Planctomycetes as a Source for Cytotoxic Activity Towards Cancer Cells

Anne Lunde

Master of Science in Biology – Microbiology
Department of Biological Sciences, University of Bergen

Supervised by:
Prof. Lise Øvreås, Department of Biological Sciences, University of Bergen
Prof. Lars Herfindal, Department of Clinical Science, University of Bergen

Spring 2021

Acknowledges

This master thesis was created as research for the research group Marine Microbiology at Department of Biological Sciences at the University of Bergen (UiB), throughout the period of June 2020 to June 2021. The thesis was written with supervision from Lise Øvreås at the Department of Biological Sciences and Lars Herfindal at the Department of Clinical Sciences at UiB and Haukeland hospital.

I would like to thank Lise and the team at Marine Microbiology for an amazing opportunity, working with the most exciting, interesting and difficult group of bacteria I can think of. Thank you for your advice on writing style and for trusting me with several different projects at a time. Thank you for inviting me into a community where I was received with open arms and positive feedback at the PVC webinar where I had the great honour to be a speaker. Thank you also for providing access to closed labs throughout the covid-19 pandemic. A special thanks to Hilde Rief Armo at Marine Microbiology for teaching me the layout of the lab, assisting me at every step, answering my questions and helping me carry heavy desk top shakers. I could not have submitted this thesis without you.

Thank you to Lars and Reidun and the team at Haukeland for teaching me all I know about cultivation of cells, extraction of phases and fractions and for welcoming me warmly into an amazing group of pharmacists and nanotechnologists. I would have never thought that I would be welcomed with such ease and become part of the group, even receiving a Christmas gift from the lab Santa Clause. Thank you for having open arms and open ears, even at times when all I did was ask stupid questions.

Finally, I would like to thank my family and friends. A big thank you to my three older sisters who have provided support, inspiration and excellent examples of hard work. Thank you to Hans-Henrik for support through periods of hard work and long nights, and to my friend Aisha for your inspiring interest and excitement for my work.

Abstract

Planctomycetes are a ubiquitous phylum in the bacterial domain. They are known to possess unique traits distinguishing them from other bacteria, such as the formation of rosette aggregates, the lack of FtsZ-protein used in binary fission and the preferred division by budding, large genomes and crateriform structures in the cell wall. The bacteria are found in diverse habitats, including in extreme environments and in association with other organisms such as macroalgae and diatoms. Planctomycetes are therefore presumed to be productive producers of quorum sensing autoinducers and secondary metabolites, which could be useful as bioactive molecules in medicine.

In this study, Planctomycetes strains from various habitats were tested for cytotoxic potential towards acute myeloid leukaemia cell line MOLM-13, prostate cancer cell line PC3 and normal rat kidney cell line NRK. The strains were cultivated in M30 medium, harvested and freeze-dried before being tested for cytotoxic activity in aqueous phase and organic phase, using both small and large volumes of biomass. To examine where the cytotoxic potential was located and obtain some knowledge on what kinds of bioactive molecules were responsible for the cytotoxic potential, solid phase extraction (SPE) was conducted. This extracted fractions of aqueous or organic extracts, and separated chemical compounds from the samples. High performance liquid chromatography (HPLC) was conducted to visualize selected SPE on a chromatogram and further separate the SPE into smaller fractions. Experiments using the separated fractions were conducted on the cancer cell lines to investigate which fractions showed cytotoxic potential. Results showed that the Planctomycetes had cytotoxic potential towards both cancer cell lines, with aqueous extracts being most cytotoxic towards MOLM-13 and organic extracts being most cytotoxic towards PC3. Aqueous extracts were found to induce apoptosis in MOLM-13, while organic extracts presumably inhibited cell growth in PC3. The study showed polar and hydrophilic cytotoxic compounds extracted from strain TBK2h. The compounds were either variants of the same substance, or very similar substances. Both hydrophobic and hydrophilic cytotoxic substances were isolated from strain P1a, presumably working together to produce cytotoxicity for the strain. The isolated cytotoxic compounds are scheduled to be chemically analysed to identify known or unknown cytotoxic substances.

Table of contents

Abbreviations

ACN = Acetonitrile C_2H_3N

Chl = Chloroform ($CHCl_3$)

DMSO = Dimethyl sulfoxide (C_2H_6OS)

E1 – elution phase 1

E2 – elution phase 2

EOH = ethanol (C_2H_5OH)

h = hour

HPLC – high performance liquid chromatography

L+W – load and wash phase

MeOH = methanol (CH_3OH)

milliQ = ultrapure water

mL = millilitre

PBS = phosphate-buffered saline

SPE – solid phase extraction

µL = microlitre

µm = micrometre

1 Introduction

1.1 Background

According to World Health Organization, cancer is the second largest cause for mortality worldwide each year ("Cancer," n.d.). In 2020, almost 10 million people died from various types of cancer, the most common being breast cancer ("Cancer," 2021). Cancer diseases are a class of diseases which have in common the cancer cell, an abnormal cell capable of uncontrollable growth. Cancer is not necessary malignant, but when it is, it can infiltrate vital organs and cause harm, or destruct the nearby environment, at times causing death (Um, 2015). Cancer treatment exists in several forms, including chemotherapy, radiation, surgery, immunotherapy and targeted drug therapy (Abbas & Rehman, 2018). Unfortunately, therapy resistance is a common theme amongst cancer patients, and is one of the main reasons for metastasis, meaning the spreading of cancer to new areas of the body, and relapse (Rueff & Rodrigues, 2016; X. Wang, Zhang, & Chen, 2019). The causes of therapy resistance are based on changes in the cancer cells, which may result in a tolerance towards the treatment. This can happen through genetic mutations or as selection for resistance through repeated exposure (Gonzalez-Angulo, Morales-Vasquez, & Hortobagyi, 2013; X. Wang et al., 2019). Breast cancer is one of the most common cancer types, where therapy resistance is very common. At the start of treatment, targeted cytotoxic drugs are effective in 90% of the cases. Unfortunately, this percentage sinks considerably after some time, and therapy resistance is to be expected (Gonzalez-Angulo et al., 2013). The threat of cancer and therapy resistance is a calling for science today to focus on the search novel sources of cytotoxic drugs. This includes exploring new territories and new organisms, looking for natural producers of bioactive compounds. The bacterial phylum Planctomycetes are one of these still enigmatic and understudied bacterial phyla, demonstrated to have great biotechnological potential for production of novel bioactive compounds (Graça, Calisto, & Lage, 2016).

1.2 Aim and objectives

The aim of the study was to identify anticancer activity in Planctomycete bacteria from various habitats, focusing in on isolation of cytotoxic compounds in the bacteria. For this purpose, four research objectives were specified:

1. Obtaining high biomass of Planctomycetes, for use in large-scale extractions of bioactive molecules
2. Isolation of secondary metabolites from the bacteria with the potential for anticancer activity
3. Screening for anticancer activity in secondary metabolites using analysis of metabolic activity and induced apoptosis in the cell lines
4. Further isolation and characterization of chemical compounds capable of anticancer activity, using high performance liquid chromatography (HPLC)

1.3 Introduction to Planctomycetes

Planctomycetes are a phylum of Gram-negative bacteria who are unique in many ways, especially in comparison with other bacterial phyla (John A. Fuerst & Sagulenko, 2011). Their ecological, phylogenetic and morphological differences have made them great candidates for use in applied science, both within medicine (Calisto et al., 2019) and in environmental research (Helmer et al., 2001; Strous, Van Gerven, Gijs Kuenen, & Jetten, 1997). Historically, Planctomycetes were discovered in 1924 by Hungarian biologist Nador Gimesi. In a Budapest lake, he viewed what looked like planktonic ovoid or spherical cells with stalks and thought they must have been eukaryotes. He therefore interpreted them as fungi, and gave them the name *Planctomyces bekefii,* where the genus name is a description of what he saw - plancto deriving from plankton, and myces meaning fungi (Gimesi N, 1924). Independently, in 1935, Arthur T. Henrici and Delia E. Johnson of the University of Minnesota discovered their own stalked organism, which they identified as a bacteria and named *Blastocaulis sphaerica* (Henrici & Johnson, 1935). Later studies revealed that the two organisms *P. bekefii* and *B. sphaerica* were indeed the same species, a bacteria, which kept its original eukaryotic name, *Planctomyces bekefii* (Hirsch, 1972). Although, like many Planctomycetes, the bacteria has never been cultivated (Dedysh et al., 2020), the name was still recognized in The Approved List of Bacterial Names from 1980 (Skerman, McGowan, & Sneath, 1980). In fact, the first ever pure culture of Planctomycetes was isolated near 50 years after the discovery of the phylum (J. T. Staley, 1973), and surprisingly few have been obtained in axonic cultures at all (Kaboré, Godreuil, & Drancourt, 2020; Ward, 2015). To understand the relevance and importance of this difficult-to-cultivate bacterial phylum, it is necessary to dig deeper into the specifics of its uniqueness, which is described in the following subchapters.

1.3.1 Ecology of Planctomycetes

Planctomycetes, although scarcely studied in comparison to many other bacterial phyla, are known to be found in various habitats. They are widespread around the world and can be observed in various and vastly different natural environments. The first discovered specimen was collected from freshwater lakes in Budapest, Hungary (Gimesi N, 1924). Previously, it was known that the phylum was restrictively found in freshwater, which has since been disproved. Although it has commonly been found in freshwater ecosystems (Brümmer, Felske, & Wagner-

Döbler, 2004; Franzmann & Skerman, 1984; Pollet, Humbert, & Tadonléké, 2014), the phylum has also been detected in various other habitats, including some more extreme ones.

Planctomycetes were firstly successfully isolated from soil in Australia in the year 1992 (Liesackt & Stackebrandt, 1992). Since then, they have been reported in terrestrial environments all over the world (Buckley, Huangyutitham, Nelson, Rumberger, & Thies, 2006). They have been isolated from soils in farmed land (Borneman et al., 1996; Derakshani, Lukow, & Liesack, 2001), on rain forest floors (Borneman & Triplett, 1997), and amongst *Sphagnum* moss (Dedysh, Pankratov, Belova, Kulichevskaya, & Liesack, 2006). A number of strains have also been found living in marine environments, for example in the Indian ocean and on the coasts of Oregon and Namibia (Jian Wang et al., 2020; Woebken et al., 2007). Marine environments also includes on the surfaces of marine eucaryotes, where the Planctomycetes thrive (Kaboré et al., 2020). The bacteria have been discovered living in biofilms on, among many examples, algae and kelp (Bengtsson & Øvreås, 2010; Longford et al., 2007), on sponges and corals (Pimentel-Elardo, Wehrl, Friedrich, Jensen, & Hentschel, 2003; N. S. Webster, Wilson, Blackall, & Hill, 2001; Nicole S. Webster & Bourne, 2007) and even on the giant tiger prawn (*Penaeus mondon)* (J. A. Fuerst, Sambhi, Paynter, Hawkins, & Atherton, 1991). More extreme environments where Planctomycetes are found include the salt lakes of the Atacama desert in Chile (Drees et al., 2006), deep sea hydrothermal vents and iron hydroxide deposits (Kato et al., 2010; Lanzén et al., 2011; Julia E. Storesund & Øvreås, 2013), the acidic habitats of wetlands (Ivanova & Dedysh, 2012), and lastly in the human gut (Caroline Cayrou, Sambe, Armougom, Raoult, & Drancourt, 2013). The phylum is commonly found in these various habitats organized in biofilms or amongst other microorganisms in microbial mats (Prieto-Barajas, Valencia-Cantero, & Santoyo, 2018; Wiegand et al., 2020).

This diversity in habitats and environmental factors including temperature, pH and salinity, suggests a strong capability of adaptation within the phylum. It is also suggested that planctomycetes thrive in environments which include other organisms, such as in biofilms or microbial mats, which points to the bacteria being highly capable of communication and also defence.

1.3.2 Phylogeny of Planctomycetes

Looking at Planctomycetes in a phylogenetical light, there are some interesting hypothesises and facts to be found. Planctomycetes were originally classified as eukaryotes, but when it was known that they were indeed bacteria they were for some time taxonomically classified as a genus (James T. Staley & Bauld, 1981). In 1986, Schlesner and Stackebrandt described their findings of a new family, *Planctomycetaceae* which combined the two genera *Planctomycetes* and *Pirella*. They also described a novel order, which they named Plancomycetales (Schlesner & Stackebrandt, 1986). Later findings based on more modern 16s rRNA analysis have concluded that Planctomycetes are indeed a phylum (Ward, 2010).

Planctomycetes are also a part of the bacterial superphylum Planctomycetes, Verrucomicrobia and Chlamydiae (PVC phylum), which encompasses the phyla it is named after in addition to the phylum Lentisphaerae and some uncultured candidate phyla. The Planctomycetes phyla is of particular interest due to the fact that research on members of the group have shown that they can be of medical and biotechnological importance (Wagner & Horn, 2006). It is also speculated if Planctomycetes or members of the PVC superphylum might be in some way ancestral to all bacteria (Brochier & Philippe, 2002), or share a common descendant with eukaryotes due to their homology (John A. Fuerst & Sagulenko, 2012). This last statement has been questioned and compromised in later research, where the Gram-negative ancestry of Planctomycetes has been strengthened (Devos, 2014a, 2014b; Jeske et al., 2015).

The planctomycete phylum, although widely spread and abundant, consists of few described individuals and groups. The phylum includes, according to the National Centre for Biotechnology Information (NCBI) Taxonomy Browser as of spring 2021, 3 classes, 9 orders, 13 families, and 68 genera, wherein a large number of the genera are represented by only one species (Schoch et al., 2020). The strains mentioned in this study belong to the genera *Blastopirellula, Gimesia, Rhodopirellula and Rubinisphaera.* Strains P2s, P61, P62 and P1a were unclassified, but they seem to be belonging to the family of *Planctomycetacea* (Sæbø, 2018). Planctomycetes are known to have especially large genomes with a median of around 7,4 Mb, the norm being somewhere around 3-4 Mb for other sequenced bacteria (Andrei et al., 2019; Reva & Tümmler, 2008).

1.3.3 Morphology of Planctomycetes

The Planctomycete phylum is known to have distinct appearances, and uncommon features which are scarcely found in other phyla. For quite some time it was thought that Planctomycetes did not have a cell wall that included the protein peptidoglycan, and it was believed that they might be encased in a unique protein envelope instead (König, Schlesner, & Hirsch, 1984; Liesack, König, Schlesner, & Hirsch, 1986). It was later found that the phylum indeed possesses peptidoglycan cell walls, making it fit more into the category of Gram-negative bacteria (Jeske et al., 2015; Van Teeseling et al., 2015). Another theory that has been questioned is the claim that some planctomycetes, namely the strain *Gemmata obscuriglobus,* has a nucleus that is membrane-encompassed, much like a eukaryote cell (John A Fuerst & Webb, 1991). It was later proposed that this claim was faulty, and that the membrane that was found did not encompass a nucleus, but it was rather invaginations of the inner membrane found in the cytoplasm (Santarella-Mellwig, Pruggnaller, Roos, Mattaj, & Devos, 2013). These discoveries may have hindered the illusion that the Planctomycetes are exceptionally unique fairy-tale-like creatures, but the phylum is nonetheless different form others in many ways. The likenesses and differences between previous and current descriptions of a Planctomycete cell, eukaryote cells and ordinary Gram-negative cells are detailed in figure 1.

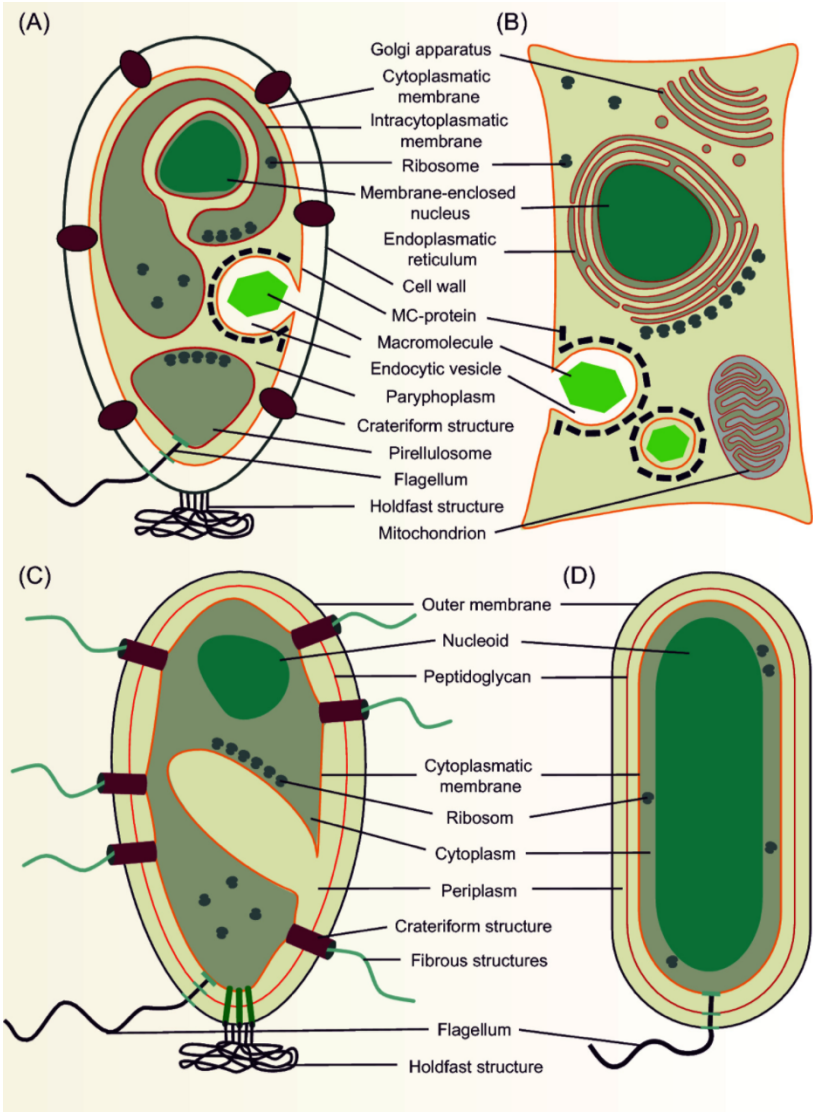


Figure 1. The morphology of a Planctomycete cell in comparison. (A) visualizes the previous consensus and thoughts on how Planctomycete cells could look like, which were thought to be more similar to eukaryotic cells (B). (C) shows the consensus now, which is much more like ordinary Gram-negative bacteria (D), although it still has some unique features, like the crateriform structures and invaginated periplasm. Figure from (Wiegand, Jogler, & Jogler, 2018).

Planctomycetes are often oval or tear shaped coccus, and they possess what are called crateriform structures in the outer cell wall, meaning they have circular craters on their exterior (Youssef & Elshahed, 2014). The craters can be spread all around the cell surface, or concentrated in one spot, often at a polar end (Youssef & Elshahed, 2014). Planctomycete cells are also thought to contain an enlarged periplasmic space (Boedeker et al., 2017; Wiegand et al., 2018). The purpose of these crateriform structures and the enlarged periplasm could be to engulf macromolecules (Boedeker et al., 2017; Wiegand et al., 2020). Crateriform structures and enlarged periplasm are visualized in figure 1. Although the idea of a membrane-bound nucleus was debunked, Planctomycetes are still thought to have some kinds of membranous

organelles (John A. Fuerst & Sagulenko, 2012; Lindsay, Webb, & Fuerst, 1997; Lindsay et al., 2001). This includes the annamoxosome organelle, which is used in energy production by annamox Planctomycetes (Saier & Bogdanov, 2013; van Teeseling, Neumann, & van Niftrik, 2013). The majority of new swarming Planctomycete cells are mobile, using flagella to propel forward. This appendix can appear singularly or pairwise and is often lost when the cell reaches maturity (Youssef & Elshahed, 2014). It is common for Planctomycetes to have a pili or stalk on the cell surface, which is used to attach itself to surfaces, or often to other individuals. This attachment in groups is called rosettes, due to the visual appearance of the grouping (Ward, 2010). Rosette formation is a good way to identify planctomycetes when looking for them in a microscope, as they are unique in this trait.

1.3.4 Cultivation, and the metabolisms of Planctomycetes

As Planctomycetes are so varied in their habitats of choice, a contingency must be that they are adapted to these habitats in various ways, including their metabolism. It is known that Planctomycetes utilize different metabolism pathways, both aerobic and anaerobic (Elshahed et al., 2007; Fuerst & Sagulenko, 2011; Youssef & Elshahed, 2014). The majority of isolated Planctomycetes cultivated in axenic samples, are chemoheterotrophic aerobes or facultative anaerobes (Kaboré et al., 2020). Unfortunately, cultivating contrasting strains have proven to be difficult (Kaboré et al., 2020; Wiegand et al., 2018). The aforementioned annamox bacteria, for example, are a contrasting group of chemoautotroph obligate anaerobes, who oxidise dinitrogen from ammonia with the help of their unique cellular compartment called the annamoxosome (Niftrik et al., 2004; Strous et al., 1999; van De Graaf et al., 1995). These bacteria are highly specialized, some being thermophilic as well, and they have not been obtained in pure culture as yet (Kuenen, 2020). Other metabolic pathways for Planctomycetes includes the ability to reduce sulphur to sulphide, possibly conducting sulphur respiration (Elshahed et al., 2007), and the conduction of fermentation using carbohydrates in anoxic environments (Slobodkina et al., 2015), amongst others. The bacteria are able to use various carbon and nitrogen sources, ranging from glucose to methanol and gelatine to urea (Youssef & Elshahed, 2014).

The ratio of cultivated to uncultivated Planctomycete bacteria is very low, with most strains being known only through molecular techniques (Olga M. Lage & Bondoso, 2012). This may be due to difficulty in specializing medium, in addition to the growth rate of most

Planctomycetes being exceptionally low, with examples of typical generation times being 11 hours for *Gemmata obscuriglobus* or two weeks for typical annamox Planctomycetes (Fuerst, 2017). This low growth rate means that Planctomycetes are susceptible to being overtaken by other fast-growing bacteria (Kaboré et al., 2020). The Planctomycetes require specialized media, where antibiotics are often added to address this issue of being outnumbered by bacteria with rapid generation times (Kaboré et al., 2020; Olga M. Lage & Bondoso, 2012). As the Planctomycetes have little peptidoglycan in their cell walls, they are more resistant to the types of antibiotics that target this specifically than many other bacteria (C. Cayrou, Raoult, & Drancourt, 2010), and so these types of antibiotics are often added to Planctomycete-targeted media (Schlesner, 1994; J. Wang, Jenkins, Webb, & Fuerst, 2002). Common antibiotics that target peptidoglycan cell walls are for example β-lactam antibiotics like ampicillin, which target cell wall synthesis (Katzung, Kruidering-Hall, & Trevor, 2018), or aminoglycoside antibiotics like streptomycin, which target protein synthesis (Krause, Serio, Kane, & Connolly, 2016). Another cultivation challenge is the overgrowth of fungi, which cannot be stopped by antibiotics targeting peptidoglycan cell walls, but rather with specialized fungicides like Pevaryle and Benlate (Lage & Bondoso, 2011). Other specifications made to media to accommodate Planctomycetes are the use of low levels of nutrients to target oligotrophs (Lage & Bondoso, 2012; J. T. Staley, 1973), and the substitution of agar with gelrite or gellan as a solidifying ingredient (Julia E. Storesund & Øvreås, 2013). The nutrient sources used in cultivation mediums for Planctomycetes are varied, but the addition of N-acetyl D-glucosamine, which can be used as both a carbon source and nitrogen source, has proven to be effective in the isolation of marine strains (Schlesner, 1994; Wiegand et al., 2020).

1.3.5 The cell cycle and propagation of Planctomycetes

A trademark sign of Planctomycete bacteria is their cell cycle and cell division. In comparison with almost all other bacterial phyla, they do not inhabit the *ftsZ* gene (Erickson & Osawa, 2010; Wiegand et al., 2020). This gene encodes the protein FtsZ, which is important in cell division by binary fission, the most common method for propagation in bacteria (Löwe & Amos, 1998; Margolin, 2000). In comparison, Planctomycetes display a cell cycle which is often based on budding, which is uncommon for bacteria, and more often seen in eukaryotes (Jogler et al., 2012).

In the mechanisms of binary fission, where an organism duplicates its genetics and separates from the middle of the cell, the FtsZ protein forms what is called a Z ring around or possibly inside the cell where division is to happen (Reyes-Lamothe & Sherratt, 2019). Although no Planctomycetes have ever been known to possess the gene capable of producing this protein (Erickson & Osawa, 2010), some strains can indeed divide by binary fission, either as their exclusive division method, or in addition to budding (Rivas-Marín, Canosa, & Devos, 2016; Wiegand et al., 2020). The main cellular division method for Planctomycetes is still budding, which mainly happens at a polar end of ovoid cells or arbitrarily in round ones (Wiegand et al., 2020; Youssef & Elshahed, 2014). A recent study discovered a novel division strategy in the strain *Kolteria novifilia* which was observed to be budding laterally, with the daughter cell emerging from the side of the mother cell (Wiegand et al., 2020). In polar budding Planctomycetes, it is common that daughter cells emerge from the opposite polar end to where the mother cell is fastened with a stalk (Lee, Webb, & Fuerst, 2009). The different methods of reproduction are visualized in figure 2.

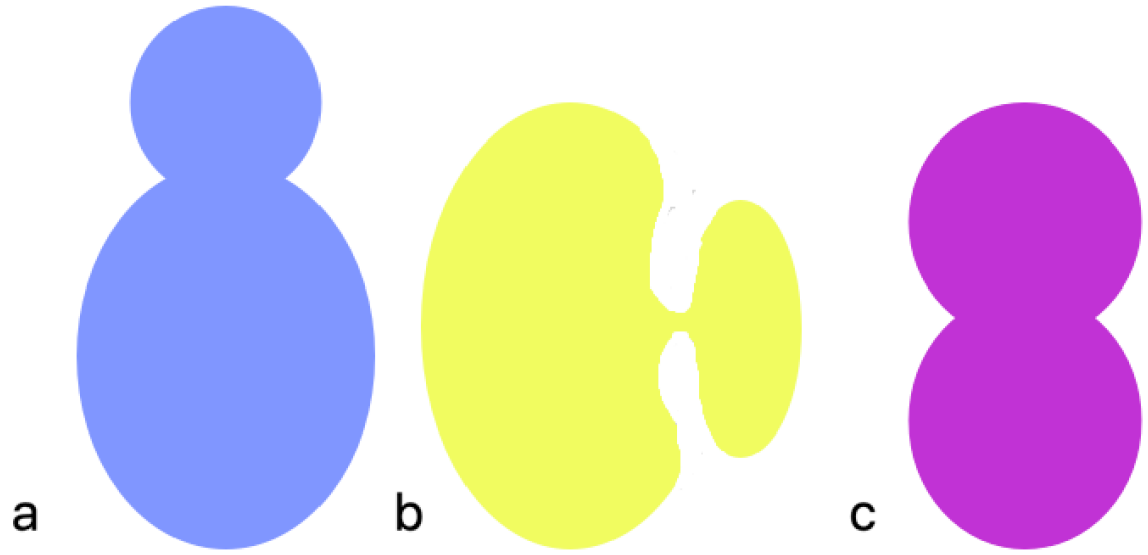


Figure 2. The different cell division methods of Planctomycetes. a) budding on polar end of an ovoid cell, b) lateral budding of K. novifilia, c) binary fission without the use of FtsZ. Figure made with inspiration from (Wiegand et al., 2020).

The aforementioned cell cycle or life cycle of the majority of the Planctomycete phylum is one based on differences in the properties of mother and daughter cells, which is called a developmental programme (Reyes-Lamothe & Sherratt, 2019). The sessile mother cell replicates, creating a swarmer daughter cell (Tekniepe, Schmidt, & Starr, 1981). The daughter cell is often motile by the use of flagella, which is lost when the swarmer cell becomes a sessile

cell, fastening to a surface or another cell (Hirsch, 1974; Tekniepe et al., 1981). Already sessile cells are able to divide several times (Tekniepe et al., 1981). This cycle includes a genetic difference in the two stages, where DNA replication is being repressed in the swarmer cells and is active in the sessile, stalked cells (Reyes-Lamothe & Sherratt, 2019). The life cycle of the majority of Planctomycetes is detailed and visualized in figure 3.

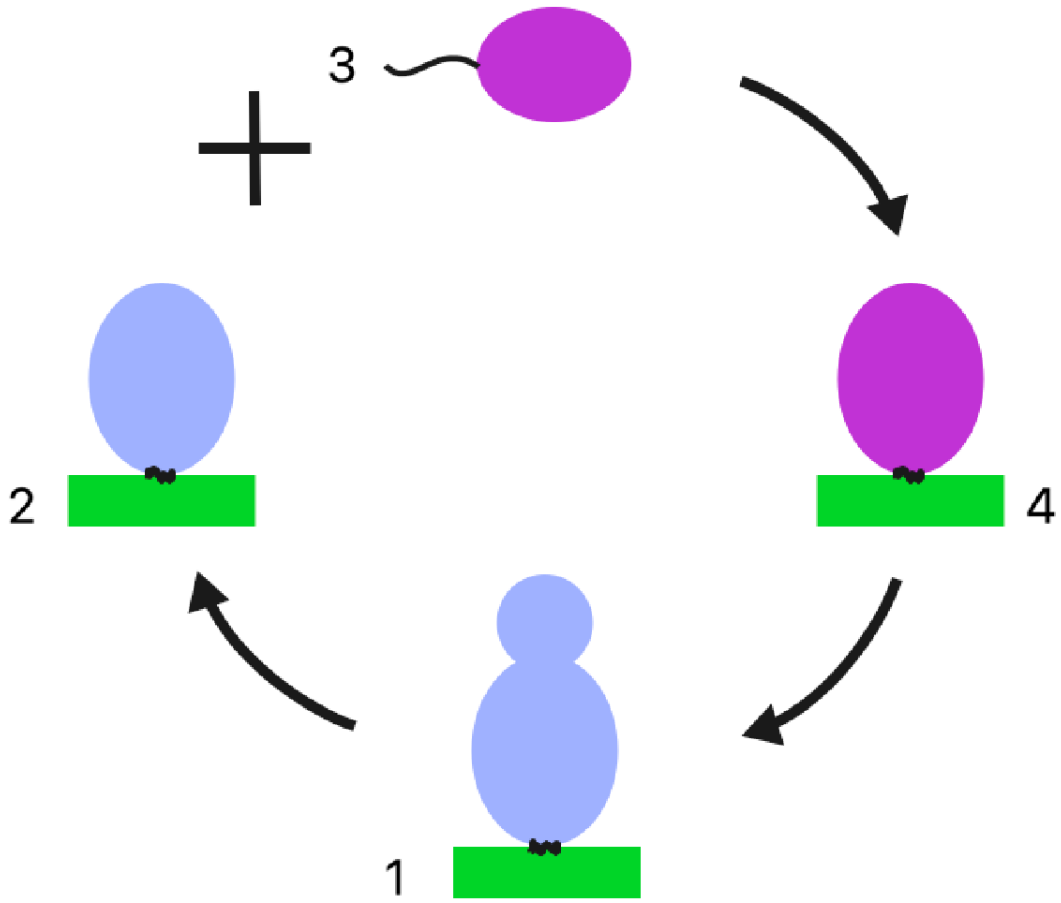


Figure 3. Life cycle of an ordinary Planctomycete bacteria. 1. cellular budding on polar end of stalked cell, 2. after separation, mother cell stays holdfast, while 3. daughter cell is motile, using flagella to swarm around while it matures, before 4. fastening to a surface where budding can occur. Figure made with inspiration from (Wiegand et al., 2018).

