

Microbiological drinking water quality evaluation in a technical analyses laboratory

Susana Afonso João

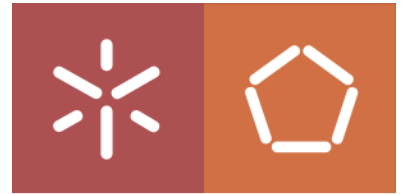
Mestrado em Tecnologia e Ciência Alimentar

Departamento de Química e Bioquímica

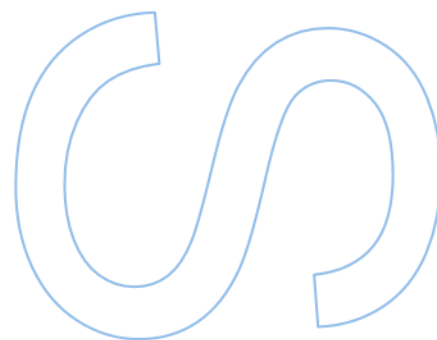
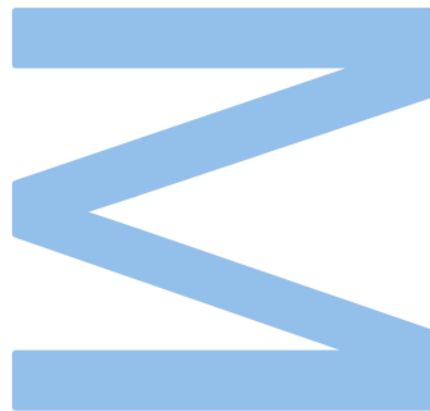
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Universidade do Minho
Escola de Engenharia





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Susana Afonso João

Relatório de Estágio realizado no âmbito do Mestrado em
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"The glory of the heavens was ever beyond the grasp of those who never thought to reach it. But if I gained anything from all of this, it is the courage to stretch out my hand"

– G'raha Tia, Final Fantasy XIV © Square Enix

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Resumo

O acesso a uma fonte de água de consumo segura é considerado um dos direitos humanos básicos uma vez que, é necessário para a sobrevivência humana. Por esse motivo, água utilizada para consumo humano não deve apresentar nenhum risco para a saúde de nenhum indivíduo ao longo do seu período de vida, apresentando-se como sendo incolor, inodora e insípida.

Alterações nas características organoléticas da água são indicativas de contaminação, nomeadamente a nível microbiológico. Relativamente a este aspeto, a maioria dos contaminantes microbiológicos são originários de fezes de animais de sangue quente e de humanos. No entanto, outras bactérias que não têm como origem matéria fecal são também relevantes, tais como os agentes patogénicos oportunistas de canalização, por exemplo *Legionella* spp..

Deste modo, é necessário utilizar métodos padronizados para a deteção e quantificação de agentes patogénicos aquáticos. Diversos meios de cultura seletivos e diferenciais são utilizados de forma a determinar as características morfológicas e bioquímicas típicas de certos grupos bacterianos através de testes de confirmação. A expressão dos resultados obtidos deve ser fiável e precisa.

Assim, para assegurar a qualidade dos resultados, são executados controlos laboratoriais ao longo de todo o processo analítico. Os controlos variam desde avaliações ambientais e de performance de meios de cultura até verificações aos próprios profissionais e testes de esterilidade. Estes procedimentos laboratoriais contribuem derradeiramente para a promoção da saúde e para as medidas de prevenção de doença.

Palavras-chave: Água de consumo humano, Agentes patogénicos aquáticos, Enterobacteriaceae, *Legionella*, Controlo de qualidade.

Abstract

Safe drinking water access is considered one of the basic human rights since it is necessary for human survival. Hence, water used for human consumption should not present any health risks over the lifetime of any individual, presenting itself as colourless, odourless and tasteless.

Changes in organoleptic features greatly implicate water contamination, namely microbial wise. Regarding this aspect, most microbial contaminants originate from human and warm-blooded animal faeces. However, other bacteria that do not derive from faecal matter also have an important role, such as opportunistic plumbing pathogens like *Legionella* spp..

This way, it is necessary to employ standard methods for waterborne pathogen detection and enumeration. Several selective and differential culture media are used to ascertain typical morphological and biochemical features of certain bacterial groups by confirmatory tests. The expression of the obtained results must be reliable and accurate.

Therefore, to ensure result quality, laboratory controls are employed along the whole analytical process. Controls range from environmental and culture media performance evaluation to sterility and professional personnel testing. These laboratorial procedures ultimately contribute to health promotion and disease prevention measures.

Keywords: Drinking water, Waterborne pathogens, Enterobacteriaceae, *Legionella*, Quality control.

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List of Abbreviations

ALS	Australian Laboratory Services
ATCC	American Type Culture Collection
BCYE Agar	Buffered Charcoal Yeast Extract Agar
BP-RPF Agar	Baird Parker - Rabbit Plasma Fibrinogen Agar
BPW	Buffered Peptone Water
CCA	Chromogenic Coliform Agar
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Units
CN Agar	Cetrimide Agar
DAEC	Diffusely Adherent <i>Escherichia coli</i>
EAEC	Enteraggregative <i>E. coli</i>
EHEC	Enterohemorrhagic <i>E. coli</i>
EIEC	Enteroinvasive <i>E. coli</i>
EPEC	Enteropathogenic <i>E. coli</i>
EPS	Extracellular Polymeric Substances
ERSAR	Entidade Reguladora dos Serviços de Águas e Resíduos
ETEC	Enterotoxigenic <i>E. coli</i>
ExPEC	Extraintestinal Pathogenic <i>E. coli</i>
FIB	Faecal Indicator Bacterium
GVPC Agar	Glycine, Vancomycin, Polymyxin B, Cycloheximide Agar
HCl	Hydrochloric Acid
HUS	Haemolytic Uremic Syndrome
ILCs	Interlaboratory Comparisons
InPEC	Intestinal Pathogenic <i>E. coli</i>
IPAC	Instituto Português de Acreditação
ISA	Iron Sulphite Agar
ISO	International Organization for Standardization
IU	International Unit
KCl	Potassium Chloride
MAC	<i>Mycobacterium avium</i> Complex
MEVAG Agar	<i>Milieu d'Étude de la Voie d'Attaque des Glucides</i> (Medium for Studying the Path of Carbohydrate Attack)
MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>

N/A	Non applicable
NCTC	National Collection of Type Cultures
NF	Norme française
NP	Norma Portuguesa
OPPs	Opportunistic Plumbing Pathogens
PCA	Plate Count Agar
PCQA	Programas de Controlo da Qualidade da Água
QC	Quality Control
RM	Reference Material
rpm	Rotations Per Minute
RSV Broth	Rappaport-Vassiliadis Broth
S	Standard Deviation
SDG	Sustainable Development Goal
TSA	Tryptic Soy Agar
TSC	Tryptose Sulphite Cycloserine Agar
TSI Agar	Triple Sugar Iron Agar
TTC	Tri-phenyltetrazolium Chloride
UN	United Nations
UV	Ultraviolet
WDCM	World Data Centre of Microorganisms
WHO	World Health Organization
XLD Agar	Xylose Lysine Desoxycholate Agar

1. Introduction

1.1. ALS – Life Sciences Portugal S.A.

Australian Laboratory Services (ALS) Pty Ltd initiated its operations in Brisbane, Queensland, Australia in 1974 in analytical analyses services for mineral and oil shale exploration industries. Seven years later, ALS was acquired by Campbell Brothers Limited, a chemical company focused on soap manufacturing, initially established in Australia in 1863 (ALS, 2024a). In 1990, the company had already become a major player as a laboratory services provider in Australia, undergoing a period of great growth in the sector, and ten years later it underwent a global expansion period reaching North America, Africa and Europe (ALS, 2024b). Nowadays, ALS Global possesses an international presence with over 350 locations in more than 70 countries, having acquired 9 locations in Portugal (ALS, 2024c).

This way, ALS Global operates in several industrial sectors with a focus on four main areas: life sciences, minerals, energy and industry. The life sciences sector includes a wide range of environmental, pharmaceutical, food, animal health and electronics assays (ALS, 2024d). The corporation's vision is to become the worldwide leader in laboratory analytical services while completing its mission of aspiring to achieve a better, healthier and safer world for all individuals. ALS intends to deliver this by using its passion for science to solve complex problems (ALS, 2023). With this in mind, the core values of ALS consist of showing honesty and integrity through constructive and open debates; exceeding expectations by putting the client's needs first, while striving to achieve constant improvement to be a leader in the market and following the best practices, having safety as its number one priority. All this without overlooking the success celebration (ALS, 2024e).

In Portugal, business activities were initiated in 2008 through an office for commercial and technical support for Portuguese customers (ALS, 2024b). Later on, in 2021, ALS Global acquired Sagilab S.A., a company based in Porto city focused on chemical and microbiological laboratory services in several sectors, such as health, food and environmental (ALS, 2024f). This organic unit is accredited by Instituto Português de Acreditação (IPAC) in several scopes, including cosmetics, textiles, ambient air, medical activities, liquid effluents and waters.

Regarding drinking water microbiological analyses, this unit is certified in (ALS, 2024g; IPAC, 1999):

- Enumeration of microorganisms at 30 °C by inoculation;
- Enumeration of viable microorganisms with colony count at 22 ± 2 °C by inoculation using ISO 6222 method;
- Enumeration of viable microorganisms with colony count at 36 ± 2 °C by inoculation using ISO 6222 method;
- Enumeration and detection of *Legionella pneumophila* by membrane filtration with elution using ISO 11731 method;
- Enumeration and detection of *Legionella* spp. by membrane filtration with elution using ISO 11731 method;
- Enumeration of *Clostridium perfringens* by membrane filtration using ISO 14189 method;
- Enumeration of coagulase-positive staphylococci by membrane filtration using NF T 90-412 method;
- Enumeration and detection of total staphylococci by membrane filtration;
- Enumeration of faecal coliforms by membrane filtration;
- Enumeration and detection of *Pseudomonas aeruginosa* by membrane filtration using ISO 16266 method;
- Enumeration of *Escherichia coli* and total coliforms by filtration using ISO 9308-1 method;
- Enumeration and detection of sulphite reducing clostridia spores by membrane filtration using NP EN 26461-2 method;
- Enumeration and detection of intestinal enterococci by membrane filtration using ISO 7899-2 method;
- Detection of *Salmonella* spp. by membrane filtration using ISO 19250 method.

Furthermore, ALS is considered to be a suitable laboratory to carry on drinking water analyses by Entidade Reguladora dos Serviços de Águas e Resíduos (ERSAR) in Portugal (ERSAR, 2024).

1.2. Drinking water quality

Safe drinking water access is considered one of the basic human rights since it is necessary for human survival and its quality affects our health. According to the Guidelines for Drinking Water Quality outlined by the World Health Organization (WHO, 2011a), safe drinking water includes water used for human consumption that does not present any health risks over the lifetime of any individual, even when the individual's health is compromised (e.g. immunocompromised or elderly individuals). Furthermore, safe potable water should be colourless, odourless and tasteless since organoleptic changes in these parameters are usually associated with microbial or chemical contaminations and physical properties modifications (WHO, 2011b).

Nowadays, it is already estimated that millions of people die globally due to waterborne diseases, with 3.3 % of the world's deaths being related to this problem. Additionally, 50 % of worldwide diseases are caused by poor-quality water (WHO, 2019). Therefore, contaminated or poor water sources pose a global public health problem responsible for pathologies such as cholera, poliomyelitis, dysentery and other gastric diseases caused by waterborne pathogens (UN-Water, 2021). Besides, it was reported by WHO that, worldwide, no less than 2 billion people drink water from a contaminated source with faeces (WHO, 2023).

This tendency will become more noticeable as the years go by, since the worsening of climate change across the globe combined with the rising growth of human population is expected to decrease access to safe drinking water, with presumed loss in water quality associated with increasing pollution levels. It is estimated that by the year 2050 more than 40 % of the human population will experience the effects of severe water stress (European Investment Bank, 2023).

To eradicate poverty and encourage sustainability, the United Nations (UN) proposed the 2030 Agenda for Sustainable Development which comprises 17 sustainable development goals and 169 target objectives. From these, goal 6 emphasizes the need to ensure water availability, sustainable management and sanitation for all (Table 1). This agenda was accepted unanimously by all the 193 member states (UN, 2015).

Since 2015 there have been several updates to this agenda, namely the 2021 update which determined that the number of people that did not possess access to safe drinking water in 2020 was around 2 billion, which corresponds to 26 % of the planet's population. However, since the 2030 Agenda for Sustainable Development was implemented in 2015, more than 600 million people were able to gain access to safe drinking water. This data shows that most state members are off track to complete the

target 6.1 from Sustainable Development Goal 6 (SDG 6) as it would be necessary to reach a fourfold speed increase in the process in order to achieve universal and safe access to drinking water by 2030 (UN-Water, 2021).

Table 1 - UN 2030 Agenda for Sustainable Development Goal 6 and respective targets. Adapted from UN, (2015).

Goal 6	Guarantee access and responsible management of water and sanitation resources for everyone
6.1	Secure universal access to safe and affordable drinking water for everyone by the year 2030.
6.2	Ensure access to proper and fair sanitation and hygiene for everyone, eliminating open defecation, with a focus on the needs of people in vulnerable situations namely women and girls by the year 2030.
6.3	Enhance water quality by reducing pollution, stopping waste dumping, and minimizing the release of harmful chemicals and materials, cutting untreated wastewater by half, and significantly boosting recycling and safe reuse worldwide, by the year 2030.
6.4	Significantly improve water use efficiency in all sectors, ensure sustainable freshwater withdrawals and supply to combat water scarcity, and greatly reduce the number of people affected by it by the year 2030.
6.5	Apply integrated water resources management at all levels, including through cross-border cooperation when needed, by the year 2030.
6.6	Safeguard and restore water-related ecosystems by the year 2020.
6.a	Strengthen international cooperation and provide capacity-building support to developing countries for water and sanitation initiatives, such as water desalination and efficiency and wastewater treatment, by the year 2030.
6.b	Encourage and enhance the involvement of local communities in improving the management of water and sanitation.

1.3. Drinking water quality indicators and contaminants

As it was said above, drinking water should be colourless, odourless and tasteless, since changes to these organoleptic features may indicate contamination by chemical or biological agents, although drinking water is never 100 % clean as it always contains impurities and contaminants (Fish & Boxall, 2018). However, most contaminants cannot be easily detected through optical analyses of the water's properties, requiring further testing (Sharma & Bhattacharya, 2017). Moreover, water

contaminants can be classified as one of four types: organic, inorganic, biological or radiological. The detection of inorganic contaminants requires chemical analyses of the water. These compounds can be naturally occurring in water, such as water hardness, which varies according to geographic location, or anthropogenic wastes such as pharmaceutical products, disinfectants and insecticides, which contain fluorides, for example (Sharma & Bhattacharya, 2017). Regarding organic contaminants, these include pesticides derived from agriculture, pharmaceuticals, dyes and other industrial by-products (Pal et al., 2014; Schweitzer & Noblet, 2018). As for radiological contaminants, these originate from radioactive elements that can be incorporated due to industrial pollution or from rocks present in the waterbed (Lytle et al., 2014). Lastly, biological contaminants are constituted by living organisms including bacteria, viruses and protozoa. These microorganisms can be pathogenic, with a big impact on human health, as infections caused by pathogens are considered to be the most commonly associated health risk in drinking water (Schweitzer & Noblet, 2018).

Additionally, more than 500 waterborne pathogens of importance to human health have been identified, with an emphasis on enteric bacteria, including *E. coli*, *Salmonella* spp. and *Shigella* spp., which originate from faecal matter (Rath, 2021). These bacteria belong to the Enterobacteriaceae family, which is widely known for including coliforms, a group mainly constituted by *Escherichia*, *Citrobacter*, *Enterobacter* and *Klebsiella* genera. Enterobacteriaceae are gram-negative, facultative anaerobic rod-shaped bacteria that may possess motility and can ferment glucose with gas or acid production (Patel et al., 2014). This bacterial family can be divided in different groups. Total coliforms can also ferment lactose with acid and gas production at 37 °C in 48 hours (Li et al., 2021), while faecal coliforms, a subgroup of total coliforms, originate from the gastrointestinal system of warm-blooded animals and are capable of fermenting lactose at 44 °C (Kabler & Clark, 1960).

The main representative of this group is *E. coli*, an organism that not only is considered to be a faecal coliform but also a faecal indicator bacterium (FIB). FIB presence in drinking water indicates the possible presence of other pathogens that are a risk to human health, since FIB are easily detected and can remain in water for a period of 4 to 12 weeks (Price & Wildeboer, 2017). Likewise, FIB represents a reliable microbial water quality indicator as faecal matter is one of the major sources of health-related problems caused by compromising of water quality (Ishii & Sadowsky, 2008).

To be considered an ideal FIB, bacteria must follow certain criteria, namely:

- Detection should only occur in environments contaminated with faeces, hence possessing a high correlation with faecal matter pollution;
- Detection should occur in hosts that are infected by other faecal pathogens, possessing a high correlation between the amount present in a determined matrix and the pathogens' numbers present in the same matrix;
- Detection should occur in high numbers in the gastrointestinal system of hosts as well as faeces and environments with faecal pollution, possessing a high detection sensibility through simple and affordable methodologies;
- Multiplication should only occur in the host's gastrointestinal tract. However, FIB should remain in a viable and/or detectable state similar to faecal pathogens;
- FIB should not be pathogenic to human beings (Cabral, 2010; Rochelle-Newall et al., 2015).

1.3.1. Drinking water pathogens

According to Bradley's classification, diseases that originate in contact with water can be divided into several groups: waterborne illnesses, water illnesses related to vector insects, water-based, and water-washed diseases (White et al., 2002). In Table 2 it is possible to observe some examples of pathogens that belong to each category. Water-washed infections originate essentially from water used for personal and domestic hygiene. In these cases, the water does not act as a vehicle for pathogens, unlike other water-related diseases. Thus, an incrementation in hygiene and sanitation conditions can help prevent these infections (Forde, 2023). As for water-based illnesses these propagate due to pathogens that include an aquatic phase in their life cycle with the inclusion of an intermediary host, for example a snail in dracunculiasis case. Water illnesses related to insects such as malaria include insects that propagate or bite near water bodies as vectors (Oleiwi, 2020). Finally, diseases related to the consumption of contaminated water are considered to be waterborne (Bartram & Hunter, 2015).

In Table 3 it is possible to observe several groups of waterborne pathogenic bacteria as well as important characteristics that mark their significance as waterborne pathogens. As it was mentioned above, waterborne pathogens are of great importance to human health and include Enterobacteriaceae such as *Shigella* spp., *Salmonella* spp. and *E. coli*. The most representative member of the Enterobacteriaceae family is *E. coli*, a bacterium that is considered to be a model organism as well as a FIB. *E. coli* are

compromised by non-pathogen commensal bacteria, naturally present in the intestinal microbiota of animals and humans as well as pathogenic strains that are divided into two main groups: intestinal pathogenic (InPEC) and extraintestinal pathogenic (ExPEC) *E. coli* (Braz et al., 2020). InPEC transmission occurs through the oral faecal route and is usually associated with diarrheal symptoms. Besides, this group is divided into 6 pathovars according to their different virulence factors: enterohemorrhagic (EHEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), enteroaggregative (EAEC), enteropathogenic (EPEC) and diffusely adherent (DAEC) *E. coli* (Rojas-Lopez et al., 2018).

EHEC are known for their higher infectivity when compared to other pathovars (WHO, 2022), causing fatal conditions such as haemolytic uremic syndrome (HUS), where blood vessel damage and inflammation occurs, causing clot formation, potentially leading to renal failure (Sheerin & Glover, 2019). HUS is normally associated with Shiga toxin-producing *E. coli* such as *E. coli* O157:H7 (Y. Liu et al., 2021), an important pathogen usually associated with large outbreaks of gastrointestinal nature, presenting high mortality and morbidity rates worldwide (Manning et al., 2008; Reiss et al., 2006). In addition, infections caused by this pathovar and therefore this serotype are characterized by the occurrence of bloody diarrhoea that can vary between mild and severe with several other gastrointestinal symptoms, leading to gastroenteritis (Saxena et al., 2015). Contaminated drinking water is a known reservoir of *E. coli*, with EHEC and EIEC possessing a great relevance in this infection route (Cabral, 2010). Unlike EHEC, EIEC infections only sporadically trigger bloody diarrhoea and are similar to those caused by *Shigella* spp., causing bacillary dysentery and watery diarrhoea, while multiplying and causing inflammation in the intestinal epithelium (Pasqua et al., 2017).

Shigella spp. transmission can also occur through the consumption of contaminated drinking water or by the oral-faecal route. This genus is composed by gram-negative rod-shaped closely associated with the genus *Escherichia*. These bacteria are capable of producing Shiga toxins and cause bacillary dysentery similarly to certain *E. coli* pathovars. Bacillary dysentery features several gastrointestinal symptoms such as watery diarrhoea and cramp abdominal pain preceded by malaise and fever (Mumy, 2024). Shiga toxins invade the intestinal epithelium, suffering endocytosis, and stop protein synthesis causing cellular death. To a certain extent, *Shigella* spp. share several virulence and pathogenesis factors with the *Escherichia* genus (Liu et al., 2021), although when compared to other enteric bacteria, *Shigella* features a lower infectious dose, with only 10 cells needed to initiate the infectious process (WHO, 2022).

Table 2 - Transmission mechanism and examples of water related illnesses. Adapted from Cairncross & Feachem, (2018).

