Environmental Biotechnology
Prof. Pinaki Sar
Department of Biotechnology
Indian Institute of Technology, Kharagpur

Lecture – 29
Methods in Microbial Ecology with Relevance to Environmental Biotechnology (Contd.,)

Welcome to the next lecture of this environmental biotechnology course and this lecture is on methods in microbial ecology with relevance to environmental biotechnology. So, we have been discussing these different methods which are used in environmental biotechnology to identify characterize and find out the best application of microbial communities within different environmental systems.

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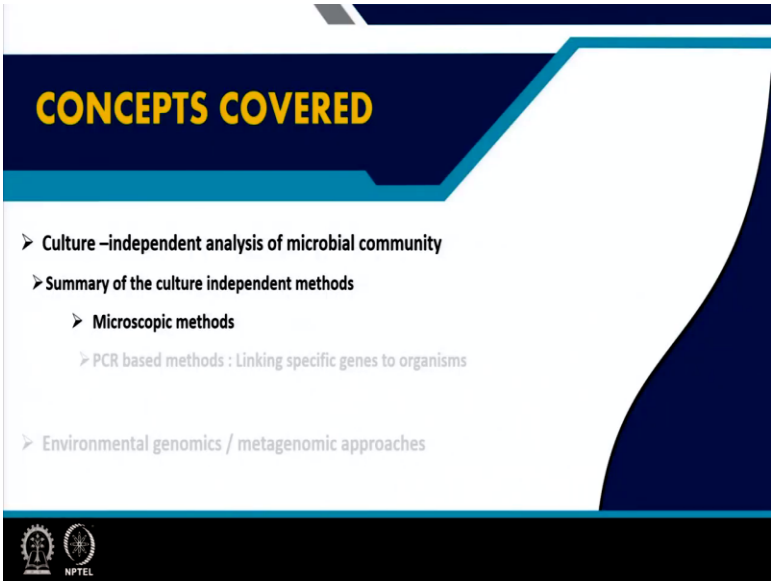

In today's lecture I am going to cover this following aspects and these are we will introduce ourselves to the the culture independent approaches that are being practiced a broad overview of the summary of different culture independent methods will be discussed. And followed by the discussion on different microscopic methods particularly the fluorescent microscopic methods which have been used widely to describe the importance of specific group of microorganisms.

Or the general abundance and presence of the living or dead microbial strains or microbes and

linking the function to different taxonomic group of microorganism present within a different environmental biotechnology system.

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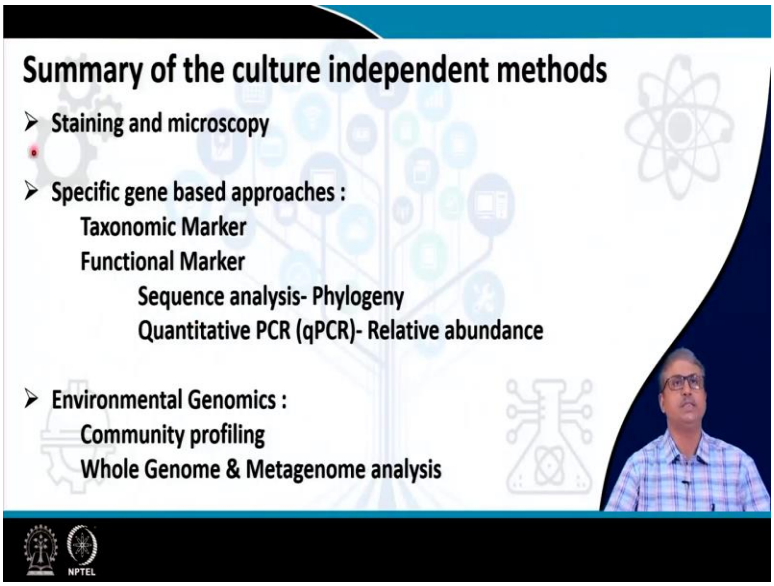

So, here we present the summary of the culture independent methods this is a kind of a broad layout of our next couple of lectures that we will be going through. the first is the staining and microscopy which will be discussed in the today's lecture. The second important approach is based on specific genes and the specific genes present in the microorganisms of environmental relevant communities could be the taxonomic marker genes like the genes which give us information about the taxonomic identity of the organism and the markers for their functional properties.

So, with respect to functional marker properties we basically use different protein coding genes or enzyme coating genes. and these enzymes or proteins are selected based on their the function or the relevant function within a particular environmental system. And as we can understand that depending upon the research question and the process of the technology that is being targeted or the process which is being investigated in context to the environmental biotechnology system these functional markers are selected.

So, there are actually very well known marker genes which are involved in very specific pathways including the carbon fixation to a carbon metabolism to methane metabolism to the

degradation of different pollutants to heavy metal resistance heavy metal transformation and different other environmental relevant processes including sulphur metabolism nitrogen metabolism etcetera.

So, specific genes are selected based on the process that we are trying to investigate. and this functional gene based or the taxonomic marker gene based analysis both are used to obtain two sets of information. One is the sequence based analysis where the specific gene of interest is PCR amplified and then sequenced and following the sequence analysis we can derive the the phylogeny of the organism or phylogeny of the gene often the catalytic abilities of the protein coding genes are also deciphered.

When we convert the nucleic acid sequence to amino acid sequence and then try to look at the conserved amino acid residues and find out the possible functionalities of those enzymes coding sequences or the protein coding sequences. The other major way of deriving information from these specific gene based approaches is the quantitative PCR based method or the qPCR based method.

Where in the relative abundance of a particular organism or multiple organisms are certain because these are specific to individual organism because the taxonomic marker genes could be selected based on the organisms that are of interest or the functional genes multiple functional genes can be selected based on the role of those or importance of those genes in the given system. So, this quantitative PCR based approach is very well standardized and well practiced across different environmental biotechnology settings.

To identify the relative abundance to ascertain the relative abundance of the organism and also to characterize the functional genes in terms of their relative abundance for example we can assume that there could be many many bacteria or archaea in a particular environment. But in order to identify that which species or which members of which genera are actually most abundant these qPCR based methods which actually target 16s ribosomal RNA genes specific to different taxonomic groups are found to be very useful.

Similarly with respect to functional genes in a given environment there could be many different functions occurring at a given point of time like sulphur reduction to nitrogen nitrate reduction or ammonia oxidation or carbon fixation by multiple pathways. So, when we look at this qPCR based approaches these are often found to be very useful because it can easily give us data on exactly what process is more abundant.

There may be presence of many genes but presences of many genes are not enough gene may not be functioning. So, qPCR data often used with respect to the total RNA that we extract from the sample. So, when we extract the total RNA and then adopt the qPCR based method then this is very going to be very useful to have a kind of real time data about the gene expression status of the of the selected targets.

So, thereby the sequence based analysis and quantity PCR based analysis of marker genes are found to be very useful and in a in a specific lecture we are going to talk about these in detail. The last approach is the environmental genomics approach wherein again there are actually three sub approaches one is community profiling. Community profiling refers to profiling of all the organisms present in the community.

As we know the community is basically composed of numerous populations and each of the populations are basically group of organisms those belong to same species. So, ideally there are numerous species in the community. So, it may be a biofilm it may be a planktonic it may be any type of community. So, if you want to know what are the organisms presents one of the most suitable technique is the profiling.

Profiling means what are the species presents a quick overview of this can be obtained when we use this 16s ribosomal RNA genes present within the community and try to ascertain the how many different types of 16s ribosomal RNA genes are there. So, there are many many procedures to do this profiling and we will talk about those methods in a lecture in a separate lecture rather. And then the whole genome and meta genome analysis the whole genome are basically the sequencing the entire genome of specific organisms including those like particularly the pure culture bacteria that we discussed earlier.