1.4 On cellular signals and bioactive molecules

It is known that small bioactive molecules and secondary metabolites are a part of the natural cell signalling ways in nature. In the following subchapters I will detail what bioactive molecules secondary metabolites are, what they are used for in nature, what kinds we can expect to find in Planctomycetes, and how they can be of relevance to humans in biotechnology and importantly medicine.

1.4.1 Cellular signalling, quorum sensing and secondary metabolites

In microbial communities, there exists cell to cell, intraspecies and interspecies communication, referred to as quorum sensing (Miller & Bassler, 2001). This quorum sensing mechanism is a way for bacteria to measure and understand the cell density in the area and know how to respond (Bassler & Losick, 2006; Federle & Bassler, 2003). This is done using small molecules called autoinducers (AI). These AI molecules are produced by bacteria, and diffuse freely in and out of cells, fluctuating in levels depending on the number of individuals present (Federle & Bassler, 2003; Rutherford & Bassler, 2012)(figure 4). When the AI reach a certain density, they can trigger a genetic response in the bacteria cell (Rutherford & Bassler, 2012). This response can be anything from the formation of biofilm, expression of bioluminescence, or indeed the production of antimicrobial compounds such as antibiotics (Boyer & Wisniewski-Dyé, 2009; Davies et al., 1998; Nealson, 1977).

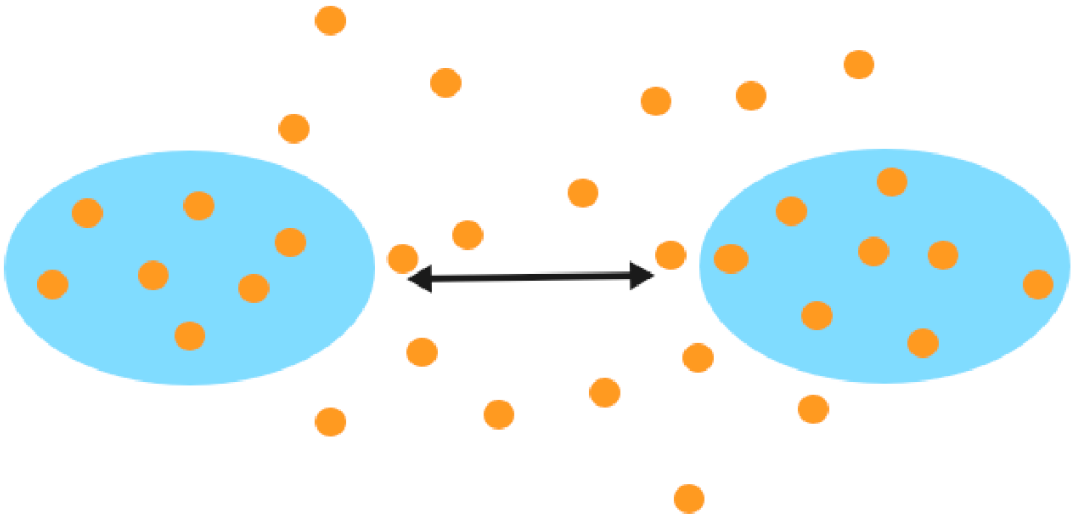


Figure 4. Quorum sensing in bacteria. Orange circles represent autoinducers diffusing in and out of the cells. Figure made with inspiration from (Bassler & Losick, 2006).

Metabolism in organisms is the combination of biochemical reactions the organism carries out Metabolism is another possible mechanism to obtain small bioactive molecules (Thirumurugan, Cholarajan, Raja, & Vijayakumar, 2018). These are called secondary metabolites, and can be produced not only by bacteria, but also by other microbes like fungi, in addition to plants (Mosunova, Navarro-Muñoz, & Collemare, 2020; Thirumurugan et al., 2018). Secondary metabolites are called such, as they are produced as a biproduct of metabolism, but do not contribute integrally to cell growth (Sanchez & Demain, 2019; Thirumurugan et al., 2018). This is in contrast to the primary metabolites, which includes vital molecules like lipids, amino acids and nucleic acids which make up the cellular structure, proteins and genome (Mosunova et al., 2020). Instead, secondary metabolites have functions primarily in protection and survival, but also in transport or in sporulation and reproduction in organisms that do such (Demain & Fang, 2000). These secondary metabolites can be induced by quorum sensing, or be separate from the phenomenon, and they are known to include antimicrobial molecules (Calisto et al., 2019; Sanchez & Demain, 2019). It is speculated that only organisms lacking their own immune system are capable of producing these secondary metabolites, as these aid greatly in their protection (Demain & Fang, 2000).

1.4.2 Bioactive molecules in medicine

Bioactive molecules from quorum sensing bacteria or the metabolisms of organisms are known to be of use and importance in human medicine. Amongst others, the bioactive molecules can be used as anticancer agents, antiparasitic agents, immunosuppressants and cholesterol-lowering drugs (Sanchez & Demain, 2019). They also include antibiotics, which from the chance discovery of penicillin by Alexander Fleming in 1928 has been a staple and a life saver within modern medicine (Gaynes, 2017). Antibiotics are antimicrobial molecules, which can be produced by microorganisms to protect themselves and their environment or host from other microorganisms (Sanchez & Demain, 2019; Teasdale, Liu, Wallace, Akhlaghi, & Rowley, 2009). Today, over 6000 antibiotics have been described (Thirumurugan et al., 2018), and they have helped raise life expectancy and lowered the death rate for infectious diseases tremendously (Sanchez & Demain, 2019).

Unfortunately, there is a glooming struggle of antibiotic resistance on the horizon, claimed by the World Health Organization (WHO) to be amongst the largest threats to global health in the year 2020 ("Antibiotic resistance," 2020). Antibiotic resistance as a mechanism means that a microorganism evolves through generations to be able to fight the drugs that were originally effective killers (Morier, 2021). Antibiotic resistance happens presumably due to overuse, misuse and overproduction, both in medicine and in agriculture (Larsson, 2014; Ventola, 2015), and has unfortunately happened to alarmingly many antibiotic drugs (Ventola, 2015) (figure 5). To fight this growing problem, a major solution is finding novel antibiotics (Gould & Bal, 2013). This includes searching for new sources of bioactive molecules.

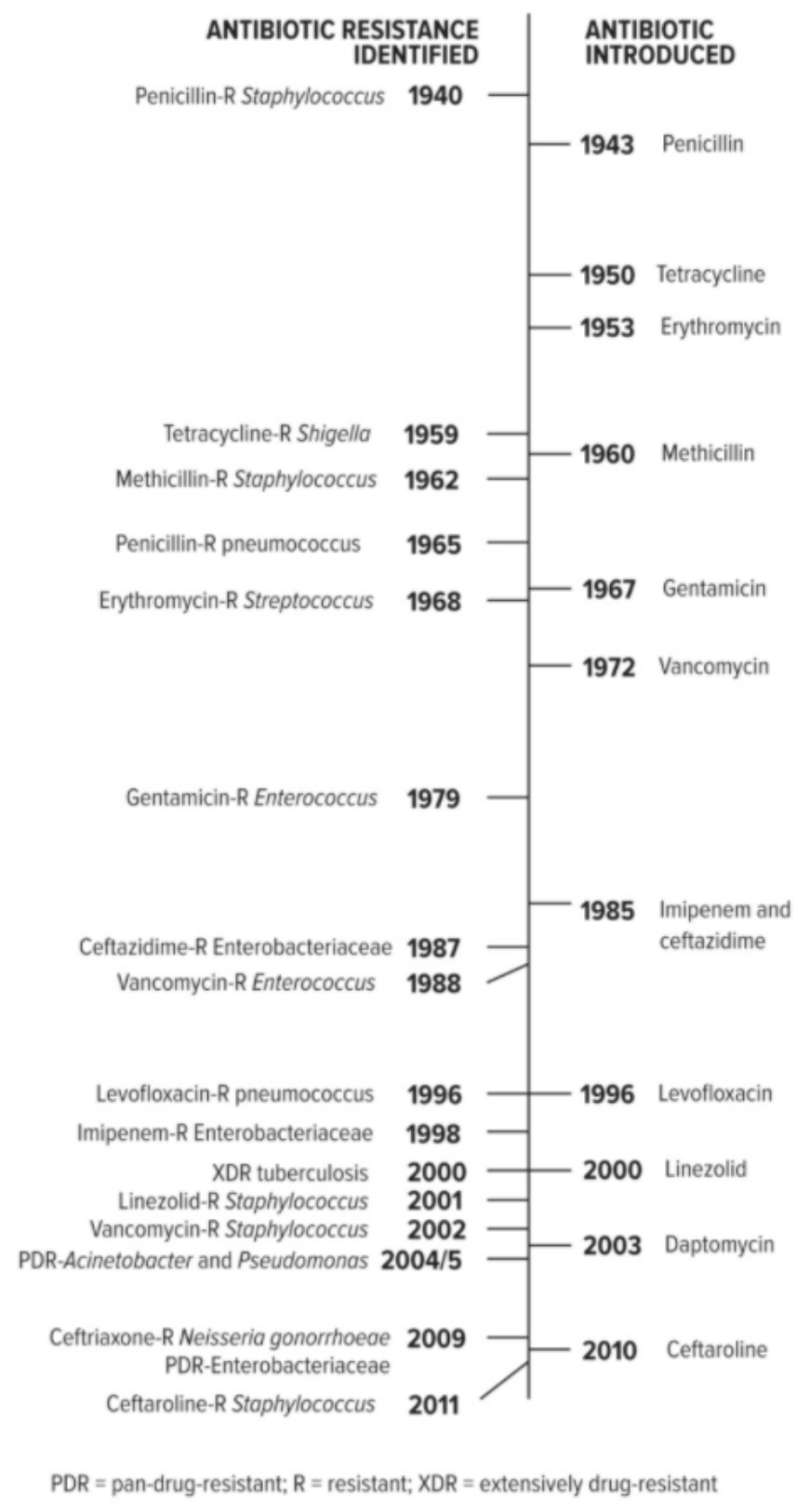


Figure 5. Key events in the history of antibiotics and antibiotic resistance from the year 1940 to 2011. PDR = pan-drug-resistant (meaning resistant to all antibiotics,) R= resistant, XDR = extensively drug-resistant. The figure is taken from (Ventola, 2015)*, and it uses data from Centres for Disease Control and Prevention (CDC), Office of Infectious Disease, in the United States, 2013.*

Bioactive molecules can sometimes be used in cancer therapy (Sanchez & Demain, 2019). At times, the compounds can have antibiotic properties in addition to exhibiting cytotoxic activity (Karpiński & Adamczak, 2018; Soldatkina et al., 2018). Anticancer activity has been reported in bioactive molecules from both prokaryotes and eukaryotes, and in various organisms within the domains, from diatoms to fungi (Evidente et al., 2014; Prestegard et al., 2009). As an example, Wall and Wani discovered the anticancer drug they called Taxol from the tree *Taxus brevifolia*. This drug has been shown to be effective against ovarian cancer, breast cancer, lung cancer and prostate cancer (Wall & Wani, 1996). Anticancer activity has also been found in mesopelagic species, where the fish species *Myctophym punctatum* and the crustacean *Meganyctiphanes norvegica* possessed compounds that were active against strains of lung and breast cancer, and a liver cancer cell line, respectively (Lauritano et al., 2020). Importantly, anticancer activity has also been described in microorganisms, including bacteria (Calisto et al., 2019; Forli, 2014; Gong, Wei, Huang, & Chen, 2014; Karpiński & Adamczak, 2018; Wrótniak-Drzewiecka, Brzezińska, Dahm, Ingle, & Rai, 2016). As bacteria are vivid producers of bioactive molecules from quorum sensing and metabolism, it is sensible to believe that they might be producers of cytotoxic compounds. The Gram-negative myxobacteria is one such group of bacteria who are known to be producing a great number of secondary metabolites (Gong et al., 2014; Wrótniak-Drzewiecka et al., 2016). Some myxobacteria produce epothilones, which are bioactive compounds that can induce apoptosis in cancer cells by targeting the eukaryotic cell wall, and are used in the treatment of breast cancer (Forli, 2014; Weissman & Müller, 2010; Wrótniak-Drzewiecka et al., 2016). It has also been shown that myxobacteria can have a negative effect on liver, prostate, bone, cervix and pancreas cancer cell lines (Kumar, Yadav, Chambel, & Kaur, 2017).

1.4.3 Planctomycetes in the light of medicine

From what we know about Planctomycetes, and what we know about the production of bioactive molecules and their use in medicine, there is a conclusion to draw that they are possible sources for anticancer compounds (Kaboré et al., 2020). Planctomycetes are known to be living in biofilm or microbial mats (Bengtsson & Øvreås, 2010; Prieto-Barajas et al., 2018). It is logical to believe that they have reason to protect their environment from overpopulation of other organisms, and to protect themselves from pathogenic organisms as well. It is also known that they participate in quorum sensing, for example it has been found that they produce a class of signalling AI called stieleracines (Kallscheuer et al., 2020). Alike to the myxobacteria,

the Planctomycetes possess unusually large genomes, and complex life cycles (Andrei et al., 2019; Reichenbach, 2001; Reyes-Lamothe & Sherratt, 2019). These are factors that are known to be correlate with the production of bioactive molecules (Jeske, Jogler, Petersen, Sikorski, & Jogler, 2013), and as mentioned, it has been proven that myxobacteria do produce these compounds and that they can have cytotoxic effect (Gong et al., 2014; Wrótniak-Drzewiecka et al., 2016). Planctomycetes are also known as producers of large proteins without known functions, which could be sources for novel pathways for bioactive molecules (Wiegand et al., 2018). In addition to this, Planctomycetes are resistant to many types of antibiotics (C. Cayrou et al., 2010). A common trait amongst resistant organisms is the ability to produce their own antibiotics (Jeske et al., 2013). Ecological diversity is a factor strongly contributing to the production of bioactive molecules, as variety in habitat may correlate to heterogeneity in metabolism and thereby secondary metabolites (Graça et al., 2016). Although they can inhabit a plethora of natural environments, many Planctomycetes are marine bacteria, and studies have shown that marine microorganisms are large sources for bioactive compounds, with increasing numbers found every year (Blunt, Copp, Munro, Northcote, & Prinsep, 2006; Graça et al., 2016; Teasdale et al., 2009).

In conclusion, there is reason to believe that Planctomycetes are potential sources for novel bioactive compounds, and there is indeed conclusive evidence to support the statement. The bacteria have been known for some time to be able to produce antibiotics, some being lantibiotics, cypemycin and bacteriocins (Jeske et al., 2013). Calisto et. al showed in 2019 that the phylum was also capable of the production of anticancer compounds (Calisto et al., 2019). Genomics had shown that Planctomycetes had the ability to synthesize epothilones like myxobacteria (Graça et al., 2016), but the 2019 article showed that Planctomycetes can indeed have a cytotoxic effect on cancer cells of prostate and leukaemia strains (Calisto et al., 2019). What is unknown today is the chemistry of the compounds causing this anticancerous effect, and if they are known or novel substances, or indeed if it is a case of several substances working in cooperation.

1.5 Description of technology used in the study

1.5.1 High performance liquid chromatography (HPLC)

HPLC is a technique within chemistry which is used to separate and/or identify different components in a liquid sample. A HPLC typically consists of a sample, one or several mobile solvents, a column, a gradient pump, a detector and a computer (Figure 6). The sample is injected into a mobile phase, and subsequently leaded onto a column with a stationary phase, by the use of liquid pumps that generate high pressure. The absorbent matter is usually either silica or alumina, which are coated with hydrophobic carbon chains of certain length, for instance 18 carbon (C18-column) which was used in the present study. The analytes in the sample will interact with the hydrophobic carbon chains and be retained with the stationary phase in the column. By generating a gradient of the mobile phase, from polar to non-polar solvents, different analytes will elute from the stationary phase at different times, which is the basis of separation. This allows the components to be separated based on when they exit the column, at which point they are analysed by a detector (Calvin Giddings & Keller, n.d.). The output can be visualized as a chromatogram, where the different analytes appear as peaks. This chromatogram is a visualization of the separations that have occurred within the machine, with each peak on the graph illustrating the detector response to a compound.

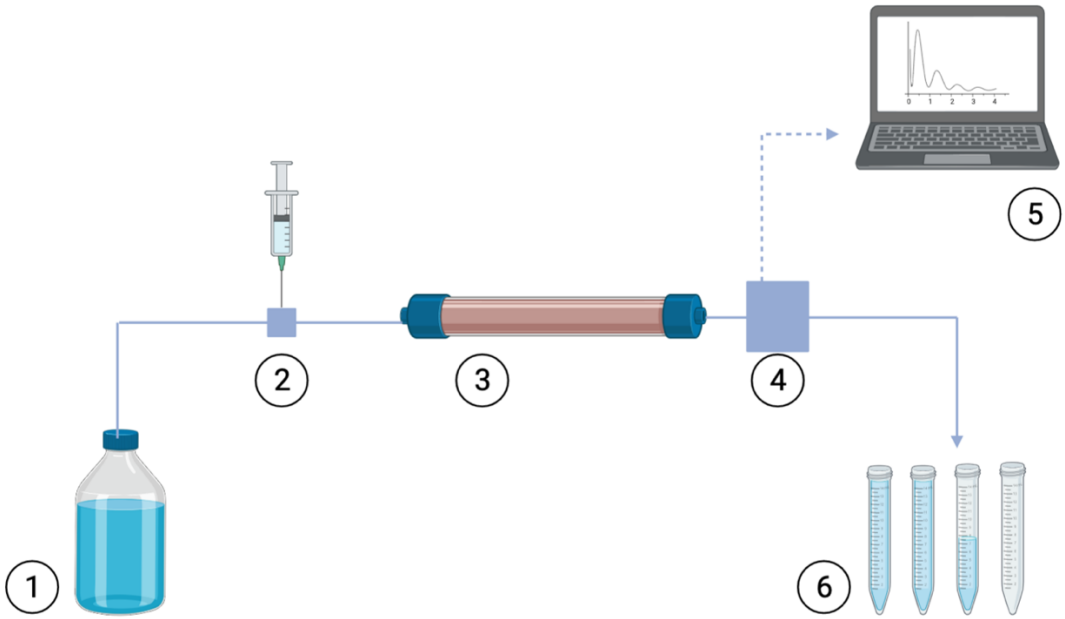


Figure 6. Steps of HPLC analysis. 1. mobile solvent or solvents, 2. injection of sample, 3. solvents and sample are pumped through an absorbent column, 4 analysation of sample components, which results in a chromatogram, 5. 6. sample collection or waste. Figure made using the online programme "BioRender" ("BioRender," n.d.)

1.5.2 WST-1 assay and microplate reader

WST-1 assay is a technique used to measure metabolic activity in a cell population. This can then be used to estimate rate of proliferation in a sample, or the the viability of the cell population. The WST-1 agent contains a tetrazolium salt, which can be cleaved enzymatically in the sample only by metabolic active cells. When the tetrazolium salts are cleaved, the compound formazan is made. Formazan is a red dye, which stains the medium, and the relative metabolic activity in a cell population can be estimated based on the amount of dye ("Protocol Guide: WST-1 Assay for Cell Proliferation and Viability | Sigma-Aldrich," n.d.). The amount of formazan dye produced is determined in a microplate reader. This technology uses a light source and measures absorbance through the wells, which will give information concerning the coloration of the wells, and therefore the metabolic activity of the cells within them ("Microplate Reader | Plate Reader - BMG LABTECH," n.d.).

1.6 Previous results from anticancer testing

Examination of cytotoxic activity has been done previously on the majority of the isolates included in this study (Calisto et al., 2019). The results from this study showed high negative effects (70-100% cell apoptosis) towards the monocytic leukaemia cell line MOLM-13 from aqueous phase of three strains (TBK2h, VLbF2 and 1mW). Some negative effect towards MOLM-13 (40-70% cell apoptosis) was found using the organic phase of five strains (P61, VLbF2, L1, 7mR and 1mW), in addition to the aqueous phase of two strains (P61 and P62). Finally, slight negative effect (20-40% cell apoptosis) was found using the organic phase of TBK2h. High negative effects were produced on the prostate cancer cell line PC3 from the organic phase of strain L1, whilst moderate negative effect on PC3 was achieved using the organic phase of strain 7mR and the aqueous phase of strain 1mW. The normal rat kidney cells (NRK) experienced high negative effect from organic phase of strain 7mR. The cytotoxic potential of strains in different phases on the cell lines is described in figure 7. Cell lines P2S and P1a was not previously included in the screening for cytotoxic activity.

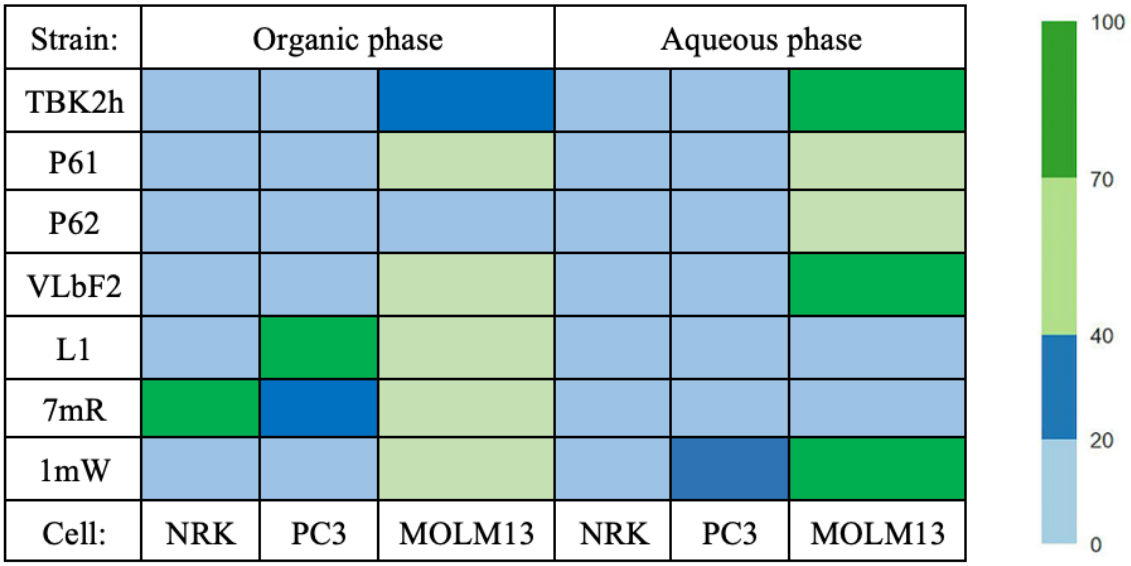


Figure 7. Detailing previous anticancer activity in some strains used in this study. Data from (Calisto et al., 2019).

2. Materials and methods

2.1 Bacterial material used in the study

The bacterial strains used were collected from all over the world, from different habitats. Strains TBK2h and VLbF2 were derived from the VaiLili hydrothermal vent field in the South Pacific Ocean at coordinates 176°36′W, 22°10.82′S. Descriptions on sampling, cultivation and isolation of planctomycetes from this area are detailed by Storesund et. al (2018). Strains 7mR and 1mW was obtained in Norway, more specific from the meromictic lake Sælenvannet at coordinates south of Bergen (60°19′N, 05°16′E). The specifics surrounding sampling of these strains, including methods for sampling at both aerobic and anaerobic layers above and below the salinity gradient called the chemocline can be found detailed by Ovreås, Forney, Daae, and Torsvik (1997). Strain 7mR was collected at 7 metres, while 1mW was collected at 1 metre below the lake surface (Julia E Storesund et al., 2020). Strains P61, P62, P1a and P2S were collected from the ice cave Svarthammarhola in Fauske located at 67°13′N, 15°31′E. Collection of the strains, cultivation and isolation, in addition to descriptions of optimal growth are detailed in Sæbø (2018). Strain L1, was isolated in Kongsfjorden in Svalbard, specifically from Hansneset at coordinates 78°59′N, 11°57′E. The strain was collected from the surface of the marine macroalgae *Saccharina nigripes*. The sampling of L1 was done using previously described methods by Bengtsson & Øvreås (2010).

2.2 Preparation of medium

The bacteria strains were cultivated in M30 growth medium (Schlesner, 1994). The medium was added different percentages of aged seawater based on what habitat the bacteria was isolated from. Strains 7mR, 1mW, TBK2h, VLbF2, Plm2, L1 and L2 were collected from marine environments, and were hence grown in medium added 70% seawater. The strains from ice cave Svarthammarhola (P1a, P2S, P61 and P62) were cultivated using medium added 18% seawater. The medium was made by combining distilled water and aged seawater and adding 20mL/L Hutners basal salts (Appendix I) (Cohen-Bazire, Sistrom, & Stanier, 1957) and 50mL/L 0.1M Tris buffer with pH of 7.5. The solution was autoclaved at 121°C for 20 minutes. A second solution was made by dissolving 2g N-Acetyl-D-Glucosamine, 10mg Na_2HPO_4 x $2H_2O$, 200mg ampicillin in 50mL distilled H_2O. To this, 1mL of vitamin solution no. 6 – 10 x solution was added (Appendix II) (J. T. Staley, 1968). The second solution was filter sterilized through a 2µm filter and added to the initial solution. Liquid M30 18% and 70% SW was stored in sterilized flasks at 4°C in the dark.

To produce solid medium for petri dishes, 5g gelrite was added to the first solution before autoclaving in a table autoclave. After combining with the second solution, the medium was poured into petri dishes in 20mL aliquots, and left overnight to dry. Solid medium was stored in sealed bags at 4°C in the dark.

2.3 Cultivation of bacteria

Bacterial strains were inoculated on to petri dishes on M30 medium with appropriate seawater percentage. When there was visible separate colony growth (approx. 7 days), colonies were transferred to liquid medium for further growth. The strains were grown at small volumes at first (50mL in 100mL flasks or 100mL in 250mL flasks) and then transferred to larger volume flasks (500-1000mL) and added fresh medium when the growth was in exponential phase (between 3-5 days). This was determined by visual inspection in light microscope. The flasks containing bacterial culture were kept around half full, giving high air space. The top was screwed loosely on to inhibit an anoxic environment. Smaller 100mL flasks were kept on a smaller analogue desk top shaker at room temperature (VWR, n.d.), while larger flasks were stored at room temperature on a high load orbital desk top shaker (Edmund Bühler GmbH, n.d.).

2.4 Harvesting and freeze drying of bacteria

After incubation in 500mL or 1000mL flasks for between 7-14 days, depending on individual growth rate, the cultures were centrifuged to harvest the bacterial cell biomass. The strains were harvested during exponential growth, determined by visual inspection in light microscope. Centrifugation was done using a high-performance centrifuge fitted with a JS 7.5 rotor (Beckman Coulter, "Avanti J-26S XP" n.d.). 50mL culture was aliquoted into falcon tubes and centrifuged at room temperature for 15 minutes at 10400xg. The supernatant was discarded, while the pellet was kept. Additional bacterial culture was aliquoted into the same falcon tubes. This was repeated between 2-6 times based on the volume needed to be centrifuged. The bacterial pellets were washed twice with 50mL sterile distilled water and stored at -20°C. Frozen pellets were placed in a freeze-drier with vacuum for between 20-24h (Edwards, 1983). The pellets were weighed and stored at -20°C.

2.5 Extraction of aqueous and organic phases from bacteria

2.5.1 Phase extractions using 10mg samples of bacteria

Phase extractions were done to get an overview of the cytotoxic potential of bacterial strains 7mR, L1, P62, P2S, P61, 1mW, TBK2h, P1a and VLbF2 towards acute myeloid leukaemia cell line MOLM-13, prostate cancer cell line PC3 and normal rat kidney cells (NRK). Aqueous and organic phases were extracted from the bacteria. To achieve this, 0.5mL chloroform (Chl), 0.5mL methanol (MeOH) and 0.5mL ultrapure water (milliQ) was added to 2mL Eppendorf tubes containing 10mg freeze-dried bacteria. The mixture was vortexed and placed on ice in the dark for 1h to allow time for extraction. Next, the samples were incubated for 40 minutes in an ultrasound-bath to homogenize the bacterial biomass with the liquid additives (Branson, n.d.). The homogenized samples were centrifuged at 12000RPM at 4°C for 20min (Beckman Coulter «Allegra X-22R», n.d., Beckman Coulter «FX301,5», n.d.), to separate into three extract layers; an organic phase, an aqueous phase and a solid phase. Samples of 850µL of the aqueous phase and 300µL of the organic phase were transferred into separate Eppendorf tubes. Vortexing in between centrifuging, the samples were washed twice with 250µL methanol, chloroform and milliQ. After both washes, 450µL of the aqueous phase and 200µL of the organic phase were collected and added to the extract. The extracts were evaporated using settings for aqueous solutions in vacuum at a temperature of 30°C (Eppendorf "Concentrator Plus", n.d., Eppendorf "Rotor F45-48-11", n.d.). To prepare the evaporated extractions for cellular experiments, 25µL of the solvent dimethyl sulfoxide (DMSO) was used to dissolve the organic phases, whereas a mixture of 25µL DMSO and 75µL milliQ was used to dissolve the aqueous phases. The samples were stored at -20°C until use in cell experiments.

2.5.2 Phase extractions using larger samples of bacteria

Larger scale extractions were performed with bacteria that showed promising results from the initial screening for cytotoxic activity.

