Developing Elemental & Isotope Analytical Methods for Bacterial Investigations

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Abstract

The threat of an attack using biological warfare agents is becoming an ever increasing concern. Targets such as highly populated areas and food stocks are at high risk due to the current inefficiency in detecting bacteria. A method that can achieve this will be a transferable asset to the food and health and safety industry. This study uses Stable Isotope and Trace Element (SITE) analysis to differentiate between bacteria *B. subtilis* and *B. cereus*. This will also aid in the identification of the production method and determine the geographical origin of the bacterium. The study used Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) to create inorganic fingerprints of the samples in conjunction with Elemental Analyser-Isotope Ratio Mass Spectrometry (EA-IRMS) analysis of the isotopes δ13C, δ14N and δ2H to achieve this.

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List of Abbreviations

ELISA	Enzyme linked immunosorbent assay
PCR	Polymerase chain reaction
MS	Mass spectrometry
QD	Quantum dot
ICP	Inductively coupled plasma
IRMS	Isotope ratio mass spectrometry
SITE	Stable isotope mass spectrometry
GFAA	Graphite furnace atomic absorption
OES	Optical emission spectroscopy
dc	Direct current
rf	Radio frequency
ppb	Parts per billion
IAEA	International atomic energy agency
m/z	Mass to charge ratio
FERA	Food and Environment Research Agency

Chapter 1- Introduction

1. Bacteria

Bacteria surround and affect every person in the world. The discovery of bacteria did not occur till the 17th century after the invention of the microscope. Often bacteria go unnoticed due to many displaying little or no activity.[1] The number of harmless or beneficial bacteria significantly outnumbers those which are deemed to be harmful or fatal. Bacteria have been used extensively for soil enhancement and for the breaking down of organic and toxic waste.[2] The phrase "good bacteria" has become a commercial asset for many companies promoting their pro-biotic drinks.[3] Despite this the fact still remains that harmful bacteria still exists with the potential to be used for negative means.

1.1 Potential for Harm

Over 1,000 naturally occurring disease agents that exist are infectious to humans. These include: 538 bacterial species, 307 fungal species, 217 viruses and 66 species of parasitic protozoa. The advancement of biotechnology had led to many breakthroughs in microbiology research however as it becomes easier to grow and manipulate bacteria, it also makes it easier for bacteria to be produced for illicit activity also. Biological warfare was considered for a long time as being the last form of attack an enemy would use and therefore was inconceivable that they would ever be used; the same way the use of nuclear missiles is often described as inconceivable. However during the Cold and Gulf Wars, reality hit home as a biological warfare attack may have been a real possibility.

Sixteen years ago in Japan, Tokyo's subway came under attack from the apocalyptic cult, Aum Shinrikyo. The terrorist group used chemical weapons in the attack on the subway however, the cult were putting resources in biological weapon research for new potential

targets. The group cultured *Clostridium botulinum*, a bacterium most commonly associated with the cosmetics industry. The group's choice to culture their own bacteria rather than buy-in was their downfall. The scientists of Aum Shinrikyo tried to produce variable strains of the bacterium for the most lethal effect however, cult members had begun to realise that no one was dying or even harmed by their attacks. Even a worker, who had slipped and nearly drowned into store of almost 10,000 litres of the bacterial solution, had not been infected in any way.[4] The most recent case of bioterrorism occurred autumn 2001, just weeks after the devastating 9/11 attacks. Letters laced with *Bacillus anthracis* (anthrax*)* were sent directly through the U.S mail to senators as well as news editors. Five died and a further 17 sickened.[5]

The extremely low toxic levels of biological warfare agents shows the need for better detection and characterisation of bacterium, rather than just identifying the bacterial strain at high concentrations. At present the time it takes for a bacterial sample to be taken, cultured and then identified is far too long. Areas such as hazard management need to be able to rapidly identify biological hazards. It was also quickly realised that when biological warfare agents are present in variable backgrounds they are harder to detect. It is important to have analytical techniques which can characterise the inorganic fingerprint of bacteria by producing a rapid multi-element analysis and can analyse down to low detection limits from pg g^{-1} – ng g^{-1}.[6] Low detection limits are extremely important as far as biological warfare agents are concerned. To understand why, the lethality and sensitivity of a biological warfare agent must be taken into account. *Bacillus anthracis,* the causative agent of anthrax, is particularly dangerous due to the very low toxic threshold in humans. The amount of anthrax to be lethal can be easily calculated. Taking the minimum lung capacity to be 4 litres and a minimum toxicity of 8,000 spores means that only 2 spores/cm^3 of air is required to be lethal.[7]

As well as bacteria being manipulated in the form of biological warfare or bioterrorism, harmful bacteria can be harboured through improper preparation and storage of food causing food poisoning.[8] A common example of food poisoning occurs from fried rice which is kept warm. *Bacillus cereus* is the bacterium present in fried rice and very uncontrollable due to a short-incubation time of 1-6 hours. The bacterium causes nausea, vomiting and stomach cramp and after long incubation can cause diarrhoea. *Bacillus cereus* has also been subject to an outbreak from milk-powder used to make macaroni and cheese.[9] Deadly bacterium was also found in Taiwanese baby-milk powder and was among 852 other cases in food, beverage and cosmetics companies. The virus, which can coexist and spread as a bacterium, was responsible for outbreaks of Meningitis.[10]

To resolve these dangerous scenarios, analytical methods must be developed within forensic microbiology to determine a number of things. Firstly can a method be developed to easily differentiate between bacteria at very low detection limits? In order to treat possible patients or an infected area correctly, a rapid bacterium identification method is needed. Where bacterium has been used as a biological warfare agent there is two pieces of evidence needed; where the bacteria was being produced and its production method. If this can be concluded then a connection can be made between the biological attack and the production centre.

1.2 Current detection methodologies

First developments of biosensors were created as early warning alarms for biological attacks. Enzyme-linked immunosorbent assays (ELISA) as well as antibody-based chromographic assays were used as they were portable and usable in the field.[11] Both are considered to be primitive detection methods now especially as more popular methods of detection exist. Three of the most common detection methods have had criticism. The first method is Polymerase Chain Reaction (PCR). PCR involves the amplification of DNA to increase the quantity and therefore increase the concentration of the analyte.[12] The second analytical technique used is Antibody sensors. As the biological sample binds to a present antigen a signal is given. The signal is then transduced by electrochemical, thermometric, optical or piezoelectric components into a quantitative result. However problems arise with selectivity due to nonspecific-biding.[13] Lastly Mass Spectrometry (MS) based sensors which use the mass to charge ratios of ions present. Recent literature has shown that the three biosensors described are not sensitive enough. The detection of low concentrations was unachievable, even though the concentration had more than enough potential to infect, spread and harm.[14]

Previously resources were put into the development of an *in situ* test to detect biological warfare agent detectors. Particular interest has been shown in the research conducted for the detection of *Bacillus anthracis* using Fluorescence Immunoassay. The bacterium is labelled with quantum dots (QDs). The labels are for each specific bacterium which gives this process a high level of selectivity. A detector quantifies the results in terms of fluorescence intensity and its relationship with logarithmic count of *B. anthracis*.[15] The impact of fluorescence immunoassay is more easily understood by comparing the images from the fluorescence microscope as seen in Fig 1.

The images from the fluorescence microscope show how many bacteria cells would not be counted if they were examined without quantum dot labels. This method has potential for portable versions as it is very easy to use and has detection times of less than one hour. Currently detecting only the *bacillus* strain, QD fluorescence has the potential to detect various biological warfare agents. However Fluorescence Immunoassays are severely hindered by their inability to analyse coloured or cloudy samples. As the biological warfare agents will present in variable background complexes, this process is inapplicable. Fluorescence does have a very bright future though.[16]

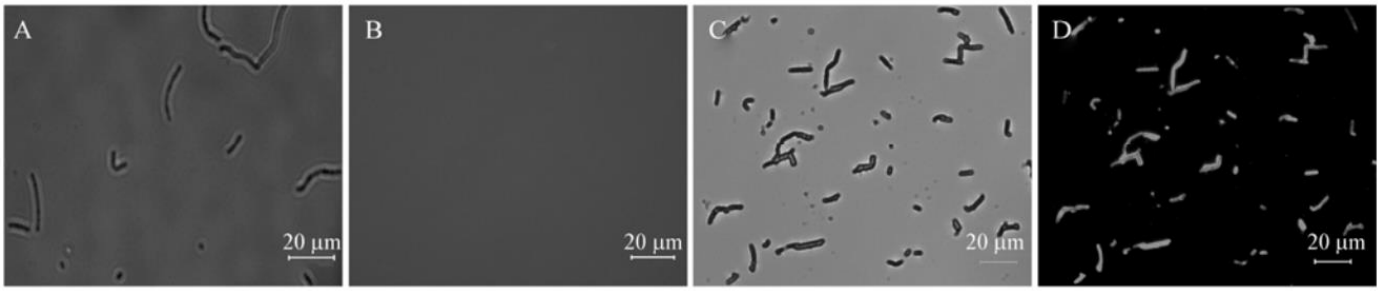

Figure 1- A) B.anthracis without QDs B) Fluorescence image without QDs C) B.anthracis with QDs D) Flourescence image with QDs[17]

A quality analytical technique should be achieved first with future views to scale down to be useful in the field. Currently samples are still sent back to defence labs around the world for analysis. In a society where knowing where your food originates from has become an important issue, an analytical technique which could provide information of place of production as well as provide data on all inorganic and heavy metals contained within would be optimal. Data on food from all around the European Union has been compiled together using various analytical techniques, predominantly using inductively coupled plasma-mass spectrometry (ICP-MS).[17] This analytical technique can be easily transferred into the

progression of biological warfare agent detection. ICP-MS has the sensitivity to analyse bacteria in aerosols and also the ability to characterise the inorganic fingerprint of the analyte whilst in complex matrices. International communication can lead to a vast database of bacteria and their inorganic fingerprints. One will be able to state the georgraphic origin of any biological warfare agent concerned. However the sample may contain varying isotopes of an element causing a difference in mass to charge ratio. Isotope Ratio Mass Spectrometry (IRMS) is an analytical method which can enable greater accuracy for locating the geographical location of a sample. Isotope ratios of particular elements such as carbon, hydrogen, oxygen, sulphur, and nitrogen can locally increase or decrease by a variety of kinetic and thermodynamic factors. The measurement of the isotope ratios can be used to differentiate between samples which may be identical in there chemical composition.[18]

The detection, identification and characterisation of biological warfare agents is made more difficult by the low detection level needed. As well as this, the analytical technique needs to be able to identify and detect organisms which are airborn. This is complicated by bacteria's ability to change its inorganic fingerprint depending on the growth conditions.[19] The ability to identify the geographical origin of a bacterial source would also be extremely useful as well as determining the production method by analysing the water and liquid broth media.