So, when we identify certain organisms as a key player or we are able to isolate them as pure culture often sequencing the whole genome is found to be very useful because then when you have the complete genome sequence with us we are able to identify what all genes and what all possibilities this particular organism might have and how they are regulated. So, there are multiple components in whole genome sequencing it is not merely the identification of genes present there.

But also the functionalities of the genes the how these genes could be could be controlled because when you look at the promoter sequences and other controlling elements of those sequences the genes because the whole genome gives us the opportunity to look at the entire architecture of the ORF and other things the meta genome analysis is basically sequencing the entire community's genome like extraction of the total total DNA which represents the genomes of all the organisms present.

So when I say all the organisms it is truly all the organisms because we do not try to isolate any specific bacteria here we try extract the DNA from the sample like soil sludge waste water or whatever kind of sample we extract the total DNA and then that total DNA is subjected to DNA sequencing and then when we sequence the DNA and then obtain the entire genomic information then that that is that is something called meta genome analysis.

And we will talk in detail about that so apart from meta genome analysis we can also extract the total RNA and go for the the meta transcriptome analysis or we can extract the total protein of an environmental sample and go for meta proteome analysis or we can extract the total metabolites from any environmental sample and go for a metabolic profiling of the entire community as well. So, all these different methods of culture independent approaches have gained popularity and also make significant advances with respect to their technicalities.

Both at the hardware and the software like the instrumentation part as well as the the softwares and the bioinformatic tools which are being used to decipher the processes based on these information. So, in our subsequent lectures we will talk in detail about these environmental

genomics and specific gene based approaches.

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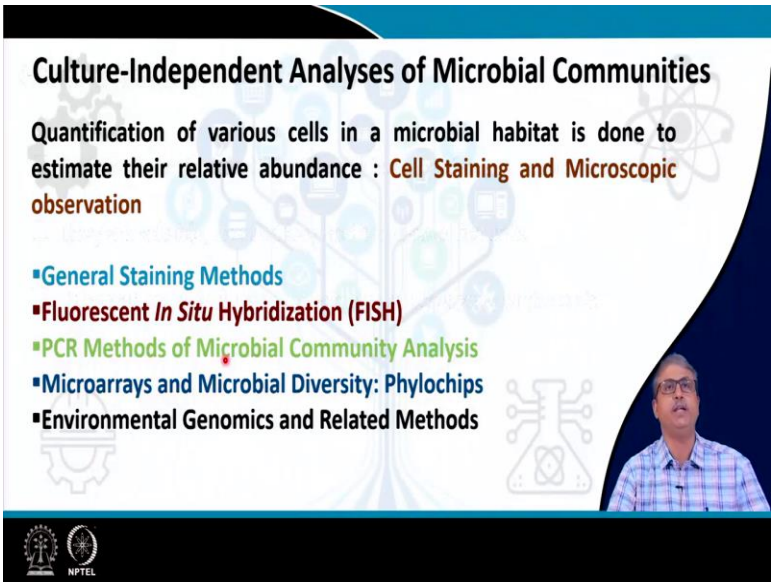

In today's lecture we are going to talk about a particular aspect that is called straining and microscopic based observation. Now this is one of the first attempt to have the culture independent analysis because if we remember through our great plate count anomaly kind of concepts when we observe that under the microscope always there are more number of counts more number of cells from any given environmental samples rather than the the colonies which appear.

So, microscopy remain a one of the very important tools for progressing the culture independent way of looking into the microbial community. So, quantification of various cells because there are many cells many types of cells many species are there in a microbial habitat or microbial community. And these quantification of various type of cells is done to estimate their relative abundance as well.

So, if we have lets an activated sludge or a sample from a bioreactor used for producing something or a biofuel system or maybe a petroleum waste remediation system. So, we want to know what are the type of cells are there or what are the type of organisms are there and what are the relative abundances of these. So, one way of looking into these questions like what is the relative abundance and what are the different types is basically a simple method of cell staining

and microscopic observation.

So, these are divided into some sub heading like the general straining method and then the fluorescent in situ hybridization based method there are other methods which are included these are basically as discussed earlier these are different aspect of aspects of the culture independent methods.

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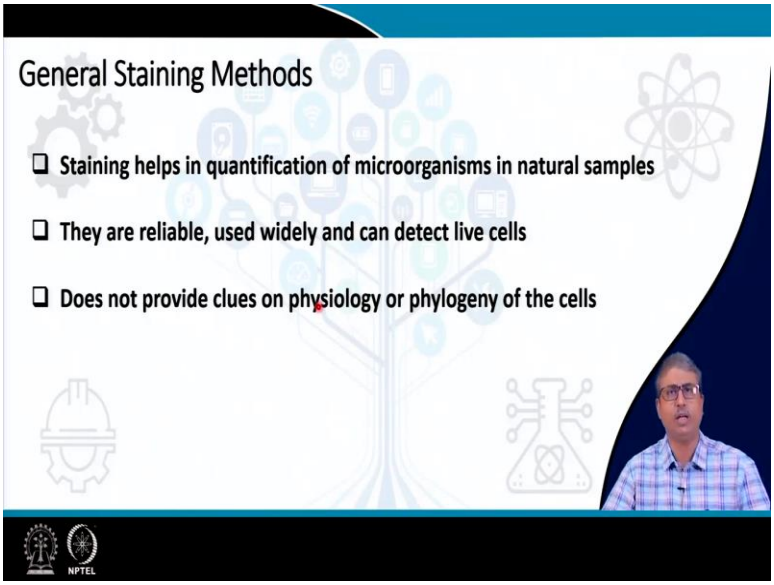

So, coming to the; general staining method staining helps in quantification of microorganisms in natural samples. So, we take the samples and samples means the water waste water sediment soil rock sludge or any kind of environmental sample which is which is a subject of the investigation. Then we add different stain stains are the dyes or coloring agents chemicals which give colors to the cells because from our basic knowledge microbiological knowledge we know that microbes are in general colorless except few cells.

Particularly when you look at them through light microscope many of them appears to be colorless and then unless we put color on them it is very difficult to have a contrast and see them properly with respect to the shape and morphology or the number of cells even. Now there are different staining techniques. So, these techniques are found to be quite reliable and used widely for a long period of time because for last maybe 30 years or so.

The staining methods have been developed from light microscopy to fluorescent microscopy both and they are used widely across different environmental samples and also they have been improved continuously. So, as we will see that initially the fluorescent microscopy or other microscopic methods were basically to enumerate the number of cells how many cells are there. So, just like a crystal violet staining method or a gram staining method improved staining method based on the DNA staining dye a kind of dyes were used to find out how many cells are there.

So, those are fluorescent stain based method or fluorescent microscopy based method. So, they have been developed and used widely primarily to find out how many cells are there and then what are the; morphologies of these cells as much as possible in a fluorescent microscope because the resolution of fluorescent microscope may not be very high compared to the electron microscope. So, these fluorescent dye based microscopy is basically done using fluorescent microscope.

So, and this later can be what was developed to discriminate between the live cells and the dead cells because when initially we were doing or the scientists were doing this of fluorescent microscopy to find out the cells present in an environmental sample they were staining with dyes which stain all cells. So, it was not possible to discriminate whether the all the cells are dead or living because in environmental context this is very important to know that whether cells are living or dead because there may be dead cells.

And unnecessarily we will be counting them for our purposes particularly may be for drinking water and many other waste water treatment systems. So, having cells and having live cells are always not equal actually there may be many cells which are dead. So, later techniques were developed to detect live cells and as we detect techniques for live cells the discrimination is can be achieved like how many cells are dead and how many cells are lying.