Water related diseases	Transmission mechanism	Diseases examples
Waterborne	Ingestion of contaminated water containing the pathogen	Cholera, typhoid fever, bacillary dysentery
Water washed	Person to person transmission, caused by sanitation and hygiene problems	Scabies, trachoma
Water based	Transmission by an intermediary host	Dracunculiasis, schistosomiasis
Water related to insect vectors	Transmission by insect vectors near water	Dengue, malaria, yellow fever

Another member of the Enterobacteriaceae family is *Salmonella*, a bacterium that is naturally present in the gastrointestinal microbiota of animals and that is responsible for the majority of gastroenteritis cases related to food consumption, being responsible for over 93 million gastroenteritis cases annually worldwide (Majowicz et al., 2010). *Salmonella* cells can withstand the action of the human stomach acid and can injure the human intestines, colonizing the gut epithelium cells and provoking watery diarrhoea, bacteraemia and septicaemia. Unlike non-typhoid *Salmonella*, typhoid *Salmonella* present a lower inflammatory response by the host since they produce toxins that can decrease inflammation (Luby, 2014). One of the typhoid variants of *Salmonella*, *Salmonella enterica* serovar Typhi can survive in water for long periods of time, however, its multiplication in the water matrix is rare (Mitscherlich & Marth, 2012). *S. enterica* serovar Typhi and Paratyphi are the etiological agents of typhoid fever, a febrile illness accompanied by nausea, headaches, malaise, chills, dry cough and myalgia (Parry et al., 2002).

Other bacteria that may be considered FIB are intestinal enterococci, since they are not able to multiply in water and their survivability in these environments is fundamentally higher than *E. coli* or other thermotolerant coliforms, revealing resistance to drying conditions and chlorination treatments (Gilmore et al., 2014; WHO, 2022). Intestinal enterococci typically originate from human and warm-blooded animal faeces. However, some representatives of this group have been observed when faecal contamination was not present (Gilmore et al., 2014). Furthermore, enterococci are gram-positive, ubiquitous bacteria, which do not produce spores and morphologically portray themselves as ovoid cells that may pair themselves, form chains or appear individually (He et al., 2018).

This group of bacteria represents one of the leading causes for human infections related to health care due to multiple antibiotic resistance, mainly existing in its two most representative species *Enterococcus faecalis* and *Enterococcus faecium* (Arias & Murray, 2012). Enterococci are the most common cause for endocarditis, an infection that triggers endocardium inflammation (McCormick et al., 2000).

Table 3 - Pathogenic bacteria transmitted through drinking water. Adapted from WHO, (2022).

Pathogenic Bacteria	Species, genus or group	Health impact	Water persistence	Infectivity	Importance of animal sources
Diarrhoeagenic <i>E. coli</i>	---	High	Moderate	Low	Yes
Enterohaemorrhagic <i>E. coli</i>	<i>E. coli</i> O157	High	Moderate	High	Yes
<i>Legionella</i>	<i>L. pneumophila</i>	High	Possible multiplication	Moderate	No
Mycobacteria	<i>Mycobacterium avium</i>	Low	Possible multiplication	Low	No
<i>S. enterica</i> serovar Typhi	---	High	Moderate	Low	No
Other Salmonellae	<i>S. enterica</i> <i>Salmonella bongori</i>	High	Possible multiplication	Low	Yes
<i>Shigella</i>	<i>Shigella dysenteriae</i>	High	Short	High	No
<i>Vibrio</i>	<i>Vibrio cholerae</i> O1 and O139	High	Short to long	Low	No

Additional examples of pathogens present in water environments are Clostridia, bacteria capable of producing spores belonging to the Bacillaceae family and the *Clostridium* genus, that are mainly dominant in water sediments and soil, inhabit the gastrointestinal tract of several warm-blooded animals and can be associated with intermittent faecal contamination. These microorganisms are rod-shaped obligatory anaerobes, capable of reducing sulphite with sulphide production. Spores can remain in water for extended periods of time presenting high resistance to chemical treatments used in water, namely common disinfectants, as well as to extreme physical conditions

such as radiation, heat, and drying (Lorenzo et al., 2017). Many bacteria belonging to this group are human pathogens causing prevalent pathologies related to neurotoxins and necrosis such as tetanus, botulism, gas gangrene and gastrointestinal diseases (Schmidt, 2019).

Moreover, it is necessary to emphasize the importance of a member of this bacterial group, *C. perfringens*. This bacterium is recognized as an important FIB, being capable of spore production that may remain viable in water for extended periods of time, presenting a higher survivability than other vegetative FIB cells and, therefore, indicating intermittent or remote faecal contamination (Prescott et al., 2022). Similar to other Clostridia, *C. perfringens* is capable of causing gas gangrene, a muscle infection distinguished by widespread tissue necrosis (Uzal et al., 2015).

Other representatives of common pathogens responsible for drinking water illnesses that often result in outbreaks include *L. pneumophila* and *M. avium*. These two bacteria are opportunistic plumbing pathogens (OPPs), sharing characteristics that allow them to proliferate in water and plumbing systems such as, being capable of forming biofilms and resisting to disinfectants as well as being oligotrophic, growing in environments with a low nutrient load (Falkinham, 2015; Falkinham et al., 2015). The stagnation that occurs in these water systems also contributes to OPPs growth as it allows for biofilm production (Nisar et al., 2020). Contrasting with other waterborne pathogens such as *Salmonella* spp. or *E. coli*, OPPs grow naturally in drinking water, not being contaminants but instead being adapted and persistent in drinking water distribution and plumbing systems (Donohue et al., 2014; Falkinham et al., 2001).

Regarding *Mycobacterium* spp., the main transmission route for non-tuberculous or atypical *Mycobacterium* species of this bacterium consists in inhalation, ingestion or contact with contaminated water, especially drinking water (WHO, 2022). Epidemiological data collected over the years suggest that contaminated hospital water represents a great risk associated with mycobacterial infections (Nishiuchi et al., 2017). The resulting mycobacterial diseases comprehend a wide spectrum of symptomatology, involving genito-urinary, gastrointestinal and respiratory symptoms while also affecting the skeletal, soft and skin tissues. The most studied non-tuberculous *Mycobacterium* is *M. avium*, known for its resilient growth in a wide range of physical water parameters, such as salinity, pH, oxygen levels and temperature (Loret & Dumoutier, 2019). Furthermore, *M. avium* is an opportunistic intracellular pathogen that can survive inside macrophages and be resilient against the hosts immune defences (Schorey & Sweet, 2008). Together with other non-tuberculous mycobacterial species, such as

Mycobacterium chimaera and *Mycobacterium intracellulare*, *M. avium* forms a complex known as the *M. avium* complex (MAC) (Falkinham, 2009). MAC infections are relatively rare and commonly occur in the respiratory tract with diseases related to cavitory, pulmonary, bronchiectatic and pneumatic symptoms. Symptomatology ranges from dyspnoea, cough, malaise to weakness, and occasionally haemoptysis (Meier et al., 2017).

Another OPP responsible for water-related illnesses is *Legionella*, a microorganism that is responsible for respiratory infections in humans, such as Pontiac fever and Legionnaires' disease, which are the result of aerosol inhalation in contaminated water sources containing these microorganisms (Blatt et al., 1993). Person-to-person transmission is rare, with only one case reported with probable transmission between two humans (Correia et al., 2016). Hence, Legionnaires' disease consists in pneumonia that includes symptoms such as diarrhoea, fever, delirium, myalgia and non-productive cough. However, there are no exclusive symptoms associated with this disease and some patients will not develop symptoms when exposed to the etiological agent (Kanarek et al., 2022). Legionnaires' disease can lead to death, with respiratory and organ failure (Pepper & Gerba, 2015). On the other hand, Pontiac fever is characterized by a shorter incubation period (Winn, 2015) and unlike Legionnaires' disease, it is not associated with pneumonia and patients recover quite rapidly, in few days. Despite this fact, Pontiac fever affects 100 % of the individuals exposed to the etiological agent. Usually, mortality is not associated with Pontiac fever epidemics and complications rarely occur (Bennett et al., 2015).

As it was said above, *Legionella* is known for biofilm production. When growing in biofilm, bacteria attach themselves to a matrix made from extracellular polymeric substances (EPS), produced by them (Rumbaugh & Sauer, 2020). In *Legionella*'s case, the biofilms produced are composed of several different species, including protozoa, the natural host for this microorganism, therefore fuelling its growth (Murga et al., 2001).

Besides the waterborne microorganisms referred above, other microorganisms may be transmitted through drinking water. Yet, evidence confirming this infection route is either lacking or inconclusive. Such bacteria include *Staphylococcus* and *Pseudomonas*, both with an important impact on human health (WHO, 2022).

For example, *P. aeruginosa*, an opportunistic pathogen, is usually detected in drinking water (Lu et al., 2016; Vaz-Moreira et al., 2012) causing chronic and acute infections, mainly in immunocompromised individuals, that may affect the respiratory and urinary tract or soft tissues (Tümmler & Klockgether, 2017). Moreover, in the last few

years there has been an emergence in carbapenem-resistant strains of *P. aeruginosa*, which exacerbates the importance of detection methods for these microorganisms (Liu et al., 2022; Wang et al., 2021) that possess a high mortality and morbidity rate (Cassini et al., 2019).

Regarding *Staphylococcus* spp., these bacteria constitute an innocuous colonizer and commensal of human skin (Severn et al., 2022). Despite that, they are capable of infecting almost any tissue of the human body, being responsible for several infections such as endocarditis and septicaemia (David & Daum, 2010). In this genus, it is important to highlight *Staphylococcus aureus*, a human pathogen responsible for nosocomial and community-based infections such as osteomyelitis and pneumonia (Kang et al., 2017; Tong et al., 2015). Furthermore, *S. aureus* is resistant to multiple antibiotics, with a focus on methicillin-resistant *S. aureus* (MRSA). MRSA are associated with skin and soft tissues infections and sepsis with the possibility of being fatal since infections caused by MRSA have a 50 % mortality rate (Klevens et al., 2007; Qiu et al., 2021).

1.4. Microbiological water quality legislation

Legislation for water for human consumption in Europe started with the Council Directive 80/778/EEC of 15 July 1980, the first drinking water directive by the European Communities. This official document intended to establish safe and equal access to drinking water while avoiding water contamination to assure human health (Council Directive 80/778/EEC of 15 July 1980, 1980).

According to Portuguese law, water considered to be suitable for human consumption comprehends all water in its original state or after undergoing treatment that is used for cooking, food handling or drinking, used for personal hygiene and other domestic purposes regardless of water source or distribution method (Decreto-Lei No. 69/2023, 2023). Safe drinking water is not only defined by the lack of contamination but also by the presence of certain minerals and other substances naturally present in water (2020/2184 EU Directive, 2020). Other important documents regarding drinking water in Portuguese law include Decreto-lei nº 306/2007 de 27 de agosto, which establishes certain criteria and guidelines for drinking water quality. This document would be overwritten on January 1st of 2018 by Decreto-lei nº 152/2017 de 7 de dezembro. The update brought a new perspective regarding the monitorization of drinking water quality

parameters, offering more affordable prices without compromising human health (Decreto-Lei No. 152/2017, 2017).

Hence, drinking water quality is evaluated by fixed values for quality parameters set out in the current legislation. Parameters can be considered to be either obligatory or indicators. Obligatory parameters include chemical and microbiological characteristics that cannot surpass the values fixed by law for safe drinking water. Values considered acceptable for safe drinking water regarding microbiological parameters are shown in Decreto-Lei nº. 152/2017 and Decreto-Lei nº. 69/2023 (Decreto-Lei No. 69/2023, 2023; Decreto-Lei No. 152/2017, 2017). This information can be observed in Table 4 in more detail.

Regarding water treatment and monitorization of *Legionella*, Lei nº. 52/2018 addresses Legionnaires' disease prevention and control, referring procedures, systems and equipment that may assist in *Legionella*'s propagation and dissemination and setting prevention strategies (Lei No. 52/2018, 2018). This law was later updated in Lei nº. 40/2019, that in addition to setting a control and prevention regime for these infections also allows for environmental health technicians to perform water and biofilm sample collection (Lei No. 40/2019, 2019). Risk classification and minimum necessary measures that need to be implemented according to contamination and dissemination risk are established by Portaria nº. 25/2021 (Portaria No. 25/2021, 2021).

In Portugal, ERSAR is responsible for water sanitation and urban residues regulation as well as the regulation of public services for water supply, proceeding with surveillance of water management entities according to the Decreto-Lei nº306/2007 de 27 de agosto. As it was mentioned above, this edict was later updated by Decreto-Lei nº152/2017 de 7 de dezembro providing insight concerning drinking water quality, avoiding water contamination to protect human health and presenting universal access to safe drinking water (Martins et al., 2023). Similarly, ERSAR monitors drinking tap water quality according to the Water Quality Control Programme (Programas de Controlo da Qualidade da Água - PCQA). The periodicity between drinking water analyses is defined according to biological, chemical and organoleptic water characteristics in this programme. PCQA topics include water treatment, origin and volume, as well as population features in that supply zone and the entity responsible for performing water quality analyses (Decreto-Lei No. 69/2023, 2023).

As an example, ERSAR reported 50 cases of non-compliance for water quality in 2022. Of these situations, 24 were regarding problems related to pH, 10 were related to coliform levels, 6 to *E. coli* and 3 to enterococci (Martins et al., 2023).

Table 4 – Microbiological parameters and corresponding parametric values for safe drinking water. Adapted from Decreto-Lei No. 69/2023, (2023) and Decreto-Lei No. 152/2017, (2017).

Parameters	Parametric value	Unit
<i>E. coli</i>	0	Number/100 mL
<i>Enterococcus</i>	0	Number/100 mL
<i>P. aeruginosa</i>	0	Number/100 mL
<i>C. perfringens</i>	0	Number/100 mL
Culturable microorganisms at 22 °C	No abnormal changes	Number/mL
Culturable microorganisms at 37 °C	No abnormal changes	Number/mL
<i>Legionella</i> spp.	< 1000	Colony Forming Units (CFU)/L

1.5. Laboratory Quality Management System

Laboratory quality is important as it impacts consistency and accuracy of test results. Despite always possessing a certain level of inaccuracy, microbiological assay methods should guarantee the maximum level of meticulousness in their results. Since errors regarding quality control may have notable negative consequences, namely treatment complications or unnecessary treatment due to false positive or false negative results or even result delay. These consequences ultimately affect cost and time efficiency. To minimize laboratory errors, all procedures and processes should be executed in a way that affords the best laboratory performance. This is not an easy progression route, since laboratories consist in complex systems that implicate several professionals and steps (Carey, 2018; WHO, 2011a).

Therefore, quality management systems should be employed. These systems are defined by the International Organization for Standardization (ISO) as coordinated activities that are used to achieve quality in laboratories, by controlling and focusing organizations. This way, quality assurance occurs along the whole analytical process as each step has associated errors. Elements monitored include corporation structure and management in general, professional personnel, equipment, reagents and inventory

overseeing, record and information administration and most importantly, process control. With the latter requiring a dedicated quality control throughout testing procedures, including sample handling and collection, method validation and verification (ISO 9001, 2015).

Furthermore, another way of guaranteeing and manage quality is by acquiring accreditations. Accreditation consists in recognition and evaluation of technical competences for certain activities and represents a way to determine laboratory competence, being provided by external competent entities, namely IPAC in Portugal. For laboratories, conformity evaluation is put in course for assays, calibrations and clinical tests. Besides, the major purpose of accreditation is to gain the client's trust in analytical processes that have been evaluated concerning technical proficiency and are recognized according to international standards (IPAC, 2022).

1.6. Internship scope

This curricular internship was carried out in the Microbiology Department of the ALS organic unit located in Porto city, Portugal. It focused on microbiological water analyses, specifically drinking water with an emphasis on *Legionella* detection and enumeration methods. More than ever, drinking water quality presents a challenging objective to achieve worldwide. This is mainly due to progressively older societies with increasing numbers of immunocompromised individuals, but also changes in environmental conditions provoked by global warming that contribute to increasing microbiological growth. Consequently, it is important to ensure safe drinking water access, a basic human right. To accomplish that, it is necessary to carry out routine water quality analyses.

2. Testing procedures

2.1. Water sample handling and general considerations

Water sample collection for microbiological analyses must be performed in sterilized flasks. This way, flasks obtained by ALS are already sterile and prepared accordingly when acquired from the supplier. These containers may possess neutralizing agents that can act against disinfectants used in water treatments.

After being collected in sterile flasks, water samples should be analysed and processed in the same day, unless there are schedule constraints that do not allow for handling. If these situations occur, samples should be stored in refrigeration temperature at 5 ± 3 °C.

Regarding preparation, testing of the most contaminated samples should be conducted after all the other samples to avoid contamination. While preparing for inoculation or filtration, water bottles need to be vigorously stirred with fast inversion movements, allowing for even microorganism distribution. If correct stirring is not possible due to water overfilling, bottles must be opened to discard the excess water.

When inoculating by culture medium incorporation, samples should always include at least two dilutions. Decimal dilution preparation is carried out in 9 mL of buffered peptone water (BPW) and incorporation occurs by adding 18 ± 2 mL of Yeast Extract Agar to Petri dishes where the samples have been inoculated. All culture media are kept in a water bath at 45 ± 1 °C to achieve the right inoculation temperature without media solidification.

Furthermore, several water analyses techniques include the membrane filtration method. This practice requires numerous preparation steps. Firstly, it is necessary to sterilize filtration ramps with a 96 % ethanol solution and drying the stations with a heat gun for 30 s with the vacuum pump on and then let sterile water flow in the system to cool the filtration surface and finally turning off the system and pump (Figure 1).



Figure 1 - Filtration ramp sterilization with a heat gun.

Afterwards, a sterile water membrane is positioned in each of the filtration stations with the help of sterile tweezers and a filtration cup is set on top of the membrane base. The final setup can be observed in Fig. 2.

The same cup can be used for the same sample. However, if each parameter requires a different amount of water to be filtered, it is recommended to initiate testing by the parameters that require less water or by higher dilutions. After finishing the filtration process, the membrane can be transferred by removing the filtration cup and with the help of sterile tweezers. If the membrane is then directly placed in culture media, it should be placed against it without bubble formation between the culture medium and the membrane. The filtration ramp must be sterilized with a heat gun between different samples. After filtration has ended, the equipment must be cleaned, the ramp discs are removed and deposited in a container with a 70 % ethanol solution. These procedures are executed according to ISO 8199 for water quality and general guidance and requirements for microbiological test procedures (ISO 8199, 2018).



Figure 2 – Final filtration ramp setup with water samples collected in sterile flasks ready to be processed by the membrane filtration method.

2.2. Culture media used for common waterborne pathogens

To study microbiological growth, it is necessary to use adequate culture media according to the organisms' nutritional requirements. Likewise, culture media can be categorized according to its functional objectives into the following groups: non-selective, selective, differential and enrichment culture media. Therefore, selective media are utilized to isolate specific groups of microorganisms, while inhibiting other microorganisms' growth by employing chemicals, antimicrobials or other substances. On the other hand, non-selective media allow for any microorganism present in biological matrices to grow. Differential media make the distinction between several groups of microorganisms in the same inoculum possible by using dyes, chemicals or other elements such as salts. Finally, enrichment media are employed to emphasize certain microorganisms' growth in matrices with a low microbial load (Bonnet et al., 2020).

Most of the culture media used by ALS Porto laboratory arrive from an external supplier ready to be used or is prepared by ALS Portugal headquarters in Tondela. However, Chromogenic Coliform Agar (CCA) and Slanetz and Bartley Agar are prepared in the Porto laboratory. In the following chapters the culture media employed for the detection of waterborne pathogens are described in more detail.

2.2.1. Tryptic Soy Agar (TSA)

TSA is a nutritive, non-selective nutritive culture medium that can be applied to either fastidious or non-fastidious and aerobic or anaerobic microorganisms. Therefore, TSA main use is work culture maintenance and subculturing in confirmation tests, while being considered a reference testing medium (Zimbro et al., 2009). Further details on culture medium composition can be observed in Supplementary Table 2.

2.2.2. Plate Count Agar (PCA)

PCA is a solid, nutritive, non-selective culture medium used as a general plating medium for certain bacterial population detection and for enumerating aerobic bacteria populations in water samples. Some of its components include tryptone and yeast extract, required by a wide range of microorganisms and spent as nitrogen sources (Corry et al., 2011). Further details on culture medium composition can be observed in Supplementary Table 2.

2.2.3. Yeast Extract Agar

Yeast extract Agar is a nutritive, non-selective culture medium employed in microorganism enumeration at 36 and 22 °C in water samples. The utilization of this medium allows for water treatment efficiency evaluation, particularly in water intended for human consumption. Moreover, Yeast Extract Agar is a variation of the PCA medium that does not contain glucose (Corry et al., 2011). Further details on culture medium composition can be observed in Supplementary Table 2.

2.2.4. Lowenstein-Jensen Agar

Lowenstein-Jensen Agar is a selective culture medium for *Mycobacterium* spp. that reduces contamination in mycobacterial samples since it contains malachite green, a compound known for its antimicrobial properties (Kassaza et al., 2014). Furthermore,

Lowenstein-Jensen Agar contains several antimicrobial compounds in its composition to limit contamination by other microorganisms, namely cycloheximide and lincomycin. Medium formulation contains homogenized egg, an ingredient that provides fatty acids, protein and nitrogen to the bacteria and allows for the growth of a wide range of mycobacteria. Another component of this medium is glycerol, a carbon source that favours mycobacterial growth (Schaefer, 1952). Culture medium composition information can be visualized in Supplementary Table 3.

