The Living Brines of Dracula

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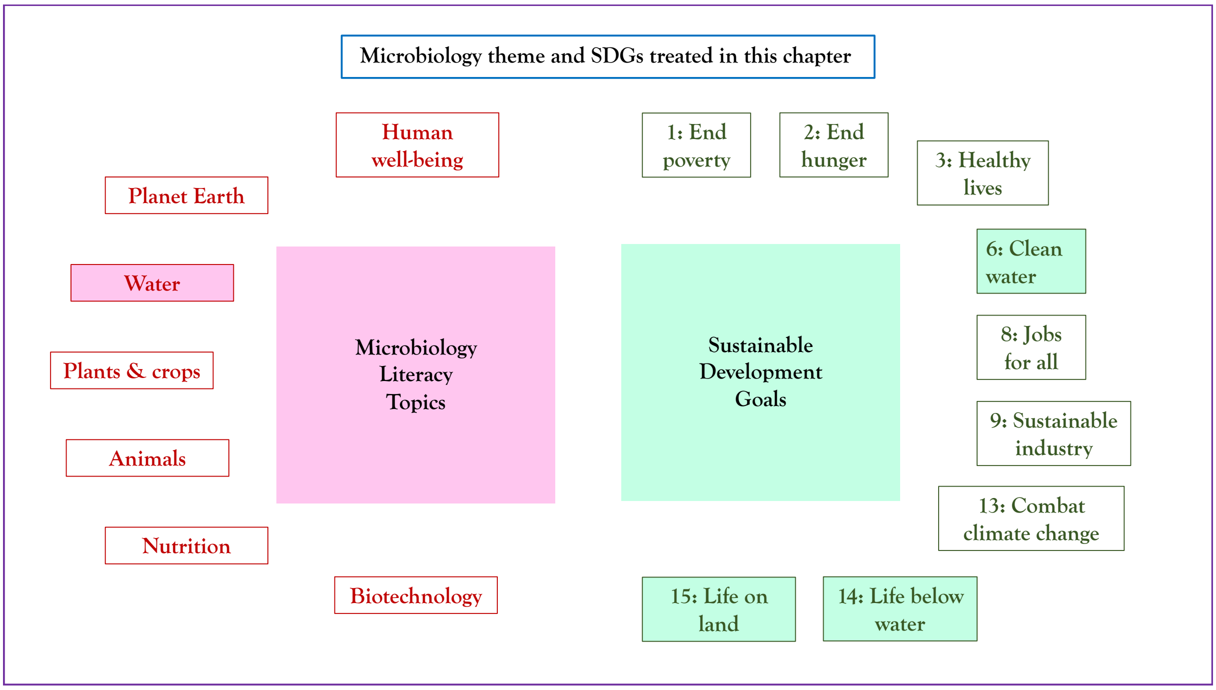
Storyline

Saline lakes and their shores are ecosystems favouring a specialized biodiversity consisting of salt-tolerant and salt-loving microorganisms, fungi, plants and animals. The distinctive biodiversity of saline lakes is equally important for healthy/complete nutrient cycling within the ecosystem, as carbon sink/traps, and as sources of scientific discoveries. Moreover, the biodiversity of saline lakes is a bioresource treasure trove for non-agricultural biomass production or microbes that can produce economically relevant substances. Saline ponds and lakes are managed by humans for manufacturing table salt, therapeutic and wellness (bathing) purposes, therefore representing tourist attractions and thus promoting the development of local economy. Climate warming, however, stimulates the evaporation of water and concentration of the dissolved salts, making salty lakes even saltier or drying them out. This chain of events triggers biodiversity decline towards a smaller number of highly specialized organisms and subsequent impairment of biogeochemical cycling of nutrients. Another side effect is the salinization of surrounding soils and ultimately of nearby groundwaters that can no longer be used as drinking water sources. The mitigation of climate change effects on saline lakes needs implementation and enforcement of science-based sustainable management and conservation policies. Exploration and sustainable management of saline lakes have important implications for Sustainable Development Goals 6. (Clean Water and Sanitation), 14 (Life below Water), and 15 (Life on Land).

The Microbiology and Societal Context

The microbiology: microbial diversity; biogeochemical cycles; biotechnology resources and prospection; pollution; eutrophication and toxic algal blooms; microbial greenhouse gas production. *And, peripherally for completeness of the storyline*: non-agricultural resource attribution; the business of salt mining. *Sustainability issues:* biodiversity; natural resources; health; tourism; economy and employment; environmental pollution; global warming.



The living brines of Dracula: the microbiology (and the adventure!)

1. ***Is Transylvania the land of Dracula? It could be…*** Transylvania, surrounded by the beautiful Carpathian Mountains, is a region in Central-Eastern Europe. It was called 'the land beyond the forests' (*Terra Ultra Silvam*) around a thousand years ago because at that time it was covered in trees.