So, that was a big jump in quantitative microbiology and environmental microbiology particularly. However these general staining methods which are adopted and developed to enumerate the type of microorganisms present or the microorgan total number of microbes of the

living and dead microbes present in any samples are they are generally they are not capable of providing any clues on physiology or phylogeny of the cells.

So, what I mean that they are not capable of producing any physiological information or phylogenetic information is that if we stain a sample may be worst water treatment plant sample. We stain with some fluorescent dye and see under the microscope we can see how many cells are there. We can also count how many cells are living and how many cells are dead but it is not possible with simple staining technique that to say that what is the physiology of these cells.

Whether they are carbon fixing they are hydrocarbon degrading they are ammonia oxidizing they are nitrate reducing or whatever may be the relevant physiology that is that is of a question or point of interest for a particular environmental engineering system or the phylogeny of the cells. Phylogeny of the cells means they are affiliation what type of are they belong to some kind of taxa or taxonomy group which are considered to be pathogenic like a vibrio type of organisms.

Or they are organisms who are generally associated with producing certain other chemicals which might be toxic to the human or other organisms. So, we are unable to ascertain any such information during this general scaling based method.

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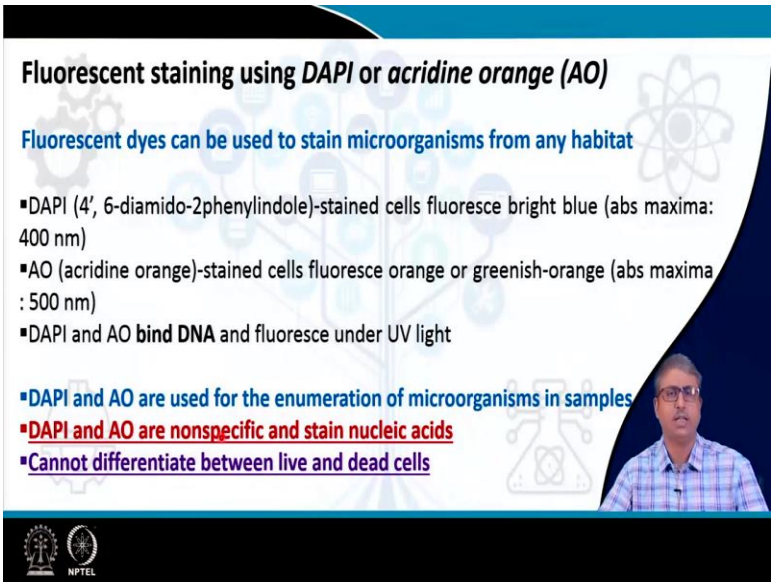

However as I mentioned earlier the general straining with methods remain very useful because

they were the first type of methods which allow us to have a thorough enumeration of the cells firstly how many cells are there. And then next how many cells are live and how many cells are dead. So, first begin with this fluorescent staining using two very popular dyes the DAPI and AO or acridine orange these fluorescent dyes can be used to stain microorganisms from any habitat.

So, you can take samples means the environmental samples from any habitat and then prepare them according to the protocols as per the protocols and stain them and following staining you can see the samples under the microscope. So, here I just want to remind that no isolation of bacteria no pure culture no medium nothing is required you can have the samples straight away taken from the your system or the may be the reactor or the tank or activated sludge system erasing tank anaerobic digester or any other kind of systems.

And then stain them with fluorescent dyes following the protocols and then take them to the fluorescent microscopy. So, two dyes one is the DAPI that is the four six diameter two phenyl indole stained cells the fluoros bright blue. So, absorbent maxima is 200, 400 nanometer and other is the actin orange which stain the cells with fluorescent orange or greenish orange color maximum absorbance is at 500 nanometer.

So, these two strains basically the DAPI and accurate orange are very popular and they bind DNA and fluoros under UV light. So, here is a point that since they bind DNA they enter inside the cell. So, during the sample preparation we need to make some treatment to these samples. So, those are counted under the sample preparation steps. So, that the DNA is exposed to the stain. So, cells remain intact. So, we are not going to lyse or destroy the cells the cells remain intact only these cells will be made.

So, that the dyes are able to enter inside the cells and they bind to the DNA and when we expose these samples under the UV light they fluoros and looking at the fluorescence we can actually count how many cells are there. So, they may be blue color cells or there may be orange color cells based on the or greenish orange colour of the cells based on the type of dyes that we are using the two dyes are used separately.

So, either you use DAPI or you use activity in orange based on your choice and the nature of samples. So, it is based on the kind of standardization that any lab might have done. So, these two dyes are DAPI and acridine orange are basically used for the enumeration of microorganism in sample. So, I said microorganism that means all microorganism in fact it will stain any or any cells present in that any cells it will not discriminate between bacteria and archaea and fungi and microalgae or algae or protozoa or anything.

According to the treatment that are that we are giving that is a procedure to be followed all cells will be exposed to the dyes and the dyes will enter into the cell and will stain the nucleic acids. So, they are non-specific. So, they do not discriminate between the type of organism like bacteria or archaea or algae or something like that. So, whatever organisms are there in your sample they will be strained and it you will be able to see or will be able to see them under the microscope.

So, now it is also important that they cannot differentiate between live and dead cells because all cells as I said are having their DNA as long as the DNA is there the cell will be able to get the color and you will be able to see them.

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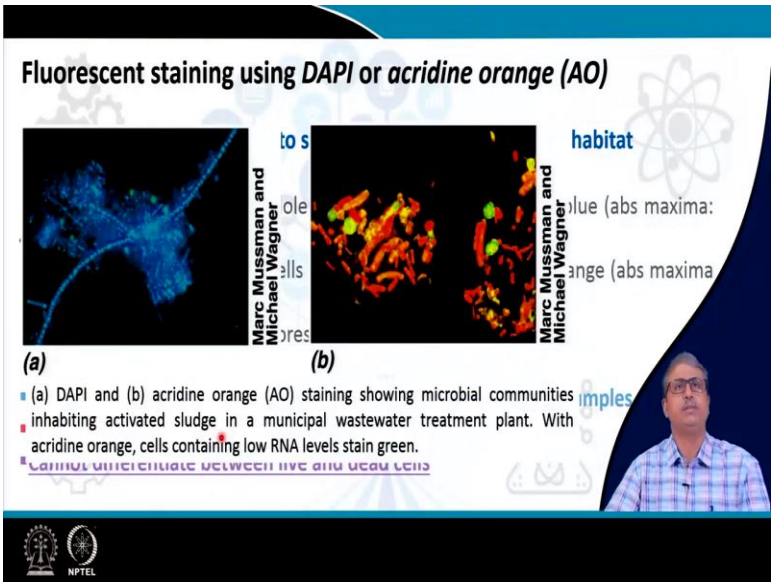

For example these are the two very elegant pictures the picture on the left side marked as a is a DAPI stained sample and on the right marked as b is acted in orange strain samples. These are showing microbial communities inhabiting activated sludge in a municipal wastewater treatment

plant. So, as in activated sludge we know that there are multiple organisms present. So, you can see that a different type of cell morphologies could be could be located.

And if we go for may be zooming into the or zoom into a particular spot on the field we will be able to see some more details of course due to the background often the contrast of the image or the cells may not be very high always but for that you need some further standardization to have very clear image in order to look at the cell morphology but generally this is not done for morphological purposes this is done only to have a count on the cells.

So, whether we are able to see the cells and then if we are able to see the cells then how many cells are there. So, you can we can use this kind of techniques either dapistrain or acridine orange strain cells and these are very popular techniques to have a total count like enumerating the total number of cells.