2.2.5. Selenite Cystine Broth

Selenite Cystine Broth is a *Salmonella* spp. selective enrichment culture medium. Since *Salmonella* can be present in small amounts and detection can be hindered due to the interference of other microorganisms present in samples, Selenite Cystine Broth is formulated in a way to favour this microorganism growth. One component of the medium, sodium selenite, inhibits the growth of gram-positive bacteria and most enteric gram-negative bacteria without impairing *Salmonella* growth (Difco Laboratories, 1998; Zimbro et al., 2009). Further details can be observed in Supplementary Table 4.

2.2.6. Rappaport-Vassiliadis (RSV) Broth

RSV Broth is a liquid selective enrichment culture medium intended for *Salmonella* spp. detection. Aiming at inhibiting the growth of other microorganisms, this medium contains malachite green and magnesium chloride, two growth inhibitors. While magnesium chloride augments medium osmotic pressure, malachite green hinders the growth of non-salmonellae microorganisms. With these conditions gathered, plus lower medium pH, a selection of highly resistant *Salmonella* is obtained (Zimbro et al., 2009). Further details can be observed in Supplementary Table 5.

2.2.7. Chrom ID *Salmonella* Agar

Chrom ID *Salmonella* Agar is a differential and selective culture medium for isolation of presumptive *Salmonella* spp.. This medium contains chromogenic substrates

that allow for species distinction based on esterase enzymatic activity, with *Salmonella* colonies displaying a mauve colour that may vary from pink to purple and other microorganisms showing colourless or blue to green coloured colonies. Besides, Chrom ID *Salmonella* Agar includes several antimicrobial agents in its composition to inhibit other microorganisms (Difco Laboratories, 1998). More details on culture medium composition can be observed in Supplementary Table 6.

2.2.8. Xylose Lysine Desoxycholate (XLD) Agar

XLD Agar is a differential and selective culture medium specially designated for the differentiation and isolation of enteric bacteria. Sodium desoxycholate is implemented in its formulation as a selective agent inhibiting gram-positive bacteria growth. Xylose is an ingredient fermented by almost all enteric bacteria and lysine differentiates *Salmonella* species that perform lysine decarboxylation to cadaverine, turning medium pH to alkaline. With culture medium alkalinization, *Salmonella* can demonstrate a purple shade around the colonies.

Other XLD Agar elements include sodium thiosulfate, a sulphur source and ferric ammonium citrate an indicator. With this conjugation it is possible to observe colonies with black centers due to hydrogen sulphide production that causes the formation of a black iron sulphide precipitate in the colonies (Corry et al., 2011). Further information on culture medium composition can be observed in Supplementary Table 7.

2.2.9. Urea Agar

Urea Agar is a selective and differential medium used for distinguishing enterobacteria based on urea hydrolysis. It is specifically useful for the distinction between *Salmonella* spp. and *Proteus* spp.. The main ingredient, urea, is added as a nitrogen source for urease activity detection. Microorganisms capable of hydrolysing urea with ammonia formation increase medium pH that then changes colour from light yellow to pink red. Fluctuations in pH are visible due to phenol red, a pH indicator (Zimbro et al., 2009). Further details on culture medium composition can be observed in Supplementary Table 8.

2.2.10. L-lysine decarboxylation medium

This medium concept is based on the ability of certain bacteria, such as *Salmonella*, to hydrolize L-lysine to cadaverine. When a positive reaction is displayed cadaverine is created, turning the pH of the medium to alkaline. This outcome is visible by medium coloration change from yellow to purple and thanks to the pH indicator bromocresol (Collee et al., 1996). Culture medium composition data can be observed in Supplementary Table 10.

2.2.11. Cetrimide (CN) Agar

CN Agar is a *P. aeruginosa* selective culture medium containing magnesium chloride and potassium sulphate, which stimulate pigment production by *P. aeruginosa*. This species is known for pyocyanin (blue green), fluorescein (yellow green), pyorubrin (red brown) and pyomelanin (brown black) expression, pigments usually related to virulence, quorum sensing or other phenomena such as iron acquisition (Kothari et al., 2022). Other *Pseudomonas* species or even other gram-negative rod-shaped bacteria are unable to produce pyocyanin. In addition, the selective agent used is cetrimide, a compound that causes phosphorus and nitrogen release from other microbial cells (Corry et al., 2011; Difco Laboratories, 1998; Zimbardo et al., 2009). Further details on culture medium composition can be observed in Supplementary Table 11.

2.2.12. Acetamide Broth

Acetamide Broth is a differential liquid culture medium used to identify non-fermentative gram-negative bacteria, particularly, *P. aeruginosa*. This microorganism is capable of deaminating acetamide while producing ammonia. When this reaction is present in the Acetamide Broth after adding Nessler's reagent, positive reactions are observed when the medium changes colour to orange or brick red due to its interaction with ammonia (Zimbardo et al., 2009). Details on culture medium composition can be observed in Supplementary Table 12.

2.2.13. King B Agar

King B Agar is a solid culture medium used for confirmation and identification of *P. aeruginosa* based on fluorescein production. Fluorescein is a green to yellow pigment that possesses fluorescence and is insoluble in chloroform unlike pyocyanin. Other ingredients comprise glycerol, dipotassium phosphate and magnesium sulphate which enhance pigment production. Furthermore, King B Agar does not contain any added iron, a condition that enhances pigment production, since pigments are related to iron uptake systems (Vindeirinho et al., 2021). Further details on culture medium composition can be observed in Supplementary Table 13.

2.2.14. MacConkey Agar

MacConkey Agar is a differential and selective medium for lactose fermenting and non-fermenting gram-negative enteric bacteria. In its composition this medium contains bile salts and crystal violet, two gram-positive bacteria inhibitors that enhance gram-negative bacteria growth. When coliform bacteria are present in MacConkey Agar, they display a pink to red pigmentation possibly with a bile precipitate shade around colonies that indicates medium acidification due to lactose fermentation. Other non-target bacteria are colourless (Corry et al., 2011; Zimbro et al., 2009). Culture medium composition can be observed in Supplementary Table 14.

2.2.15. Triple Sugar Iron (TSI) Agar

TSI Agar is a culture medium used to differentiate gram-negative enteric bacilli. The procedure principle is based on carbohydrate fermentation and hydrogen sulphide production. This medium contains three sugars: sucrose, glucose and lactose. So, when these carbohydrates are fermented there is gas formation and changes in the medium coloration from red to yellow, due to medium acidification perceived by phenol red, a pH indicator. Besides, hydrogen sulphide production is detected by a black precipitate resulting from thiosulfate reduction (Difco Laboratories, 1998; Zimbro et al., 2009). TSI Agar result interpretation can be observed in more detail in Table 5. Culture medium composition is detailed in Supplementary Table 9.

Table 5 - TSI Agar medium changes interpretation. Adapted from ISO 19250, (2010).

Culture medium modifications	Result interpretation
Butt	
Yellow	Medium acidification due to glucose fermentation
Red or no colour changes	Alkaline pH. No carbohydrate fermentation
Black	Hydrogen sulphide formation
Bubbles or cracks	Gas formation from glucose
Slant	
Yellow	Lactose and/or sucrose fermentation
Red or no colour changes	Lack of lactose and sucrose fermentation

2.2.16. CCA culture medium

CCA is a differential and selective culture medium employed for *E. coli* and total coliforms enumeration, specifically in water samples containing a low microbial load. CCA action principle consists of distinguishing enzymatic activity for β -galactosidase and β -glucuronidase. While coliforms possess β -galactosidase and hence are pink to red in colour, *E. coli* owns both β -galactosidase and β -glucuronidase which translates in a dark blue to violet pigmentation (Turner et al., 2000). Culture medium composition can be observed in Supplementary Table 15.

2.2.17. Lactose Tergitol 7 Agar

This culture medium can differentiate and select coliforms present in water samples. Lactose fermentation is visible by colour change of the culture medium due to the pH indicator bromothymol blue.

Faecal coliforms can be identified by the presence of tri-phenyltetrazolium chloride (TTC) in the medium that is reduced to formazan, an insoluble red pigment. TTC is a growth indicator since most lactose fermenting organisms are capable of reducing it to formazan, except for *E. coli*, and certain species of *Enterobacter*. Tergitol 7 is the

selective agent, inhibiting gram-positive bacterial growth and preventing *Proteus* swarming. This way, it is possible to identify presumptive faecal coliform colonies by their yellow to amber or red colonies (Difco Laboratories, 1998). Further information on culture medium composition can be observed in Supplementary Table 16.

2.2.18. Bile-Aesculin-Azide Agar

Bile-Aesculin-Azide Agar is a differential and selective culture medium for intestinal enterococci. Bile is added as an ingredient to suppress other gram-positive bacteria growth, while azide inhibits the growth of gram-negative bacteria. Positive reactions consist in aesculin hydrolyzation to aesculetin and dextrose. The attained aesculetin reacts with the ferric citrate present in the culture medium forming a dark brown to black area (Corry et al., 2011). Further information on culture medium composition can be observed in Supplementary Table 18.

2.2.19. Slanetz and Bartley Agar

Slanetz and Bartley Agar is a selective culture medium intended for intestinal enterococci detection and enumeration present in water samples by the membrane filtration method. This way, sodium azide hinders gram-negative bacterial growth and TTC is a growth indicator that is reduced to insoluble formazan resulting in pink and red to brown coloration of the enterococci colonies (Corry et al., 2011; Difco Laboratories, 1998; Zimbro et al., 2009). More details on culture medium composition can be observed in Supplementary Table 17.

2.2.20. Iron Sulphite Agar (ISA)

ISA is a selective culture medium for thermophilic anaerobic bacteria detection and enumeration. The presence of sulphite-reducing bacteria is observed by black colony formation. These colonies reduce sulphite-producing sulphide, which in turn reacts with iron (III) citrate causing the formation of a black precipitate (Corry et al., 2011).

Further details on culture medium composition can be observed in Supplementary Table 19.

2.2.21. Tryptose Sulphite Cycloserine (TSC) Agar

TSC Agar supplemented with D-cycloserine is a selective culture medium for *C. perfringens* enumeration. Overall, TSC Agar represents the most well-suited culture medium for *C. perfringens* recovery since it is capable of inhibiting facultative anaerobe growth. Furthermore, this culture medium contains sodium disulphite, a sulphite source that is reduced to sulphide by presumptive *C. perfringens* colonies with a resulting black coloration from interaction with a ferric salt. The supplement added, D-cycloserine is an antibiotic capable of preventing interfering flora development and is responsible for a smaller size in colonies (Corry et al., 2011; Difco Laboratories, 1998). Culture medium composition details can be visualized in Supplementary Table 19.

2.2.22. Mannitol Salt Agar

Mannitol Salt Agar is a selective and differential culture medium for total staphylococci enumeration and detection, preventing other bacterial growth with high salinity. Sodium chloride in conjugation with the phenol red pH indicator facilitates the distinction between pathogenic coagulase-positive staphylococci colonies, that present themselves as being yellow and staining the surrounding medium due to acidification and smaller white coagulase-negative staphylococci colonies, that do not acidify the medium. Mannitol is only fermented by pathogenic coagulase-positive staphylococci (Corry et al., 2011; Difco Laboratories, 1998; Zimbro et al., 2009). Culture medium composition can be explored in Supplementary Table 20.

2.2.23. MEVAG (Milieu d'Étude de la Voie d'Attaque des Glucides/Medium for Studying the Path of Carbohydrate Attack) Agar

MEVAG Agar is a medium used for respiratory type determination, allowing for discrimination between oxidative and fermentative pathways, during total staphylococci detection and enumeration confirmation tests. High carbohydrate concentrations present in MEVAG Agar enable fermentation with medium acidification that results in colour change to yellow thanks to bromothymol blue, a pH indicator. Since staphylococci are facultative anaerobes, glucose fermentation should occur throughout the medium both anaerobically and aerobically and, therefore, the medium should be entirely yellow (Candeias, 2014). More information on culture medium composition is visible in Supplementary Table 21.

2.2.24. Baird Parker - Rabbit Plasma Fibrinogen (BP-RPF) Agar

BP-RPF Agar is a selective and differential medium for enumeration of coagulase-positive staphylococci. When rabbit plasma fibrinogen is added as a supplement to BP culture medium it is possible to proceed with enumeration and confirmation of presumptive colonies with only one step. What is more, BP-RPF Agar contains sodium pyruvate and glycine that improve staphylococci growth, with the latter also suppressing other microbial growth. Other components such as lithium chloride and potassium tellurite diminish the appearance of other microorganisms as well, acting as selective elements. With the employment of rabbit plasma, coagulase activity can be easily spotted by clot formation that causes halo development around presumptive colonies. Typical staphylococci colonies exhibit a black colour thanks to potassium tellurite hydrolysis (Corry et al., 2011; Zimbardo et al., 2009). Further details on culture medium and supplement composition and can be observed in Supplementary Table 22 and 23.

2.2.25. Buffered Charcoal Yeast Extract (BCYE) Agar

BCYE Agar is a selective medium for *Legionella* detection and enumeration, employed thinking about their specific growth requirements. With that in mind, BCYE Agar includes L-cysteine and ferric pyrophosphate in its formulation since these elements are essential for *Legionella* growth. While L-cysteine represents an important amino acid and energy source required as a sulphur source for metabolite biosynthesis

and enzyme functioning (Takagi & Ohtsu, 2017), iron is related to pathogenesis, oxygen metabolism and enzyme functioning (Cianciotto, 2015; Percival & Williams, 2013). Finally, activated charcoal is added to function as a detoxicant for toxic metabolism products (Corry et al., 2011; Zimbro et al., 2009). Further details on culture medium composition can be observed in Supplementary Table 24.

2.2.26. Glycine, Vancomycin, Polymyxin B, Cycloheximide (GVPC) Agar

This medium is an adaptation from BCYE Agar containing antimicrobials for better *Legionella* selection. It still retains BCYE main components namely, activated charcoal, L-cysteine and ferric pyrophosphate. Concerning antimicrobials, vancomycin is an antibiotic for gram-positive bacteria while polymyxin B and glycine are employed for gram-negative bacteria and cycloheximide is a fungicide (Corry et al., 2011). More details regarding composition can be observed in Supplementary Table 24.

2.3. Enumeration and detection methods

2.3.1. Culturable microorganisms' enumeration by inoculation at 22, 30, and 36 °C

Enumeration of culturable microorganisms at 22 and 37 °C is executed according to ISO 6222 while enumeration at 30 °C is executed according to ALS internal protocol. Culturable microorganisms are defined as all aerobic bacteria, yeasts and moulds that can establishing colonies in a certain non-selective culture medium in specified conditions. For total microorganism enumeration, sample preparation is accomplished as referred in the above chapters, dilutions are made accordingly and the pour plate method is performed according to ISO 8199 using Yeast Extract Agar. The time interval between dilution execution and plating must not exceed 15 min. Petri dishes are left to dry on a flat surface and then collected to be incubated at 22 ± 2 °C, 30 ± 2 °C for 68 ± 4 h and 36 ± 2 °C for 21 ± 3 h or 44 ± 4 h (ISO 6222, 1999).

2.3.1.1. Culturable microorganisms' enumeration results

The number of culturable microorganisms is given by the total amount of colonies present in the Petri dishes. Results are expressed in CFU/mL of sample volume, for each incubation temperature. If the plates that were inoculated with an undiluted portion of the sample do not present any colonies, then the results should be expressed as “no detection in one milliliter”. On the other hand, if there are more than 300 colonies on plates with the highest sample dilution, the results should be expressed as “> 300” or should be given as an approximate number (ISO 6222, 1999). Example results obtained in a drinking water sample can be observed in Fig. 3.



Figure 3 - Example results found for culturable microorganisms enumeration using Yeast Extract Agar.

2.3.2. *Mycobacterium* spp. detection

Mycobacterium is a genus constituted of immotile bacillary bacteria, which are also aerobic and incapable of producing spores. One of the most important characteristics of these bacteria is their resistance to discoloration when exposed to acid-alcohol and their fastidious growth, which can vary between 3 to 8 weeks. Additionally, as it was said above, mycobacteria are OPPs naturally present in aquatic environments, also associated with diseases such as tuberculosis, presenting a substantial importance in immunocompromised individuals (Ulmann et al., 2021). Furthermore, these

microorganisms are resistant to most common disinfectants, possessing a complex lipidic cell wall (Maitra et al., 2019).

This way, *Mycobacterium* spp. identification is performed by membrane filtration, where the obtained pellet undergoes decontamination treatment to eliminate other microorganisms and promote mycobacterial growth (Neumann et al., 1997; Ulmann et al., 2021).

Firstly, 5 mL of the water sample are placed in a sterile bottle and then 1 L of the sample is filtered using a 0.45 µm membrane, according to the proceedings described in other chapters. Then the membrane is transferred to the bottle containing the 5 mL of the sample and shaken during 20 min at 90 rpm. Meanwhile, 50 mg of L-cysteine chlorohydrate are dissolved in 10 mL of benzalkonium chloride. These two reagents are used as part of the chemical decontamination process since L-cysteine chlorohydrate is responsible for the digestion, emulsification and decontamination of mucous components and other naturally occurring aquatic microorganisms, while benzalkonium chloride is a common disinfectant used for its antimicrobial properties to which mycobacteria are resistant (Ulmann et al., 2021).

Following the 20 min incubation at 90 rpm, 5 mL of the mixture referred above are added to the flask containing the concentrated sample, which is stirred during 30 s in the vortex. Afterwards, the concentrate is left to rest during 30 min and 5 mL of phosphate buffer are then placed in this mixture, which is then centrifuged at 3000 rpm during 20 min. Phosphate buffer is used with the objective of neutralizing the sample after decontamination. Subsequently, the supernatant is discarded and 1 mL of bovine albumin is deposited in the sediment, letting it rest for 5 min. This step is important since bovine albumin is capable of stimulating mycobacterial growth (Mavi et al., 2023). Finally, the concentrated and decontaminated sample is inoculated in Lowenstein-Jensen Agar and incubated at 22 ± 2 °C and 36 ± 2 °C horizontally for 24 to 48 h with the tube cap unscrewed. After this period of time, the tubes are incubated vertically with the cap totally closed for 30 days.

2.3.2.1. *Mycobacterium* spp. confirmation tests and results

Regarding results obtained following incubation, suspicious bacterial colonies possess a pallid to strong yellow colour and are flat or cauliflower-shaped (Fig. 4 A and B). In case the culture medium changed colour to turquoise blue, this may indicate the

presence of other microorganisms. Suspicious colonies are assessed using the Ziehl-Neelsen stain method. This procedure is utilised for the detection of acid-fast bacteria with an emphasis on mycobacteria and consists in executing a smear in a slide and proceed to perform heat fixation, applying 1 to 2 mL of carbol-fuchsin for 5 min and then washing the slide with water. The slide is then covered with a decolourant for 15 min, this process is repeated until fuchsin is removed, then it is washed with water again and methylene blue reagent is applied for 2 min. Next, the reagent excess is removed with water and the slide is left to dry before advancing to microscopic observation of the Ziehl-Neelsen stain, where mycobacteria present themselves as being stained in red to purple (Fig. 4 C). The Ziehl-Neelsen stain method is employed since mycobacteria are acid-fast microorganisms, being resistant to discoloration when exposed to acid-alcohol. This happens due to the complexity of mycobacterial waxy lipidic cell walls. Other bacteria cannot retain fuchsin after applying a decolourant and appear stained in blue since methylene blue is employed afterwards, while mycobacteria retain the fuchsin and are stained in red to purple (Talbot & Raffa, 2015). To continue with the confirmation methods, presumptive colonies are sent to an external entity.

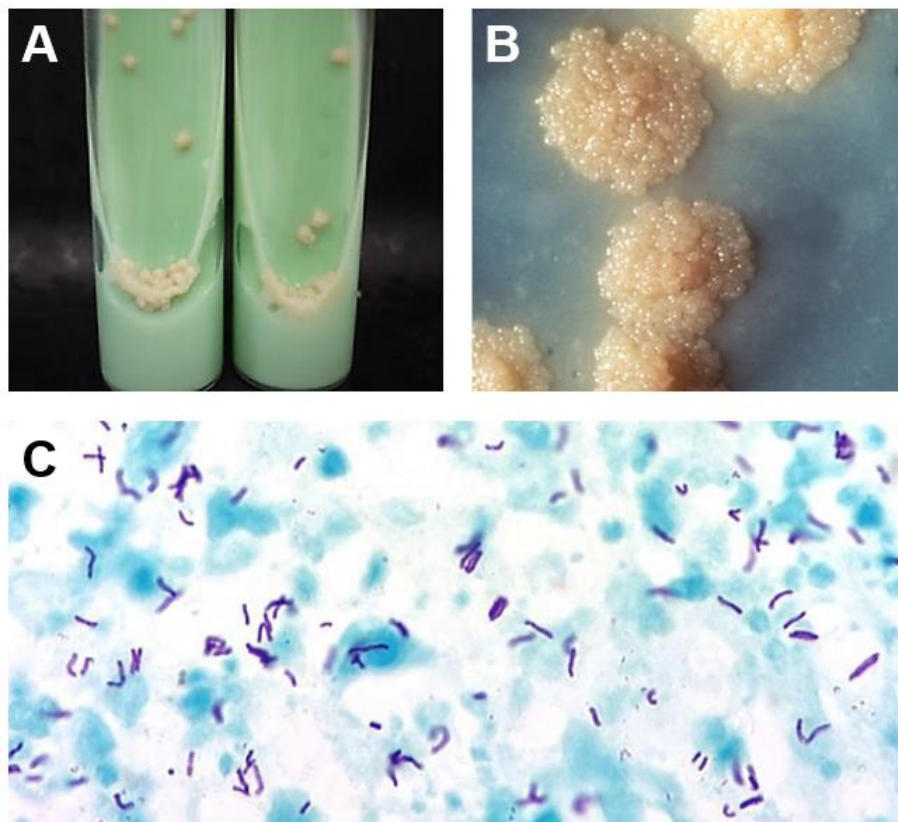


Figure 4 - Positive reactions for *Mycobacterium* spp. A) Typical pale yellow mycobacteria colonies on Lowenstein-Jensen Agar. B) Detail of characteristic cauliflower shaped mycobacterial colonies. C) Ziehl-Neelsen stain microscopic observation with purple acid-fast bacilli. Adapted from Agarwal et al., (2005), Centers for Disease Control and Prevention (CDC), (1976) and Vargas Rodríguez et al., (2023).