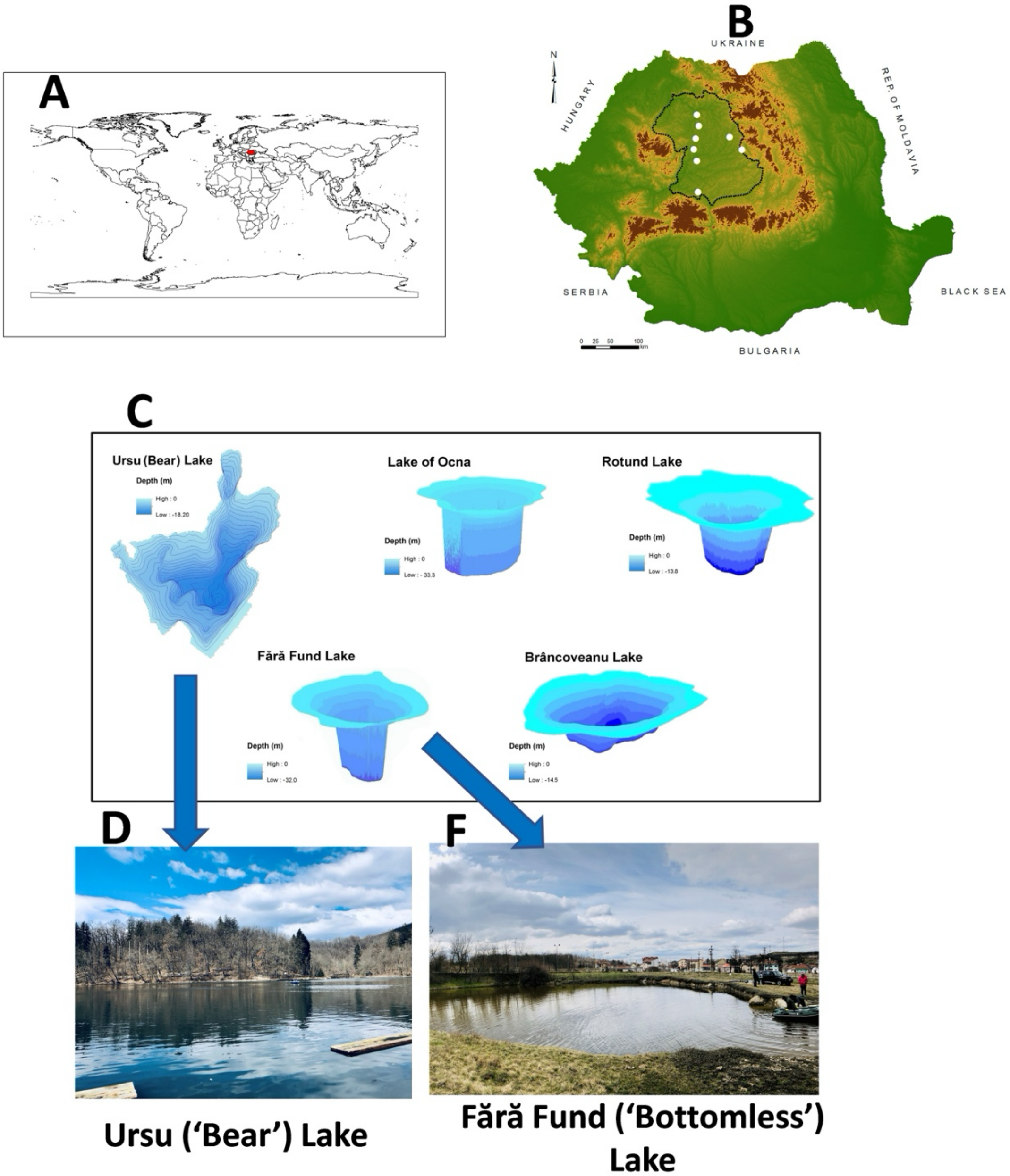
Transylvanian natives are a multi-ethnic and multi-cultural group having Hungarian, Romanian and German origins, sharing a common geographical (Transylvanian Basin surrounded by the Carpathians) and historical setting (established for more or less 1000 years). Moreover, they share an old belief that their world is haunted by vampires (called *virkolaks*), wicked fairies (*iele*), or ghosts. The vampires are best known for their need to feed off the blood of living people, and the most famous of these is the character Dracula.

2. ***The legend of Dracula.*** Dracula's legend began about 600 years ago, when the ruler of Transylvania (Sigismund of Luxembourg) established the Knight Order of the Dragons and appointed Vlad the Second as a 'Dragon' Knight to defend the region. Vlad the Second and then his son, Vlad the Third, were given the nickname *Dracul* (meaning 'of the Dragons'). In a 120-year-old story inspired by Transylvanian folklore, entitled "Dracula", the Irish author Bram Stoker weaved a horror story about Vlad the Third – also known as Vlad the Impaler – referring to him as 'Count Dracula', and conferring upon him the character of a vampire! We could consider Dracula as an 'international' brand but one that has become inseparably associated with Transylvania. It also makes a captivating backdrop for our story to begin!

3. ***So what are Dracula's brines?*** About 13 million years ago, the shallow Paratethys Sea that once covered present-day Central and Eastern Europe, including the Transylvanian region, eventually evaporated due to movement of the Earth's crust (the African and European plates). As the seawater slowly evaporated, a large, flat deposit of salt was left behind.

This salt deposit is deep, 300 metres (m) deep in some places, and has been mined by people for at least 2000 years. Interestingly, the word 'salary' (that means pay or wages) originates from the Latin word *salarium*, meaning 'salt currency', because Roman soldiers and workers (~2000 years ago) were often paid in salt blocks. Even today, the land of Dracula sits on top of vast salt deposits, is dotted with numerous salt mines, and saline lakes formed in abandoned salt mines and caves (see title-page image). One of these lakes is called Bear Lake (or 'Ursu' Lake} and another, that is very deep, is called Bottomless Lake ('Fără Fund' Lake). Even though they are so salty, Dracula's lakes are in fact full of life!