2.5.2.1 Aqueous extraction

Approximately 100mg freeze-dried samples of bacterial strains TBK2h, VLbF2, L1 and P2S, was added to 50mL falcon tubes. 15mL of a 70%/30% solution of MeOH and milliQ was added to the samples, which were subsequently homogenized at 15 000 RPM for three cycles of 20 seconds, cooling the samples on ice between each cycle (VirTis, n.d). The homogenizer was run for a few seconds in MeOH and thereafter EtOH to remove debris and wash between the samples. The samples were then stored at 4°C in the dark overnight to extract, before being transferred to new 50mL centrifuge tubes to prepare for centrifugation. The samples were centrifuged at 7000 RPM at 20°C for 15 minutes (Beckman Coulter "Avanti J-26S XP", n.d., Beckman Coulter "JA 25,50", n.d.). The pellets were washed twice on the same centrifuge settings with 10mL 70/30% MeOH and milliQ. Supernatants were combined in 250mL round bottomed flasks to prepare for evaporation. Approximately 10% of the combined supernatants were aliquoted into Eppendorf tubes and evaporated in a concentrator as previously described (Eppendorf "Concentrator Plus", n.d., Eppendorf "Rotor F45-48-11", n.d.). This was done to perform test experiments for cytotoxicity on small portions of the extracts, while keeping the remainder for solid phase extractions. The small samples were stored at -20°C until use. To prepare the samples for cell experiments, 5uL DMSO and 20uL milliQ was added to dissolve the precipitates. Strain P2S was gelatinous, meaning the precipitate of a 10% sample was difficult to dissolve. Cell experiments for P2S were therefore performed using a sample representing 3,33% of the original large extraction. The remainder of the extractions were evaporated in round bottomed flasks using a rotary evaporator at 37°C at 120 RPM (Heidolph Instruments, n.d., Vacuubrand n.d.). The evaporated samples were prepared for solid phase extractions by dissolving in 0,5mL MeOH and 2mL milliQ. Strain P2S had to be added 0,5mL additional MeOH to dissolve completely. The liquid was transferred to 50mL falcon tubes and stored at -20°C.

A second large scale extraction was performed on strain TBK2h due to the available high abundance of biomass. Here, 1000mg of freeze-dried bacteria was divided into six 50mL falcon tubes. The samples were added 25mL of a 70/30% MeOH/milliQ solution. The product was homogenized and centrifuged as previously described for large samples (VirTis, n.d., Beckman Coulter "Avanti J-26S XP", n.d.). The supernatants from centrifuging and washing were combined, and evaporated in a rotary evaporator as previously described (Heidolph

Instruments, n.d., Vacuubrand n.d.). To prepare for solid phase extractions 10mL MeOH and 20mL milliQ was added to dissolve the precipitate. This product was stored at -20°C.

2.5.2.2 Organic extraction

A 500mg sample of biomass from bacterial strain P1a was weighed into a 50mL falcon tube, and added 10mL MeOH and 10mL Chl. The sample was homogenized as previously described (VirTis n.d.) and kept at 4°C in the dark for approximately 1 hour to extract. The homogenized sample was divided into two 50mL centrifuge tubes and centrifuged at previously described settings (Beckman Coulter "Avanti J-26S XP", n.d.). The pellets were washed twice with 10mL of a 50%/50% solution of MeOH and Chl at the same centrifuge settings. Supernatants were combined in a 250mL round-bottomed flask, and 10% percent was allotted out in six parallels of 1mL into smaller Eppendorf tubes. The smaller samples were evaporated in a concentrator, while the remaining larger extraction was evaporated in a rotary evaporator as previously described (Eppendorf "Concentrator Plus", n.d., Heidolph Instruments, n.d., Vacuubrand n.d.). The evaporated samples were stored at -20°C before preparation for cell experiments. To prepare for experiments, 20µL DMSO was added to one of the 1mL parallels, with the intent of transferring the liquid from one sample to the next and dissolving the precipitate of all six parallels corresponding to 10% of the original extraction. This was difficult, as the samples were gelatinous. Therefore, only 1mL sample (corresponding to approximately 1,67% of the original large extraction) was used in experiments at a time. The larger evaporated sample was prepared for solid phase extraction by dissolving the precipitate in 2mL MeOH and 2mL milliQ.

Organic phase extractions were done as described before using a 150mg sample and a 200mg sample of P1a. Contrarily, 10% of the supernatants following centrifuging was not aliquoted into smaller Eppendorf tubes for evaporation, as smaller scale experiments on cell lines were not performed. The supernatants were evaporated in a rotary evaporator as described (Heidolph Instruments, n.d., Vacuubrand n.d.). The precipitate of the 150mg sample was dissolved in 2mL MeOH and 2mL milliQ to prepare for solid phase extraction using small size cartridge. The precipitate of the 200mg sample was dissolved in 10mL MeOH and 20mL milliQ to prepare for solid phase extraction using larger size cartridge.

2.6 Solid phase extraction (SPE) of aqueous and organic phases of selected bacterial strains

2.6.1 SPE of strains VLbF2, L1, P2S and P1a using small cartridge

SPE using small cartridge was performed on previous aqueous phase extractions of strains VLbF2, L1, P2S and organic phase extractions of strain P1a. SPE consisted of extraction of three phases; load and wash (L+W), elution phase 1 (E1) and elution phase 2 (E2). The SPE cartridge (Waters "Sep-Pak C18 plus", n.d.) was activated with 1mL MeOH followed by 2mL milliQ equilibration. Next, 2mL of the extraction was loaded onto the cartridge, followed by a wash of 2mL milliQ. The eluate was collected as the L+W-fraction. E1 consisted of injection of 2 mL MeOH, collecting the eluate in a separate tube. For E2, 2mL acetonitrile (ACN) was injected through the cartridge and collected. The three fractions were evaporated in a concentrator as previously described (Eppendorf "Concentrator Plus", n.d.). To prepare the evaporated extracts for experiments on cell lines, 20µL milliQ and 5µL DMSO were added to the three the fractions from the aqueous extracts to dissolve the precipitate, while 10µL DMSO and 5-10µL milliQ was added to the fractions from the organic extract of P1a. As L1 and P2S were gelatinous in nature, they were added milliQ and DMSO according to Table 1.

Table 1. amount of DMSO and milliQ added to extraction steps of different strains after solid phase extraction

Strain, extraction step	milliQ (in µL)	DMSO (in µL)
P2S L+W	95	30
P2S E1	75	25
P2S E2	20	5
L1 L+W	60	15
L1 E1	20	5
L1 E2	20	5
VlbF2 L+W	20	5
VlbF2 E1	20	5
VlbF2 E2	20	5
P1a L+W	10	10
P1a E1	5	10
P1a E2	5	10

2.6.2 SPE of strains TBK2h and P1a using large cartridge

SPE using large cartridges were done with strain TBK2h using aqueous extract from a 1000mg biomass sample and strain P1a using organic extract from a 200mg biomass sample.

For SPE of larger extractions of strains P1a and TBK2h, a 10g SPE cartridge was used, in addition to a peristaltic pump fitted with a plastic tube to feed sample and solvents to the cartridge (Waters "Sep-Pak C18 35cc, 10g", n.d., Gilson, n.d.). The tube and cartridge were activated with 70mL MeOH and equilibrated with120mL milliQ before the sample was loaded. For L+W, the 30mL extraction was loaded onto the cartridge, followed by a wash of 30mL milliQ which was both collected. E1 consisted of 50mL MeOH which was collected in a separate flask. For E2, ACN was run through the system at different volumes, 50mL for P1a, and 100mL for TBK2h, as the original biomass samples were different in size, and collected in a separate flask. For strain TBK2h, 10% of the collected liquids from the three fractions were aliquoted into Eppendorf tubes for evaporation in a concentrator as previously described (Eppendorf "Concentrator Plus", n.d.). The samples were prepared for cell experiments by dissolving the precipitate in 20µL milliQ and 5µL DMSO. The remaining collected fractions from TBK2h in addition to SPE of P1a were evaporated in a rotary evaporator as previously described (Heidolph Instruments, n.d., Vacuubrand n.d.). The evaporated SPE of the strains were prepared for high performance liquid chromatography (HPLC) by dissolving the precipitate in 20% MeOH and 80% milliQ.

2.7 Cultivation of cells

The extracts and fractions were tested on an acute myeloid leukaemia cell line (MOLM-13) a prostatic cancer cell line (PC3) and a normal rat kidney cell line (NRK).

2.7.1 MOLM-13 cells

MOLM-13 cells were cultivated in RPMI-1640 medium (Sigma-Aldrich R5886, n.d.), supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich F9665, n.d.), 0,2mM L-glutamine, 50 IU/mL penicillin and 0,1mg/mL streptomycin. The cell suspensions were kept in a 50mL, 25cm^2 suspension culture flask, and incubated at 37°C in a humidified atmosphere with 5% CO_2.

The cell suspensions were diluted after 3-5 days, to keep the cellular amount at around 400-800 000 cells/mL.To dilute the suspensions, the contents of a flask were transferred to a 15mL falcon tube. The suspension was centrifuged at approximately 150xg for 5 minutes (Hettich n.d.). The supernatant was discarded, and the cells resuspended in 5mL room temperature culture medium. Cells/mL were counted by use of a haemocytometer in an inverted microscope (Olympus n.d.). The resuspended cell culture was diluted with medium to fit required cell density.

2.7.2 PC3 and NRK cells

PC3 and NRK cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich D-6429, n.d.) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich F724, n.d.), 0,2mM L-glutamine, 50 IU/mL penicillin and 0,1mg/mL streptomycin. The cell suspensions were kept in a 50mL, 25cm^2 tissue culture flask and were incubated at 37°C in a humidified atmosphere with 5% CO_2.

The suspensions were diluted approximately every three days, when reaching around 80% confluency. As the NRK and PC3 cells are adherent, the liquid in the flasks was first discarded. The flasks were then rinsed twice with 5mL PBS. 1mL 0.30 mg/L trypsin was added to the flasks, and they were incubated for 2 minutes at 37°C in a humidified atmosphere with 5% CO_2.

Five millilitres of medium was added while washing the sides of the flask. The medium and trypsin were transferred to a 15mL falcon tube and centrifuged at 150-200xg for 5 minutes (Hettich, n.d.). The supernatants were discarded while the cells were resuspended in 1mL room temperature medium, before adding up to 5mL. A counting of cells using a haemocytometer, or a visual inspection of cell abundance was performed in an inverted microscope (Olympus n.d.). Depending on the cell density, 500µL or 1mL of the cell suspension was added to a new 50mL, 25cm^2 tissue culture flask, with either 9,5 or 9mL medium.

2.8 Experiments on cell lines

Experiments were performed on all small-scale extractions to test the strain for cytotoxic activity against NRK, PC3 and MOLM-13 cells. Both 24h and 72h experiments were done for all extractions on all cell lines using 96-well microplates. For all cell lines, the aqueous phases were tested at 4% added extract, and the organic phases at 1% added extracts.

2.8.1 Cell experiments on MOLM-13

For experiments of 24h incubation, 400 000cells/mL was used. For experiments of 48-72h incubation, between 100 000-200 000 cells/mL was used. For cell experiments using MOLM-13 cells, 100µL medium was added to four wells as a blank control, and 50µL of cell culture was added to the remaining wells. In addition to the cell culture, 50µL medium was added to four wells as a cellular growth control. 46µL medium was added to the wells that were going to contain aqueous extractions, in addition to 4µL of the extractions. 49µL medium was added to the wells that were going to contain organic extractions, in addition to 1µL of the extraction. A 1% DMSO control was made using 50µL cell culture, 49µL medium and 1µL DMSO. This was done as the solvent DMSO may be cytotoxic for some strains. The microplates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for either 24 or 72h. For experiments with samples from large-scale extractions and SPE, MOLM-13 cells were exposed to three different concentrations of extracts, 5%, 2% and 0.5% for between 48-72h. This was to detect differences in cytotoxic potential between the different strains and fractions from the solid phase extraction.

2.8.2 Cell experiments on PC3 and NRK

For experiments of 24h incubation, 50 000cells/mL of cell lines PC3 and NRK was used. For experiments of 48-72h incubation, 20 000 cells/mL was used. As PC3 and NRK cells were adherent, 100µL cell culture was added to the microplates 24h before the extracts to allow the cells to attach to the bottom of the wells. This volume was removed prior to addition of fresh medium. 100µL medium was added to the growth controls. For aqueous phases, 96µL medium and 4µL extract was added. For organic phases and DMSO control, 99µL medium and 1µL extract or DMSO was added. The microplates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for either 24 or 72h. For experiments with samples from large-scale

extractions and SPE, PC3 cells were exposed to three different concentrations of extracts, 5%, 2% and 0.5% for between 48-72h.

2.9 High performance liquid chromatography (HPLC) separation and cellular experiments using HPLC fractions

HPLC separation of SPE and experiments using HPLC separated fractions were performed using strains TBK2h and P1a. SPE E1 was used, extracted from the aqueous phase of a 1000mg sample of TBK2h. SPE E2 was used, extracted from the organic phase of a 200mg sample of P1a. The HPLC system was fitted with a C18 column intended for reverse phase chromatography. H_2O and ACL were used as solvents, to carry the samples through the system. The solvents were added in varying amounts described in table 2.

Table 2. Describing percentage of H_2O and ACN injected through HPLC system at different times

Time (min)	% H_2O	%ACN
0.0	90	10
2.0	90	10
25.0	0	100
30.0	0	100
33.0	90	10

A 3mL sample of TBK2h SPE E1 was injected into the HLPC system. Liquid fractions were collected in falcon tubes from the output of the HPLC system. The fractions were collected for two minutes each, gathering 16 samples in the space of 35 minutes. A 4mL sample was used for strain P1a SPE E2, and was injected through the system. A total of 20 samples were collected for the strain, at two minutes per fraction. Chromatograms detailing detector response were produced.

The samples were evaporated in a concentrator as previously (Eppendorf “Concentrator Plus”, n.d.), and the precipitate was dissolved using 5µL DMSO. All fractions were diluted 1/10 using

cellular cultivation media RPMI medium (Sigma-Aldrich R5886, n.d).for the TBK2h samples and DMEM (Sigma-Aldrich D-6429, n.d.) for the P1a samples. The media were supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich F724, n.d.), 0.2mM L-glutamine, 50 IU/mL penicillin and 0.1mg/mL streptomycin. Cell experiments were performed using the separated fractions at varying dilutions. Fractions of strain TBK2h were used for experiments on cell line MOLM-13, and added at 0.1% (1µL of 1/10 dilution) and 0.5% (5µL of 1/10 dilution) extraction. Fractions of strain P1a were used for experiments on cell line PC3 and were added at 1% (1µL of undiluted sample) and 0.3% (3µL of 1/10 dilution) extraction. Setups for cellular experiments were as previous, using a blank control without added cells, a cell control with no additives and a 1% DMSO control to obtain an overview of the DMSO effect on the cells. Experiments were performed for 48h incubation.

2.10 Metabolic assay and microscopy

After incubation of cells and extractions, 10µL WST-1 was added to each of the wells. The plate was then placed in the incubator for 2 hours and the absorbance at 450nm was read using a plate reader, with a reference read at 620nm. The average of the blank control was calculated and subtracted from all wells containing cells. The readings of the metabolic activity in the wells were then related to the control wells containing only cells and medium. Equation 1 describes the calculation steps used to obtain the results from the metabolic assay.

Equation 1.

$$Step\ 1.\ output\ from\ one\ well - average\ blank\ control$$

$$Step\ 2.\ \frac{result\ from\ step\ 1}{average\ control}$$

Bar graphs were produced using Microsoft Excel (2016). After the metabolic activity was measured, the cells were fixated by adding 100µL of a fixing medium consisting of phosphate-buffered saline (PBS), 4% formaldehyde and 0,5mg/L of the DNA dye Hoechst 33342 solution. The plates were sealed using parafilm and stored at 4°C.

As the results from WST-1 assay could be interfered by the presence of pigments or particles in the extracts, the cells were also studied under a fluorescent microscope. Microscopy was conducted to calculate induced apoptosis in the cell lines. The cells were viewed through a fluorescence microscope at a magnification of 20, and photographs were acquired to be able to study nuclear morphology. Using the image processing program ImageJ (Schneider, Rasband & Eliceiri, 2012), and a cell counter plugin (De Vos, n.d.), the number of normal and apoptotic/necrotic nuclei were counted. Calculations were performed to obtain a percentage of apoptotic cells, adjusted to the average of apoptotic cells in the control (Equation 2).

Equation 1.

$$\%\ apoptopic\ cells - (average\ contol * \frac{100 - \%\ apoptopic\ cells}{100 - average\ control})$$

3 Results

3.1 Harvesting and freeze drying of bacteria

Harvesting of bacteria occurred when the bacterial strains were at the top of exponential growth, which was determined by use of light microscopy. The different strains had different growth patterns, ranging from around 7 days (for example TBK2h) to 14 days (for example P1a and P2S) before reach of peak growth. Due to the density of the cultures at harvesting, in addition to the weight of the strains after freeze-drying, similar volumes yielded different weights of freeze-dried product. Highest freeze-dried biomass yield per mL culture was found in strain TBK2h, with 0.73mg freeze-dried matter per mL of culture harvested. Lowest yield was found in strain P2S, with 0.086mg freeze-dried material per mL culture. Strains P1a, TBK2h, L1, P2S and VLbF2 were used in larger scale aqueous and organic phase extractions than the remaining strains, in addition to solid phase extractions, and were therefore cultivated at larger volumes to obtain additional biomass for the additional experiments (Table 3).

Table 3. Volume of bacterial culture from strains P1a, TBK2h, P62, L1, 7mR, 1mW, P2S and P61 harvested, and total weight of freeze-dried bacteria

Bacterial strain:	Volume harvested (in mL):	Weight freeze-dried matter (in mg):
P1a	5550	1005.4
TBK2h	3750	2743.4
P62	1850	457.9
L1	3350	428.0
7mR	1950	370.0
1mW	2050	690.9
P2S	3350	289.3
P61	1850	277.3

3.2 Obtaining and categorizing results from cell experiments

Results from experiments performed on acute myeloid leukaemia cell line MOLM-13, prostate cancer cell line PC3 and normal rat kidney cells (NRK) were obtained through WST-1 assays and calculations of percentage of apoptotic cells visualized on microscope pictures. Apoptotic cells were viewed in fluorescence microscope as having strongly coloured nuclei, due to fixation including the colourant Hoechst 33342, which binds to DNA. Apoptosis causes cells to shrink and DNA to condense, making the stained nuclei more visible (Figure 8 a). As the cells shrink, fragments of nuclei and organelles in the cell form apoptotic bodies (Figure 8 b). Viable, healthy cells were rounder, not as bright in colour and with whole nuclei (Figure 8 c).

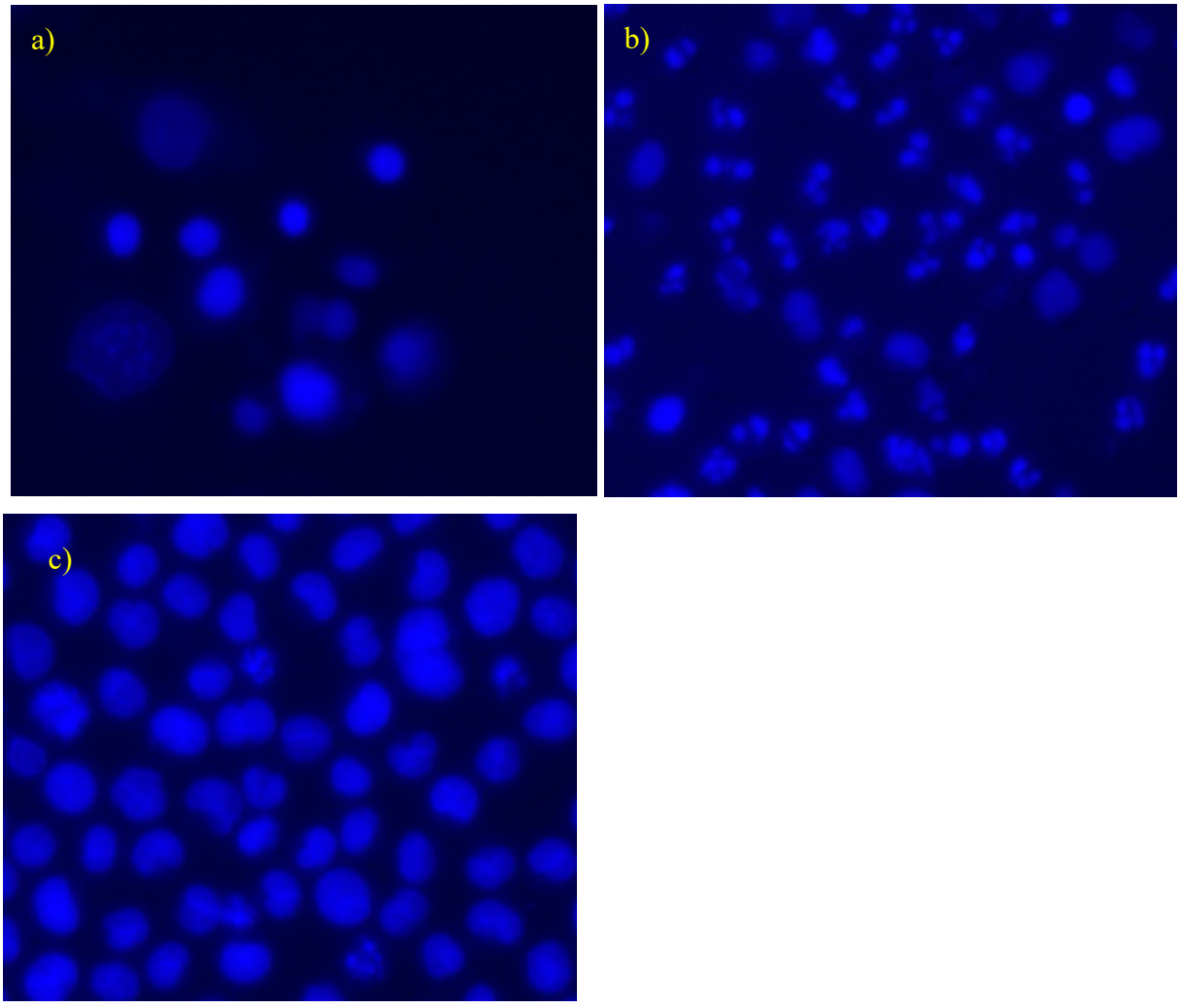


Figure 8. Pictures taken in fluorescence microscope showing treated and untreaded cancer cells dyed with Hoechst 33342. a) PC3 cells treated with organic phase of P2S for 24h, showing condensed nuclei as small stained circles next to faint whole, healthy cancer cells. b) MOLM-13 cells treated with aqueous phase of strain 1mW after 72h, showing apoptotic bodies after nucleic condensation and collapsing. c) Visualizing untreated viable cells in MOLM-13 control after 72h. Cells are viewed as healthy, round and whole.

To categorize the cytotoxic potential of the bacteria based on the percentage of apoptosis, high potential was set at 70-100% apoptotic cells, intermediate potential was set at 40-70%, low potential at 10-40%, and no potential at less than 10% apoptosis. This same categoric system was implemented on the WST-1 assays, high potential being >70% less metabolic activity compared to the untreated control, intermediate being 40-70% less metabolic activity, and so on.

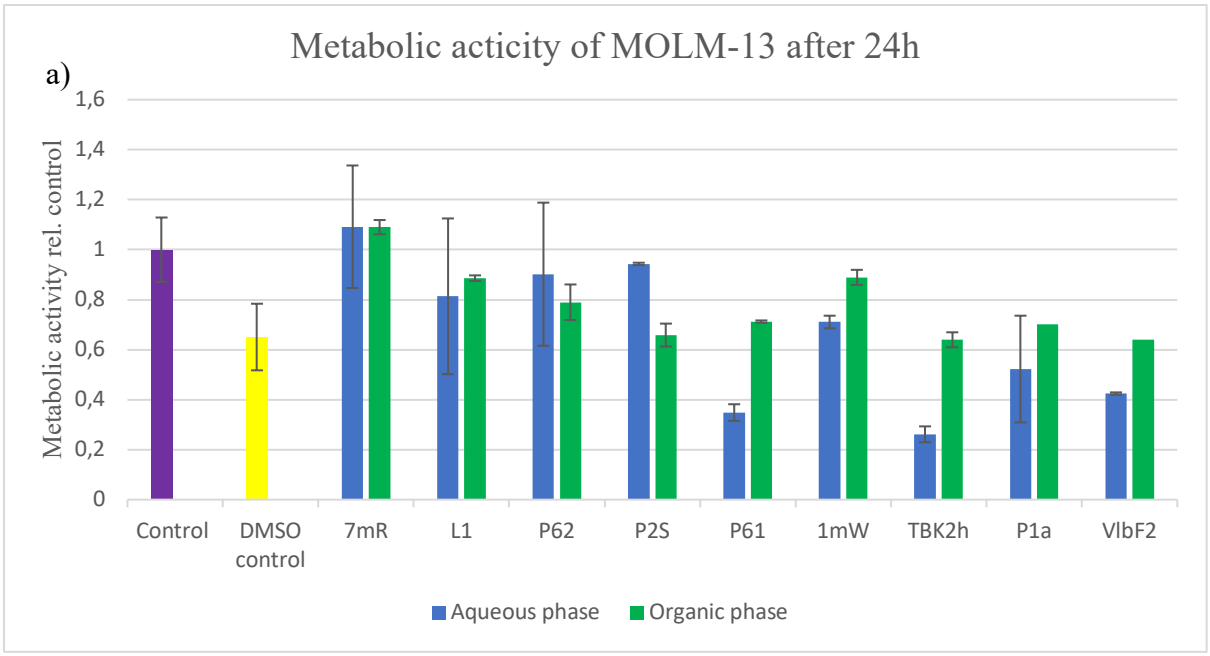
3.3 Cell experiments using small scale extractions of aqueous and organic phases

Samples of 10mg from bacterial strains 7mR, L1, P62, P2S, P61, 1mW, TBK2h, P1a and VLbF2 were tested for their cytotoxic activity against leukaemia cell line MOLM-13, prostate cancer cell line PC3 and normal rat kidney cells (NRK) for 24 and 72 hours exposure.

3.3.1 MOLM-13 cell line

WST-1 assay showed some toxicity caused by extracted phases of the bacteria after 24 hours. Aqueous phases of P61 and TBK2h were most active, showing intermediate and high cytotoxic potential respectively. Organic phases showed low or no cytotoxic activity towards MOLM-13 after 24h according to metabolic activity analysis (Figure 9a). Looking at percentage of apoptotic cells, aqueous phases of TBK2h and VLbF2 had highest cytotoxic activity, with 73% and 52% induced apoptosis respectively, in comparison to the control which showed 5% apoptosis and the DMSO control which showed 18% apoptosis. All organic extracts and the remaining aqueous extracts showed low or no effect on percentage of apoptosis (Figure 9b). After 24h the aqueous extraction of strain P61 induced only 16% apoptosis, low efficiency, according to percentage of apoptosis, contrary to the WST-1-assay where the strain showed intermediate efficiency (Figure 9a and b).

After 72 hours, metabolic activity was low relative to the control in the aqueous and organic phases of all the strains as well as in the DMSO control. Aqueous phases of strain 1mW, TBK2h and VLbF2 exhibited the most cytotoxic abilities after 72h, all showing high cytotoxic potential towards MOLM-13 according to metabolic activity analysis. Organic phases of 7mR and TBK2h also showed high cytotoxic abilities, while the remaining organic and aqueous phases exhibited intermediate potential according to WST-1 analysis (figure 10a). The calculated percentage of apoptosis showed similar results to the WST-1 assay, with aqueous phases of TBK2h and VLBf2 being most cytotoxic, in addition to organic phase of 7mR. The strains showed high potential for cytotoxicity, with 71%, 90% and 78% induced apoptosis respectively (figure 10b). Contrary to the metabolic activity analysis the DMSO control and strain 1mW showed intermediate cytotoxic potential, compared to high potential in WST-1 analysis. The remaining aqueous and organic extractions (bar aqueous phase of 7mR) showed reduced cytotoxic abilities compared to analysis of metabolic activity, going from intermediate to low or no cytotoxic potential towards MOLM-13 (Figure 10a and b).



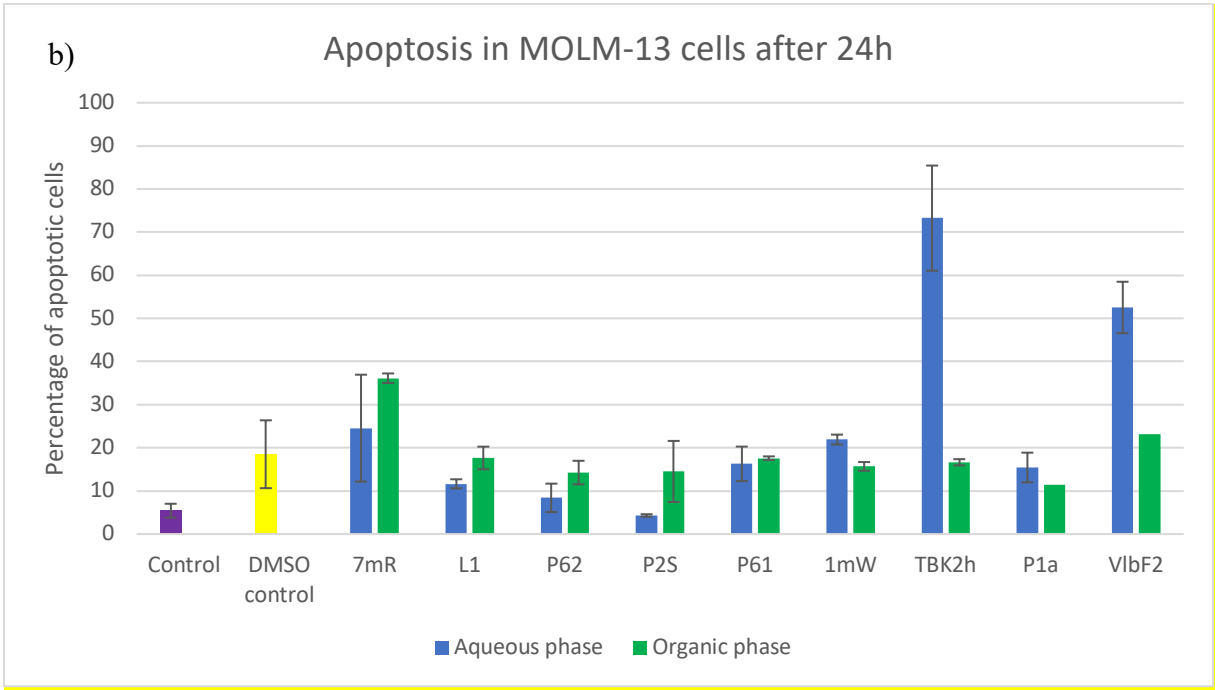


Figure 9. a) Results from WST-1 assay of aqueous (blue) and organic (green) extractions of strains 7mR, L1, P62, P2S, P61, 1mW, TBK2h, P1a and VLbF2 tested for cytotoxicity against leukaemia cell line MOLM-13 for 24h, relative to a control (purple). A DMSO control added at 1% was also tested for cytotoxicity (yellow). b) Calculated percentage of apoptotic MOLM-13 cells in samples added aqueous and organic phases of the same strains after 24h. Apoptosis was calculated from pictures taken in fluorescence microscope.