2. Stable Isotope and Trace element analysis

The forensic application of SITE (stable isotope and trace element) analysis has increased due to the processes ability to determine the sample itself through trace element fingerprinting as well as the geographical origin. SITE analysis can be extremely diverse and be used in numerous scenarios to achieve results.

Trace element analysis can be used to create a complete inorganic fingerprint of the sample from the elemental concentrations. It can also be used to distinguish between similar samples which may have come from different origins or contain differing trace elements through environmental exposure or production. Trace element analysis is predominately done using mass spectrometry based analytical techniques such Inductively Coupled Plasma- Mass Spectrometry (ICP-MS).

Compared to similar analytical techniques, ICP-MS exhibits far more advantages available for the user. ICP-MS has the combination of the very high detection limits of Graphite Furness atomic absorption (GFAA), as well as the rapid multi-element analysis of Inductively Coupled Plasma-Optical emission spectrometry (ICP-OES). It is then a surprise to say that ICP-MS only represents 7% of the overall atomic spectroscopy market. The number of sales in the first 5 years of commercial availability was much lower than machines such as ICP-OES. The credentials for ICP-MS are second to none and the extra expense is definitely worthwhile for the complete package. Unfortunately for this analytical technique, the user interface still remains complicated for novices and scientists who are set in their ways and very comfortable with previous Atomic absorption techniques. Robert Thomas has more than 20 years' experience in the development and application of atomic spectroscopy instrumentation. He recognised the complicated nature in which the processes of ICP-MS were being presented and simplified it so anyone can learn the benefits of ICP-MS, its mechanistic parts as well as its application in all manners of things from environmental chemistry to taking a role the inorganic fingerprinting of biological warfare agents.[20]

The sample first needs to be generated into an aerosol which can then be easily swept into the plasma. There are a couple of other methods to this but the most common, and the one at the University's analytical suite, is a nebuliser and a spray chamber. The nebuliser consists of a high flow gas which collides with the liquid sample breaking it apart into an

aerosol. The steady flow of the liquid insures an even amount of aerosol is produced no matter what the viscosity is. The function of the spray chamber is to ensure only the smallest droplets enter the plasma. Large droplets are harder for the machine to dissociate and the main reason why only 2-7% of the sample is actually processed. As the aerosol travels through the spray chamber, the larger, heavier droplets drop down through the chamber due to gravity. It also removes the pulsating occurrence of aerosol produced by the peristaltic pump.[21]

The sample is then introduced to the plasma at high velocity where the sample undergoes a transformation. The water surrounding the droplet is first evaporated to leave only tiny solid particles. The sample particles are then vaporised into a gas and then atomized into its ground state atom conformation. Finally the sample atoms are continuously bombarded by the argon electrons still being produced. The collisions between the sample ion and electrons cause ionisation. This is represented in Fig. 2.[22]

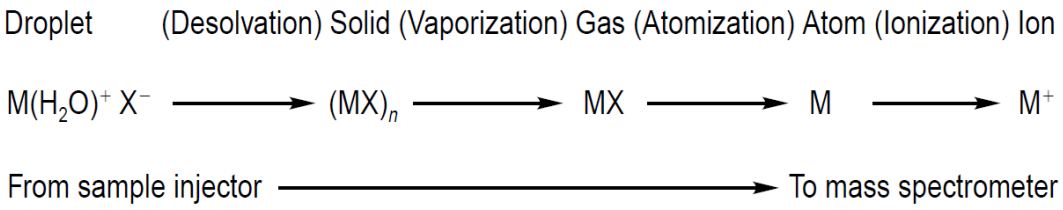

Figure 2- Ionisation process of sample[23]

Now that the elemental ions have been produced, they will be analysed through the mass spectrometer. MS will provide data on the µg/g of each element present in the sample. However, how can each element be distinguishable between one and another? The answer is separation of the ions using a quadrupole. A quadrupole consists of four metal rods where

one pair is charged with a direct current (dc) whilst the other pair has been given a radio frequency (rf) field. Now that the rods have a dc/rf voltage passing through, the ions can be electrostatically steered though the centre of the rods. Ions which are being analysed at this time drop out of the system. Only the ion with the chosen mass-to-charge ratio may pass through. This process is repeated for each desired ion wishing to be analysed simply by changing the voltage and frequency of the current flowing through the quadrupole as seen in fig 3[23]. This is changed automatically by the computer as each repeat is taken according to the ions selected for analysis. The ions then enter the detector which calculates the parts per billion (ppb) as well as relative concentration (mg/kg) of the element.[24]

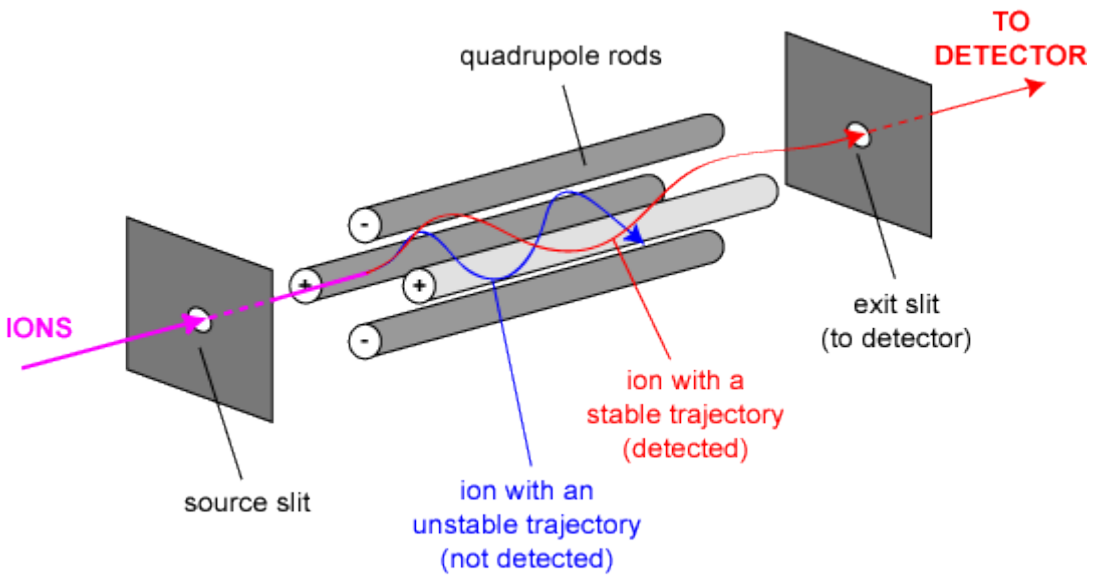

Figure 3- Magnetic Quadrupole of the ICP-MS[1]

The other half of SITE is the analysis of stable isotopes. IRMS became commercially available around 10 years ago it has become a viable asset for many scientific purposes. From archaeology to food authenticity, medicine to forensic science, the number of published articles has increased significantly.[25] IRMS analysis allows the precise measurement of

naturally occurring isotopes. It is able to measure a mixture of isotopes without having to previously select specific isotopes to analyse.[26] Isotopes of light and abundant elements such as $^{13}C/^{12}C$, $^{18}O/^{16}O$, $D/^{1}H$, $^{15}N/^{14}N$, and $^{34}S/^{32}S$ are most easily measured by IRMS. The value first given is the ratio between the heavy and light isotopes. (Eq.1). This ratio is then compared to the international standard ratio for each element set out by the International Atomic Energy Agency (IAEA) to give the value, δ. (Eq. 2).[27]

(Eq.1) $$ratio\ (R) = \frac{Abundance\ of\ Heavy\ Isotope}{Abundance\ of\ Light\ Isotope}$$

(Eq. 2) $$\delta = 1000(R_{sample} - R_{standard})/R_{standard}$$

Depending of the composition of the sample, the analyte is converted thermally or through combustion into simple gases such as H_2, CO_2, CO and N_2. The IRMS can then measure the mass to charge ratio (m/z) of the ions to their corresponding gases. For example the analysis of Carbon ions the sample would be converted first to CO_2 where the mass spectrometer would give (m/z) values of 44, 45 and 46. This can be related to the different combination of CO_2 molecules containing ^{12}C, ^{13}C, ^{16}O, ^{17}O and ^{18}O.[28] IRMS set-up can be divided into five separate parts: sample introduction; electron ion source; Faraday-cup detector array and computer for data acquisition.[29]

The two main types of sample introduction used for IRMS is an elemental analyser (EA) and gas chromatography (GC). Other introduction systems like liquid chromatography (LC) are used however recent publications have only shown variable degrees of success. For this study EA sample introduction was used.

Precise sample preparation is required for EA-IRMS as the average isotopic signal is given for the entire sample. If there were variable sample masses it would significantly skew the results. 1mg of sample is measured into a tin or silver capsule, sealed and placed into the auto-sampler ready to be dropped into the combustion oven. Depending on the isotope being analysed, the combustion products will be treated differently. For Carbon isotope analysis Nitrous oxide compounds are converted to N_2 and surplus O_2 removed. Excess water produced from the combustion is then removed through a chemical trap. The analyte is then passed through a gas chromatograph where N_2 and CO_2 are separated.

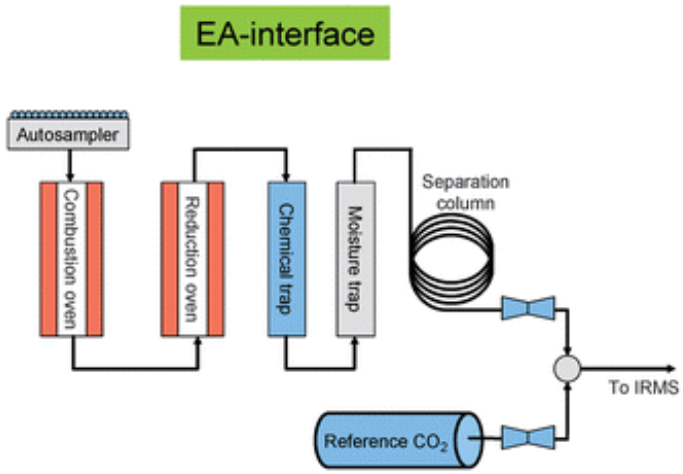

Figure 4 - Schematic depicting the EA-IRMS induction system[26]

The effluent undergoes electron ionisation where a filament produces excited electrons which collide with the analyte causing them to ionise. Next the individual ions pass a magnet. The magnet repels the ions away from itself towards Faraday cup detectors. The Faraday cups are spaced out in an array with m/z ratio determining which Faraday cup the ion travels into. The greater the m/z ratio, the more inertia it will experience due to it being heavier because of extra neutrons however with no change to its charge. The conductive metal Faraday cup catches the ions and converts them into a current.[30] This current can then be amplified before entering a computer for processing. This interface can be seen in fig. 7.

