The measured live content of the initial bacteria suspensions were also higher for *E. coli* with 89 % compared to 57 % for *B. subtilis*.

4.6 Discussion

4.6.1 Preparation of Bacteria Suspensions

For more predictable results, the initial concentration of bacteria suspension could have been estimated by measuring the optical density of the suspension with a spectrophotometer prior to preparation. Since this equipment was not available to use in this project it was a bit uncertain if the adequate amount of stain was used for each sample.

Additionally, bacteria are living organisms that divide and increase in number under the right conditions. This phenomenon makes it somewhat harder to decide the concentration of bacteria in solution through flow cytometry as it changes if a sample is stored in room temperature or if it contains nutrients. It is not known how much influence this had on the concentration measurements in this project but the aim was always to reduce the effect by performing the measurements as soon as possible after the sample preparation and if not possible, storing them in a refrigerator for a short time. For further measurements a bacteriostatica such as sodium azide, NaN_3, can be used to prevent bacterial growth after preparation, thus resulting in difficulties with plate counting comparisons.

4.6.2 Autofluorescence

From the autofluorescence experiments one can conclude that detection of bacteria by their autofluorescence is hardly executed with the FACSCanto II since the fluorescent intensity peak for the unstained samples is found around zero, as displayed in Figure 4.4.

To succeed with the detection, it would be preferable with a much lower flow rate and a more sensitive and accurate apparatus, like the one used in the previously cited study [46]. A lower flow rate increases the excitation time of each bacteria and would facilitate the detection of the autofluorescence signal. Additionally, it would be favorable to calibrate the PMT amplification of the autofluorescence signals with non-fluorescent particles and particles with known fluorescence intensity. In this study, no such calibration was performed and adjustment of the PMT voltage was only done with respect to the strong fluorescent signal from the stained samples. However, even with the current equip-

ment an eventual autofluorescence signal could have been amplified to a detectable level, but unlikely distinguished from the synchronously amplified background fluorescence and electronic noise.

The lower concentration attained in the SYTO 9-stained sample is most likely a result of the pipetting during the cleaning process where a certain amount of bacteria might have accompanied the removed supernatant. Consequently a more exact method to clean and prepare bacteria suspensions could be helpful when searching for such weak signals as the intrinsic fluorescence of minuscule microbes.

4.6.3 Concentration Determination

Five of the concentration measurements in Figure 4.5 are somewhat unreliable because of their low event rate. First of all, the *Bacillus subtilis* sample generated an excess of the recommended maximum count of 1000 events/s. Too high event rates may cause interference through simultaneously registered particles and the high concentration increases the risk of aggregation between bacteria. Such bacteria clusters appears as larger particles in the scattering plot, which could explain the outlying ”tail” of events in the upper right corner of the graph.

Moreover, a common problem with the FACSCanto II is that it only records and plots multiples of the least-event-limit, which for the used settings was 47 events. Even lower event numbers were counted but not plotted which becomes an issue for low concentrations as in the four measurements diluted 1:100 and 1:1000, where the plotted events amount to either 47 or 94. This software problem must be bypassed in order to decrease the detection limit and obtain a sufficiently high resolution. However, the results are not useless but less accurate when calculating concentrations of the suspensions.

An important error source was discovered after the measurements, when analyzing the recorded data. It was found that not all bacteria in the suspensions had been registered since the threshold for the measured signals to be treated as a single event was set to be height dependent of the forward- and side-scattered signals. This loss was not visible during the measurements since the events were plotted dependent on the area of the intensity signals. This is clearly visible in Figure 4.10 when plotting

them as dependent on the height of the scattering intensities instead.