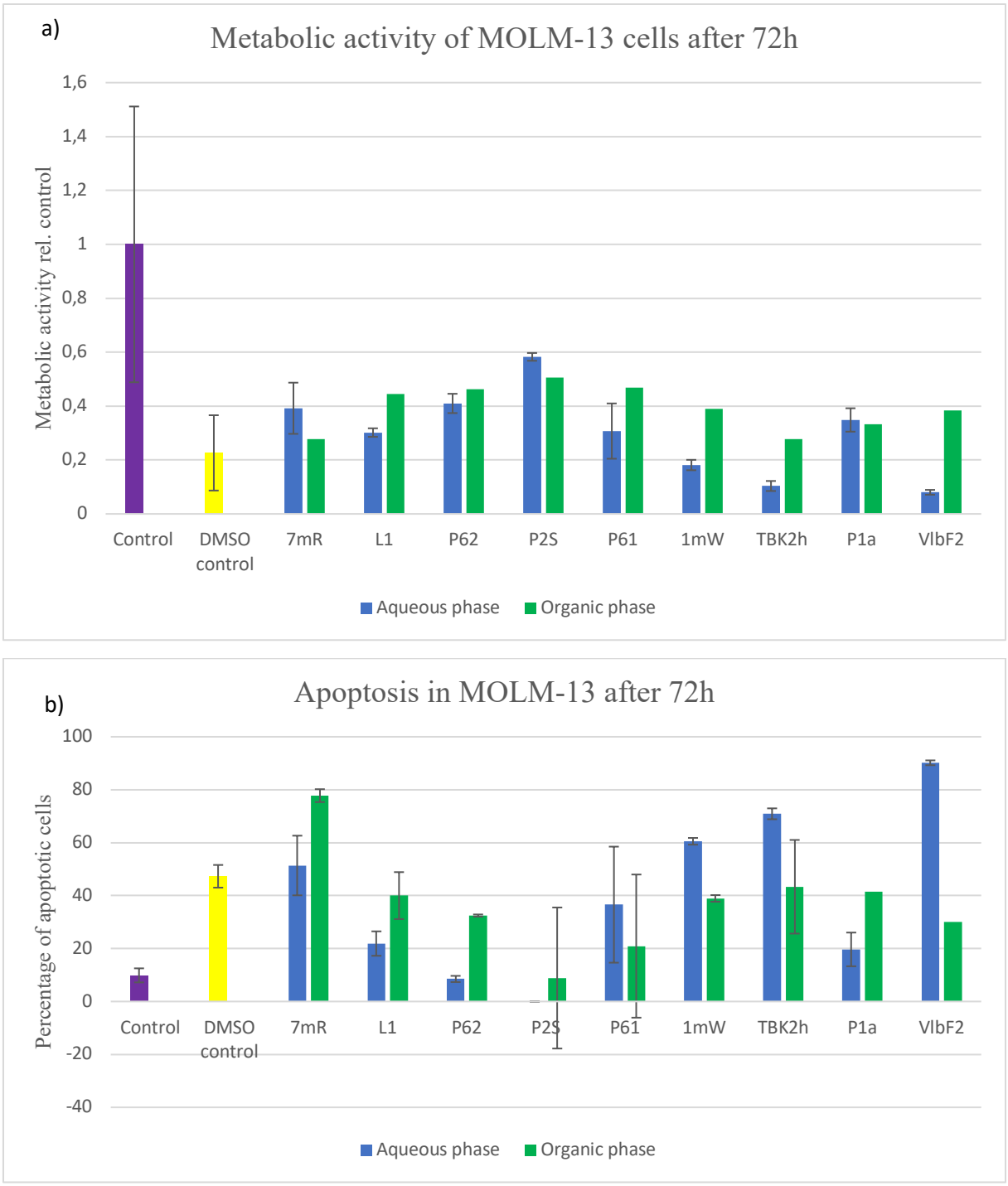


Figure 10. a) WST-1 assay of aqueous (blue) and organic (green) extractions of strains 7mR, L1, P62, P2S, P61, 1mW, TBK2h, P1a and VLbF2 tested for cytotoxicity against leukaemia cell line MOLM-13 for 72h, relative to a control (purple). A DMSO control added at 1% was also tested for cytotoxicity (yellow). b) Calculated percentage of apoptotic MOLM-13 cells in samples added aqueous and organic phases of the same strains after 72h. Apoptosis was calculated from pictures taken in fluorescence microscope.

3.3.2 PC3 cell line

After 24 h, organic phases had the most cytotoxic potential towards the PC3 cell line (Figure 11a and b). The output from WST-1 analysis showed that organic phases of strains P2S and VLbF2 had the highest potential towards the cell line, with 89% and 93% less metabolic activity than the untreated control respectively. Organic phases of strains 7mR, 1mW, L1 and P1a showed intermediate potential, with >40% less metabolic activity than the control. Aqueous phase of strain L1 showed intermediate cytotoxic potential, while the remaining aqueous extracts showed low or no potential after 24h according to analysis of metabolic activity. DMSO control had no cytotoxic potential towards PC3 cells (Figure 11 a). Percentage of apoptosis showed that the organic phases were more cytotoxic active than aqueous phases after 24h. Aqueous phases showed low or no potential on the cells according to percentage of apoptosis, and DMSO control showed no potential (Figure 11b). P2S was most cytotoxic, showing intermediate potential, which was contrary to the high potential seen in WST-1 analysis. Organic phases of strains 7mR, 1mW, L1, VLbF2 and P1a had no more than low potential on the PC3 cell line, also contrary to metabolic analysis (Figure 11a and b).

After 72h organic phases were still most cytotoxic active towards cell line PC3 (Figure 12a and b). The WST-1 assay showed high cytotoxic potential from the organic phases of strains L1, P2S, 1mW, P1a and VLbF2. Strain P1a was found to induce 96% less metabolic activity compared to the control. Intermediate potential was seen from organic phases of strains P61, 7mR and TBK2h, in addition to the aqueous phase of 7mR, while the remaining aqueous phases and organic phase of P62 showed low or no potential (Figure 12a). Percentage of apoptosis after 72h, documented a lower potential overall from the strains, with the most cytotoxic being the organic phases of strains 1mW and P1a with 38% and 35% induced apoptosis respectively (Figure 12b). Strains L1, P2S and VLbF2 showed low potential towards PC3 from percentage of apoptosis, contrary to the high potential shown in WST-1 analysis. DMSO control showed no potential in both metabolic activity and percentage of apoptosis (Figure 12a and b).

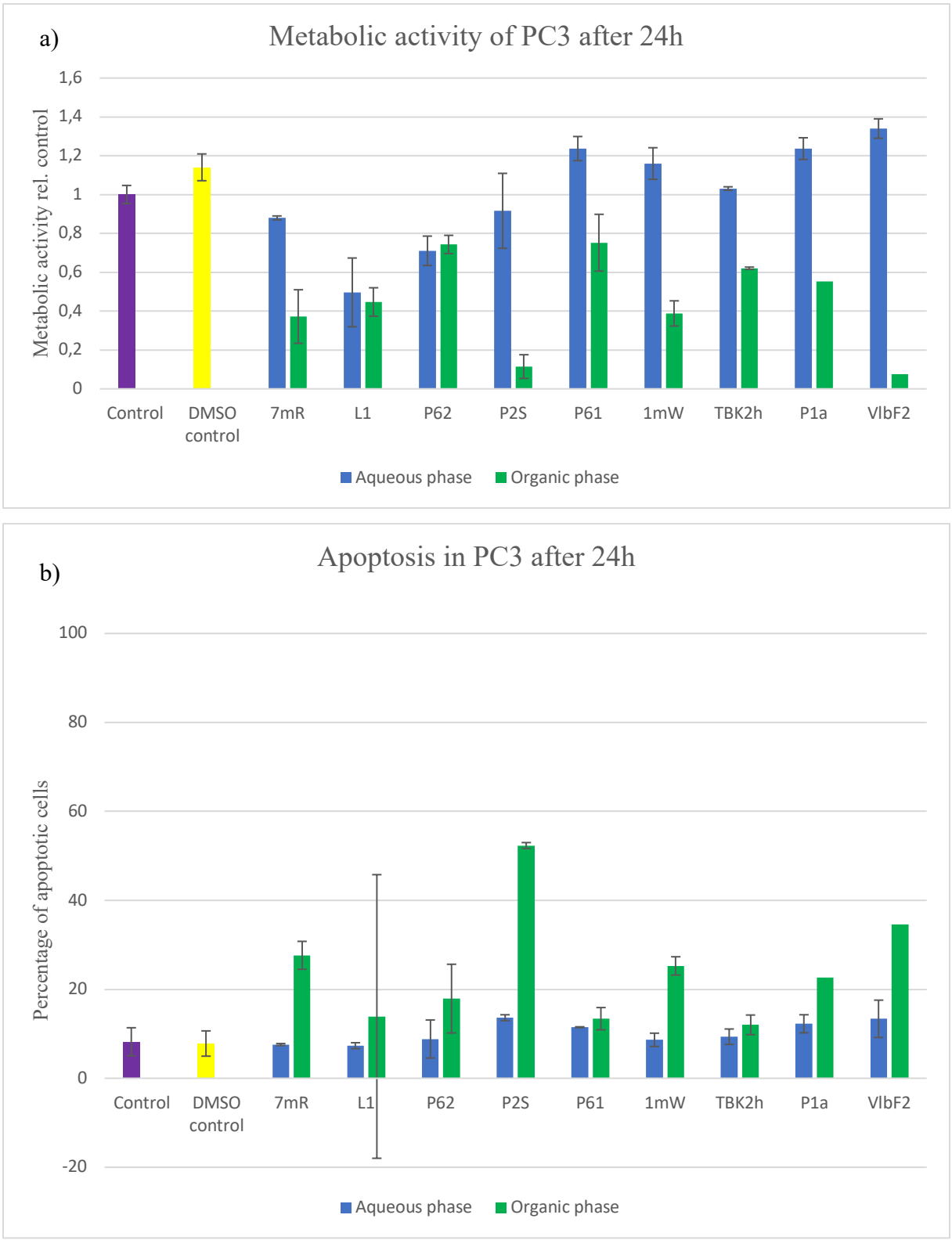
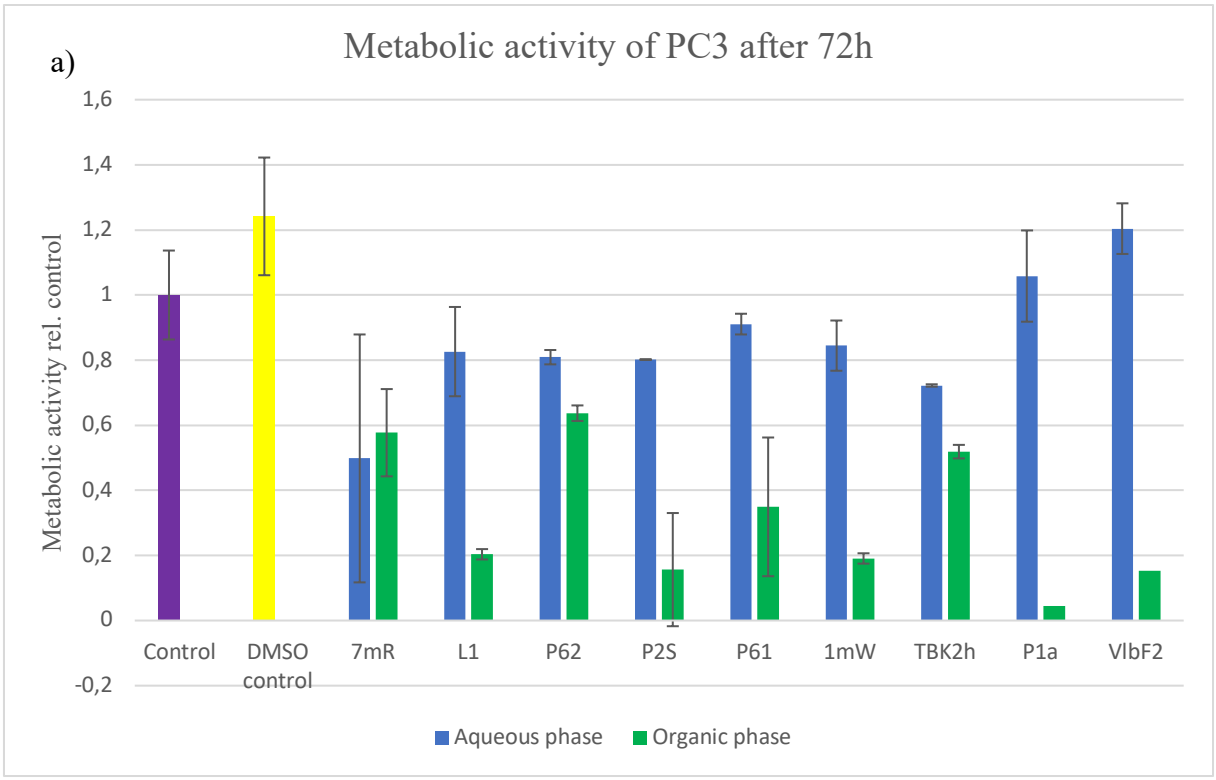


Figure 11. a) WST-1 assay of aqueous (blue) and organic (green) extractions of strains 7mR, L1, P62, P2S, P61, 1mW, TBK2h, P1a and VLbF2 tested for cytotoxicity against prostate cancer cell line PC3 for 24h, relative to a control (purple). A DMSO control added at 1% was also tested for cytotoxicity (yellow). b) Calculated percentage of apoptotic PC3 cells in samples added aqueous and organic phases of the same strains after 24h. Apoptosis was calculated from pictures taken in fluorescence microscope.



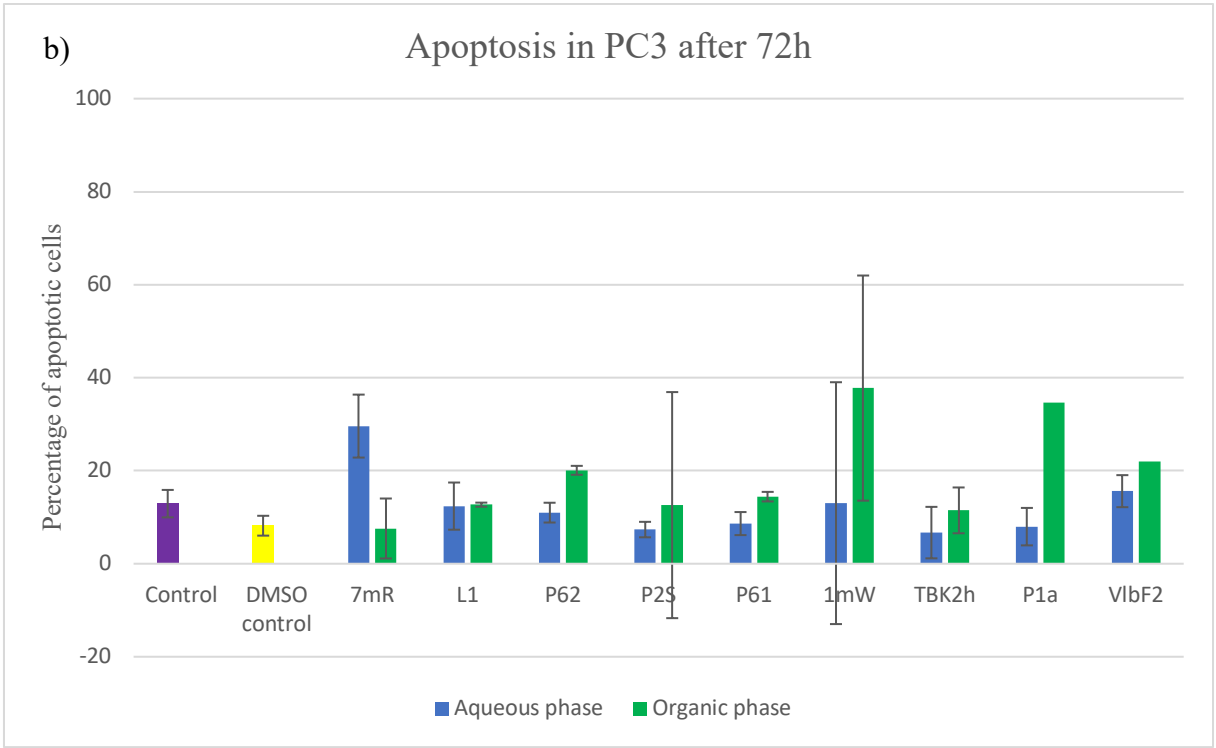


Figure 12. a) WST-1 assay of aqueous (blue) and organic (green) extractions of strains 7mR, L1, P62, P2S, P61, 1mW, TBK2h, P1a and VLbF2 tested for cytotoxicity against prostate cancer cell line PC3 for 72h, relative to a control (purple) A DMSO control added at 1% was also tested for cytotoxicity (yellow). b) Calculated percentage of apoptotic PC3 cells in samples added aqueous and organic phases of the same strains after 72h. Apoptosis was calculated from pictures taken in fluorescence microscope.

3.3.3 Normal rat kidney cells (NRK)

After 24h incubation, WST-1 analysis showed that organic phases induced a higher cytotoxic effect on NRK cells than aqueous phases. Organic phases of all strains excluding P62 and P61 were classified as high in cytotoxic potential. Strains P62 and P61 showed intermediate potential in organic phase. TBK2h showed intermediate potential in aqueous phase, while the remaining aqueous extracts showed low or no potential after 24h according to analysis of metabolic activity (Figure 13a). In analysis of apoptosis, organic phase of strain P2S was the only extract showing intermediate cytotoxic potential, with 43% induced apoptosis. The remaining organic phases showed low potential towards NRK. Aqueous phases had no potential towards NRK after 24h (Figure 13a). DSMO control showed low potential t in WST-1 analysis, and no potential in percentage of apoptosis (Figure 13a and b).

After 72h exposure, organic phases were more cytotoxic active than aqueous phases (Figure 14 a and b). WST-1 analysis showed that organic phases of strains 7mR, L1, 1mW, P1a and VLbF2 had high cytotoxic potential towards NRK cells. The remaining organic phases showed intermediate potential. Only aqueous phase of strain VLbF2 showed intermediate potential, with the remaining aqueous phases having low or no potential. The DMSO control showed intermediate potential after 72 hours (Figure 14a). Percentage of apoptosis showed only low or no potential induced by the organic phases. The DMSO control was also equivalently low, showing no potential. Aqueous phases showed no or low potential towards the cells, excluding strain P61 (Figure 14b). Contrary to the WST-1 analysis of metabolic activity, aqueous phase of P61 showed high potential on NRK cells in percentage of apoptosis after 72h with over 85% apoptosis (Figure 14a and b).

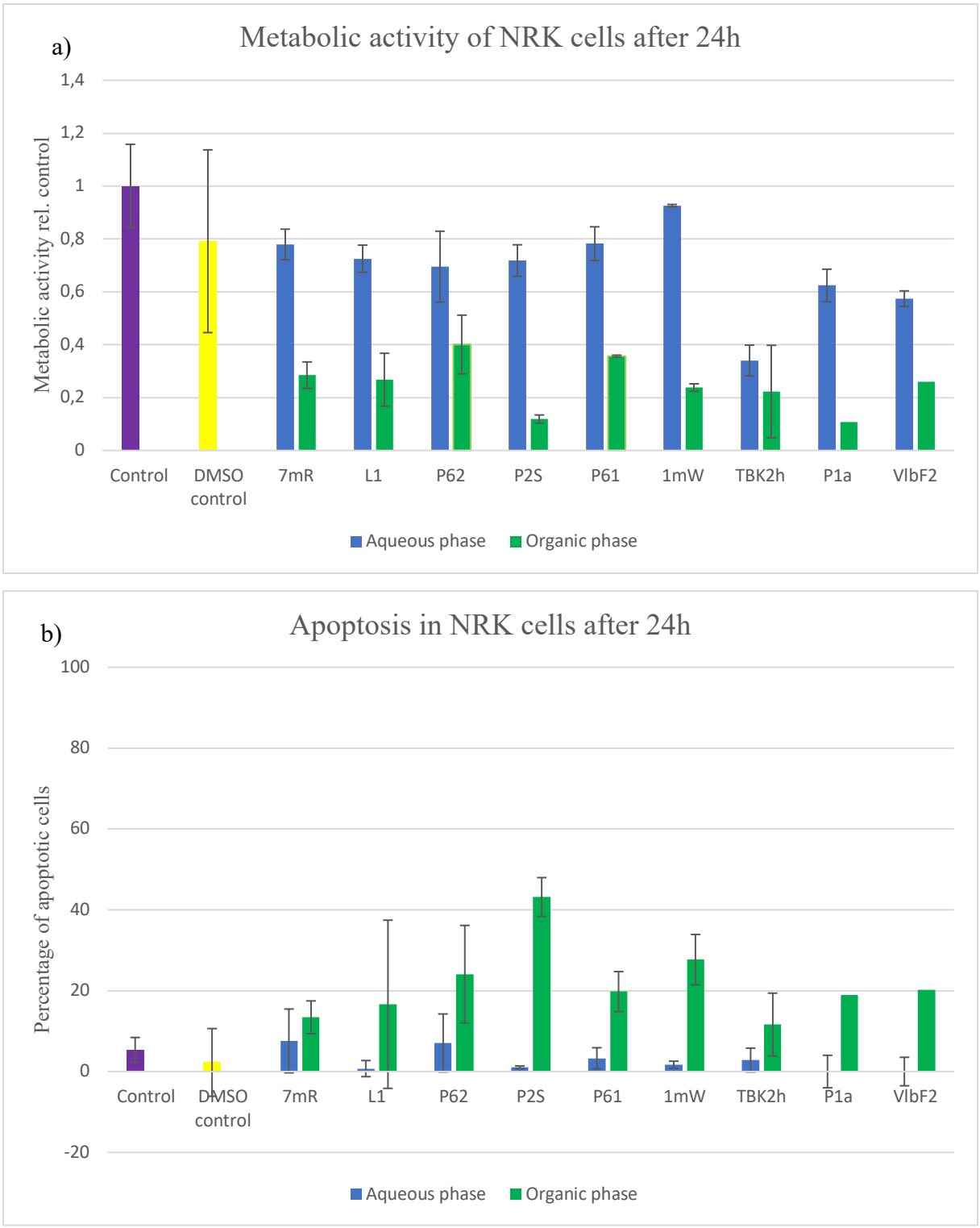
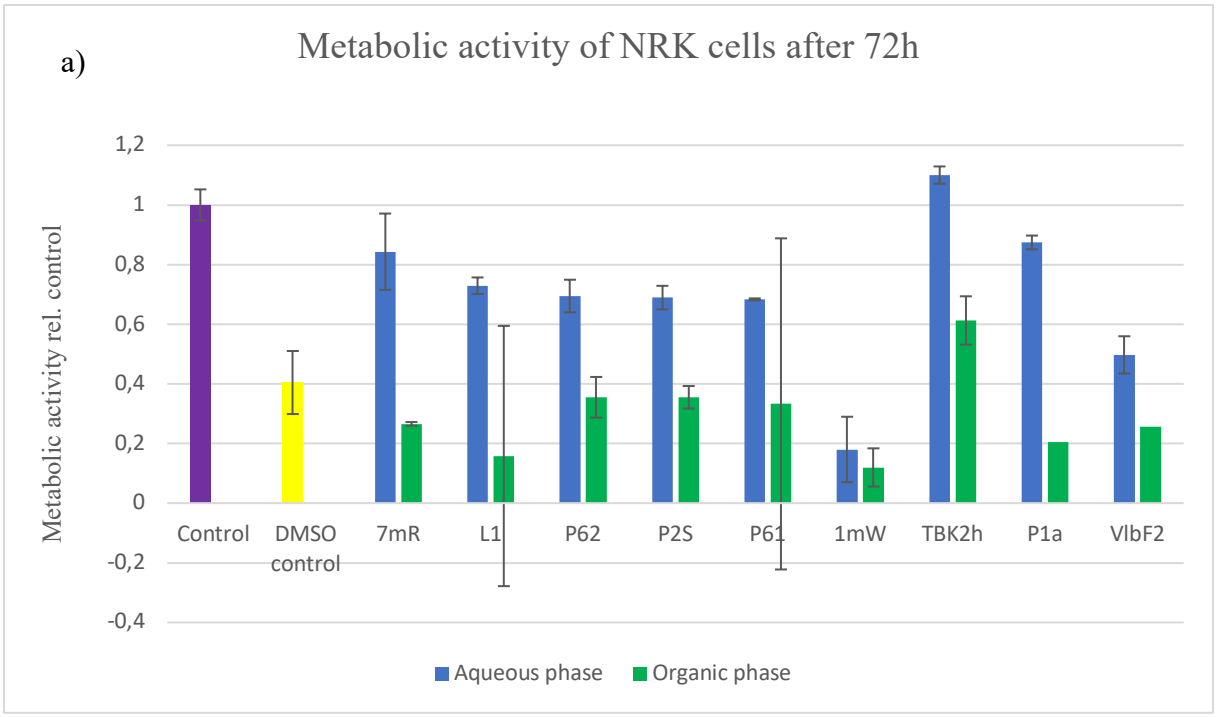


Figure 13. a) WST-1 assay of aqueous (blue) and organic (green) extractions of strains 7mR, L1, P62, P2S, P61, 1mW, TBK2h, P1a and VLbF2 tested for cytotoxicity against normal rat kidney cells (NRK) for 24h, relative to a control (purple). A DMSO control added at 1% was also tested for cytotoxicity (yellow). b) Calculated percentage of apoptotic NRK cells in samples added aqueous and organic extracts of the same strains after 24h. Apoptosis was calculated from pictures taken in fluorescence microscope.



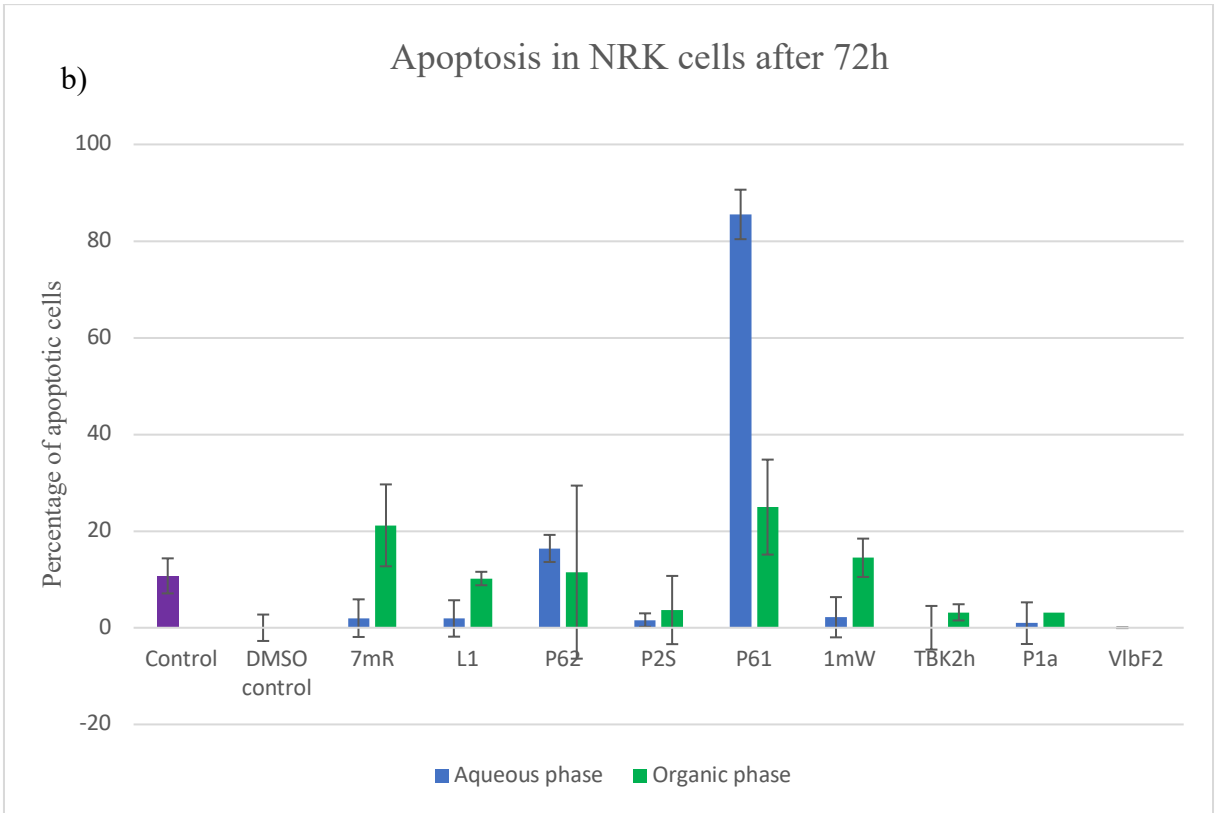


Figure 14. a) WST-1 assay of aqueous (blue) and organic (green) extractions of strains 7mR, L1, P62, P2S, P61, 1mW, TBK2h, P1a and VLbF2 tested for cytotoxicity against normal rat kidney cells (NRK) for 72h, relative to a control (purple). A DMSO control added at 1% was also tested for cytotoxicity (yellow). b) Calculated percentage of apoptotic NRK cells in samples added aqueous and organic extracts of the same strains after 72h. Apoptosis was calculated from pictures taken in fluorescence microscope.

