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To all, thanks you very much.

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I. Abbreviations and Acronyms

°C – degrees Celsius
% – percentage
µm – micrometer
µL – microliter
µM – micromolar
µg.µL^{-1} – microgram per microliter
µg.L^{-1} – microgramа per liter
µA – microampere
µ – specific growth rate
bp – base pairs
BSA – Bovine Serum Albumin
$CuSO_4$ – copper sulfate
cps – count per second
dNTPs – deoxynucleoside triphosphates
EDXRF – X-ray Fluorescence spectrometer scattered energy
eV – electronvolt
EMEPC – Estrutura de Missão para a Extensão da Plataforma equipa Continental
XRF – X-Ray Fluorescence
g – gram
g.cm^{-3} – grams per cubic centimeter
h – hours
H_2O_2 – hydrogen peroxid
HCl – hydrochloric acid
HNO_3 – nitric acid
kV – kilovolt
KH_2PO_4 – monopotassium sulfate
KCl – potassium chloride
L – liter
LB – Luria-Bertani
MA – Marine Agar
MB – Marine Broth
m – meter
MM – Minimal salt (M9) medium
MIC – Minimum Inhibitory Concentrations
min – minutes
mg – milligram
mg.mL^{-1} – milligram per millilitre
mL – milliliter
mm – millimeter
mM – millimolar
M – molar
nm – nanometers
NB – Nutrient Broth
Na_2HPO_4 – dissodium phosphate
NaCl – sodium chloride
NaOH – sodium hydroxide
$Na_2WO_4.2H_2O$ – sodium tungstate dehydrate
OD – Optical Density
pH – -log[H^+]
PBS – Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
RAST – Rapid Annotation using Subsystem Technology
R2A – Reasoner's 2A
RS – Reasoner's 2A with Seawater filtrate
rpm – rotation per minute
SWF – seawater filtrate

sec – seconds

TMPD - tetramethyl-p-phenylenediamine dihydrochloride

TAE – tris-acetate

TBE – tris-borate

$U.\mu L^{-1}$ – unit per microliter

v/v – volume/volume concentration

w/v – weigh/volume concentration

W – tungsten

YNB – Yeast Nitrogen Base

II. Abstract

Tungsten (W) is a heavy metal with an atomic number of 74, and it is the sixth group within the transition elements in the periodic table. This metal is the heaviest element that have a biological function. Worldwide, this heavy metal can be present in a few mines, and Portugal has one of the largest mines with W. Besides, it can also be found in higher concentrations, comparatively to seawater concentration, in hot black smokers, formed by hydrothermal vents (temperatures reaching 400°C). Because of the mix of high temperature hydrothermal fluids with cold oxygenated seawater, minerals precipitates to form mineral deposits. These mineral deposits have a rich microscopic diversity with organisms belonging to *Bacteria* and *Archaea* phylo.

W is the only third-row transition element that have biological activity being part of enzymes that catalyse oxygen atom transfer reactions. Tungstate (WO_4^{2-}) is the oxidized form of elemental W and the form that is internalized into the bacterial cells. W is internalized through a specific ABC-type family transporter. W can be taken up into the cells through three different transporter protein systems: TupABC (specific for WO_4^{2-}), WtpABC (transports WO_4^{2-} and molybdate (MoO_4^{2-})) and ModABC. The oxyanion WO_4^{2-} shows similar geometry to other oxyanion, namely to MoO_4^{2-}. Due to their similarities, MoO_4^{2-} is taken up into the cells by the same ABC-type transporter as WO_4^{2-}. Although, the chemical properties of these metals are sufficiently different to be distinguished by living organisms at their incorporation into enzymes.

Once the metal is inside the cell, the element binds to a molecule called pterin. In the case of W, it is coordinated to two pterines moieties molecules, forming the bis-pterin cofactor (Wco or tungstopterin). For molybdenum (Mo), it is coordinated by sulphur atoms in two dithiolene of a pterin molecule, called molybdopterin cofactor (Moco). Consequently, Mo and W only gain their biological activity upon incorporation into cofactors that serve as an anchor to provide the appropriate environment enabling efficient control of the redox properties of the metal.

The present work focus on one ABC-type transporter for W, namely TupABC transporter system. The objective was characterizing previously isolated W resistant strains, from Lucky Strike hydrothermal vents and uranium mine from

Urgeiriça, by determining their MIC and the presence of genetic determinants coding for protein transporters responsible for the uptake of W into the cells. At the end, this work will allow the selection of the more suit microorganism to construct of a whole-cell W bioacumulator.

Keywords: Tungsten, accumulation, *tupABC* operon, bacterial resistance and hydrothermal vents.

III. Resumo

Tungsténio (W) é um metal pesado com um número atómico de 74 e é o sexto grupo dos elementos de transição na tabela periódica. Este metal é o maior elemento que contém uma função biológica. Mundialmente, este metal pode estar presente em algumas minas, e Portugal tem uma das maiores minas com W. Além disso, também pode ser encontrado em elevadas concentrações, comparativamente com as concentrações da água do mar, em fumarolas negras quentes, formadas por fontes hidrotermais (temperaturas atingindo 400 °C). Devido à mistura de fluidos hidrotermais de elevadas temperaturas com água do mar oxigenada fria, minerais precipitam para formar depósitos de minerais. Nestes depósitos de minerais são encontrados alguns organismos pertencendo ao filo *Bacteria* e *Archaea*.

W é o único elemento de transição da terceira fila que tem uma actividade biológica, sendo parte de enzimas que catalisam reações de transferência de átomos de oxigénio. Tungstato (WO_4^{2-}) é a forma oxidada do elemento W e a forma como é internalizado para o interior das células bacterianas. W é internalizado através de um sistema transportador da família ABC. W pode ser transportado através três sistemas diferentes de transportadores proteicos: TupABC (específico para WO_4^{2-}), WtpABC (transporta WO_4^{2-} e molibdato (MoO_4^{2-})) and ModABC. O oxianião WO_4^{2-} apresenta uma geometria semelhante a outro oxianião, nomeadamente a MoO_4^{2-}. Contudo, as propriedades químicas destes metais são suficientemente diferentes para que os organismos vivos consigam distinguir um do outro na sua incorporação nas enzimas.

Uma vez que o metal esteja no interior da célula, o elemento liga-se a uma molécula designada por pterina. No caso de W, é coordenado a duas metades de moléculas pterinas, formando o cofator bi-pterina (Wco ou tungstopterina). Para molibdénio (Mo), é coordenado por dois átomos de enxofre em ditioleno de uma molécula pterina, chamada de cofator molibdopterina (Moco). Consequentemente, Mo e W apenas ganham a sua actividade biológica após incorporação em cofatores que servem como âncora para proporcionar o ambiente adequado que permita um controlo eficiente das propriedades redox do metal.

O presente trabalho tem como foco um dos transportadores ABC para W, nomeadamente o sistema transportador TupABC. O objetivo foi caracterizar estirpes resistentes a W previamente isoladas, de fontes hidrotermais Lucky Strike e de minas de urânio da Urgeiriça, por determinação do seu MIC e a presença de determinantes genéticos para transportadores responsáveis pela acumulação de W para o interior das células bacterianas.

Palavras-chave: Tungsténio, acumulação, operão *tupABC*, resistência bacteriana e fontes hidrotermais.

IV. Introduction

1. Raw materials and their importance

Raw materials and their applications are vital for the reindustrialization and competitiveness of Europe, in a world where a large proportion of primary resources are produced outside Europe and controlled by a small number of countries, with an important impact in their economies. In the past, raw materials exploitation was focused in the economically high grade ore deposits, extracted and processed by conventional techniques. The metal recovery efficiency of these techniques was variable and not very high, and, consequently, is expected that a significant amount was discarded to the tailings dams (Kikuchi, T., *et al.* 2012).

Tailings from mining and metallurgical processes represent a potential environmental problem because of their metal content. Tailings used to be considered as mine waste to be stored for perpetuity. This view is now obsolete in the light of research efforts to recover or remove residual metals and define end points for tailings management, they should be seen as a potential ore able to be recovered, preferable by more ecofriendly methods, and not as pure waste (McCarthy, S., *et al.* 2014).

Microbes interact with metals and minerals in natural and synthetic environments, altering their physical and chemical state. On the other hand, metals and minerals are also able to affect directly and/or indirectly the microbial growth, activity and survival. Metals and their compounds interact with microbes in various ways depending on metal species, organism and environment. While structural components and metabolic activity influence metal speciation and, therefore, solubility, mobility, bioavailability and toxicity (Gadd, G. 2010). Microbes produce substances that can disturb the speciation of inorganic compounds. Biominerals are the products of the selective uptake of elements from the local environment and their incorporation into functional structures under strict biological control (Gadd, G. M., 2010).

2. Bacteria included in this study and their origin

2.1. Sediments from hydrothermal vents

During the oceanographic mission to the Mid-Atlantic Ridge hydrothermal vent field, Lucky Strike, with the *Estrutura de Missão para a Extensão da Plataforma equipa Continental* (EMEPC) team in EMEPC/Luso/2009 campaign (including the ROV pilots and the crew on board NRP Gago Coutinho) sediments samples were collected (at 37° 17 N, 32° 16 W and at a depth of 1695 meters (m)). Different cultivation methods showed that these sediments contained different bacteria.

Near hydrothermal vents, the mixture of hot and cold fluids lead to dissolved materials to precipitate and form energy-rich and heavy metal-rich solid surfaces (Zierenberg, R. A., *et al.* 2000). The general geochemical conditions consist of:

- elevated temperatures (between 300 and 400 degrees Celsius (°C));
- absence of sulphate;
- high and variable concentrations of sulphite, methane, hydrogen and metals.

The release of cations like divalent calcium (Ca^{2+}), divalent magnesium (Mg^{2+}) and sodium (Na^{+}) lead to the formation of mineral containing hydroxyl groups consequently hydrogen ions are released into solution. The water, hot and acidic, releases reduced sulphur (H_2S) and metals iron (Fe), manganese (Mn), zinc (Zn), arsenic (As) and copper (Cu), trough redox reaction, from the volcanic rock (Zierenberg, R. A., *et al.* 2000). This geological events lead to high concentrations of heavy metals in the water environment. Heavy metals are metals with a high density metal (≥ 5 grams per cubic centimeter ($g.cm^{-3}$)), that constitutes the majority of the naturally occurring elements in the periodic table. Of the nighty occurring elements from the periodic table, twenty-one are nonmetals, sixteen are light metals and fifty-three are heavy metals. Not all elements are relevant in a biological context, but those that have a role in an organism can be diverse and reach over several biochemical reactions. Any metal that have a biochemical function, in other words, biometals, can easily form complex compounds, which may, or may not, be redox-active. Because they can form complex compounds,

some heavy metal ions are essential trace elements, but essential or not, most of them are toxic at higher concentrations (Nies, D. H. 1999).

Concentrations of tungsten (W) in seawater are 0.1 microgram per liter ($\mu g.L^{-1}$) but are dramatically increased in hydrothermal vents, where many organisms expressing W-dependent enzymes are found. From the evolutionary point of view, such high affinity and specificity uptake systems allow bacteria to scavenge the less abundant molybdate (MoO_4^{2-}) or tungstate (WO_4^{2-}) oxyanions in the presence of competing anions, such as sulfate, whose concentration in sea water is about 10^5 times higher than that of MoO_4^{2-} (Schawarz, G., *et al* 2007).

The isolation and characterization of bacteria from the hydrothermal sediments, showed that they belonged to different species, namely: *Sulfitobacter dubius*, *Sulfitobacter pontiacus*, *Sulfitobacter litoralis*, *Mesoflavibacter zeaxanthinifaciens*, *Halomonas meridiana*, *Staphylococcus saprophyticus*, *Dokdonia donghaensis*, *Erythrobacter citreus, Aurantimonas litoralis*, *Gaetbulibacter jejuensis*, *Maribacter dokdonensis*, *Maribacter forsetii* and *Winogradskyella poriferorum*.

2.2. Sediments from uranium mine

Other sediments samples were collected prevenient from contaminated groundwater uranium mine of Urgeiriça (Central Portugal). After various tests, it was found that they contained bacteria. After their isolation, it was found that these bacteria belonged to different genus, such as: *Williamsia*, *Rhodococcus*, *Paenibacillus*, *Bacillus*, *Micrococcus, Paenibacillus, Rhodanobacter, Arthrobacter, Exiguobacterium, Sphingomonas, Terrabacter, Aeromonas and Flavihumibacter*. Former uranium mines are a source of environmental contamination, since the leaching of acid water resulting from mining activity, which can transfer heavy metals and radioisotopes to the surrounding environmental compartments With this, the W heavy metal may be involved in the transfer to the surrounding environmental compartments.

3. Bacterial metal resistance mechanisms

The introduction of heavy metals in the environment, in various forms, can produce modifications of the microbial communities and their activities. Heavy metals generally exert an inhibitory action on microorganisms. This could happen by blocking essential functional groups, displacing essential metal ions or modifying the active conformations of biological molecules (Hassen, A., *et al.* 1998). Nevertheless, to have any physiological or even toxic effect, most heavy metal ions have to enter the cell. Most cells solve this problem by using two types of uptake system, one is fast and it is unspecific, and the other system is slower and has a high substrate specificity. The unspecific systems are constitutively expressed, since they are used by a variety of substrates, and they are usually driven only by the chemiosmotic gradient across the cytoplasmic membrane of bacteria. In other hand, the specific systems used ATP hydrolysis as the energy source, sometimes in addition to the chemiosmotic gradient, and they are inducible. These kind of expensive uptake systems are only produced by the cell in times of need, starvation or a special metabolic situation. The tolerance to heavy metals depends on numerous factors, such as (Nies, D. H. 1999; Hasse, A., *et al.* 1998):

- environment in which they are found;
- metal bioavailability;
- their concentration.

3.1. Tungsten

WO_4^{2-} oxyanion is the main source of essential metal W, with an atomic number of 74 (Russ, H. 2002). W (Swedish: "tung + sten" = heavy stone) is the sixth group within the transition elements in the periodic system like chromium and molybdenum (Mo) (Andreesen, J. R., *et al*. 2008), and has been also reported as an essential trace element for some *Bacteria* and *Archaea*, as we can see in Figure 1 (Bevers, L. E., *et al.* 2006; Barajas, E.A., *et al.* 2011).

W is found as oxo-rich tungstate minerals (W(VI)), as scheelite ($CaWO_4$) and as wolframite ($(Fe/Mn)WO_4$) while the more reduced tungstenite (WS_2 (W(IV)) is very rare (Kietzin, A., *et al.* 1996).

This metal is the heaviest element with a biological function in some special habitats, namely hot black smokers or sulfide-enriched waters, which contain higher concentrations of W. However, it is not a universal bioelement and it is the only third-row transition element that exhibits biological activity in enzymes (Bevers, L. E., *et al.* 2006; Andreesen, J. R., *et al*. 2008; Bevers, L., *et al.* 2009). For some species, this metal is essential, where their life depends on the presence of the element. For other species, W is a facultative bioelement, where they choose to make biological use of the element but only when they experience specific environmental constraints. For the remaining species, W is biochemical indifferent or possibly a foreign chemical substance: they have not developed a functional use of the element, although, upon its inadvertent intake, their physiology may well be affected (Bevers, L., *et al.* 2009).

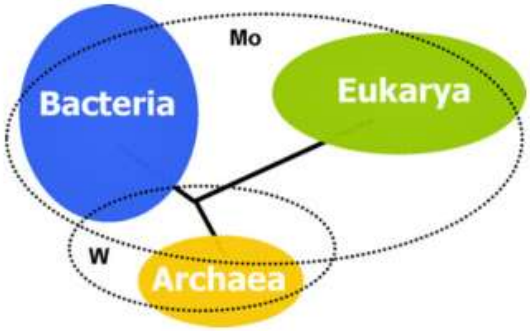

Figure 1. The tree of the three domains of life and the distribution of tungsten- and molybdenum-containing enzymes: molybdenum enzymes are found in all forms of life whereas the occurrence of tungsten enzymes appears to be restricted to *Archaea* and some *Bacteria* (Adapted from Bevers, L*., et al.* 2009).

3.2. Tungsten *vs.* Molybdenum (Mo) – commonalities and differences

The WO_4^{2-} oxyanion shows similar geometry to other oxyanion, namely molybdate (MoO_4^{2-}). The close chemical and physical similarities (Table I) to each of them are based on equal atomic and ionic radii, similar electronegativity, and coordination characteristics. The similarities between these oxyanions lead to a lack of discrimination by biological systems and, thus, to the toxicity of WO_4^{2-} (Bevers,

L. E., *et al.* 2006; Andreesen, J. R., *et al*. 2008). Generally, the content of Mo is somewhat higher in natural abundance than W, being the 54th and 55th elements when ranking them according to their general abundance on earth. Whereas, sulfides of Mo are generally insoluble, sulfides of W are more soluble, especially in their form as thio-tungstates (precursors of Mo and W sulfide) (Andreesen, J. R., *et al*. 2008).

Table I. Physical and chemical properties of the tungsten and molybdenum (Adapted from Kietzin, A.*, et al.* 1996).

Element	Tungsten (W)	Molybdenum (Mo)
Atomic number	74	42
Average atomic weight	183.85	95.94
Stable isotopes (above 9% abundance)	182, 183[a], 184, 186	92, 94, 95, 96, 97, 98
Unstable isotopes used in labelling	185 (β^-)	99 (β^-)
Electronic configuration of outer shell	$4f^{14}\ 5d^4\ 6s^2$	$4d^5\ 5s^1$
Atomic radii (Å)	1.40	1.40
Ionic radii for IV oxidation state (Å)	0.68	0.68
Electronegativity	1.4	1.3
pK_a of oxo acid (MO_4^{2-}/HMO_4^-)[b]	4.60	3.87
Solubility (g/L) of Na_2MO_4 (25 °C)[c]	0.86	1.33
M = O bond length (Å)	1.76	1.76
Melting point (°C)	3410	2610
Density (g/cm^3)	19	10

[a] ^{183}W has an unstable nuclear isomer; [b] M represents W or Mo; [c] There is great variability in the reports on the solubility of tungstate salts.

W has been regarded as an antagonist of the biological functions of Mo. Due to the great similarities in the properties of the two elements, it was reasoned that insight into the catalytic role of Mo in various enzymes might be provided by replacing Mo with W. Clearly, the chemical properties of both metals are sufficiently different that living organisms can distinguish between them, either at the levels of their incorporation into enzymes or in the properties of themselves, which function with Mo but not with W (Johnson, M. K., *et al.* 1996). It is reported that small

cytoplasmic molybdate and tungstate Mop proteins are produced from some bacteria, which is involved in oxyanion storage or homeostasis (Barajas, E. A., *et al.* 2011).

The W content of most rock formations is similar to that of Mo, but in surface waters the solubility and mobility of W is low when compared to Mo, due to intensive sorption precipitation. In sea water, the concentration of W is extremely low and about 500 000 fold less than that of Mo, as we can see in Table II. Despite some marine sediments contain quite high W concentrations (Kietzin, A., *et al.* 1996).

Table II. Distribution of tungsten and molybdenum in natural environments (Adapted from Kietzin, A., *et al.* 1996).

Element	Tungsten	Molybdenum
Earth's crust (mg/kg)	1.55	1.55
Rank	54th	53 th
Soils (mg/kg)	0.1 – 3.0	0.3 – 39
Seawater (µg/kg)	0.0002	8.9 – 13.5
Freshwater (µg/kg)	< 0.1	0.1 – 5.0
Terrestrial hot springs (µg/kg)	15 – 300	3 – 60
Keough hot sping (µg/kg)	300	N.R.[a]
Searles lake brines (µg/kg)	70.000	N.R.
Juan de Fuca, vent fluids (µg/kg)	0.37	< 0.002
Juan de Fuca, chimney walls (mg/kg)	N.R.	6 – 120
Juan de Fuca, vent flanges (mg/kg)	180 – 585	47
TAG, mid-Atlantic relict zones (mg/kg)	N.R.	40 – 240
Most abundant valance state (complex)	VI (WO_4^{2-})	IV (MoS_2)

[a] Not Reported.