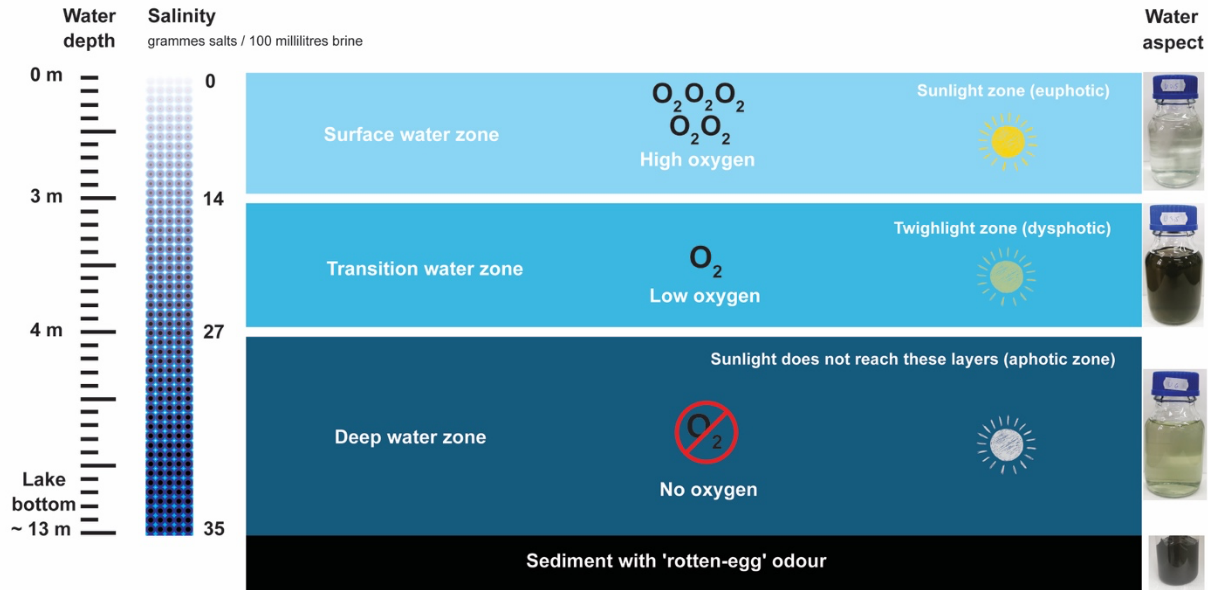
The approximately 40 salt lakes of Transylvania range in size from 2000 cubic metres (similar to Olympic-sized swimming pool) to 500,000 cubic metres (a volume similar to that of the famous ship, the Titanic). Most of the salt lakes look dark so it is not possible to see the bottom. In fact, many are very deep, going down to 100 metres. Some have fairly low salinity (slightly more salty than seawater) but some are saturated with salt (about 10 times the salinity of seawater), meaning that the water cannot dissolve any more salt and any extra added would precipitate out as solid crystals. Some of the lakes have a gradient of salt, so they are slightly salty at the top but reach salt-saturation at the bottom. Others are very salty all the way down; these are called brine lakes. The main salt present is sodium chloride (NaCl) - the salt that we know as table salt. Whereas the idea of table salt may not sound scary, the salt lakes are so salty that if we drank too much we would get sick, or could even die!



Location of Romania on the world map (A), approximate location and markings of saline lakes in the Transylvanian Basin (B), shapes and depths of different salt lakes in Transylvania (C), with an arrow indicating the explored saline Ursu ('Bear') Lake (D) and Fără Fund ('Bottomless') Lake (F).

4. Transylvanian salt lakes seem as mysterious and scary as Dracula. Not only is very salty water not good for humans: even most kinds of microbes found on Earth cannot cope with living there. There are thought to be more than 1 trillion species of microbes on Earth: yes, 1 trillion – a million times a million, or 10^{12} species! But, of these, only about a thousand species of microbes can live in saturated sodium chloride brine (that has about 35 grammes of NaCl per 100 millilitres of brine).

Dracula's salt lakes differ in size and salt concentration. Those with a salt gradient are permanently layered (stratified) and their layers do not mix freely, so different kinds of life are found in different layers (see below and ***More interesting titbits about Dracula's brines*** at the end of this story). But what are these forms of life?



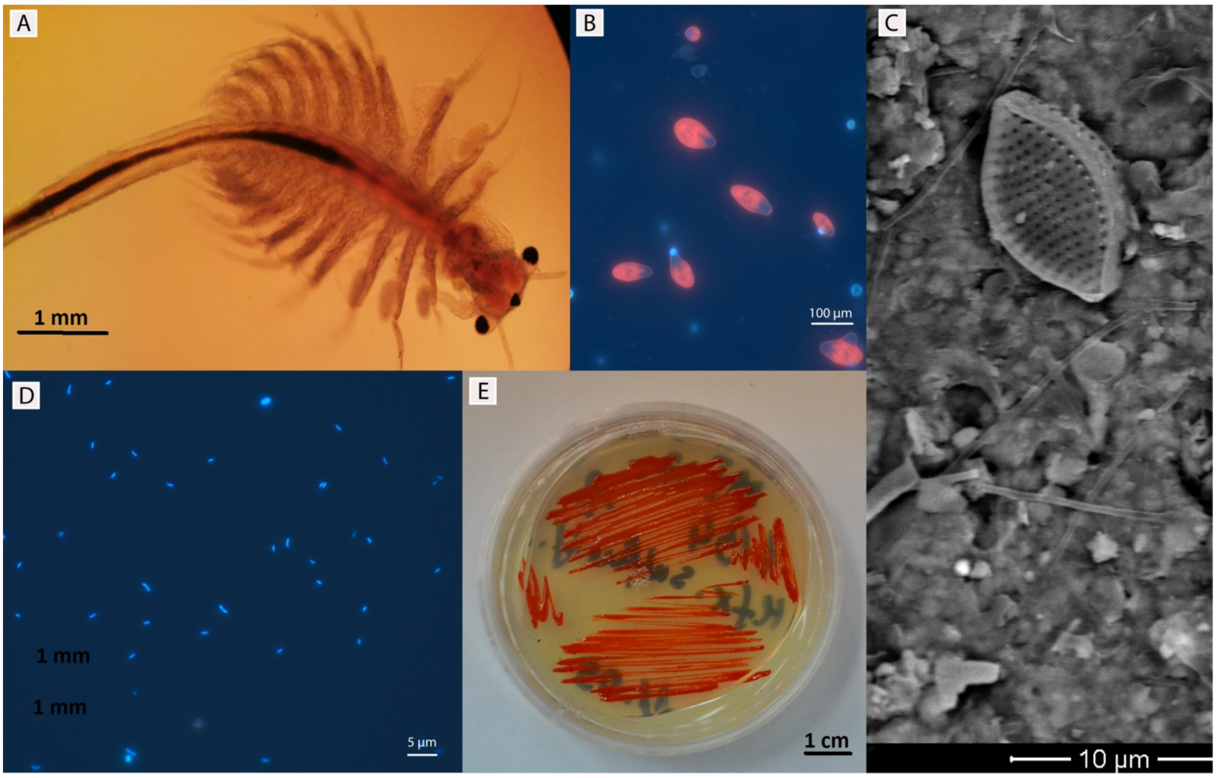
Scheme of the stratified water body typical to many deep salty lakes in Transylvania. The main environmental characteristic is the salinity. Salinity is defined as the concentration of salts dissolved in water (or brine) and is expressed as grammes of salts per 100 millilitres of brine (or litres of water in the case of seawater). The water collected from these contrasting layers have different transparencies (see the bottles at the right). At the bottom of such salty lakes, a black, soft and strange-smelling sediment is present.