3.3 Cell experiments using larger scale extractions of selected strains

Large scale extractions using between 100-500mg bacterial biomass were done on strains which showed cytotoxic activity towards cancerous cells, or on strains that had not been tested for cytotoxic abilities in previous published work. Aqueous phases were extracted from strains P2S, L1, TBK2h and VLbF2, and organic phase was extracted from strain P1a. The strains were tested for cytotoxic abilities against either acute myeloid leukaemia cell line MOLM-13 or prostate cancer cell line PC3 in a dilution series of 5%, 2% and 0.5% extract to 95%, 98% and 99.5% cell culture. A 1% DMSO control was used for the experiments. Cytotoxic abilities of the strains were measured after 72h by a combination of WST-1 assay and calculations of percentage of apoptosis from morphological inspections of cell nuclei in fluorescence microscope.

3.3.1 Aqueous phases of P2S, L1, TBK2h and VLbF2 on MOLM-13

Aqueous phases were extracted from bacterial samples of 100mg from strains P2S, L1, TBK2h and VLbF2. Samples of 10% were collected from the extractions of L1, TBK2h and VLbF2, while a 3.33% sample was collected of P2S. The samples were analysed for cytotoxic abilities towards MOLM-13. Analysis of metabolic activity after 72h showed that all strains showed high cytotoxic potential at 5%, while only strain TBK2h showed high potential at 2%. Strain VLbF2 showed intermediate potential at 2%, while the remaining strains showed low or no potential at 2%. At 0.5%, all the strains showed low or no potential. The 1% DMSO control exhibited intermediate potential in WST-1 analysis (Figure 15a). Analysis of apoptosis showed intermediate potential from the DMSO control, with 40% induced apoptosis. Strains P2S, L1 and VLbF2 showed high potential at 5%, while TBK2h only exhibited intermediate potential, with 66% induced apoptosis. TBK2h also showed intermediate potential at 2%, as did strain VLbF2. The remaining strains showed no potential at 2% and 0.5% dilution. VLbF2 was the only strain that showed low potential at 0,5% (Figure 15b)

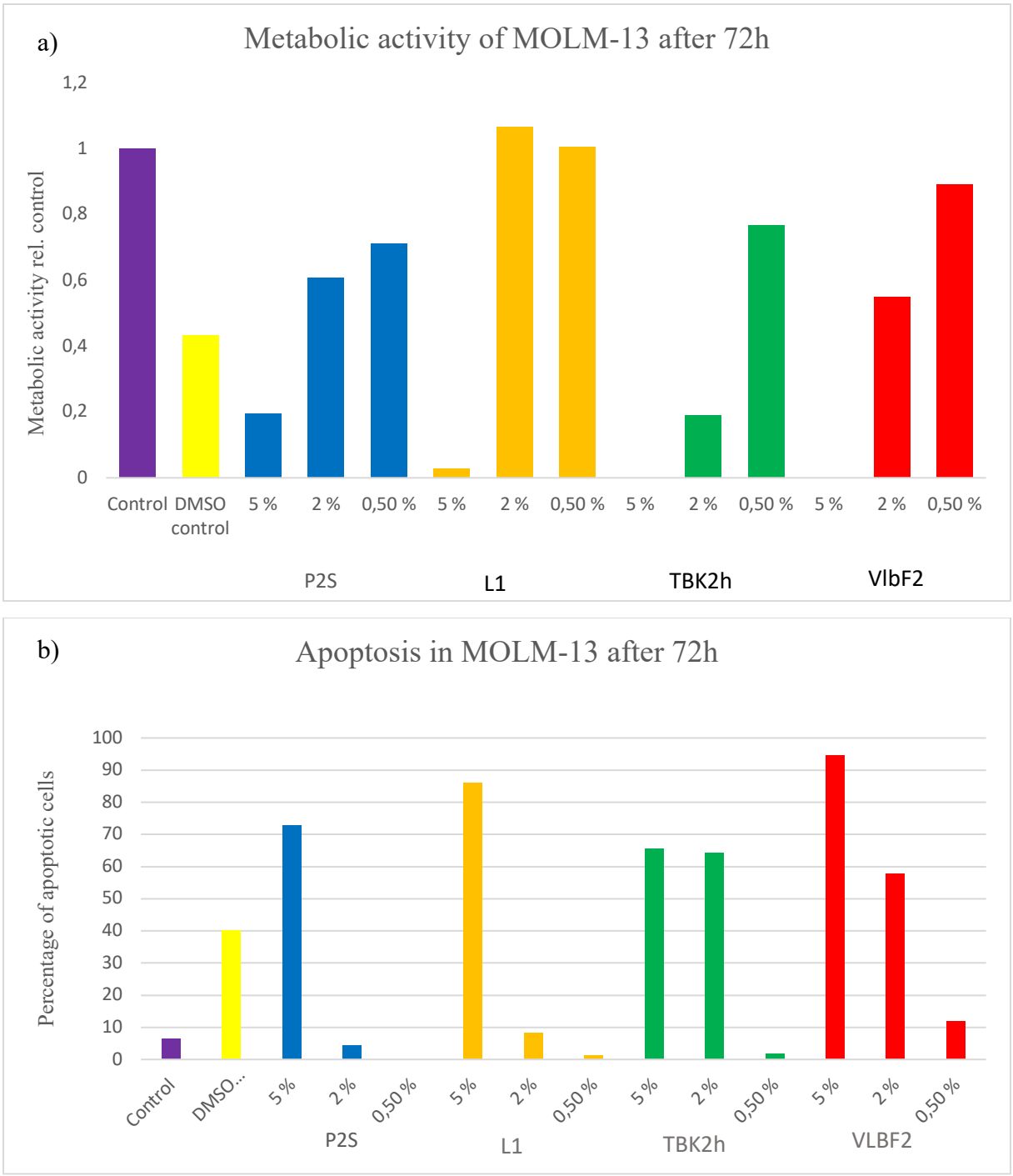


Figure 15. a) WST-1 assay of aqueous phase extracted from 100mg biomass samples of strains P2S (blue), L1 (orange), Tbk2h (green) and VLbF2 (red) tested for cytotoxicity against leukaemia cell line MOLM-13 at a dilution series of 5%, 2% and 0.5% for 72h, relative to a control (purple). A DMSO control added at 1% was also tested for cytotoxicity (yellow). b) Calculated percentage of apoptotic MOLM-13 cells in samples added aqueous phases of the same strains after 72h. Apoptosis was calculated from pictures taken in fluorescence microscope.

3.3.2 Organic phase of P1a on PC3

A 500mg bacterial sample was used to extract organic phase of strain P1a. A 1,67% sample of the extraction was collected and tested for cytotoxic abilities towards cell line PC3. After 72h, WST-1 analysis showed that the 2% dilution had intermediate cytotoxic potential, while the remaining dilutions showed no potential. The 1% DMSO control showed intermediate potential (Figure 16a). Analysis of apoptosis showed high potential in the 5% dilution, with a percentage of apoptosis of 77%. The 2% dilution showed low potential, with 34% induced apoptosis, while the 2% dilution and the DMSO control showed no potential on apoptosis (Figure 16b).

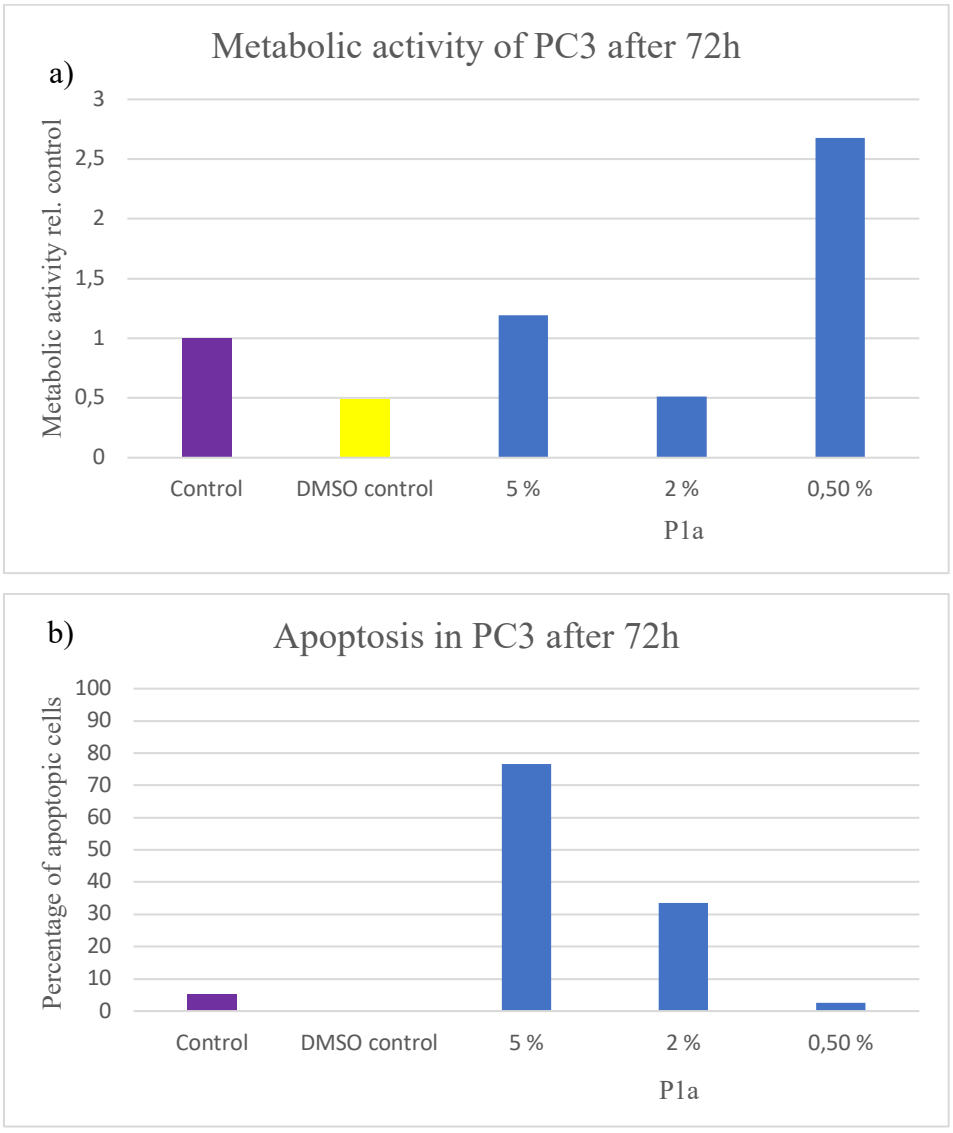


Figure 16. a) WST-1 assay of organic phase extraction using 500mg biomass sample of strain P1a tested against prostate cancer cell line PC3 for 72h at a dilution series of 5%, 2% and 0.5% relative to a control (purple). A DMSO control added at 1% was also tested for cytotoxicity (yellow). b) Calculated percentage of apoptotic PC3 cells in samples added aqueous phases of the same strain after 72h. Apoptosis was calculated from pictures taken in fluorescence microscope.

3.4 Cell experiments using solid phase extractions (SPE) of selected strains

SPE were performed on the selected strains where larger phase-extractions of different volumes had been performed. SPE were conducted on the selected strains to separate chemical substances from impurities in the three fractions load and wash (L+W), elution phase 1 (E1) and elution phase 2 (E2). SPE were tested on acute myeloid leukaemia cell line MOLM-13 and prostate cancer cell line PC3 in dilution series of 5%, 2% and 0.5% extractions to 95%, 98% and 99.5% cell culture. The cells were exposed for 48h, as this was found through light microscopy to be sufficient for full effect of the strains. The results were collected by determination of metabolic activity and calculation of cell apoptosis.

3.4.1 SPE from aqueous phases of VLbF2, L1, P2S and TBK2h on MOLM-13

SPE were conducted on aqueous phases derived from 100mg bacterial samples of strains VLbF2, L1 and P2S, in addition to a 1000mg bacterial sample of strain TBK2h. The extractions were tested against cell line MOLM-13.

Metabolic activity analysis on strains VLbF2, L1 and P2S showed that VLbF2 had high cytotoxic potential at 5% and 2% in all three fractions. The strain also showed intermediate potential at 0.5% for all fractions (Figure 17a) This was supported by percentage of apoptosis, where all dilutions of L+W showed high potential, in addition to E1 at 5% and 2% and E2 at 5%. E1 showed intermediate potential at 0.5%, while E2 at exhibited no potential 0.5% (Figure 17b). Strain L1 was more cytotoxic in the later fractions E1 and E2, with WST-1 analysis showing high potential from E1 at 5% and E2 at 5% and 2%. L+W showed intermediate potential at 5%, while the remaining samples showed low (E2 at 0,5%) or no (L+W and E1 at 2% and 0.5%) potential (Figure 17a). Percentage of apoptosis showed that E1 and E2 was most cytotoxic in strain L1. E2 exhibited high potential at 5% and 2%, and E1showed high potential at 5%. L+W showed intermediate potential at 5%, but low at 2% and no potential at 0.5%. E1 showed low potential at 2%, and no potential at 0.5%. This was also true for E2 at 0.5% (Figure 17b). Strain P2S was more cytotoxic in fractions E1 and E2 than in L+W. E2 showed high potential at all dilutions, while E1 showed high potential at 5% and 2%. E1 showed intermediate potential at 0.5%, the same did L+W at 5%. The remaining dilutions of L+W showed low potential (Figure 17a). Percentage of apoptosis showed high potential in E1 at 5% and E2 at

5% and 2%. L+W showed low potential at 5% and no potential at 2% and 0.5%. E1 showed intermediate potential at 2%, and no potential at 0.5%. E2 at 0,5% was also low in potential (Figure 17b). The 1% DMSO control showed high potential in WST-1 assay and percentage of apoptosis (Figure 17a and b)

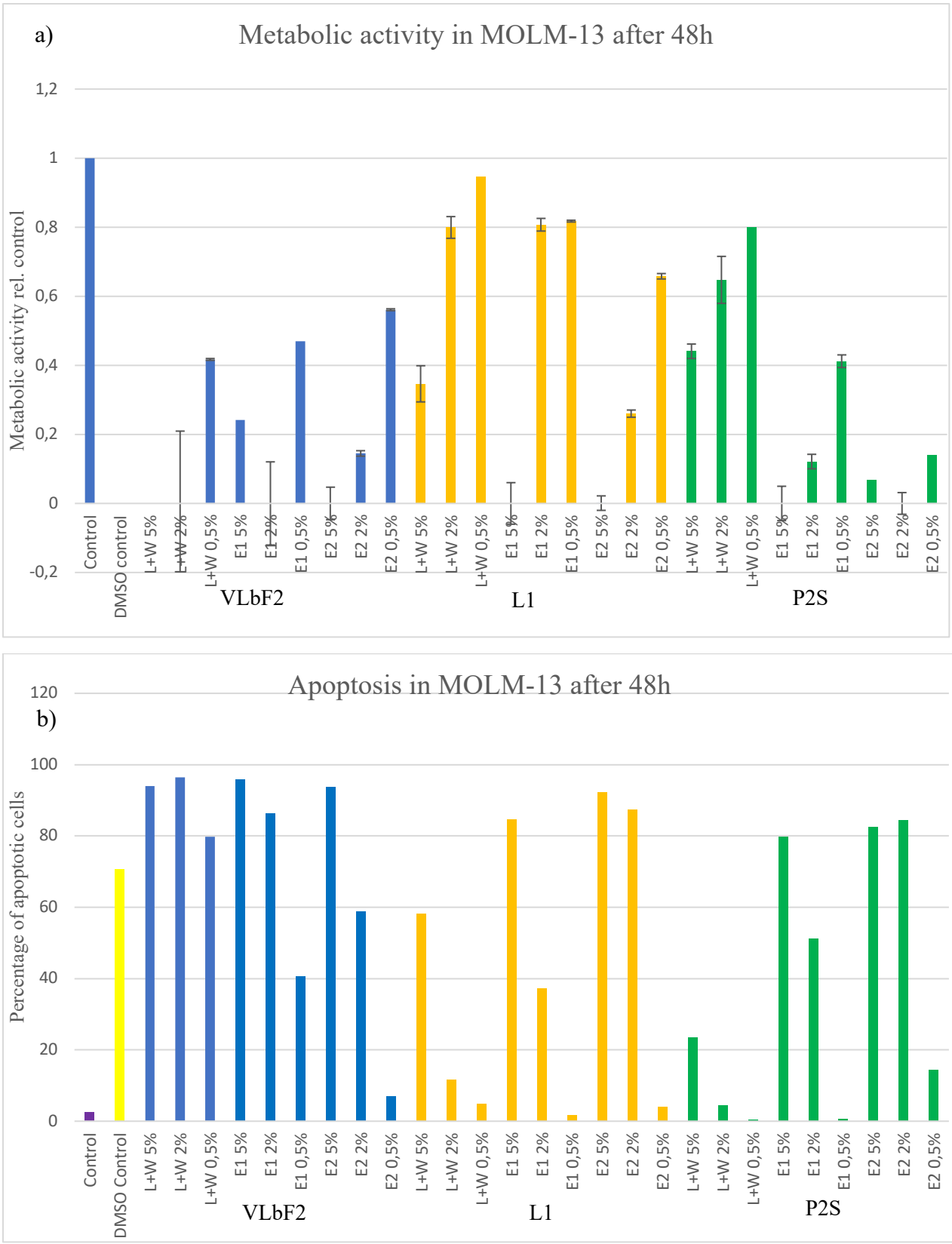
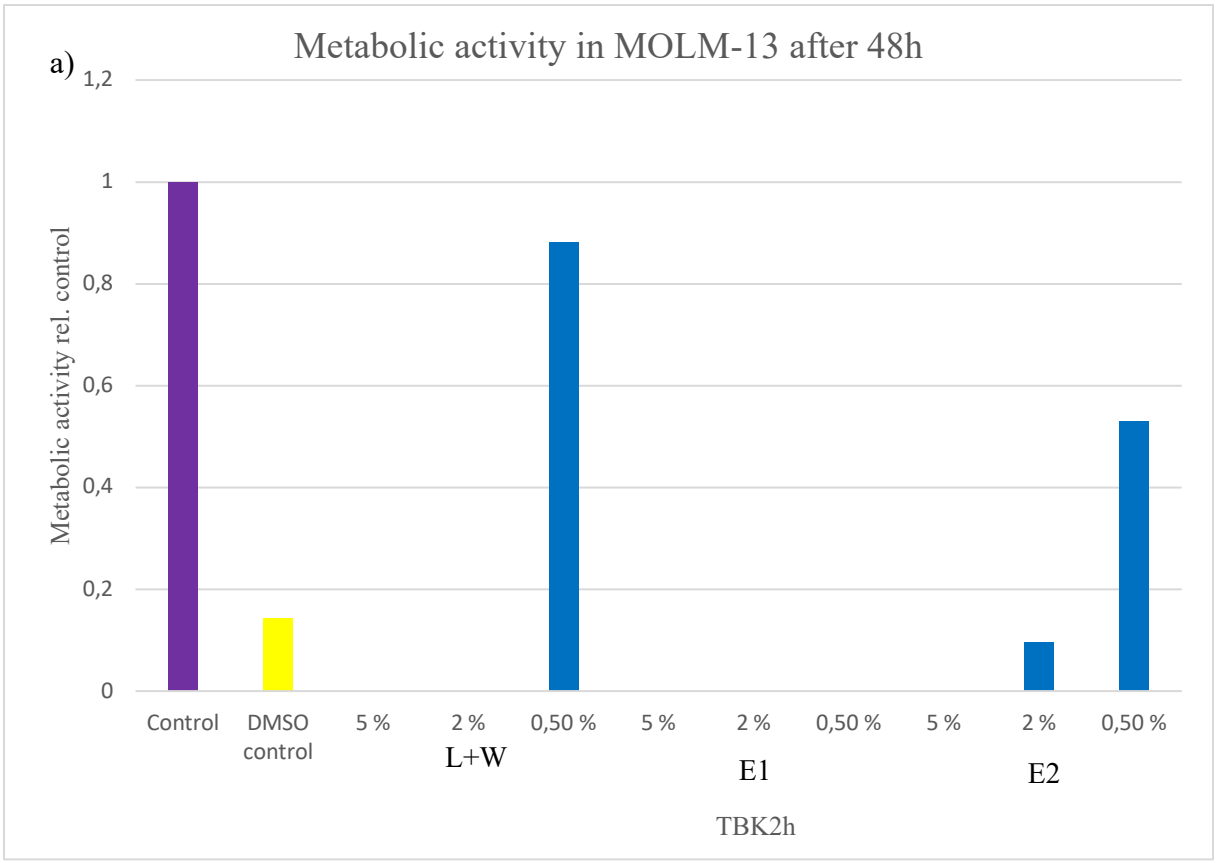


Figure 17. a) WST-1 assay of solid phase extractions of aqueous phase derived from 100mg biomass samples of strains VLbF2 (blue), L1 (orange) and P2S (green) tested for cytotoxicity against leukaemia cell line MOLM-13 in a dilution series of 5%, 2% and 0.5% for 48h, relative to a control (purple). A DMSO control added at 1% was also tested for cytotoxicity (yellow). b) Calculated percentage of apoptotic MOLM-13 cells in samples added SPE fractions of the same strains after 48h. Apoptosis was calculated from pictures taken in fluorescence microscope.

Metabolic activity analysis of SPE from strain TBK2h showed high cytotoxic potential in all fractions. L+W and E2 showed high potential at 5% and 2%, while E1 showed high potential at all dilutions. L+W showed low potential at 0.5%, while E2 exhibited intermediate potential at 0.5% (Figure 18a). Percentage of apoptosis showed that fraction L+W showed less cytotoxic activity than in WST-1 analysis, showing high potential at 2%, intermediate at 5% and low at 0.5%. E1 showed high potential at 5% and 2%, and intermediate potential at 0.5%. E2 exhibited high potential at 5% and at 0.5%, while only exhibiting intermediate potential at 2% (Figure 18b). The 1% DMSO control showed high cytotoxic potential both in the analysis of metabolic activity and in percentage of apoptosis (Figure 18a and b).



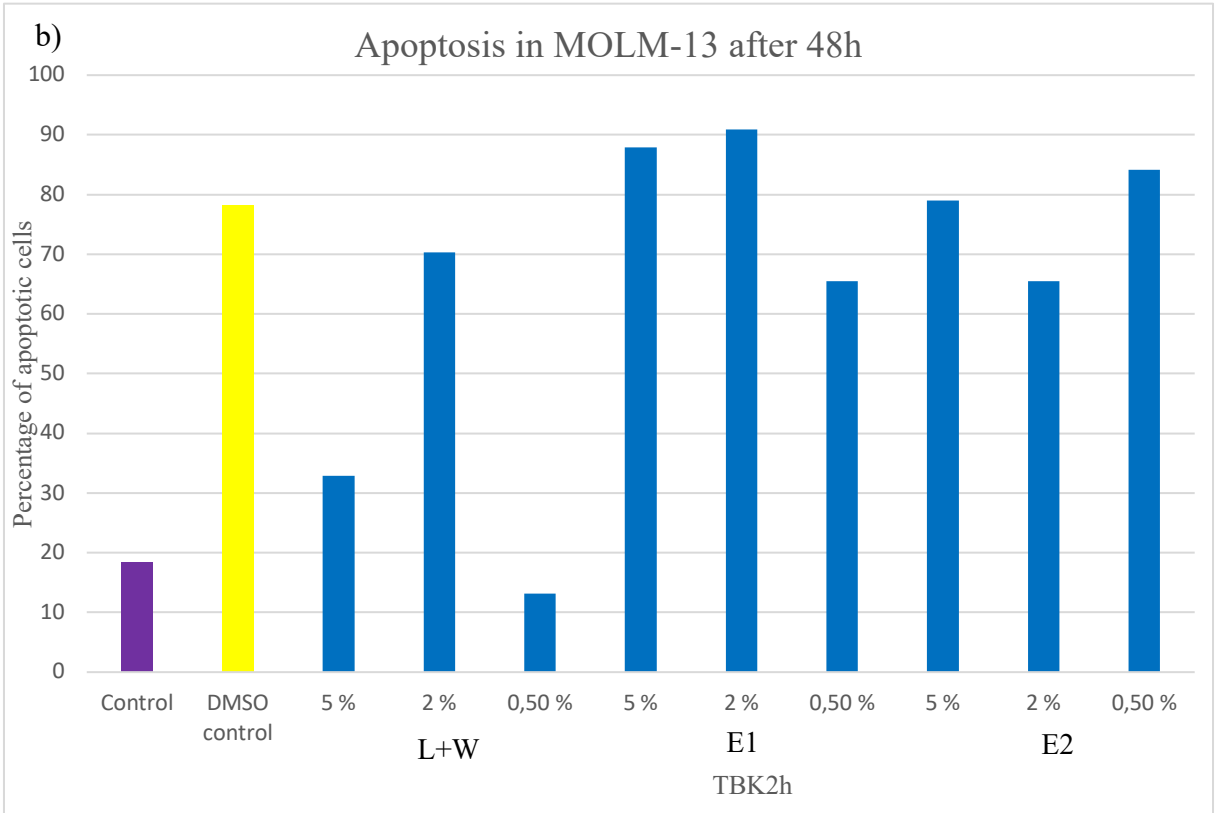


Figure 18. WST-1 assay of solid phase extraction phases of aqueous phase derived from a 1000mg biomass sample of strain TBK2h tested for cytotoxicity against leukaemia cell line MOLM-13 in a dilution series of 5%, 2% and 0.5% for 48h, relative to a control (purple). A DMSO control added at 1% was also tested for cytotoxicity (yellow). b) Calculated percentage of apoptotic MOLM-13 cells in wells added SPE phases of the same strain after 48h. Apoptosis was calculated from pictures taken in fluorescence microscope.

3.4.2 SPE from organic phase of P1a on PC3

SPE was performed using the organic extract of strain P1a derived from a bacterial sample of 150mg. SPE were tested for cytotoxic potential on the prostate cancer cell line PC3 after 48h. The cytotoxic abilities of the extractions were determined by metabolic activity analysis and calculations of percentage of apoptosis.

From WST-1 metabolic analysis, P1a showed high cytotoxicity towards PC3. L+W and E1 showed high potential at 5% and 2%, while E2 showed high potential at all dilutions. L+W and E1 showed low potential at 0.5% (Figure 19a). Calculations of apoptosis showed highest cytotoxic activity at intermediate potential, in L+W and E1 at 5% and 2% and in E2 at 5%. The remaining dilutions L+W and E1 at 0.5% and E2 at 2% and 0.5% showed low potential of cytotoxicity towards PC3 (Figure 19b). WST-1 analysis and percentage of apoptosis showed that the 1% DMSO control had low cytotoxic potential, with the WST-1 analysis showing no potential, and the apoptosis showing low potential (Figure 19a and b).

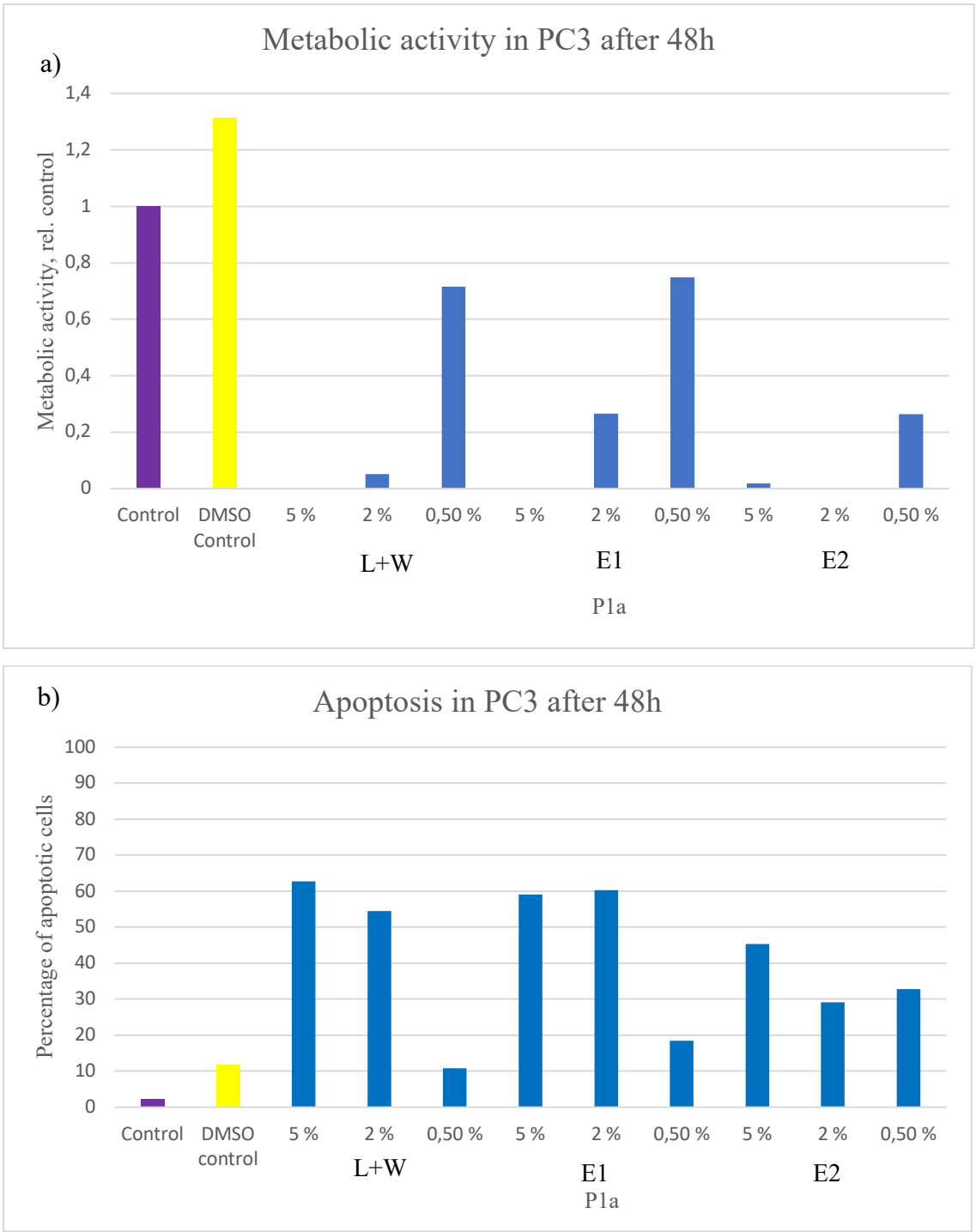


Figure 19. WST-1 assay of solid phase extraction phases of organic phase extracted from a 150mg biomass sample of strain P1a tested for cytotoxicity against prostate cancer cell line PC3 in a dilution series of 5%, 2% and 0.5% for 48h, relative to a control (purple). A DMSO control added at 1% was also tested for cytotoxicity (yellow). b) Calculated percentage of apoptotic PC3 cells in wells added SPE phases of the same strain after 48h. Apoptosis was calculated from pictures taken in fluorescence microscope.