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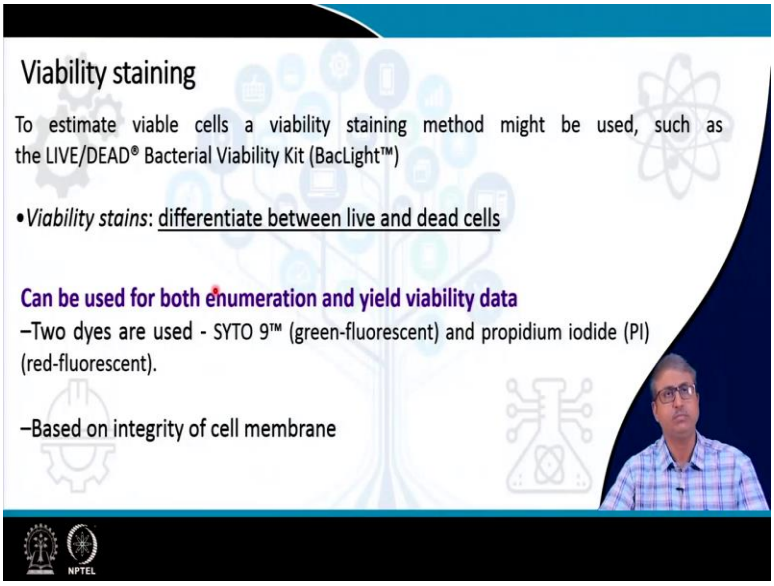

Now total number of cells is good because that gives us a kind of approximation that how many cells are there in a particular environment particularly wastewater treatments or a drinking water system or maybe a system where some kind of bioenergy or biodegradation process is going on. So, in order to know what is the load biomass load they are in. So, this is it is kind of a first approximation comes from that the total staining.

But in order to have a kind of a count on how many cells are viable actually in a particular environmental sample we need to have this viability standing. So, viability staining is basically made to estimate the viable cells through using again a set of methods which are very streamlined method. Particularly a live dead kind of detection systems which are some of them are proprietary in nature and some of them can be used in the laboratory itself by because these are these are made of two dyes are mixed together like earlier we talk about DAPI and acridine orange I said that they are to be used separately.

Because they give just the color to the cell and we do not want to mix up. So, either we use DAPI or we use activity in orange we get orange or blue colors we just count them but in case of the viability testing or viability of determination we use a combination of two dies now this combination of two dies are allowing us to differentiate between the live cells and the dead cells. And these can be used for both the enumeration purpose and to yield the viability data because together we can have a count on how many cells are there no matter they are dead or alive but at the same time will be able to say how many cells are actually living in that particular sample.

So, two common dyes which are used are SYTO 9 which is a green protein dye and the propdium iodide or pi which is a red fluorescent die. And it uses the concept of the integrity of the cell membranes. Now why integrity of cell membrane is considered here because a dead cell is considered to compromise with his membrane integrity and that means the membrane integrity is disturbed for a for a dead cell.

So, that means the dead cell will be relatively open with respect to the dyes molecules whereas living cell will have more compact and intact rather the cell membrane and some dye molecules may not be able to penetrate inside the cell.

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So, like SYTO 9 dye it is able to penetrate all membranes it is the chemical nature of this dye that it penetrate all the membranes. So, it ideally stain all the cells. So, all the cells will be stained with green colour. However the property of iodide can only penetrate cells with damaged membrane that means cells which are potentially or cells which are actually dead or damaged. So, that with damaged membrane.

So, they stain the dead cells because the dead cells are often now the dead cells means the cells which are dead but still partly intact because totally decomposed or live cells are not counted. So, it is the only the cell which is visible as a cell now cells with the compromised membrane with it will eventually be red because PI can only penetrate the damaged membrane cells. So, the dead cells will have the red colour whereas the cells with undamaged membrane will stain green.

Now the green stain will enter in all cells dead or live in both but in case of a dead cell since the red is entering into that over the green. So, red and green together it will appear as a dark red colour. So, eventually we can have actually a very clear distinction within a particular sample that the cells which are red are dead cells and cells which are green are actually the living cells. So, we can count on them and then make a an assessment and then make subsequent decision about this particular sample.

Now however it can have some issues with non specific straining in environmental samples. So,

that is true for most of this fluorescent dye based technique that some non specific binding etcetera are there. So, with environmental sample we need to have some kind of optimization of procedures.

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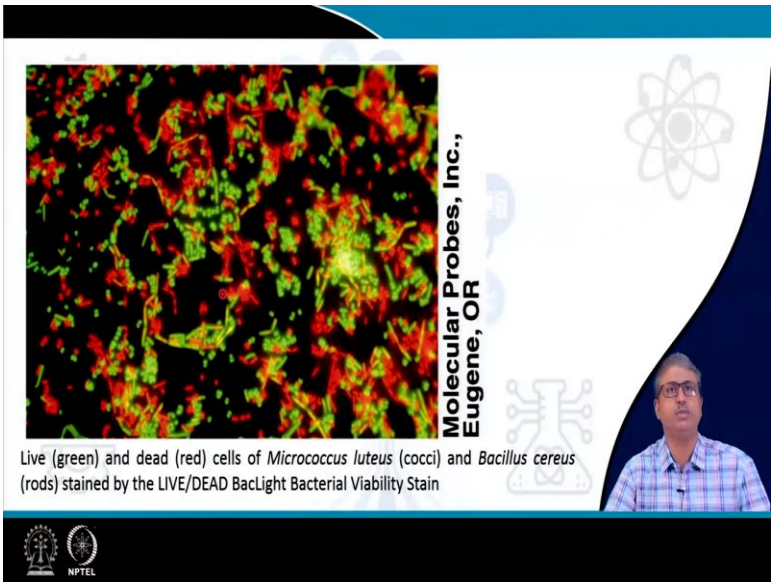

So, here is the picture for a live cells and dead cells of two organisms micrococcus luteas which are the cocky and the bacillus areas which are the rods. So, you can see there are rods and cocktail both and they are stained by this live date back like viability bacterial viability stain. So, we can clearly distinguish that out of the total number of cells how many cells are living and how many cells are dead.

This becomes very very useful in order to have a true assessment on the efficacy or the performance or the processes which are going on in an environmental system. So, this is one of the one of the very, very robust system to detect the living and dead cells.

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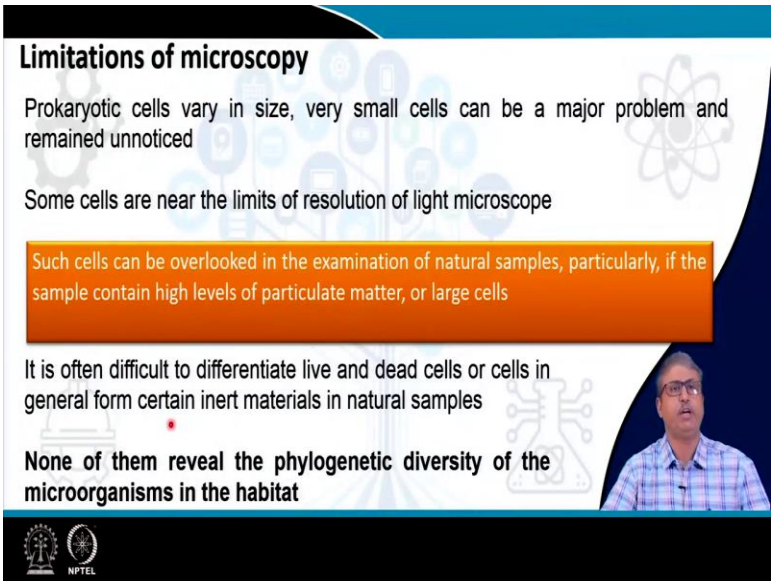

Now we will talk briefly about the limitations of different microscopy before we move forward to the next method which is the fluorescent density hybridization method. So, the general limitation of this microscope is art that the prokaryotic cells are they vary in size they could be very small. And these small cells can be a major problem and remain unnoticed because some cells are beyond could be beyond the resolution of this fluorescent microscope they may take the stem but they will not appear as a distinct cell.