4. Utilization in biological systems

The WO_4^{2-} and MoO_4^{2-} oxyanions, despite showing similar geometry, also present the metalliferous precursor molecules for cofactors that use Mo and W as catalytically active metals for a diverse array of redox reactions. Mo and W gain their biological activity upon incorporation into cofactors that serve as an anchor to provide the appropriate environment enabling efficient control of the redox properties of the metal. These cofactors have in common that in most cases sulfur atoms form three ligands of the respective metal together with two oxygen atoms (Schawarz, G., *et al.* 2007).

Mo and W are present mainly in enzymes that catalyze oxygen atom transfer reactions (Bevers, L. E., *et al.* 2006). In these enzymes they are coordinated by the sulfur atoms in two dithiolene of a pterin molecule, called molybdopterin cofactor (Moco) and, in the case of W, the metal is always coordinated by two pterin moieties, forming the so called bis-pterin cofactor (Wco or tungstopterin) (Bevers, L. E., *et al.* 2006; Barajas, E.A., *et al.* 2011). Both metals are also required for assembly and function of molydoenzymes and tungstoenzymes, respectively (Barajas, E.A., *et al.* 2011). Tungstoenzymes are considered to be more ancient than molybdoenzymes (Andreesen, J. R., *et al*. 2008).

4.1. Tungstoenzymes

W-containing enzymes, or tungstoenzymes, are mainly found in anaerobic prokaryotes, as well as hyperthermophilic archaea (obligatory tungsten-dependent) and have been also identified in aerobic proteobacteria (Barajas, E.A., *et al.* 2011). These enzymes can be divided into different categories, namely the aldehyde oxidoreductases (AORs) and the formate dehydrogenases (FDHs) (Bevers, L. E., *et al.* 2006; Johnson, M. K., *et al.* 1996).

5. ABC transporter

ABC-type transporter family is a transporter that allows the uptake of several substrates into the cells. Mo and W are two heavy metals that are transported by this system.

These kind of transporters allow bacteria to scavenge the less abundant MoO_4^{2-} or WO_4^{2-} ions in the presence of sulfate, whose concentration in see water is ca.10^5 times higher than that of MoO_4^{2-}. Due to the similarity in charge, shape and size between MoO_4^{2-} and WO_4^{2-} oxyanions, these may be transported by the same carrier (Barajas, E.A., *et al.* 2011).

All systems are members of the adenosine triphosphate (ATP) binding cassette (ABC) transporter family. The majority of these oxyanion transporters consist of three proteins (Figure 2). The "A" protein is responsible for the recognition and binding of the substrate and it is located in the periplasm, which is the space between the cytoplasmic membrane and:

i. The cell wall in Gram-positive bacteria;
ii. The outer membrane in Gram-negative bacteria;
iii. The S-layer in archaea.

The "B" component forms the transmembrane pore through which the substrate is transported into the cell. This transport is facilitated by the ATP hydrolyzing activity of component "C" on the inner surface of the membrane of the respective cell (Bevers, L., *et al.* 2009).

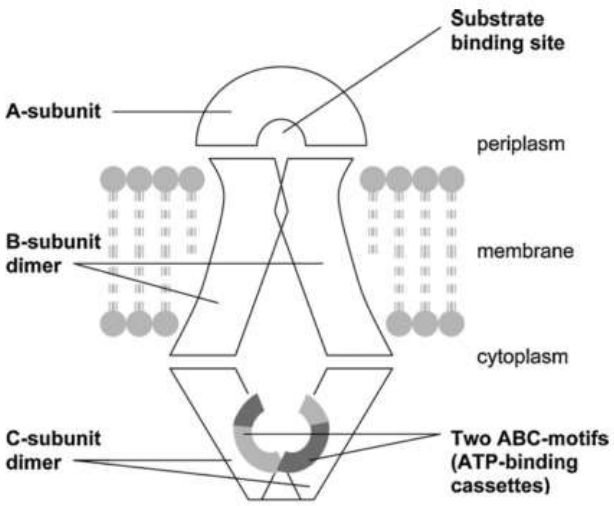

Figure 2. Schematic drawing of a molybdate ABC transporter. This import system has subunit stoichiometry AB_2C_2. The soluble, periplasmic A-protein scavenges the oxyanion and then binds to the membrane complex, which consists of a pair of translocating, transmembrane B-subunits bound to a cytoplasmic pair of C-subunits with ATPase activity (Adapted from Hagen, W. 2011).

These metals can be taken up into the cells by different transporters, as we can see in the Table III and in Figure 3. MoO_4^{2-} may be taken up through three transport systems, namely:

- High-affinity ModABC system (Maupin-Furlow, J. A., *et al.* 1995);
- CysPTWA (SulT) sulfate-thiosulfate permease (Rosentel, J. K., *et al.* 1995);
- Non-specific low-efficiency anion transport system, which requires high MoO_4^{2-} concentrations (Rosentel, J. K., *et al.* 1995).

WO_4^{2-} is taken up by bacterial cells through tree ABC-type transporters, such as:

- TupABC, which is highly specific for WO_4^{2-} (Makdessi, K., *et al.* 2001);
- WtpABC, which transports both WO_4^{2-} and MoO_4^{2-} (Bevers, L. E., *et al.* 2006);
- ModABC transporter (Grunden, A. M., *et al.* 1997).

Table III. Bacterial transporters of molybdate and tungstate (Adapted from Barajas, E.A., *et al.* 2011).

Transporter	Family	TC number[a]	Substrates	Organisms
ModABC	Molybdate uptake transporters MolT	3.A.1.8	Molybdate Tungstate	*Escherichia coli*
Sulfate/Thiosulfate permease	Sulfate/tungstate uptake Transporter SulT	3.A.1.6	Sulfate Chromate Molybdate Tungstate	*Escherichia coli*
Nonspecific anion transporter system	N.R.[b]	N.R.	Molybdate Sulfate Selenate Selenite	*Escherichia coli*
TupABC	Sulfate/tungstate uptake Transporter SulT	3.A.1.6	Tungstate	*Eubacterium acidaminophilum*
WtpABC	Sulfate/tungstate uptake Transporter SulT	3.A.1.6	Tungstate Molybdate	*Pyrococcus furiosus*

[a] According to the Transport Classification Database (TCDB); [b] No Report in the TCDB (gene not identified).

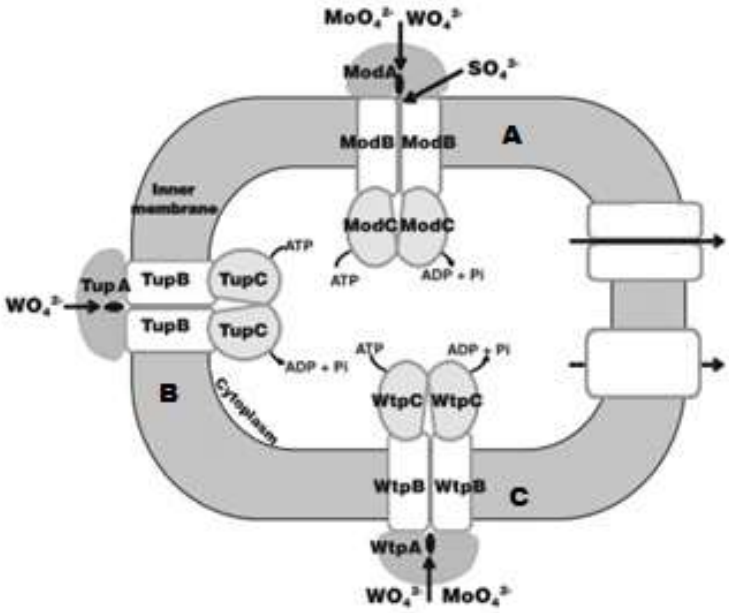

Figure 3. Bacterial oxyanion transporters. **A:** molybdate transporters; **B:** tungstate transporters; **C:** tungstate and molybdate transporters (Adapted from Barajas, E.A., *et al.* 2011).

The WO_4^{2-} transporter, namely TupABC, is not present in all organisms that produce tungstoenzymes. The hyperthermophilic *Archaea* seem to contain neither TupABC nor ModABC on their genome and, with this, opens the possibility

for a new transporter system, which is different from TupA (periplasmic component of the TupABC transporter) and ModA (periplasmic component of the ModABC transporter), namely WtpABC. This was confirmed when WtpABC transporter has been identified in the genome of *Pyrococcus furious*. This type of transporter is present in organisms that express tungstoenzymes and do not contain TupABC and is almost exclusively present in *Archaea*, while TupA is predominantly present in *Bacteria* (Schawarz, G., *et al.* 2007).

ModA, WtpA (periplasmic component of the WtpABC transporter) and TupA were used to build a phylogenetic tree, as we can see through Figure 4. In addition, to examine the evolutionary relationship of Mo/W transport system in different organisms. The periplasmic components of sulfate and Fe^{3+} transporters, which have a low level of similarity to ModA, were used as a reference.

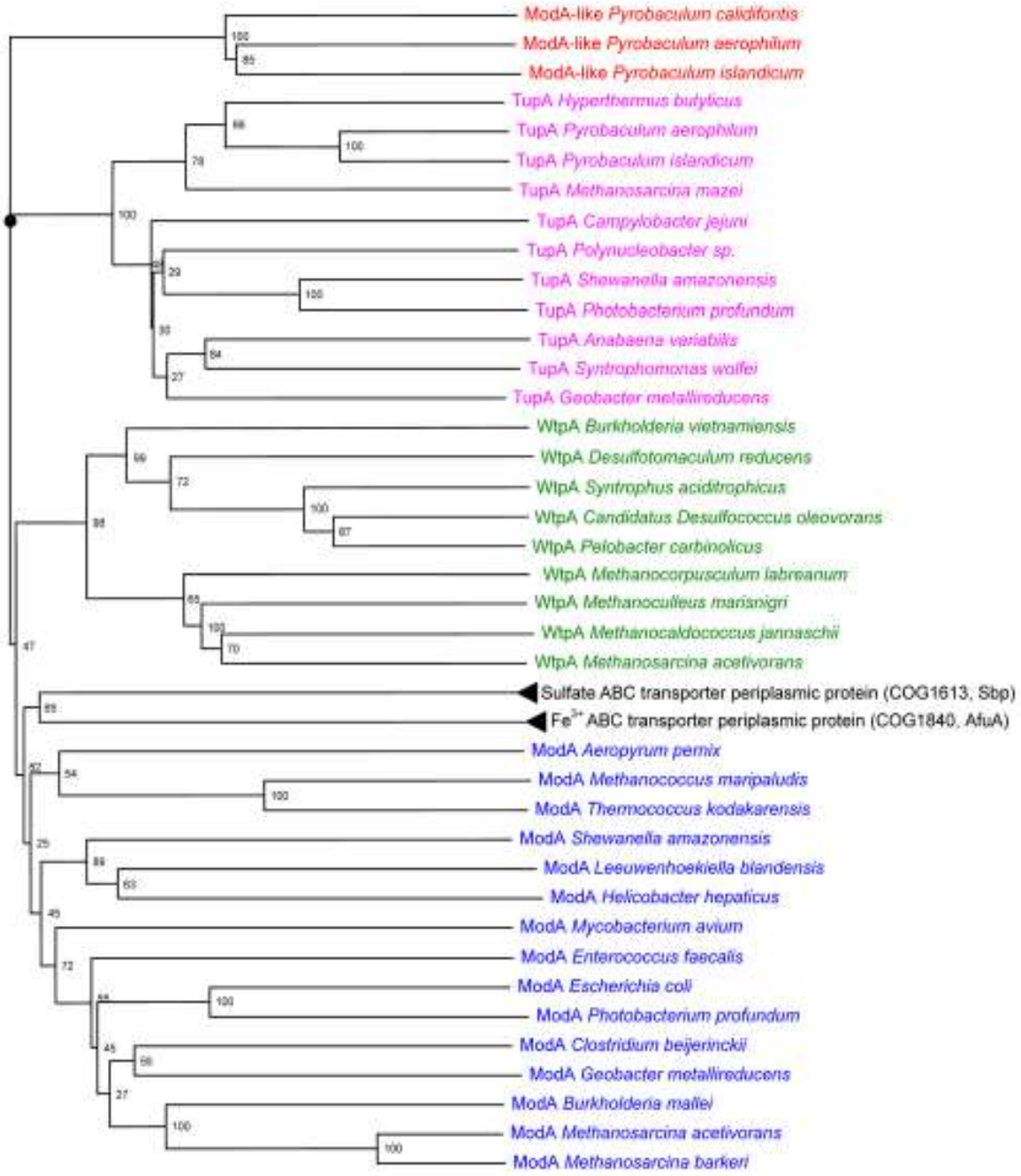

Figure 4. Phylogenetic tree of periplasmic components of Mo/W transporters in prokaryotes. ModA-like proteins are shown in red and bold, TupA in purple, WtpA in green and ModA in blue. Representative sequences were selected from a large number of orthologous proteins based on sequence similarities. The sulfate and Fe^{3+} ABC transporter branches were compressed and represented by family names. The measurement of distance for the branch lengths *(shown by a bar) (Adapted from Zhang, Y., et al. 2008).*

With this figure, we can see that they cluster in different branches, suggesting that all of them may be derived from a common ancestral gene. Then, they have diverged from the parent copy by mutation and selection or drift. Because of the homology in their sequences, it is possible that this could be involved in the ligation of the substrate.

5.1. ModABC transporter system

The high-affinity ModABC system, Figure 3A, that also transport WO_4^{2-} and sulfate, belongs to the molybdate uptake transporter family. This transporter has been well characterized in *Escherichia coli* and it is encoded by the *modABC* operon (Barajas, E.A., *et al.* 2011; Bevers, L., *et al.* 2009).

5.2. WtpABC transporter system

WtpABC, at Figure 3, was discovered in the hyperthermophilic archaeon *Pyrococcus furiosus* and, as said before, it is able to take up both WO_4^{2-} and MoO_4^{2-} (Barajas, E.A., *et al.* 2011; Bevers, L., *et al.* 2009). WtpA has a higher affinity for W than ModA and its affinity for Mo is similar to that of ModA (Zhang, Y., *et al.* 2008).

5.3. TupABC transporter system

TupABC, at Figure 3B, was identified for the first time in the mesophilic bacterium *Eubacterium acidaminophilum* (Bevers, L., *et al.* 2009). TupA, as said before, is a binding protein, in this case for WO_4^{2-}, being highly specific (Bevers, L. E., *et al.* 2006). It does not bind molybdate or even sulfate, chromate, selenite, phosphate or chlorate (Zhang, Y., *et al.* 2008). Indeed, it shows only weak binding of molybdate and sulfate when present in more than 1000-fold molar excess (Schawarz, G., *et al.* 2007).

6. Quantification of W by X-ray Fluorescence spectrometer scattered energy (EDXRF)

X-ray Fluorescence (XRF) is the emission of characteristic secondary, or fluorescent, X-rays from a material that has been excited by bombarding with high-energy X-rays or gamma rays. This technique is widely used for elemental and chemical analysis, particularly in the investigation of metals, glass, ceramics and building materials. In the present work, it was used the Epilson 3^{XLE} instrument, which is an EDXRF bench (Figure 6). EDXRF is also used for elemental analysis. The difference from this to XRF is that all of the elements presented in the sample are excited simultaneously. An energy disperse detector in combination with a multi-channel analyzer is used to simultaneously collect the fluorescence radiation emitted from the sample and then separate the different energies of the characteristic radiation from each of the different sample elements.

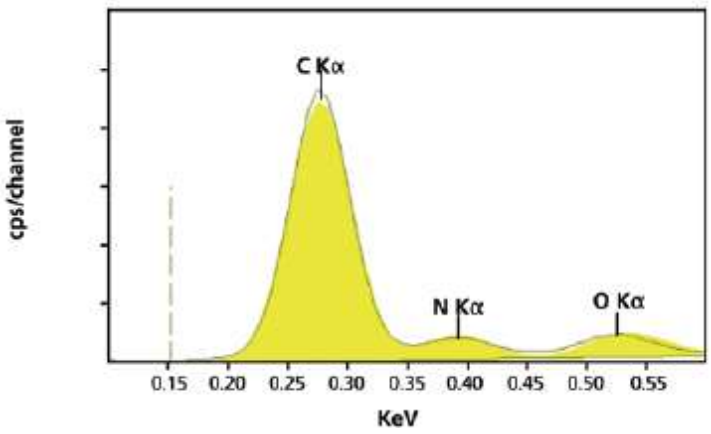

Figure 5. Fluorescent well defined peak of 85 % of carbon in a polymer sample.

EDXRF is a technique that as advantages, such as non-destructive; adequate for solids, liquids and powders; simple preparation of samples, fast and secure; precise and highly reproducible data; wide range of analytic concentration; very low operating costs and, precise and reliable analysis. The XRF spectroscopy does not require dissolution of the sample. To avoid the potential inaccuracies caused by incomplete dissolution, the complete analysis offered by XRF helps to ensure the accuracy and reliability of the results.

Figure 6. X-ray fluorescence spectrometer scattered energy (EDXRF), Epilson 3^{XLE}, bench used in this study (adapted from http://www.panalytical.pt/Epsilon-3XLE.htm).

V. Objectives

The main objective of this work was to detect and characterize strains with the ability to accumulate tungsten (W) and for those showing W accumulation ability, cells were screened for the *tup* genetic determinants and their organization in the operon determined.

To fulfil this objective, the first task in this work focused on obtaining the best growth conditions (varying the medium, the temperature and also the salinity), for two strains (*Sulfitobacter dubius* NA4 and *S. dubius* As(V)4) that we know to have *tupABC* operon. The next task was to screen *tup* genes in all strains previously isolated from Lucky Strike hydrothermal vent and from uranium mine of Urgeiriça. The organization of the *tup* determinants in the *tupABC* operon was studied in the third task. The genes were identified using molecular typing tools and bioinformatics analysis. The last task focused in the characterization of strains resistance to W and, finally, in testing some strains to their ability to accumulate W into their cells.

VI. Materials and Methods

1. Samples

In the next tables, are listed the different bacteria used from Mid-Atlantic Ridge hydrothermal vent field, Lucky Strike (Table IV), and from uranium contaminated groundwater from Urgeiriça mine (Central Portugal) (Table V).

Table IV. Isolated strains from Mid-Atlantic Ridge hydrothermal vent field, Lucky Strike, and their isolated name.

Bacterial strain	Isolate name
Sulfitobacter dubius	As(V)4
	NA4
	Sb5
	Cr2
	Gm5
Sulfitobacter pontiacus	Amp1
Sulfitobacter litoralis	Cu7
Mesoflavibacter zeaxanthinifaciens	Te13
	Kn1
	Gm11
Halomonas meridiana	Cr1
	Te14
Staphylococcus saprophyticus	Cu4
Dokdonia donghaensis	As(III)2*
Erythrobacter citreus	Kan3
	Gm3
	Gm6
	NA16
	NA19
	NA6
	Zn1
Aurantimonas litoralis	As(V)2
Gaetbulibacter jejuensis	Te11
Maribacter dokdonensis	Te7
	Te2
Maribacter forsetii	Te4
Winogradskyella poriferorum	Te6

Table V. Isolated strains from uranium contaminated groundwater from Urgeiriça mine (Central Portugal) and their isolated name.

Bacterium	Isolated name
Williamsia sp.	A2 614
Rhodococcus sp.	A2 937
	A2 67
Paenibacillus sp.	7B 841
Bacillus sp.	7B 635
	7B 900
	7B 645
Micrococcus sp.	A2 984
Paenibacillus sp.	7B 637
Rhodanobacter sp.	7B 583
Arthrobacter sp.	A2 64
	7B 214
Exiguobacterium sp.	A1 19
Sphingomonas sp.	A2 49
Terrabacter sp.	A2 62
Aeromonas sp.	7A 204
Flavihumibacter sp.	7B 231

2. Best conditions of growth

The two strains, *Sulfitobacter dubius* NA4 and *S. dubius* As(V)4, were tested in different solid media (with exception of Marine Agar (MA)), namely Reasoner's 2A agar (R2A), Nutrient Broth (NB) (normal and diluted 1:10), Luria-Bertani (LB) (normal and diluted 1:10) and Minimal salt (M9) medium (MM). R2A with seawater filtrate (RS) was used as control. With this, each medium was tested in different temperatures, namely at 22 degree Celsius (°C), 25°C, 30°C and 37°C and also in different salinities, 1 percentage (%), 2% and 3% of chloride sodium (NaCl). The media was placed for incubation in its appropriate temperatures, for a maximum of 5 days.