2.3.3. *Salmonella* spp. detection

The *Salmonella* spp. detection method used is based on ISO 19250 protocol. The technique employed is the membrane filtration method by filtering 1000 mL with a 0.45 µm pore membrane. To correctly identify the presence of *Salmonella* spp. it is usually necessary to proceed with a pre-enrichment since a low microbial load may remain undetected (Taskila et al., 2012). This pre-enrichment is performed with 50 mL of BPW, a non-selective enrichment liquid medium, with incubation of the inoculum at 36 ± 2 °C for 18 ± 2 h. Afterwards, an enrichment is achieved by utilising 10 mL of selective liquid medium, RSV Broth and Selenite Cystine Broth to where 0.1 mL of the previous inoculum is transferred. RSV Broth is incubated at 41.5 ± 1 °C for 48 ± 4 h, while Selenite Cystine Broth is incubated at 36 ± 1 °C for 21 ± 3 h. Then, the next step involves a recognition phase where 10 µL of the culture are cultivated in two selective solid media. The first culture medium to be used is XLD Agar and the second medium employed is chosen by the laboratory. In ALS Porto's Unit the chosen culture medium is Chrom ID *Salmonella* Agar. The incubation period for XLD Agar is 36 ± 2 °C for 24 ± 3 h while for Chrom ID *Salmonella* Agar is 36 ± 2 °C for 21 ± 3 h (ISO 19250, 2010).

2.3.3.1. *Salmonella* spp. confirmation tests and results

All purple colonies in Chrom ID *Salmonella* Agar and all pink colonies or colonies that possess yellow margins and/or with a black center in XLD Agar are to be considered presumptive (Fig. 5). At least one typical or presumptive *Salmonella* colony obtained from both media is then cultivated in PCA medium and incubated for 24 ± 3 h at 36 ± 2 °C for oxidase testing, with negative reactions expected since *Salmonella* spp. are oxidase negative. If after further confirmation steps the first colony is not considered *Salmonella*, then four more suspect or typical colonies need to be analysed.

Further biochemical analyses include testing on TSI Agar where one colony is inoculated in a tube containing this culture medium by streaking and stabbing the slant surface. The sample is then incubated at 36 ± 2 °C for 24 ± 3 h. Typical *Salmonella* colonies display red slants due to medium alkalization and yellow butts caused by acidification of the medium with gas formation and medium blackening due to hydrogen sulphide production (Fig. 6 A). When lactose-positive *Salmonella* spp. are present tube slants are yellow.



Figure 5 - Positive reactions in Chrom ID *Salmonella* Agar and XLD Agar. Typical *Salmonella* purple-coloured colonies on Chrom ID *Salmonella* Agar and characteristic black *Salmonella* colonies on XLD Agar.

If the previous test results turn out to be indicative of *Salmonella* presence, then confirmation proceeds to urea tests, where one colony is inoculated in Urea Agar and incubated at 36 ± 2 °C for 24 h. The results should be negative since *Salmonella* spp. cannot hydrolyze urea. When other microorganisms that can hydrolyze urea are present, ammonia liberation occurs and phenol red, present in the medium changes colour to rose pink and to deep cerise later on (Fig. 6 B).

After obtaining negative results for the urea test, one colony is then inoculated in L-lysine decarboxylation medium and incubation occurs at 36 ± 2 °C for 24 ± 3 h. Typical *Salmonella* cultures possess a purple colour due to lysine hydrolyzation to cadaverine (Fig. 6 A). Finally, serological tests are executed using *Salmonella* polyvalent antisera for serotyping according to flagellar antigens (Poly-H) (Fig. 7 A) or omnivalent antisera for somatic antigens (Omni-O) (Fig. 7 B). These tests can identify presumptive *Salmonella* colonies by agglutination (ISO 19250, 2010).

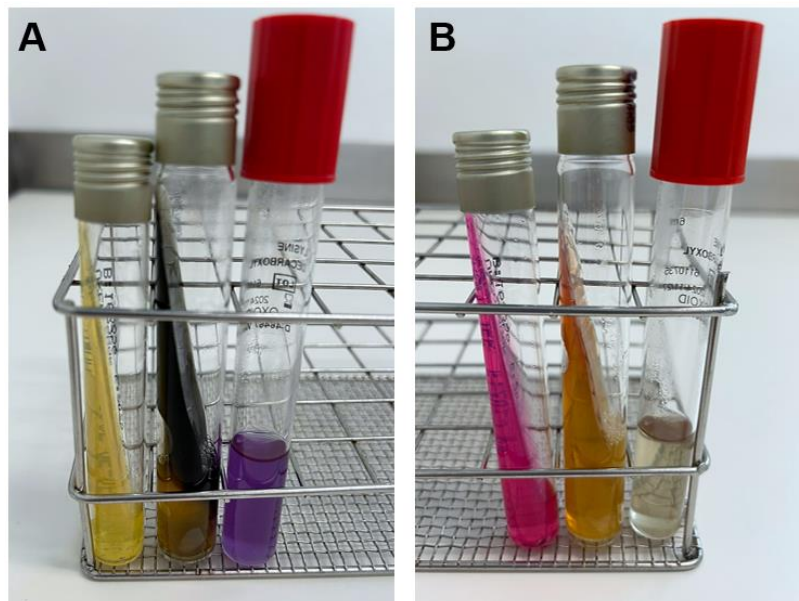


Figure 6 - Confirmatory test results for *Salmonella* spp. detection. A) Positive results for Urea Agar, TSI Agar and L-lysine decarboxylation medium, respectively. On the left side, a characteristic negative reaction for *Salmonella* in Urea Agar, demonstrating lack of medium colour modifications and hence no urease activity. In the middle, a typical TSI Agar reaction for *Salmonella* displaying a yellow butt, which indicates glucose fermentation and medium blackening, indicative of hydrogen sulphide formation. Finally, on the right side, a positive reaction for lysine decarboxylation is achieved by medium colour change to purple. B) Negative results for Urea Agar, TSI Agar and L-lysine decarboxylation medium, respectively. On the left side, there is coloration change to pink, indicative of urea hydrolysis. In the middle, the TSI Agar tube lacks any change in medium colour in the butt and presents a red slant, revealing lack of fermentation. Lastly, the L-lysine decarboxylation medium does not have colour alteration and, therefore, represents a negative reaction.

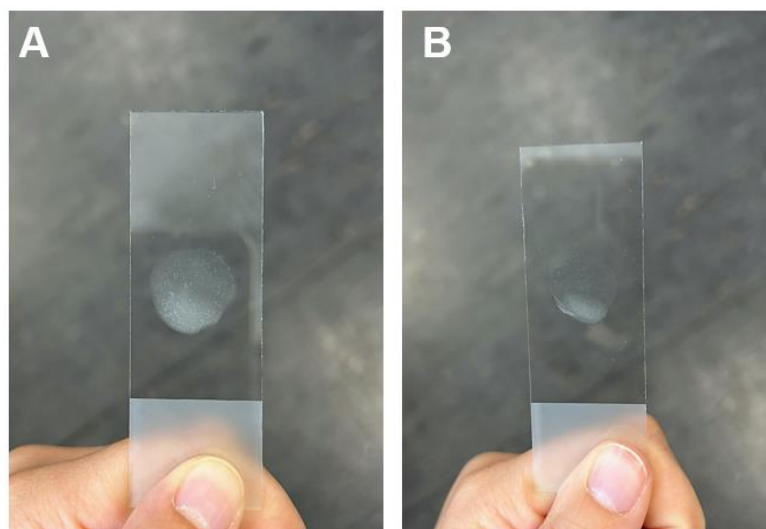


Figure 7 - *Salmonella* antisera positive results showing agglutination. A) Polyvalent antisera for flagellar antigen serotyping (Poly-H). B) Omnivalent antisera for somatic antigen serotyping (Omni-O).

2.3.4. *P. aeruginosa* detection and enumeration

P. aeruginosa detection method is described in ISO 16266. Therefore, the method employed is done by membrane filtration using a 0.45 µm pore membrane. Filtration volume is usually 100 mL, however, in some cases, such as bottled water volume may correspond to 250 mL. The membrane is then positioned in a Cetrimide (CN) Agar Petri dish and incubated at 36 ± 2 °C for 44 ± 4 h (ISO 16266, 2006).

2.3.4.1. *P. aeruginosa* confirmation tests and results

All colonies that present a green or blue colour can produce pyocyanin, a pigment produced by this microorganism and are, therefore, confirmed as *P. aeruginosa* (Fig. 8). When applying an ultraviolet (UV) light, fluorescent colonies are considered to be presumptive and other non-fluorescent red to brown colonies are also presumptive *P. aeruginosa* (Fig. 9). At least 10 colonies of those that advance to further confirmation methods are cultivated on PCA and incubated at 36 ± 2 °C for 22 ± 2 h, to obtain subcultures.

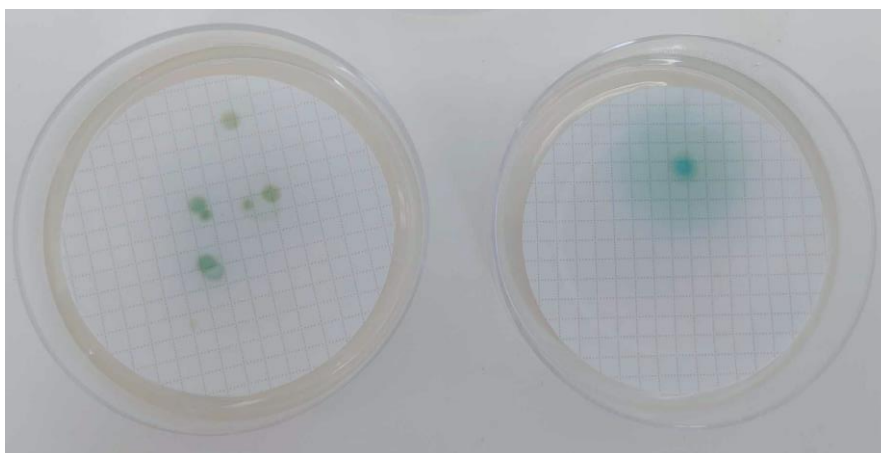


Figure 8 - CN Agar pyocyanin producing *P. aeruginosa* colonies.

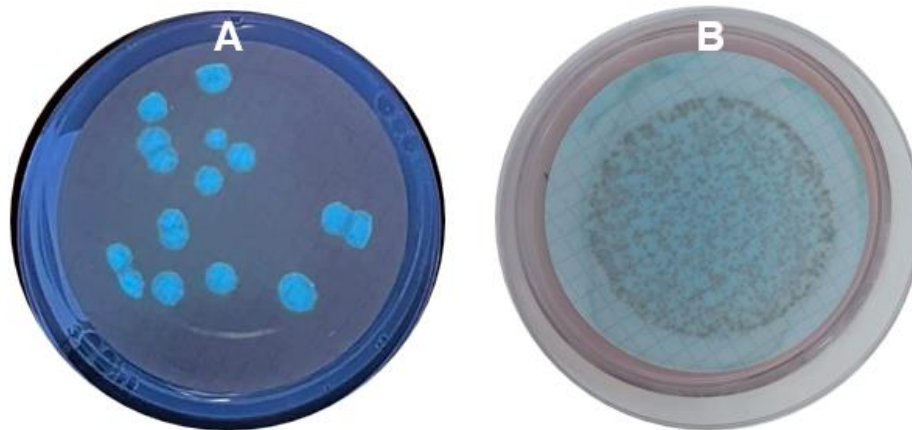


Figure 9 - Presumptive *P. aeruginosa* colonies on CN Agar. A) Fluorescent presumptive colonies under a UV light. B) Presumptive *P. aeruginosa* colonies in CN Agar with a brown coloration, indicative of pyrorubrin or pyromelanin production.

Then, fluorescent colonies are confirmed using Acetamide Broth and are incubated at 36 ± 2 °C for 22 ± 2 h. Afterwards, 1 or 2 droplets of Nessler's Reagent are pipetted into the Acetamide Broth. Ammonia production is shown by the colour that is obtained since Nessler's reagent reacts in the presence of ammonia. *P. aeruginosa* can produce ammonia from acetamide so positive results present a brick colour while negative reactions show a yellow coloration (Fig. 10).

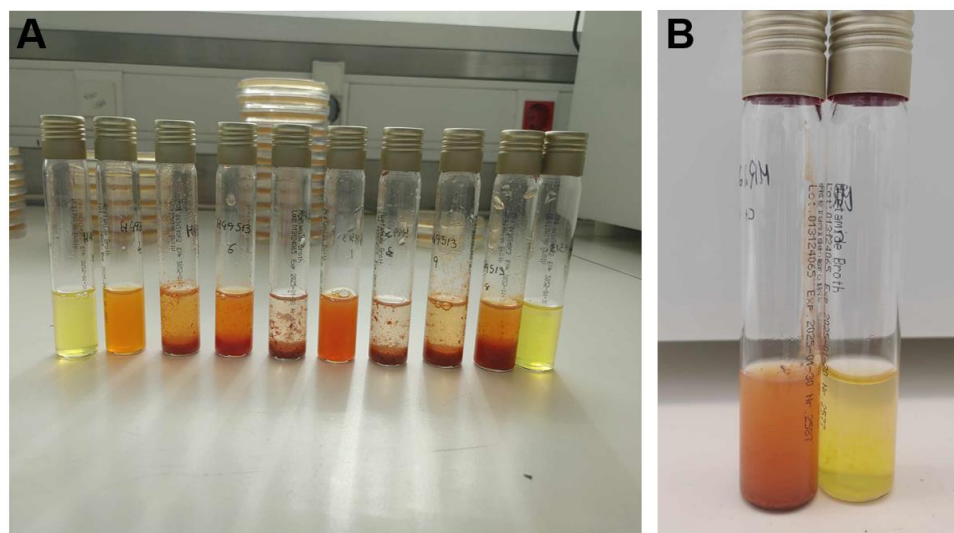


Figure 10 – A) Results for 10 presumptive *P. aeruginosa* colonies from a water sample after incubation in Acetamide Broth and adding Nessler's reagent. B) Negative control on the right side, presenting a yellow coloration (RM3) and positive control on the left side with a brick colour (RM26), indicative of ammonia production.

On the other hand, red to brown colonies present in CN Agar are confirmed by the oxidase test, where a positive reaction is obtained when there is a blueish to purple coloration in a 10 s period using a commercial kit (Fig. 11). This bacterium is oxidase

positive, and so colonies that present a positive result are subject to a fluorescence production test, where at least three colonies are cultivated on King B Agar and incubated at 36 ± 2 °C for 5 days with daily fluorescence examination utilizing a UV light (Fig. 12). Positive results are obtained for fluorescent samples. Results are given as the total number of characteristic colonies per sample volume (ISO 16266, 2006).



Figure 11 - Oxidase test results from PCA subcultures originating from CN Agar red to brown presumptive *P. aeruginosa* colonies. The swab from the commercial kit turned purple indicating that the corresponding colony is oxidase positive.

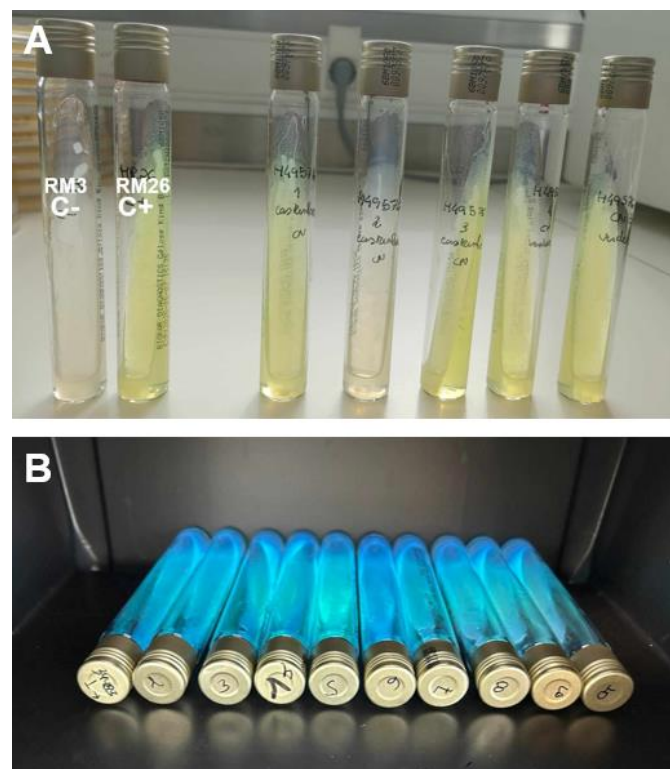


Figure 12 - Appearance of King B Agar tubes after incubation. A) On the left side of the image, it is possible to observe the negative control (C-) with a white colour (RM3) and the positive control (C+) exhibiting a greenish coloration (RM26). The remaining tubes correspond to 5 different colonies that displayed positive results in the oxidase test and were then subjected to inoculation in King B Agar. B) Positive reactions for King B Agar fluorescence test.

2.3.5. Enterobacteriaceae detection and enumeration

The detection and enumeration technique performed by ALS is based on an internal protocol. Water samples that require analyses of their enteric bacteria load are processed by the membrane filtration method. For this, at least 100 mL of sample are employed for filtration with a 0.45 µm pore filter that is set in a MacConkey Agar Petri dish and incubated at 36 ± 2 °C for 44 ± 4 h.

2.3.5.1. Enterobacteriaceae confirmation tests and results

After incubation, lactose-positive colonies present themselves as being pink to red (e.g. *E. coli*) while lactose-negative colonies are colourless (Fig. 13). At least five colonies must be cultivated in PCA Petri dishes and incubated at 36 ± 2 °C for 24 h.

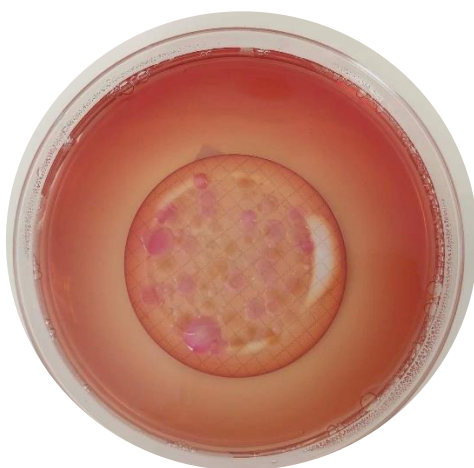


Figure 13 - Results for Enterobacteriaceae detection and enumeration in MacConkey Agar. It is possible to observe pink to red presumptive colonies.

Subsequently, an oxidase test is executed and oxidase-negative colonies continue in the next confirmation steps, being cultivated in TSI Agar with slant stabbing and incubated at 37 ± 1 °C for 24 ± 3 h. All colonies that ferment glucose, lactose or sucrose and therefore, change culture medium colour to yellow, are considered to be enterobacteria (Fig. 14). Results are given as “detected” or “not detected” by sample volume for detection analyses and expressed in CFU per sample volume.



Figure 14 - TSI Agar results for Enterobacteriaceae detection and enumeration. It is possible to observe a yellow slant, indicative of lactose and/or sucrose fermentation with bubble formation due to gas production and medium blackening revealing hydrogen sulphide formation.

2.3.6. Total coliform enumeration

The total coliform enumeration protocol utilized is more suitable for water containing a low microbial load that will result in the presence of less than 100 total colonies on CCA culture medium. These types of water include water destined for human consumption, since analyses to more heavily polluted water will translate in higher background growth that may interfere with reliable results due to the low selectivity of the differential culture medium employed.

The method applied to enumeration of these microorganisms can be consulted in ISO 9308-1 and is accomplished by membrane filtration with a 0.45 µm pore filter. Filtration volume is usually 100 mL, however, in some cases, such as bottled water (250 mL), other volumes may be filtered. Following filtration, the membrane filter is moved to a CCA plate and incubated at 36 ± 2 °C during 21 ± 3 h (ISO 9308-1, 2014).

2.3.6.1. Total coliform confirmation tests and results

Afterwards, examination of membrane filters is performed with colonies exhibiting a pink to red pigmentation being positive for β -D-galactosidase and consequently, presumptive coliform bacteria other than *E. coli*. On the other hand, colonies presenting a dark blue to violet coloration possess a positive reaction for β -D-galactosidase and β -D-glucuronidase and are considered to be *E. coli* (Fig. 15). At least 10 of the presumptive pink to red colonies follow further confirmation tests and subcultures are prepared using TSA culture medium and incubated at 36 ± 2 °C for 21 ± 3 h.

Next, oxidase tests are performed in isolated colonies and the expected results corresponds to absence of swab colour modification, since coliform bacteria are oxidase negative. Expression of results is given by the total number of coliform bacteria present in 100 mL of the water sample. This includes all dark blue to violet colonies and all oxidase negative pink to red colonies (ISO 9308-1, 2014).