5. ***Brine lakes were thought to be lifeless.*** Many people bathing in such salty water will tell you that no fish are swimming there, so they typically believe that nothing lives in the lake. But microbiologists have tools (including microscopes, culture media to grow microbes from lake samples, and methods for genetic analyses of samples) to examine the water for the tiny forms of life that do exist - in some cases even swimming - in their salty home. Indeed, these saline lakes are populated by organisms of many sorts and behaviors that differ according to salt concentration, oxygen level, and light intensity.

6. ***Brine lakes are now known to be full of life, mostly microbial life!*** The low salinity and shallow lakes are inhabited by microbes similar to those found in freshwaters and soils, but having the ability to tolerate the presence of salts; they are called ‘halotolerant’ microbes. Salts have the property to attract water molecules and thus immobilise some of the water molecules and make them unavailable to cells. What is more, high concentrations of ions (the parts of salts that separate when dissolved in water) make the concentration of water molecules lower. So, the higher the salt concentration is, the less ‘free’ water is available, and thus cells tend to dehydrate and in some cases they become inactive or even die.

7. ***And there are some animals in brine lakes!*** The very salty lakes (brine lakes) that are up to 10 times saltier than the seawater are called ‘hypersaline’ lakes: *hyper* comes originally from the Greek language meaning *over* or *beyond*. They are worlds for life where only the specialised salt-loving or ‘halophilic’ organisms can live. The animals that live in hypersaline lakes around the world, including Transylvanian’s brines, are invertebrates (they lack the spinal cord and internal skeleton) and are typically small, ranging from the size of a sand grain (few hundreds micrometres) up to the size of a rice grain (1-3 millimetres). Examples of such halophilic animals are the rotifer *Brachionus plicatilis*, and the crustaceans named *Moina salina* (saltwater daphnia), and *Artemia salina* (brine shrimp: A in the figure below). These invertebrates feed on unicellular

algae such as species of green algae called *Dunaliella* (**B**) and diatoms (microscopic algae that have a silica shell**; C**). *Artemia* brine shrimps are found in many salty lakes around the world; they are orange or pink in colour due to the carotene stored in their body. That is why flamingoes living around salty lakes in Africa have their feathers wonderfully pink: they store carotene from the brine shrimps that they lunch on! Perhaps you are not surprised to learn that carotene, which is a kind of Vitamin A, is also the pigment that gives carrots their colour.



Images of some invertebrate animals, algae and microbes living in Transylvanian's saline lakes: (A) brine shrimp (*Artemia salina*); (B) microscopic *Dunaliella* green alga; (C) diatoms ('shelled' algae); (D) microbes have different cell shapes and sizes and these can be observed by colouring them with special dyes that glow under dark (a technique called 'fluorescence microscopy'); and (E) colonies (many, many cells produced by reproduction of halophilic archaea that are red in colour. These colonies are growing here on a solid culture medium (here, 'solid' means that it has seaweed extract added – agar – to make it gel-like rather than liquid), which contains nutrients that were added to sustain them.

8. ***Brine lakes contain very special microbes.*** In addition to invertebrates and algae that love salt, the brines of Transylvania are home to special kinds of bacteria and archaea – unicellular forms of life that lack the nucleus. Many of these microorgansims have names beginning '*Sali*' or '*Halo*' that show how much they love living in salty conditions. These include *Halobacterium*, *Haloferax*, *Halomicrobium*, *Halorubrum*, *Salinibacter*, *Salinicola*, *Salinivibrio* and many others. So, if you will discover a new salt-loving microbe, perhaps you could name it something beginning with *Halo-* or *Sali-*?

9. ***Microbes keep brine lakes healthy.*** Did you know that, collectively, the microbes in these lakes play a crucial role in keeping saline ecosystems in balance, even though each is on its own so small (invisible to the naked eye)? They do this by recycling organic materials/substances, in much the same way as we humans recycle food waste or garden waste (well, in fact, it is also the microbes that recycle food- and garden wastes). Scientists have learned a lot about the chemistry and ecology of salt lakes, but there are still so many mysteries to unravel! Imagine this: are there undiscovered life-forms living in these lakes? Could there be strange and amazing things

happening that we don't even know about yet? How do the different invertebrates, algae, fungi, bacteria, and archaea work together or compete? It's a hidden world waiting to be explored!

10. ***But human activities can harm brine lake ecosystems.*** But that's not all. Human activities, especially destructive works, like mining salt, and pollution like disposing of waste in the lakes, can harm these unique habitats. It's important to understand how our actions affect the health of their ecosystems.

11. ***The microbes of brine lakes can be useful to us.*** And guess what? Some of these incredible microbes might even produce substances that could be helpful for us! They could be used to make natural and safe food additives, like carotene to colour foods, and ingredients for medicines or cosmetics, but also to degrade or neutralise harmful substances like hydrocarbons or heavy metals.