So, you will not be able to you will not be able to see them distinctly. So, maybe 5 or 10 small cells will group together naturally often they are also clubbed together. So, they may appear as a single red or green dot and we will count them or we may count them as a a single organism but they are actually five or ten different organisms which are or same organisms they are staying together that is one issue particularly when we when we know that or sometimes we know that some environmental samples are having actually very small cells within them.

So, we need to take again some precautions and the sampling procedures and the sample preparations are done accordingly and that is basically the some cells are always there which are near the limits of the resolution of the light microscope and they are very difficult to resolve under this normal microscope. So, such cells can be overlooked in the examination of the natural samples particularly if the sample contains high level of particulate matter or large cell.

So, particular matters are often there in different waste water treatment or the effluents or the contaminated river or contaminated sediments. So, the particulate matter will appear as a big relatively bigger chunk or bigger size structure and compared to that the smaller size cells will be out of focus and it will be very hard to get them into the countable form. Now it is often difficult also to differentiate live and dead cells or cells in general form containing in inert material in natural samples that is also a point of concern.

Again for all these things we need to have some kind of standardization and this thorough standardization is always found to be very useful in improving the efficacy of any kind of these microscopic methods. Now another very crucial limitation again is even if this backlight type of system or acquired in sorry propidium iodide and the cytogreen based methods are able to discriminate the living and dead cells none of them they reveal the phylogenetic diversity of the microorganisms in the habitat.

So, we are unable to define or unable to find out what are these organisms are we just know that or just able to say that they are living cells or they are dead cells or how many cells are there. But what are these cells what is the name of this organism. Can we can we obtain this data we can obtain this data but not through this methods we have to go for some advanced microscopic method.

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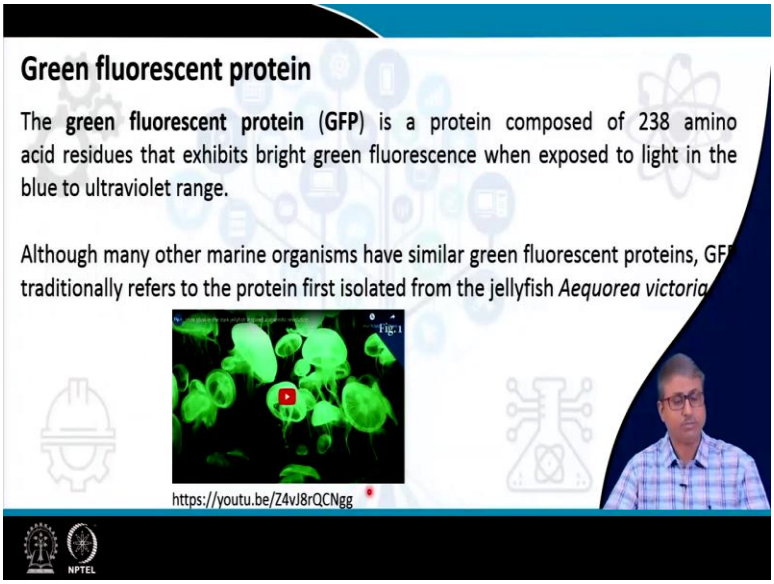

Now in recent past there is also some important contribution from the very well known protein which is called a green fluorescent protein and this in microscopy or microscopic detection of the cells. Now GFP or green fluorescent protein is a protein composed of 238 amino acid residue and it exhibits a bright green fluorescence when exposed to light in the blue to ultraviolet range. So, here is the picture which is showing the Jellyfish which is basically the aquarium Victoria from where originally the the green protein protein was first discovered.

And subsequently the green protein is discovered in many other organisms and the gene coding that protein has been identified cloned and then used in different purposes for these microscopic uses. So, if interested you can go to this particular site and look at the the functioning that how actually GFP works.

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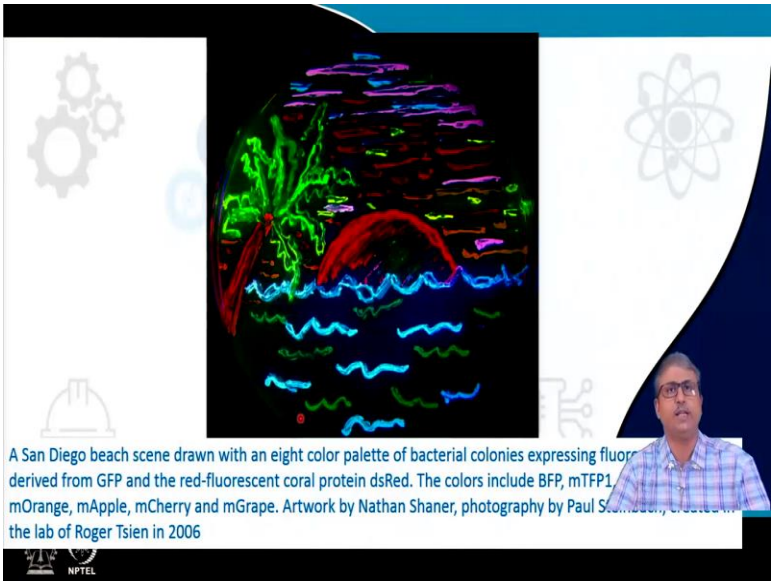

So, subsequently as I mentioned there many different type of fluorescent proteins have been isolated and the encoding genes encoding them have been obtained. So, here is an the picture of microbiological art where a San Diego Beach scene is drawn with an eight colour pellet of bacterial colonies expressing the fluorescent proteins derived from GFP and the red fluorescent coral protein dsRed.

So, these are all bacterial cells with engineered with GFP and then the red fluorescent protein and different type of colours are developed by actually engineering those GFP and red protein

and this is an artwork by Nathan Saner and photography by Paul Sinback and created in the lab of Roger Sein in 2006.

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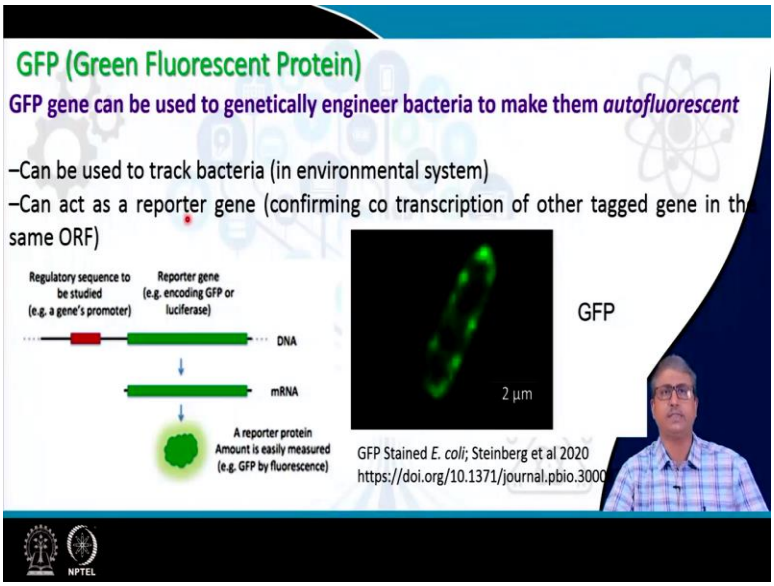

So, this GFP is becoming very important or becomes very important with respect to detection and characterization of microbes through fluorescent microscopy because GFP itself is a fluorescent protein and as I mentioned under the microscope fluorescent microscope you can see the cells which are stained with GFP. So, what is being done actually the GFP gene is taken out and that is the reporter gene we called.