2.1. Growth test

The others isolated strains were inoculated to plates of LB medium with 3% of NaCl, to keep it under the same conditions.

The isolated strains that grew in LB agar with 3% of NaCl were used to the next test.

3. Bacterial Characterization

3.1. API ZYM

API ZYM test strips (bioMérieux) is a semi-quantitative method, which was used for the detection of enzyme activities (Table XXIII in annexes), according to the manufacture's instructions and incubated at 22°C.

3.2. API 20NE

API 20NE test strips (bioMeriéux) is a standardized system that was used for characterization of non-fastidious, non-enteric Gram-negative rods with the combination of several tests (Table XXIV in annexes), according to the manufacture's instructions and incubated at 22 °C.

3.3. Biolog GN2 MicroPlates

The ability to oxidize different carbon sources (Figure 33 in annexes) were determined by Biolog GN2 MicroPlates, incubated at 22°C for 2 days. For the realization of this test, a bacterial suspension with sterilized milli-Q water was made.

3.4. Gram staining

The cells staining were made by Hucker method. First, the preparation of blades and fixation were made. A drop of sterile NaCl 0.85% was placed in the center of the blade and the respective bacteria was transferred into the middle blade with a sterile loop, the cells were spread through circular movements. The swab was left to air dry completely. Then, the cells were fixed to the blade by heat, passing them two a three times through the flame. The respective blades with swabs were covered up with crystal violet for 1 minute (min) and then washed gently with running water. They were covered up with lugol solution for about 1 min and then washed with running water. For 30 seconds (sec), they were covered up with alcohol 95% or ketone, and then with safranin for 1 min. Between the two solutions, the blades were washed with running water. The excess water was removed with paper and then dry to air dry completely. The results were observed under an optical microscope bright field at a magnification of 1000x, using the oil immersion. The cells stained of violet were found to be gram-positive bacteria and the cells stained of red or pink were found to be gram-negative bacteria.

3.5. Endospore staining

For the detection of endospores it was followed the Schaeffer-Fulton method. The preparation of the blades and the fixation were made as before. The blades were placed on the top of a cup with boiling water on a hot plate and it was covered up with malachite green 0.5% weigh/volume concentration (w/v), leaving act for 2 a 3 min. After this, they were gently washed with running water. The preparations were covered up with safranin, leaving act for 30 sec. The swabs were washed again with water and after drying the preparations were observed under an optical microscope bright field at a magnification of 1000x, using the oil immersion. With this, the cells show a red color and the endospores a green color.

3.6. Flagella staining

The flagella staining was performed according to the Kodaka method. In a blade, the cellular material was prepared and this between the blade and the flap. A drop of Ryu was placed at one end of the blade. With a filter paper at the other end of the blade, the dye was spreading with the cellular material. The results were observed under an optical microscope bright field, at a magnification of 1000x.

3.7. Capsule staining

The capsule staining methodology was adapted from Anthony, E. E. Jr. (1931). The organism was not grown in milk broth or litmus milk, so serum protein was used to prepare the swab. The bacterial suspension was prepared in a solution containing Bovine Serum Albumin (BSA) 0.05% (milligram per milliliter ($mg.ml^{-1}$)) and from this suspension a volume of 40 microliter (µL) was stricken on a blade and left to air dry. The swab was covered up with crystal violet for 2 min and then it was rinsed gently with a solution of copper sulfate ($CuSO_4$) 20%. The blade was left to air dry completely. After this, the preparation was observed under an optical microscope bright field at a magnification of 1000x, using the oil immersion.

3.8. Oxidase test

Oxidase test was made with the objective to see if a bacterium produces cytochrome c oxidase.

Some drops of a solution of tetramethyl-p-phenylenediamine dihydrochloride (TMPD) 1%, prepared immediately before used, was added to a piece of filter paper. Some colonies were transferred, with a toothpick, to a filter paper and spread through circular movements. Develops a violet or purple color within 10 sec.

3.9. Catalase test

A small amount of organism from well-isolated 48 hours (h) colony was placed into the slide using a sterile inoculated loop. Then, using a dropper or Pasteur pipette, one drop of 3% hydrogen peroxide (H_2O_2) volume/volume (v/v) onto the organism. Develops, immediately, bubble formation.

4. DNA extraction

DNA from isolated organisms were extracted as described below.

First, a suspension was made where one loop, with the respective bacteria, was resuspended in an eppendorf of 1.5 milliliter (mL) with 100µL of Lysozyme. The suspension was placed in a bath at 37°C overnight. In the next day, 500µL of GES was added, at room temperature, and then it was agitated and incubated 10 min. 250µL of ammonium acetate (previously placed on ice) was added and, once again, it was agitated and incubated 10 min, 500µL of chloroform-isoamyl (24:1) was added. It was made a vortex and centrifuged for 15 min at maximum speed. Then, the supernatant was discarded for a new eppendorf of 1.5mL. These last steps, since the addition of chloroform-isoamyl (24:1), were repeated 3 times. After this, 600µL of isopropanol was added for 1h at 4°C. The eppendorf was inverted 2 a 3 times and then it was centrifuge for 15 min at maximum speed. The eppendorf was dropped and dried in a paper. To the same eppendorf, 500µL of ethanol 70% was added and then centrifuge for 15 min at maximum speed. The eppendorf was dropped and placed for incubation at 60°C. After drying the ethanol from the eppendorf, 30µL of sterile water was added and left to hydrate overnight. Then, the resulting DNA was stored at -20°C.

5. Minimum Inhibitory Concentration (MIC) test

MIC test is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after incubation. Luria-Bertani (LB) medium with 3% of NaCl was prepared with different concentrations of sodium tungstate dehydrate ($Na_2WO_4.2H_2O$), namely 0.5 millimolar (mM), 1mM, 10mM, 25mM, 50mM, 80mM and 100mM. Sodium tungstate dehydrate ($Na_2WO_4.2H_2O$) was added at sterilized medium after autoclaving at 121°C, for 20 min. For this test and the others $Na_2WO_4.2H_2O$ was used, we have used the abbreviation of tungsten (W) to facilitate the work.

The preparation of suspensions with distilled water were made. Then, these suspensions were added to the respective plates. After applying the suspensions, they were incubated at 22°C, during five days. As control, plates without bacteria inoculates of the described, W concentrations, were used.

6. Quantification of W by X-ray fluorescence spectrometer scattered energy (EDXRF)

6.1. Calibration curve

The FRX technique demands first the elaboration of calibration curve. To this end, standards with different concentration of W in milli-Q water were performed. The concentrations used was 0mM, 50mM, 100mM, 300mM, 500mM, 700mM, 1000mM and 1500mM of W. With this, there was obtained the following standard curve (Figure 7).

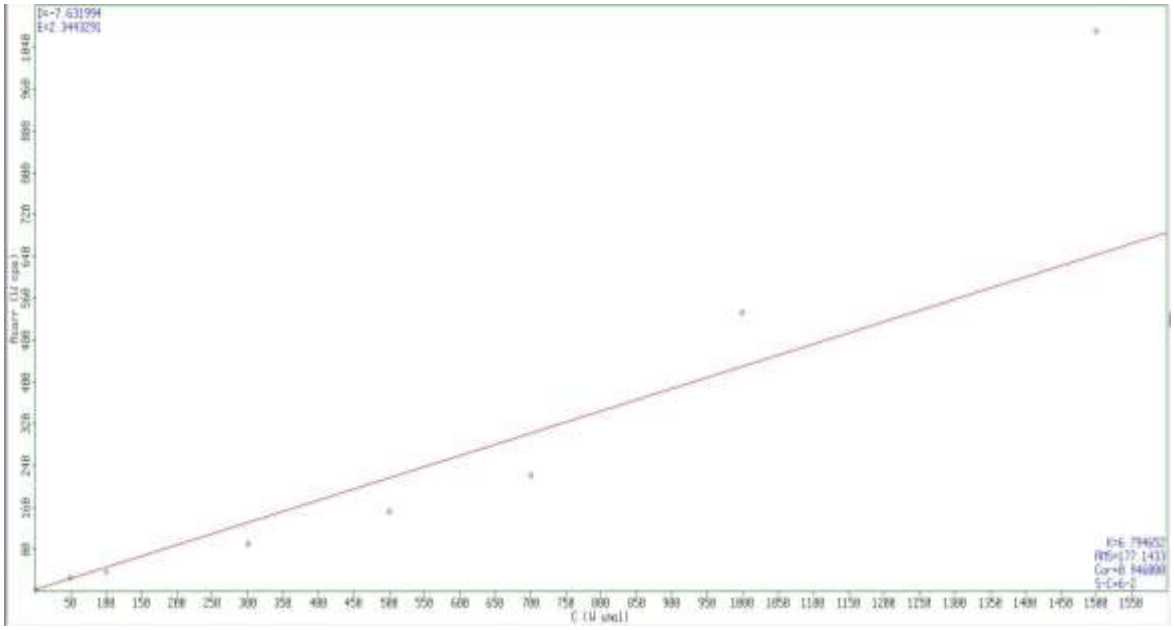

Figure 7. Calibration curve measured with the standards of tungsten at Epsilon 3^{XLE}.

6.2. Cells

6.2.1. Cell digestion

To each pellet, it was added 2mL of nitric acid (HNO_3) 10% and mixed by vortex to homogenise. They were incubated in a 50°C bath for 30 min. Then they were centrifuged, during 20 min at maximum speed and at 4°C. Having the pellet, the supernatant was discarded.

6.2.2. W measurements

After cell lysis, 2mL of Phosphate Buffered Saline (PBS) was added and vortexed to homogenise. With this, we were able to measure the accumulation of W into the cells through EDFRX at Epsilon 3^{XLE}.

The samples, after addition of PBS, were transferred to the samples support. These samples supports were previous prepared with a filter of Mylar thin-film, which have a thickness of 3.6 micrometers (µm), a size of 63.5 millimeters (mm) and a format of precut circles. Each of them was placed on Epilson 3^{XLE} using the following conditions (Table VI). The unit of measurement obtained on each sample was micromolar (µM).

Table VI. Conditions used at Epilson 3^{XLE}.

Corrent	80 microampere (µA)
Voltage	50 kilovolt (kV)
Filter	Ag
Measuring means	Air
Measuring time	60 sec
Condition name	Ni/Nb

6.3. Protein quantification through Bradford Method

For this work, it was made the protein quantification to examine the quantity of W concentration with the biomass. The quantification of protein, using BSA as standard, was made through Bradford Method. This method is commonly used to determine the total protein concentration of a particular sample.

To a cuvette, 1mL of Bradford reagent, 99µL of milli-Q water and 1µL of the respective sample, if it is concentrated (or 100µL if it is diluted), were added. These cuvettes were agitated and incubated, at room temperature, for 15 min. Then, the absorbance was measured at 595 nanometers (nm) and the amount of protein, namely BSA, was obtained through the standard curve line (Figure 8). As control, it was used milli-Q water with Bradford reagent.

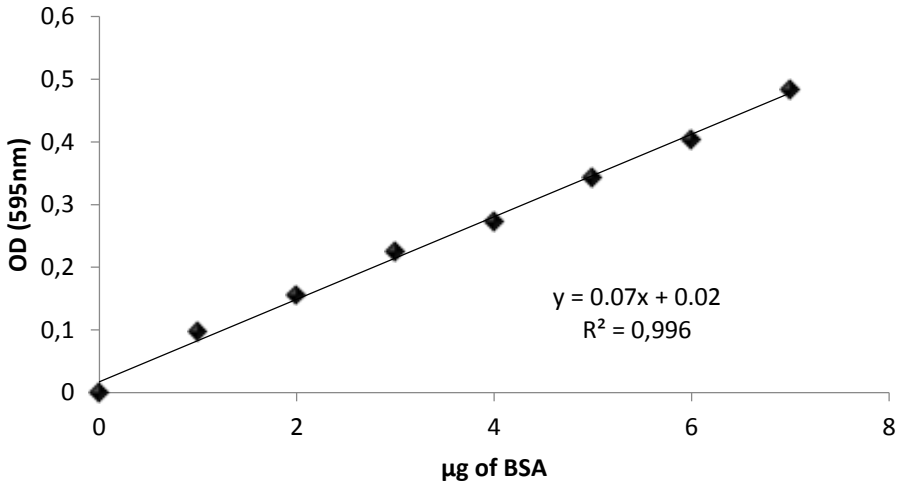

Figure 8. Curve prepared for the quantification of protein (BSA) by the Bradford method (y: absorbance; x: µg of protein).

7. Assays for bacterial tungsten accumulation

The W accumulation was determined for the following strains: *S. dubius* As(V)4, *S. dubius* NA4, *S. dubius* Cr2, *S. dubius* Sb5, *Halomonas meridiana* Cr1, *Staphylococcus saprophyticus* Cu4 and *Ochrobactrum tritici*.

7.1. Growth curve study in batch system

Firstly, the growth curve in batch system of these strains, in the presence and in the absence of the metal, was followed. In order to do it this, a pre-inoculum was made by growing the culture cells in RS medium. The strains *S. dubius* Cr2, *S. dubius* Sb5, *H. meridiana* Cr1, *S. saprophyticus* Cu4 and *O. tritici* were replicated to LB medium with 3% of NaCl and the others two (*S. dubius* As(V)4 and *S. dubius* NA4) were inoculated in RS medium. From the last growth, it was prepared the cell suspension with milli-Q water. The cell suspension (200µL) was ressuspended in Erlenmeyer flask of 100mL with 50mL of LB with 3% of NaCl (liquid medium) and incubated in a shaker with an agitation of 130 rotation per minute (rpm) for 24 h, at 22°C. In the case of *S. dubius* As(V)4 and *S. dubius* NA4, the cell suspension was ressuspended with Marine Broth (MB) and incubated in a shaker in the same conditions for 48 h, at 22°C. Subsequently, each culture in stationary phase of growth was transferred to three different 100mL Erlenmeyer flasks (one control and the others with 1mM and 20mM of W) with the same medium, and in an appropriate volume to obtain an initial turbidity of 0.06 Optical Density (OD) at 600nm. The different Erlenmeyer flasks were incubated in the shaker with the same conditions as above. Periodically, culture samples were collected and the OD at 600nm determined in a spectrophotometer.

7.2. Uptake of W during growth

Having the graphic of the growth curve of the respective strain, the next step was to verify the uptake of W of *S. dubius* NA4, *S. dubius As(V)4, H. meridiana Cr1, S. dubius Cr2, S. saprophyticus Cu4 and S. dubius Sb5* during its growth at the beginning, middle of the exponential phase and the end/beginning of the stationary phase of growth.

The pre-inoculum and culture inoculation were prepared the same as before, to six different 100mL Erlenmeyer flasks (three with 1 or 5mM of W and the other three with 20mM of W) to an initial turbidity of 0.06 OD at 600nm. They were incubated in the same conditions (130rpm; 22°C). Reaching the time of each point of the exponential curve (beginning, middle and end), one Erlenmeyer, of each concentration, was taken and the total medium was transferred to a falcon tube of 50mL.

All the falcons obtained were centrifuged during 20 min, at maximum rotation and at 4°C. The supernatant was discarded and 2mL of PBS were added to each falcon tube. This washing process was repeated 3 times in the same conditions. The quantification of W was made as described in point 6. in Materials and Methods.

7.3. Maximum uptake of W

In the previous chapter, the amount of W take up by the cells was related with the physiological state of the cells (different points of the growth curve). The point of the growth curve where cells had higher amounts of W was identified. The next objective was to determine the maximum W at that point of the growth curve, at different concentrations of W.

The pre-inoculum and the inoculation of the culture with an initial turbidity of 0.06 OD at 600nm were prepared as before. Two different 100mL Erlenmeyer flasks with 10mM and 30mM of W were used, and incubated in the shaker at 130rpm; 22°C. Reaching the time of maximum uptake of W (determined before), which is at the end of the exponential curve, the

Erlenmeyers flasks were taken and the total medium was transferred to a falcon tube of 50mL. Then, they were proceeded the same way like before (centrifuged and washing process). The quantification of W was made as described in point 6. in Material and Methods.

8. Detection of specific genes by polymerase chain reaction (PCR) amplification

8.1. Screening of genes from tungsten operons: *tupA* and *tupB* genes

Amplification of *tupA* and *tupB* genes from all strains of bacteria mentioned (Table VII and Table VIII) were performed by PCR, using the following bacterial degenerated primers (Table IX):

Table VII. Forward and reverse degenerated primers sequence for amplification of *tupA* and *tupB* gene, from isolated bacteria.

	Gene	**Designation**	**Sequence**
Forward Primer	*tupA*	tupA_F	5′ - GCGGTSACCACSTCSTTCAAC - 3′
Reverse Primer		tupA_R	5′ - ATATTGGTTGAACAYKACRGGATCGCC - 3′
Forward Primer	*tupB*	tupB_F	5′ - ATGGGCTTGCCGCCSGTCGTG - 3′
Reverse Primer		tupB_R	5′ - GCGGCCAAAGCCRGCCAG - 3′

The degenerated primers were purchased from Macrogen (Macrogen.Inc). The PCR reaction was performed in a 200µL eppendorf with a final volume of 30µL. Table X shows the composition of the PCR reaction.

Table VIII. Composition of PCR reaction for amplification of *tupA* and *tupB* genes from isolated bacteria.

Composition	Volumes (µL)
Sterile water (performed)	30
Reaction Buffer 10x[a]	3
Deoxynucleoside triphosphates (dNTPs), 1 mM each	6
Primer tupA_F/tupB_F, 0.5 microgram per milliliter (µg.µL^{-1})	0.3
Primer tupA_R/tupB_R, 0.5 µg.µL^{-1}	0.3
Taq DNA polimerase, 5 unit per microliter (U.µL^{-1})	0.3
DNA	4

[a] Reaction Buffer 10x: 670mM Tris-HCl (pH 8.8 at 25°C), 160mM $(NH_4)_2SO_4$, 100mM KCl, 0.1% stabilizer.

For the PCR reaction, a thermocycler MyCycler™ (Bio-Rad) was used. For the amplification of *tupA* and *tupB* genes, the conditions and expected length of the amplification product are listed in the table below (Table IX).

Table IX. Detailed PCR program.

Gene target	Amplification conditions			Expected amplified fragment length
tupA	**1 cycle**	**Initial denaturation**	94°C for 5 min	547 base pairs (bp)
	30 cycle	**Denaturation**	94°C for 45 sec	
		Annealing	48°C for 1 min	
		Extension	72°C for 35 sec	
	1 cycle	**Final extension**	72°C for 5 min	
		Final hold	4°C for an indefinite time	
tupB	**1 cycle**	**Initial denaturation**	94°C for 5 min	274bp
	10 cycle	**Denaturation**	94°C for 45 sec	
		Annealing	55°C (0.5 ↓) for 1 min	
		Extension	72°C for 15 sec	
	20 cycle	**Denaturation**	94°C for 45 sec	
		Annealing	50°C for 1 min	
		Extension	72°C for 15 sec	
	1 cycle	**Final extension**	72°C for 5 min	
		Final hold	4°C for an indefinite time	

In order to detect contamination, a negative control sample was prepared simultaneous with the reaction mixture without the DNA. The PCR products were analyzed through electrophoresis on agarose gel, stained with ethidium bromide (10 mg.mL^{-1}; Bio-Rad). Amplification products from *tupA* were analyzed on a 1% (w/v) agarose gel in TAE 1x buffer for 50 min (75 volts (V)) and those from *tupB* were analyzed on a 2% (w/v) agarose gel in TBE 1x buffer for 40 min (68V).