12. ***Bioprospecting.*** We all know the term *prospecting*, as in *prospecting for gold*, sieving stream water for tiny nuggets of gold? Well, we use a similar term – *bioprospecting* – for searching for microbes that can provide us with a valuable product or service.

We often think of bioprospecting for species that can be useful to humans in terms of sampling in biodiversity hotspots, like the Amazonian forest or other tropical rainforests, or biodiverse parts of the ocean such as coral reefs. However, because halophilic microorganisms have peculiar metabolisms and strange proteins, or produce weird substances (metabolites) to cope with living at high salt, these often have properties that can also be highly useful for biotechnological applications.

13. ***Robust enzymes that function under harsh conditions.*** One example is the enzymes – proteins that catalyse chemical reactions – of some halophiles that are stable not only at high salt, but also with little water, including in solvents such as alcohol. In biotechnology, this can mean that some of these enzymes are useful to convert hydrophobic substances (those substances that do not dissolve freely in water) to a new and useful form. One type of enzyme that degrades proteins, and hence are called proteases, is used in the formulations of commercial detergents to help in washing out organic substances. They are also included in some paints to repel microorganisms (a phenomenon called 'antibiofouling'). Enzymes called cellulases and xylanases that degrade plant materials, such as cellulose and hemicelluloses, are used to break up plant matter so that it can then be fermented by alcohol-producing microbes to synthesise biofuels. Other enzymes called lipases that degrade lipids/fats/oils can be used in the production of nutritional supplements like dietary fatty acids. Indeed, halophilic microbes are a remarkable source of substances that (even if we do not realise it) are of use in our everyday lives!

14. ***The excitement of bioprospecting, exploration and discovery: sampling campaigns.*** Thinking of new questions, finding the answers to the questions listed above, and identifying new applications via bioprospecting are not always easy, but things that are challenging can also be exciting!

To bioprospect, we need to collect samples that may contain the microbes having the desired properties/activities – the gold nuggets. Collecting samples is usually done on a *sampling campaign*, where we go to places – often exotic, fascinating habitats that are sometimes difficult to reach – expected to have microbes of interest. Microbiologists and biotechnologists, and you too, are explorers when involved in these activities, uncovering the secrets of these fascinating lakes and discovering things that no one else knows. If you live near a salt lake (or even seawater, freshwater, or soil), you too can think about these questions, explore the environment, and even study some samples. So, get ready for an adventure that's full of excitement and discovery!

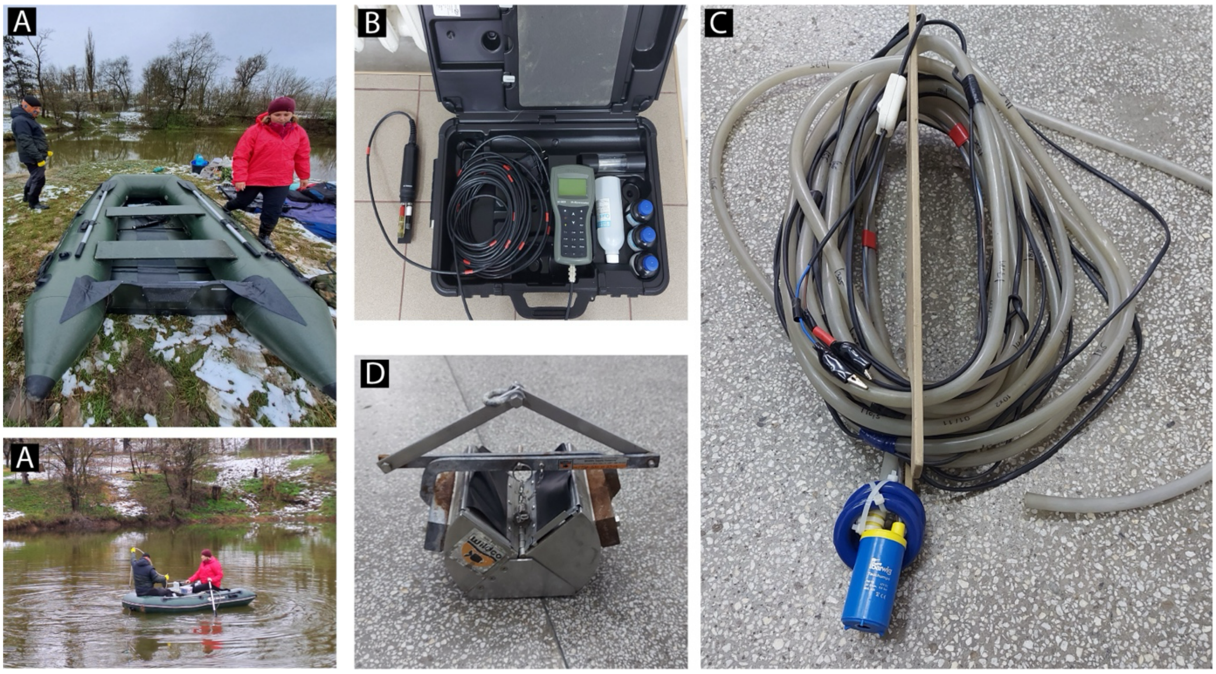
Let's go on a virtual sampling campaign to discover the life in Dracula's brines!

Getting started. Are you ready for the thrilling journey to Transylvanian brines? We'll be exploring some of the most challenging lakes out there that are quite large and very deep, so are difficult to navigate. They are the Bottomless ('Fără Fund') and Bear ('Ursu') lakes; see also the ***More interesting titbits about Dracula's brines*** below.

Here's a fun fact about these lakes: because of their high salt content, the brine is more dense than the human body. That means you don't have to worry about being a great swimmer to enjoy these lakes because you will float on the surface. Isn't that cool?