And this gene is actually recombined or joined with another gene of interest this gene of interest is generally a regulatory sequence to be studied that is some genes promoter. For example if you want to study the organisms which are involved in ammonia oxidation for example or organisms who are involved in sulphate reduction or organisms who are involved in a heavy metal tolerance or the organisms who are involved in petroleum hydrocarbon degradation.

So, in case of ammonia oxidation for example the AMO the AMO gene promoter is we taken and then that has to be fused with the GFP and this will be within a plasmid and this plasmid will be transferred to a bacterial cell and then this bacterial cell will be or the cells will be released into the this environmental sample where ammonia is there. Now since ammonia presence of ammonia will activate this promoter which is involved in the ammonia oxidation process.

So, this will switch on the transcription of this entire stage and eventually the in this case the GFP will be transcribed the mRNA will be produced and from mRNA the gap protein will produce. So, we will be able to see whether the ammonia oxidation is potentially being possible in this environment or not with using the particular set of genes that are being used. So, there have been numerous cases where environmental samples are being studied with GFP because this is now considered as a reporter gene to report certain events which are environmentally relevant event.

So, on the right side here is a GFP state E.coli which is again engineered with a GFP protein to have certain kind of functional attributes to be obtained. Now GFP gene actually used in a genetically engineered format to make them autofluorescent and they can be used to track a bacteria in an environmental system or can act as a reporter gene to confirm the co transcription of other tagged gene in the same way.

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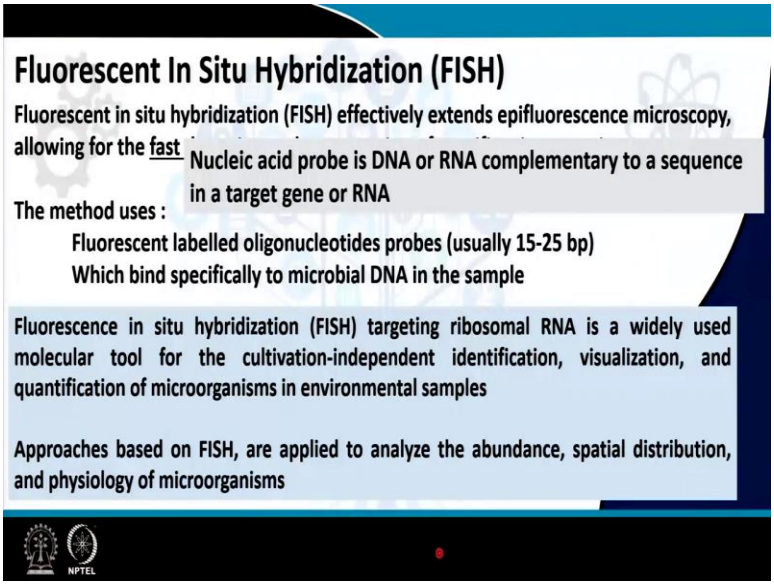

Now we will talk about the fluorescency to hybridization or FISH. The fluorescent in situ hybridization or fish is a very effective technique which is basically based on the epifluorescent microscopy and it allows the fast detection and enumeration of specific microorganism. So, here the important point is this specific microorganism. So, we can be very specific it is it is beyond the limit of the DAPI or acted in orange or SYTO green kind of combination technique that

backlight.

So, it is more specific we can have a very specific detection of individual organisms and even we can improve it further to have a function to a specific group of organism like which organism is doing what that also we can we can determine. So, the method uses a fluorescent level of oligonucleotide probe which is actually a 15 to 25 nucleotide long stretch of p short piece of DNA and which is labeled with a fluorescent molecule.

And when this molecule that is the probe is allowed to hybridize with its target molecule the target molecule could be a DNA or could be RNA present in a sample or present within a cell of a sum within a sample it binds specifically because it will rely on the specific nucleotide complementarity a tgc binding pattern. So, only when the probe will get its complementary DNA or RNA it will bind and it will bind and then it is fluorescently tanned.

So, if it has bound to the particular cell within a system and this is exposed to a fluorescent microscope we will be able to detect it now this probe is basically a short stretch of nucleic acid could be a DNA or RNA molecule which is complementary to a sequence in a target gene or a RNA molecule and these are synthesized in the laboratory and they are tagged with fluorescent molecules.

Now following the hybridization that is the probe is allowed to hybridize find out the complementarity and hybridize the specific hybridization of probe to its target allows the visualization of the cells using an epifluorescent microscope or a confocal laser scanning microscope. So, you can see which cells have the target molecule. So, that the probe has bound to them and the if the probe has bound to the target molecule that means it is confirming that this particular organism or this particular functional gene is there.

So, suppose we want to see whether the very well known pathogen in water system vibrio or vibrio calorie is there or not. So, you can make a vibrio quality specific 16 ribosomal RNA gene probe and then take the water sample and allow the water sample to have this probe interact with the cells present there. So, any cells who is positive to vibrio quality or is bibliocaly will have the

complementary region.

So, that probe will be able to go there and hybridize and following hybridization if we are able to if we take the sample to a flow of epifluorescent microscope or a confocal microscope we will be able to see this now fluorescent in situ hybridization targeting the ribosomal RNA basically for the taxonomy purpose or identifying the specific group as I gave the example of the vibrio calorie. So, is a widely used molecular tool for the cultivation independent identification visualization and quantification.

So, it carries 3 information all together it is cultivation independent it is it is capable of giving us the identification that whether a particular group of organism or particular groups of organisms. So, multiple organisms can be detected if we have multiple probes with multiple fluorophore attached to them. Visualization we see them that which cell is where particularly if you consider a system where maybe a plastic degradation is occurring.

So, if you want to know which bacteria are actually physically associated with the plastic molecule and which are the bacteria who are not physically associated but they might be degrading the compounds which are released following the the plastic degrading bacteria actually initiate the first level of degradation reactions. So, so we can visualize those things very clearly and then the quantification.

So, for example the vibrio that I was referring to we can quantify how many biblio positive cells are there in a particular sample. Now approaches based on the fish are also applied to analyze the abundance and moreover the special distribution how the organisms are distributed within the sample and the physiology of the organisms physiology of the organisms can be connected further to different other genes because beyond attack 16s marker gene if we use a functional gene and then use that gene as a probe we will be able to find out the function that is the physiological function and as well as the taxonomic function or taxonomic affiliation of the organism.

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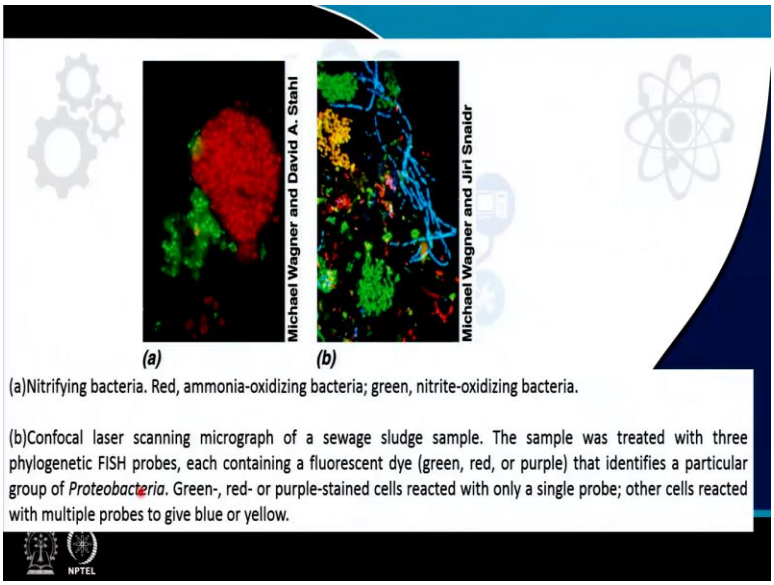

So, here is again the picture of a nitrifying bacteria which is panel a and coloured as red because red coloured fluoresce fluorophores were used for this and on the ammonium oxidizing bacteria are coloured the green. And and also you have the b panel this panel is showing the convocal laser scanning micrograph of a sewage sludge sample. The sample was treated with three phylogenetic FISH probes as I mentioned that you can actually use multiple probes but of course each of the probes will be different in terms of they are capable of detecting different organisms.