8.2. Organization of *ABC operon*: *tupBC* and *tupCA* genes

Amplification of *tupBC* and *tupCA* genes from the bacterial strains mentioned (*S. dubius* As(V)4, *S. dubius* NA4, *Erythrobacter citreus* NA16, *Dokdonia donghaensis* As(III)2* and *S. dubius* Sb5) were performed by PCR, using the following bacterial specific primers (Table X), based on the strain *S. dubius* As(V)4:

Table X. Forward and reverse specific primers sequence for amplification of *tupBC* and *tupCA* gene, from isolated bacteria.

	Gene	Designation	Sequence
Forward Primer	*tupBC*	tupBC_F	5′ - CCGCGAGATGGTCGATAT - 3′
Reverse Primer		tupBC_R	5′ - GCGCCGATCATCCAATGT - 3′
Forward Primer	*tupCA*	tupCA_F	5′ - CGGTGGTGACGCAACTT - 3′
Reverse Primer		tupCA_R	5′ - GCGCGGATGGTCTCTAAC - 3′

The specific primers were purchased from Macrogen. The PCR reaction was performed in a 200µL eppendorf with a final volume of 30µL. The composition of the PCR reaction is showed in Table VIII, with the difference in primers used, which were: primer tupBC_F/tupCA_F (0.5µg.µL^{-1}) and primer tupBC_R/tupCA_R (0.5µg.µL^{-1}).

For the amplification of *tupBC* and *tupCA* genes, the PCR reaction was submitted in the same Thermocycler. The conditions and expected length of the amplified fragment are listed in Table XI.

Table XI. Detailed PCR program.

Gene target	Amplification conditions			Expected amplified fragment length
tupBC	**1 cycle**	**Initial denaturation**	94°C for 5 min	1441bp
	30 cycle	**Denaturation**	94°C for 45 sec	
		Annealing	49°C for 1 min	
		Extension	72°C for 2 min	
	1 cycle	**Final extension**	72°C for 10 min	
		Final hold	4°C for an indefinite time	
tupCA	**1 cycle**	**Initial denaturation**	94°C for 5 min	1551bp
	29 cycle	**Denaturation**	94°C for 45 sec	
		Annealing	51°C for 1 min	
		Extension	72°C for 2 min	
	1 cycle	**Final extension**	72°C for 10 min	
		Final hold	4°C for an indefinite time	

A negative control was prepared as before. The PCR products were analyzed through electrophoresis on a 1% (w/v) agarose gel, stained with ethidium bromide, in TAE 1x buffer for 1 h (80V). The PCR products were purified (E.Z.N.A.® Gel Extraction Kit) and then sent for sequencing (Stabvida).

Amplification of *tupBC* and *tupCA* genes from *S. dubius* NA4 was performed by PCR, using the following bacterial specific primers (Table XII), based on the strain *S. dubius* NA4:

Table XII. Forward and reverse specific primers sequence for amplification of *tupBC* and *tupCA* gene, from isolated bacterium.

	Gene	Designation	Sequence
Forward Primer	*tupBC*	tupBCna_F	5' - CCCGCCCGATGAATGATA - 3'
Reverse Primer		tupBCna_R	5' - CAAGTGCGAGAACAAGAG - 3'
Forward Primer	*tupCA*	tupCAna_F	5' - CAGATGGTGACCCCCCTG - 3'
Reverse Primer		tupCAna_R	5' - AAAATCACTGCTCGGCGT - 3'

The specific primers were purchased from Macrogen. The PCR reaction was performed in a 200µL eppendorf with a final volume of 30µL. The composition of the PCR reaction is showed in Table VIII, with the difference in primers used, which were: primer tupBCna_F/tupCAna_F (0.5µg.µL^{-1}) and primer tupBCna_R/tupCAna_R (0.5µg.µL^{-1}).

For the amplification of *tupBC* and *tupCA* genes, the PCR reaction was submitted in the same Thermocycler mentioned before. The conditions and expected length are listed in the table below (Table XIII).

Table XIII. Detailed PCR program.

Gene target	Amplification conditions			Expected fragment length
tupBC	**1 cycle**	**Initial denaturation**	94°C for 5 min	1414bp
	35 cycle	**Denaturation**	94°C for 45 sec	
		Annealing	50°C for 1 min	
		Extension	72°C for 2 min	
	1 cycle	**Final extension**	72°C for 10 min	
		Final hold	4°C for an indefinite time	
tupCA	**1 cycle**	**Initial denaturation**	94°C for 5 min	1513bp
	35 cycle	**Denaturation**	94°C for 45 sec	
		Annealing	46°C for 1 min	
		Extension	72°C for 2 min	
	1 cycle	**Final extension**	72°C for 10 min	
		Final hold	4°C for an indefinite time	

A negative control was prepared as before. The PCR products were analyzed, purified and sent for sequencing in the same conditions as before.

8.3. Sequencing

Amino acid sequence was performed by the Sanger Sequencing Technique, being obtained as a service by Stabvida.

8.3.1. Gene Sequencing analysis

Reference genes, *tupABC*, from S. dubius As(V)4 and S. dubius NA4, our laboratory strains, were previously obtained by next-gen seq (IIIumina) and analysed using the RAST (Rapid Annotation using Subsystem Technology) software package. All sequences were compared with sequences available in NCBI database, using BLAST network services (Blastx) (Altschul, S. F., *et al.* 1997).

The sequences obtained were identified from the following parameters: closest sequence and % of similarity.

VII. Results

1. Phenotypical and morphological characterization

1.1. Cell morphology

Sulfitobacter dubius As(V)4 and *S. dubius* NA4 were characterized through different tests.

Table XIV present the different staining tests made of both strains. With this, we can see that, both of them, when submitted to gram staining, resulted in bacils Gram-negative cells (stained of pink to red). Regarding the presence of flagella, *S. dubius* As(V)4 and *S. dubius* NA4 did not show flagella and both had endospores. *S. dubius* NA4 showed capsule, as opposed to *S. dubius* As(V)4 that did not show the presence of capsule.

Table XIV. Results of different tests of *Sulfitobacter dubius* As(V)4 and *Sulfitobacter dubius* NA4.

Bacteria	Gram staining		Flagella staining	Endospore staining	Capsule staining
***S. dubius* As(V)4**	Bacils	Gram-negative	ND	D	ND
***S. dubius* NA4**			ND	D	D

ND: Not detected; D: Detected.

1.2. Phenotypical characterization

The biochemical profile of both Gram-negative strains tested through API ZYM are shown in the following table (Table XV).

Strain *S. dubius* NA4 have the presence of the enzymes Alkaline phosphatase, Esterase, Esterase lipase, Leucine arylamidase, Valine arylamidase, Cystine arylamidase, Trypsine, Acid phosphatase, Naphthol-AS-BI-phosphohydrolase, α-galactosidase and N-acetyl-β-glucosaminidase. The other strain, *S. dubius* As(V)4, have the enzymes Alkaline phosphatase, Esterase lipase, Leucine arylamidase, Valine arylamidase, Acid phosphatase, Naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase. The color level for Alkaline phosphatase and Acid phosphatase, relatively to strain *S. dubius* NA4, is 5 and 4, respectively.

Table XV. API ZYM test of Gram-negative strains, *Sulfitobacter dubius* As(V)4 and *Sulfitobacter dubius* NA4, after 4 hours (h) of incubation at 22 degrees Celsius (°C).

	Detected enzyme																		
Strains analyzed	**Alkaline phosphatase**	**Esterase (C4)**	**Esterase lipase (C8)**	**Lipase (C14)**	**Leucine arylamidase**	**Valine arylamidase**	**Cystine arylamidase**	**Trypsin**	**α-chymotrypsin**	**Acid phosphatase**	**Naphthol-AS-BI-**	**α-galactosidase**	**β-galactosidase**	**β-glucuronidase**	**α-glucosidase**	**B-glucosidase**	**N-acetyl-β-glucosaminidase**	**α-mannosidase**	**α-fucosidase**
S. dubius As(V)4	1	0	2	0	5	3	0	0	0	1	4	0	0	0	0	0	1	0	0
S. dubius NA4	5	2	2	0	5	5	3	4	0	4	3	3	0	0	0	0	1	0	0

The API 20NE results are shown in Table XVI.

The results for most tests were negative for both strains. *S. dubius* NA4 presented a positive result for β- galactosidase. *S. dubius* As(V)4 was only positive after 48 h of incubation for the assimilation of glucose.

Table XVI. API 20NE test of Gram-negative strains, namely *Sulfitobacter dubius* As(V)4 and *Sulfitobacter dubius* NA4, after 48 h of incubation at 22°C.

		Realized tests*																					
		Spontaneous reactions									Assimilation tests												
Strains analyzed		NO_3/NO_2	NO_3/N_2	TRP	GLU	ADH	URE	ESC	GEL	PNG	|GLU|	|ARA|	|MNE|	|MAN|	|NAG|	|MAL|	|GNT|	|CAP|	|ADI|	|MLT|	|CIT|	|PAC|
S. dubius	**24 h**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
As(V)4	**48 h**	/	/	/	/	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
***S. dubius* NA4**	**24 h**	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	48 h	/	/	/	/	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

*No_3/NO_2: Reduction of nitrates into nitrites; NO_3/N_2: reduction of nitrates into nitrogen; TRP: tryptophan metabolism with formation of indole; GLU: glucose fermentation; ADH: presence of arginine dihidrolase; URE: presence of urease; ESC: hydrolysis of esculin; GEL: hydrolysis of gelatin; PNG: presence of β-galactosidase; |GLU|: assimilation of glucose; |ARA|: assimilation of arabinose; |MNE|: assimilation of mannose; |MAN|: assimilation of mannitol; |NAG|: assimilation of N-acetylglucosamine; |MAL|: assimilation of maltose; |GNT|: assimilation of gluconate; |CAP|: assimilation of caprate; |ADI|: assimilation of adipate; |MLT|: assimilation of malate; |CIT|: assimilation of citrate; |PAC|: assimilation of phenyl-acetate.

BIOLOG GN2 MicroPlates test was made to characterize these two strains. *S. dubius* As(V)4 were negative for L-arabinose and *S. dubius* NA4 for Glucuronamide and also for L-arabinose. Strains were able to oxidase the rest of carbon sources.

S. dubius As(V)4 and *S. dubius* NA4 were negative to oxidase test and positive to catalase test.

2. Optimal conditions of growth

Firstly, the two strains of the *S. dubius* species, namely *S. dubius* NA4 and *S. dubius* As(v)4, were characterized in order to determine the best growth conditions. This species has an optimal growth in Marine Agar (MA), containing 1 to 2 percentage (%) of sodium chloride (NaCl) and at a temperature between 10 and 30°C. For the growth, the following conditions were optimized: medium (namely the solid), temperature and salinity. The objective was to verify in which medium the strains grew better, in other words, to check which was its optimal medium growth, its optimal temperature growth and which was its optimal concentration of NaCl.

Tables XVII-A and XVII-B presents the growth of each strain at different media, different percentage of salinities and different temperatures, having as control Reasoner's 2A with seawater filtrate (RS) medium.

Table XVI-A. Optimal conditions of growth of *Sulfitobacter dubius* NA4 and *Sulfitobacter dubius* As(V)4 in different nutrient media, after 24 h and 48 h, and varying the concentration of salt and temperature.

			24 h				48 h			
Medium	**NaCl (%)**	**Temperature (°C)**	***S. dubius* NA4**		***S. dubius* As(V)4**		***S. dubius* NA4**		***S. dubius* As(V)4**	
			NA1	***NA2***	**A1**	**A2**	**NA1**	**NA2**	**A1**	**A2**
RS		**22**	+	+	+	+	+	+	+	+
		25	+	+	+	+	+	+	+	+
		30	+	+	+	+	+	+	+	+
		37	+	+	+	+	+	+	+	+
LB	**1**	**22**	-	+/-	+/-	+	+/-	+/-	+/-	+/-
		25	+/-	+/-	+/-	+/-	-	-	+/-	+/-
		30	+/-	-	+/-	+/-	+/-	-	+/-	+/-
		37	+/-	+/-	-	+/-	+/-	+/-	-	+/-
	2	**22**	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
		25	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
		30	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
		37	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
	3	**22**	+/-	-	+	+	+	+/-	+	+
		25	-	-	+/-	+/-	-	-	+/-	+/-
		30	-	-	+/-	-	-	-	+/-	-
		37	-	+/-	-	-	-	+/-	-	+/-
NB	**1**	**22**	-	-	+/-	-	-	-	+/-	-
		25	+/-	+/-	+/-	+/-	-	-	+/-	+/-
		30	-	-	+/-	+/-	-	-	+/-	+/-
		37	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
	2	**22**	+/-	-	+/-	-	+/-	-	+/-	-
		25	-	+/-	-	-	-	+/-	-	-
		30	+/-	+/-	-	-	+/-	+/-	-	-
		37	-	-	-	-	-	-	-	-
	3	**22**	-	-	+/-	-	+/-	+/-	+/-	-
		25	-	-	-	-	-	-	-	-
		30	-	-	-	-	-	-	-	-
		37	-	-	-	-	-	-	-	-

Table XVII-B. Optimal conditions of growth of *Sulfitobacter dubius* NA4 and *Sulfitobacter dubius* As(V)4 in different diluted media and minimal media, after 24 h and 48 h, and varying the concentration of salt and temperature.

			24 h				48 h			
Medium	**NaCl (%)**	**Temperature (°C)**	***S. dubius* NA4**		***S. dubius* As(V)4**		***S. dubius* NA4**		***S. dubius* As(V)4**	
			NA1	**NA2**	**A1**	**A2**	**NA1**	**NA2**	**A1**	**A2**
RS		**22**	+	+	+	+	+	+	+	+
		25	+	+	+	+	+	+	+	+
		30	+	+	+	+	+	+	+	+
		37	+	+	+	+	+	+	+	+
Diluted LB (1:10)	**1**	**25**	+	+	+	+	+	+	+	+
		30	+	+	+	+	+	+	+	+
		37	+	+	+	+	+	+	+	+
		37	-	-	-	-	-	-	-	-
	2	**22**	+/-	+/-	-	-	+/-	+/-	-	-
		25	+/-	+/-	-	+/-	+/-	+/-	-	+/-
		30	+/-	+/-	+/-	+/-	+/-	+/-	-	+/-
		37	-	+/-	+/-	+/-	+/-	+/-	-	-
	3	**22**	-	+/-	-	+/-	-	+/-	-	+/-
		25	+/-	-	-	+/-	+/-	+/-	+/-	+/-
		30	+/-	-	+/-	-	+/-	-	+/-	-
		37	-	-	-	-	-	-	-	-
Diluted NB (1:10)	**1**	**22**	+/-	+/-	-	-	+/-	+/-	-	-
		25	+/-	+/-	-	-	+/-	+/-	-	-
		30	-	+/-	-	-	+/-	+/-	-	-
		37	-	-	-	-	-	-	-	-
	2	**22**	+/-	+/-	-	-	+/-	+/-	-	+/-
		25	+/-	+/-	-	-	+/-	+/-	+/-	+/-
		30	+/-	+/-	+/-	-	+/-	+/-	+/-	-
		37	-	-	-	-	-	-	-	-
	3	**22**	+/-	-	-	-	+/-	-	-	+/-
		25	-	+/-	+/-	-	-	+/-	+/-	-
		30	-	+/-	-	-	-	+/-	+/-	-
		37	-	-	-	-	-	-	-	-
R2A	**1**	**22**	-	-	-	-	-	-	-	-
		25	-	-	-	-	-	-	-	-
		30	-	+/-	+/-	+/-	-	-	-	+/-
		37	+/-	-	-	+/-	+/-	-	-	-
MM	**1**	**22**	+/-	+/-	+/-	-	+/-	+/-	+/-	-
		25	+/-	+/-	+/-	+/-	+/-	-	+/-	-
		30	+/-	+/-	-	-	+/-	+/-	-	-
		37	+/-	+/-	-	-	+/-	+/-	+/-	-
	2	**22**	-	+/-	-	-	+/-	+/-	-	-

	25	+/-	+/-	-	+/-	+/-	+/-	-	-
	30	+/-	+/-	-	-	+/-	+/-	-	-
	37	+/-	+/-	-	+/-	-	+/-	-	-
3	**22**	-	-	-	-	-	-	-	-
	25	-	-	-	-	-	-	-	-
	30	-	-	-	-	-	-	-	-
	37	-	-	-	-	-	-	-	-

In both tables presented, Table XVII-A and Table XVII-B, we can see that the two strains grown in RS medium. Relatively to Table XVII-A, Nutrient Broth (NB) and Minimal salt (M9) (MM) medium, at 1 and 2% of NaCl, it was verified some growth, in particular half of the plate. In the case of MM medium, at 3% of NaCl, it was not verified growth in any temperatures. In NB medium, at 3% of NaCl, it was verified some growth but only at 22°C. In Reasoner's 2A (R2A) medium, we can see that at lower temperatures (22 and 25°C) there were no growth. Therefore, at higher temperatures (30 and 37°C) there was verified some growth, covering half of the plate. In Luria-Bertani (LB) medium, at 1 and 2% of NaCl, it was verified some growth in both bacteria in all temperatures. Only at 3% of NaCl and at 22°C it was verified a better growth in the plates then the others media.

2.1. Growth test of isolated strains

Strains *S. dubius* Sb5, *Mesoflavibacter zeaxanthinifaciens* Te13, *Dokdonia donghaensis* As(III)2*, *Erythrobacter citreus* Kan3, *E. citreus* Gm3, E. citreus Gm6, *E. citreus* NA16, *E. citreus* NA19 and *E. citreus* NA6 from Lucky Strike did not grow. Strains *Rhodanobacter* sp. 7B 583 and *Flavihumibacter* sp. 7B 231 from uranium mine of Urgeiriça did not grow.

3. Characterization of isolates as resistant to tungsten

Isolated strains from Mid-Atlantic Ridge hydrothermal vent field and from contaminated groundwater uranium mine of Urgeiriça were characterized regarding to their behaviour as resistant, relatively to tungsten (W). For this, the Minimum Inhibitory Concentration (MIC) test was made.

Table XIX present the resistance of the different isolates.

Table XVIII. MIC's of Bacteria samples from Mid-Atlantic Ridge hydrothermal vent field, Lucky Strike, and from contaminated groundwater uranium mine of Urgeiriça at different concentrations of tungsten.