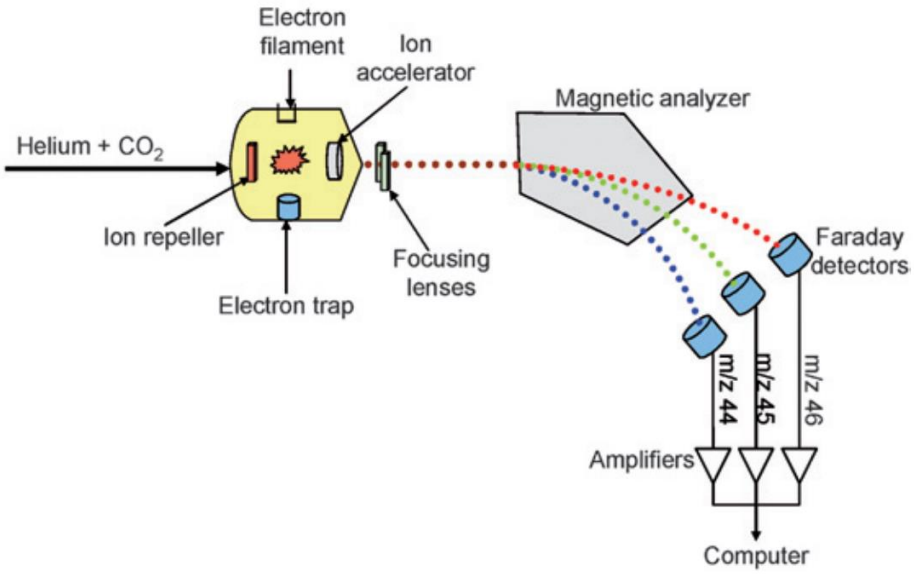

Figure 5- Schematic of IRMS interface[26]

The use of a magnet with Faraday cup detectors is much more desirable than a conventional quadrupole and "organic" mass spectrometry set up. Although this interface has the ability to detect different ions, it has a number of draw backs. The main reason is because the mass spectrometer only has a single detector interface. This means that only one ion can be detected at a time. Elements with a pair or a triplet of ions (X^+ and $X+1^+$/ $X+2^+$) cannot be analysed simultaneously and equates to approximately 50% ions being unaccounted for. The

accuracy required to detect the natural abundance of isotopes should be ±0.001 atom%. MS and even continuous flow MS, in selected ion monitoring (SIM) only ±0.1 atom% is achievable. Therefore quantitative results using this technique would not be reliable enough.

3. SITE analysis around the world

The forensic application of SITE analysis is increasingly being used around the globe for the identification of compounds as well as commercial products. In recent years more and more people have grown concerned about where their products originate from. This was brought to the forefront of the public's attention when horse meat was found to be contained in many beef products around the UK and Ireland.[31] The ability to determine the geographical origin using SITE analysis has become a valuable forensic asset in order to find those who are accountable or to confirm the timeline of events. SITE analysis has been used in a paper by K. Heaton *et al.* to verify the geographical origin of beef around the world. It was observed that intensive maize and/or C_4 pasture feeding, during cattle production, gave rise to significant differences in the ^{13}C content of beef produced in Brazil and the USA compared to British beef cattle fed predominantly on C_3 pasture and fodder. The mean δ^2H‰ and δ^{18}O‰ values of beef lipid related well with the latitude of production regions and the relationship between the H and O isotopic contents were found to parallel the Meteoric Water Line.[32]

It has been long established that the chemical composition of an organism reflects the chemical composition of the environment it is grown in. An early example was when marine phytoplankton was discovered to have an elemental ratio between C, N and P.[33] As well as elements such as H, O and S it has also been established that the metal content in an organism can also reflect the growth environment and conditions. Metals are required by microbes for a number of purposes which include cell structure, growth, maintenance as well as charge balancing. On the whole cell membranes are impermeable to charged metals and are therefore

acquired one of three ways: via endocytosis; in ionic form through membrane pumps, channels or transporters or similar transport as an organic-metal complex.[34]

It is not just enough to compare the elemental signatures of bacteria without taking into account their signature stability. Sorption of metals by dead biomass can occur [35] and due to the sorption process being reversible it is a question whether or not a stable signature can be achieved from a forensic point of view.[36] In order to analyse bacteria successfully it needs to be either shown to retain a stable signature of an appropriate time scale or be confined to a predictable pattern. Such scenarios which may have effect could be temperature, humidity or post-processing. Ghosal *et al.* proved that the signature could be manipulated by exposing the cells to water and aqueous salt solutions. This emphasized the need for stringent sample collection and storage protocol.[37]

The first research to use Inductively Coupled Plasma-Mass Spectrometry for the identification and detection of bateria was by C. M. Gikunju *et al.* The paper aims to achieve an inorganic chemical characterisation of three bacteria, *Bacillus subtilis* spores (BG), *Bacillus subtilis* vegetative cells (Bg) and *Bacillus thuringienisis* (Bt). The log concentration (mg/kg) is graphically represented against each fingerprint component. Fig 4. This shows the bacterium have differing compositions of inorganic elements. Not only does this graph show it is possible to collect data at a low detection threshold but it is also easy to distinguish the difference between each result. It shows how vastly different *Bacillus subtilis* spores are to the other two bacterium.

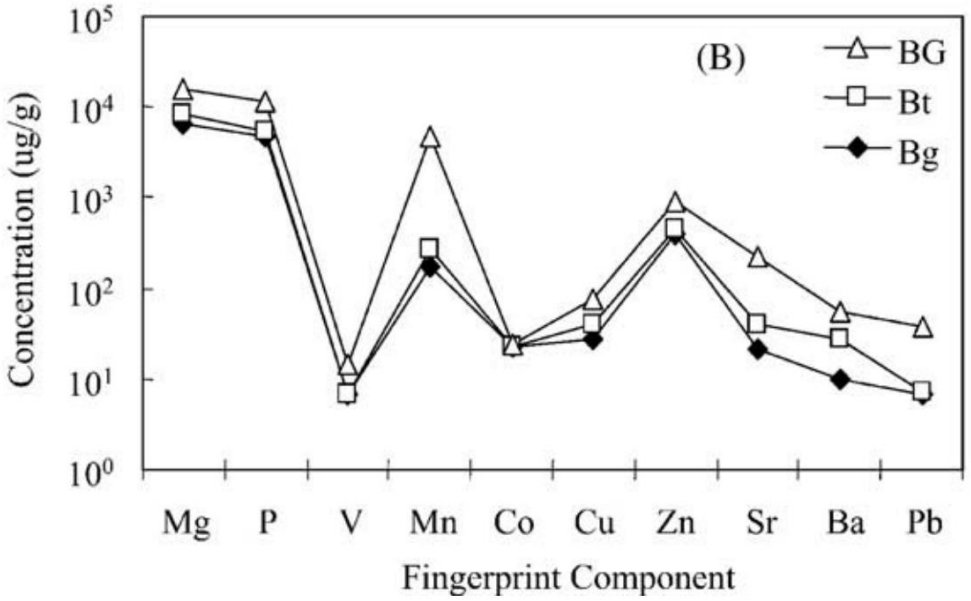

Figure 6- Log Concentration vs fingerprint component for each Bacteria strain[7]

Bacteria incorporate C, N, O and H atoms from the nutrient broth from which they are grown from. O and H atoms are additionally acquired from the water in which the nutrient broth is made up with. The medium in which bacteria are grown in are therefore the prolific source of isotopic variation. Agar is commonly used as a solidifying agent due to it not being consumed by the bacteria and hence has no contributing factor to the isotopic ratios within the bacteria.[38]

Kreuzer-Martin *et al.* completed a study of *Bacillus subtilis* grown in 30 different media. It showed that although the bacterium did incorporate C into their overall biomass, it did not change the overall stable isotope content. However, the isotope ratio for N did increase positively by approximately 4.5 ‰ than that of the culture medium. *Bacillus subtilis* was also grown in four different nutrient media using 5 isotopically different water sources. Analysis showed that around 30% of the H originated from the water used for culture. Lastly the study showed the overall contributing factor by growing 12 *B. subtilis* samples (from the

same strain as before) using only one water source. The resulting regression analysis showed that 70% of the H content came from the nutrient growth media.[39]

4. Study Aims

The study should go some way into addressing current issues with microbial forensics as well as the validation of previous research. The study comprises of three key aims: the differentiation of bacteria; identification of production method and determination of geographical origin. The research will involve the analysis of *B. subtilis* and *B. cereus* which have been grown in different nutrient media broths furthermore analysis will be done on *B. subtilis* and *B. cereus* which been grown with different water sources. ICP-MS will be used to produce inorganic fingerprints of the samples. The variance in elemental concentration will be used to differentiate between the bacteria, water source and nutrient liquid broth media used. Analysis of the isotopes will be done using EA-IRMS. Water sourced from different locations will be used when growing the bacterium samples to show whether or not the geographical origins can be determined by this analytical method. *Bacillus subtilis* and *Bacillus cereus* will be used as they belong to the same genus as anthrax but are only low level biological hazards.

Chapter 2- Materials and Methods

1. Prepartion of Bacteria samples

A total of 18 bacterial samples were analysed. Half the bacterial strains used in this study were *B. subtilis* and the other half *B. cereus.* Strains were first cultured in four different liquid broth (LB) nutrient media. As well as a variance in growth media *B. subtilis* and *B. cereus* was grown in five different water sources. The water used was from Preston, Lytham, Manchester as well as bottled waters, Evian and Highland Spring, were used.

TABLE 1- LB Nutrient Media

#	Supplier	Content (g/l)
1	Difco	Beef extract 3g Peptone 5g
2	Oxoid	'Lab Lemco' powder 1g Yeast extract 2g Peptone 5g Sodium Chloride 5g
3	Fluka	Meat extract 1g Yeast extract 2g Peptone 5g Sodium Chloride 5g
4	Formedium	Peptone 38.56% Yeast extract 15.38% Sodium Chloride 38.46% 'lab lemco' powder 7.8%

For analysis, cells were grown in 250ml of culture until they reached late exponential phase, before being centrifuged at 4,000 rpm for 10 minutes at room temperature. The cell pellets were washed twice in distilled water to remove any residual media before freeze drying. Cell numbers were determined by plate counts and optical density at 600nm. Bacteria grown in the different water sources all used LB 3 as their media.

