Tutorial Class 1 Environmental Engineering 2 2022/23

Example Exam questions- answers and notes

1a. What are the key microbial factors that you would consider in order to provide some measure of the potential for an industrial contaminant to undergo intrinsic attenuation by the communities present on a site?

Biomass (number of cells present). In order for the microbial community to have any impact, in terms of contaminant remediation, the biomass of cells within the community has to be sufficiently high to have significant impact in terms of degrading the contaminant. A contaminated groundwater, which had a microbial biomass of $<10^4$ ml/g, is very unlikely to demonstrate the potential for significant extent/rates of contaminant degradation. Community densities $>10^6$ hold greater promise for attenuation of the contaminant at realistic time frames. In the case of bacteria biomass is usually expressed as Colony Forming Units, can be indicative of a contaminant which is very contaminated.

One factor that needs to be considered with regards to microbial biomass-contaminant interactions, is that the contaminant itself can alter the biomass microbial. Contaminants that have low toxicity and nutritional value can stimulate microbial community, whilst a toxic chemical can inhibit or indeed kill cells on exposure. Some sites are so highly toxic, they are essentially devoid of microbial life, and certainly those with the capacity to biotransform the contaminant.

Microbial resistance to toxic contaminants. Some microbes can degrade contaminants that are toxic to them, but that is rare. In most instances, even if a microbial cell is sufficiently resistant to the toxic impact of the contaminant, it is unlikely to have the capacity to utilise the contaminant as a nutrient or energy source. In this case the contaminant remains un-metabolised. Even if a microbial population is resistant to the toxic impact of a contaminant, its biodegradation activity may still be inhibited by the presence of other contaminants present on the site. Only about 50% of sites in the UK are contaminated by one chemical, the rest are co-contaminated by other organics and inorganics. For instance, heavy metals are commonly found in contaminated sites and these are typically very toxic to microbial communities so inhibit remediation.

Activity. Even in the presence of microbial communities with the genetic capacity to degrade the contaminant, this does not guarantee long term and consistent attenuation of the contaminant. You need to have the right type of microbes (with the genetic capacity) present, they need to be present in sufficient numbers (biomass) and be active. The presence of toxic heavy metals will typically inhibit microbial activity. In the case of aerobic bacteria, exposed to a contaminant which is readily degradable, the community can rapidly degrade the contaminate, leading to rapid consumption of the oxygen present and even build up of toxic break-down products. Thus, microbial activity can itself inhibit continuation of the attenuation process.

Genetic capability and community composition. In order for microbial communities to have the capacity to attenuate an organic contaminant, it has to contain populations that

have the genetic make-up, most notably possession of the appropriate catabolic genes. These encode for the generation of specific enzymes that bio-transform the contaminant so breaking down. In some cases the microbial cell has a complete set of genes which enable the complete mineralization of the contaminant to CO_2. For other more complex chemical, specific populations may only be able to undertake one step of the degradation pathway, which leads to breakdown products which in turn can be degraded by other populations with the appropriate gene sets. Several contaminants, such as atrazine (herbicide), require microbial consortia of complementary populations that works as a community to mineralise the contaminant.

1b. In the case of a chemical spill into a habitat, what are the key factors (environmental, chemical and microbial) you would need to access to decide whether or not a site would recover naturally or require intervention?

The key factors that determine the potential for natural attenuation of an organic contaminant in a site include:
Environmental

- Temperature- in extreme cold <4°C cells are inactive whilst above 40°C many stop functioning. Some forms of bacteria produce extremely resistant spores that can survive up to 100°C.
- pH- some contaminants can make sites extreme in terms of pH, where as most microbial have optimal activity ranges around pH 7. Each bacterial population has its optimal pH range for growth, some can survive at pH 1 whilst others pH 13.
- Presence of oxygen or another electron acceptor is essential for cellular energy production.
- Presence of toxic co-contaminants can inhibit microbial activity.
- Presence of co-substrates- in order to grow and be active, microbes require other nutrients (e.g. trace elements), they and cannot survive solely on the target contaminant. They need an appropriate mixture of P, N and C plus other trace elements.
- No microbe can survive in the absence of water- ideally, they need moisture conditions that is suitable for plant growth.

Contaminant chemistry

- Concentration, if this is very high it can kill all living things, too low and there is insufficient substrate to switch on degradation activity.
- Some contaminants have extreme pH which can inhibit activity. Most microbes demonstrate optimal activity around neutral.
- Bioavailability, in order to be susceptible to microbial attack the contaminant needs to be accessible to microbial action. For optimal degradation performance both the cell and contaminant need to be in close proximity so that the contaminant can be exposed to enzyme digestion.
- Chemistry, the chemical nature of the contaminant, including its hydrophobicity, which influences its interaction with the cells and the environment. This in turn will dictate such factors as its solubility, mobility, and bioavailability.