But if you're planning to study them up close, it's a good idea to use a boat. Our team has a very practical inflatable boat designed for saltwater conditions, but boats can also be rented. The inflatable boat is useful for sailing on the sea as well as on the brines, and it can carry up to four people.

Now, remember to pack a few important things including paddles, so that you can navigate the lake-surface smoothly. You'll also need an air pump that is different from the ones you use for bicycles because it is designed for heavy-duty use. Some lakes are not very salty at the surface, meaning that people may not float, so wearing life jackets is important too. It's important to have the right equipment to make your adventure safe and enjoyable.



Toolkit for lake water and lake sediment sampling in Transylvanian brines: (A) inflatable boat; (B) a special kit called 'field multiparameter probe' that scientists use outdoors to measure different environmental characteristics including temperature, pH, and oxygen levels; (C) a special pump called "submersible pump" that can pull water from different depths of the lake; and (D) a scooping tool called 'grab dredge' that helps getting sediment from the bottom of the lakes.

Sampling kit. Before we start to decide what tools and equipment we need for discovering the life-forms living in these deep brines, it is really important to decide how we wish to collect them, what things we should measure, how many samples to take, whether we also wish to analyse the chemicals in the lake water, and how much of each sample we need to study back in a laboratory or classroom. All of this planning is part of what we call *experimental design*, how we will carry out the exercise from beginning to end. Experimental design is based on what we want to find out, so often starts by considering what should be the outcome. Experimental design is at the heart of sampling campaign planning, because it determines for example how many samples we take at different locations. Because the results will often be analysed statistically, we often involve in the campaign planning a statistician – an expert in statistics and a key person who will be involved in drawing conclusions from our work (if you would like to know more about this, read the Topic Framework *The importance of statistics and replication in microbiology* by James I. Prosser in Section 19: How we study microbes).

Besides the basic equipment (such as a boat), there are three specialist tools we have to bring on this field trip. The first is a device that can make important measurements in the water including temperature, oxygen, how acidic, neutral or basic it is (or 'pH'), how clear it is (known as 'turbidity'), and how salty it is (**B**). The second is a pump that can suck up water from different parts of the lake (**C**). And the third is a handheld tool that helps us scoop up sediment from the bottom of the lake (**D**). Sometimes, interesting organisms live in this muddy sludge (see ***More interesting titbits about Dracula's brines***).

In addition to this kit, we also need gloves to keep our hands clean (i.e. to protect us) and, crucially, to avoid microbes from our hands from contaminating the samples or sampling equipment (i.e. to protect the samples from us!), sterile tubes and bottles to collect lake-water and lake-sediment samples, a notebook to write down all the things we see and learn, and special markers containing ink that is not washed away by water when we label the sample bottles.

Thinking carefully when taking the samples, storing them, and bringing them back for analysis. *Showtime*: it is sampling time! The team boards the boat and, having all the sampling tools packed, must carefully row to the central point of the lake. Here, the bottom of the lake is deep enough for our purpose, and so is the water column. We really need to sample the lake water at least 5 or 6 depth points (for example, down to depths of 1, 2, 3, 4, 6 and 9 metres), Thus, representative samples are collected to help us in exploring most of the lake's biodiversity.

Teamwork! Once the central point of the lake's surface is reached, the team members should divide their tasks (assign responsibilities):

- one takes care of measuring the environmental characteristics, using the multiparameter probe,
- one is in charge of collecting samples of lake water and pouring them into separate clean (sterile) bottles according to each sampling depth,
- and the third (equally important) person writes down details of sampling activity, and also helps the colleagues during the measuring or sampling (for example by organising/cataloging/storing the filled bottles, or unwinding the cables or ropes).

The group's teamwork is important to the success of the sampling mission.

Sampling. It is a delicate task to slowly immerse the multiparameter probe and the pump hose so that turbulence is not created in the water that would cause the different layers (that have a different chemistry and different microbiology) to mix together. If we accidentally mix the water layers, then there is a likelihood of mixing the different kinds of microbial communities living in these different layers. This will not give us a true picture of the complex microbiology that is specific to each water layer/zone.

The last samples taken should be the lake sediment samples because, once the water samples have been taken, there is no need to worry about the accidental mixing of water layers. (However, even for sediment sampling, we try to avoid mixing layers, by being slow and gentle, although we know from experience that, after a few days, the stratification of lake water that has been mixed returns to its normal condition.) Sediment sampling is done using the grab dredge, a tool that is quite heavy (around 10 kilograms) so must be lowered into the lake carefully, by a strong team member, otherwise it will sink too rapidly. A long and thick rope is needed to hang the dredge, which of course has to be longer than the greatest depth of the lake to reach down to the sediment.

Sample handling. We learn in our first ecology lesson that samples start to change the moment they are removed from their natural environmental context (*bottle effect*). To minimize such changes, once we have put the lake-water samples and sediment samples in clean sterile bottles, we need to keep them under conditions as close to their natural conditions as possible, i.e. cool (preferably around 4°C) and dark, and to bring the samples as quickly as possible (within 4-6 hours) to our laboratory,

Characterising life in samples from Dracula's lakes back in the laboratory

Sample work-up. Once back in the laboratory, the samples should be divided into subsamples – aliquots – for different tests. Some aliquots of lake-water or sediment samples are used for microscopic analyses, to count and try to identify microbial cells, and other subsamples are used to study genes that identify what living things are present; this is important because some microorganisms look alike under the microscope so cannot be identified visually. Other subsamples are used to determine what kinds of chemicals are in the water: ions (salts), nutrients, and other substances.

To analyse the number and shapes of microbial cells, we use a method that uses colourful dyes that glow in the dark. This helps us see the cells better and count them (**D** in the third figure). We also add a few drops of lake-water samples to liquids or agar plates that help certain types of microorganisms grow, to obtain larger amounts of microbial material, gain information about their growth requirements, or study individual microbes separately (**E** in the same figure).