			W concentration (mM)						
Isolated strains		**Control**	**0.5**	**1**	**10**	**25**	**50**	**80**	**100**
Bacillus sp.	**7B 645**	+	+	+	+	+	+	+	+
Williamsia sp.	**A2 614**	+	+	+	+	+	+	+	+
Terrabacter sp.	**A2 62**	+	+	+	+	+	+	+	+
Rhodococcus sp.	**A2 937**	+	+	+	+	+	+	+	+
Arthrobacter sp.	**7B 214**	+	+	+	+	+	+	+	+
Rhodococcus sp.	**A2 67**	+	+	+	+	+	+	+	+
Arthrobacter sp.	**A2 64**	+	+	+	+	+	+	+	+
Paenibacillus sp.	**7B 637**	+	+	+	+	+	+	+	+
Sphingomonas sp.	**A2 49**	+	+	+	+	+	+	+	+
Bacillus sp.	**7B 900**	+	+	+	+	+	+	+	+
Aeromonas sp.	**7A 204**	+	+	+	+	+	+	+	+
Micrococcus sp.	**A2 984**	+	+	+	+	+	+	+	+
A. litoralis	**As(V)2**	+	+	+	+	+	+	+	+
H. meridiana	**Cr1**	+	+	+	+	+	+	+	+
S. dubius	**Cr2**	+	+	+	+	+	+	+	+
S. saprophyticus	**Cu4**	+	+	+	+	+	+	+	+
Exiguobacterium sp.	**A1 19**	+	+	+	+	+ / -	+	+	+
S. dubius	**Sb5**	+	+	+	+	+	+ / -	+ / -	+ / -
Paenibacillus sp.	**7B 841**	+	+	+	+	+ / -	+ / -	+ / -	+ / -
W. poriferorum	**Te6**	+	+	+	+/-	+/-	+/-	+/-	+/-
S. dubius	**As(V)4**	+	+	+	+ / -	+ / -	+ / -	+ / -	+ / -
Bacillus sp.	**7B 635**	+	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -	+ / -
S. dubius	**NA4**	+	+	+	+ / -	-	-	-	-
E. citreus	**NA6**	+	+	+	+	+	-	-	-
S. pontiacus	**Amp.1**	+	+	+	+	-	-	-	-
M. dokdonensis	**Te2**	+	+	+	-	-	-	-	-
S. litoralis	**Cu7**	+	-	+	-	-	-	-	-
M. zeaxanthinifaciens	**Te13**	+	x	x	+	-	-	x	x

	Gm11	+/-	+	+/-	+/-	-	-	-	-
	Kn1	+/-	+	+/-	-	-	-	-	-
E. citreus	**Zn1**	+/-	+/-	+/-	-	-	-	-	-
M. dokdonensis	**Te7**	+/-	+/-	+/-	-	-	-	-	-
H. meridiana	**Te14**	-	+	+/-	-	-	-	-	-
M. forsetti	**Te4**	-	+	-	-	-	-	-	-
G. jejuensis	**Te11**	-	-	-	-	-	-	-	-

The resistance results shown that sixteen bacteria out of thirty-five were resistant to W at all concentrations tested (0.5mM up to 100mM). *Exiguobacterium sp.* A1 19 grow at 25mM less to its control. Five bacteria on thirty-five resisted at all concentrations tested but with different growth levels. *S. dubius* NA4, *S. pontiacus* Amp.1 and *Mesoflavibacter zeaxanthinifaciens* Te13 were resistant up to 10mM of W, while *Erythrobacter citreus* NA6 was resistant up to 50mM. The strains that did not grow at any concentrations were *Halomonas meridiana* Te14, *Maribacter forsetii* Te4 and *Gaetbulibacter jejuensis* Te11.

4. Characterization of bacterial isolates as accumulators

Strains were characterized considering their ability to accumulate W, according to the Material and Methods described in 7. The next figures (Figure 10 to Figure 16) present the growth curve of each strain at two different concentrations of W and without W (negative control). The strains *S. dubius* As(V)4, *S. dubius* NA4, *S. dubius* Sb5, *Ochrobactrum tritici* and *Staphylococcus saprophyticus* Cu4 were grown at 1mM and 20mM, whereas the strains *H. meridiana* Cr1 and *S. dubius* Cr2 were grown at 5mM and 20mM. Table XX shows the specific growth rate (μ) of each strain at the different concentrations of W.

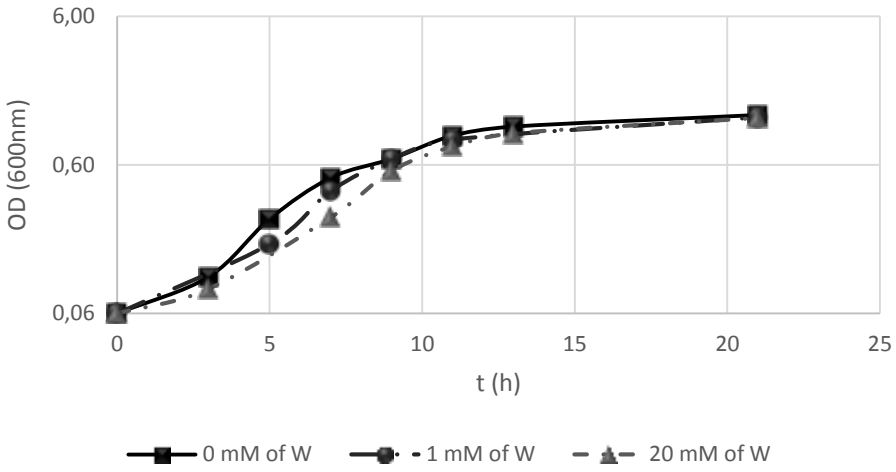

Figure 9. Growth curve of *Sulfitobacter dubius* NA4.

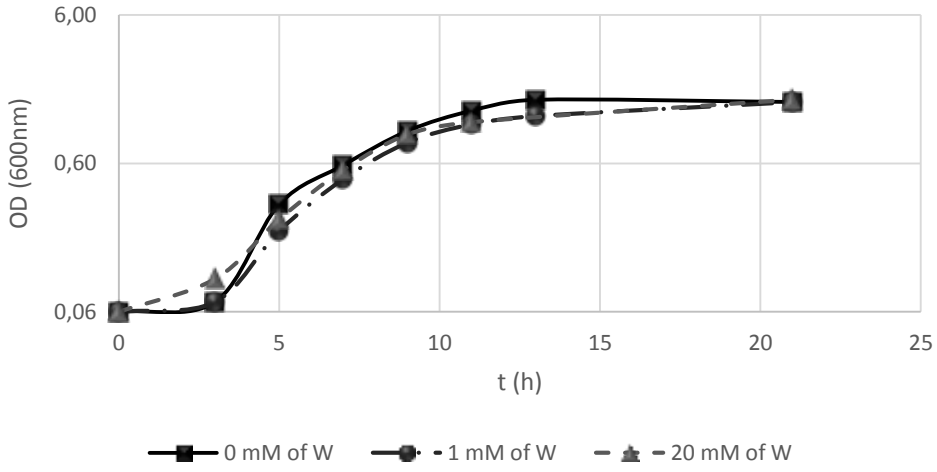

Figure 10. Growth curve of *Sulfitobacter dubius* As(V)4.

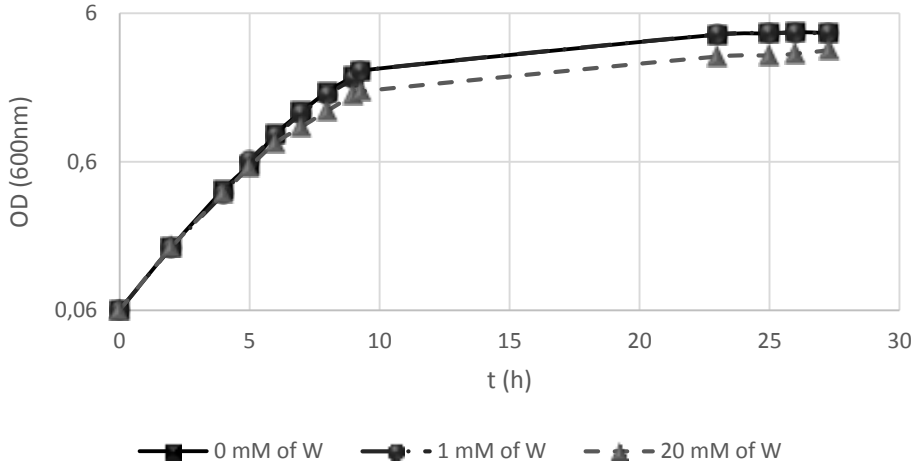

Figure 11. Growth curve of *Sulfitobacter dubius* Sb5.

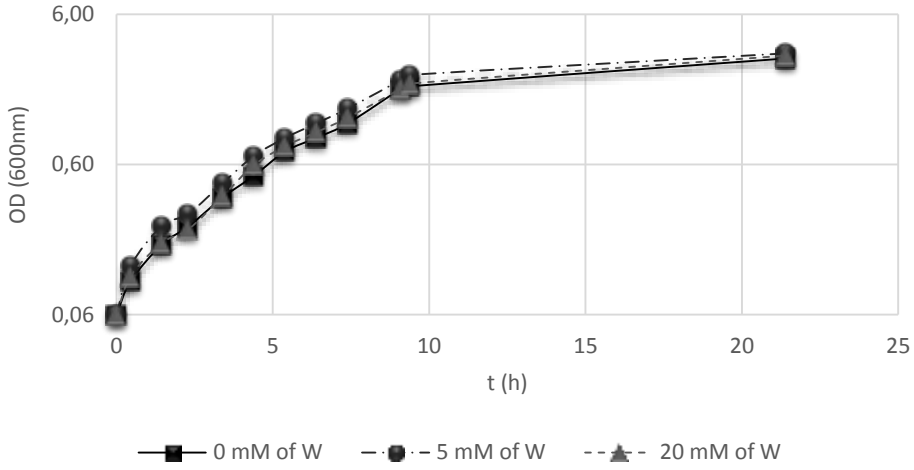

Figure 12. Growth curve of *Halomonas meridiana* Cr1.

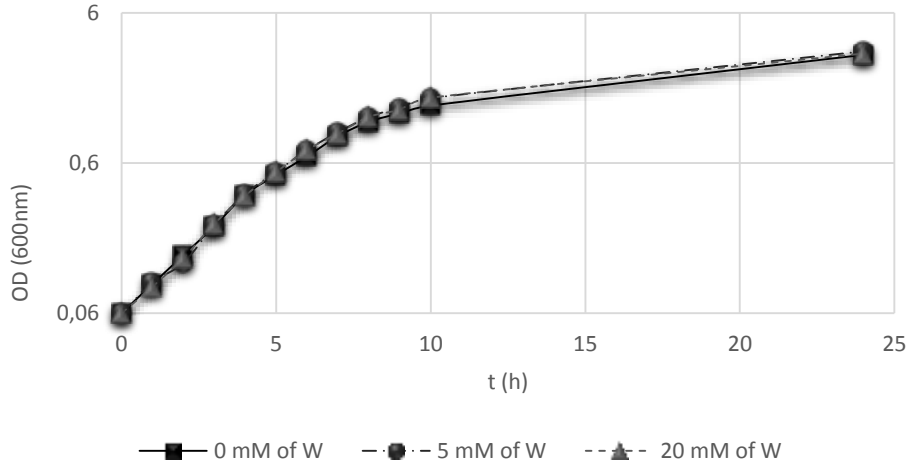

Figure 13. Growth curve of *Sulfitobacter dubius* Cr2.

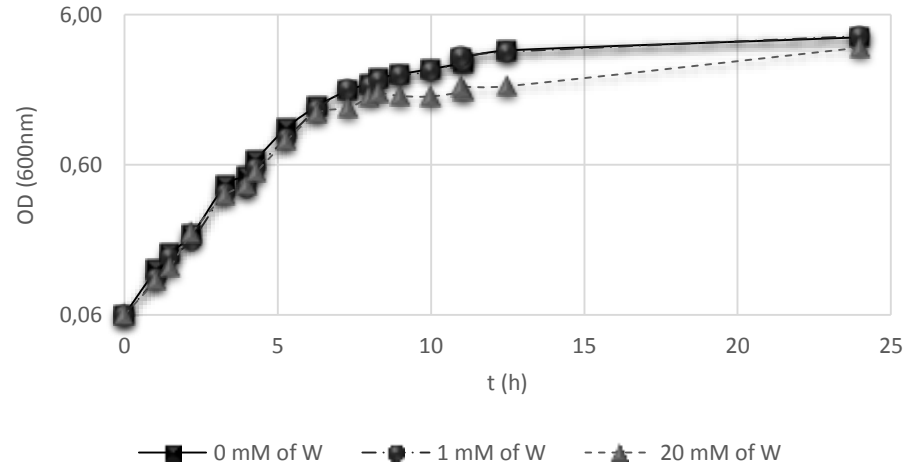

Figure 14. Growth curve of *Staphylococcus saprophyticus* Cu4.

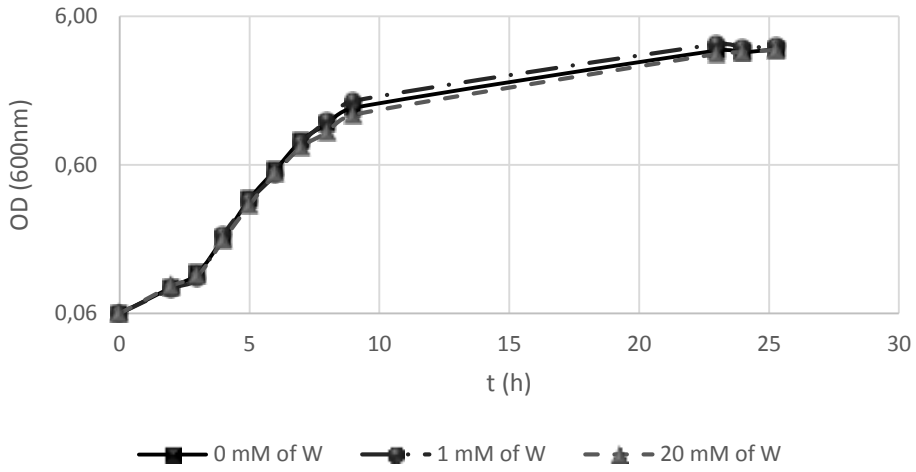

Figure 15. Growth curve of *Ochrobactrum tritici*.

Table XIX. Values of specific growth rate from growth curve of each strain tested, at different concentration of tungsten.

Bacterial strain	Concentration of W (mM)	Slope of the line	R^2	Specific growth rate (μ; h^{-1})
S. dubius **NA4**	**0**	y = 0.100x - 0.229	0.992	0.10
	1	y = 0.122x - 0.450	0.999	0.12
	20	y = 0.134x - 0.664	0.999	0.13
S. dubius **As(V)4**	**0**	y = 0.194x - 0.764	0.999	0.19
	1	y = 0.151x - 0.559	0.996	0.15
	20	y = 0.173x - 0.629	0.992	0.17
S. dubius **Sb5**	**0**	y = 0.479x - 2.049	1	0.48
	1	y = 0.453x - 1.908	0.999	0.45
	20	y = 0.328x - 1.275	0.997	0.33
H. meridiana **Cr1**	**0**	y = 0.204x - 0.383	0.997	0.20
	5	y = 0.238x - 0.368	0.997	0.24
	20	y = 0.212x - 0.346	0.999	0.21
S. dubius **Cr2**	**0**	y = 0.233x - 0.731	0.999	0.23
	5	y = 0.241x - 0.718	0.999	0.24
	20	y = 0.211x - 0.533	1	0.21
S. saprophyticus **Cu4**	**0**	y = 0.413x - 1.125	0.999	0.41
	1	y = 0.454x - 1.434	0.992	0.45
	20	y = 0.396x - 1.186	0.993	0.40
O. tritici	**0**	y = 0.182x - 0.541	0.995	0.18
	1	y = 0.157x - 0.430	0.996	0.16
	20	y = 0.234x - 0.867	0.994	0.23

When W was added to the media, all strains were affected. The growth of *S. saprophyticus* Cu4 (Figure 15) and *S. dubius* Sb5 (Figure 12) were affected at high concentration (20mM of W). *H. meridiana* Cr1 (Figure 13) was affected at low concentration (5mM of W). The μ for strains *S. dubius* As(V)4 and *S. dubius* Sb5 were high when W was not present in the media. For *H. meridiana* Cr1, *S. dubius* Cr2 and *S. saprophyticus* Cu4 it was high at low concentrations of W (1 or 5mM). For *S. dubius* NA4 and *O. tritici* it was high at high concentrations (20mM of W).

The next figures (Figure 17 to Figure 23) shows the results of uptake determined in three different points of the growth curve: beginning, middle and end of exponential growth curve. The W quantification was performed using X-ray fluorescence spectrometer scattered energy (EDFRX), according to Material and Methods described in 6.

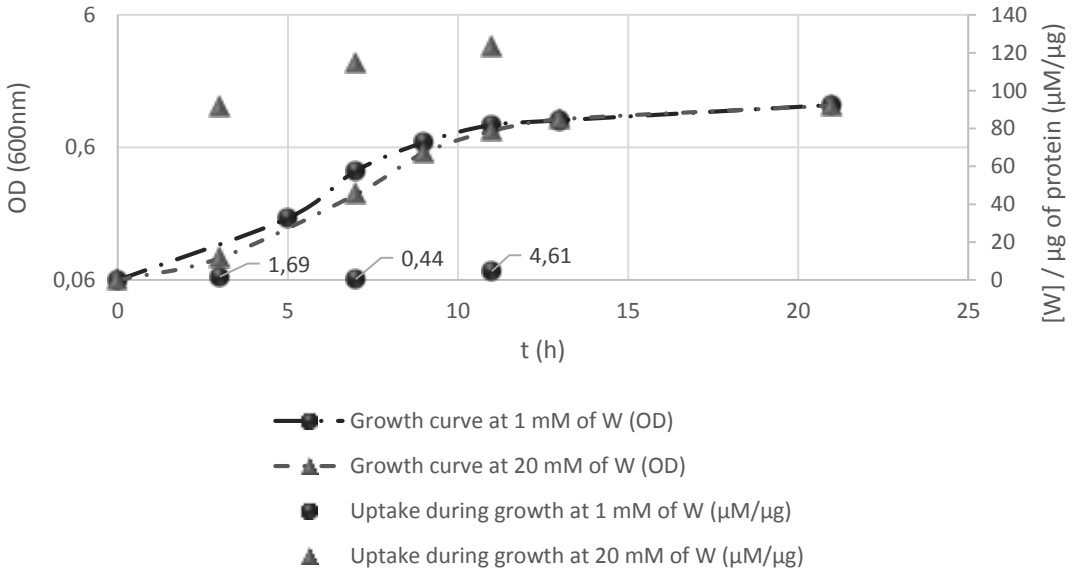

Figure 16. Growth curve of *Sulfitobacter dubius* NA4 versus uptake during growth.

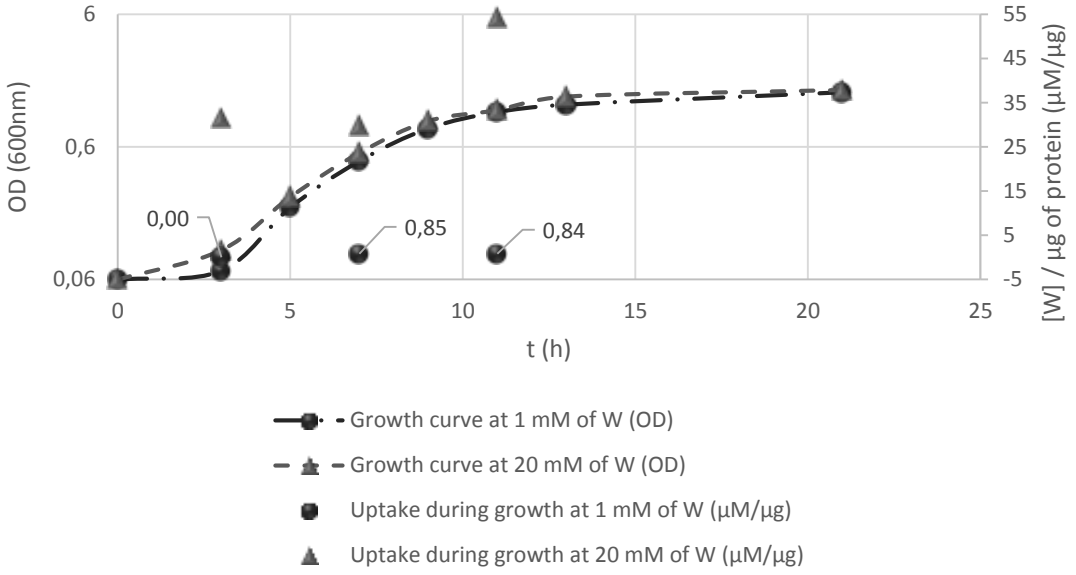

Figure 17. Growth curve of *Sulfitobacter dubius* As(V)4 versus uptake during growth.