2. ICP-MS

2.1 Sample Digestion procedure

5mg of each sample was digested with 5ml HNO_3 70% in microwavable Teflon® vessels. The samples were added to the CEM-Mars 5 microwave and ramped to 150°C for 10 minutes then plateaued at 150°C for a further 20 minutes for complete digestion. Once digested, 10ml sample tubes were filled with 9.5ml of 1% HNO_3 water with 500µl of the sample. The sample list for the ICP-MS was done with multi-element samples and acid blanks and even intervals to ensure new sample analysis was not affected by previous runs.

2.2 Sample analysis

Initial data analysis was performed using Thermo plasmalab X-series ICP-MS to calculate the concentration (µg/g) for each element. The elements were selected using the results of survey runs of the bacteria as well as on the approach used by Gikunju *et al.* (2004). The elements showed relative abundance in the bacterium analysed incorporating the ability of ICP-MS to analyse them specifically. Calibration standards were carried out using a multi-element standard and with no standard reference materials some samples had been spiked with In to assure the validity of the analysis. Following this, the data was exported to Microsoft Excel where the averages, standard deviation and relative standard deviation were calculated. The resulting average for each sample was constructed into comparative line graphs with logarithmic scales.

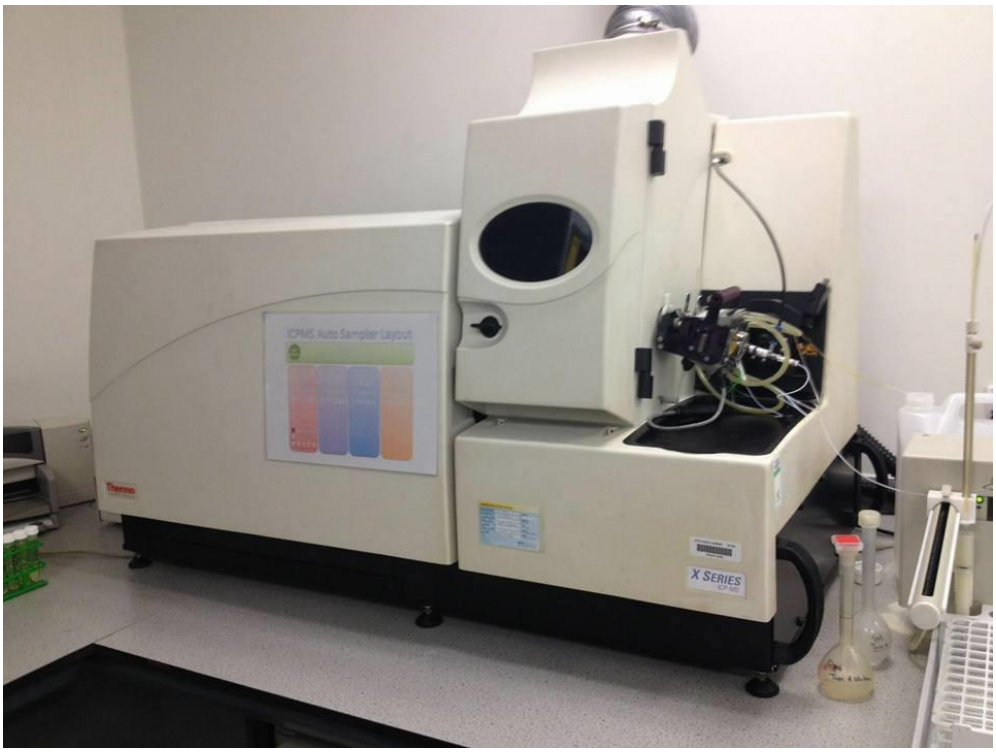

Figure 7- Thermo Plasmalab X-series ICP-MS

3. IRMS

3.1 Carbon (δ13C) and nitrogen (δ15N) isotope analysis

1mg of sample was weighed into 8x5mm tin capsules, sealed and placed into the auto sampler. Carbon (δ13C) and nitrogen (δ15N) isotope ratios were determined using a Fissons 1108 Elemental Analyser at a temperature of 1020°C and 650°C coupled to a GV Instruments Isoprime 100 in continuous flow mode. Included were regularly spaced laboratory standards in all sample runs. Porcine collagen and JCR sucrose were chosen as standards as their (δ13C) and (δ15N) isotope ratios have values close the results predicted for bacteria. These standards, which the samples were calibrated by, were not international approved standards however are used for quality control by the Food and Environment Research Agency (FERA) and have the validity to be published in research journals. Before samples were run, stability checks were carried out which included a linearity test for quality control. All samples were run in duplicate and in triplicate where memory effect was apparent. Values are reported in delta notation (δ): δ(‰) = (Rsample / Rstandard – 1) x 1000, where Rsample and Rstandard are the ratio of heavy to light isotopes of the sample and standard respectively. Long term in-house repeatability for each element is as follows: 0.2‰ (δ13C), 0.3‰ (δ15N).

Figure 8- Fissons 1108 Elemental Analyser

3.2 Hydrogen (δD) analysis

Solid samples for hydrogen (δ2H) analysis were run using the same procedure as for δ13C and δ15N. After running a test batch Paraguayan and Bavarian horse hair was chosen as standards. Casein was chosen as the quality control standard and holds equal validity as a standard as porcine collagen did for δ13C and δ15N analysis.

In order to analyse the water samples, 1µl of sample was syringed into 10 x 5mm smooth wall flat base tin capsules. Interantional standards were used for the calibration of the liquid samples. The standards used were VSMOW2 (Vienna Standard Mean Ocean Water 2), GISP (Greenland Ice Sheet Precipitation) and SLAP2 (Standard Light Antarctic Precipitation 2). After running the δ13C and δ15N analysis of the samples, the Fissons 1108 Elemental Analyser was replaced with a new Vecstar pyrolysis furnace at a temperature of 1350°C connected to the GV Instruments Isoprime 100.

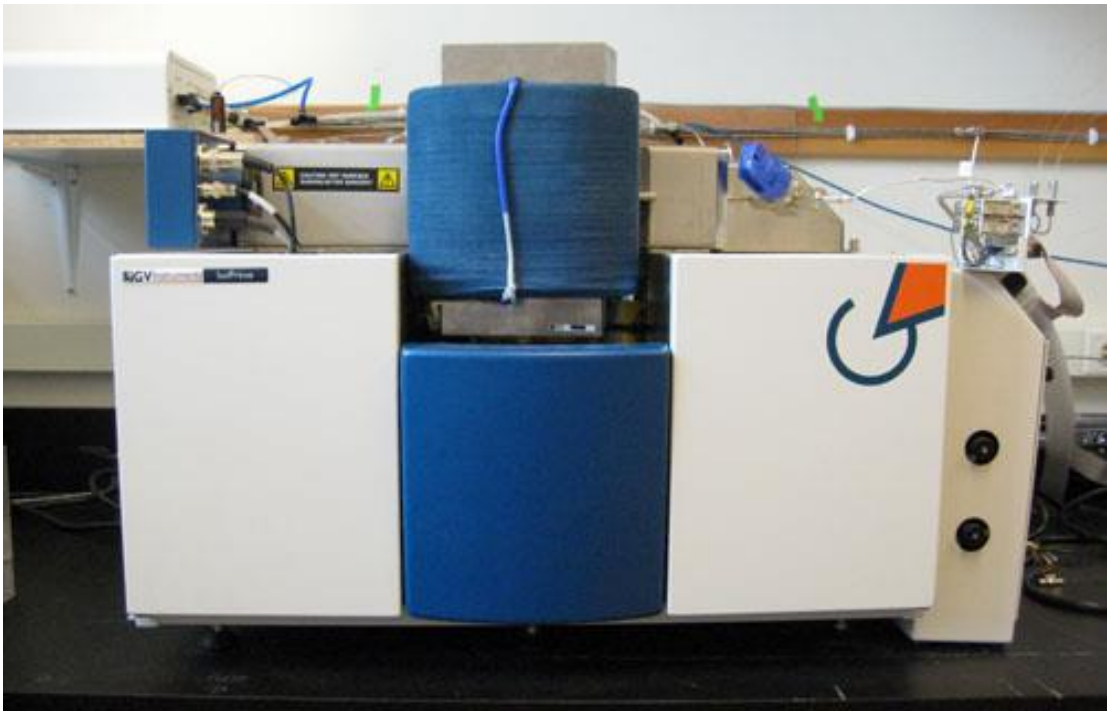

Figure 9- Vecstar Pyrolysis Furnace

3.3 Data Processing

Linear shift normalisation or stretch-shift correction was used to determine the final δ-values. The average difference between the true and measured δ-values of the standard is calculated. This is added to the measure δ-value of the sample to give the true δ-value of the sample. This method has a significant advantage over single point anchoring as the calculation is independent of the isotopic composition of the working gas. In order to achieve the best accuracy the standards bracketed the samples to encompass any changes the IRMS may experience during the full run of sample analysis.

Chapter 3 - Results and Discussion

1. ICP-MS

LB media 1 has the lowest concentration of Na when analysed solely by itself. The results for *B. subtilis* grown in LB media 1 also show it to have the lowest concentration of Na. The same can also be said of the concentration of Mg. LB media 1 has the highest concentration of Mg and in turn the *B. subtilis* results show the bacteria has the highest concentration. This indicates that these elements have been encompassed by the bacteria in order to grow. This also translates to the concentrations of Cu and Pb in LB media 4 showing that the concentration of an element correlates to the concentration of an element retained by the bacteria however this cannot be said of all the elements. Ca is relatively abundant within LB media 1 however it is not transferred to the bacteria in the same way as the other elements. The other LB mediums show little to no (in the case of LB media 4) concentration of Ca. This gives an indication that Ca is not consumed by *B. subtilis* during the growth process. This is probable reason why only LB media 1 has a concentration of Ca and can be linked to the relatively high quantity of beef extract in its composition. The result of the LB media samples show no concentration of Mn at all but is present in the *B. subtilis*. The concentration of Mn within the bacteria is very low though ($\lesssim$1µg/g) which could indicate a different source of Mn from the environment, a contamination or possibly a memory effect from the ICP-MS. The results for *B. cereus* will show whether or not it behaves in a similar fashion or differently to that of *B. subtilis*.