3.5 HPLC separations of SPE and cancer cell experiments using HPLC fractions

HPLC separation and analysis of separated fractions was conducted using SPE E1 extracted from aqueous phase of strain TBK2h, in addition to SPE E2 extracted from organic phase of strain P1a. HPLC fractions from the two strains were measured for cytotoxic abilities against the cancerous cell lines MOLM-13 and PC3 respectively after treatments of 48h. TBK2h fractions were added 0.5% and 0.1%, while P1a was added at 1% and 0.3%. The cytotoxic activity of the extracted HPLC fractions was measured by metabolic activity analysis and calculations of apoptosis.

3.5.1 HPLC separation of TBK2h and HPLC fractions of TBK2h on MOLM-13

HPLC fractions from SPE E1 of strain TBK2h showed activity on the chromatogram at 2-8 minutes, forming two peaks which represent the detector response to one or several chemical compounds. These peaks appeared at around 70-80% polarity, meaning the corresponding compound or compounds were hydrophilic and polar (Figure 20). The peaks corresponded to cytotoxic activity on the cell line MOLM-13, which was found in both WST-1 analysis and percentage of apoptosis. Fractions 2 and 3, collected at 2-4 minutes and 4-6 minutes showed high potential at 0.5% (Figure 21a and b). This corresponded to the first peak in the chromatogram (Figure 20). Fraction 4, collected at 6-8 minutes, showed intermediate potential at 0.5% (Figure 21a and b). This corresponded to the second peak in the chromatogram, which appeared at around 70% polarity (Figure 20). Fractions 1 and 14, at 0-2 minutes and 26-28 minutes respectively, both showed intermediate potential at 0.5% in metabolic activity analysis. This was not seen in percentage of apoptosis, where the fractions showed no potential towards the MOLM-13 cell line. The 1% DMSO control showed low potential in metabolic activity analysis and percentage of apoptosis (Figure 21a and b).

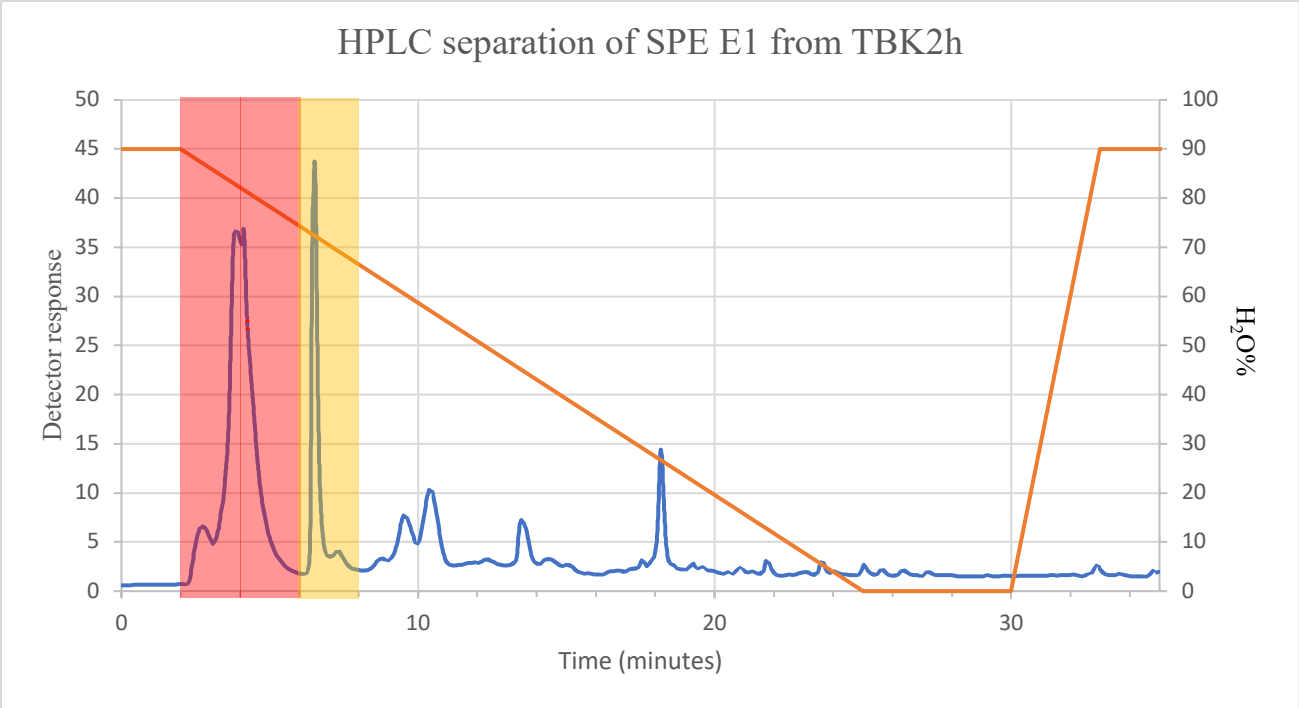


Figure 20. Chromatogram detailing HPLC separation of SPE E1 from aqueous phase of strain TBK2h at a gradient of mobile solvent H_2O (secondary axis). Peaks corresponding to HPLC fractions cytotoxic to cell line MOLM-13 are highlighted red for high cytotoxic effect and orange for intermediate cytotoxic effect.

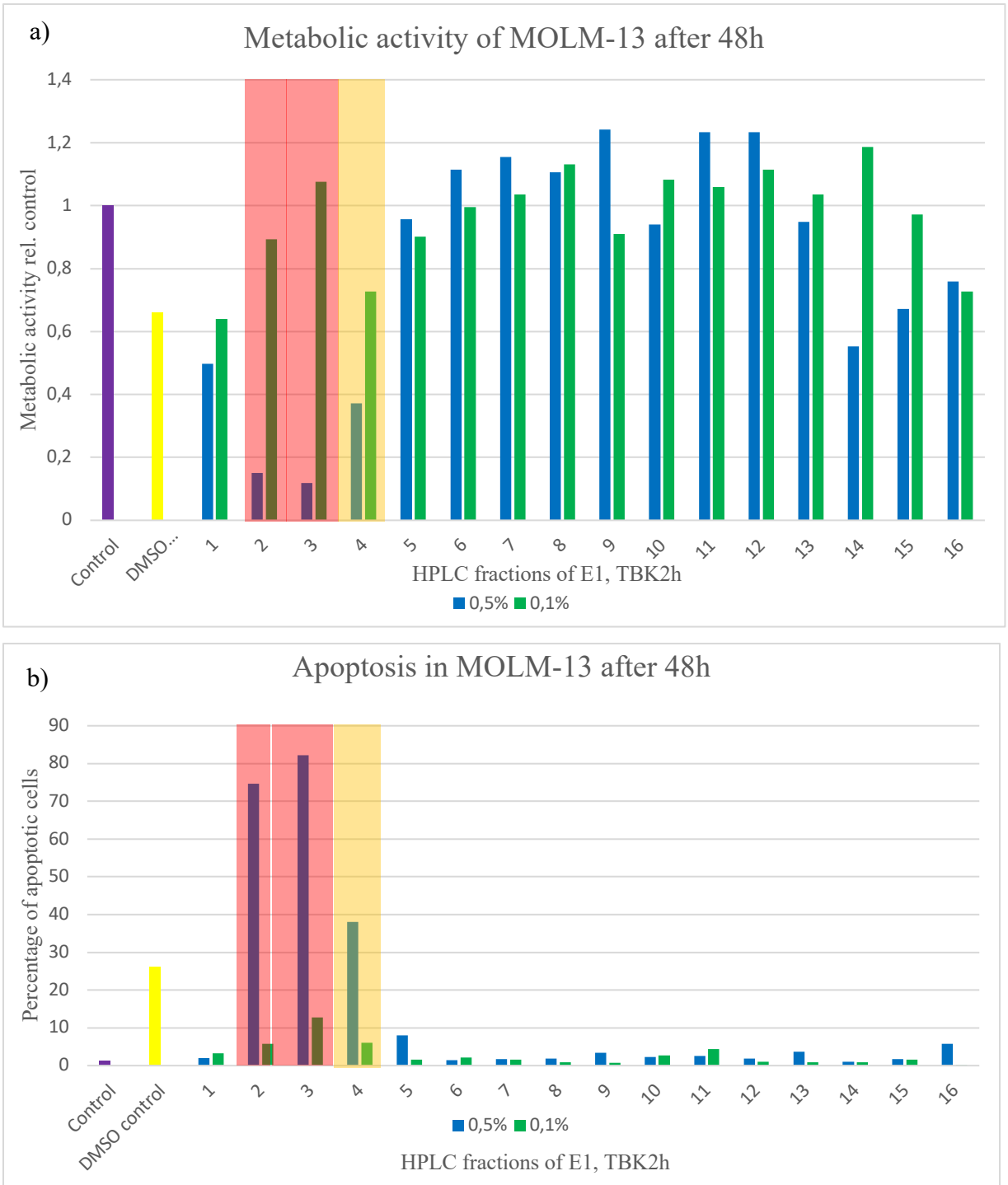
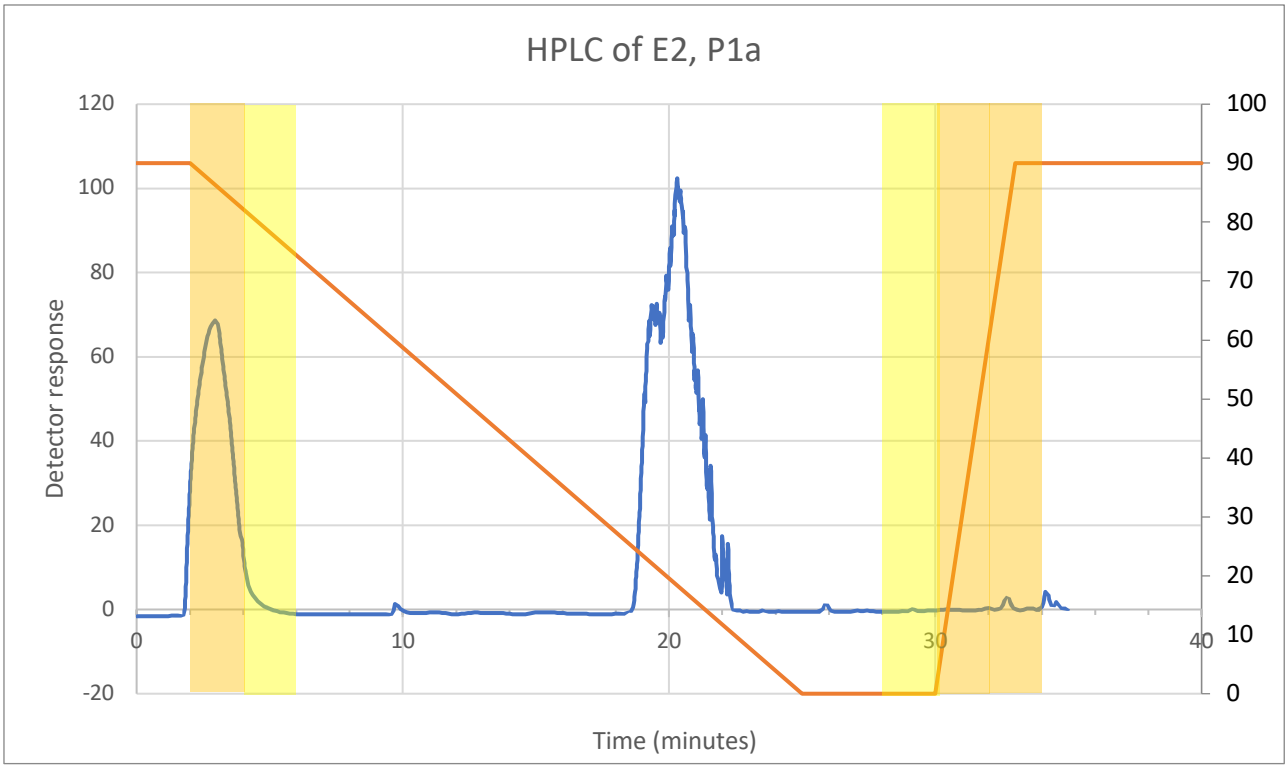


Figure 21. Analysis of cytotoxic effect of HPLC separated fractions of SPE E1 from strain TBK2h on cancer cell line MOLM-13. Fractions added at 0.5% (blue) and 0.1% (green) for 48h. A DMSO control added at 1% was also tested for cytotoxicity (yellow). a) Detailing metabolic activity of MOLM-13 cells through WST-1 analysis after 48h exposure to HPLC fractions in relation to a control (purple). b) Detailing apoptosis in MOLM-13 cells after 48h exposure to HPLC fractions. Apoptosis was calculated from pictures taken in fluorescence microscope. Fractions corresponding to peaks in chromatogram (Figure 20) are coloured to highlight cytotoxic effects on MOLM-13. Red = high cytotoxic effect, orange = intermediate cytotoxic effect.

3.5.2 HPLC separation of P1a and HPLC fractions of P1a on PC3

HPLC chromatogram showing separated fractions of SPE E2 from strain P1a showed the presence of one peak at 2-6 minutes at high polarity, in addition to several smaller peaks at 28-36 minutes at lower polarity (Figure 22a and b). The peaks corresponded to cytotoxic activity towards the cell line PC3 in both WST-1 analysis and percentage of apoptosis (Figure 23a and b). A large peak was visible on the chromatogram at 18-22 minutes, not corresponding to cytotoxic activity according to cytotoxicity analysis (Figure 22a and b, Figure 23a and b). Fraction 2, collected at 2-4 minutes, showed high potential in metabolic activity analysis at 1% and intermediate potential at 0.3% (Figure 23a). The fraction displayed intermediate potential in the percentage of apoptosis at 1%, and low potential at 0.3% (Figure 23b). Fraction 3 displayed low effect at 1% in percentage of apoptosis, but did not show any cytotoxic potential in analysis of metabolism (Figure 23a and b). High potential was found in fractions 15 and 16 collected between 28-32 minutes at 1%, according to metabolic activity analysis (Figure 23a). Fraction 15 showed low potential, and fraction 16 showed intermediate potential at 1% from percentage of apoptosis. Fraction 17 also showed intermediate potential at 1% according to analysis of apoptosis, but no potential in WST-1 analysis. DMSO control showed no cytotoxic potential at 1% in WST-1 analysis and analysis of apoptosis (Figure 23a and b).



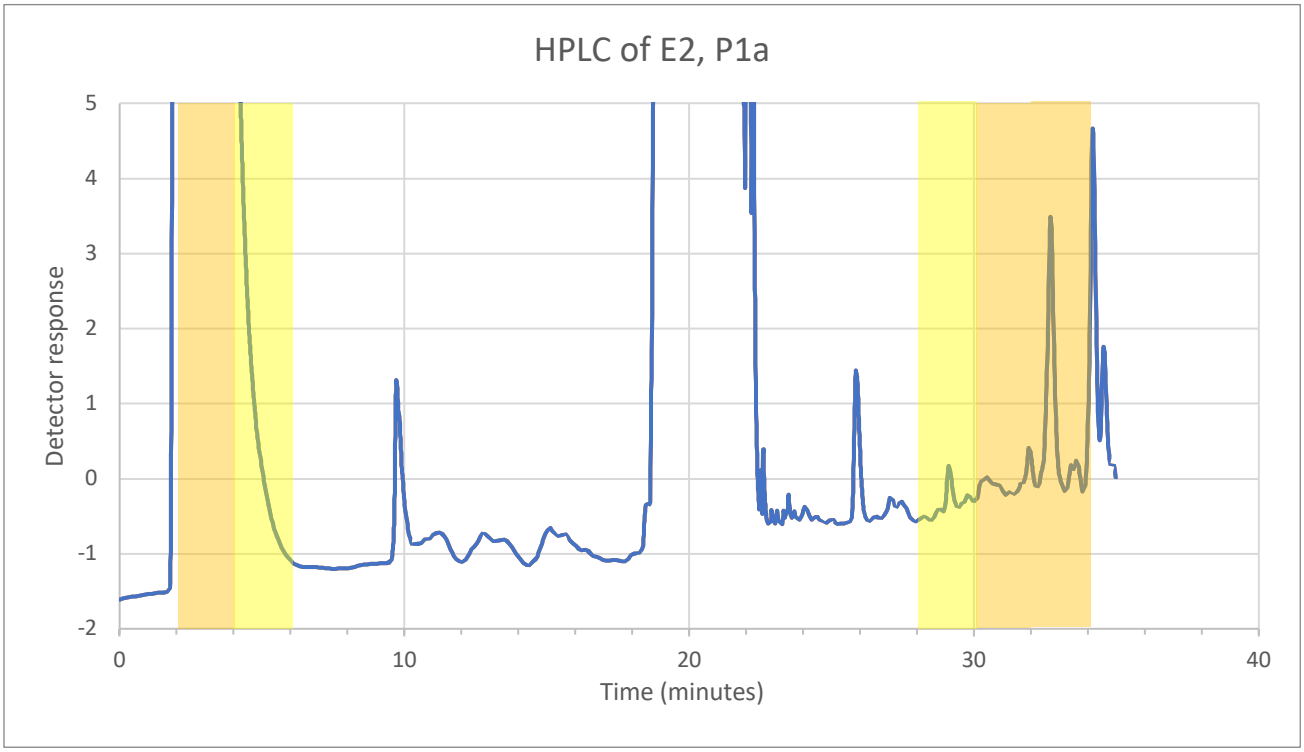


Figure 22. Chromatogram detailing HPLC separation of SPE E2 from organic phase of strain P1a at a gradient of mobile solvent H_2O (secondary axis). Peaks corresponding to HPLC fractions cytotoxic to cell line PC3 according to analysis of apoptosis are highlighted orange for intermediate cytotoxic effect and yellow for low cytotoxic effect. a) Full scale chromatogram with H_2O gradient at secondary axis. b) Chromatogram with focus on showing smaller peaks at 28-36 minutes.

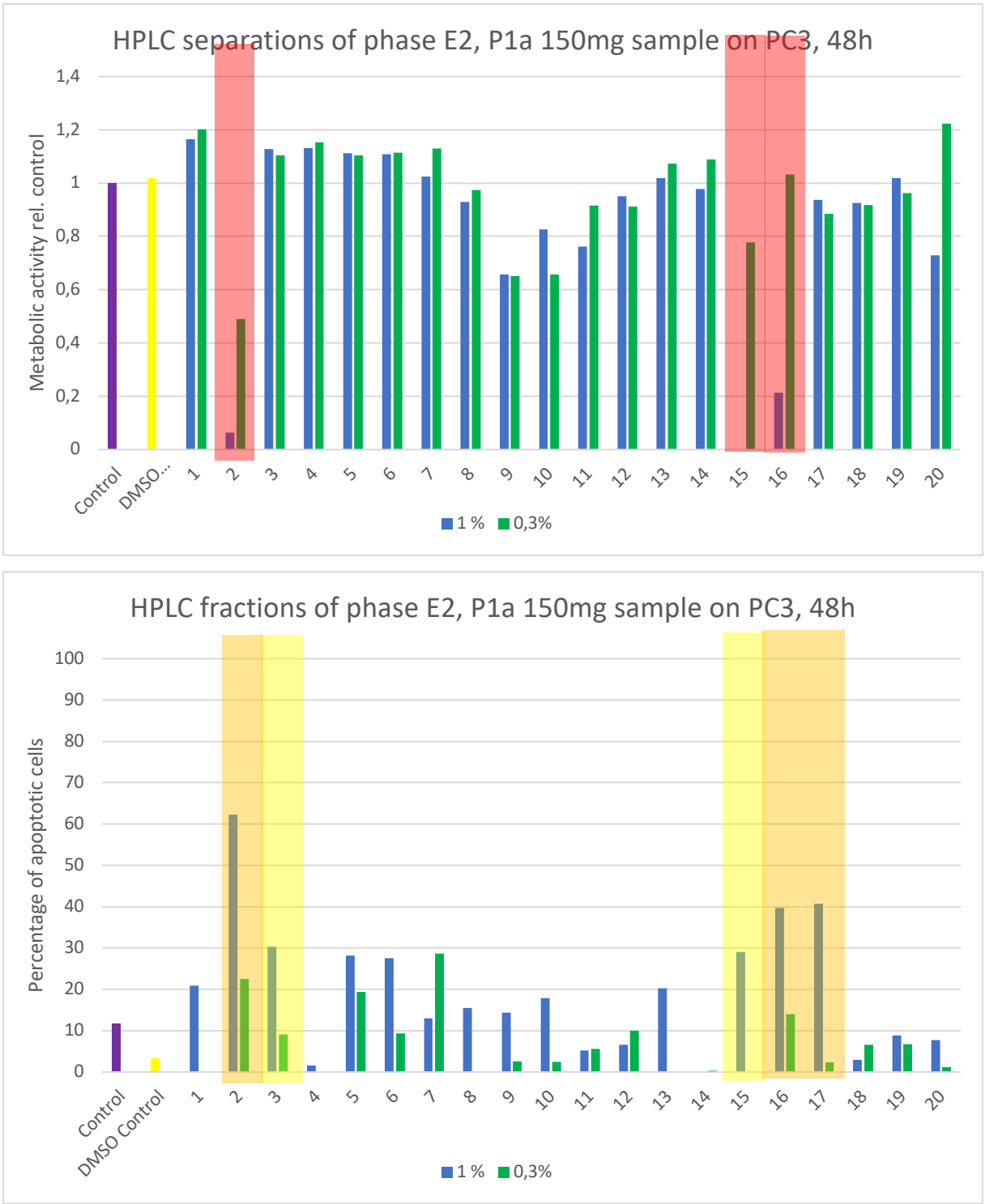


Figure 23. Analysis of cytotoxic effect of HPLC separated fractions of SPE E2 from strain P1a on cancer cell line PC3. Fractions added at 1% (blue) and 0.3% (green) for 48h. A DMSO control added at 1% was also tested for cytotoxicity (yellow). a) Detailing metabolic activity of PC3 cells through WST-1 analysis after 48h exposure to HPLC fractions, in relation to a control (purple). b) Detailing apoptosis in PC3 cells after 48h exposure to HPLC fractions. Apoptosis was calculated from pictures taken in fluorescence microscope. Fractions corresponding to peaks in chromatogram (Figure 22) are coloured to highlight cytotoxic effects on PC3. Red = high cytotoxic effect, orange = intermediate cytotoxic effect, yellow = low cytotoxic effect.

4 Discussion

Bacteria are known to be producers of bioactive molecules, and these bioactive compounds have previously been shown to be useful in human medicine (Sanchez & Demain, 2019). Strain L1 was previously sampled from biofilm on the surface of macroalgae *Saccharina nigripes*. Biofilms are known to encourage production of bioactive compounds (Boyer & Wisniewski-Dyé, 2009; Davies et al., 1998; Nealson, 1977). Strains TBK2h, VLbF2, P61, P62, P1a and P2S were sampled from extreme environments, namely hydrothermal vent systems and ice caves (Sæbø, 2018; Julia Endresen Storesund et al., 2018). Extreme environments induce metabolic adaptions to the habitats, which could lead to an intricate metabolism with the possibility for production of secondary metabolites. Therefore, the belief was that the strains in this study would produce bioactive molecules able to act as anticancer drugs, which could be isolated and analysed.

Planctomycetes have previously been shown to possess the ability to negatively affect cell growth in the acute myeloid leukaemia cell line MOLM-13 and in the prostate cancer cell line PC3 (Calisto et al., 2019). In this study, it was found that most of the highly or intermediate cytotoxic strains were active on the MOLM-13 cell line, whilst the minority affected cell line PC3. The majority of the cytotoxicity towards MOLM-13 came from aquatic extractions, while the cytotoxic activity against PC3, in general came from organic extracts.

4.1 Cultivation and harvesting bacterial strains

Cultivation of the different bacterial strains showed that the strains varied in growth patterns, not only in growth rate, but also in growth tactics and the appearance of the cultures. For example, strains TBK2h and VLbF2 yielded quite homogenous cultures, with uniform distribution of bacteria throughout the culture. Strain P1a on the other hand, grew mostly as aggregates, which meant that it needed vigorous shaking to separate the aggregates and stimulate growth. Strains L1 and P2S were quite gelatinous in nature. They were both quite homogenously distributed in their cultures, but became viscous and slime-like when harvested in a centrifuge. This meant that there was difficult separating the culture fluid and distilled reverse osmosis water from the actual bacteria. Consequently, this entailed that there was still some liquid left in the samples from these strains when they were freeze-dried, meaning that a portion of the overall freeze-dried material from these strains was freeze-dried liquid.

Generally, the more homogenous cultures were easier to harvest than the less homogenous and uniform ones. P1a, which grew in aggregates was on the difficult side to harvest. The aggregates were problematic to transfer from the culture to the centrifuge tubes, as they tended to remain near the bottom of the flask, even after stirring, so the aggregates at times needed to be pipetted.

4.2 Conflicting results

There can be different reasons contributing to dissimilarities or at times contrasting outcomes from the metabolic activity assays and the percentage of apoptosis. For instance, cells can have intact mitochondria despite being in the beginnings of apoptosis process. This is presumably because apoptosis is an energy requiring pathway for the cells (Eguchi, Shimizu, & Tsujimoto, 1997; Shiraishi et al., 2001). The intact mitochondrion would mean that the cell could exhibit metabolic activity through WST-1 assay. The cell would look apoptotic in phase microscopy, and therefore results could differ. In addition to this, colouration of the strains may have an impact on metabolic assays, being that WST-1 analysis is based on the measuring of colour waves in microplate wells containing cells. Strains with red or pink colouring, like strain L1 or P61 might show skewed results in metabolic analysis, and would consequently have to be judged solely on percentage of apoptosis. This has been done in previous studies using similar methods for cytotoxicity analysis (Calisto et al., 2019; Sæbø, 2018). A third reason for difference between metabolic analysis and apoptosis is the appearance of the cells in phase microscopy. There are different stages to apoptosis, including the condensation of the nuclei, but also fragmentation of the nuclei (Kerr, Wyllie, & Currie, 1972). This splitting of the nuclei into fragmented apoptotic bodies can skew results in two ways – by appearing as several different apoptotic cells, making the percentage of apoptosis higher than expected, or by disappearing in more cloudy samples as they are small in size, making them difficult to distinguish from the surrounding environment. In this last case, the counted non-apoptotic cells will count higher due to imbalance with apoptotic cells. A fourth, common reason for difference in the results, is that treatment of cells using bioactive matter caused inhibition of growth, instead of apoptosis. Such cell growth inhibition is a common anticancer tactic has previously been seen from bioactive compounds and cancer drugs towards prostate cancer cells (Afsar et al., 2016; Sidana et al., 2012).

4.3 Cytotoxic potential of dimethyl sulfoxide (DMSO) on results from cellular experiments

DMSO was added to the different strains and extractions in varying amounts to dissolve the samples, as the substance has been proven an effective polar and a non-polar solvent, fitting for both aqueous and organic extracts (Santos, Martins-Silva, & Saldanha, 2005; Verheijen et al., 2019). DMSO was in general added in similar amounts to the aqueous and organic extracts, though some strains were added additional DMSO to help with dissolving. This applied for both aqueous and organic extracts, and was done when the strains showed gelatinous behaviour, to dissolve the precipitate sufficiently to get exact measurements using pipettes.

DMSO is known to possess cytotoxic abilities towards cancer cells (Collins, Ruscetti, Gallagher, & Gallo, 1978). In this study, DMSO was more cytotoxic active towards the leukaemia cell line MOLM-13 than towards the prostate cancer cell line PC3 and normal rat kidney cells (NRK). A DMSO control was prepared for all cellular experiments using 1% DMSO, which gave an indication of the cytotoxic abilities of the solvent to compare the cytotoxic potential of the different bacterial strains. The extracts were in initial small-scale experiments added to the cells with 1% DMSO, organic extracts being dissolved in 100% DMSO and added at 1%, and aqueous extracts being dissolved in 25% DMSO and added at 4% to cell experiments. In cases where additional DMSO was added to extracts, distinguishing the cytotoxic potential of the extract from the potential of the DMSO-additive was challenging. This was particularly challenging in experiments using the MOLM-13 cell line, where the cytotoxic activity of both the DMSO solvent and a number of strains was relatively high.

4.4 Cell experiments using small scale extractions of aqueous and organic phases

4.4.1 Potential towards cell line MOLM-13

Experiments using aqueous and organic extractions on MOLM-13 cells after 24h showed contrasting results regarding the metabolic assay and percentage of apoptosis for the aqueous phase of strain P61. WST-1 assay showed intermediate potential, bordering on high potential, while percentage of apoptosis exhibited low potential (Figure 9a and b). This may be due to the colouring of the strain being red. Aqueous extracts of TBK2h and VLbF2 exhibited the most cytotoxic activity of all strains after both 24h and 72h. After 24h, these strains caused high induction of apoptosis, with a lower cytotoxic DMSO control. After 72h, the strains were able to induce a high percentage of apoptosis, as did the DMSO control, meaning the results after 72h could be interpreted as being caused by the cytotoxic abilities of the DMSO (Figure 10a and b). As the strains were active against MOLM-13 cells at low DMSO cytotoxicity, it is likely the strains have cytotoxic abilities separate from the DMSO.

4.4.2 Potential towards cell line PC3

Experiments on PC3-cells showed a slight difference in cytotoxic potential for organic phases of strains 7mR, L1, 1mW and P1a after 24h between the WST-1 analysis and percentage of induced apoptosis. The strains showed intermediate potential on the WST-1 analysis and low potential on percentage of apoptosis. Strains P2S and VLbF2 exhibited high potential in metabolic analysis and intermediate and low potential respectively in the percentage of apoptosis (Figure 11 a and b). The underlying reason for the difference was difficult to identify, but could be due to the strains being growth inhibitors towards the PC3 cell line, blocking cell growth instead of inducing apoptosis. A previous study has shown growth inhibition of PC3-cells by bioactive molecules derived from the shrub *Acacia hydaspica* R. Parker (Afsar et al., 2016). The divergence between methods of analysis was consistent, with most of the organic phases of the strains exhibiting higher potential on WST-1 analysis than on percentage of apoptosis (Figure 11 a and b). After 72h, the same contrast in results could be seen in the organic phase of all the strains, similarly probably induced by inhibition of cellular growth (Figure 12 a and b).