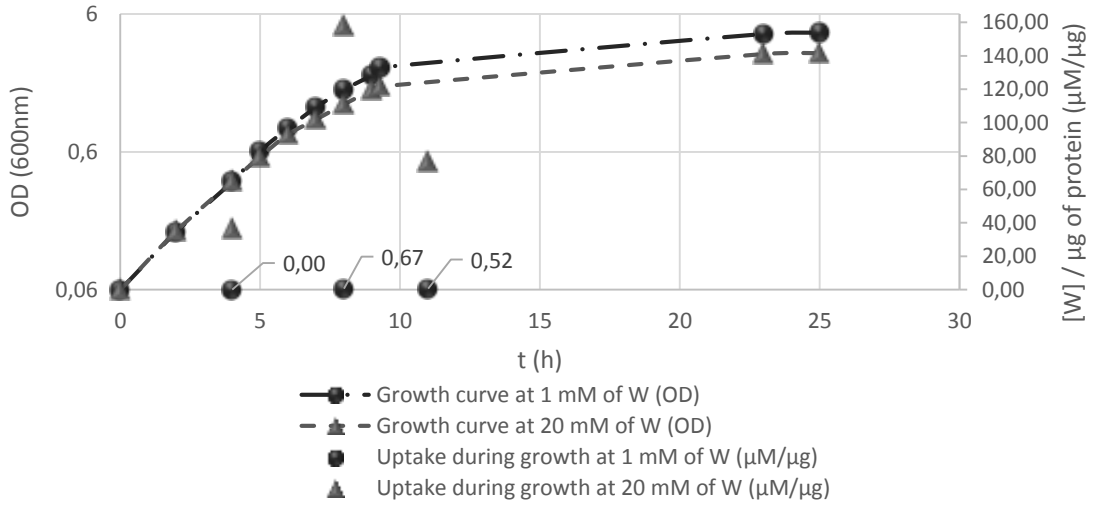

Figure 18. Growth curve of *Sulfitobacter dubius* Sb5 versus uptake during growth.

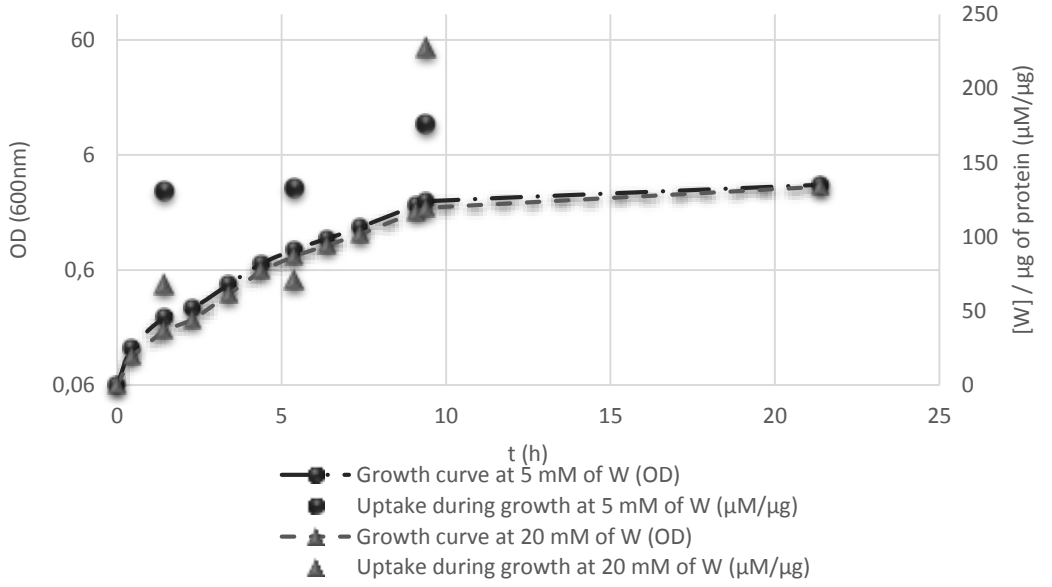

Figure 19. Growth curve of *Halomonas meridiana* Cr1 versus uptake during growth.

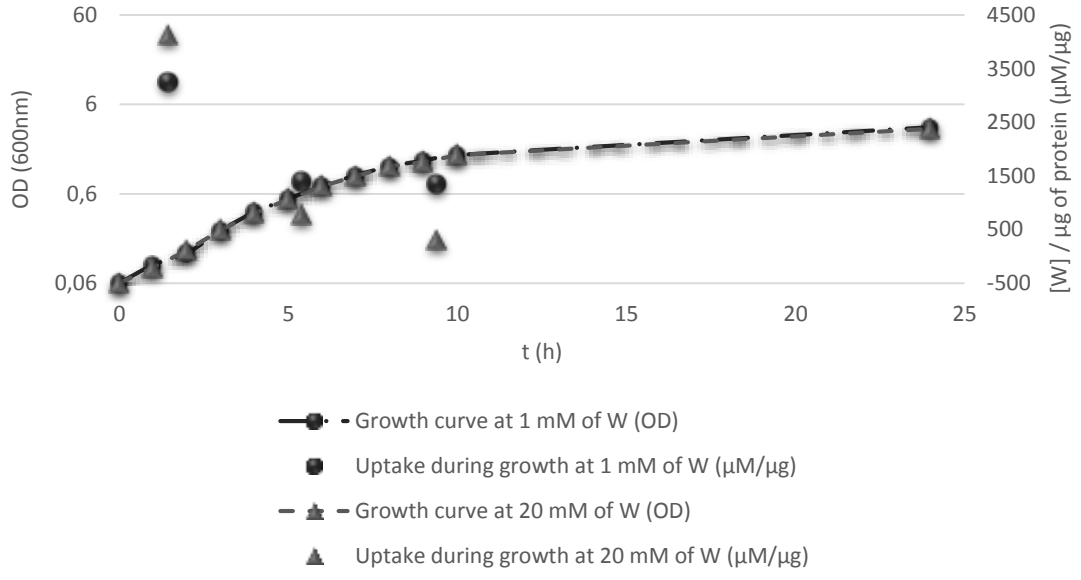

Figure 20. Growth curve of *Sulfitobacter dubius* Cr2 versus uptake during growth.

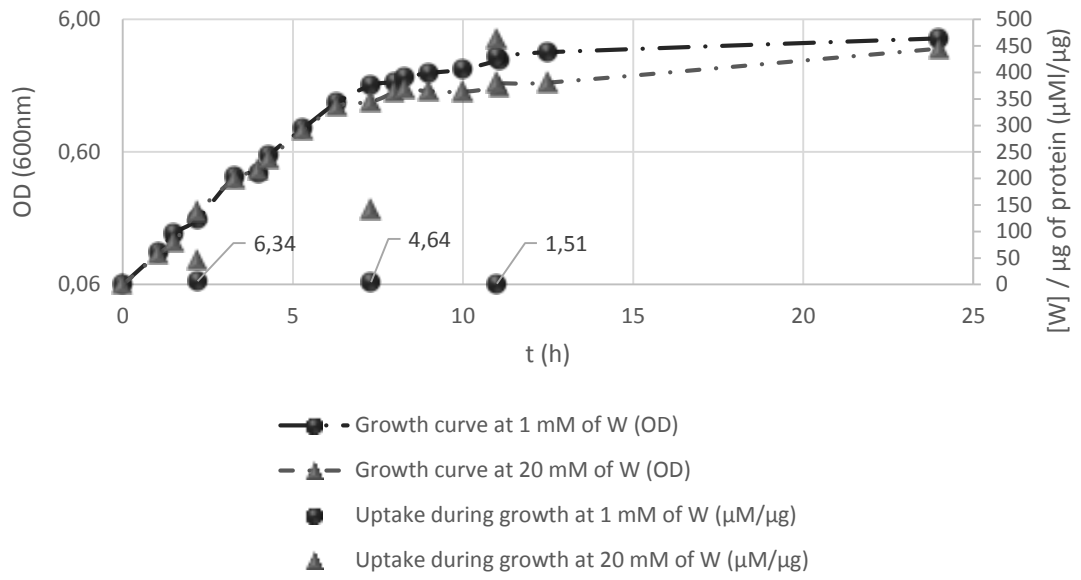

Figure 21. Growth curve of S*taphylococcus saprophyticus* Cu4 versus uptake during growth.

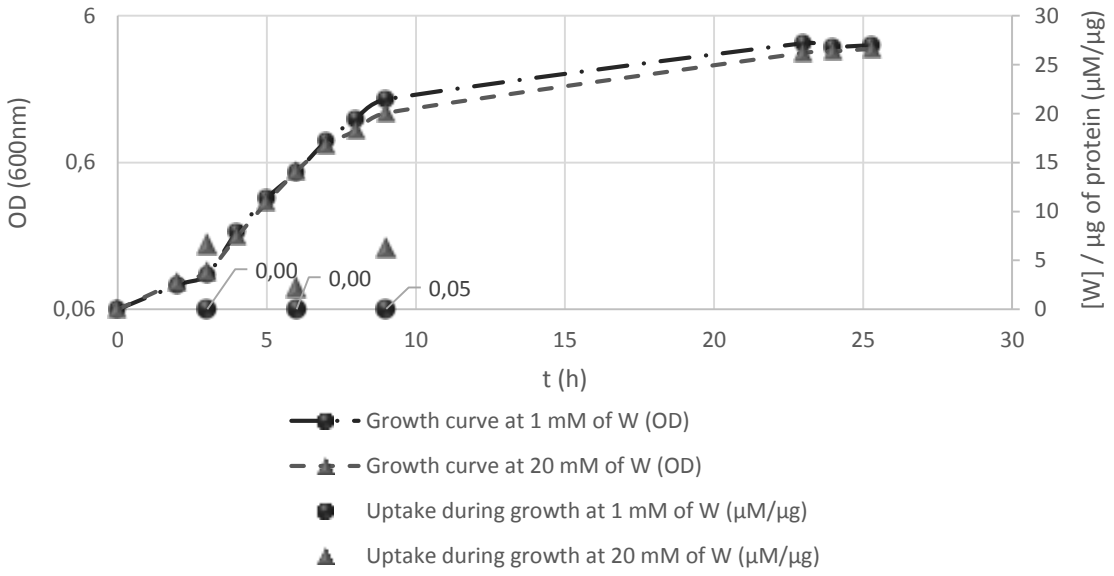

Figure 22. Growth curve of *Ochrobactrum tritici* versus uptake during growth.

Results from EDFRX shows that most bacteria have a specific accumulation pattern. The strains *S. dubius* NA4 (Figure 17), *S. dubius* As(V)4 (Figure 18), *S. dubius* Sb5 (Figure 19) and *S. saprophyticus* Cu4 (Figure 22) have a lower uptake of W at low concentrations than at high concentrations. At low concentrations, *S. dubius* As(V)4 and *S. dubius* Sb5 does not accumulate in the beginning of exponential phase of growth curve. Within these four, *S. dubius* NA4 and *S. dubius* As(V)4 have an maximum uptake until the end of exponential curve

of growth curve for both concentrations. *S. dubius* Sb5 accumulates more W until the middle of exponential growth curve. *S. saprophyticus* Cu4 at 1mM accumulates more in the beginning of exponential phase of growth curve and at 20mM in the end of exponential phase of growth curve. Relatively to the other strains, the accumulation pattern of W is different. *H. meridiana* Cr1 (Figure 12) and *S. dubius* Cr2 (Figure 13) have a high accumulation at low concentrations of W and a low uptake at high concentrations. *H. meridiana* Cr1 accumulates more W until the end of exponential phase of growth curve. *S. dubius* Cr2 accumulates more in the beginning of exponential phase of growth curve. *O. tritici* (Figure 23) is the only strain that did not accumulate W (or too little). At low concentrations, *O. tritici* does not accumulate and at high concentrations, the accumulation is not significant, when compared with the other strains.

The maximum uptake of W by the cells was determined at various concentrations of W (1 or 5mM, 10mM, 20mM and 30mM) at the growth curve phase when the maximum uptake was observed. The next figures (Figure 24 to Figure 29) shows the results of total uptake of W of each bacteria with the increasing in W concentration in the growth medium.

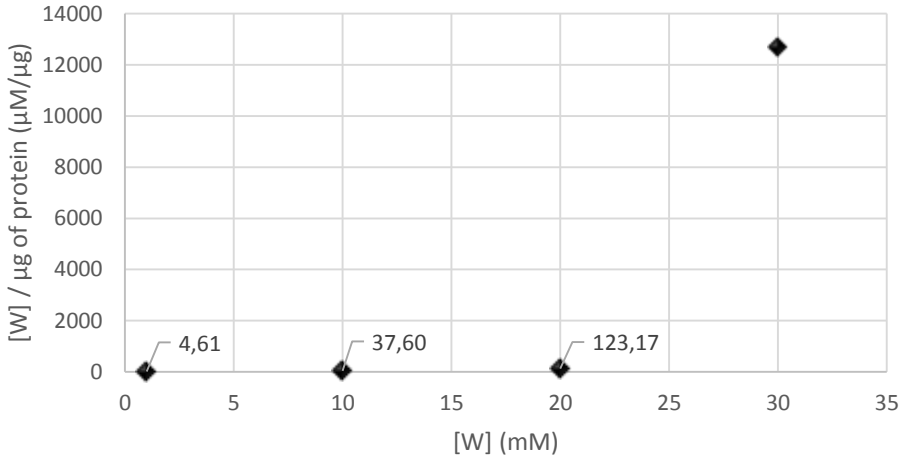

Figure 23. Maximum uptake of tungsten of *Sulfitobacter dubius* NA4.

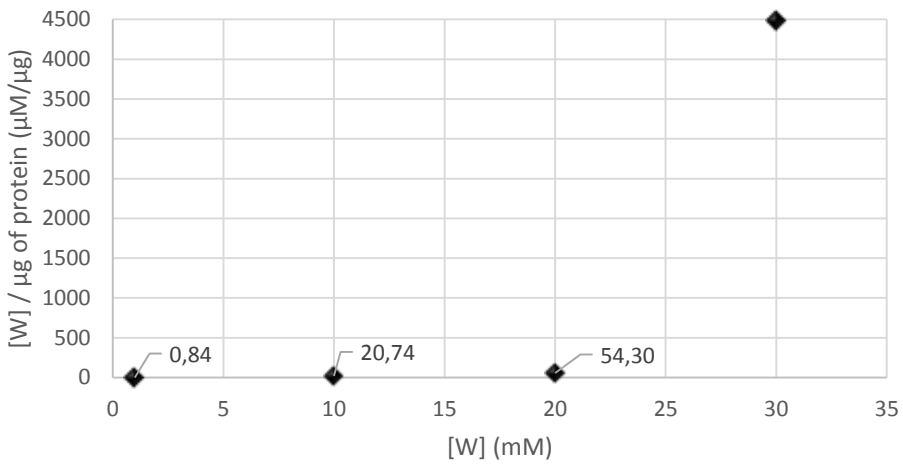

Figure 24. Maximum uptake of tungsten of *Sulfitobacter dubius* As(V)4.

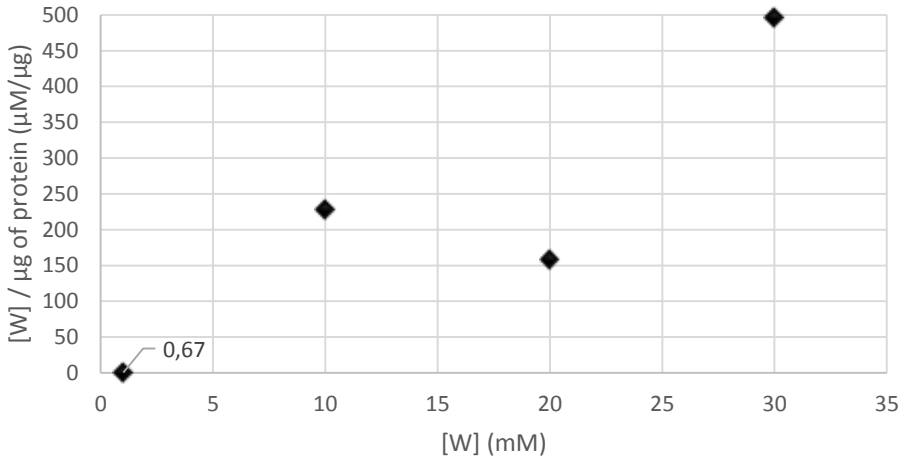

Figure 25. Maximum uptake of tungsten of *Sulfitobacter dubius* Sb5.

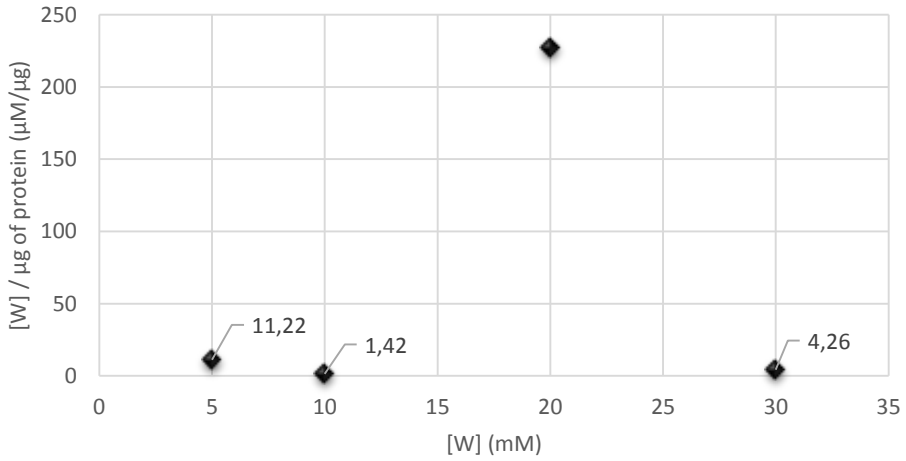

Figure 26. Maximum uptake of tungsten of *Halomonas meridiana* Cr1.

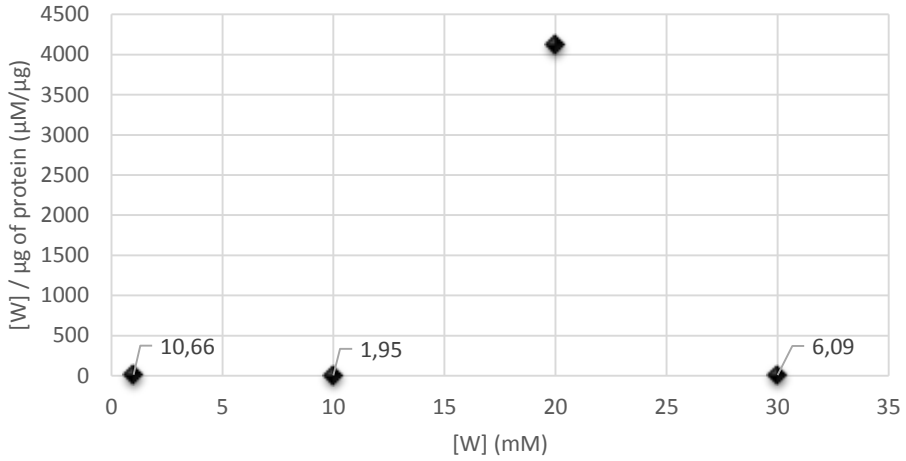

Figure 27. Maximum uptake of tungsten of *Sulfitobacter dubius* Cr2.

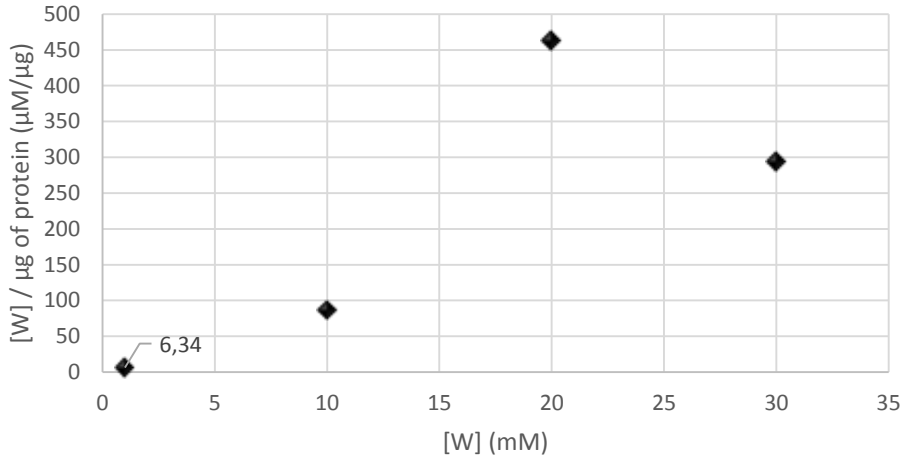

Figure 28. Maximum uptake of tungsten of *Staphylococcus saprophyticus* Cu4.