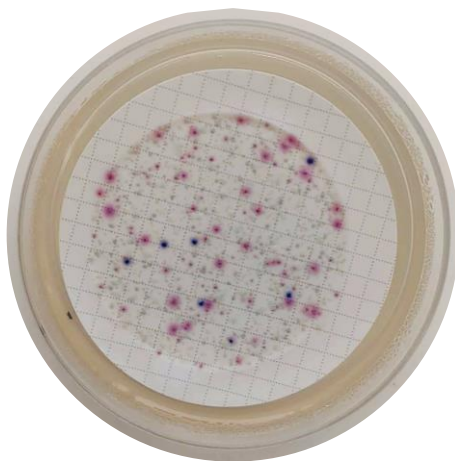


Figure 15 - CCA plate for total coliform detection and enumeration with both pink to red and dark blue colonies, suggesting *E. coli* (blue colonies) and coliform (pink to red colonies) presence.

2.3.7. Faecal coliform enumeration

Faecal coliform enumeration is performed using an internal protocol as reference. The method employed consists in membrane filtration with a $0.45 \mu\text{m}$ pore filter that is later set on a plate containing a selective culture medium, Lactose Tergitol 7 Agar. Incubation occurs at 44 ± 0.5 °C, with plate examination at 21 ± 3 h and 44 ± 4 h, since prolonging incubation until 44 ± 4 h may improve result sensibility for samples that do not present typical colonies after 21 ± 3 h.

2.3.7.1. Faecal coliform confirmation tests and results

Concerning plate examination, all colonies that stain the culture medium with a yellow pigmentation, regardless of shape and size, are considered to be presumptive faecal coliform bacteria, since that indicates lactose fermentation (Fig. 16). Subsequently, at least 10 typical yellow to amber and red colonies must be cultivated in PCA Petri dishes and incubated at 36 ± 2 °C for 21 ± 2 h. After subculture incubation, oxidase tests must be performed with an expected negative reaction since faecal coliforms are oxidase negative. The obtained results are expressed in CFU per sample volume.

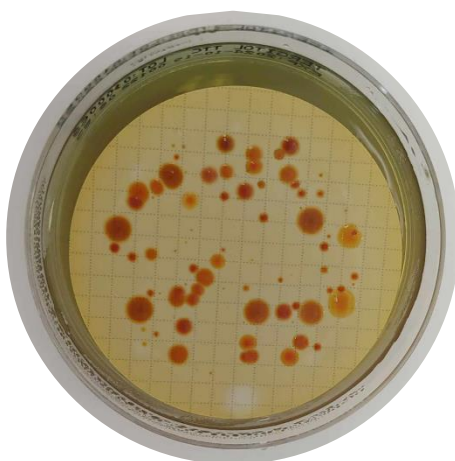


Figure 16 – Lactose Tergitol 7 Agar plate after incubation displaying yellow to amber and red presumptive colonies.

2.3.8. Intestinal Enterococci detection and enumeration

Detection and enumeration of intestinal enterococci in water samples is accomplished according to ISO 7899-2. The technique used is based on membrane filtration with a $0.45 \mu\text{m}$ pore membrane that is then set on a Slanetz and Bartley Agar Petri dish that incubates for 36 ± 2 °C during 44 ± 4 h (ISO 7899-2, 2000).

2.3.8.1. Intestinal Enterococci confirmation tests and results

Typical enterococci colonies are pink, red or brown in colour throughout all of the colony or only in the centre with a raised morphology in Slanetz and Bartley Agar (Fig. 17). Colonies with these characteristics undergo confirmatory steps where the membrane is transferred to a Bile-Aesculin-Azide Agar Petri dish along with the colonies with the help of sterile forceps. Petri dishes containing this culture medium must be preheated in advance at 44 °C. Incubation of the culture occurs at 44 ± 0.5 °C for 2 h. The results must be read immediately after the incubation period (ISO 7899-2, 2000). Typical enterococci colonies present a black colour in their surroundings and count as a positive reaction as shown in Fig. 18.

Result expression is reported according to ISO 8199 parameters for enumeration in selective culture media. This way, only characteristic colonies are counted and results are expressed following the formula below:

$$C_s = \frac{Z}{V_{tot}} \times V_s$$

Where:

- C_s is the estimated CFU number in a sample's reference volume (usually 100 mL);
- Z is the total sum of counted colonies observed in the plates or membranes of the corresponding sample;
- V_{tot} is the total tested sample volume in mL;
- V_s is the reference volume chosen to express microbial concentration.

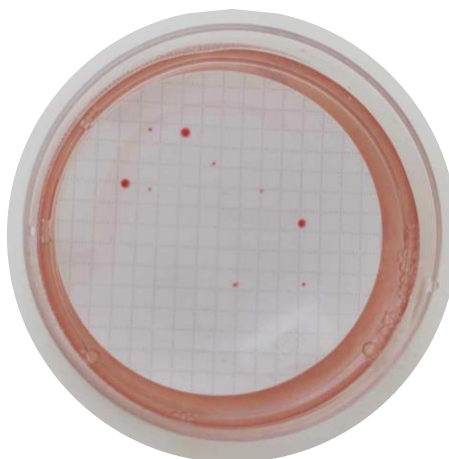


Figure 17 - Presumptive intestinal enterococci red colonies in Slanetz and Bartley Agar.

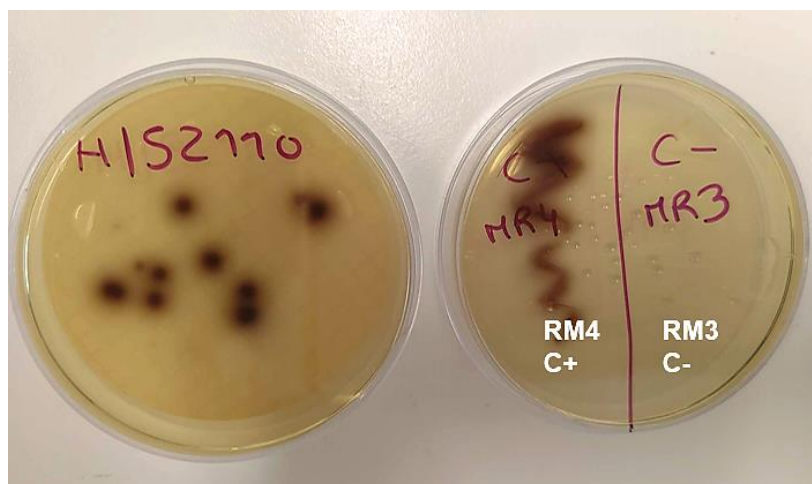


Figure 18 – Typical intestinal enterococci colonies in Bile-Aesculin-Azide Agar present in a water sample paired with the correspondent positive and negative controls for comparison. A) Positive reaction in a drinking water sample presenting a black surrounding area around colonies. B) A Petri dish was used for both assay controls with RM4 serving as the positive control (C+) on the left and RM3 as the negative control (C-) on the right side.

However, this formula is best applied to the membrane filtration method when the total number of colonies is between 10 and 100 for typical colonies found in selective media. When the plates show less than 10 colonies result accuracy decreases, especially if the number is between 1 to 3 colonies. In these situations, it should only be stated that enterococci are present in the sample. If the total colony number is equal or superior to four, then the formula above is still employed, despite the result being reported as an estimation. When no colonies are observed the results are given as “non-detected” per sample volume.

When the total amount of colonies surpasses 200 colonies and typical colony number is greater than 100, results are expressed as the total number of colonies per sample volume and number of typical colonies per sample volume (ISO 8199, 2018).

2.3.9. Clostridia detection and enumeration

The detection and enumeration method that is under operation for Clostridia is executed according to NP EN 26461-2 by membrane filtration with a 0.22 µm pore filter. This pore size is adequate to retain the spores produced by Clostridia. The necessary filtered sample volume corresponds to 100 mL for water that has low pollution levels such as drinking water. Before filtration, spore selection must be achieved. For that, water samples are heated in a water bath at 75 + 5 °C for 15 min and then the protocol

advances with sample filtration. Next, the membrane is transferred to a Petri dish containing ISA culture medium that is then incubated in anaerobiosis at 37 ± 1 °C for 20 ± 4 h and 44 ± 4 h (NP EN 26461-2, 1994).

2.3.9.1. Clostridia results

Presumptive colonies possess a black pigmentation in ISA culture medium (Fig. 19). Results for this method are conveyed according to ISO 8199 parameters for enumeration in selective culture media (ISO 8199, 2018). The formula and parameters used for the result statement are referred above in the intestinal enterococci detection and enumeration method.

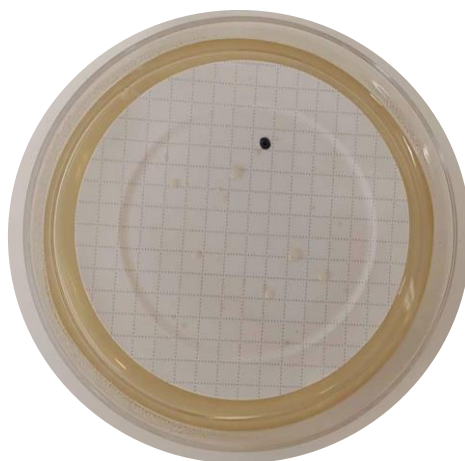


Figure 19 - Typical black coloured sulphite reducing Clostridia colony on ISA culture medium.

2.3.10. *C. perfringens* enumeration

The protocol used in *C. perfringens* detection is stated in ISO 14189 and consists of membrane filtration by a 0.45 µm pore filter. This pore size is adequate to retain this bacterium spores. It is necessary to emphasize that after sample collection, appropriate analyses are only possible for a maximum recommended period of 12 h for vegetative cells and 24 h for spores. Filtration volume varies according to the sample in question, with 100 mL being the volume required for water intended for human consumption. After filtration, there is membrane transfer to a Petri dish containing TSC Agar supplemented

with D-Cycloserine. Petri dishes are then incubated anaerobically at 44 ± 1 °C for 21 ± 3 h (ISO 14189, 2013).

2.3.10.1. *C. perfringens* confirmation tests and results

All colonies with a black or grey to yellow-brown pigmentation viewed either by the surface of the membrane or below its surface are considered presumptive *C. perfringens* colonies (Fig. 20). Enumeration must be achieved within 30 min after the anaerobic incubation since the black coloration fades rapidly.

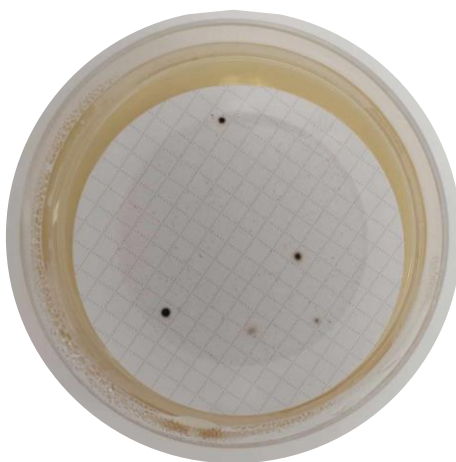


Figure 20 - TSC Agar results obtained for a water sample containing black coloured presumptive *C. perfringens* colonies.

For confirmation procedures, at least 10 colonies must be inoculated into a nutrient-rich culture medium to obtain subcultures. In this case, TSA is used for that purpose and subsequently incubated anaerobically at 36 ± 2 °C for 21 ± 3 h. Colonies that grow on this culture medium are subject to the acid phosphatase test, which consists in spreading these colonies on filter paper and adding 2 to 3 drops of acid phosphatase on top. Positive reactions are obtained when a purple to red coloration appears after 3 to 4 min (Fig. 21). Results are expressed by the total number of *C. perfringens* colonies (with blackish coloration and possessing acid phosphatase) in 100 mL of sample (ISO 14189, 2013).

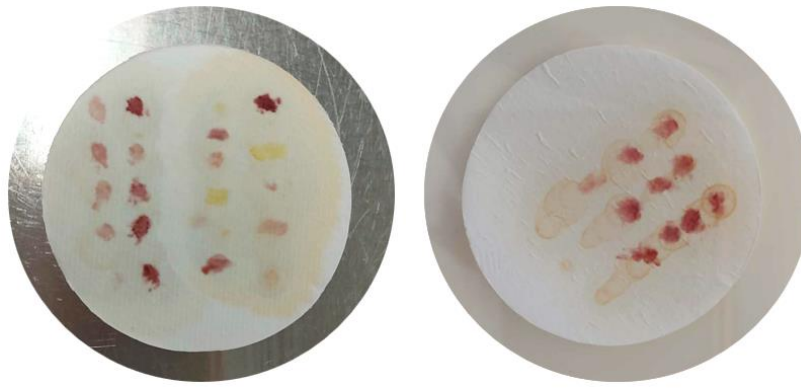


Figure 21 - Results obtained for acid phosphatase test. On the left it is possible to view a combination of positive reactions with a darker purple to red coloration and negative reactions presenting a yellow pigmentation. While on the right picture, it is possible to observe only positive reactions obtained for *C. perfringens* colonies.

2.3.11. Total staphylococci detection and enumeration

Total staphylococci detection and enumeration is performed using an internal ALS protocol as reference. The method used is executed by membrane filtration with a 0.45 μm pore membrane filter that is subsequently placed in a Mannitol Salt Agar plate and incubated at 37 ± 1 °C during 48 ± 4 h.

2.3.11.1. Total staphylococci confirmation tests and results

All yellow or white colonies present in Mannitol Salt Agar that may or may not show a yellow halo are considered presumptive staphylococci (Fig. 22). Other mucous and big colonies do not need to undergo confirmation studies since they belong to the *Bacillus* genus. This way, presumptive colonies that have developed in the membrane surface are counted and at least 3 colonies are chosen for inoculation in PCA to create subcultures and are incubated at 37 ± 1 °C for 24 ± 4 h.

These colonies undergo morphological and biochemical studies. Firstly, a Gram stain is performed and if bacteria portray themselves as gram-positive cocci, confirmation continues. Next a catalase test is performed by placing a catalase reagent droplet in a slide and then mixing a presumptive colony with it. A positive reaction is achieved when hydrogen peroxide degradation in oxygen and water occurs, forming small bubbles (Fig.

23). For catalase-positive colonies, respiratory-type tests are implemented. This test consists of inoculating presumptive colonies in MEVAG Agar tubes by stabbing the culture medium and incubating them at 36 ± 1 °C for 21 ± 3 h. A positive reaction is displayed when the culture medium acquires a yellow coloration due to glucose fermentation that acidifies the medium along the entirety of the culture medium (Fig. 24). This result indicates the presence of *Staphylococcus* spp. since fermentation occurred in both anaerobiosis and aerobiosis and staphylococci are facultative anaerobes.

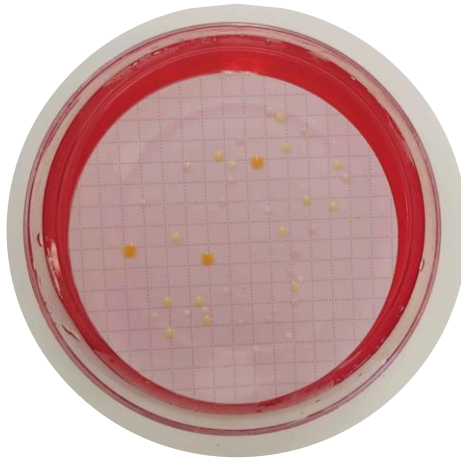


Figure 22 - Results obtained for total staphylococci in Mannitol Salt Agar containing yellow and white presumptive colonies.



Figure 23 - Positive result for catalase tests. It is possible to observe oxygen bubbles, a sign of hydrogen peroxide hydrolysis.

Attained results are expressed in CFU per sample volume and if the number of CFU is higher than 80, the results are expressed as "> 80 CFU per filtered sample volume".

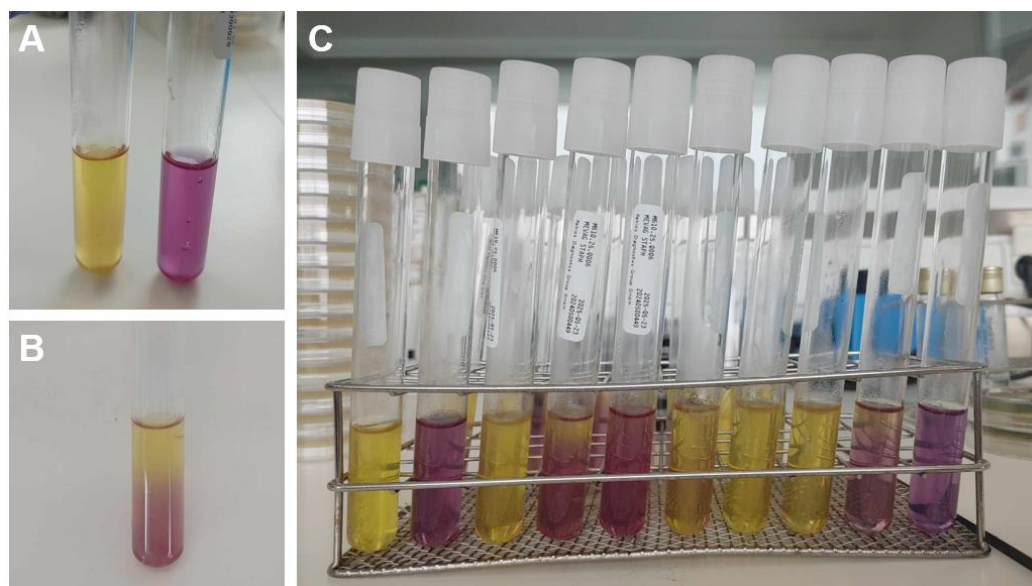


Figure 24 – MEVAG Agar results. A) Positive control (RM21) with medium colour change to yellow on the left side and negative control (RM47) without any coloration changes on the right side. B) Example of a negative result where glucose fermentation only occurred in aerobiosis. C) Several water matrix results with partial and total glucose fermentation indicated by a yellow pigmentation.

2.3.12. Coagulase-positive staphylococci enumeration

Coagulase-positive staphylococci enumeration is performed according to NF T 90-412 protocol utilizing the membrane filtration with a 0.45 μm pore membrane filter. Usually, the filtered sample volume corresponds to 100 mL. After filtering the required water volume, the membrane is placed in a BP-RPF Agar plate that is incubated at 36 ± 2 °C for 44 ± 4 h. Two readings of the Petri dishes are performed, one at 21 ± 3 h and the last one at 44 ± 4 h (NF T 90-412, 2016).

2.3.12.1. Coagulase-positive staphylococci confirmation tests and results

For correct examination it is advised to lift the membrane from the culture medium while searching for fibrin halos without moving or removing the membrane. Presumptive coagulase-positive staphylococci display a black or grey colour whilst being surrounded by an opaque fibrin halo that indicates coagulase activity (Fig. 25). Additionally, at least

five colonies that possess these morphological characteristics are selected for further microscopic examination and if the microorganisms observed take a cocci appearance, this reveals the presence of coagulase-positive staphylococci. Finally, results are expressed by the total number of coagulase-positive staphylococci per sample filtered volume (NF T 90-412, 2016).

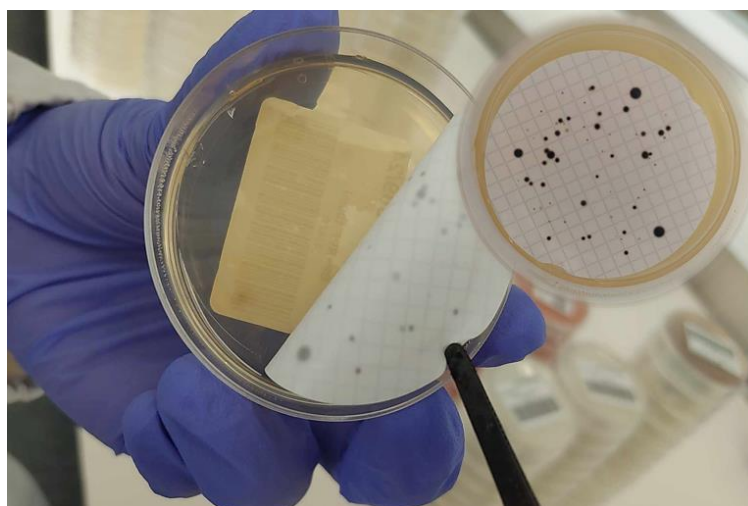


Figure 25 - Typical coagulase-positive staphylococci colonies on BP-RPF Agar with fibrin halo formation under the membrane filter, indicating plasma coagulation.

2.3.13. *Legionella* spp. and *L. pneumophila* detection and enumeration

Legionella is a genus comprised by fastidious gram-negative rod-shaped bacteria which requires the existence of the L-cysteine amino acid and soluble iron (III) for initial and optimal growth.

After sampling, it is recommended to perform the correspondent analyses under a 24 h period, however, this time interval can be extended up to 48 h.

The technique utilized for *Legionella* spp. and *L. pneumophila* detection and enumeration can be found in ISO 11731 and is performed by membrane filtration with elution, in the case of water intended for human consumption, with a 0.2 µm pore membrane filter.

Regarding drinking water, the advised filtration volume should be 1000 mL or the volume that is allowed by membrane capacity. When it is not possible to filter the advised

volume, the total volume that was filtered must be registered since that information is important for data treatment and result expression. When the membrane filter cannot filter the designed water volume due to particle accumulation it is necessary to resort to concentration by centrifugation. In these situations, a volume of 200 ± 5 mL is centrifuged at 3000 rpm for 30 min, then the supernatant is discarded and the acquired pellet is resuspended in 2 to 10 mL of sterilized water. This mixture corresponds to a sample prepared to undergo the consequent treatments.