4.4.3 Potential towards cell line NRK

Experiments on the normal rat kidney cells gave contrasting results between the WST-1 analysis and the percentage of apoptosis. This contrast was similar to the deviation found in metabolic and apoptosis analysis of cell line PC3. After 24h the organic phases of all strains with the exception of P62 and P61 were highly cytotoxic according to metabolic activity analysis, but exhibited no or low potential according to percentage of apoptosis (Figure 13a and b). Similar results were visible after 72h (Figure 14a and b). This difference in analytic results points to the organic extracts being growth inhibitors towards NRK-cells. The aqueous phases showed no substantial contrasting results, with the exception of strain P61, which after 72h induced high metabolic activity in addition to very high percentage of apoptosis. This could be a result of the colouring of the strain, light red, skewing the WST-1 results.

4.5 Cell experiments using larger scale extractions of aqueous and organic phases

4.5.1 Selection of strains for cell experiments

The strains involved in cellular experiments using larger amounts of bacterial biomass were chosen based on different criteria. There was a focus on negative potential, meaning the strain should be able to inhibit metabolic activity and induce apoptosis in a cell line. The strains were additionally chosen on the basis of whether the bacteria had been tested for anticancer activity previously.

Strains TBK2h and VLbF2 showed high cytotoxicity towards percentage of apoptosis and on metabolic activity in the cell line MOLM-13 after 72h using their aqueous extractions. They had both been tested for cytotoxic activity against the cell line in previous published work, but not at larger scales (Calisto et al., 2019). The strains were therefore candidates for cellular experiments using higher amounts of freeze-dried biomass, against the cell line MOLM-13.

Strain P1a exhibited some negative potential towards cell line PC3 using organic phase extraction. Metabolic activity showed high potential, while percentage of apoptosis showed low to intermediate potential, possibly explained by the strain being a growth inhibitor. The organic phase of the strain also showed some negative potential on the control normal rat kidney cells after both 24h and 72h. At the time of selection of strains for larger scale extractions, the possibility of growth inhibition was not known. This led to WST-1 analysis of PC3 cells and NRK cells being disregarded, and P1a was viewed as not cytotoxic towards NRK cells in organic phase. Although anticancer activity towards PC3 cells was not high, the strain had not been tested for cytotoxic behaviour previously and was therefore tested against PC3 on a larger scale.

Strain P2S showed high induction of cell apoptosis and high cytotoxic potential on metabolic activity on cancer cell line PC3 after 24h using the organic phase of the strain. The strain also showed abilities to induce apoptosis in control cell line NRK, using the organic phase after 24h, which meant the organic phase was not selected for further analysis. P2S was not exceedingly cytotoxic using the aqueous phase in smaller scale extraction experiments, but the strain had never been tested for cytotoxic activity before. This meant the strain was implemented in further

research. P2S was tested using larger scale extractions of aqueous phase on the cell line MOLM-13. MOLM-13 was chosen as this cell line was deemed to be most susceptible to anticancer activity from aqueous phase extractions.

Strain L1 was previously reported as having high potential towards PC3 and intermediate potential towards MOLM-13 in organic phase extraction (Calisto et al., 2019). This was not clearly visible from the results obtained in this study. There were no substantial negative effects from the organic extract to go forward with, so the decision was made to use the aqueous phase against cell line MOLM-13. The aqueous phase of the strain had in experiments with smaller scale extractions showed low effect towards MOLM-13, so the expectation was for it to express a stronger cytotoxicity at larger extraction volumes.

4.5.2 Potential of L1, P2S, VLbF2 and TBK2h towards MOLM-13

Aqueous phases were extracted from ten times higher biomass than previously and added to MOLM-13 cells in a dilution series of 5%, 2% and 0.5%. There were no major conflicting results between the metabolic activity analysis and the percentage of apoptosis (Figure 15). Overall, the higher volume of biomass used in the extractions induced a stronger negative response in the cell line. Strains TBK2h and VLBF2 showed stronger effects from the higher biomass than P2S and L1. TBK2h and VLbF2 exhibited higher cytotoxic abilities than P2S and L1 using smaller scale extractions and continued this trend using larger extractions. The strains can therefore be viewed as more cytotoxic towards MOLM-13 than P2S and L1, although these bacteria were also capable of cytotoxicity in high doses.

4.5.2 Potential of P1a towards PC3

Organic phase was extracted from strain P1a at 50 times the previous volume of biomass. The extraction was added in a dilution series of 5%, 2% and 0.5% extraction to cell culture. The results showing metabolic activity differed greatly from the calculated percentage of apoptosis. Contrary to in initial small-scale experiments, WST-1 analysis showed lower cytotoxic potential than analysis of induced apoptosis (Figure 16a and b). Strain P1a is not coloured, which means this should not affect WST-1 analysis. There was a possibility of the gelatinous nature of the strain causing impurities in the wells, so the decision was made to disregard the

WST-1 metabolic analysis. When regarding only calculations of apoptosis, the P1a showed strong cytotoxicity towards PC3 at high doses of the organic phase.

4.6 Cell experiments using solid phase extractions (SPE)

4.6.1 Potential of SPE from L1, P2S, VLbF2 and TBK2h on MOLM-13

A 48h experiment was conducted on cell line MOLM-13 using SPE of VLbF2, L1 and P2S. Strain VLbF2 showed strong cytotoxic abilities towards the cell line in all fractions, with no notable contrasting results between the metabolic activity analysis and percentage of apoptosis. L1 and P2S showed stronger cytotoxic abilities in the two elution fractions than in the load and wash phase with no outstanding outliers between the methods of cytotoxicity measurement (Figure 17a and b). Lack of early activity in the load and wash-cycle was expected of all the strains, as the chemical substances in the solid phase is eluded out in the later phases. VLbF2 being strongly active in all the phases points to the strain being highly capable of cytotoxicity against the cell line. Concluding, all strains showed strong cytotoxicity in aqueous extract towards MOLM-13, VLbF2 showing highest cytotoxic abilities.

TBK2h was tested using a biomass sample of 1000mg, and showed cytotoxic activity in all the fractions, including the load and wash-phase. There was some dissimilarity between the two methods of metabolic activity and apoptosis. Results of phase L+W at 5% exhibited higher potential according to the WST-1 assay than percentage of apoptosis (Figure 18a and b). The skewed results may be due to difficulty in distinguishing apoptotic cells from viable cells, as they were similar in appearance (Figure 24).

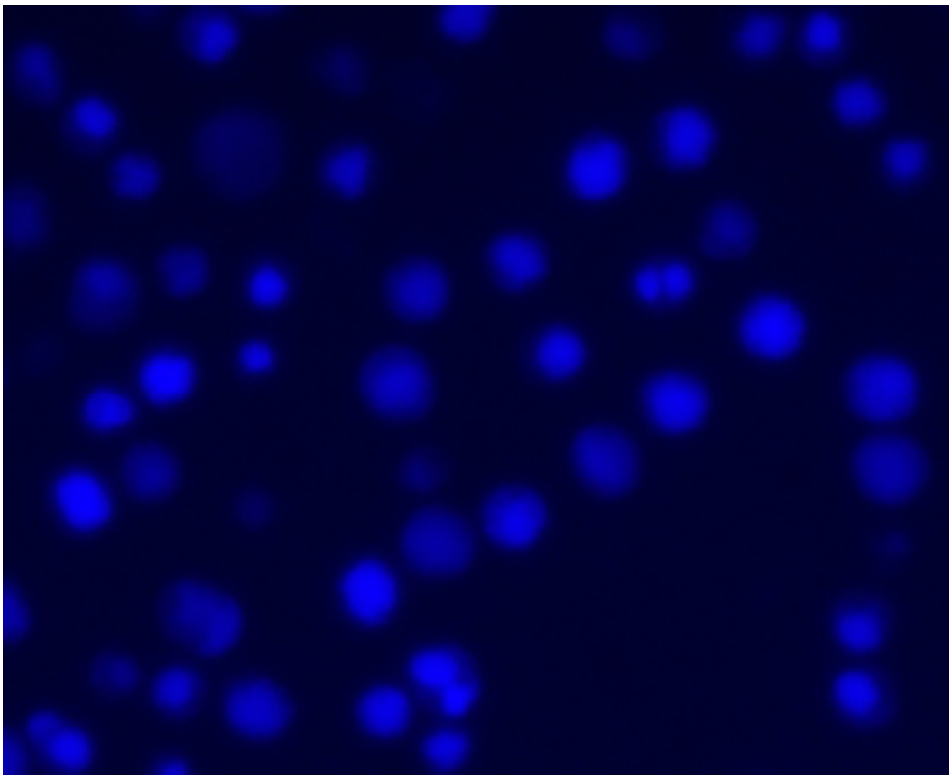


Figure 24. MOLM-13 cells treated with 5% L+W fraction from aqueous extraction of 1000mg biomass sample of TBK2h after 48h incubation. Picture taken in fluorescence microscope at 20x magnification. Apoptotic and normal cells are similar in appearance, with apoptotic cells being slightly brighter and rounder, indicating condensed nuclei.

In conclusion, TBK2h was strongly cytotoxic in the aqueous phase against cell line MOLM-13. As the strain was easy to cultivate and the collection of biomass was fast, this strain was selected for further HPLC-analysis. Phase E1 was deemed to be the most active fraction and was chosen for the HPLC-analysis.

For the SPE experiments using aqueous extractions, the 1% DMSO control showed high cytotoxicity in both methods of analysis. This was a factor contributing to the cytotoxic activity in the strains, although at which level is an uncertainty. The SPE were added 20µL milliQ and 5µL to dissolve the precipitate after evaporation, but P2S and L1 were added additional amounts of both substances to combat the gelatinous nature of the strains (Table 3). This was done with fraction L+W for both strains in addition to phase E1 for P2S. These phases did not show exceptionally high cytotoxic abilities compared to the remaining phases. Therefore, there was a presence of cytotoxic abilities within these strains in addition to the effect of the DMSO control. This is presumably true for the strain TBK2h and VLbF2 as well.

4.6.2 Potential of SPE from P1a towards PC3

Experiments using SPE of organic phase of P1a towards PC3 showed contrast between the results of metabolic activity analysis and the percentage of apoptosis, presumably caused by inhibition of growth as opposed to induction of apoptosis. P1a showed intermediate to high potential on the PC3 cell line, with the highest activity for all dilutions combined being registered in E2 (Figure 19a and b). The previous cellular experiment using larger scale extractions was done with a sample of 500mg, while experiments with SPE was done using a 150mg sample. In the larger-scale experiment, P1a exhibited higher cytotoxic potential than with the use of SPE, showing the strain would be more cytotoxic at higher volumes of biomass. P1a was used in HPLC analysis, where the chosen fraction was E2.

4.7 Interpretation of HPLC chromatograms and results from cancer cell experiments using HPLC fractions

4.7.1 HPLC analysis of TBK2h

From HPLC chromatogram detailing detector response of phase E1 from strain TBK2h, two peaks were visible at 2-6 minutes, corresponding to cytotoxic activity towards the leukaemia cell line MOLM-13. Fractions 2 and 3, collected at 2-4 minutes and 4-6 minutes respectively represented the same chemical compound, while fraction 4 collected at 6-8 minutes appeared to represent a different compound to the previous fractions (Figure 20). The peaks were determined as hydrophilic and polar substances, as they were eluted at high percentages of the mobile phase H_2O. The compound or compounds depicted by the detector response were capable of intermediate to high cytotoxic effect towards the cell line MOLM-13. This was found through metabolic activity analysis and calculation of percentage of apoptosis, without substantial dissimilarities between the two methods of analysis (Figure 21a and b). The compounds were either variants of the same substance, or very similar substances. Conclusively, fraction E1 was shown to contain polar, hydrophilic substances capable of high cytotoxic activity against acute monocytic leukaemia cell line MOLM-13.

4.7.2 HPLC analysis of P1a

HPLC separation and analysis of phase E2 from strain P1a showed some divergent results in method analysis, with the WST-1 assay showing higher cytotoxic potential than percentage of apoptosis (Figure 23a and b). This could again be due to growth inhibition by the strain, although difficulties were also found distinguishing apoptotic cells from viable cells in fluorescence microscopy. It is previously known that adherent cells can act differently during apoptosis than other cells, at times increasing in volume instead before condensation, which could result in apoptotic cells looking similar to normal cells (Desjardins & MacManus, 1995).

At 18-22 minutes, a peak was formed on the chromatogram that did not correspond to any cytotoxicity. This was presumably caused by air infiltrating the column, due to improper de-gassing of the sample. The earlier cytotoxic fractions 2 and 3 were fractions of the same substance, as they were part of the same peak. This compound was polar and hydrophilic, as it

was eluted at a high concentration of the polar mobile phase H_2O (Figure 22a). Fractions 15-17 were represented by several smaller peaks, indicating separate substances. These substances were eluted at low polarity, meaning they were hydrophobic (Figure 22b). The spacing between the different areas of activity on the chromatogram invited the thought that several hydrophilic and hydrophobic substances were working together to provide cytotoxicity for the P1a SPE. A second explanation could also be that the cytotoxic compound or compounds were hydrophobic, and were eluted at the beginning of the HPLC cycle as a flaw. This would mean that the bioactive substance or substances eluted in later stages were remnants of the previously eluted substance or substances.

5 Conclusion and suggestions for further research

Concluding, cytotoxic activity from Planctomycetes bacteria was confirmed in this study. Planctomycetes bacteria were found able to produce bioactive compounds that were cytotoxic towards acute myeloid leukaemia cell line MOLM-13 and prostate cancer cell line PC3. This was known from previous studies of Planctomycetes bacteria (Calisto et al., 2019; Sæbø, 2018), although this study showed that greater initial biomass extracted could induce greater cytotoxic activity. Also seen previously, aqueous extractions were more cytotoxic towards leukaemia cells, while organic extracts were more cytotoxic towards prostate cancer cells (Calisto et al., 2019). This study showed cytotoxicity towards MOLM-13 cells as induced apoptosis, while the theory for PC3 cells was that the cytotoxic active strains could act as growth inhibitors, not inducing cell death, but blocking further cell growth. This had previously been seen in prostate cancer cells treated with bioactive compounds and anticancer drugs (Afsar et al., 2016; Sidana et al., 2012). A study by Afsar et al from 2016 had shown that cytotoxic compounds could work as both growth inhibitors and induce apoptosis in the PC3 cell line. This was supported by results from this study which showed that greater initial biomass extracted of the Planctomycetes strain P1a could induce apoptosis in PC3 cells instead of working as a growth inhibitor for the cancer cells, which was seen using less biomass in initial extraction of phases.

The use of multiple anticancer drugs is common in anticancer therapy, as different compounds target different mechanisms of cancerous cells (Gale, 2020; Lu, Chen, Lu, & Ding, 2015). HPLC analysis executed in this study of cytotoxic separated fractions from strains showed the possible ability for several substances of different polarity working together to produce cytotoxicity within a bacterial strain. The separation of bacterial extractions exhibiting cytotoxic abilities into HPLC fractions is a good start for understanding the understudied topic regarding the chemistry behind anticancer activity in bacteria. Further chemical investigations and liquid chromatography should be performed.

The strain TBK2h was found to possess cytotoxic compound capable of inducing high percentage of apoptosis and inhibiting metabolic activity in cancer cell line MOLM-13. Further work should be done testing the remaining SPE fractions L+W and E2 in HPLC-separated fractions against the same cell line to compare results and observe the presence or absence of similar substances. This should also be done with strain P1a, which was shown to have intermediate to high potential against prostate cancer cell line PC3. Further experiments should

be done on the existing fractions of SPE E2 from P1a to obtain additional results for stronger determination of where cytotoxicity is found in E2. This should be done to confirm or contradict the presence of several polar and non-polar substances. Furthermore, it would be beneficial to extract P1a at a larger volume of biomass to examine if this could amplify the cytotoxic abilities of SPE and induce apoptosis as results from larger scale extractions showed. Larger biomass extractions and solid phase extractions of the organic phase of P1a should be examined for cytotoxic abilities towards control cells NRK to investigate further whether the strain is viable for medical use. Fraction E1 of TBK2h and E2 of P1a are in the process of being sent to the University of Porto for further chemical analysis. This may determine the identity of the cytotoxic compounds and whether they are known or unknown substances.

References

Abbas, Z., & Rehman, S. (2018). An Overview of Cancer Treatment Modalities. In *Neoplasm*. InTech. https://doi.org/10.5772/intechopen.76558

Afsar, T., Trembley, J. H., Salomon, C. E., Razak, S., Khan, M. R., & Ahmed, K. (2016). Growth inhibition and apoptosis in cancer cells induced by polyphenolic compounds of Acacia hydaspica: Involvement of multiple signal transduction pathways. *Scientific Reports*, *6*(1), 1–12. https://doi.org/10.1038/srep23077

Andrei, A. Ş., Salcher, M. M., Mehrshad, M., Rychtecký, P., Znachor, P., & Ghai, R. (2019). Niche-directed evolution modulates genome architecture in freshwater Planctomycetes. *ISME Journal*, *13*(4), 1056–1071. https://doi.org/10.1038/s41396-018-0332-5

Antibiotic resistance. (2020, July 31). Retrieved April 20, 2021, from https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance

Bassler, B. L., & Losick, R. (2006, April 21). Bacterially Speaking. *Cell*. Elsevier B.V. https://doi.org/10.1016/j.cell.2006.04.001

Beckman Coulter. (n.d.) Allegra X-22R Refrigerated Benchtop Centrifuge. [Apparatus].

Beckman Coulter. (n.d.). Avanti J-26S XP (B22987). [Apparatus]. https://www.beckman.com/centrifuges/high-speed/avanti-j-26s-series/b22987

Beckman Coulter. (n.d.) FX301.5 Fixed-Angle Aluminum Rotor (392274). [Apparatus]. https://www.beckman.com/centrifuges/rotors/fixed-angle/392274

Beckman Coulter. (n.d.). JA 25,50 fixed angle rotor (363055). [Apparatus]. https://www.beckman.com/centrifuges/rotors/fixed-angle/363055

Bengtsson, M. M., & Øvreås, L. (2010). Planctomycetes dominate biofilms on surfaces of the kelp Laminaria hyperborea. *BMC Microbiology*, *10*(1), 261. https://doi.org/10.1186/1471-2180-10-261

BioRender. (n.d.). Retrieved April 21, 2021, from https://biorender.com/

Blunt, J. W., Copp, B. R., Munro, M. H. G., Northcote, P. T., & Prinsep, M. R. (2006, February 2). Marine natural products. *Natural Product Reports*. The Royal Society of Chemistry. https://doi.org/10.1039/b502792f

Boedeker, C., Schüler, M., Reintjes, G., Jeske, O., Van Teeseling, M. C. F., Jogler, M., … Jogler, C. (2017). Determining the bacterial cell biology of Planctomycetes. *Nature Communications*, *8*. https://doi.org/10.1038/ncomms14853

Borneman, J., Skroch, P. W., O'sullivan, K. M., Palus, J. A., Rumjanek, N. G., Jansen, J. L., … Triplett, E. W. (1996). *Molecular Microbial Diversity of an Agricultural Soil in*

Wisconsin. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* (Vol. 62). Retrieved from http://aem.asm.org/

Borneman, J., & Triplett, E. W. (1997). *Molecular Microbial Diversity in Soils from Eastern Amazonia: Evidence for Unusual Microorganisms and Microbial Population Shifts Associated with Deforestation*. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* (Vol. 63). Retrieved from http://rdp.life.uiuc.edu

Boyer, M., & Wisniewski-Dyé, F. (2009, September 3). Cell-cell signalling in bacteria: Not simply a matter of quorum. *FEMS Microbiology Ecology*. Blackwell Publishing Ltd. https://doi.org/10.1111/j.1574-6941.2009.00745.x

Branson. 3510 Ultrasonic Bath. [Apparatus].

Brochier, C., & Philippe, H. (2002). Phylogeny: A non-hyperthermophilic ancestor for bacteria. *Nature*, *417*(6886), 244. https://doi.org/10.1038/417244a

Brümmer, I. H. M., Felske, A. D. M., & Wagner-Döbler, I. (2004). Diversity and seasonal changes of uncultured Planctomycetales in river biofilms. *Applied and Environmental Microbiology*, *70*(9), 5094–5101. https://doi.org/10.1128/AEM.70.9.5094-5101.2004

Buckley, D. H., Huangyutitham, V., Nelson, T. A., Rumberger, A., & Thies, J. E. (2006). Diversity of Planctomycetes in soil in relation to soil history and environmental heterogeneity. *Applied and Environmental Microbiology*, *72*(7), 4522–4531. https://doi.org/10.1128/AEM.00149-06

Calisto, R., Sæbø, E. F., Storesund, J. E., Øvreås, L., Herfindal, L., & Lage, O. M. (2019). Anticancer Activity in Planctomycetes. *Frontiers in Marine Science*, *5*(JAN), 499. https://doi.org/10.3389/fmars.2018.00499

Calvin Giddings, J., & Keller, R. A. (n.d.). Chromatography . In *Encyclopedia Britannica*. Retrieved from https://www.britannica.com/science/chromatography/additional-info#contributors

Cancer. (n.d.). Retrieved April 20, 2021, from https://www.who.int/health-topics/cancer#tab=tab_1

Cancer. (2021, March 3). Retrieved April 20, 2021, from https://www.who.int/news-room/fact-sheets/detail/cancer

Cayrou, C., Raoult, D., & Drancourt, M. (2010). Broad-spectrum antibiotic resistance of Planctomycetes organisms determined by Etest. *Journal of Antimicrobial Chemotherapy*, *65*(10), 2119–2122. https://doi.org/10.1093/jac/dkq290

Cayrou, Caroline, Sambe, B., Armougom, F., Raoult, D., & Drancourt, M. (2013). Molecular diversity of the *Planctomycetes* in the human gut microbiota in France and Senegal.

APMIS, *121*(11), 1082–1090. https://doi.org/10.1111/apm.12087

Cohen-Bazire, G., Sistrom, W. R., & Stanier, R. Y. (1957). Kinetic studies of pigment synthesis by non-sulfur purple bacteria. *Journal of Cellular and Comparative Physiology*, *49*(1), 25–68. https://doi.org/10.1002/jcp.1030490104

Collins, S. J., Ruscetti, F. W., Gallagher, R. E., & Gallo, R. C. (1978). Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. *Proceedings of the National Academy of Sciences of the United States of America*, *75*(5), 2458–2462. https://doi.org/10.1073/pnas.75.5.2458

Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W., & Greenberg, E. P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science*, *280*(5361), 295–298. https://doi.org/10.1126/science.280.5361.295

Dedysh, S. N., Henke, P., Ivanova, A. A., Kulichevskaya, I. S., Philippov, D. A., Meier-Kolthoff, J. P., … Overmann, J. (2020). 100-year-old enigma solved: identification, genomic characterization and biogeography of the yet uncultured *Planctomyces bekefii*. *Environmental Microbiology*, *22*(1), 198–211. https://doi.org/10.1111/1462-2920.14838

Dedysh, S. N., Pankratov, T. A., Belova, S. E., Kulichevskaya, I. S., & Liesack, W. (2006). Phylogenetic analysis and in situ identification of Bacteria community composition in an acidic Sphagnum peat bog. *Applied and Environmental Microbiology*, *72*(3), 2110–2117. https://doi.org/10.1128/AEM.72.3.2110-2117.2006

Demain, A. L., & Fang, A. (2000). The natural functions of secondary metabolites. *Advances in Biochemical Engineering/Biotechnology*. Springer, Berlin, Heidelberg. https://doi.org/10.1007/3-540-44964-7_1

Derakshani, M., Lukow, T., & Liesack, W. (2001). Novel bacterial lineages at the (sub)division level as detected by signature nucleotide-targeted recovery of 16S rRNA genes from bulk soil and rice roots of flooded rice microcosms. *Applied and Environmental Microbiology*, *67*(2), 623–631. https://doi.org/10.1128/AEM.67.2.623-631.2001

Desjardins, L. M., & MacManus, J. P. (1995). An adherent cell model to study different stages of apoptosis. *Experimental Cell Research*, *216*(2), 380–387. https://doi.org/10.1006/excr.1995.1048

Devos, D. P. (2014a). PVC bacteria: Variation of, but not exception to, the Gram-negative cell plan. *Trends in Microbiology*, *22*(1), 14–20. https://doi.org/10.1016/j.tim.2013.10.008

Devos, D. P. (2014b, February 1). Re-interpretation of the evidence for the PVC cell plan supports a Gram-negative origin. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*. Springer. https://doi.org/10.1007/s10482-013-

0087-y

De Vos, Kurt (n.d.). Cell Counter [Computer Software]. University of Sheffield, UK.

Drees, K. P., Neilson, J. W., Betancourt, J. L., Quade, J., Henderson, D. A., Pryor, B. M., & Maier, R. M. (2006). Bacterial community structure in the hyperarid core of the Atacama Desert, Chile. *Applied and Environmental Microbiology*, *72*(12), 7902–7908. https://doi.org/10.1128/AEM.01305-06

Edmund Bühler GmbH. (n.d.). Universal shaker SM 30 A control. [Apparatus]. https://www.edmund-buehler.de/en/laboratory-equipment/shakers-up-to-30-kg/universal-shaker-sm-30-a-control

Edwards. (1983). EF4 Modulyo. [Apparatus].

Eguchi, Y., Shimizu, S., & Tsujimoto, Y. (1997). Intracellular ATP Levels Determine Cell Death Fate by Apoptosis or Necrosis. *Cancer Research*, *57*(10).

Elshahed, M. S., Youssef, N. H., Luo, Q., Najar, F. Z., Roe, B. A., Sisk, T. M., … Krumholz, L. R. (2007). Phylogenetic and metabolic diversity of Planctomycetes from anaerobic, sulfide- and sulfur-rich Zodletone Spring, Oklahoma. *Applied and Environmental Microbiology*, *73*(15), 4707–4716. https://doi.org/10.1128/AEM.00591-07

Eppendorf. (n.d.). Concentrator Plus. [Apparatus]. https://online-shop.eppendorf.no/NO-en/Centrifugation-44533/Concentrator-62468/Concentrator-plus-PF-25748.html

Eppendorf. (n.d.). Rotor F45-48-11. [Apparatus].

Erickson, H. P., & Osawa, M. (2010, October). Cell division without FtsZ - A variety of redundant mechanismsmmi. *Molecular Microbiology*. NIH Public Access. https://doi.org/10.1111/j.1365-2958.2010.07321.x

Evidente, A., Kornienko, A., Cimmino, A., Andolfi, A., Lefranc, F., Mathieu, V., & Kiss, R. (2014). Fungal metabolites with anticancer activity. *Natural Product Reports*. Royal Society of Chemistry. https://doi.org/10.1039/c3np70078j

Federle, M. J., & Bassler, B. L. (2003). Interspecies communication in bacteria. *Journal of Clinical Investigation*. The American Society for Clinical Investigation. https://doi.org/10.1172/JCI20195

Forli, S. (2014). Epothilones: From Discovery to Clinical Trials. *Current Topics in Medicinal Chemistry*, *14*(20), 2312–2321. https://doi.org/10.2174/1568026614666141130095855

Franzmann, P. D., & Skerman, V. B. D. (1984). Gemmata obscuriglobus, a new genus and species of the budding bacteria. *Antonie van Leeuwenhoek*, *50*, 261–268.

Fuerst, J. A., Sambhi, S. K., Paynter, J. L., Hawkins, J. A., & Atherton, J. G. (1991). Isolation of a bacterium resembling Pirellula species from primary tissue culture of the giant tiger

prawn (Penaeus monodon). *Applied and Environmental Microbiology*, *57*(11), 3127–3134. https://doi.org/10.1128/aem.57.11.3127-3134.1991

Fuerst, John A. (2017). Planctomycetes-New Models for Microbial Cells and Activities. In *Microbial Resources: From Functional Existence in Nature to Applications* (pp. 1–27). Elsevier Inc. https://doi.org/10.1016/B978-0-12-804765-1.00001-1

Fuerst, John A., & Sagulenko, E. (2011, June 16). Beyond the bacterium: Planctomycetes challenge our concepts of microbial structure and function. *Nature Reviews Microbiology*. Nature Publishing Group. https://doi.org/10.1038/nrmicro2578

Fuerst, John A., & Sagulenko, E. (2012). Keys to eukaryality: Planctomycetes and ancestral evolution of cellular complexity. *Frontiers in Microbiology*, *3*(MAY), 167. https://doi.org/10.3389/fmicb.2012.00167

Fuerst, John A, & Webb, R. I. (1991). *Membrane-bounded nucleoid in the eubacterium Gemmata obscuriglobus (prokaryote/eukaryote distinction/electron microscopy)*. *Proc. Nati. Acad. Sci. USA* (Vol. 88).