Results from EDFRX shows that the maximum uptake of W pattern varies with from strain to strain. *S. dubius* NA4 (Figure 24) have an uptake pattern very similar to *S. dubius* As(V)4 (Figure 25). The uptake increases with increasing the concentration of W, accumulating less until a concentration of 20mM. *H. meridiana* Cr1 (Figure 27) and *S. dubius* Cr2 (Figure 28) have similar patterns of uptake to each other. They accumulate more at a concentration of 20mM and, at 10mM, they have a low uptake of W. The other two strains, *S. saprophyticus* Cu4 (Figure 29) and *S. dubius* Sb5 (Figure 26), are the only strains that have a different and variable pattern of uptake from the other strains tested. *S. dubius* Sb5 accumulates less at low concentrations (1mM) and more at high concentrations (30mM). *S. saprophyticus* Cu4 also accumulates less at low

concentrations of W and this accumulation increases until 20mM. This concentration, 20 mM of W, is the limit of uptake for this strain because at 30mM the accumulation decreases.

5. Screening of isolated strains of *tupA* and *tupB*

Strains were screened for the presence of *tupA* and *tupB* genes. Isolated strains were subjected to DNA extraction and polymerase chain reaction (PCR) amplification. The results shown next is only relatively to *tupA* gene. Results relative to *tupB* gene were not very conclusive and for this reason, they were not shown.

S. dubius As(V)4 and *S. dubius* NA4, have *tupA* gene (Figure 30-A). The expected fragment length of *tupA* gene is 547 base pairs (bp) and in the PCR the bands obtained correspond to that value (between bands of 400bp and 600bp but closer to 600bp). Therefore, the PCR product of *S. dubius* As(V)4 was purified and then sent for sequencing. The fragment showed a sequence similarity of 77% with extracellular tungstate binding protein, the closest relative. Both strains, *S. dubius* As(V)4 and *S. dubius* NA4, were used as positive control relatively to the rest of the isolates. We verified that seven bacteria (including the positive controls) of thirty-four shown to have *tupA* gene (Figure 30), namely *S. dubius* As(V)4, *S. dubius* NA4, *Erythrobacter citreus NA16, S. dubius* Sb5, *Dokdonia donghaensis* As(III)2*, *Rhodococcus sp.* A2 67 and *S. litoralis Iso* Cu7. One PCR fragment in seven, namely *S. dubius* Sb5, was purified and sent for sequencing. The sequence was compared with Blastx and the closest relative was ABC tungstate transporter permease, substrate-binding protein with 74% similarity.

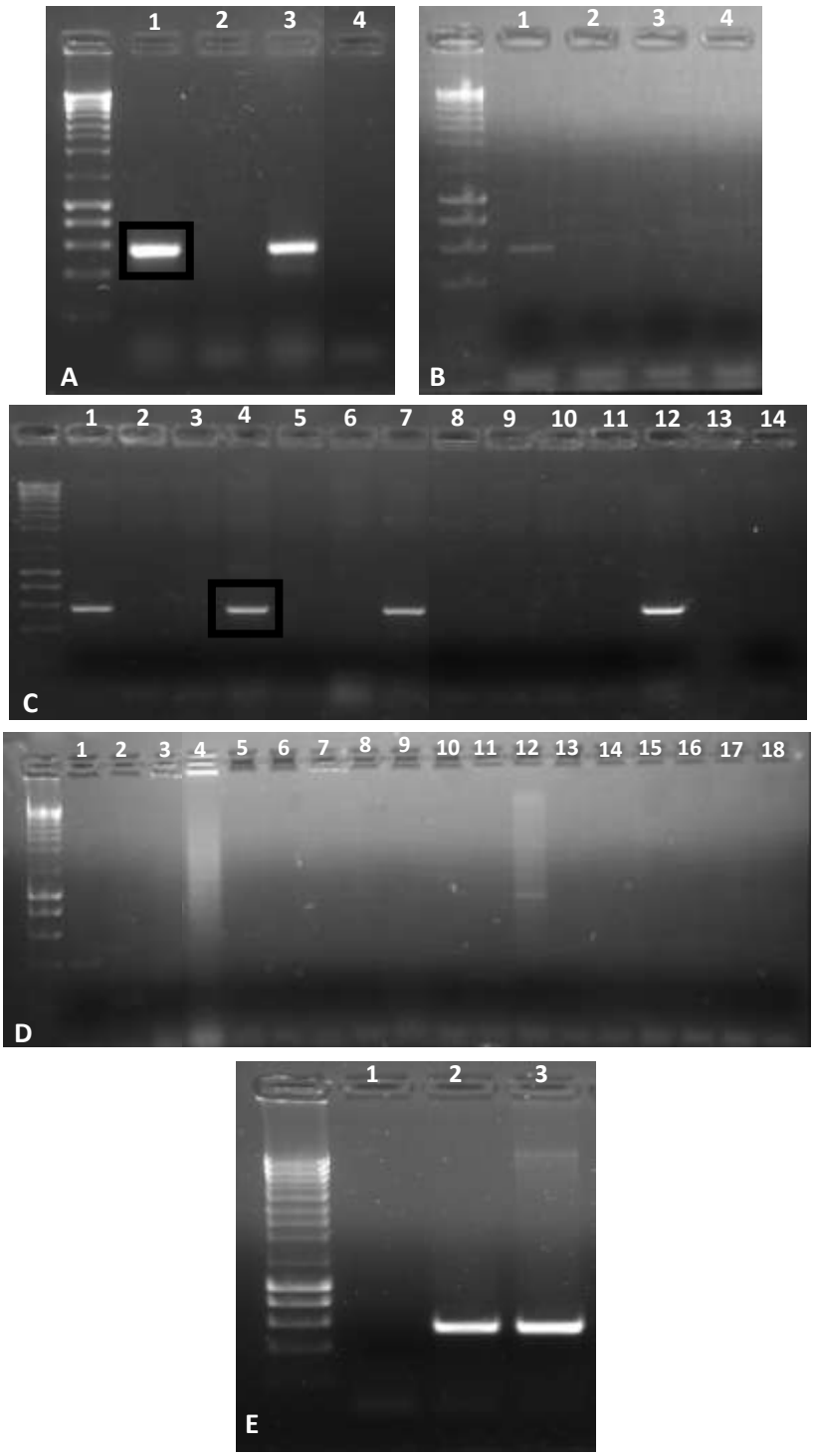

Figure 29. PCR of isolated strains on a 1 % agarose electrophoresis gels in TAE 1x, stained with ethidium bromide: A – well 1: As(V)4, well 2: Kan 3, well 3: NA4, well 4: negative control; B – well 1: NA4, well 2: Kan 3, well 3: NA6, well 4: negative control; C – well 1: NA16, well 2: Gm3, well 3: Gm6, well 4: Sb5, well 5: Gm5, well 6: Cr1, well 7: As(III)*2, well 8: Cr2, well 9: Cu4, well 10: NA19, well 11: Amp1, well 12: NA4, well 13: negative control; D – well 1: A2 64, well 2: A2 49, well 3: 7B 204, well 4: 7B 231, well 5: 7B 900, well 6: A2 937, well 7: A2 984, well 8: A2 62, well 9: 7B 841, well 10: 7B 637, well 11: Te7, well 12: A2 67, well 13: Te6, well 14: Kn1, well 15: Te2, well 16: Te14, well 17: As(V)2, well 18: Zn1; E – well 1: negative control, well 2: Cu7, well 3: As(V)4. All unmarked wells contain 4 µL of NZYTech DNA ladder III.

6. Analysis of strains for the organization of *ABC operon*

Strains showing a band of *tupA* and *tupB* genes, it was made another deeper analysis. The objective was the identification of the order of the genes namely *tupA*, *tupB* and *tupC*, in the *ABC operon*.

The organization of *tup* genes of *S. dubius* As(V)4 and *S. dubius* NA4 is known based on the analysis of the sequence of the plasmid, using the RAST (Rapid Annotation using Subsystem Technology) Software package. In these strains, the genes in the operon were shown as *tupBCA*. To screen the other strains to determine the order of the genes in the operon, primers were designed amplifying the genes *tupBC* and the genes *tupCA*. *S. dubius* As(V)4, *S. dubius* NA4, *E. citreus* NA16, *D. donghaensis* As(III)2* and *S. dubius* Sb5 were subjected to PCR.

S. dubius As(V)4, *D. donghaensis* As(III)2* and *S. dubius* Sb5 showed to have bands of high intensity (Figure 31) after PCR using primers tupBC_F and tupBC_R to amplify the genes B and C of the *tup operon*. The expected amplified fragment length of this fragment, *tupBC*, is 1441bp. In this PCR, two of the three bands obtained correspond to that value (between bands of 1400bp and 2000bp, but more closer to 1400bp). The PCR products of *S. dubius* As(V)4 and *S. dubius* Sb5 were purified and then sent to sequencing to confirm. Relatively to *S. dubius* As(V)4, the analysis of the sequence showed 84% similarity with *tupB* gene and 86% similarity with *tupC* gene, for the first and the second part of the fragment, respectively. The first part of the fragment revealed to be a tungstate ABC transporter system, permease TupB, and the other part a tungstate ABC transporter, ATP-binding protein. Relatively to *S. dubius* Sb5, the sequence showed 81% similarity with *tupB* gene and 95% similarity with *tupC* gene, for the first and the second part of the fragment, respectively. The first part revealed to be an ABC-type tungstate transport system, periplasmic component and the second a tungstate ABC transporter, ATP-binding protein.

The band with high weight (from well 5), had similarity of 99% with a cystathionine gamma-synthase.

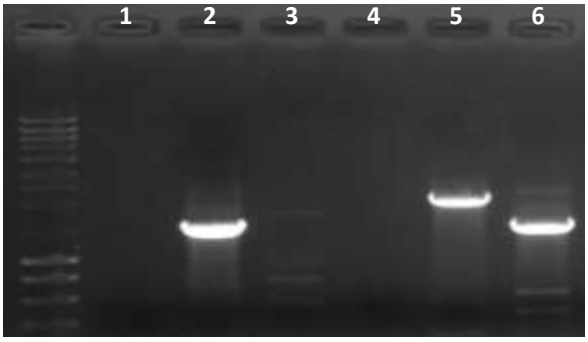

Figure 30. PCR of isolated strains on a 1 % agarose electrophoresis gels in TAE 1x, stained with ethidium bromide. Well 1: Negative control, well 2: As(V)4, well 3: NA4, well 4: NA16, well 5: As(III)*2, well 6: Sb5. The unmarked well contain 4 µL of NZYTech DNA ladder III.

After looking for *tupBC* gene in these strains, they were submitted to another PCR, using primers tupCA_F and tupCA_R, to see the presence of *tupCA* fragment. The expected amplified fragment length of this fragment, *tupCA*, is 1551 bp. The PCR amplification of the fragment, only the strain *S. dubius* As(V)4 showed a band. The PCR product of *S. dubius* As(V)4 was purified and then sent to sequencing. The analysis of the respective sequence showed 88% similarity with *tupC* and 67% similarity with *tupA*, for the first and the second part of the fragment, respectively. The first part of the fragment revealed to be a tungstate ABC transporter, ATP-binding protein, and the other part a tungstate ABC transporter, substrate-binding protein TupA.

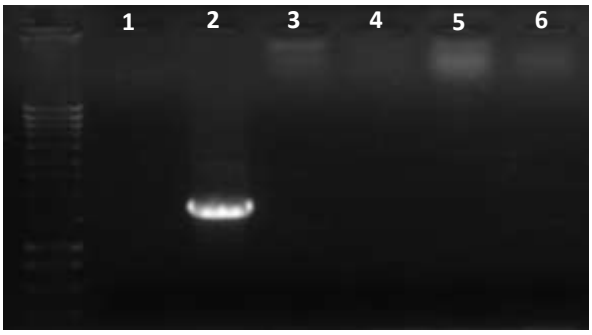

Figure 31. PCR of isolated strains on a 1 % agarose electrophoresis gels in TAE 1x, stained with ethidium bromide. Well 1: negative control, well 2: As(V)4, well 3: NA4, well 4: NA16, well 5: As(III)*2, well 6: Sb5. The unmarked well contain 4 µL of NZYTech DNA ladder III.

Since strain *S. dubius* NA4 did not present an amplified fragment on either PCR described, new specific primers were made from this strain. *S. dubius* NA4 was subjected to a new PCR with the objective to verify the presence of genes *BC* and *CA*.

Under the new PCR conditions, using primers tupBCna_F/tupCAna_F and tupCAna_R/tupCAna_R, *S. dubius* NA4 have bands on both genes (Figure 13). The expected amplified fragment length of these fragments, *tupBC and tupCA*, is 1414bp and 1513bp, respectively. The bands obtained corresponds to those values (between bands of 1400bp and 2000bp, but more closer to 1400bp). The PCR products were purified and then sent to sequencing to confirm.

Relatively to *tupBC* fragment (Figure 33-A), the analysis of the sequence showed 60% similarity with *tupB* and 55% similarity with *tupC*, for the first and the second part of the fragment, respectively. The first part of the fragment revealed to be a tungstate ABC transporter system, permease TupB, and the other part a tungstate ABC transporter, ATP-binding protein. Relatively to the other PCR, which was made for *tupCA* fragment (Figure 33-B), the analysis showed 58% similarity with *tupC* and 69% similarity with *tupA*, for the first and the second part of the fragment, respectively. The first part of the fragment revealed to be a tungstate ABC transporter, ATP-binding protein, and the second a tungstate ABC transporter, substrate-binding protein TupA.

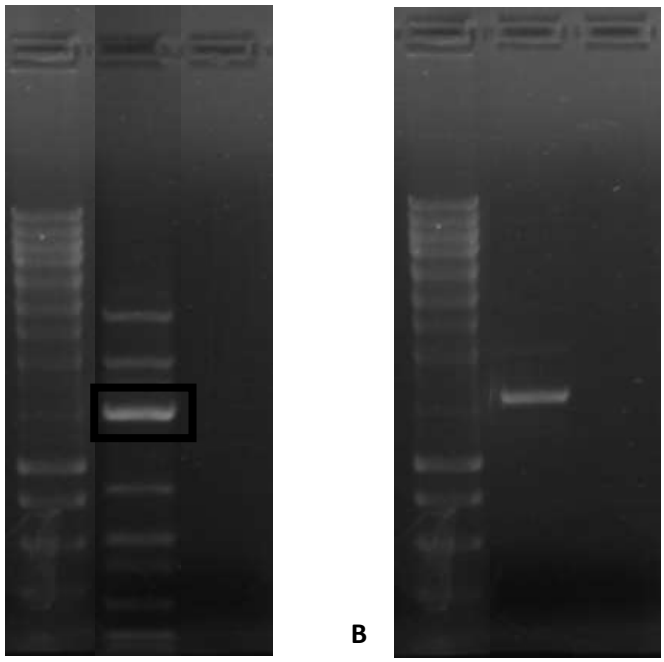

Figure 32. PCR of isolated strains on a 1 % agarose electrophoresis gels in TAE 1x, stained with ethidium bromide. A: *tupBC* fragment; B: *tupCA* fragment. Well 1: NA4, well 2: negative control. The unmarked well contain 4 µL of NZYTech DNA ladder III.

VIII. Discussion

Tungsten (W) is a heavy metal that, industrially, has been valued, mostly, for its high-melting point. Worldwide there are few mines that contain W, and Portugal has one of the largest mines with W. Besides, it is found also in hydrothermal vents, about 1000 times the level found in seawater. Hydrothermal vents are geologically and biologically unique features on Earth. They support highly productive biological communities comparable, in total biomass production, to the most prolific marine ecosystems. Due to their geological characteristics, minerals precipitate to form vent mineral deposits. These mineral deposits formation are consequence of the high temperature hydrothermal fluids that mixes with cold oxygenated seawater. In consequence, they are rapidly colonized by a diversity of *Archaea* and Bacteria, which utilizes the abundant geochemical energy available in the hydrothermal fluids (Flores, G. E., *et al.* 2011).

Most of bacteria tested in this study came from the Lucky Strike vent field, which is one of the largest hydrothermal areas known to date. This area has twenty-one active chimney sites distributed over approximately 150.000 km^2 at around 1700 meters (m) depth. The physical and chemical characteristics of the vent gases and waters are distinct from other Mid Atlantic Ridge sites due to low sulphur and high methane contents (Bertini, I., *et al.* 2007). Lucky Strike is a very oxidized hydrothermal vent and, as said before, contains high concentrations of W. Therefore, in this type of environment, W is predominantly founded in oxidized form tungstate (WO_4^{2-}), which is the form how this metal is internalized into the cells.

This work aimed to characterize previously isolated W resistant strains from Lucky Strike and from Urgeiriça, in order to achieve information to allow the construction of a whole-cell W bioaccumulation. The first task was to determine the best conditions of growth (varying the temperature, salinity and culture medium), for strains belonging to the genus *Sulfitobacter*, namely *Sulfitobacter dubius* NA4 and *S. dubius* As(V)4. These strains grow in Marine Agar (MA) and in Reasoner's 2A agar (R2A) with seawater filtrate (RS). Although, both media contain high concentration of salts and numerous minerals to simulate seawater composition. The drawback of using these media relates with their ability to react

with other constituents in the media and form a precipitate under high temperatures and pressures used in sterilization. This is a limiting factor when evaluating growth in liquid media, since the turbidity is not only dependent on the growth of the bacteria but also on the dispersion of the precipitate.

S. dubius NA4 and *S. dubius* As(V)4 grow better in Luria-Bertani (LB) medium, which is a nutritionally rich medium. Both have grown better in LB with 3 % of NaCl, which was expected since they have an ocean origin, where the concentration of salt is high. Relatively to the temperature, their optimal growth was at 22 °C, which was the temperature that was used on isolation of these strains, from this environment. These results confirmed the description of these species by Ivanova, E. P., *et al.* (2004), where strains are described as requiring seawater or Na^+ for growth, in 1-2% of NaCl and optimal growth at 25°C. Having all the optimal conditions, all the other isolated strains from Lucky Strike hydrothermal vent and from uranium contaminated groundwater from Urgeiriça mine were grown in this medium. The growth results showed that all strains are able to grow in the presence of salt.

Morphological and biochemical characteristics of strains *S. dubius* As(V)4 and *S. dubius* NA4 were compared with the type strain *Sulfitobacter dubius* KMM 3554^T. Differences were observed in the ferment carbon sources, such as glucose, maltose and citrate.

Heavy metal resistance to W was another characteristic determined for both strains. The Minimal Inhibitory Concentration (MIC) of W results showed that the resistance was variable and different in strains from the same species. Strains from uranium mine of Urgeiriça were resistant up to 100mM of W. On the other hand, from Lucky Strike hydrothermal vent, different strains had resisted to different concentrations of W. *Aurantimonas litoralis* As(V)2, *Halomonas meridiana* Cr1, *S. dubius* Cr2 and *Staphylococcus saprophyticus* Cu4 are resistant up to 100mM. For this group of strains, the highest resistance to W was not determined. *S. dubius* Sb5 resisted to less than 50mM and *Winogradskyella poriferorum* Te6 and *S. dubius* As(V)4 resisted to less than 25mM of W. Ghazvini, and co-workers (2009) tested the growth of a bacterium (Bacillus sp. GT-83) with different concentrations of W. With this study, the author showed that this

species grow up to 2208 mg/L of W, which corresponds to a concentration of 12.01mM. Strains from Lucky Strike and from uranium mine grew at higher concentrations. Only *H. meridiana* Te14, *Maribacter forsetii* Te4 and *Gaetbulibacter jejuensis* Te11 did not grow in the presence of W.