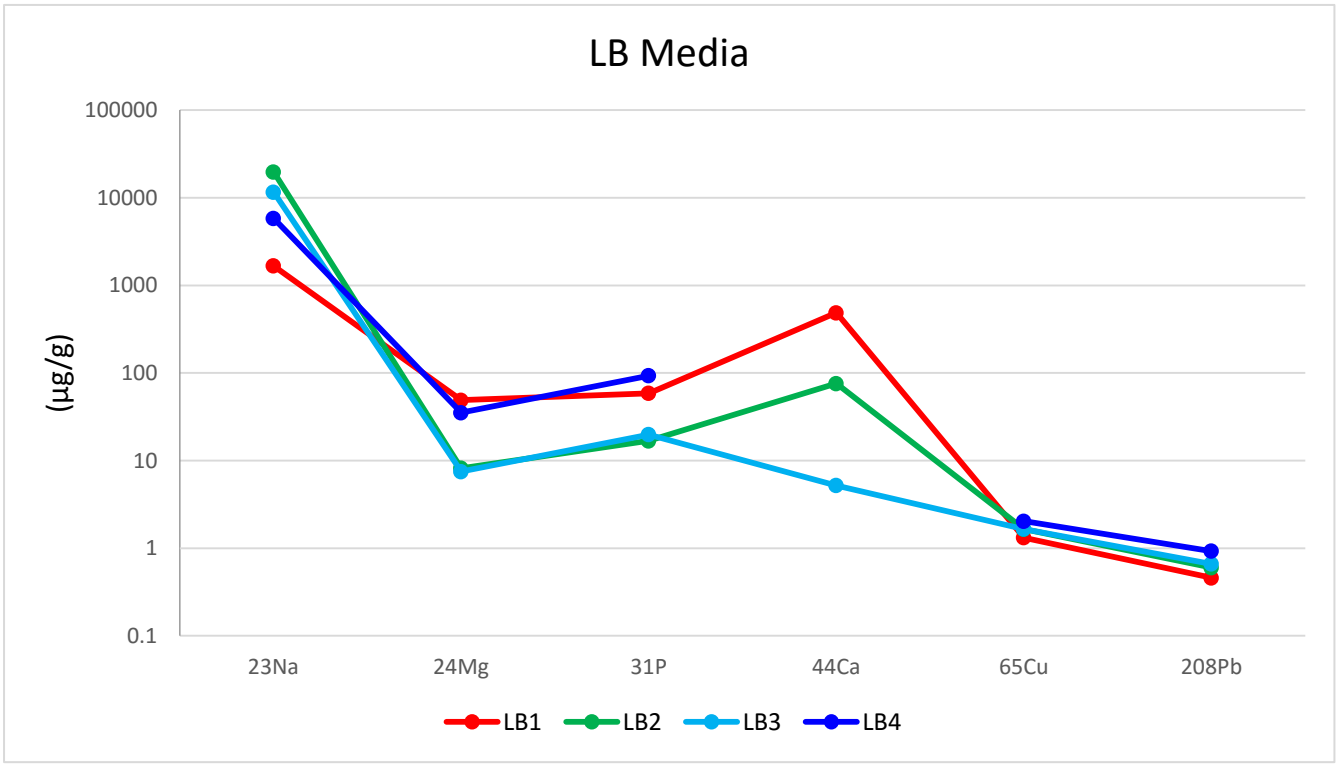

Figure 10- A graph depicting the (µg/g) concentrations of Liquid Broth media on a logarithmic scale (85-105% error)

TABLE 2- ICP-MS data for LB Media

(µg/g)	23Na	24Mg	31P	44Ca	65Cu	208Pb
LB1	1670.00	49.02	58.73	488.03	1.32	0.46
LB2	19770.00	8.19	16.84	75.81	1.65	0.60
LB3	11641.00	7.51	19.84	5.21	1.65	0.66
LB4	5830.33	35.25	93.04	-22.80	2.03	0.93

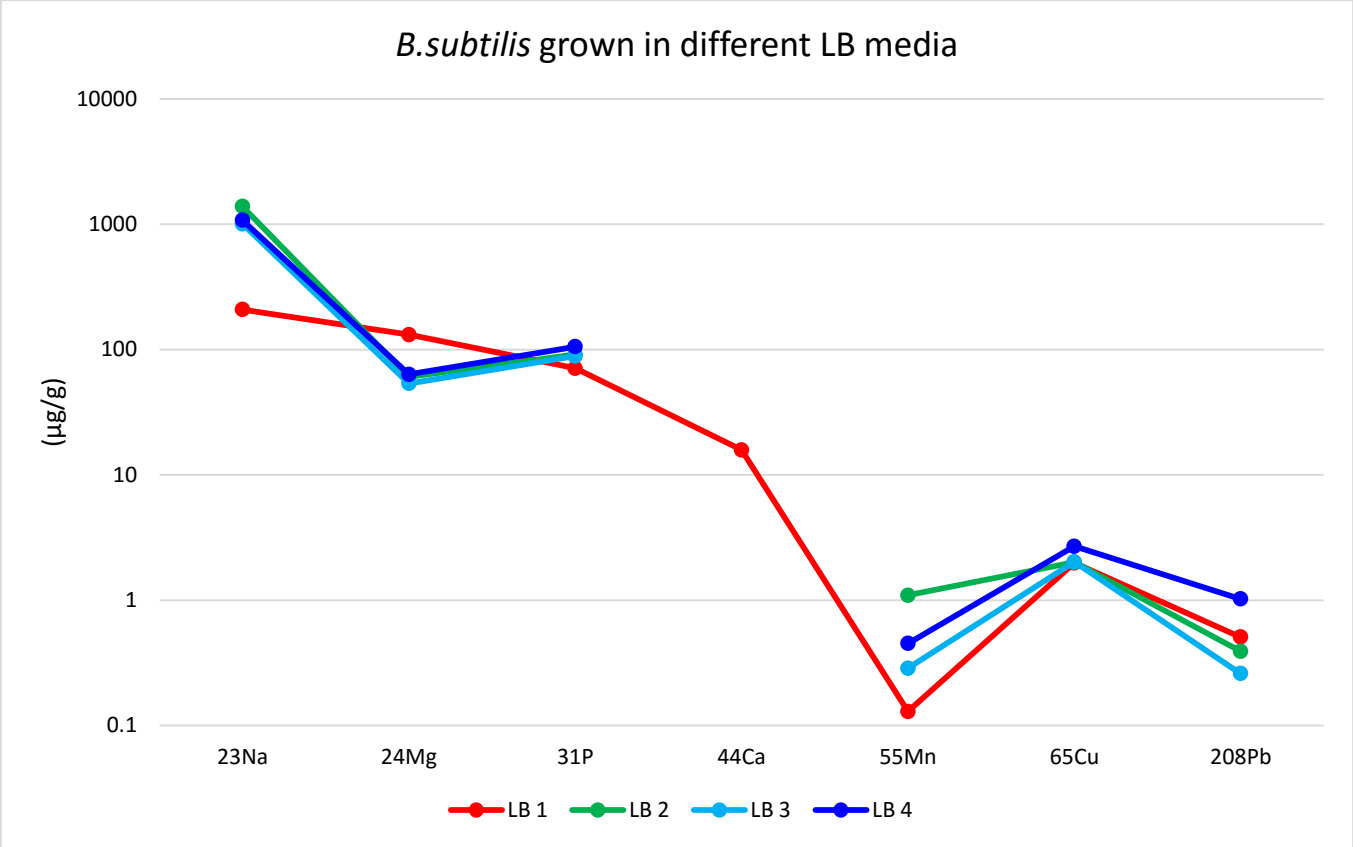

Figure 11- A graph depicting the (µg/g) concentrations of B. subtilis grown in different Liquid broth media on a logarithmic scale. (85-105% error)

TABLE 3- ICP-MS data for *B. subtilis*

(µg/g)	23Na	24Mg	31P	44Ca	55Mn	65Cu	208Pb
LB 1	208.83	131.70	70.87	15.81	0.13	1.98	0.51
LB 2	1388.00	60.62	91.34	-16.87	1.09	2.01	0.39
LB 3	1007.00	53.65	88.66	-36.84	0.29	2.02	0.26
LB 4	1077.80	63.44	105.57	-46.83	0.45	2.69	1.02

Na exhibits the same behaviour in *B. cereus* as it did in *B. subtilis* however this cannot be said for the other elements. The concentration of Mg has actually dropped for LB media 1 whereas the other bacteria have remained at a very similar concentration. This could be due to measurement error however it is notable to point out the concentrations of Cu and Pb are in fact larger than the other bacterium. LB media 1 produced the least yield across the board when trying to produce the bacteria suggesting that the overall composition of the media has a greater effect on the bacterium's ability to consume the inorganic elements needed. LB media 1 is the only media not to include yeast extract which can speed up the cell production process considerably.

LB media 4 has a much greater concentration of Mg than LB media 2 and 3 however the concentration of Mg within *B. cereus* is very similar. A proposed reason could be that the composition of LB media 4 is not suited to the consumption of Mg by *B. cereus* however a more probable hypothesis would be that *B. cereus* has a saturation point of Mg, where the bacterium cannot uptake anymore Mg causing the excess to be washed away during the sample production process. This can be seen in fig.11.