When the filtration process is complete, the membrane is transferred to a sterile container with 10 mL of a diluted Ringer's solution, a mixture composed of a 1:10 dilution prepared with Ringer's solution and distilled water. This diluent is used to wash the membrane filter. Afterwards, the containers are shaken in an ultrasonic water bath for 6 min, which is the time required to achieve maximum recovery of microorganisms that may be attached to the membrane (Fig. 26).

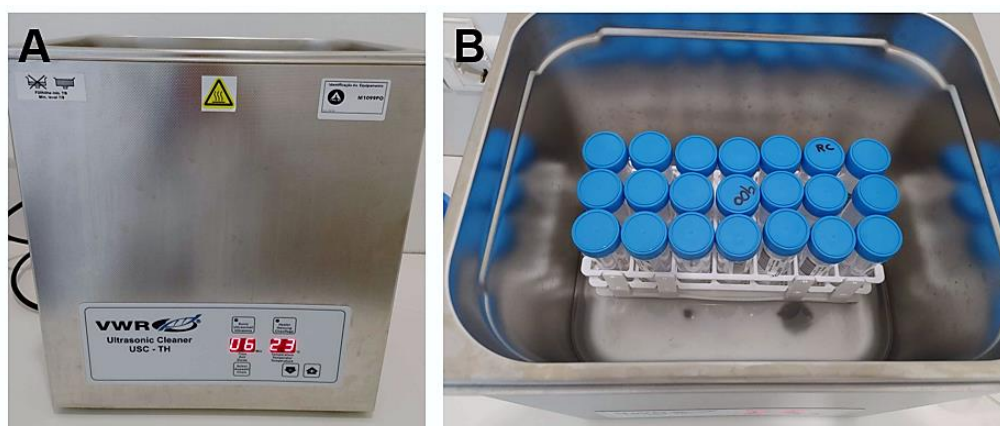


Figure 26 - Ultrasonic water bath. A) Ultrasonic water bath exterior and settings. B) Ultrasonic bath interior with a rack containing several samples ready for shaking.

The concentrate that is obtained after agitation represents the prepared sample. Thenceforth, this mixture is separated into 3 portions: one untreated portion, one portion for heat treatment and one portion for acid treatment. For the heat treatment, sample portions of 2 mL are added to sterile containers and placed in a water bath at 50 ± 1 °C during 30 ± 2 min. Sample temperature is controlled by adding a similar separate container and monitoring its temperature. For the acid treatment a 1:10 dilution is performed with 1 mL of the sample and 9 mL of an acid solution, and left to incubate for 5 ± 0.5 min.

The acid solution employed for *Legionella* spp. and *L. pneumophila* enumeration and detection is prepared in the ALS laboratory by the combination of 3.9 parts of solution

A and 25 parts of solution B. The pH of the resultant acid solution is adjusted to 2.2 ± 0.2 by adding a potassium hydroxide solution of 1 mol/L and then it is autoclaved and stored in a glass flask at room temperature, protected from light for the maximum period of one month. Regarding the components of the solutions used, solution A is a 0.2 mol/L hydrochloric acid (HCl) solution achieved by adding 17.4 mL of concentrated HCl to one liter of sterile distilled water and solution B is a 0.2 mol/L potassium chloride (KCl) solution achieved by dissolving 14.9 g of KCl in one liter of sterile distilled water.

Moreover, 500 μ L of the three prepared sample portions (untreated, heat and acid treatment portions) are inoculated and spread in BCYE and GVPC Agar Petri dishes. The setup used for *Legionella* sample processing can be observed in Fig. 27. When the inoculate has been absorbed by the culture media plates are inverted and incubated at 36 ± 2 °C for 10 days, with an intermediate reading at the fifth day (ISO 11731, 2017).

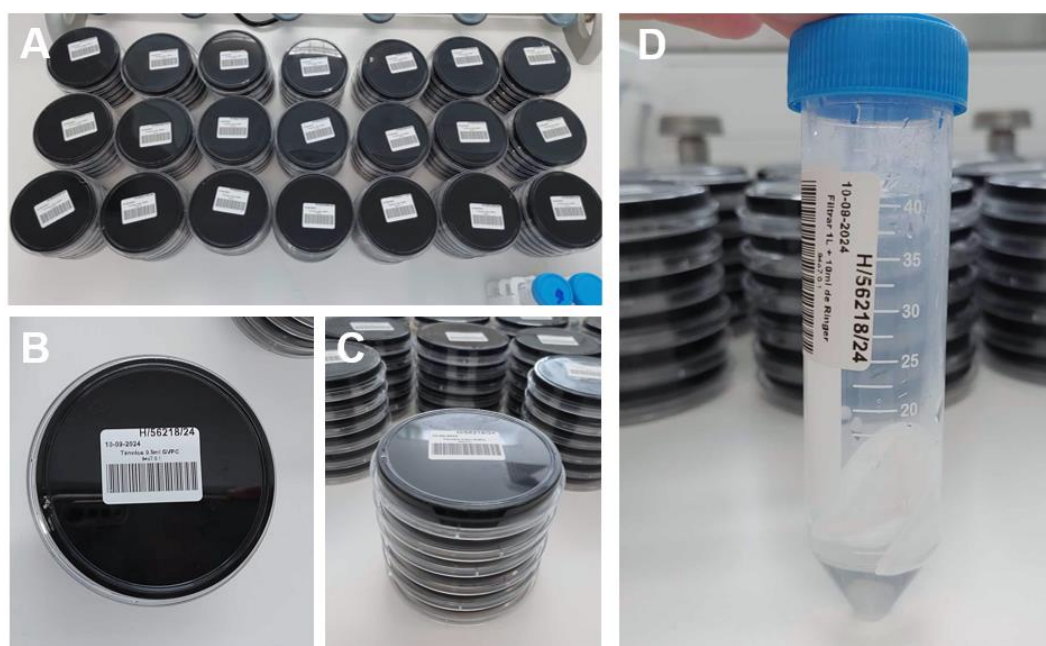


Figure 27 - Final set up for *Legionella* sample processing. A) Sample positioning by sample number. B) Petri dish labelling. C) Sample pile detail. Each sample pile is constituted by 3 plates from each culture media. D) Ringer solution tube with membrane filter after ultrasound bath incubation.

2.3.13.1. *Legionella* spp. and *L. pneumophila* confirmation tests and results

When examining the plates, colonies that exhibit white-grey coloration, smooth borders with a ground glass appearance and an entire edge are typical of *Legionella* species (Fig. 28). Nevertheless, other colony pigmentation might occur. The distinction between *Legionella* may be achieved through the usage of UV light observation since some species have brilliant white autofluorescence, others appear as red and *L. pneumophila* is green tinged with yellow.

Confirmatory studies are initiated by preparing subcultures of three presumptive *Legionella* colonies, when there is only one type of colony or at least one colony of each type when there are different presumptive *Legionella* colonies. These subcultures are performed using BCYE Agar and TSA culture media, since *Legionella* species cannot grow without the presence of L-cysteine. Therefore, colonies that only grow in BCYE Agar, advance in the confirmation studies, while colonies that grown in both culture media are discarded from further confirmation (Fig. 29). Incubation occurs at 36 ± 2 °C for 48 h.

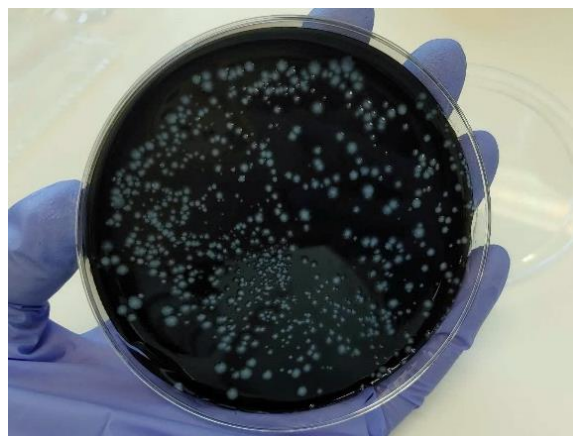


Figure 28 – BCYE Agar plate containing presumptive *Legionella* colonies with a white-grey coloration.

Finally, selected colonies are subject to serological tests using a commercial *Legionella* latex bead agglutination test kit. Colonies are spread on the reaction card using a buffer and then the correspondent serological solution is added. The kit used contains three solutions: one for *L. pneumophila* serotype 1 identification, one for *L. pneumophila* serotypes 2 to 14 and the last for other Legionellaceae identification. When agglutination is visualized for a certain solution, it means the colony being tested belongs to the *Legionella* group associated with that solution (Fig. 30).

Results for the membrane filtration method with elution are expressed using the following formula:

$$C_s = \frac{a \times V_c}{V \times V_{tot}} \times V_s$$

Where:

- C_s is the number of *Legionella* in CFU/L;
- a is the number of calculated confirmed *Legionella*;
- V_c is the sample volume in mL;
- V is the sample volume that is inoculated per plate in mL;
- V_{tot} is the total tested sample volume in mL;
- V_s is the reference volume chosen to express *Legionella* concentration in the sample which is 1000 mL for drinking water (ISO 11731, 2017).



Figure 29 - Confirmation results obtained after incubation of three *Legionella* presumptive colonies subcultures in BCYE Agar (left) and TSA (right) culture media. Since all colonies selected from the sample grew in both culture media, this sample was discarded from further confirmation tests.

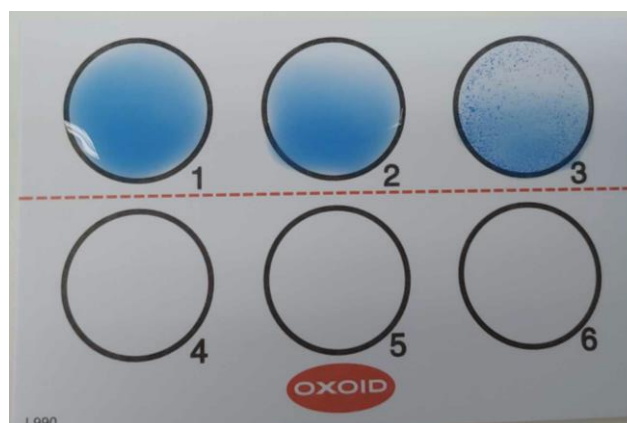


Figure 30 - Results obtained for the *Legionella* latex test. Agglutination can be observed in the third circle test, which corresponds to the *Legionella* species solution kit.

3. Laboratory Quality Control

Laboratory quality control (QC) is an essential step of the process to obtain consistent and reliable results as well as to ensure the quality of all the procedures performed (Magnusson & Tsimillis, 2023; Nwose, 2013). In ALS, several QC tests are executed periodically, including sterility controls, performance evaluation and productivity control charts.

3.1. Internal quality control

At ALS, several internal quality control assays are implemented with distinctive periodicities according to method requisites. Internal QC controls are compromised by several assessments namely, sterility tests, neutralizing agent controls and culture media, diluent and environmental controls.

3.1.1.Environmental control

To avoid contamination sources that may endanger the testing procedures aseptic conditions and result quality, it is essential to proceed with the correct disinfection of surfaces and perform a regular verification of sterility conditions in the laboratory (Sandle, 2015).

This way, in ALS Porto's Unit, environmental control is done every week after all analytical procedures have been completed. This control is executed by verifying surface disinfection and air quality. Environmental controls are made in pre-defined laboratory areas with rotating locations to ensure reliable test conditions. In Porto's unit case, laboratory zones used correspond to the readings room, laminar flow chamber, filtration, balances, sample processing and bench areas.

Regarding surface disinfection, this procedure is done after applying a 70 % ethanol solution to the surface that is going to be tested. After cleansing, a 10 cm² area should be delimited utilizing a sterile liquid swab (Fig. 31 B). For optimal results, horizontal, vertical and oblique swab rotation is necessary. Then, the swab solution is

homogenized in a vortex and 1 mL of this sample is inoculated in Yeast Extract culture medium. Incubation of the resulting plates occurs at 30 ± 1 °C during 72 h.

Later, the results obtained consist of the total number of colonies present in the plates. If the plates do not exhibit any microbial growth, the results should be expressed as “< 1 CFU/cm²”. If the results achieved are equal or superior to 5 CFU/cm², it is necessary to repeat surface disinfection and environmental control, while reviewing the disinfection procedure. When 15 or more CFU/cm² exist in the plates it is obligatory to proceed with cause evaluation and open a non-conformity process.

When executing this type of control in laminar flow chambers, non-conformities are observed when 1 or more CFU/cm² are visualized. Again, it is required to open a non-conformity process and proceed with cause evaluation, while scheduling cleansing and surface disinfection, with control repetition.

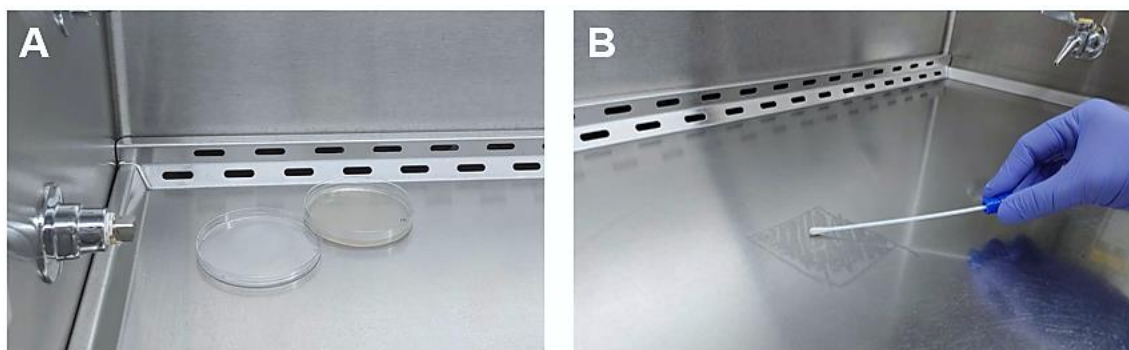


Figure 31 – Environmental control. A) Exposition PCA plate. B) Area delimitation of the tested surface location with a sterile liquid swab.

For air quality assessment, PCA Petri dishes are exposed to the air in the locations determined to be assessed each week for 30 min (Fig. 31 A). When testing the laminar flow chamber for air quality, the equipment must be turned on for at least 3 min before plate exposition. Incubation of exposition plates is done at 30 ± 1 °C for 72 h.

When no colony growth is observed after plate incubation, the result expression is given as “< 1 CFU” or “0 CFU”. In laminar flow chambers, results should not be equal or superior to 1 CFU, while in the laboratory rooms colony counting should be inferior to 8 CFU. When 8 to 10 CFU are seen, disinfection evaluation and control repetition are required. Furthermore, it is also advised to ensure that there are not air circulation flows that may interfere with the results (ISO 17025, 2017; Lipps et al., 2022).

3.1.2. Culture media control general remarks

Culture media preparation, production, storage and performance testing meet the control requirements stated by ISO 11133.

This way, culture media are evaluated regarding their performance in the established quality controls, namely Reference Materials (RMs) analyses, interlaboratory comparisons (ILCs), positive and negative controls and sterile controls. Culture media acquired from external suppliers suffer a quantitative evaluation monthly.

When the culture media purchased are ready to use after supplementation, quality controls consist of sterility tests and qualitative evaluation, accomplished for each new product batch. Regarding dehydrated culture media, original packaging should be identified with opening date and the media should then be distributed in tubes, flasks or Petri dishes. After distribution, Petri dishes should be stored at 5 ± 3 °C for a maximum period of one month (ISO 11133, 2014). Plates should be stored upside down and placed in containers possessing a lid, with correct labelling, containing information regarding culture media identification, preparation and expiration dates and batch number. Culture media and other reagents storage conditions are shown in Fig. 32.

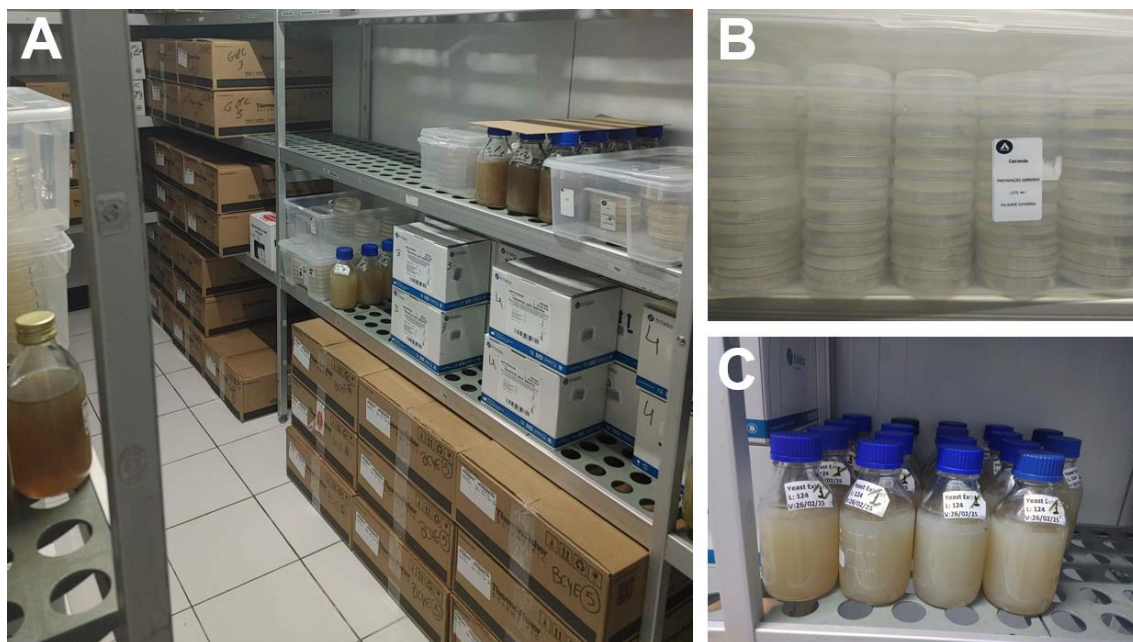


Figure 32 - Storage conditions for culture media and other reagents. A) Freezer room where all culture media and reagents that require refrigeration temperature are stored. B) Example of culture media storage container correctly labelled. C) Culture media flasks supplied by Tondela's unit labelled with media identification, batch number and expiration date.

Culture media or diluent batch numbers should be consumed in sequential order and an indication of the sterilization cycle should also be registered, especially when the same batch has been separated into different cycles. Concerning lifetime, when presented in flasks, culture media can usually be used for 3 months. When a batch attains its maximum lifetime, culture media can still be employed if there are no physical changes and it obtains the required values in quality controls (ISO 8199, 2018).

Most culture media utilized by ALS Porto's unit is either bought from an external supplier already plated or ready to use after supplementation or supplied by Tondela's unit in flasks or plates. Culture media and diluents distributed by other units are accompanied by an evaluation report containing quality control parameters referred in ISO 11133 (ISO 11133, 2014).

Hence, media that is not supplied by external or internal entities is prepared in Porto's unit and, therefore a physical culture media control is mandatory. These exceptions correspond to Slanetz and Bartley Agar and CCA culture media.

3.1.3. Physical culture media control

The physical culture media control implemented at Porto's unit consists of pH evaluation for each batch of media prepared from scratch in this laboratory or media that request supplementation with components that trigger pH fluctuations. For pH evaluation, the culture media analysed must be at room temperature. If values differ from those that are established, then the batch must be discarded.

Likewise, pH values for the tested media should be:

- 6.8 ± 0.2 for CCA;
- 7.2 ± 0.2 for Slanetz and Bartley Agar;
- 7.6 ± 0.2 for TSC Agar after supplementation.

Moreover, pH calibration is executed using three standard solutions corresponding to acid (pH 4), neutral (pH 7) and basic (pH 10) pH to ensure the correct reading of culture media pH. Together with the culture media in the test, the pH of a reference pH 7 solution is also read (Fig. 33) (ISO 7218, 2024; ISO 11133, 2014).

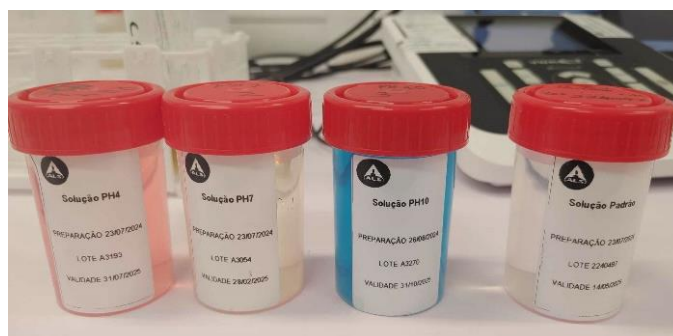


Figure 33 - Standard solutions employed for pH calibration.

3.1.4. Sterility culture media control

To perform sterility tests, one plate or tube from each batch produced is tested by incubation at the recommended temperature and time period for the methods employed in the respective media. For the results to be considered satisfactory there cannot be any colour changes, turbidity, deposit or gas formation or colony growth (ISO 11133, 2014).

3.1.5. Culture media performance evaluation

After culture media performance evaluation, an internal evaluation certificate with the assessed parameters is emitted for each external supplier batch, when culture media are used by another ALS unit and after six months from the opening of a flask. This certificate must be emitted in the two weeks following batch production.