So, different phylogenetic probe means different taxonomic groups are to be targeted each containing a fluorescent dye. So, in this case the green red and purple dyes are attached. So, you can see very clearly and that identifies a particular group of proteobacteria green red purple strain cells reacted with only a single probe other cells reacted with multiple probes to give us blue or yellow. So, these are some of the examples. So, similarly there are innumerable number of cases where we see that the fluorescent simple fluorescent density hybridization based techniques have been used.

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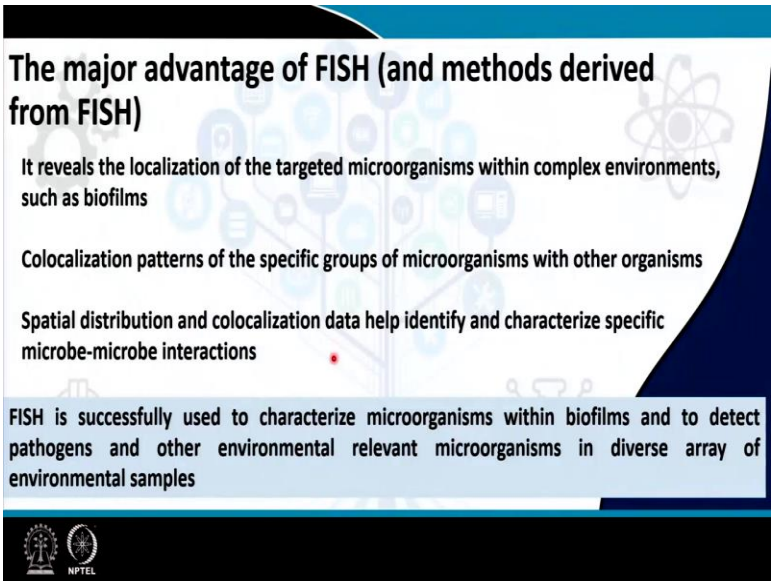

Now the major advantages of the fish apart from that they are able to give you a view of the cells they are able to identify or they are able to see tell you the abundance of the cell. It reveals the localization of the targeted microorganism within a complex environment such as a biofilm. A particular type of organism is located where within a community that you can identify very well co-localization patterns of the specific groups of microorganism with other organisms because we know that microbes interact with other species.

So, how a particular species is interacting with other species that is also revealed by or that is the co-localization pattern will reveal that thing the second and third is the spatial distribution and co-localization data will further help to identify and characterize specific micro interaction that how a specific micro were the two different or more than two species are interacting. From environmental biotechnology context these interactions are very important.

FISH is successfully used to characterize microorganism within biofilm and to detect pathogens and other environmental relevant microbes in diverse array of environmental settings.

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So, conceptually as I mentioned it is a very straightforward technique. So, within the DNA you have some target region. So, you make a probe which can actually by bind to the target and then when you allow this probe to bind. So, and denature the DNA. So, in the denature near DNA that target DNA straight strands are separated and the in the and the hybridization condition is created.

So, these probe molecules will be able to able to find out and if they find out the complementarity they will bind.

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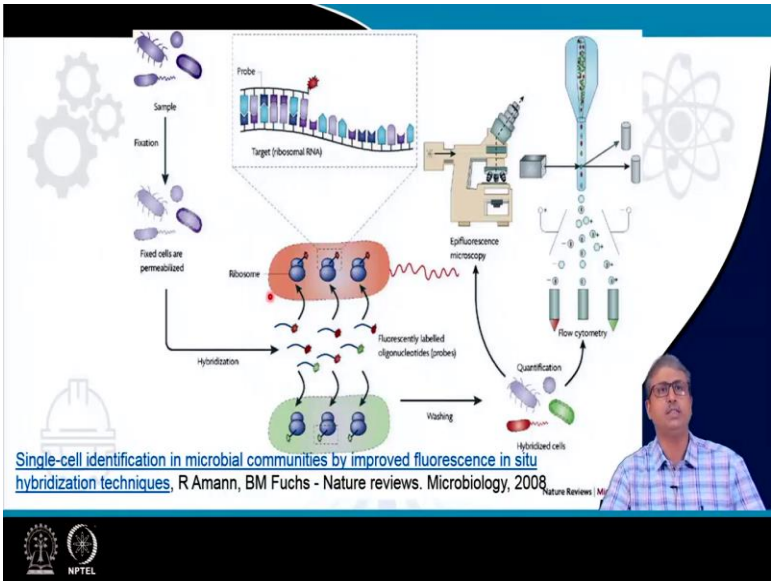

So, in a microbiological sense you can see here schemes of events are given. So, you have the

samples then fix the samples through permeabilization permeabilization. So, that the probe can enter inside. So, you have already probes you have two probes here one is green color and one red color. So, you hybridize them. So, the probes will enter into the cell and will find the ribosomal RNA to hybridize with them and following hybridization you give some treatment what is called washing.

So, washing will move all the unbound probe molecules because that otherwise will give you false positive results. So washing is also very critical in step following washing what you expect that some cells are not taking any color. So, they remain blue some cells are red they have taken the red probe or red probe rather could bind to the DNA present in them or the ribosomal RNA in this case or some are green.

Now you take this entire sample into a bifolus fluorescent microscope or a confocal microscope or a kind of a flow cytometry machine or a fax machine. So, that you can discriminate and see that how many cells are red how many cells are green and if you use a microscope like a confocal microscope you will be able to see the co localization pattern and the interaction between the multiple cells together.

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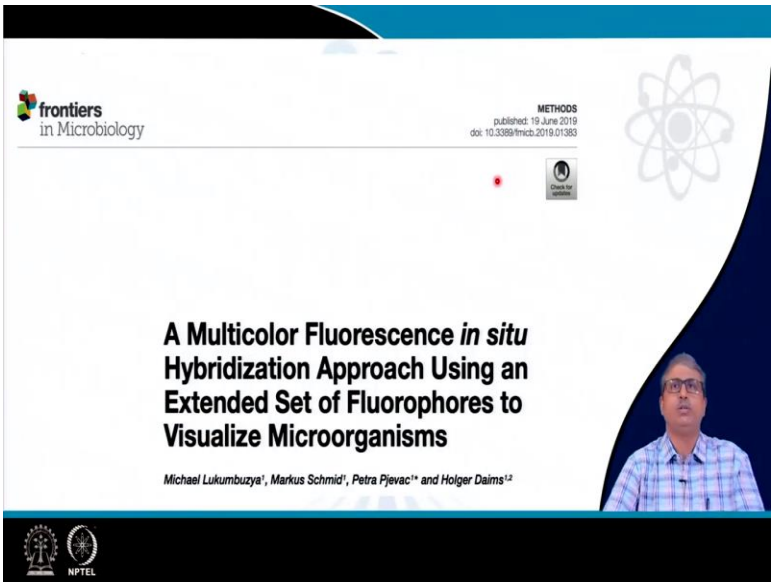

So, recently there is a very interesting method developed using the fish which is a multicolored fluorescent fluorescence insidious hybridization approach which is used to detect actually seven

or visualize seven different microorganisms.