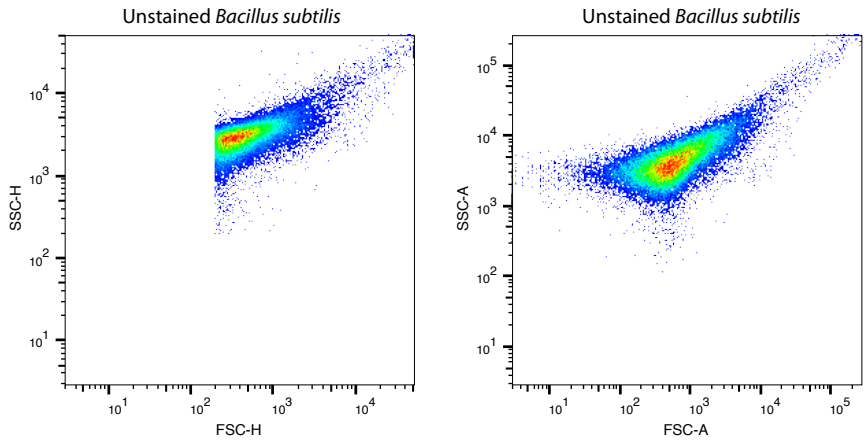

Figure 4.10: A comparison between the same sample of *B. subtilis* when plotting the heights of the scattered intensities and when plotting the intensity areas in the right figure. Since the threshold is set to "SSC-H 200 AND FSC-H 200-H" a fraction of the bacteria is not detected.

Whenever this detection loss occurred during the experiments a lower bacteria concentration than the real was calculated. Therefore, the comparison between the bacteria concentrations determined with flow cytometry would differ from those determined with plate counting of colony forming units. To avoid this phenomenon, the PMT voltages should have been adjusted during data acquisition with the same parameters as the threshold values had been set to. A further amplification of the scattering signals might however have resulted in an amplification of the background signal above the threshold limits. A slower flow rate would then have been necessary to increase signal to noise ratio but this was not possible with the FACSCanto II cytometer.

Anyway, even if a part of the counts was aborted, the concentration through plate counting is lower than the results acquired from the flow cytometry measurements regardless dilution, as seen in Figure 4.6. It is therefore essential to realize that two related but still different parameters have been measured with the two methods; CFU concentration and bacteria concentration. The correlation between them is complex, since not all cells will result in a colony forming unit and since some cells might merge in to one colony forming unit. Beside this, as was shown

in the viability measurements, the bacteria suspensions commonly contain a proportion of dead cells and only live cells can reproduce.

The fact that the percentage of events inside the gates, belonging to the bacteria population, decreases with lower concentrations indicates that a certain amount of noise is registered in each acquisition. The noise level could preferably be confirmed and taken into account through slightly longer background measurements of the medium.

4.6.4 Comparison Between Bacteria in Ultrapure Water and Isotonic Buffer

For some reason the bacteria concentration was significantly lower when suspended in Milli-Q water than in NaCl and BSA. The difference is most likely an effect of an improper cleaning process as already described in section 4.6.2. Less likely but still reasonable theories are either that the hypotonicity of the Milli-Q water has increased the internal osmotic pressure of the cells and caused lysis of a proportion of them or that the environment of the bacteria in NaCl and BSA contained nutrients enough to allow cell division.

However, these are only guesses and more measurements with same method at different occasions must be performed to obtain any statistical significance and to verify if the effect is reproduced.

If considering the two blank samples, they still generated particle counts both inside and outside of the bacteria gate. It is always hard to say without further investigations if these background signals belong to actual particle contamination of the medium or if it comes from electronic noise. Nevertheless, the Milli-Q water claimed a lower background content than the NaCl-BSA-solution and can therefore be considered purer. The Milli-Q water was thought to exemplify the WRO water for the dialysis application whereas the NaCl-BSA-solution was prepared to nourish the bacteria.

4.6.5 Viability

Two rather distinct populations could be identified for both bacteria even though a somewhat too high amount of events landed up in the

outlying and uncharacterized region. Since the gates were set manually, this could have been prevented by including more events in the regions but that would probably have lead to inclusion of false counts to the population in a higher extent. Thus, more explicit constrains would have been useful when deciding the sizes and shapes of the population gates.