W is the heaviest element with a biological function (Andreesen, J. R., *et al.* 2008). This metal is part of a cofactor of proteins structure and, with this, it is considered a biological relevant metal. W is incorporated into a molecule of pterin cofactor that is required for assembly and function of enzymes, such as tungstoenzymes (Makdessi, K., *et al.* 2001; Barajas, E. A., *et al.* 2011). The two enzyme families that are dependent of tungsten are the formate dehydrogenase (FDH) family and the aldehyde oxidoreductases (AOR) family (Schawarz, G., *et al.* 2007). The metal to be able to connect to pterin molecule and then to have a biological functional, it has to enter the cell. This is achieved by the presence of ABC-type transporter system.

In this work, we can showed that all growth of all strains were affected, with the addition of W into LB medium with 3% of NaCl, because of the variations in their growth curves. In a study conducted by the author Ghazvini, and co-workers (2009), it was found that growth of Bacillus sp. GT-83 was also affected in the presence of W. All strains tested in the present work grew at higher concentration and it was less affected than in that study.

Membrane transport proteins provide molecule-specific pathways in and out of the cell. These proteins, besides de transport of nutrients into the cell, they are responsible for pumping toxic compounds out of the cell. These proteins are dominated by transporters belonging to the resistance-nodulation-cell division (RND) superfamily, which are able to recognize and actively export these substances out of bacterial cells (Delmar, J. A., *et al.* 2013).

In the literature, there is few reports related to Tungsten and bacteria. One of the reports is related with uptake of W into the cells. The only report founded by us, is the one from Ghazvini, and collaborators (2009). Ghazvini determined the biosorption and bioaccumulation of WO_4^{2-} by a bacterial strain, namely Bacillus sp. GT-83. Therefore, in the present study it was an objective to deepen the study relatively to the uptake of W into the cells, during the cell

growth and at different concentrations. In addition, the maximum W concentration accumulated by bacteria in their cells was determined. *S. dubius* NA4, *S. dubius* As(V)4 and *H. meridiana* Cr1 accumulated W into their cells until the end of the exponential phase of their growth curve. *S. dubius* Sb5 accumulated W until the middle of the exponential phase of growth curve, since the maximum W accumulated was detected in cells removed at the middle of the exponential phase as expected. Increasing the growth time resulted in the decrease of the W accumulated by the population that still increasing. *S. dubius* Cr2 accumulated W in the beginning of the exponential phase of growth curve. Then, with the increase of the growth time, a decrease of W accumulated by the population, which continued to grow, was verified. Relatively to *S. saprophyticus* Cu4, the concentration of W affected the W accumulation pattern. At low concentrations, the accumulation of W was maximum in the beginning of the exponential phase of growth curve and at high W concentrations, the accumulation was maximum until the end of the exponential phase of its growth curve. *O. tritici* did not accumulate W and it was used as a control.

The strain *S. dubius* NA4, based on the analysis of the sequence of one of its plasmids using RAST (Rapid Annotation using Subsystem Technology) software package, the gene coding for *formate dehydrogenase* (upstream on the sequence) it is plasmidic. Of the strains tested, *H. meridiana* Cr1 showed a similar W accumulation pattern, comparatively to *S. dubius* NA4, and different from the others. In consequence, it is possible that this strains have also the enzyme dependent of W: formate dehydrogenase.

The results from maximum accumulation of W showed that strains *H. meridiana* Cr1, *S. dubius* Cr2 and *S. saprophyticus* Cu4 had a maximum accumulation when grown in the presence of 20 mM of W. When growing at higher W concentrations, the strains accumulated less. Therefore, W seems to have a toxic effect at high concentrations in these strains which seem also to have an accumulation limit. *S. dubius* NA4*, S. dubius* As(V)4 and *S. dubius* Sb5 had a maximum accumulation when grown at the highest concentration of W in the growth media. Therefore, we did not determined the accumulation limit for these strains.

In the literature, there is few reports related to the organization of *tupABC* operon responsible for uptake of W into the cells. One report founded describes the overexpression of the binding protein TupA. Through this study of Makdessi and collaborators (2001) the extracytoplasmic binding protein TupA (W transporter) of the *tupABC* operon, was overexpressed in the strain *Escherichia coli* to demonstrate the specificity of the binding of WO_4^{2-} by TupA. In the present study, in order to justify the ability of the strains to take up W, the gene coding *tupA* was screened. Of all strains tested, only *S. dubius* NA4, *S. dubius* As(V)4, *Erythrobacter citreus* NA16, *S. dubius* Sb5, *Dokdonia donghaensis* As(III)2* and *S. litoralis* Cu7 had the gene coding *tupA*. Since we could not find, in the literature, description of the presence of tupA gene in the species mentioned above, this work describes probably for the first time the presence of *tupA* gene in these species. We also found strains belonging to the species mentioned below, that were able to take up W but did not showed the presence of the tupA gene. However, it is possible that *H. meridiana* Cr1, *S. saprophyticus* Cu4 and *S. dubius* Cr2 have other ABC transporters systems then TupABC, like ModABC transporter and WtpABC transporter.

The study was also deepened the study of the organization of *tupABC* operon. The analysis of the organization of the operon results showed that *S. dubius* NA4 and *S. dubius* As(V)4 had the *tupABC* operon in the following order: *tupB*, *tupC* and then *tupA*. On the other hand, *S. dubius* Sb5 showed to had *tupB gene* upstream to *tupC* gene, but we were not able to detect *tupA* gene downstream to *tupC* gene. Nevertheless, the tupA gene was detected in this strain by PCR. In the future, more tests will be done to localize this gene coding *tupA*.

Based on all these results and from all strains tested, *S. dubius* NA4 would be chosen as a template for the construction of a bioacumulator of W. This construct will be based on DNA modifications for the improvement of W uptake system.

IX. Conclusions

From the results presented, some conclusions can be drawn:

- Strains *S. dubius* NA4 and *S. dubius* As(V)4, varying the media, the temperature and the percentage of salinity, grown better in LB medium with 3% of NaCl;
- All strains isolated from Lucky Strike hydrothermal vents and from uranium contaminated groundwater from Urgeiriça grew in the presence of salt;
- Some strains presented the capacity to grow at high concentrations of W in liquid medium. However, W showed to induce toxicity since it reduced bacterial growth rate when it is added to the medium;
- *S. dubius* NA4, *S. dubius* As(V)4 and *H. meridiana* Cr1 presented a maximum accumulation of W at the end of the exponential phase of their growth curve;
- *S. dubius* Sb5 presented a maximum accumulation per gram of biological material, in cells from the middle of the exponential growth phase;
- Cells from *S. dubius* Cr2 accumulated W maximally in the beginning of the exponential phase of the growth curve and then the accumulation per gram of cells decreased;
- The concentration of W in the medium affected the W accumulation pattern of *S. saprophyticus* Cu4;
- *O. tritici* did not accumulate W into their cells;
- Varying the concentrations of W, *H. meridiana* Cr1, *S. dubius* Cr2 and *S. saprophyticus* Cu4 cells had a maximum accumulation ability when grown in the presence of 20 mM W;
- *S. dubius* NA4, *S. dubius* As(V)4, *Erythrobacter citreus* NA16, *S. dubius* Sb5, *Dokdonia donghaensis* As(III)2* and *S. litoralis* Cu7 presented the *tupA* gene, coding for tungstate-binding protein;
- *S. dubius* NA4 and *S. dubius* As(V)4 had the *tupABC* operon, with the genes in the following order: *tupB*, *tupC* and then *tupA;*
- *S. dubius* Sb5 showed to have the *tupBC* genes and the tupA gene but its order in the operon was not solved.

X. References

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XI. Annexes

1. Culture media

1.1. Luria-Bertani (LB) medium

LB is a nutritionally rich medium, which is used for the growth of pure cultures. To 1 liter (L) with distilled water, 10 grams (g) of tryptone, 5g of yeast extract and 5g of sodium chloride (NaCl) were weigh and dissolved. Then, it was sterilized by autoclaving at 121 degrees Celsius (°C), for 20 minutes (min).

1.2. Nutrient Broth (NB) medium

NB is used for the cultivation of many species of non-fastidious microorganisms. To 1L with distilled water, 8g of the powder (Merck) was weigh and dissolved. After this, the medium was sterilized by autoclaving at 121°C, for 20 min.

1.3. Reasoner's 2A (R2A) medium

R2A is a low nutrient medium and is used for enumerating heterotrophic organisms in treated potable water. To 1L with distilled water, 18.2g of the powder (Merck) was weigh and dissolved. Heat with frequent agitation and boil for 1 min to completely dissolve the powder. Then, it was sterilized by autoclaving at 121°C, for 20 min.

1.4. Reasoner's 2A medium with Seawater filtrate (RS)

RS is the same medium than the previous. The only difference is that the powder was dissolved in seawater filtrate instead of distilled water.

1.5. Minimal salt (M9) (MM) medium

MM is a medium that contains the essentials minimum nutrients possible for colony growth, generally without the presence of amino acids. It is used to define if a particular microbial species is a heterotroph (an organism that cannot fix carbon and uses organic carbon for growth and energy from complex organic compounds). Auxotrophs (organisms with nutritional requirements) will not be able to grow in this medium. To 1L, 100 milliliter (mL) of macronutrients (Table XXI), 10mL of micronutrients (Table XXII), 11.44g of HEPES, 100mL of Yeast Nitrogen Base (YNB) 10x and 690mL of water were weigh and dissolved. Apart, 100mL of glucose 5 percentage (%) was weigh and dissolved. Then, both solutions were sterilized by autoclaving at 121°C, for 20 min. After this, glucose was added to the first solution through filtration.

Table XX. Composition of macronutrientes solution (for 1L).

Compound	**Formula**	**Amount (g)**
Calcium sulfate	$CaSO_4$	0.6
Sodium chloride	NaCl	0.08
Potassium nitrate	KNO_3	1.03
Sodium nitrate	$NaNO_3$	6.98
Magnesium sulfate heptahydrate	$MgSO_4.7H_2O$	1
Nitrilotriacetic acid	NTA	1
Sodium phosphate	$NaHPO_4$	1.11

Table XXI. Composition of micronutrientes solution (for 0.5L).

Compound	**Formula**	**Amount**
Zinc sulfate monohydrate	$ZnSO_4.H_2O$ (100 millimolar (mM))	174 microliter (µL)
Manganese sulfate monohydrate	$MnSO_4.H_2O$ (100 mM, MnII)	88.5µL
Copper sulfate pentahydrate	$CuSO_4.5H_2O$ (100 mM, CuII)	20µL
Nickel chloride hexahydrate	$NiCl_2.6H_2O$ (100 mM, NiII)	38µL
Sodium molybdate dihydrate	$NaMoO_4.2H_2O$ (100 mM)	73µL
Boric acid	H_3BO_3 (100 mM)	2,426µL
Cobalt sulfate heptahydrate	$CoSO_4.7H_2O$ (100 mM, CoII)	427µL
Iron sulfate heptahydrate	$FeSO_4.7H_2O$ (100 mM, FeII)	0.01µL
Ethylenediaminetetraacetic acid	EDTA	0.25g

1.6. Marine Broth (MB) medium

MB is used for cultivating heterotrophic marine bacteria. To 1L with distilled water, 37.4g (Difco™ 2216) was weigh and dissolved. Heat with frequent agitation and boil for 1 min to completely dissolve the powder. Then, it was sterilized by autoclaving at 121°C, for 20 min.

2. Solutions and Reagents

2.1. Stock solution of Glucose 5%

5g of glucose was weigh and dissolved with 100mL of distilled water and sterilized by filtration.

2.2. Tungsten solution

329.87g of sodium tungstate dehydrate ($Na_2WO_4.2H_2O$) was weigh and dissolved with 1L of distilled water and then it was sterilized by filtration, with a filter of 0.2 micrometer (µm).

2.3. TMPD (tetramethyl-p-phenylenediamine dihydrochloride) 1%

1g of TMPD was weigh and dissolved with 100mL of distilled water.

2.4. Solution of copper sulfate ($CuSO_4$) 20%

20g of $CuSO_4$ was weigh and dissolved with 100mL of distilled water.

2.5. Nitric acid (HNO_3) solution 10%

10mL of HNO_3 was dissolved with 100mL of distilled water.

2.6. Lysozyme solution

10 milligram (mg) of lysozyme was weigh and dissolved with 1mL of sterile water.

2.7. GES (100mL)

20mL of EDTA (0.5 Molar (M); pH 8) and 60g of guanidine thiocyanate were weigh and dissolved by heating at 65°C, in part with milli-Q water. Once cool, 1g of N-laurylsarcosine was added. The volume was adjusted to 100mL with milli-Q water. Then, the solution was sterilized by filtration, with a filter of 0.45µm.

2.8. Phosphate Buffered Saline (PBS) 10x stock solution

80g of NaCl, 2g of potassium chloride (KCl), 14.4g of dissodium phosphate (Na_2HPO_4) and 2,4g of monopotassium sulfate (KH_2PO_4) was weigh and dissolved in distilled water. The pH value was adjusted into 7.4 with hydrochloric acid (HCl) and then was added distilled water to 1L. The PBS stock solution was stored at room temperature.

2.9. Tris-Borate (TBE) 5x stock solution

54g of Tris base (Merck) and 27.5g of boric acid were dissolved in 800mL of distilled water. To this solution, 20mL of EDTA (0.5M; pH 8) was added and the volume was adjusted to 1L. The stock solution was stored at room temperature.

2.10. TBE 0.5x solution

The solution was prepared by dilution of TBE 5x stock solution in distilled water.

2.11. Tris-Acetate (TAE) 50x stock solution

121g of Tris Base (Merck) was weigh and dissolved in two solutions, namely in 50mL of EDTA solution (0.5M; pH 8; 3.4.1.2) and in 28.55mL of glacial acetic acid (Merck). The pH value was adjusted to 8, using sodium hydroxide (NaOH) solution (5M). Then, the volume was adjusted to 500mL with distilled water. Finally, the stock solution was stored at room temperature in a dark bottle.

2.12. TAE 1x buffer Solution

The solution was prepared by dilution of TAE 50x stock solution in distilled water.

2.13. Ethidium bromide (3.5.1.10) solution (5 milligram per milliliter ($mg.mL^{-1}$))

0.5g of ethidium bromide was weigh and dissolved in 100mL of sterile water and the solution was stored at 4°C, in a dark bottle wrapped in aluminum foil, in order to preserve from the light.

2.14. Loading Buffer

All the components, namely bromophenol blue (0.25%), sucrose (40%) and sterile water were mixed to dissolve.

3. Agarose gel 1% weigh/volume (w/v)

Agarose (1g) was weighted into a 250mL Erlenmeyer flask. TAE 1x solution (100mL) was added into the respective Erlenmeyer flask and was swirled to mix. To dissolve the agarose, this solution was heated in a microwave for about 3 or 4 min (the vial was shake a couple of times in order to facilitate dissolution of the agarose) and was left to cool at room temperature, stirring for 5 min. During heating, the electrophoresis equipment was prepared, putting the combs. After cooling, the ethidium bromide (8µL) was added to the mixture and was stirred to mix.

The gel was placed slowly into the tank and all the bubbles formed were pushed away to the side carefully, using the combs. Then, the gel was leaved to set at least 45 min. Before use, the combs were removed from the gel carefully.

Table XXII. Detected enzymes, with the respective substrate, used in API ZYM test.

Detected enzyme	Substrate
Alkaline phosphatase	2-naphthyl phosphate
Esterase (C4)	2-naphthyl butyrate
Esterase lipase (C8)	2-naphthyl caprylate
Lipase (C14)	2-naphthyl myristate
Leucine arylamidase	L-leucyl-2-naphthylamide
Valine arylamidase	L-valyl-2-naphthylamide
Cystine arylamidase	L-cyctyl-2-naphthylamide
Trypsin	N-bensoyl-DL-arginine-2-naphthylamide
α-chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide
Acid phosphatase	2-naphthyl phosphate
Naphthol-AS-BI-phophohydrolase	Naphthol-AS-BI-phosphate
α-galactosidase	6-Br-2-naphthol-αD-galactopyranoside
β-galactosidase	2-naphthyl-βD-galactopyranoside
β-glucuronidase	Naphthol-AS-BI-βD-glucuronide
α-glucosidase	2-naphthyl-αD-glucopyranoside
β-glucosidase	6-Br-2-naphthyl-βD-glucopyranoside
N-acetyl-β-glucosaminidase	1-naphthyl-N-acetyl-βD-glucosaminide
α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside
α-fucosidase	2-naphthyl-αL-fucopyranoside

Table XXIII. Different tests used in API 20NE test.

Tests		Active components	Reactions/enzymes	
NO_3	**NO_2**	Potassium nitrate	Reduction of nitrates to nitrites	
	N_2		Reduction of nitrates to nitrogen	
TRP		L-tryptophane	Indole production (tryptophane)	
GLU		D-glucose	Fermentation (glucose)	
ADH		L-arginine	Arginine dihydrolase	
URE		Urea	Urease	
ESC		Esculin ferric citrate	Hydrolysis (β-glucosidase) (esculine)	
GEL		Gelatin (bovine origin)	Hydrolysis (protease) (gelatin)	
PNPG		4-nitrophenyl-βD-galactopyranoside	β-galactosidase (para-nitrophenyl-βD-galactopyranosidase)	
|GLU|		D-glucose	Assimilation	Glucose
|ARA|		L-arabinose		Arabinose
|MNE|		D-mannose		Mannose
|MAN|		D-mannitol		Mannitol
|NAG|		N-acetyl-glucosamine		N-acetyl-glucosamine
|MAL|		D-maltose		Maltose
|GNT|		Potassium gluconate		Potassium gluconate
|CAP|		Capric acid		Capric acid
|ADI|		Adipic acid		Adipic acid
|MLT|		Malic acid		Malate
|CIT|		Trisodium citrate		Trisodium citrate
|PAC|		Phenylacetic acid		Phenylacetic acid

Water	α-Cyclodextrin	Dextrin	Glycogen	Tween 40	Tween 80	N-Acetyl-Dgalactosamine	N-Acetyl-Dglucosamine	Adonitol	L-Arabinose	D-Arabitol	D-Cellobiose
i-Erythritol	D-Fructose	L-Fucose	D-Galactose	Gentiobiose	α-D-Glucose	m-Inositol	α-D-Lactose	Lactulose	Maltose	D-Mannitol	D-Mannose
D-Melibiose	β-Methyl-D-Glucoside	D-Psicose	D-Raffinose	L-Rhamnose	D-Sorbitol	Sucrose	D-Trehalose	Turanose	Xylitol	Methyl Pyruvate	Mono-Methyl-Succinate
Acetic Acid	Cis-Aconitic Acid	Citric Acid	Formic Acid	D-Galactonic Acid Lactone	D-Galacturonic Acid	D-Gluconic Acid	D-Glucosaminic Acid	D-Glucuronic Acid	α-Hydroxy Butyric Acid	β-Hydroxy Butyric Acid	γ-Hydroxy Butyric Acid
p-Hydroxy Phenylacetic Acid	Itaconic Acid	α-Keto Butyric Acid	α-Keto Glutaric Acid	α-Keto Valeric Acid	D,L-Lactic Acid	Malonic Acid	Propionic Acid	Quinic Acid	D-Saccharic Acid	Sebacic Acid	Succinic Acid
Bromo Succinic Acid	Succinamic Acid	Glucuronamide	L-Alaninamide	D-Alanine	L-Alanine	L-Alanylglycine	L-Asparagine	L-Aspartic Acid	L-Glutamic Acid	Glycyl-LAspartic Acid	Glycyl-LGlutamic Acid
L-Histidine	Hydroxy-Lproline	L-Leucine	L-Ornithine	Lphenylalanine	L-Proline	L-Pyroglutamic Acid	D-Serine	L-Serine	L-Thr eonine	D,L-Carnitine	γ-Amino Butyric Acid
Urocanic Acid	Inosine	Uridine	Thymidine	Phenyethylamine	Putrescine	2-Aminoethanol	2,3-Butanediol	Glycerol	D,L-α-Glycerol Phosphate	Glucose-1-Phosphate	Glucose-6-Phosphate

Figure 33. Carbon sources in BIOLOG GN2 MicroPlates test.