3.1.5.1. Solid culture media performance evaluation

Solid culture media performance is evaluated by its productivity, selectivity and specificity rate. Concerning productivity, this concept is defined as the culture media recuperation rate for target microorganisms. According to ISO 11133, culture media productivity evaluation can be performed by quantitative or qualitative tests. By qualitative methods, the target microorganisms are inoculated by streaking in the media

to be tested with a 1 µL loop. Then incubation follows temperature and incubation conditions recommended by the respective methods. Next, the obtained growth is evaluated with:

- 0 – when no growth is observed;
- 1 – when weak growth is observed (regarding colony size and number);
- 2 – when good microbial growth is observed.

On the other hand, for quantitative evaluation of culture media, suspensions are prepared with RMs and dilutions are employed to obtain a final result of 80 to 120 CFU. Only counts with 50 CFU and higher can be considered for evaluation. A comparison test between the reference culture medium and the tested medium is executed in duplicates. Results are expressed following the formula below and the subsequent values should be between 0.5 or 0.7 to 1.4 for selective and non-selective culture media, respectively.

$$P_r = \frac{N_s}{N_o}$$

Where:

- P_r is the culture medium productivity;
- N_s is the average number of CFU obtained in the test medium;
- N_o is the average number of CFU obtained in the reference medium.

In contrast, culture media selectivity is defined as the inhibition rate of non-target microorganisms. Selectivity evaluation is performed with work cultures supplied by Tondela's unit. When the culture medium is presented in Petri dishes, suspensions used for this evaluation should contain at least 10^4 CFU. Inoculation is made by streaking in the tested medium with a 1 µL loop and incubation is executed by following the recommendations for the respective methods. Evaluated growth is categorized according to its intensity:

- 0 – when no growth is observed;
- 1 – when weak growth is observed (regarding colony size and number);
- 2 – when good microbial growth is observed.

Finally, specificity is a demonstration of atypical characteristics concerning non-target microorganisms. This method operates by using an inoculum with less than 10^3 CFU. Morphological and biochemical features of work cultures are then evaluated.

Furthermore, when employing these tests for culture media utilized for membrane filtration methods, RM dilutions must be prepared and filtered and then the membrane placed in the media. The target inoculum concentration should be around 100 CFU (ISO 11133, 2014).

3.1.5.2. Liquid culture media performance evaluation

Contrary to solid culture media, liquid media productivity is evaluated for both target and non-target microorganisms. For target microorganisms, productivity evaluation is made by inoculating the medium with a dilution containing 100 or less CFU. Incubation should proceed according to the respective methods and afterwards, a 10 µL loop inoculum is cultivated in a selective medium for the target microorganisms. Productivity is considered to be satisfactory when the target microorganism grows in the selective medium (should present at least 10 CFU). While for non-target and target mixtures, inoculates should contain ≤ 100 CFU of the target microorganisms and ≥ 1000 CFU of the non-targets. After incubation, a 10 µL loop inoculate is streaked on a selective medium. Productivity is satisfactory when targets present good growth (at least 10 CFU).

In liquid culture media, selectivity is assessed by non-target microorganisms growth. The test tube is cultivated with a $> 10^4$ CFU inoculum and then incubated following recommendations. Later, a 10 µL loop inoculum is cultivated in a non-selective culture medium for non-target microorganisms. Selectivity is considered to be satisfactory when there is no growth from non-targets.

On the topic of non-selective enrichment culture media and selective confirmation media, these evaluations are executed for each external supplier batch. Performance tests are evaluated by turbidity of work cultures and the qualitative test results are merely indicative. The conditions used for these two types of culture media can be observed in Table 6. Growth intensity evaluation is categorized according to the following groups:

- 0 – when no growth or turbidity is observed;
- 1 – when weak growth or turbidity is observed (regarding colony size and number);
- 2 – when good microbial growth or high turbidity is observed.

Other features such as gas formation or modifications in coloration may also be verified through this method.

Table 6 - Testing conditions for performance tests aimed at non-selective enrichment culture media and selective confirmation media.

	Non-selective enrichment culture media	Selective confirmation culture media
Tested volume	10 mL	10 mL
Target microorganisms	≤ 100 CFU	$\geq 10^4$ CFU

Additionally, confirmation culture media controls should be executed upon arrival by supplier batch and qualitative evaluation inoculum used should be a subculture from an adequate work culture (ISO 11133, 2014).

3.1.6. Productivity control charts

As was said above, productivity is defined as the culture media recuperation rate for target microorganisms and productivity control charts represent graphical, quantitative approaches designed to monitor culture media productivity. To facilitate the creation of a new report it is necessary to progress with at least 10 productivity analyses in different batches from the same culture medium and then calculate the mean and standard deviation (S). Analyses should ideally be executed in duplicates, by various professionals and on different days, to allow limit delimitation. Using the productivity formula previously referred, the mean and standard deviation are calculated and warning (mean ± 2 S) and action (mean ± 3 S) limits are defined to allow for chart creation. These limits are recalculated after 20 productivity assays and generated periodically after every 30 assays, also suffering a revision process after the opening of a new external provider batch where productivity is assessed quantitatively. This type of control is initiated monthly.

A non-conformity is observed when:

- Two of the values obtained repeatedly exceed the warning limit (± 2 S);
- One of the values obtained surpasses the action limit (± 3 S);
- Six consecutive values obtained are increasing or decreasing;
- Nine consecutive values obtained are situated on the same side of the mean (ISO 11133, 2014).

3.1.7. Diluent control

3.1.7.1. Quantitative diluent control

Diluent control is carried out by quantitative tests for each batch acquired or after 6 months have passed since package opening. This test determines the diluent's ability to hold the microbial load existing in samples without causing an increase or decrease to this number of cells. The procedure consists of inoculating a diluent portion with a 10^4 CFU/mL culture, then a 100 μ L aliquot is immediately transferred to a TSA Petri dish, this condition is considered to be the initial time (T_0). The diluent is maintained at room temperature for ± 45 min and another aliquot is transferred for TSA; this condition corresponds to the time after 45 min ($T_{45\text{min}}$). After incubation at 30 °C for 72 h, the difference in the number of colonies found should not be higher than 30 % (ISO 8199, 2018).

3.1.7.2. Diluent volume control

A diluent volume control is performed every 3 months before diluent distribution in tubes and after autoclavation. This test is implemented for 10 individually weighted tubes that are subsequently dispersed in the group of tubes prepared to contain the said diluent. The attained weight values are registered, and the diluent is dispensed in all tubes, after which autoclavation occurs. Later, tube weight registration is carried out. Diluent volume distribution accuracy is calculated by the following equation:

$$\frac{V_n \times V_m}{V_n} \times 1000$$

Where:

- V_n is the nominal diluent volume dispensed in each tube;
- V_m is the average volume obtained.

The results achieved should be inferior to 2, or else the batch needs to be discarded (ISO 8655-1, 2022).

3.1.8. Sterile collection flasks and Petri dishes control

Flasks and Petri dishes used for microbiological water assays are already acquired sterile and ready to use. For flask sterility verification, 20 to 50 mL of fused PCA are dispensed to each flask, which are then rotated horizontally to allow for culture medium spreading and wall adherence, before medium solidification. After this process is completed, the flasks are left horizontally for medium cooling at room temperature (Fig. 34). Consequently, incubation occurs at 22 °C for 5 days (ISO 19458, 2006). The same concept is applied to Petri dishes with the regular amount of Yeast Extract Agar used for plate pouring with plate incubation at 22 °C for 72 h (ISO 6222, 1999). After incubation, culture medium should not display any colony formation, or else the batch is discarded. Only 1 % of the flasks and Petri dishes received are evaluated for their sterility (ISO 6222, 1999; ISO 19458, 2006).



Figure 34 - Sample flasks for microbiological assays. A) Flasks enclosed before sterility evaluation. B) Flasks after PCA culture medium insertion for sterility evaluation.

3.1.9. Membrane filters control

Performance tests for membrane filters are executed to verify if a certain batch of membranes can secure consistent results for a determined assay and can be safely employed, guaranteeing correct procedures. In this process, membrane-culture media system suitability is evaluated, and the tests are executed for all culture media using membrane filters and all conditions where membranes are employed. This assessment is required for all membrane batches, as well as all culture media batches.

For obtaining satisfactory results, parameters that need to be calculated include reference count and membrane productivity, selectivity and specificity. A reference count is defined as the total colony count grown in a reference culture medium without using a membrane filter. These results are attained by streaking in a non-selective medium, all microbial strains expended in the membrane filter control and incubating them according to their growth requirements. Reference counts are necessary for productivity, selectivity and specificity evaluations.

This way, the membrane filtration method is applied to an inoculum of the selected RM strains. Inoculated microbial load should be between 80 and 120 CFU, $> 10^4$ CFU and $> 10^3$ CFU for productivity, selectivity and specificity evaluation, respectively. For the filtration process at least 20 mL of a sterile diluent (e.g. sterile water) should be added to allow for even microbial distribution. Productivity is calculated with the equation referred previously on 3.1.5.1. and results are considered to be acceptable if $P_r \geq 0.5$ or 0.7 and < 1.4 for selective and non-selective media, respectively. Specificity is evaluated according to typical morphological characteristics displayed in RM strains and, finally, selectivity is evaluated by non-target microorganism growth previously mentioned in 3.1.5.1. (ISO 11133, 2014).

3.1.10. Blank controls

Blank controls are executed using a sterile diluent to assure sterility along sample processing analytical procedures, namely sample handling and work conditions. For these controls, adequate diluent or sterile deionized water is consumed accordingly and the usual sample processing occurs.

For membrane filtration water analyses, blank controls are made every day at the end of the work session for all culture media utilized in the samples collected and processed during that day. Filtration volume and incubation times match those normally used for the corresponding culture media and methods. Results should be negative without any CFU formation (Lightfoot & Maier, 1998; Lipps et al., 2022).

3.1.11. Negative and positive controls

These controls are executed for each assay performed with a target microorganism (positive control) and negative controls for selectivity and specificity. Culture media employed for water analyses are tested daily. With this objective in mind, RM strains are streaked on adequate culture media to verify morphological colony features. The strains used are present in Tables 7 and 8 and Supplementary Table 1.

3.1.12. Laboratory Control Standard

In this control, a suspension prepared from a reference strain is subject to the whole analytical process to gauge target microorganism recovery. Inoculate concentration should be at least 10 CFU and samples should be compromised of not only target microorganisms but non-targets as well. The parameters examined vary depending on the matrix type (Lipps et al., 2022).

3.1.13. Duplicates and parallel tests

Duplicates and parallel tests are done to obtain a precision assessment of results. Likewise, duplicates consist of suspensions that suffer identical conditions to the main sample procedure, incorporating the same volume, dilutions, inoculation and incubation times executed by the same analyst. On the other hand, parallel tests are executed by different professionals with the possibility of variation to other conditions such as culture media batch or equipment.

This criterion is recalculated annually and evaluated quarterly. When appraised results surpass the precision criteria, corrective actions must be put in practice alongside a non-conformity report. At the end of each year, non-conformity reports are analysed to evaluate tendencies concerning equipment, professionals or matrix deviations, for example (Lightfoot & Maier, 1998; Lipps et al., 2022).

Table 7 - Productivity positive controls and selectivity and specificity negative controls used for daily culture media control.

Culture medium	Positive control (productivity)	Negative selectivity control	Negative specificity control
Yeast Extract Agar	<i>E. coli</i> WDCM 00012 (RM3) + <i>Bacillus subtilis</i> subsp <i>spizizenii</i> WDCM 00003 (RM10)	N/A	N/A
CCA	<i>E. coli</i> WDCM 00012 (RM3) + <i>Citrobacter freundii</i> WDCM 00006 (RM22)	<i>E. faecalis</i> WDCM 00087 (RM4)	<i>S. aureus</i> WDCM 00034 (RM26)
Lactose Tergitol 7 Agar	<i>E. coli</i> NCTC 13167 / WDCM 00179 (RM54) + <i>C. freundii</i> WDCM 00006 (RM22) + <i>Clostridium bifermentans</i> WDCM 00079 (RM31)	<i>E. faecalis</i> WDCM 00087 (RM4)	<i>P. aeruginosa</i> WDCM 00025 (RM18)
CN Agar	<i>S. aureus</i> WDCM 00034 (RM26)	<i>E. coli</i> WDCM 00012 (RM3) + <i>E. faecalis</i> WDCM 00087 (RM4)	N/A
Slanetz and Bartley Agar	<i>E. faecalis</i> WDCM 00009 (RM19) + <i>Staphylococcus epidermidis</i> WDCM 00132 (RM29)	<i>E. coli</i> WDCM 00012 (RM3) + <i>Yersinia enterocolitica</i> WDCM 00038 (RM27)	N/A
Mannitol Salt Agar	<i>Y. enterocolitica</i> WDCM 00038 (RM27) + <i>Enterobacter aerogenes</i> WDCM 00175 (RM30)	<i>E. coli</i> WDCM 00012 (RM3)	N/A
BP-RPF Agar	<i>Y. enterocolitica</i> WDCM 00038 (RM27)	<i>E. coli</i> WDCM 00012 (RM3)	<i>S. aureus</i> WDCM 00034 (RM24)
TSC Agar	<i>C. perfringens</i> WDCM 00007 (RM15)	<i>B. subtilis</i> subsp <i>spizizenii</i> WDCM 00003 (RM10)	N/A
ISA	<i>C. perfringens</i> WDCM 00007 (RM15)	N/A	<i>E. coli</i> WDCM 00012 (RM3)
BCYE Agar	<i>L. pneumophila</i> serogroup 1 WDCM 00107 (RM14)	N/A	N/A
GVPC Agar	<i>Legionella anisa</i> WDCM 00106 (RM13) + <i>L. pneumophila</i> serogroup 1 WDCM 00107 (RM14)	<i>E. coli</i> WDCM 00012 (RM3) + <i>E. faecalis</i> WDCM 00087 (RM4)	N/A

3.1.14. Blind replicates and spiked samples

For water matrix samples, accuracy is measured, monthly, by microorganism recovery levels on artificially contaminated samples that are subjected to the whole analytical analyses. Recovery levels are calculated by the following equation:

$$Recovery = \frac{\text{Productivity values obtained in a given assay}}{\text{Productivity values previously achieved for the inoculum used}}$$

These samples should be treated as regular water samples without analyst knowledge and no reference to QC, aiming for a deep evaluation of the whole analytic process as well as the responsible professionals. For this, target and non-target strains are utilized, and productivity result values are satisfactory when between 0.5 or 0.7 and 1.4 (for selective and non-selective media, respectively). In case these values are not observed, the possible causes should be investigated (Lightfoot & Maier, 1998).

3.1.15. Deionized water control

At ALS Porto's unit deionized water is produced by a purification unit that employs three refinement methods, reverse osmose, deionization and adsorption. Purified water is utilized for several assays, namely for culture media supplementation and control implementation. The water control plan for deionized water is executed with a monthly periodicity for total microorganisms' enumeration at 22 ± 2 °C for 68 ± 4 h using Yeast Extract Agar according to ISO 11133 and ISO 3696. The total CFU count should not surpass 1000 CFU/mL (ISO 3696, 1987; ISO 11133, 2014).

3.2. External quality control

External quality controls are implemented according to ILCs resulting data. ILCs consist in performing the same test with identical samples in multiple laboratories. At ALS, ILCs are programmed annually, or when required, by competent authorities acting on the microbiology field. Test sample data is examined by both ALS and the organizing entity. Furthermore, ILC participations are influenced by the number of programmes available as well as their arrangement or economic power, for example.

Data obtained in ILCs must be inspected according to a few assumptions, specifically in:

- Microorganism distribution, that is never uniform despite good homogenization with arbitrary microorganism distribution and microbial load greatly affecting resulting values that are never truly a faithful sample representation;
- Variability between subsamples and the complexity of handling living organisms that may become non-viable or unexpectedly display different behaviours;
- Attraction and repulsion phenomena that create microorganism aggregation or empty zones in samples.

Additionally, the phenomena referred above may result in misleading bad laboratory performance due to false high or low microbial counts that translate into mathematical incoherences. Therefore, overall laboratory performance should be evaluated in the long-term regarding sample microbial concentration and content, expected results, data distribution, analyst assessment, false positive and false negative results. If deviation to expected data is observed a non-conformity report should be redacted (ISO 13843, 2017; Lipps et al., 2022).

4. RM and culture preparation

4.1. General remarks

The microorganisms utilized for the several control conditions necessary to preserve good quality control are selected by Tondela's unit and given to each Portuguese unit appropriately while keeping in mind the unit's requirements. This selection represents the RMs collection and is based on representative strains for each species that possess the ideal morphological features and behaviour. An RM is considered to be a substance or material that possesses properties that are uniform enough, for it to be used as a standard for method measurements and assessments or equipment calibration (ISO 16140-1, 2016). Hence, lyophilized strains obtained belong to the World Data Centre of Microorganisms (WDCM) collection or are a result of ILCs or even from known isolates.

Likewise, Tondela's unit is responsible for the production of reference stock cultures from RMs or ILCs isolates. Reference stock cultures are in turn used for the annual preparation of stock cultures that are then sent to all units. Henceforth, stock cultures suspensions are prepared with a cryoprotective medium (Nutrient Broth with 20 % glycerol) and stored at $-20\text{ }^{\circ}\text{C}$, possessing a duration of one year. Aiming to avoid phenotypical changes, microbial cultures may only be transferred 5 times from the vials (ISO 11133, 2014).

4.2. Reference stock culture preparation

Reference stock cultures are formulated from reference strains or ILCs isolates and should be handled with care, evading cross-contamination, mutations and phenotypical changes. The process for acquiring these cultures includes adequate inoculation an incubation for each strain and purity verification (ISO 11133, 2014). Storage may happen at $-80\text{ }^{\circ}\text{C}$ or $-20\text{ }^{\circ}\text{C}$ with a lifespan of 10 years or 1 year, respectively. Obtained vials are labelled with RM number, strain name and production and expiration dates (Fig. 35).

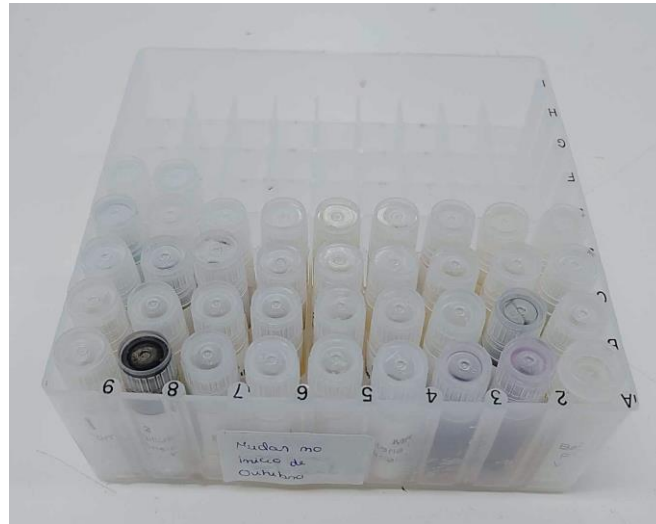


Figure 35 - Box containing RMs prepared for one month utilization provided by Tondela's unit. Vials hold a cryoprotective medium and are stored at -20 °C.

4.3. Work cultures preparation

Work cultures are prepared from stock cultures and are created in liquid and solid culture media. Preparation starts with defrosting stock cultures at room temperature allowing for uniform homogenization. Next, for liquid media cultures, 10 µL are inoculated with the help of a loop in a tube containing BPW that is then stirred in a vortex. While in solid media cultures, 1 µL inoculum is used for streaking (Fig. 36). For most strains, the culture medium employed is TSA which is incubated for 24 h at 37 °C. However, for RM15, the culture medium utilized is Columbia Agar with 5 % sheep blood and incubation is achieved in anaerobiosis, with incubation conditions being similar to other strains. Concerning *Legionella* strains, the culture medium in use is BCYE Agar and incubation occurs for 2 to 5 days at 37 °C. Finally, work cultures should provide accurate labelling containing RM number, and the preparation date using the following format: RM.Year.Month.Day.

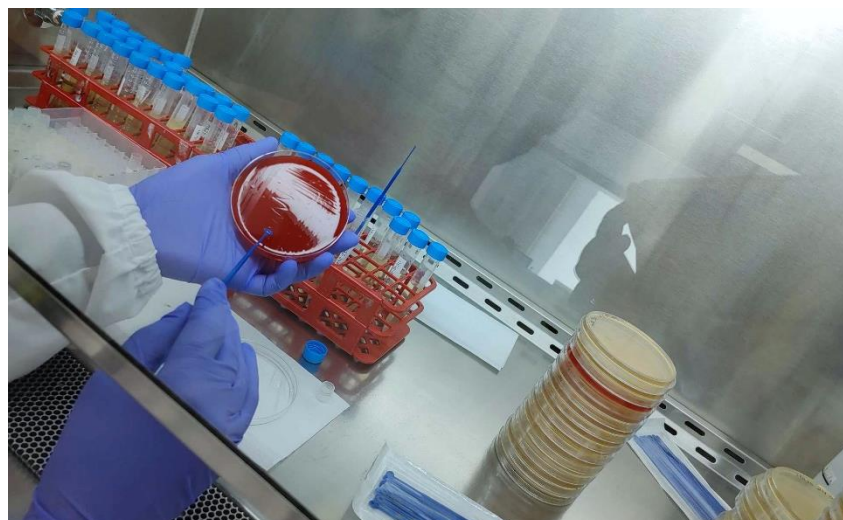


Figure 36 - Preparation of RM solid and liquid work cultures.