According to the calculations, some dead bacteria were found also in the live sample and *B. subtilis* had a larger part of dead bacteria, most likely due to issues during the cultivation. It should also be mentioned that the straight line approximation to the live proportion values in Figure 4.9 is not fitting especially well. Besides, determination of dead content in the live suspension through extrapolation of the 100 % live value of the fitted straight line to the theoretical value is not as accurate when the line is far from passing origo.

In contrast, the results from the *E. coli* measurements were both linear and behaved to a greater extent as expected in the viability test. The reason for this might be caused by the difference of the bacterial cell membrane structure. The gram-positive *B. subtilis* have one thick cell membrane whereas the gram-negative *E. coli* have two thinner membranes. This structural difference might affect the penetration level of the nucleic dyes resulting in variations in fluorescence between the two bacterial strains.

Since the dyes bind to the DNA within the bacterial cell, the emission properties change when both dyes are present due to displacement of the fluorescent molecules [66]. This occur for the dead bacteria with damaged cell membrane and because of the presence of both dyes the SYTO 9 signal is somewhat reduced for the dead bacteria which can be seen as a lower FITC intensity for the killed bacteria in Figure 4.8.

Due to the absence of the appropriate propidium iodide filter the filter for PerCP was used instead. This filter is not optimal for the propidium iodide fluorescence even though the emission intervals overlap. As a direct consequence the intensity is lower than expected and it is difficult to separate the contribution of the broad SYTO 9 emission from the PI emission in the detected red fluorescence. Especially since there were problems with the used compensation protocol that should prevent

cross-signal of one dye into another dye's channel. This is probably an explanation to why the live cell populations appear to have not only higher fluorescence in the FITC interval but also in the Per-CP interval even though it was not stained with any red fluorophore.

Also the usage of the word "dead" demands careful deliberation in this context. The propidium iodide dye stains bacteria cells with non-intact cell membrane which certainly is a sign of less viability of the organism but does not assure that it can not recover or reproduce under any circumstances.

4.7 Conclusion

During this experimental part of the project, many lessons were learned about the possibilites but also the difficulties involved when using flow cytometry for bacteria detection and water quality analysis. The extremely low detection limit needed for dialysis purposes puts the problem in a needle-in-haystack position. Also, bacterial autofluorescence detection require extremely fine-tuned equipment and it can be difficult to distinguish bacteria from similarly sized particles. On the other hand, no such objects should exist in the highly purified water, and the flow cytometer could work as a warning bell for issues with the purification process that has lead to this existence.

However, flow cytometry as a method is superior to standard plate counting in the aspect of analysis time. Furthermore, additional information about size, populations and ratios can be acquired forming a technical quantitative microbial analysis method.

Even if flow cytometry will not be used for on-line measurements in home hemodialysis in the nearest future, it is a good challenger to push down the plate counting method from its pronounced hold of the throne of microbiological quantification methods. However, to be practicable for routine analysis the method need to be fully automated, smaller, cheaper and more sensitive for bacteria application.

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Appendix A

The formulary sent to HHD-clinics

The questionnaire consisted of five sections with the following questions

1. General information

- Which clinic does it regard?
- How many patients are under HHD-treatment at the clinic?

2. Microbial analysis, 3. Analysis of endotoxins and 4. Chemical analysis

The following questions were repeated in each of the three sections

- How are the samples taken, stored and transported?
- With what frequency is the sample-taking normally performed? Does the time between the occasions vary?
- Does the clinic have any directions that dictates the routines for the sample-taking?
- Who is performing the sample-taking?
- Where are the samples analyzed?
- What analysis methods are used?
- How long time does it take between sample-taking and test answer? Does the time vary? Do you have any limits for the longest allowed response time?
- What is the approximate cost per sample divided into sample equipment, labor cost, transport cost and cost for the analysis?

5. Reflections

- Which part of the water quality verification process do you find most troublesome or demanding?
- Do you have any desires about any technical equipment that could facilitate the process?