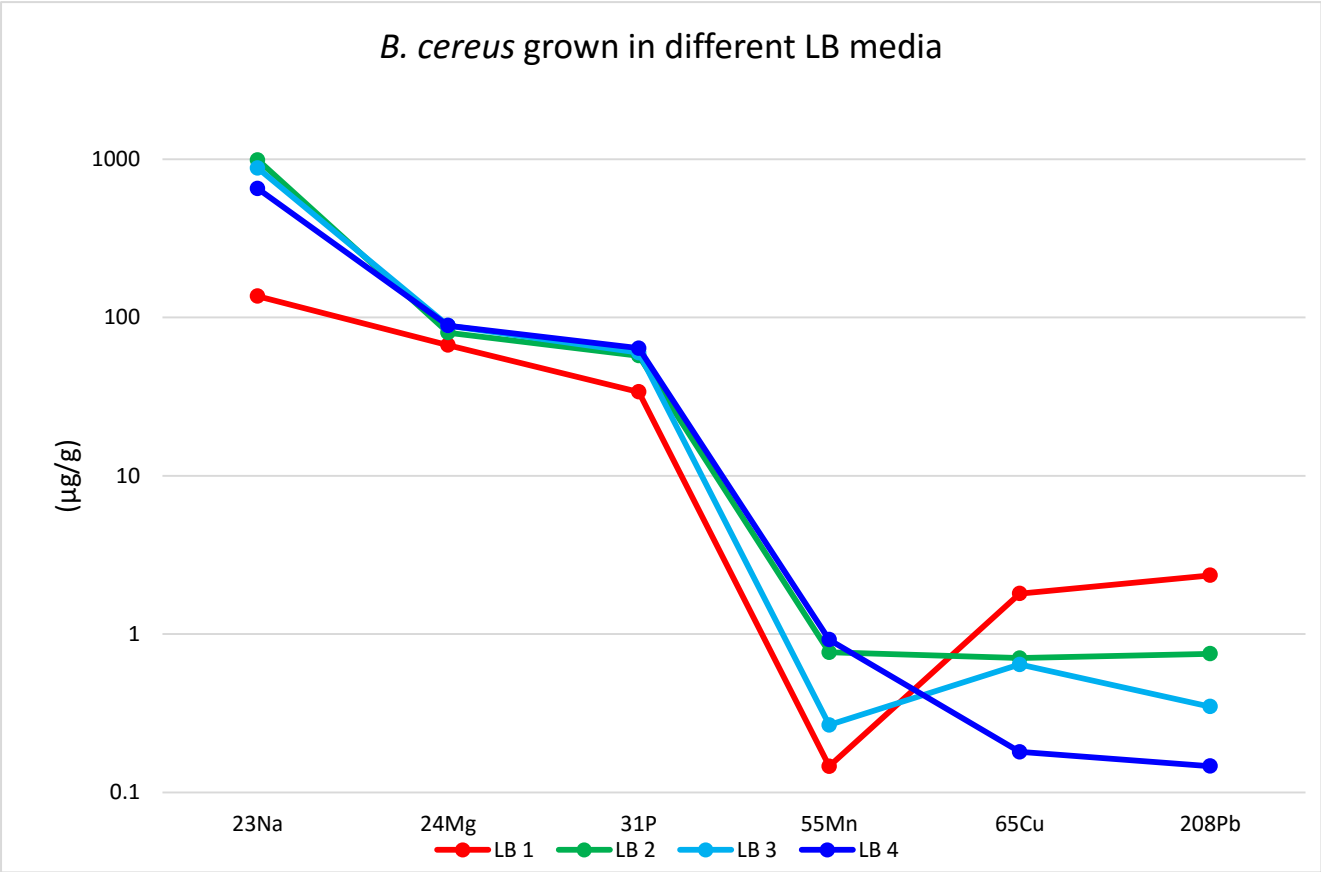

Figure 12- A graph depicting the (µg/g) concentrations of *B. cereus* grown in different Liquid broth media on a logarithmic scale. (85-105% error)

TABLE 4- ICP-MS data for *B. cereus* with different LB media

(µg/g)	23Na	24Mg	31P	55Mn	65Cu	208Pb
LB 1	136.17	66.76	33.83	0.15	1.80	2.35
LB 2	988.00	80.10	57.29	0.77	0.71	0.75
LB 3	877.40	88.78	59.93	0.27	0.64	0.35
LB 4	651.80	88.70	63.98	0.92	0.18	0.15

The (µg/g) concentrations of the water sources on their own show no significant differences across any of the samples. Evian and Highland spring water display very little concentrations for the heavier elements (fig.12). This is most likely due to the extra water treatment bottle water undergoes compared to tap water. It is evident from the results of *B. subtilis* and *B. cereus* that the bacteria's main source of inorganic elements is not from the water source but from the nutrient broth it is grown in. As the LB media does have a lot higher concentrations of inorganic elements the effect the water and its elements becomes negligible.

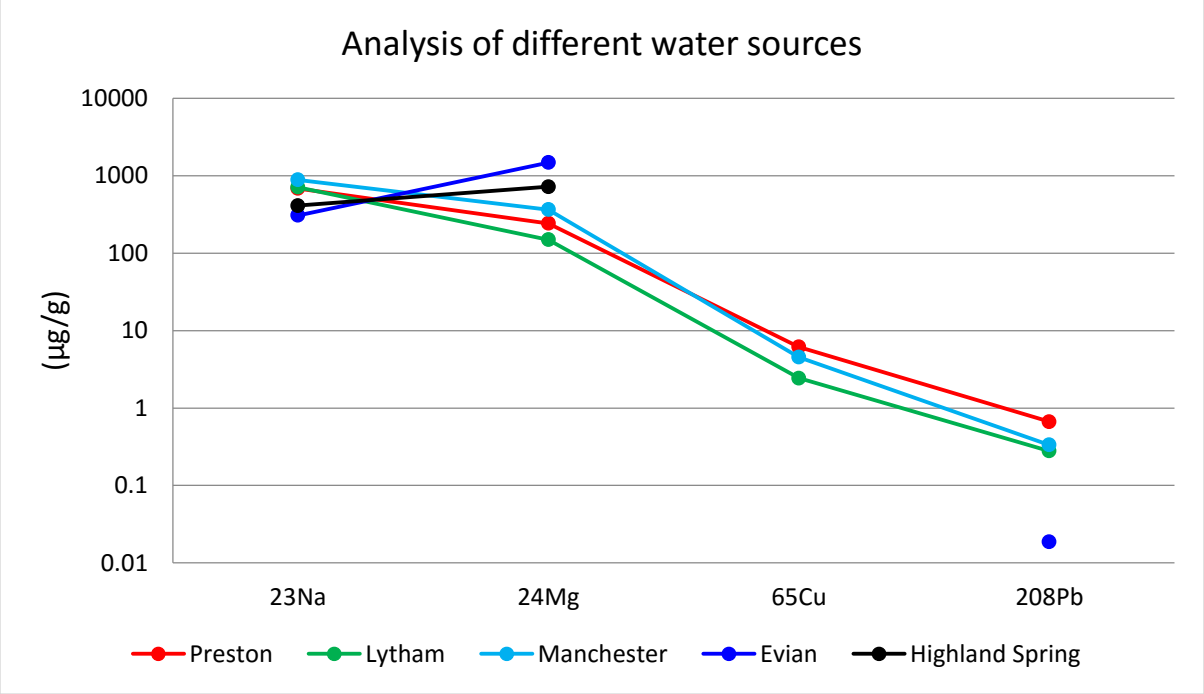

FIGURE 13- A graph depicting the (µg/g) concentrations of water sources on a logarithmic scale. (85-105% error)

TABLE 5- ICP-MS data of water sources

(µg/g)	23Na	24Mg	65Cu	208Pb
Preston	683.43	242.47	6.14	0.67
Lytham	711.93	148.97	2.43	0.28
Manchester	883.2	365.45	4.53	0.33
Evian	307.9	1480.33	-0.13	0.019
Highland Spring	411.5	720.9	-0.38	-0.074

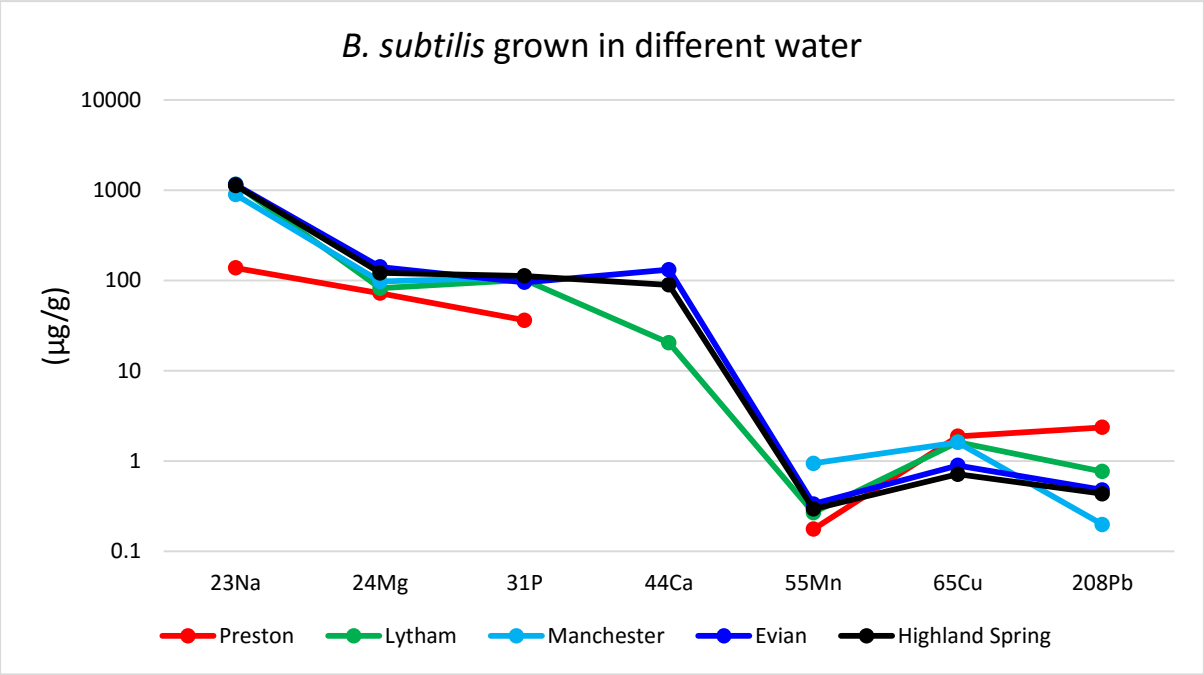

Figure 14- A graph depicting the (µg/g) concentrations of *B. subtilis* grown in different water on a logarithmic scale. (85-105% error)

TABLE 6- ICP-MS data of *B. subtilis* with different water sources

(µg/g)	23Na	24Mg	55Mn	65Cu	208Pb
Preston	137.83	72.51	0.18	1.89	2.37
Lytham	1162	82.07	0.27	1.63	0.77
Manchester	896.47	97.56	0.94	1.62	0.2
Evian	1154.13	141.5	0.33	0.9	0.48
Highland Spring	1131	121.43	0.3	0.71	0.44