Microbial

- There needs to be sufficient microbial cells present.
- The right type of population needs to be present- that is cells containing the right gene information.
- They need to be able to be adaptable and modify their behaviour to suit changing conditions in the habitat.
- The microbial populations need to be resistant to any toxic co-contaminants present.

1c. If intrinsic rates of contaminant degradation in a habitat are slow, what engineered interventions can be applied to stimulate microbial bioremediation of the site and how do they work?

The most common reason for slow degradation includes:

- The concentration of the contaminant is so high it kills those cells that could potentially degrade them. In the case of soils this problem can be overcome by diluting contaminated soil with clean soil or another substrate, such as saw-dust. Bioslurping is another approach, in this case combining elements of bioventing and vacuum-enhanced pumping of free-product to recover free-product from the groundwater and soil (so reducing the concentration), whilst at the same time reducingthe concentration in the soil, so favouring microbial activity. The bioslurper system uses a "slurp" tube that extends into the free-product layer. The pump draws liquid (including free-product) and soil gas up the tube in the same process stream. Pumping lifts light non-aqueous phase liquids (LNAPLs), such as oil, off the top of the water table. The LNAPL is brought to the surface, where it is separated from water and air. The biological processes, in the term "bio-slurping", refers to aerobic biological degradation of the hydrocarbons, when air is introduced into the unsaturated zone. When free-product removal activities are completed, the bioslurping system is easily converted to a conventional bio-venting system to complete the remediation.
- There is insufficient oxygen to stimulate the favoured aerobic mode of biodegradation. In deep groundwater aquifers this can be alleviated by sinking wells and injecting air or oxygen release granules.
- There are insufficient microbial populations with the genetic capacity to degrade the contaminant or resistant to the toxicity of co-contaminants. This is a rare situation, which when it does occur can be overcome by bio-augmentation of specific selected populations originating from culture collections, other sites and even generically modified. These can be injected into soils or introduced into sunken wells in the case of aquifers.
- Heterogeneity of contaminant distribution on the site. In this case, the contaminant is present unevenly as hot spots, at such high concentrations they kill or inhibit all the cells present. Alternatively, other spots are clear of all contamination or present at such low concentrations, that degradation activity is not expressed. This condition can be alleviated by several physical mixing approaches, including electrokinetics. With this, electrodes are placed into

contaminated soil and a DC current introduced. This induces several process including electro-migration and electro-osmosis between the cathode and anode. The resulting effect is that particles of different charges move through the soil at different rates, including bacteria which are normally negatively charged, and this results in a more even distribution of contaminants, and more intimate contact between the contaminant and microbial cells. The additional advantages are that the process slightly warms up the soil and splitting (hydrolysis) water generates oxygen and H_2 which leads to a pH gradient between the electrodes, so potentially stimulating microbial activity by optimising pH conditions.

2a. Summarise two complementary approaches for assessing the microbial biomass of waterway contaminated by toxic chemical leak and highlight their relative strengths, and weaknesses of the approaches.

i) **Culture plate counts**. With this method samples are put through a dilution series which runs from 10^0 to 10^7. Then 0.1 ml is spread using a sterile glass rod onto agar media contained in Petri dishes- a processes called 'lawn plating'. With lawn plating colonies grow on the surface of the agar like a lawn. Alternatively, 0.1 ml of the suspension is aliquoted into 10 ml of liquid agar which has been kept at 45°C which is then mixed by rolling and then poured into a Petri dish. With this approach bacterial colonies grow on the surface and within the agar giving a 3-D spread of colonies. There are different types of agar which contain nutrients and supplements which favour growth of specific types of bacteria or be broad spectrum enabling a broad range of bacteria, in term of species diversity to grow. Typically, the plated sample should be incubated for 24-48 hours at a control temperature of 20-37ºC until microbial colonies develop. A gross indication of the extent of diversity in the sample can be obtained by determining the number of different colony types growing on the agar. Examination of the different colony types, based on colour, provides some indication of the degree of diversity within the community Samples taken from sites where only one of two colony types develop are indicative of s site contaminated by a very toxic chemical.

By employing a more general growth medium the same sample can provide an estimate of the total of culture bacteria in the sample. The total is expressed as the number of colonies growing on the medium per ml of water plated.

Strength- the method is reasonable easy to perform, requiring little training and experience. Materials are also reasonably cheap.