4.4. Test suspensions preparation

Dilutions planned for testing should be determined from previous tests performed in standard conditions. To ascertain work culture concentration in liquid media, serial dilutions are made with BPW and then 100 μL from several dilutions are inoculated in TSA or Blood Agar for aerobic and anaerobic microorganisms, respectively. Incubation proceeds at 36 ± 2 $^{\circ}\text{C}$ during 21 ± 3 h. Moreover, work culture inoculates may only be used in a 2 h period after preparation at room temperature or 24 h when refrigerated at 5 ± 3 $^{\circ}\text{C}$. Adequate inoculation volumes and dilutions required for quality controls are defined based on CFU/mL concentration established by this method (ISO 11133, 2014).

4.5. RMs used for water matrix samples

The RMs chosen for water matrix samples and that are employed in controls in microbiological assays and confirmation tests are present in Table 8.

Table 8 - RM affiliation and strain number used in water matrix assays and confirmation tests as controls.

RM number	Microorganism	Reference
RM3	<i>E. coli</i>	WDCM 00012
RM4	<i>E. faecalis</i>	WDCM 00087
RM7	<i>S. enterica</i> serovar Enteritidis	WDCM 00030
RM9	<i>S. enterica</i> serovar Typhimurium	WDCM 00031
RM10	<i>B. subtilis</i> subsp <i>spizizenii</i>	WDCM 00003
RM13	<i>L. anisa</i>	WDCM 00106
RM14	<i>L. pneumophila</i> serogroup 1	WDCM 00107
RM15	<i>C. perfringens</i>	WDCM 00007
RM17	<i>E. coli</i>	WDCM 00013
RM18	<i>P. aeruginosa</i>	WDCM 00025
RM19	<i>E. faecalis</i>	WDCM 00009
RM21	<i>S. aureus</i>	WDCM 00032
RM22	<i>C. freundii</i>	WDCM 00006
RM24	<i>S. aureus</i>	WDCM 00034
RM26	<i>S. aureus</i>	WDCM 00034
RM27	<i>Y. enterocolitica</i>	WDCM 00038
RM29	<i>S. epidermidis</i>	WDCM 00132
RM31	<i>C. bifermentans</i>	WDCM 00079
RM40	<i>Proteus mirabilis</i>	WDCM 00023
RM47	<i>Micrococcus luteus</i>	ATCC 4698
RM50	<i>Pseudomonas paraaeruginosa</i>	WDCM 00026 or ATCC 027
RM54	<i>E. coli</i>	NCTC 13167 or WDCM 00179

5. Conclusion

The internship theme carried out at ALS Life Sciences Portugal S.A. emphasized the importance of drinking water microbiological quality evaluation. This assessment must be performed regularly to maintain satisfactory microbiological quality standards, which are essential to achieve safe drinking water access. With the latter being considered a basic human right, since water quality greatly affects human health.

This way, health promotion and disease prevention measures are ultimately a reflection of assays implemented in laboratories and the results obtained for these assays. Hence, the importance of reliable and accurate results, since false positive or false negative evaluations may cause unnecessary treatment implementation and other complications, such as delay in results. Therefore, testing procedures must be executed according to pre-determined methods, guaranteeing quality in data expression. At ALS, most methods employed are based on international or national standards and all follow the applicable legislation, to ensure result uniformization. Furthermore, laboratory quality controls are performed for quality appraisal during the whole analytical process and are applied to the organization as a whole.

Regarding microbial contamination, most waterborne pathogens present in drinking water originate from faecal pollution, which must be controlled periodically due to human health danger of the resulting pathologies. Despite this, some other hazardous bacteria are not contaminants, but instead natural inhabitants of water environments. Such is the case of *Legionella* spp., an OPP that is adapted and persistent in drinking water distribution and plumbing systems. Keeping this in mind, most of the drinking water samples analysed over the internship period did not display high degrees of microbial load, which indicates treatment efficiency for this type of matrix.

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7. Annexes

Supplementary Table 1 - RMs used as controls in confirmation tests, their expected reaction in the respective culture medium or test reagent and the method employed in confirmation.

Confirmation culture medium or reagent	Test principle	Assay	Method	Positive control	Negative control	Characteristic reaction
Acid phosphatase	Acid phosphatase detection	<i>C. perfringens</i> enumeration	ISO 14189	<i>C. perfringens</i> WDCM 00007 (RM15)	<i>C. bifermentans</i> WDCM 00079 (RM31)	Positive reaction: Red or purple coloration. Negative reaction: Lack of colour change to red or purple.
		Coliform enumeration	ISO 9308-1			
Oxidase	Cytochrome detection	Faecal coliform enumeration	Internal protocol	<i>E. coli</i> WDCM 00012 (RM3) or <i>E. coli</i> WDCM 00013 (RM17)	<i>P. aeruginosa</i> WDCM 00025 (RM18) or <i>P. paraaeruginosa</i> WDCM 00026 or ATCC 027 (RM50)	Positive reaction: Violet, purple or blue colour. Negative reaction: Lack of colour change to violet, purple or blue.
		<i>Salmonella</i> spp. detection	ISO 19250			
		Enterobacteriaceae enumeration and detection	Internal protocol			
		<i>P. aeruginosa</i> detection and enumeration	ISO 16266	<i>P. aeruginosa</i> WDCM 00025 (RM18) or <i>P. paraaeruginosa</i> WDCM 00026 or ATCC 027 (RM50)	<i>E. coli</i> WDCM 00012 (RM3) or <i>E. coli</i> WDCM 00013 (RM17)	
Bile-Aesculin-Azide Agar	Aesculin hydrolysis detection	Intestinal enterococci detection and enumeration	ISO 7899-2	<i>E. faecalis</i> WDCM 00087 (RM4) or <i>E. faecalis</i> WDCM 00009 (RM19)	<i>E. coli</i> WDCM 00012 (RM3) or <i>E. coli</i> WDCM 00013 (RM17)	Positive reaction: Dark coloration around colonies. Negative reaction: Lack of dark coloration around colonies.

Coagulase test (BP-RPF Agar)	Coagulase detection	Coagulase-positive staphylococci enumeration	NF T 90-412	<i>S. aureus</i> WDCM 00032 (RM21) or <i>S. aureus</i> WDCM 00034 (RM24)	<i>S. epidermidis</i> WDCM 00132 (RM29)	Positive reaction: Plasma coagulation with halo formation around colonies. Negative reaction: Lack of plasma coagulation.
Gram stain	Morphology observation	Total staphylococci detection and enumeration	Internal protocol	<i>S. aureus</i> WDCM 00032 (RM21) or <i>S. aureus</i> WDCM 00034 (RM24)	<i>E. coli</i> WDCM 00012 (RM3) or <i>E. coli</i> WDCM 00013 (RM17)	Positive reaction: Gram-positive purple-coloured cocci. Negative reaction: Gram-negative red-coloured bacteria or other gram-positive bacteria.
Catalase test	Catalase detection	Total staphylococci detection and enumeration	Internal protocol	<i>S. aureus</i> WDCM 00032 (RM21)	<i>E. faecalis</i> WDCM 00087 (RM4)	Positive reaction: Oxygen bubble formation. Negative reaction: Lack of bubble formation.
MEVAG Agar	Respiratory type determination	Total staphylococci detection and enumeration	Internal protocol	<i>S. aureus</i> WDCM 00032 (RM21)	<i>M. luteus</i> ATCC 4698 (RM47)	Positive reaction: Colour change to yellow in the totality of the culture medium. Negative reaction: Colour change only in anaerobiosis or aerobiosis or lack of colour change.
King B Agar	Fluorescence detection	<i>P. aeruginosa</i> detection and enumeration	ISO 16266	<i>P. aeruginosa</i> WDCM 00025 (RM18) or <i>P. paraaeruginosa</i> WDCM 00026 or ATCC 027 (RM50)	<i>E. coli</i> WDCM 00012 (RM3) or <i>E. coli</i> WDCM 00013 (RM17)	Positive reaction: fluorescence presence with UV radiation. Negative reaction: lack of fluorescence with UV radiation.
Acetamide Broth	Ammonia production detection	<i>P. aeruginosa</i> detection and enumeration	ISO 16266	<i>P. aeruginosa</i> WDCM 00025 (RM18) or <i>P. paraaeruginosa</i> WDCM 00026 or ATCC 027 (RM50)	<i>E. coli</i> WDCM 00012 (RM3) or <i>E. coli</i> WDCM 00013 (RM17)	Positive reaction: colour change to brick red after adding 1 or 2 drops of Nessler's reagent. Negative reaction: coloration changes to yellow.

Legionella latex kit	Agglutination detection	<i>Legionella</i> spp. identification	ISO 11731	Control kit reagents	Control kit reagents	Positive reaction: Visible agglutination. Negative reaction: No visible agglutination.
TSI Agar	Lactose, glucose and sucrose fermentation with gas formation	<i>Salmonella</i> spp. detection	ISO 19250	<i>S. enterica</i> serovar Typhimurium WDCM 00031 (RM9)	<i>P. aeruginosa</i> WDCM 00025 (RM18)	Positive reaction: Yellow butt with glucose fermentation; black coloration indicating hydrogen sulphite formation and gas formation; yellow slant indicating lactose and/or sucrose.
		Enterobacteriaceae enumeration and detection	Internal protocol			Negative reaction: Red butt indicating lack of glucose fermentation; Red slant meaning lack of lactose and sucrose fermentation.
Salmonella antisera	Agglutination detection	<i>Salmonella</i> spp. detection	ISO 19250	<i>S. enterica</i> serovar Typhimurium WDCM 00031 (RM9)	<i>E. coli</i> WDCM 00013 (RM17)	Positive reaction: Visible agglutination. Negative reaction: No visible agglutination.
Urea test	Urea hydrolysis detection with ammonia production	<i>Salmonella</i> spp. detection	ISO 19250	<i>S. enterica</i> serovar Typhimurium WDCM 00031 (RM9) or <i>S. enterica</i> serovar Enteritidis (RM7)	<i>P. mirabilis</i> (RM40)	Positive reaction: Lack of colour change and no ammonia production. Negative reaction: Pink coloration in the medium with ammonia production.
L-lysine decarboxylation medium	L-lysine decarboxylation	<i>Salmonella</i> spp. detection	ISO 19250	<i>S. enterica</i> serovar Typhimurium WDCM 00031 (RM9) or <i>S. enterica</i> serovar Enteritidis (RM7)	<i>P. mirabilis</i> (RM40)	Positive reaction: Colour change to purple. Negative reaction: No colour changes.
Chrom ID Salmonella Agar	Morphology observation	<i>Salmonella</i> spp. detection	ISO 19250	<i>S. enterica</i> serovar Typhimurium WDCM 00031 (RM9) or <i>S. enterica</i> serovar Enteritidis (RM7)	<i>E. coli</i> WDCM 00012 (RM3) or <i>E. coli</i> WDCM 00013 (RM17)	Positive reaction: Pink to purple-coloured colonies. Negative reaction: Lack of pink to purple coloration in colonies.

XLD Agar	Morphology observation	<i>Salmonella</i> spp. detection	ISO 19250	<i>S. enterica</i> serovar Typhimurium WDCM 00031 (RM9) or <i>S.</i> <i>enterica</i> serovar Enteritidis (RM7)	<i>E. faecalis</i> WDCM 00087 (RM4) or <i>E. faecalis</i> WDCM 00009 (RM19)	Positive reaction: all pink colonies with yellow margins and with a black center. Negative reaction: Other colony pigmentation.
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Supplementary Table 2 - Non-selective nutritive culture media composition comparison Adapted from Atlas, (2010).

	Yeast Extract Agar	TSA	PCA
Tryptone	6 g	---	5 g
Yeast extract	3 g	---	2.5 g
Casein peptone	---	15 g	---
Dextrose	---	---	1 g
Sodium chloride	---	5 g	---
Soy peptone	---	5 g	---
Agar	15 g	15 g	15 g
Deionized water	1000 mL	1000 mL	1000 mL

Supplementary Table 3 – Lowenstein-Jensen Agar culture medium composition. Adapted from Atlas, (2010).

Lowenstein-Jensen Agar	
Potassium dihydrogen phosphate	2.5 g
Magnesium sulphate	0.24 g
Magnesium citrate	0.6 g
L-asparagine	3.6 g
Potato flour	30 g
Malachite green	0.4 g
Glycerol	12 mL
Fresh egg yolk and whites	1000 mL
Deionized water	600 mL
Potassium dihydrogen phosphate	2.5 g
Magnesium sulphate	0.24 g
Magnesium citrate	0.6 g

Supplementary Table 4 - Selenite Cystine Broth culture medium composition. Adapted from Atlas, (2010).

Selenite Cystine Broth	
Tryptone	5 g
Lactose	4 g
Disodium hydrogen phosphate	10 g
L-cystine	0.01 g
Sodium biselenite	4 g
Deionized water	1000 mL

Supplementary Table 5 - RSV Broth culture medium composition. Adapted from Atlas, (2010).

RSV Broth		
Solution A (1000 mL)	Soy peptone	5 g
	Sodium chloride	8 g
	Potassium dihydrogen phosphate	1.4 g
	Dipotassium hydrogen phosphate	0.2 g
	Deionized water	1000 mL
Solution B (100 mL)	Magnesium chloride hexahydrate	400 g
	Deionized water	1000 mL
Solution C (10 mL)	Malachite green oxalate	0.4 g
	Deionized water	100 mL

Supplementary Table 6 - Chrom ID *Salmonella* Agar culture medium composition. Adapted from Atlas, (2010).

Chrom ID <i>Salmonella</i> Agar	
Peptone	6.25 g
Tris	0.16 g
Lactose	6 g
Bile salts	1.5 g
Sodium chloride	5 g
Niaproof	1 mL
Chromogenic mixture	12.46 mL
Selective mixture	0.025 g
Agar	14 g
Deionized water	1000 mL
Peptone	6.25 g
Tris	0.16 g

Supplementary Table 7 - XLD Agar culture medium composition. Adapted from Atlas, (2010).

XLD Agar	
D-xylose	3.75 g
L-lysine hydrochloride	5 g
Sodium deoxycholate	1 g
Yeast extract	3 g
Sucrose	7.5 g
Lactose	7.5 g
Sodium chloride	5 g
Sodium thiosulphate	6.8 g
Iron (III) ammonium citrate	0.8 g
Phenol red	0.08 g
Agar	13.5 g
Deionized water	1000 mL

Supplementary Table 8 - Urea Agar culture medium composition. Adapted from Atlas, (2010).

Urea Agar	
Enzymatic digest of peptone	1 g
Glucose	1 g
Sodium chloride	5 g
Potassium dihydrogen phosphate	2 g
Phenol red	0.012 g
Agar	15 g
Deionized water	1000 mL

Supplementary Table 9 - TSI Agar culture medium composition. Adapted from Atlas, (2010).

TSI Agar	
Meat extract	3 g
Yeast extract	3 g
Enzymatic digest of peptone	20 g
Sodium chloride	5 g
Lactose	10 g
Sucrose	10 g
Glucose	1 g
Iron (III) citrate	0.3 g
Sodium thiosulphate	0.3 g
Phenol red	0.024 g
Agar	13 g
Deionized water	1000 mL

Supplementary Table 10 - L-lysine decarboxylation medium composition. Adapted from Atlas, (2010).

L-lysine decarboxylation medium	
L-lysine monohydrochloride	5 g
Yeast extract	3 g
Glucose	1 g
Bromocresol purple	0.015 g
Deionized water	1000 mL

Supplementary Table 11 - CN Agar culture medium composition. Adapted from Atlas, (2010).

CN Agar	
Gelatine peptone	16 g
Casein hydrolysate	10 g
Potassium sulphate	10 g
Magnesium chloride	1.4 g
Glycerol	10 mL
Agar	15 g
Deionized water	1000 mL
Nalidixic acid	0.015 g
Hexadecytrimethyl ammonium bromide	0.2 g

Supplementary Table 12 - Acetamide Broth culture medium composition. Adapted from Atlas, (2010).

Acetamide Broth		
Solution A (900 mL)	Potassium di-hydrogen phosphate	1 g
	Magnesium sulphate	0.2 g
	Acetamide	2 g
	Sodium chloride	0.2 g
	Deionized water	900 mL
Solution B (1 mL)	Sodium molybdate	0.5 g
	Iron sulphate heptahydrate	0.05 g
	Deionized water	100 mL
Deionized water (99 mL)		

Supplementary Table 13 - King B Agar culture medium composition. Adapted from Atlas, (2010).

King B Agar	
Peptone	20 g
Glycerol	10 mL
Di-potassium hydrogen phosphate	1.5 g
Magnesium sulphate heptahydrate	1.5 g
Agar	15 g
Deionized water	1000 mL

Supplementary Table 14 - MacConkey Agar culture medium composition. Adapted from Atlas, (2010).

MacConkey Agar	
Peptone	17 g
Protease peptone	3 g
Lactose	10 g
Bile salts	1.5 g
Sodium chloride	5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Agar	13.5 g
Deionized water	1000 mL

Supplementary Table 15 - CCA culture medium composition. Adapted from Atlas, (2010).

CCA	
Enzymatic digest of casein	1 g
Yeast extract	2 g
Sodium chloride	5 g
Sodium dihydrogen phosphate	2.2 g
Di-sodium hydrogen phosphate	2.7 g
Sodium pyruvate	1 g
Sorbitol	1 g
Tryptophane	1 g
Tergitol 15-S-7 surfactant	0.15 g
Salmon- β -D-galactosid	0.2 g
X-beta-G-glucuronide CHX salt	0.1 g
Isopropyl- β -D-thiogalactopyranoside	0.1 g
Agar	15 g
Deionized water	1000 mL

Supplementary Table 16 - Lactose Tergitol 7 Agar culture medium composition. Adapted from Atlas, (2010).

Lactose Tergitol 7 Agar	
Pancreatic digest of meat	10 g
Meat extract	5 g
Yeast extract	6 g
Lactose	20 g
Tergitol 7	0.1 g
Bromothymol blue	50 mg
2,3,5 triphenyl tetrazolium chloride	25 mg
Agar	10 g
Deionized water	1000 mL

Supplementary Table 17 - Slanetz and Bartley Agar culture medium composition. Adapted from Atlas, (2010).

Slanetz and Bartley Agar	
Tryptose	20 g
Yeast extract	5 g
Glucose	2 g
Dipotassium hydrogen phosphate	4 g
TTC solution (1 g of 2,3,5-triphenyltetrazolium chloride + 100 mL of deionized water)	10 mL
Sodium azide	0.4 g
Agar	10 g
Deionized water	1000 mL

Supplementary Table 18 - Bile-Aesculin-Azide Agar culture medium composition. Adapted from Atlas, (2010).

Bile-Aesculin-Azide Agar	
Tryptone	17 g
Peptone	3 g
Yeast extract	5 g
Ox-bile	10 g
Sodium chloride	5 g
Aesculin	1 g
Ammonium iron (III) citrate	0.5 g
Sodium azide	0.15 g
Agar	15 g
Deionized water	1000 mL

Supplementary Table 19 - Comparison between ISA and TSC Agar culture media composition. Adapted from Atlas, (2010).

	ISA	TSC Agar
Enzymatic digest of casein	15 g	15 g
Enzymatic digest of soya	5 g	5 g
Yeast extract	5 g	5 g
Sodium disulphite	1 g	1 g
Iron (III) ammonium citrate	1 g	1 g
Agar	14 g	14 g
Deionized water	1000 mL	1000 mL
D-cycloserine solution	---	10 mL
D-cycloserine solution : 4 g D-cycloserine + deionized water 100 mL		

Supplementary Table 20 - Mannitol Salt Agar culture medium composition. Adapted from Atlas, (2010).

Mannitol Salt Agar	
Pancreatic digest of casein	5 g
Peptic digest of animal tissue	5 g
Beef extract	1 g
D-mannitol	10 g
Sodium chloride	75 g
Phenol red	0.025 g
Agar	15 g
Deionized water	1000 mL

Supplementary Table 21 - MEVAG Agar culture medium composition. Adapted from Atlas, (2010).

MEVAG Agar	
Plant peptone	2 g
Yeast extract	0.5 g
Glucose	10 g
Sodium chloride	30 g
Bromocresol Purple	0.015 g
Agar	3 g

Supplementary Table 22 - BP-RPF Agar culture medium composition. Adapted from Atlas, (2010).

BP-RPF Agar	
Pancreatic digest of casein	10 g
Beef extract	5 g
Yeast extract	1 g
Glycine	12 g
Sodium pyruvate	10 g
Lithium chloride	5 g
Agar	17 g
RPF supplement	100 mL
Pancreatic digest of casein	10 g
Beef extract	5 g

Supplementary Table 23 - RPF supplement composition for BP-RPF Agar. Adapted from Atlas, (2010).

RPF Supplement	
Bovine fibrinogen	3.75 g
Trypsin inhibitor	25 mg
Potassium tellurite	25 mg
Rabbit plasma	25 mL

Supplementary Table 24 - BCYE and GVPC Agar culture media composition comparison. Adapted from Atlas, (2010).

	BCYE Agar	GVPC Agar
Yeast extract	10 g	10 g
Activated charcoal	2 g	2 g
α-ketoglutarate	1 g	1 g
ACES buffer (2-[(2-amino-2-oxoethyl)-amino]-ethane sulfonic acid)	10 g	10 g
Potassium hydroxide	2.8 g	2.8 g
L-cysteine hydrochloride monohydrate	0.4 g	0.4 g
Iron (III) pyrophosphate	0.25 g	0.25 g
Ammonium-free glycine	---	3 g
Polymyxin B sulphate	---	80000 IU
Cycloheximide	---	0.08 g
Vancomycin hydrochloride	---	0.001 g
Agar	12 g	12 g
Deionized water	1000 mL	1000 mL