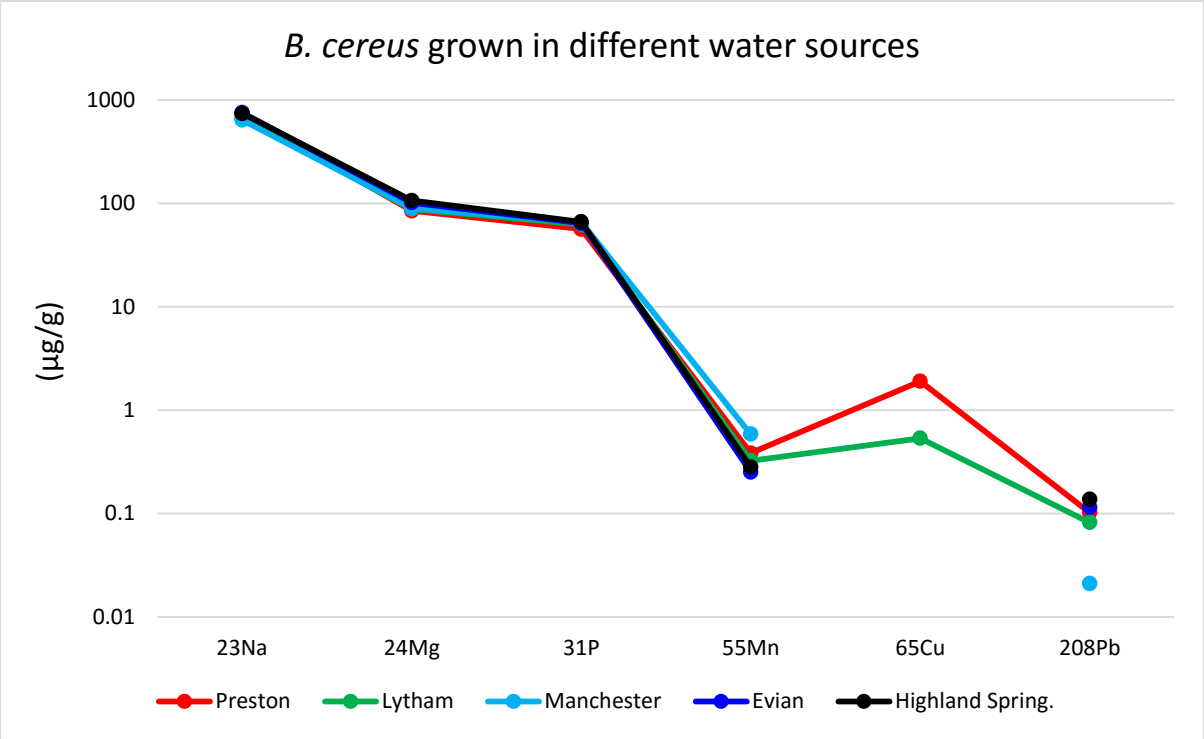

FIGURE 15- A graph depicting the (µg/g) concentrations of *B. cereus* grown in different water on a logarithmic scale. (85-105% error)

TABLE 7- ICP-MS data for *B. cereus* with different water sources

(µg/g)	23Na	24Mg	31P	55Mn	65Cu	208Pb
Preston	736.13	84.67	56.54	0.38	1.9	0.10
Lytham	665.3	88.27	62.43	0.32	0.54	0.082
Manchester	640.43	89.64	65.51	0.59	-0.021	0.021
Evian	752.51	101.67	64.24	0.25	-0.043	0.12
Highland Spring.	740.27	106.46	66.23	0.28	-0.055	0.14

1. EA-IRMS

2.1 δ13C and δ15N analysis

The isotope ratio of δ13C and δ15N between the four LB Medias shows good differentiation. LB 1 has a much lesser value of δ13C to that of LB 2, 3 and 4 which have comparatively similar values of δ13C. All show good selectivity of δ15N values varying from each sample as represented in fig.15.

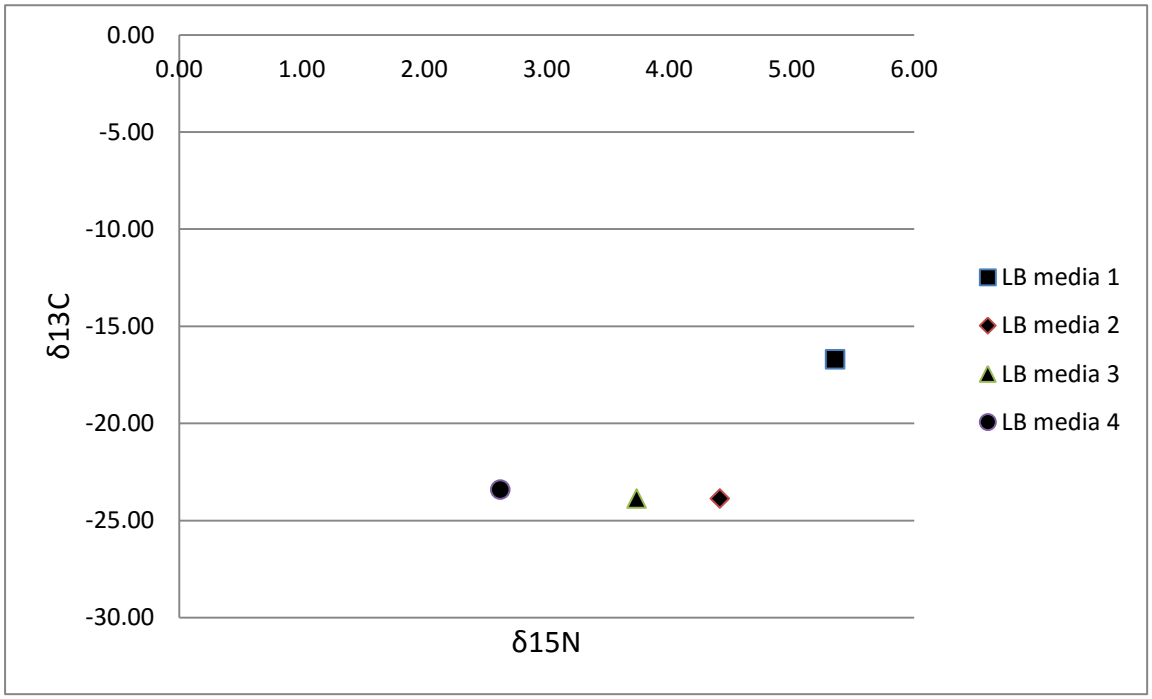

FIGURE 16- Graph showing δ15N and δ13C values for LB media

TABLE 8- δ15N and δ13C IRMS data for LB media

	δ15N	ABS	n		δ13C	ABS	n
LB media 1	5.36	0.02	2	**LB media 1**	-16.70	0.10	2
LB media 2	4.42	0.69	2	**LB media 2**	-19.40	0.44	2
LB media 3	3.74	0.02	2	**LB media 3**	-23.87	0.07	2
LB media 4	2.62	0.02	2	**LB media 4**	-23.42	0.08	2

Fig. 16 shows some interesting trends. LB Medias 1 and 2 respond in a mutual way. The δ13C and δ15N values for *B. subtilis* when cultured in the nutrient broths LB 1 & 2 are less than that of the nutrient broth powders singularly. Furthermore the δ13C and δ15N values for *B.cereus* are notably lower than *B. subtilis* as well as the nutrient broth powder itself.

Nutrient broth powders LB 3 & 4 and their constituent bacteria show a different trend. The values for δ13C do not change considerably however the values for δ15N do. The results for δ15N after bacterium growth decreases however, unlike in LB 1 & 2, the values for *B. subtilis* are lower than that of *B. cereus.*

There is high distinguishability between the bacterium present in each sample which in turn means that IRMS can be a useful tool in the determination of bacteria. The same can also be said when determining which LB media was used however only when the bacterium is known. If analysis is carried out with both unknown bacteria as well as unknown production method, the selectivity is greatly reduced.

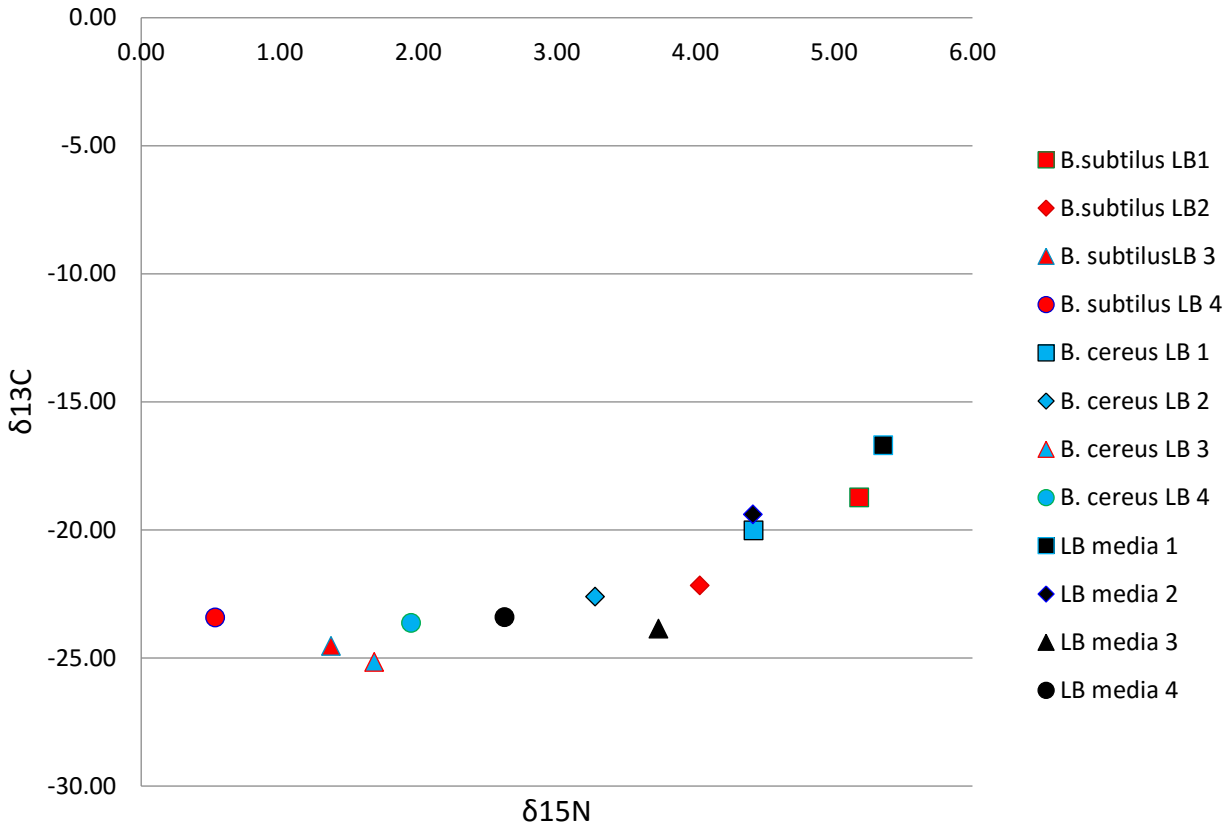

Figure 17-Graph showing δ15N and δ13C values for B. subtilis and B. cereus in different LB media.