Weakness- The limit of this approach is that it is reliant on the ability of bacteria being able to grow on the agar. It is well document that less than 1% of the total community present. It takes 2-4 days for colonies to grow is not quick

ii) **Microscope and graticule**. With this method water samples are stained with acridine orange which is a nucleic acid selective dye. Its is cell-permeable and attaches electrostatically to with both DNA and RNA. When bound to DNA, using fluorescent microscopy it shows up green and when attached to RNA it shows up blue. Since RNA

is only produced when the cells are viable and active, the technique give some indication of the proportion of cells that are viable or dead. Under a microscope with x100 objective the bacteria cells can be easily seen after the stain has been added. The suspension is added to the haemocytometer which is marked with $1mm^2$ squares with defined volume. Thus, the number of cells per in the known volume can be used to express the number of cells per ml of sample.
Strength- quick and easy to perform.
Weakness- labour intensive and does not differentiate between live and dead cells.

2b. **Summarise two complementary approaches determining how the chemical leak is impacting on species diversity and detail the relative merits of the technologies.**

i) Culture plating (also see above)- the same procedure as detailed above in 2a i) is used to grow up colonies in or on agar. Different bacterial species produce an enormous array of colony types that differ in colour, texture, general morphology and even smell. The number of different colony types enables a gross assessment of how the bacterial communities differ in samples.
Strength- cost effective, simple to perform and train staff, requires a non-specialist lab.
Weakness- Only detects those bacteria that can grow on selected agar- most less than 1% coverage. Takes 1-4 days to get colonies to grow.

i. **Molecular approach (DGGE)** - In order to get a more complete assessment of the composition of the microbial community it is now more common to employ molecular approaches. With this the community is exposed to a vigorous procedure to extract the DNA from the cells present. This is achieved by adding the samples to a bead-beater which shakes to break open the cells within a sample. Since DNA represents only present in small quantities an enzyme Polymerase chain reaction (PCR) is added which makes multiple copies of the DNA present. The PCR amplifies a single or few pieces of the indicator DNA by several orders of magnitude so that it can be detected. The resulting solution containing the amplified DNA is then analysed by Temperature Gradient Gel Electrophoresis (TGGE) or Denaturing Gradient Gel Electrophoresis (DGGE), are forms of electrophoresis which uses either a temperature or chemical gradient to denature the sample as it moves across an acrylamide gel. Denaturing gels are capable of inducing DNA to melt resulting in the DNA spreading through the gel at the rage which is proportional to its size or charge- thus the individual genetic components can be detected. The gel induce fragment melting which under an electric charge spread (speed and distance they move is determined by the fragment size) through the gel to generate a unique DNA finger-print for each species or population present which can be easily distinguished from other species. Thus, the method obviates the need for any culturing and results can be obtained much more quickly.

2c. Summarise how you would go about isolating bacteria that are effective at degrading the leaked chemical (referred to as compound X).

If the leak has been long term, the exposed microbial community will have acclimated to its toxicity increasing the chances of some populations or strains developing the ability to

degrade the compound and utilize it as a substrate. This will certainly be the case if there are signs that the compound X is undergoing degradation- in particularly when chemical analysis suggests that break down products detected. In this instance the opportunity of isolating populations able to degrade compound X are higher. In order to isolate degraders, soils are collected and 1-10 g are added to a minimal medium solution (500ml). This medium contains all the nutrients required for healthy growth with exception of either a nitrogen or carbon source which is provided by compound X. By adding the soil, the rationale is that the N(nitrogen) or C (carbon) required for microbial growth is provided by compound X since the medium is otherwise free of these elements. The solution is then incubated on a shaking incubator kept at similar temperature as is prevalent in the target site, normally between 15 -25°C until there are signs that there is growth- this normally takes a form of the solution going cloudy or showing a colour change. When is occurs 10 ml of the solution is transferred to a second flask which contains the same minimal growth medium, with compound X as the sole C or N source. The process which is called 'enrichment with isolation' which is repeated three to four times or certainly until there is some confidence that the only organisms able to grow in the solution are those able to assimilate compounds. The solution is then sampled and an aliquot then plated on an agar plate composed of the minimal medium with compound X as the sole N or C sources. Thus, the only colonies develop on the agar are those able to grow on the agar and use compound X as a sole N or C source. To confirm that the individual isolated bacteria can actually degrade compound X, minimal growth solution is inoculated with a single pure colony growing on the isolation plate. Clouding of the medium after 2-10 days confirms cells are growing so must be able to assimilate compound X. Chemical analysis of the medium and the degradation of compound X is further confirmation that the isolate can assimilate the target compound.