Gale, R. P. (2020). Combination Cancer Therapy . In *MSD Manual*. MSD Manual Consumer Version. Retrieved from https://www.msdmanuals.com/home/cancer/prevention-and-treatment-of-cancer/combination-cancer-therapy

Gaynes, R. (2017). The discovery of penicillin—new insights after more than 75 years of clinical use. *Emerging Infectious Diseases*, *23*(5), 849–853. https://doi.org/10.3201/eid2305.161556

Gilson (n.d.). MINIPULS 3. [Apparatus]. https://www.gilson.com/default/minipuls-3-peristaltic-pumps.html

Gong, G.-L., Wei, X.-M., Huang, Y.-Y., & Chen, X.-F. (2014). Preparation and regeneration of protoplast from antitumor agent epothilone-producing microbes myxobacteria. *Journal of Chemical and Pharmaceutical Research*, *6*(3), 472–476. Retrieved from www.jocpr.com

Gonzalez-Angulo, A. M., Morales-Vasquez, F., & Hortobagyi, G. N. (2013). Overview of Resistance to Systemic Therapy in Patients with Breast Cancer. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK6306/

Gould, I. M., & Bal, A. M. (2013). New antibiotic agents in the pipeline and how hey can help overcome microbial resistance. *Virulence*, *4*(2), 185–191. https://doi.org/10.4161/viru.22507

Graça, A. P., Calisto, R., & Lage, O. M. (2016). Planctomycetes as Novel Source of Bioactive Molecules. *Frontiers in Microbiology*, *7*(AUG), 1241.

https://doi.org/10.3389/fmicb.2016.01241

Helmer, C., Tromm, C., Hippen, A., Rosenwinkel, K.-H., Seyfried, C. F., & Kunst, S. (2001). *Single stage biological nitrogen removal by nitritation and anaerobic ammonium oxidation in biofilm systems*. Hannover. Retrieved from http://iwaponline.com/wst/article-pdf/43/1/311/428663/311.pdf

Henrici, A. T., & Johnson, D. E. (1935). *STUDIES OF FRESHWATER BACTERIA II. STALKED BACTERIA, A NEW ORDER OF SCHIZOMYCETES1 Station-on-Alexander, Cushing, Minnesota Downloaded from*. Minneapolis. Retrieved from http://jb.asm.org/

Heidolph Instruments GmbH (n.d). Hei-VAP core. [Apparatus]. https://heidolph-instruments.com/en/products/Rotary-Evaporators/Hei-VAP-Core~p27143

Hettich. (n.d.). Universal 320. [Apparatus]. https://www.hettichlab.com/en/product/universal-320-320-r/

Hirsch, P. (1972). Two Identical Genera of Budding and Stalked Bacteria: Planctomyces Gimesi 1924 and Blastocaulis Henrici and Johnson 1935. *International Journal of Systematic Bacteriology*, *22*(2), 107–111. https://doi.org/10.1099/00207713-22-2-107

Hirsch, P. (1974). Budding Bacteria. *Annual Review of Microbiology*, *28*(1), 391–440. https://doi.org/10.1146/annurev.mi.28.100174.002135

Ivanova, A. O., & Dedysh, S. N. (2012). Abundance, Diversity, and Depth Distribution of Planctomycetes in Acidic Northern Wetlands. *Frontiers in Microbiology*, *3*(JAN), 5. https://doi.org/10.3389/fmicb.2012.00005

Jeske, O., Jogler, M., Petersen, J., Sikorski, J., & Jogler, C. (2013). From genome mining to phenotypic microarrays: Planctomycetes as source for novel bioactive molecules. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *104*(4), 551–567. https://doi.org/10.1007/s10482-013-0007-1

Jeske, O., Schüler, M., Schumann, P., Schneider, A., Boedeker, C., Jogler, M., … Jogler, C. (2015). Planctomycetes do possess a peptidoglycan cell wall. *Nature Communications*, *6*. https://doi.org/10.1038/ncomms8116

Jogler, C., Waldmann, J., Huang, X., Jogler, M., Glöckner, F. O., & Mascher, T. (2012). Identification of proteins likely to be involved in morphogenesis, cell division, and signal transduction in planctomycetes by comparative genomics. *Journal of Bacteriology*, *194*(23), 6419–6430. https://doi.org/10.1128/JB.01325-12

Kaboré, O. D., Godreuil, S., & Drancourt, M. (2020, November 30). Planctomycetes as Host-Associated Bacteria: A Perspective That Holds Promise for Their Future Isolations, by Mimicking Their Native Environmental Niches in Clinical Microbiology Laboratories.

Frontiers in Cellular and Infection Microbiology. Frontiers Media S.A. https://doi.org/10.3389/fcimb.2020.519301

Kallscheuer, N., Jeske, O., Sandargo, B., Boedeker, C., Wiegand, S., Bartling, P., … Jogler, C. (2020). The planctomycete Stieleria maiorica Mal15T employs stieleriacines to alter the species composition in marine biofilms. *Communications Biology*, *3*(1), 1–8. https://doi.org/10.1038/s42003-020-0993-2

Karpiński, T. M., & Adamczak, A. (2018, June 1). Anticancer activity of bacterial proteins and peptides. *Pharmaceutics*. MDPI AG. https://doi.org/10.3390/pharmaceutics10020054

Kato, S., Takano, Y., Kakegawa, T., Oba, H., Inoue, K., Kobayashi, C., … Yamagishi, A. (2010). Biogeography and biodiversity in sulfide structures of active and inactive vents at deep-sea hydrothermal fields of the southern mariana trough. *Applied and Environmental Microbiology*, *76*(9), 2968–2979. https://doi.org/10.1128/AEM.00478-10

Katzung, B. G., Kruidering-Hall, M., & Trevor, A. J. (2018). Beta-Lactam Antibiotics & Other Cell Wall Synthesis Inhibitors. In M. Weitz & P. J. Boyle (Eds.), *Katzung & Trevor's Pharmacology: Examination & Board Review, 11e* (11th ed.). New York: McGraw-Hill Medical. Retrieved from https://accesspharmacy.mhmedical.com/content.aspx?bookid=1568&sectionid=95703865

Kerr, J. F. R., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*, *26*(4), 239–257. https://doi.org/10.1038/bjc.1972.33

König, E., Schlesner, H., & Hirsch, P. (1984). Cell wall studies on budding bacteria of the Planctomyces[Pasteuria group and on a Prosthecomicrobium sp. *Arch Microbiol*, *138*, 200–205.

Krause, K. M., Serio, A. W., Kane, T. R., & Connolly, L. E. (2016). Aminoglycosides: An overview. *Cold Spring Harbor Perspectives in Medicine*, *6*(6). https://doi.org/10.1101/cshperspect.a027029

Kuenen, J. G. (2020). Anammox and beyond. *Environmental Microbiology*, *22*(2), 525–536. https://doi.org/10.1111/1462-2920.14904

Kumar, S., Yadav, A. K., Chambel, P., & Kaur, R. (2017). Molecular and functional characterization of myxobacteria isolated from soil in India. *3 Biotech*, *7*(2), 112. https://doi.org/10.1007/s13205-017-0722-9

Lage, Olga M., & Bondoso, J. (2012). Bringing Planctomycetes into pure culture. *Frontiers in Microbiology*, *3*(DEC), 405. https://doi.org/10.3389/fmicb.2012.00405

Lage, Olga Maria, & Bondoso, J. (2011). Planctomycetes diversity associated with macroalgae. *FEMS Microbiology Ecology*, *78*(2), 366–375. https://doi.org/10.1111/j.1574-6941.2011.01168.x

Lanzén, A., Jørgensen, S. L., Bengtsson, M. M., Jonassen, I., Øvreås, L., & Urich, T. (2011). Exploring the composition and diversity of microbial communities at the Jan Mayen hydrothermal vent field using RNA and DNA. *FEMS Microbiology Ecology*, *77*(3), 577–589. https://doi.org/10.1111/j.1574-6941.2011.01138.x

Larsson, D. G. J. (2014). Antibiotics in the environment. *Upsala Journal of Medical Sciences*. Informa Healthcare. https://doi.org/10.3109/03009734.2014.896438

Lauritano, C., Martínez, K. A., Battaglia, P., Granata, A., de la Cruz, M., Cautain, B., … Guglielmo, L. (2020). First evidence of anticancer and antimicrobial activity in Mediterranean mesopelagic species. *Scientific Reports*, *10*(1), 1–8. https://doi.org/10.1038/s41598-020-61515-z

Lee, K. C., Webb, R. I., & Fuerst, J. A. (2009). The cell cycle of the planctomycete Gemmata obscuriglobus with respect to cell compartmentalization. *BMC Cell Biology*, *10*, 4. https://doi.org/10.1186/1471-2121-10-4

Liesack, W., König, H., Schlesner, H., & Hirsch, P. (1986). Chemical composition of the peptidoglycan-free cell envelopes of budding bacteria of the Pirella[Planctomyces group. *Arch Microbiol*, *145*, 361–366.

Liesackt, W., & Stackebrandt, E. (1992). Occurrence of Novel Groups of the Domain Bacteria as Revealed by Analysis of Genetic Material Isolated from an Australian Terrestrial Environment. *JOURNAL OF BACTERIOLOGY*, 5072–5078.

Lindsay, M. R., Webb, R. I., & Fuerst, J. A. (1997). Pirellulosomes: A new type of membrane-bounded cell compartment in planctomycete bacteria of the genus Pirellula. *Microbiology*, *143*(3), 739–748. https://doi.org/10.1099/00221287-143-3-739

Lindsay, M. R., Webb, R. I., Strous, M., Jetten, M. S. M., Butler, M. K., Forde, R. J., & Fuerst, J. A. (2001). Cell compartmentalisation in planctomycetes: Novel types of structural organisation for the bacterial cell. *Archives of Microbiology*, *175*(6), 413–429. https://doi.org/10.1007/s002030100280

Longford, S., Tujula, N., Crocetti, G., Holmes, A., Holmström, C., Kjelleberg, S., … Taylor, M. (2007). Comparisons of diversity of bacterial communities associated with three sessile marine eukaryotes. *Aquatic Microbial Ecology*, *48*(3), 217–229. https://doi.org/10.3354/ame048217

Löwe, J., & Amos, L. A. (1998). Crystal structure of the bacterial cell-division protein FtsZ.

Nature, *391*(6663), 203–206. https://doi.org/10.1038/34472

Lu, D.-Y., Chen, E.-H., Lu, T.-R., & Ding, J. (2015). Anticancer Drug Combinations, Studies from Different Pathways. *Studies from Different Pathways. Cell Dev Biol*, *4*, 166. https://doi.org/10.4172/2168-9296.1000166

Margolin, W. (2000). Themes and variations in prokaryotic cell division. *FEMS Microbiology Reviews*, *24*(4), 531–548. https://doi.org/10.1111/j.1574-6976.2000.tb00554.x

Microplate Reader | Plate Reader - BMG LABTECH. (n.d.). Retrieved April 21, 2021, from https://www.bmglabtech.com/microplate-reader/

Miller, M. B., & Bassler, B. L. (2001, November 28). Quorum sensing in bacteria. *Annual Review of Microbiology*. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA . https://doi.org/10.1146/annurev.micro.55.1.165

Morier, D. (2021). Antibiotic resistance. In *Encyclopedia Britannica*. Retrieved from https://www.britannica.com/science/antibiotic-resistance

Mosunova, O., Navarro-Muñoz, J. C., & Collemare, J. (2020). The Biosynthesis of Fungal Secondary Metabolites: From Fundamentals to Biotechnological Applications. In *Reference Module in Life Sciences*. Elsevier. https://doi.org/10.1016/b978-0-12-809633-8.21072-8

Nealson, K. H. (1977). Autoinduction of bacterial luciferase - Occurrence, mechanism and significance. *Archives of Microbiology*, *112*(1), 73–79. https://doi.org/10.1007/BF00446657

Niftrik, L. A., Fuerst, J. A., DamstÃ©, J. S. S., Kuenen, J. G., Jetten, M. S. M., & Strous, M. (2004). The anammoxosome: an intracytoplasmic compartment in anammox bacteria. *FEMS Microbiology Letters*, *233*(1), 7–13. https://doi.org/10.1016/j.femsle.2004.01.044

Olympus (n.d.). IMT-2. [Apparatus].

Ovreås, L., Forney, L., Daae, F. L., & Torsvik, V. (1997). Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Applied and Environmental Microbiology*, *63*(9), 3367–3373.

Pimentel-Elardo, S., Wehrl, M., Friedrich, A., Jensen, P., & Hentschel, U. (2003). Isolation of planctomycetes from Aplysina sponges. *Aquatic Microbial Ecology*, *33*(3), 239–245. https://doi.org/10.3354/ame033239

Pollet, T., Humbert, J. F., & Tadonléké, R. D. (2014). Planctomycetes in lakes: Poor or strong competitors for phosphorus? *Applied and Environmental Microbiology*, *80*(3), 819–828. https://doi.org/10.1128/AEM.02824-13

Prestegard, S. K., Oftedal, L., Coyne, R. T., Nygaard, G., Skjærven, K. H., Knutsen, G., … Herfindal, L. (2009). Marine Benthic Diatoms Contain Compounds Able to Induce Leukemia Cell Death and Modulate Blood Platelet Activity. *Marine Drugs*, *7*(4), 605–623. https://doi.org/10.3390/md7040605

Prieto-Barajas, C. M., Valencia-Cantero, E., & Santoyo, G. (2018, January 1). Microbial mat ecosystems: Structure types, functional diversity, and biotechnological application. *Electronic Journal of Biotechnology*. Pontificia Universidad Catolica de Valparaiso. https://doi.org/10.1016/j.ejbt.2017.11.001

Protocol Guide: WST-1 Assay for Cell Proliferation and Viability | Sigma-Aldrich. (n.d.). Retrieved April 21, 2021, from https://www.sigmaaldrich.com/technical-documents/protocols/biology/roche/cell-proliferation-reagent-wst-1.html

Reichenbach, H. (2001). *Myxobacteria, producers of novel bioactive substances*. *Journal of Industrial Microbiology & Biotechnology* (Vol. 27). Retrieved from www.nature.com/jim

Reva, O., & Tümmler, B. (2008). Think big - Giant genes in bacteria. *Environmental Microbiology*, *10*(3), 768–777. https://doi.org/10.1111/j.1462-2920.2007.01500.x

Reyes-Lamothe, R., & Sherratt, D. J. (2019, August 1). The bacterial cell cycle, chromosome inheritance and cell growth. *Nature Reviews Microbiology*. Nature Publishing Group. https://doi.org/10.1038/s41579-019-0212-7

Rivas-Marín, E., Canosa, I., & Devos, D. P. (2016). Evolutionary cell biology of division mode in the bacterial Planctomycetes-verrucomicrobia- Chlamydiae superphylum. *Frontiers in Microbiology*. Frontiers Research Foundation. https://doi.org/10.3389/fmicb.2016.01964

Rueff, J., & Rodrigues, A. S. (2016). Cancer drug resistance: A brief overview from a genetic viewpoint. In *Methods in Molecular Biology* (Vol. 1395, pp. 1–18). Humana Press Inc. https://doi.org/10.1007/978-1-4939-3347-1_1

Rutherford, S. T., & Bassler, B. L. (2012). Bacterial quorum sensing: Its role in virulence and possibilities for its control. *Cold Spring Harbor Perspectives in Medicine*. Cold Spring Harbor Laboratory Press. https://doi.org/10.1101/cshperspect.a012427

Sæbø, E. F. (2018). *Isolation and Characterization of Novel Planctomycetes from Svarthammarhola Ice Cave with Potential for Production of Bioactive Molecules*.

Saier, M. H., & Bogdanov, M. V. (2013). Membranous Organelles in Bacteria. *Journal of Molecular Microbiology and Biotechnology*, *23*(1–2), 5–12. https://doi.org/10.1159/000346496

Sanchez, S., & Demain, A. L. (2019). Secondary metabolites. In *Comprehensive Biotechnology* (pp. 131–143). Elsevier. https://doi.org/10.1016/B978-0-444-64046-8.00012-4

Santarella-Mellwig, R., Pruggnaller, S., Roos, N., Mattaj, I. W., & Devos, D. P. (2013). Three-Dimensional Reconstruction of Bacteria with a Complex Endomembrane System. *PLoS Biology*, *11*(5). https://doi.org/10.1371/journal.pbio.1001565

Santos, N. C., Martins-Silva, J., & Saldanha, C. (2005, April 1). PTEN "meets" DMSO. *Leukemia Research*. Elsevier Ltd. https://doi.org/10.1016/j.leukres.2004.09.009

Schlesner, H. (1994). The Development of Media Suitable for the Microorganisms Morphologically Resembling Planctomyces spp., Pirellula spp., and other Planctomycetales from Various Aquatic Habitats Using Dilute Media. *Systematic and Applied Microbiology*, *17*(1), 135–145. https://doi.org/10.1016/S0723-2020(11)80042-1

Schlesner, H., & Stackebrandt, E. (1986). Assignment of the genera Planctomyces and Pirella to a new family Planctomycetaceae fam. nov. and description of the order Planctomycetales ord. nov. *Systematic and Applied Microbiology*, *8*(3), 174–176. https://doi.org/10.1016/S0723-2020(86)80072-8

Schneider, C. A.; Rasband, W. S. & Eliceiri, K. W. (2012), "NIH Image to ImageJ: 25 years of image analysis", *Nature methods* 9(7): 671-675, PMID 22930834

Schoch, C. L., Ciufo, S., Domrachev, M., Hotton, C. L., Kannan, S., Khovanskaya, R., … Karsch-Mizrachi, I. (2020). NCBI Taxonomy: A comprehensive update on curation, resources and tools. *Database*. Oxford University Press. https://doi.org/10.1093/database/baaa062

Shiraishi, J., Tatsumi, T., Keira, N., Akashi, K., Mano, A., Yamanaka, S., … Nakagawa, M. (2001). Important role of energy-dependent mitochondrial pathways in cultured rat cardiac myocyte apoptosis. *American Journal of Physiology - Heart and Circulatory Physiology*, *281*(4 50-4). https://doi.org/10.1152/ajpheart.2001.281.4.h1637

Sidana, A., Wang, M., Shabbeer, S., Chowdhury, W. H., Netto, G., Lupold, S. E., … Rodriguez, R. (2012). Mechanism of growth inhibition of prostate cancer xenografts by valproic acid. *Journal of Biomedicine and Biotechnology*, *2012*. https://doi.org/10.1155/2012/180363

Sigma-Aldrich. (n.d.). DMEM (D-6429). https://www.sigmaaldrich.com/catalog/product/sigma/d6429?lang=en&region=NO

Sigma-Aldrich. (n.d.). Fetal bovine serum (F9665). https://www.sigmaaldrich.com/catalog/product/sigma/f9665?lang=en&region=NO&gclid=Cj0KCQjwktKFBhCkARIsAJeDT0hwVQ-hRkR92S06m_c_1UWf4fZVQ1Awj-WnOi1_LcMxaAsei2xih9saAhsEEALw_wcB

Sigma-Aldrich. (n.d.). RPMI-1640 (R5886). https://www.sigmaaldrich.com/catalog/product/sigma/r5886?lang=en&region=NO&gcli

d=Cj0KCQjwktKFBhCkARIsAJeDT0gxaA2Y8XxR9IcqaE5Xx7yx9ZDqhOdoSoUE3XM9FWeqIo9uo5i5-y8aAoacEALw_wcB

Skerman, V. B. D., McGowan, V., & Sneath, P. H. A. (1980). Approved lists of bacterial names. *International Journal of Systematic Bacteriology*, *30*(1), 225–420. https://doi.org/10.1099/00207713-30-1-225

Slobodkina, G. B., Kovaleva, O. L., Miroshnichenko, M. L., Slobodkin, A. I., Kolganova, T. V., Novikov, A. A., … Bonch-Osmolovskaya, E. A. (2015). Thermogutta terrifontis gen. Nov., sp. nov. and thermogutta hypogea sp. nov., thermophilic anaerobic representatives of the phylum planctomycetes. *International Journal of Systematic and Evolutionary Microbiology*, *65*(3), 760–765. https://doi.org/10.1099/ijs.0.000009

Soldatkina, M. A., Klochko, V. V., Zag rodnya, S. D., Rademan, S., Visagie, M. H., Lebelo, M. T., … Reva, O. N. (2018). Promising anticancer activity of batumin: A natural polyene antibiotic produced by Pseudomonas batumici. *Future Medicinal Chemistry*, *10*(18), 2187–2199. https://doi.org/10.4155/fmc-2018-0062

Staley, J. T. (1968). Prosthecomicrobium and Ancalomicrobium: new prosthecate freshwater bacteria. *Journal of Bacteriology*, *95*(5), 1921–1942. https://doi.org/10.1128/jb.95.5.1921-1942.1968

Staley, J. T. (1973). Budding bacteria of the Pasteuria-Blastobacter group. *Canadian Journal of Microbiology*, *19*(5), 609–614. https://doi.org/10.1139/m73-100

Staley, James T., & Bauld, J. (1981). The Genus Planctomyces. In *The Prokaryotes* (pp. 505–508). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-662-13187-9_39

Storesund, Julia E., & Øvreås, L. (2013). Diversity of planctomycetes in iron-hydroxide deposits from the arctic mid ocean ridge (AMOR) and description of bythopirellula goksoyri gen. nov., sp. nov., a novel Planctomycete from deep sea iron-hydroxide deposits. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *104*(4), 569–584. https://doi.org/10.1007/s10482-013-0019-x

Storesund, Julia E, Lanzèn, A., Nordmann, E.-L., Armo, H. R., Lage, O. M., & Øvreås, L. (2020). Planctomycetes as a Vital Constituent of the Microbial Communities Inhabiting Different Layers of the Meromictic Lake Saelenvannet (Norway). *Microorganisms*, *8*(1150), 1–18. https://doi.org/10.3390/microorganisms8081150

Storesund, Julia Endresen, Lanzèn, A., García-Moyano, A., Reysenbach, A. L., & Øvreås, L. (2018). Diversity patterns and isolation of Planctomycetes associated with metalliferous deposits from hydrothermal vent fields along the Valu Fa Ridge (SW Pacific). *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *111*(6),

841–858. https://doi.org/10.1007/s10482-018-1026-8

Strous, M., Fuerst, J. A., Kramer, E. H. M., Logemann, S., Muyzer, G., Van De Pas-Schoonen, K. T., … Jetten, M. S. M. (1999). Missing lithotroph identified as new planctomycete. *Nature*, *400*(6743), 446–449. https://doi.org/10.1038/22749

Strous, M., Van Gerven, E., Gijs Kuenen, J., & Jetten, M. (1997). Effects of Aerobic and Microaerobic Conditions on Anaerobic Ammonium-Oxidizing (Anammox) Sludge. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, *63*(6), 2446–2448. Retrieved from http://aem.asm.org/

Teasdale, M. E., Liu, J., Wallace, J., Akhlaghi, F., & Rowley, D. C. (2009). Secondary metabolites produced by the marine bacterium halobacillus salinus that Inhibit quorum sensing-controlled phenotypes in gram-negative bacteria. *Applied and Environmental Microbiology*, *75*(3), 567–572. https://doi.org/10.1128/AEM.00632-08

Tekniepe, B. L., Schmidt, J. M., & Starr, P. (1981). Life cycle of a budding and appendaged bacterium belonging to morphotype IV of the Blastocaulis-Planctomyces group. *Current Microbiology*, *5*(1), 1–6. https://doi.org/10.1007/BF01566588

Thirumurugan, D., Cholarajan, A., Raja, S. S. S., & Vijayakumar, R. (2018). An Introductory Chapter: Secondary Metabolites. In *Secondary Metabolites - Sources and Applications*. InTech. https://doi.org/10.5772/intechopen.79766

Um, P. (2015). Cancer, Definition. In F. Rodriguez-Valera & B. . White (Eds.), *Encyclopedia of Metagenomics*. Boston: Springer, Boston, MA. https://doi.org/10.1007/978-1-4899-7475-4_106

Vacuubrand (n.d.). VARIO PC 3001. [Apparatus]. https://www.vacuubrand.com/en/page1380.html

van De Graaf, A. A., Mulder, † Arnold, De Bruijn, P., Jetten, M. S. M., Robertson, L. A., & Gijs Kuenen, A. J. (1995). *Anaerobic Oxidation of Ammonium Is a Biologically Mediated Process Downloaded from*. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* (Vol. 61).

van Teeseling, M. C. F., Mesman, R. J., Kuru, E., Espaillat, A., Cava, F., Brun, Y. V., … Van Niftrik, L. (2015). Anammox Planctomycetes have a peptidoglycan cell wall. *Nature Communications*, *6*. https://doi.org/10.1038/ncomms7878

van Teeseling, M. C. F., Neumann, S., & van Niftrik, L. (2013). The Anammoxosome Organelle Is Crucial for the Energy Metabolism of Anaerobic Ammonium Oxidizing Bacteria. *Journal of Molecular Microbiology and Biotechnology*, *23*(1–2), 104–117. https://doi.org/10.1159/000346547

Ventola, C. L. (2015). The antibiotic resistance crisis: causes and threats. *P & T Journal*, *40*(4), 277–283. https://doi.org/Article

Verheijen, M., Lienhard, M., Schrooders, Y., Clayton, O., Nudischer, R., Boerno, S., … Caiment, F. (2019). DMSO induces drastic changes in human cellular processes and epigenetic landscape in vitro. *Scientific Reports*, *9*(1), 1–12. https://doi.org/10.1038/s41598-019-40660-0

VirTis. (n.d.). Tempest Virtishear I.Q.2. [Apparatus].

VWR. (n.d.). Mini shaker. [Apparatus]. https://us.vwr.com/store/product/4902576/vwr-mini-shakers

Wagner, M., & Horn, M. (2006, June 1). The Planctomycetes, Verrucomicrobia, Chlamydiae and sister phyla comprise a superphylum with biotechnological and medical relevance. *Current Opinion in Biotechnology*. Elsevier Current Trends. https://doi.org/10.1016/j.copbio.2006.05.005

Wall, M. E., & Wani, M. C. (1996). Camptothecin and taxol: From discovery to clinic. In *Journal of Ethnopharmacology* (Vol. 51, pp. 239–254). Elsevier Ireland Ltd. https://doi.org/10.1016/0378-8741(95)01367-9

Wang, J., Jenkins, C., Webb, R. I., & Fuerst, J. A. (2002). Isolation of Gemmata-like and Isosphaera-like planctomycete bacteria from soil and freshwater. *Applied and Environmental Microbiology*, *68*(1), 417–422. https://doi.org/10.1128/AEM.68.1.417-422.2002

Wang, Jian, Ruan, C. J., Song, L., Li, A., Zhu, Y. X., Zheng, X. W., … Dai, X. (2020). Gimesia benthica sp. nov., a planctomycete isolated from a deep-sea water sample of the northwest indian ocean. *International Journal of Systematic and Evolutionary Microbiology*, *70*(7), 4384–4389. https://doi.org/10.1099/ijsem.0.004301

Wang, X., Zhang, H., & Chen, X. (2019). Drug resistance and combating drug resistance in cancer. *Cancer Drug Resistance*, *2*(2), 141–160. https://doi.org/10.20517/cdr.2019.10

Ward, N. L. (2010). Phylum XXV. Planctomycetes Garrity and Holt 2001, 137 emend. Ward (this volume). In *Bergey's Manual® of Systematic Bacteriology* (Vol. 137, pp. 879–925). Springer New York. https://doi.org/10.1007/978-0-387-68572-4_14

Ward, N. L. (2015). *Planctomycetaceae*. In *Bergey's Manual of Systematics of Archaea and Bacteria* (pp. 1–2). Wiley. https://doi.org/10.1002/9781118960608.fbm00161

Waters. (n.d.). Sep-Pak C18 35 cc, 10g. [Apparatus]. https://www.waters.com/nextgen/us/en/shop/sample-preparation--filtration/wat043345-sep-pak-c18-35-cc-vac-cartridge-10-g-sorbent-per-cartridge-

55---.html

Waters. (n.d.). Sep-Pak C18 plus. [Apparatus]. https://www.waters.com/nextgen/us/en/shop/sample-preparation--filtration/wat020515-sep-pak-c18-plus-short-cartridge-360-mg-sorbent-per-cartridge-55.html

Webster, N. S., Wilson, K. J., Blackall, L. L., & Hill, R. T. (2001). Phylogenetic diversity of bacteria associated with the marine sponge Rhopaloeides odorabile. *Applied and Environmental Microbiology*, *67*(1), 434–444. https://doi.org/10.1128/AEM.67.1.434-444.2001

Webster, Nicole S., & Bourne, D. (2007). Bacterial community structure associated with the Antarctic soft coral, Alcyonium antarcticum. *FEMS Microbiology Ecology*, *59*(1), 81–94. https://doi.org/10.1111/j.1574-6941.2006.00195.x

Weissman, K. J., & Müller, R. (2010, September 18). Myxobacterial secondary metabolites: Bioactivities and modes-of-action. *Natural Product Reports*. The Royal Society of Chemistry. https://doi.org/10.1039/c001260m

Wiegand, S., Jogler, M., Boedeker, C., Pinto, D., Vollmers, J., Rivas-Marín, E., … Jogler, C. (2020). Cultivation and functional characterization of 79 planctomycetes uncovers their unique biology. *Nature Microbiology*, *5*. https://doi.org/10.1038/s41564-019-0588-1

Wiegand, S., Jogler, M., & Jogler, C. (2018, November 1). On the maverick Planctomycetes. *FEMS Microbiology Reviews*. Oxford University Press. https://doi.org/10.1093/femsre/fuy029

Woebken, D., Teeling, H., Wecker, P., Dumitriu, A., Kostadinov, I., Delong, E. F., … Glöckner, F. O. (2007). Fosmids of novel marine Planctomycetes from the Namibian and Oregon coast upwelling systems and their cross-comparison with planctomycete genomes. *ISME Journal*, *1*(5), 419–435. https://doi.org/10.1038/ismej.2007.63

Wrótniak-Drzewiecka, W., Brzezińska, A. J., Dahm, H., Ingle, A. P., & Rai, M. (2016, March 1). Current trends in myxobacteria research. *Annals of Microbiology*. Springer Verlag. https://doi.org/10.1007/s13213-015-1104-3

Youssef, N. H., & Elshahed, M. S. (2014). The Phylum Planctomycetes. In E. Rosenberg et al. (Ed.), *The Prokaryotes – Other Major Lineages of Bacteria and the Archaea* (pp. 759–810). Berlin Heidelberg: Springer-Verlag . https://doi.org/10.1007/978-3-642-38954-2_155

Appendix I – Hutners basal salts

Component	Amount
Nitrilotriacetic acid	5 g
$MgSO_4$ x $7H_2O$	13,85 g
$CaCl_2$ x $2H_2O$	1,67 g
$NaMoO_4$ x $2H_2O$	6,3 mg
$FeSO_4$ x $7H_2O$	49,5 mg
Metal salt solution «44»	50 ml
Double distilled water	450 ml

Metal salts solution «44»

Component	Amount
Ethylene diamino tetra acetate (EDTA)	25 mg
$ZnSO_4$ x $7H_2O$	109,5 mg
$FeSO_4$ x $7H_2O$	50 mg
$MnSO_4$ x H_2O	15,4 mg
$CuSO_4$ x $5H_2O$	3,9 mg
$CoCl_2$ x $6H_2O$	2,0 mg
$Na_2B_4O_7$ x $10H_2O$	1,8 mg
Double distilled water	100 ml

Appendix II – Vitamin solution no. 6 – 10x

Component	Amount
Biotin	4 mg
Pyridoxine hydrochloride	20 mg
Thiamine hydrochloride	10 mg
Ca pantothenate	10 mg
p-Aminobenzoic acis	10 mg
Folic acid	4 mg
Riboflavin	10 mg
Nicotinic acid	10 mg
Vitamin B12	0,2 mg
Double distilled water	100 ml