To explore the microbial diversity and determine their kinds of biological activity in the lake water and sediments, we need to separate the microbial cells from the rest of the sample material (water, salts, sediment particles), for example by filtration.

To separate microbes from salty water, scientists use a set-up called a 'filtration system'. This device can be made of glass (A) or stainless steel (B) and have a funnel connected to a vacuum pump. Each lake water sample is passed through a sieve with holes with diameters of 0.2 microns (that is 400 times smaller than the thickness of a human hair!) (C), which catches the microbes and separates them from the salty water.

From the microbes we collect by filtration – the microbial biomass – we extract their deoxyribonucleic acid or 'DNA'. The DNA is a linear sequence of chemical information: the genetic blueprint of life of all organisms. Because different organisms have different blueprints, its decoding provides precise information about the organisms present in a sample, and hence the microbial diversity in a sample. This DNA-encoded information can thus be used to find out exactly which microorganisms live where in the lake, and can even give clues about what kinds of activities they can carry out. For example, a microbe that has a gene sequence to make the cellulase enzyme can almost certainly break down dead plant material that contains cellulose.

***DNA sequencing, metagenomics and bioinformatics*.** To access the linear information in DNA, it must be sequenced. To determine the sequence of a DNA molecule, and to interpret the information the sequence (blueprint) contains is called genomics – the science of genes and genomes (all the genes in a DNA molecule). Powerful machines determine the sequence of DNA molecules and powerful computers help researchers decode the information obtained. Environmental samples contain many different genomes/DNA molecules. The scientific method by which DNA molecules from all the different microbes in a certain sample or environment are analysed is called 'metagenomics'.

There are tens of billions of bytes of information, that is around 50-75 gigabytes, in a single lake-water or lake-sediment sample, which may have come from hundreds of thousands of microbial cells. So, looking into the mystery of life in Dracula's brines at this level of detail needs teamwork again, teamwork that involves biologists and computer-based experts (informaticians) or of scientists that combine both skills, called 'bioinformaticians'.

But direct observation of samples is also informative and very rewarding. However, it is easy and fun to also look at the samples down a microscope, and see what is there. Some of these microbes have very beautiful geometric shapes, such as the diatoms (a kind of alga) that have cells with silica-based 'skeletons' that take many forms. Other microbes may be filamentous (such as many fungi and some bacteria), some (including algae and some bacteria) may be coloured – pigmented – because they photosynthesise, some may have large cells (fungi, algae) and some tiny cells (bacteria, archaea), some with rod-shaped ('bacillus') cells and some that have spherical ('coccus') cells, and some may be actively swimming because they have a tail-propeller called a flagellum. So just from a look down a microscope, you can get a feeling of the complexity of the microbial life there: you can do this with water from your local pond, stream, seawater, or even a bucket or other container that had water left in it for some time.

So what do we discover in Dracula's hidden world: the amazing microbes in these brines?

We study the lake-water and the sediment to understand what it's like for these microbes by counting how many cells there are, and look at their DNA to understand what activities they are involved in whilst living in the lakes! As we gather all this information, we start to see a bigger picture of the world inside the-salty lakes.

What we find is this:

- different microbes live in different layers of the lake water,
- these layers are at different depths and have different salt concentrations, and
- they also differ in their oxygen levels, light levels, and available nutrients.

At the top of the lake, where it is sunny and full of oxygen (the gas that the human body extracts from the air we breathe), we find some microorganisms that love to eat organic compounds from plants and animals; these are called air-breathing, organic substance-eaters or 'aerobic heterotrophs' (try to spell it quickly! 😊). Others, called cyanobacteria, use the sunlight to make their own food by the process called 'photosynthesis' just like plants do!

In deeper water, things start to change. Around 3 to 3.5 metres deep, there is a zone where the light starts to fade, and gases such as hydrogen sulfide and methane become more common. These gases are dangerous to humans, but some bacteria use them as food. The sulfur-eating and methane-eating bacteria are quite afraid of oxygen (well, what we really mean is that oxygen can stop them from growing or even kill them)! These bacteria are very important for the brine ecosystem because they participate in the natural cycling of carbon and sulfur elements.

Even deeper, below 4 metres, there is a dark and extreme place with no oxygen and lots of salt. It may sound unfriendly to us, and in fact it is a hostile environment for most types of microbes on Earth (this is why we call it 'extreme'), but it is the perfect home for some kinds of halophilic microbes. They use small organic molecules and turn them into gases such as methane and hydrogen sulfide (the rotten egg smell of the sediment shown in the figures of the lake structure above, and the lake sediment below). These gases then rise up and, once in higher layers of the lake, serve as food for the bacteria there.

All these microbes, therefore, work together in a cycle, changing different elements like carbon, sulfur, nitrogen and phosphorous in a way that helps them all to survive. It's like a hidden world of tiny superheroes, each with their own role in keeping the lake's ecosystem balanced.

Exploring and protecting the marvels of salty waters are our responsibilities as scientists

As scientists, we have an important worry: how people's actions might affect the special microbes and their homes in the salty waters. We feel a strong urge not just to explore these fascinating places but also to raise awareness. Our job as scientists is not just about exploring, but also about warning people on the dangers of harming these amazing habitats. In these habitats, there are so many secrets and different creatures that we haven't even discovered yet. We can't let ourselves miss out on the chance to fully appreciate and protect the incredible wonders of nature!