TABLE 9-IRMS data for bacteria is different LB media

	δ15N	ABS	n		δ13C	ABS	n
LB media 1	5.36	0.02	2	**LB media 1_1**	-16.70	0.10	2
LB media 2	4.42	0.69	2	**LB media 2_1**	-19.40	0.44	2
LB media 3	3.74	0.02	2	**LB media 3_1**	-23.87	0.07	2
LB media 4	2.62	0.02	2	**LB media 4_1**	-23.42	0.08	2
B. subtilis LB 1	5.18	0.21	2	**B. subtilis LB 1**	-18.74	0.34	2
B. subtilis LB 2	4.03	1.99	2	**B. subtilis LB 2**	-22.18	0.60	2
B. subtilis LB 3	1.37	0.10	2	**B. subtilis LB 3**	-24.54	0.03	2
B. subtilis LB 4	0.54	0.03	2	**B. subtilis LB 4**	-23.42	0.04	2
B. cereus LB 1	4.42	0.04	2	**B. cereus LB 1**	-20.03	0.36	2
B. cereus LB 2	3.28	0.15	2	**B. cereus LB 2**	-22.61	0.00	2
B. cereus LB 3	1.68	0.03	2	**B. cereus LB 3**	-25.16	0.01	2
B. cereus LB 4	1.95	0.04	2	**B. cereus LB 4**	-23.63	0.10	2
porcine collagen	5.93	0.28	2	**porcine collagen**	-16.00	0.11	2
porcine collagen	5.99	0.09	2	**porcine collagen**	-16.65	0.03	2
porcine collagen	5.93	0.08	2	**porcine collagen**	-16.87	0.26	2
				JCR sucrose	-15.83	0.13	2
				JCR sucrose	-16.19	0.39	2
				JCR sucrose	-16.50	11.78	2

The δ15N values of the samples are very similar apart from a notable difference between the δ15N values of *B. cereus* Highland Spring and Evian. Although the bacteria were grown in different water sources they were all grown using LB media 3. The results suggest that the δ15N values are influenced by the LB media far greater than the water it is grown in.

The same cannot be said for δC13 values. The general trend in fig.17 shows that the bacterium grown has the most influential effect on the resulting δC13 value. The results show *B. subtilis* causes a positive shift in δC13 to that of *B. cereus.* However the anomaly to this hypothesis is the δC13 for the bacteria grown in water from Preston exhibits the opposite effect. The absorption height for *B. subtilis* is much greater than the rest of the sample leading to believe this is the result of measurement error.

Although the results go some way to distinguishing between the different bacterium and production methods, these results hold more significance for determining the geographical origin of the bacteria. When looking at the δ15N and δC13 values of *B. subtilis* and *B. cereus* individually, there is a prominent difference between each of the bacterium grown from the varying water sources. This is especially comparable between the bottled water sources to the tap water from Preston, Lytham and Manchester. The results are not extensive enough to say for definite that the geographical origin can be determined however it does show that it is possible.

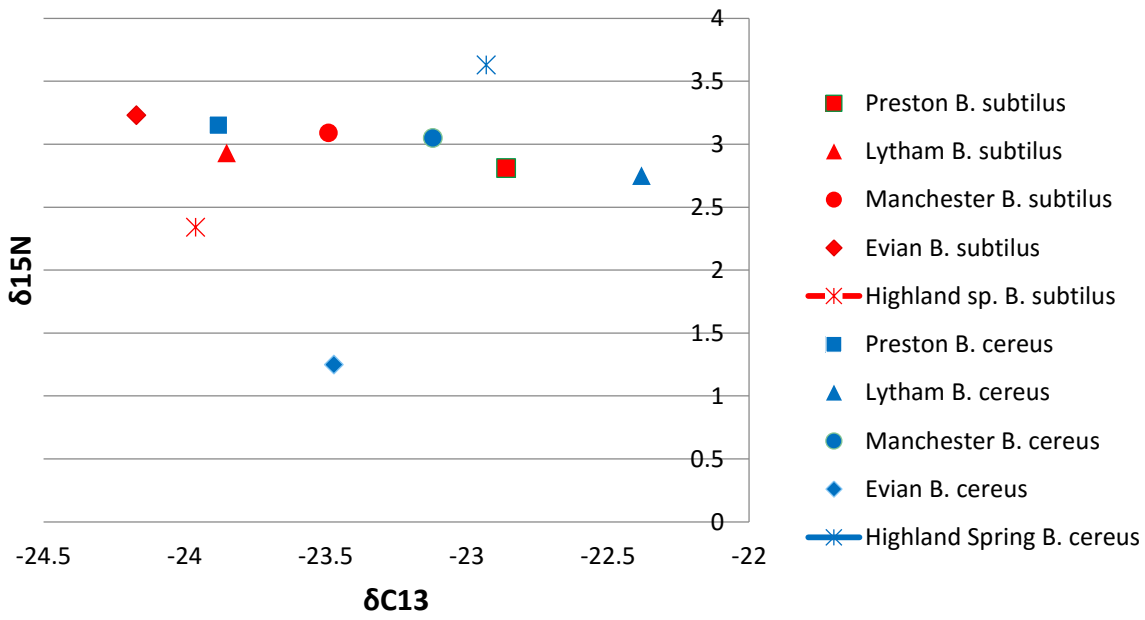

Figure 18- Graph showing δ15N and δ13C values for *B. subtilis* and *B.* cereus grown from different water sources

TABLE 10 - IRMS data for bacteria is different water sources

	δ15N	ABS	n		δC13	ABS	n
Preston B. subtilis	2.81	0.29	2	**Preston B. subtilis**	-22.86	0.56	2
Lytham B. subtilis	2.93	0.14	2	**Lytham B. subtilis**	-23.85	0.08	2
Manchester B. subtilis	3.09	0.27	2	**Manchester B. subtilis**	-23.49	0.13	2
Evian B. subtilis	3.23	0.16	2	**Evian B. subtilis**	-24.17	0.19	2
Highland sp. B. subtilis	2.34	0.11	2	**Highland sp. B. subtilis**	-23.96	0.06	2
Preston B. cereus	3.15	0.14	2	**Preston B. cereus**	-23.88	0.12	2
Lytham B. cereus	2.75	0.08	2	**Lytham B.cereus**	-22.38	0.04	2
Manchester B. cereus	3.05	0.13	2	**Manchester B. cereus**	-23.12	0.06	2
Evian B. cereus	1.25	0.2	2	**Evian B. cereus**	-23.47	0.13	2
Highland Spring B. cereus	3.63	0.09	2	**Highland Spring B. cereus**	-22.93	0.02	2
porcine collagen	5.97	0.12	2	**porcine collagen**	-18.13	0.08	2
porcine collagen	5.89	0.03	2	**porcine collagen**	-17.89	0.07	2
				JCR sucrose	-11.48	0.05	2
				JCR sucrose	-11.59	0.18	2

2.2 δ2H analysis

LB media 4 showed to have the greatest negative δ2H value compared to the other 3 LB mediums which have comparatively similar δ2H values to each other. This translates to the results of both *B. subtilis* and *B. cereus* which were grown in LB media 4 as they too have the lowest δ2H value comparatively. This exhibits the ability to determine the LB media used dependant on the initial growth media having a significant initial δ2H value. In the same fashion the results for Evian water have the lowest δ2H value and respectively the results for *B. subtilis* and *B. cereus* correlate. The other results display the same trend however not as distinguishable as the fore mentioned.

TABLE 11- δ2H IRMS data for different water sources

	δ2H	ABS	n
VSMOW 2_1	0.00	0.79	3
Preston water	-38.12	2.76	3
Manchester water	-47.22	1.42	3
GISP	-188.76	2.39	2
Lytham water	-44.61	0.33	2
SLAP2	-427.50	0.35	2
Evian	-74.51	2.32	3
Highland Spring	-55.98	1.00	3
VSMOW2_2	-1.59	1.58	3

TABLE 12- δ2H IRMS data for bacateria

	δ2H	ABS	n
LB media 1	-62.5	2.5	3
LB media 2	-68.4	0.6	3
LB media 3	-62.5	2.2	2
LB media 4	-92.6	0.3	2
B. subtilis LB1	-43.4	1.8	3
B. subtilis LB2	-63.5	2.7	3
B. subtilis LB3	-66.4	2.7	3
B. subtilis LB4	-71	0.7	3
B. subtilis Preston	-66.4	0.6	3
B. subtilis Manchester	-67.3	1.2	3
B. subtilis Lytham	-67.2	0.4	3
B. subtilis Evian	-75.8	1.1	3
B. subtilis Highland Spring	-71.5	0.5	3
B. cereus LB1	-41.5	0.3	3
B. cereus LB2	-64.7	1.2	3
B. cereus LB3	-64.1	2.1	3
B. cereus LB4	-91.7	1.2	3
B. cereus Preston	-73.2	1.1	3
B. cereus Manchester	-79.0	0.9	3
B. cereus Lytham	-74.1	1.9	2
B. cereus Evian	-85.4	1.2	3
B. cereus Highland spring	-76.1	1.6	3

Chapter 4- Conclusion

This study was carried out in order to achieve three key goals: the differentiation of bacteria; identification of production method and the determination of geographical origin using SITE analytical techniques.

The IRMS analysis in this study has shown some promising results in terms of up-stream research. δ13C and δ15N analysis has shown the ability to identify the bacterium present between *B. subtilis* and *B. cereus.* On the other hand the ICP-MS results could only conclude that the bacteria exhibited corresponding characteristics by consuming similar inorganic elements.

ICP-MS could only go so far into the identification of the production method used. The most distinguishable LB media using ICP-MS, has been LB media 1. It has a much greater variance in its inorganic fingerprint and continued to show a variance in elemental concentration within the bacteria. IRMS showed a high level of selectivity in determining which LB media was used in the production process. The problem arises when neither the bacterium nor the LB media is known as there is an overlap in δ13C and δ15N values.

This study also proved IRMS could provide promising results for determining the geographical origin from the different water sources however due to the close locations of the water sources; δ13C and δ15N values were not very specific. The analysis of the bacteria grown in differing water sources did not provide any sound data to suggest ICP-MS could be used for determining the geographical origin.

δ2H analysis has shown how the bacteria encompasses the isotopes during cell culturing and is a very viable as a tool to determine geographical origins. The δ2H values for the LB media

however did not differ greatly making it unsuitable in terms of differentiation of bacterium or production method.

SITE analysis is the incorporation of both techniques and this study shows the potential to be able to achieve the three main targets stated in the study aims.

Chapter 5- Future Work

This study has shown the great potential SITE analysis has in microbiological forensics. There are numerous ways in which this line of research can continue. Firstly international collaboration would give the opportunity to use water sources around the world. The possible larger variance in isotope ratio would lead to great selectivity and specificity for the determination of geographical origin.

So far only the *Bacillus* genus of bacteria has been analysed using SITE. Future work could expand especially to labs which have higher levels of security for the use of bio hazardous materials. As well as this more can be researched into different production methods of bacteria. This study only focused on one production method but a variance in materials used.

Often in forensics a timescale is required in order to piece together a series of events. This research can delve deeper into the exposure of bacteria over time and the environmental impact it would have on the inorganic fingerprint and isotope ratios. The analysis of other isotopes, such as Sulphur and for definite Oxygen, would give greater evidence towards both the identification and determination of geographical origin.

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