More interesting titbits about Dracula's brines

The stratified lakes are called 'sandwich' lakes:

- The surface water of these lakes is lighter because it has less salt and more fresh water mixed in. It also gets sunlight, changes in temperature, and has oxygen. But the bottom layer, which is the 'deep water zone', is much saltier and darker. It doesn't get sunlight, has a stable temperature, and has very little or no oxygen. The largest and most famous 'sandwich' water on Earth is the Black Sea located in South-Eastern Europe.
- Between the surface and deep waters, there is a special zone called the 'transition water zone'. In this zone, things blend and change quickly so it is in a way like the butter that holds the top and bottom parts of a sandwich together! The water goes from being less salty to hyper-salty, from bright to dark, and from having oxygen to not having oxygen. It's a transition zone where everything changes all at once!
- This separation of water into different layers is called 'water column stratification', Because each of the three water zones has distinct environmental conditions, they are preferred by very different microorganism communities. Some microbes thrive only in the lighter, less salty, and oxygen-rich top layer, while others prosper only in the darker, saltier, and oxygen-poor bottom layer. (This is why when we explore the microbial communities of water columns it is so important to avoid disturbing the layers and mixing up the communities.) So most of the microbes present grow best only under the conditions at the location that suits their own metabolic needs; this is what biologists call their home or habitat. The lakes in Transylvania have

this unique feature of water layering. It's like a secret world hidden beneath the surface, with different organisms preferring their own special habitats. Isn't that fascinating?

The mysterious muds are smelly sediments. At the bottom of many salt lakes in Transylvania, there are smelly sediments called 'sapropel', which can be translated as 'rotten mud'. This mud is black, soft, and it has a smell like rotten eggs. This smell comes from a gas called hydrogen sulfide (H_2S), which is made by microorganisms living in the mud. The black colour comes from a mix of lots of organic material and minerals called iron sulfides. Believe it or not, some people actually use this black mud as a skin treatment (called 'pelotherapy'). That's why these Transylvanian salt lakes with the black mud are popular among tourists, and they also support the local economy. But here's something interesting not many people know: this strange sediment is made by microorganisms slowly breaking down dead leaves, algae, and invertebrates that sink from the top of the lake.

So, next time you see this black mud in a salt lake, remember that it's a result of the activity of microbes preferring salty, no oxygen and dark conditions!

The appearance of the black, salty sediment with soft consistency and 'rotten-egg' smell that is found on the bottom of Ursu ('Bear') Lake in Transylvania.

The Evidence Base, Further Reading and Teaching Aids

Scientific literature:

- Alexe, M., Şerban, G., Baricz, A., Andrei, A.-Ş., Cristea, A., Battes, K., Cîmpean, M., Momeu, L., Muntean, V., Porav, S.A., Banciu, H.L. (2018). Limnology and plankton diversity of salt lakes from Transylvanian Basin (Romania): A review. J. Limnol., 77(1): 17-34. https://doi.org/10.4081/jlimnol.2017.1657.
- Andrei, A.-Ş. Bulzu. P.-A., Banciu, H.L. (2020) Blossoms of rot: microbial diversity of saline sapropels. In: Extremophiles as Astrobiological Models. J. Seckbach and H. Stan-Lotter (eds), Series Astrobiology Perspectives on Life of the Universe (Series editors R. Gordon and J. Seckbach), Wiley-Scivener, Beverly, MA, USA, pp. 45-82. Online ISBN: 9781119593096; Print ISBN: 9781119591689. https://doi.org/10.1002/9781119593096.ch3
- Andrei, A.-Ş., Robeson, M.S., Baricz, A., Coman, C., Muntean, V., Ionescu, A., Etiope, G., Alexe, M., Sicora, C.I., Podar, M., Banciu, H.L. (2015). Contrasting taxonomic stratification of microbial communities in two hypersaline meromictic lakes. ISME J., 9 (12): 2642-2656. https://doi.org/10.1038/ismej.2015.60

- Singh, A., & Singh, A.K. (2017). Haloarchaea: worth exploring for their biotechnological potential. Biotechnol Lett., 39, 1793-1800. https://doi.org/10.1007/s10529-017-2434-y

Further readings and useful teaching materials

On halophilic microorganisms:

- DasSarma, P., Tuel, K., Nierenberg, S. D., Phillips, T., Pecher, W.T., DasSarma, S. (2016). Inquiry-driven teaching & learning using the Archaeal microorganism *Halobacterium* NRC-1. The American Biology Teacher, 78(1), 7-13. https://doi.org/10.1525/abt.2016.78.1.7
- Halobacteria: Making Microbiology Manageable – (https://www.carolina.com/teacher-resources/Interactive/halobacteria-making-microbiology-manageable/tr10761.tr)
- Halophiles: Definition, Examples & Classification – (https://study.com/academy/lesson/halophiles-definition-examples-classification.html)
- Introduction to Saline Environments & Microbial Halophiles - (https://see.isbscience.org/modules/observing-beyond-our-senses/lesson-1-introduction-to-saline-environments-microbial-halophiles/)

On the roles of microbes in recycling the nutrients:

- Role of Microbes in Biogeochemical Cycling - (https://bio.libretexts.org/Bookshelves/Microbiology/Microbiology_(Boundless)/16%3A_Microbial_Ecology/16.01%3A_Microbial_Ecology/16.1C%3A_Role_of_Microbes_in_Biogeochemical_Cycling)

On the density-stratified lakes:

- Meromicitic Lakes (https://microbewiki.kenyon.edu/index.php/Meromictic_lakes)
- What Is A Meromictic Lake? (https://www.worldatlas.com/articles/what-is-a-meromictic-lake.html)

Video clips:

- Bear Lake (Lacul Ursu), Sovata, Romania - https://www.youtube.com/watch?v=niSNoRL-rz8
- Salt lakes of Ocna Sibiului, Romania - https://www.youtube.com/watch?v=GJMmB3mN2Gw
- The Purple Bacteria That Are Afraid of Oxygen - https://www.youtube.com/watch?v=f1P3sX1JfcA&t=109s
- Putting Brine Shrimp Under The Microscope - https://www.youtube.com/watch?v=OpdLitVsoaQ
- The Secret Life of (Deep Sea) Brine Pools https://www.youtube.com/watch?v=YTT_Tlr8Dd8
- What is environmental DNA (eDNA)? - https://www.youtube.com/watch?v=TQdTV1rAlWY