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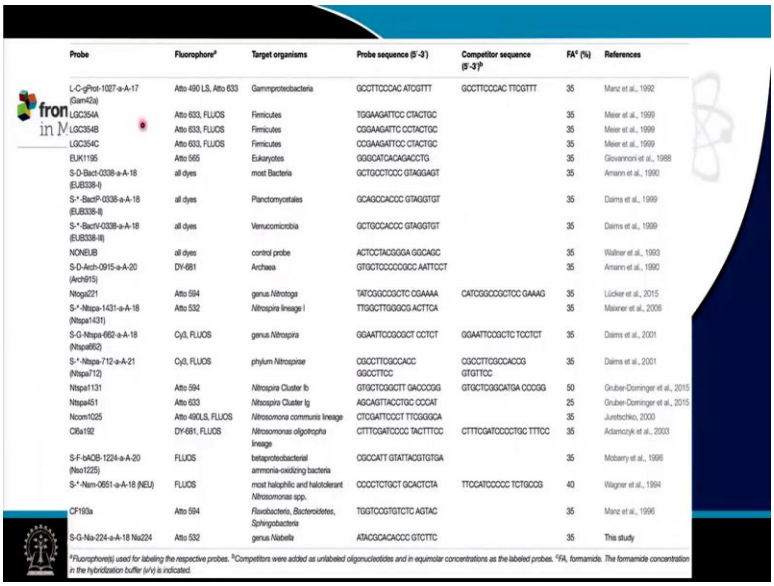

Probe	Fluorophore[a]	Target organisms	Probe sequence (5'-3')	Competitor sequence (5'-3')[b]	FA[c] (%)	References
L-C-gProt-1027-a-A-17 (Gam42a)	Atto 490 LS, Atto 633	Gammproteobacteria	GCCTTCCCAC ATCGTTT	GCCTTCCCAC TTCGTTT	35	Manz et al., 1992
LGC354A	Atto 633, FLUOS	Firmicutes	TGGAAGATTCC CTACTGC		35	Meier et al., 1999
LGC354B	Atto 633, FLUOS	Firmicutes	CGGAAGATTC CCTACTGC		35	Meier et al., 1999
LGC354C	Atto 633, FLUOS	Firmicutes	CCGAAGATTCC CTACTGC		35	Meier et al., 1999
EUK1195	Atto 565	Eukaryotes	GGGCATCACAGACCTG		35	Giovannoni et al., 1988
S-D-Bact-0338-a-A-18 (EUB338-I)	all dyes	most Bacteria	GCTGCCTCCC GTAGGAGT		35	Amann et al., 1990
S-*-BactP-0338-a-A-18 (EUB338-II)	all dyes	Planctomycetales	GCAGCCACCC GTAGGTGT		35	Daims et al., 1999
S-*-BactV-0338-a-A-18 (EUB338-III)	all dyes	Verrucomicrobia	GCTGCCACCC GTAGGTGT		35	Daims et al., 1999
NONEUB	all dyes	control probe	ACTCCTACGGGA GGCAGC		35	Wallner et al., 1993
S-D-Arch-0915-a-A-20 (Arch915)	DY-681	Archaea	GTGCTCCCCCGCC AATTCCT		35	Amann et al., 1990
Ntoga221	Atto 594	genus *Nitrotoga*	TATCGGCCGCTC CGAAAA	CATCGGCCGCTCC GAAAG	35	Lücker et al., 2015
S-*-Ntspa-1431-a-A-18 (Ntspa1431)	Atto 532	*Nitrospira* lineage I	TTGGCTTGGGCG ACTTCA		35	Maixner et al., 2006
S-G-Ntspa-662-a-A-18 (Ntspa662)	Cy3, FLUOS	genus *Nitrospira*	GGAATTCCGCGCT CCTCT	GGAATTCCGCTC TCCTCT	35	Daims et al., 2001
S-*-Ntspa-712-a-A-21 (Ntspa712)	Cy3, FLUOS	phylum *Nitrospirae*	CGCCTTCGCCACC GGCCTTCC	CGCCTTCGCCACCG GTGTTCC	35	Daims et al., 2001
Ntspa1131	Atto 594	*Nitrospira* Cluster Ib	GTGCTCGGCTT GACCCGG	GTGCTCGGCATGA CCCGG	50	Gruber-Dorninger et al., 2015
Ntspa451	Atto 633	*Nitrospira* Cluster Ig	AGCAGTTACCTGC CCCAT		25	Gruber-Dorninger et al., 2015
Ncom1025	Atto 490LS, FLUOS	*Nitrosomona communis* lineage	CTCGATTCCCT TTCGGGCA		35	Juretschko, 2000
Cfb192	DY-681, FLUOS	*Nitrosomonas oligotropha* lineage	CTTTCGATCCCC TACTTTCC	CTTTCGATCCCCTGC TTTCC	35	Adamczyk et al., 2003
S-F-bAOB-1224-a-A-20 (Nso1225)	FLUOS	betaproteobacterial ammonia-oxidizing bacteria	CGCCATT GTATTACGTGTGA		35	Mobarry et al., 1996
S-*-Nsm-0651-a-A-18 (NEU)	FLUOS	most halophilic and halotolerant *Nitrosomonas* spp.	CCCCTCTGCT GCACTCTA	TTCCATCCCCC TCTGCCG	40	Wagner et al., 1994
CF193a	Atto 594	*Flavobacteria*, *Bacteroidetes*, *Sphingobacteria*	TGGTCCGTGTCTC AGTAC		35	Manz et al., 1996
S-G-Nia-224-a-A-18 Nia224	Atto 532	genus *Nitrobella*	ATACGCACACCC GTCTTC		35	This study

[a]Fluorophore(s) used for labeling the respective probes. [b]Competitors were added as unlabeled oligonucleotides and in equimolar concentrations as the labeled probes. [c]FA, formamide. The formamide concentration in the hybridization buffer (v/v) is indicated.

So, here is a list of the different probes which are developed and maybe there are several separate database and separate literature are also available where extensive list of different probes because these probes are specific to different taxonomic groups like the sequences available and the type of target organisms are also there.

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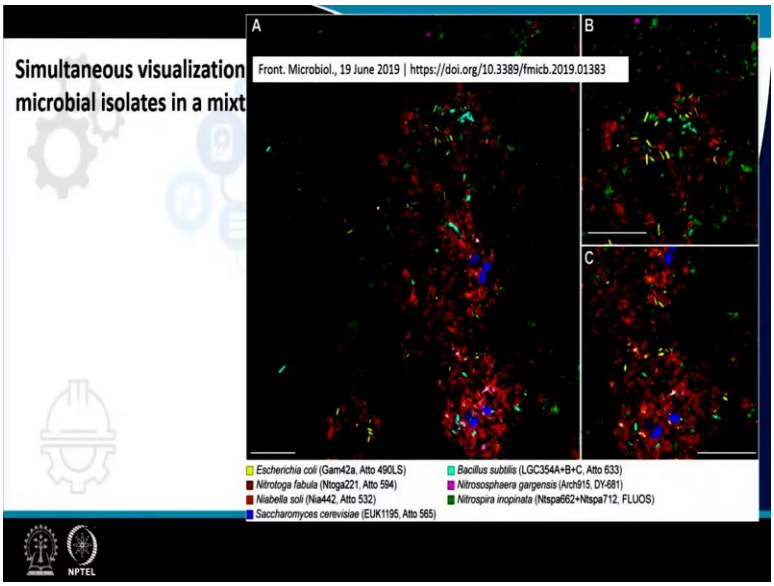

And then when we have the visualization we can see that seven different types of organisms are visible over here and according to the fluorescent dyes used you can you can follow this legend that for example the yellow color is for E.coli and the cyan color is for bacillus subtilis and so on

and so forth. So, you can actually zoom in. So, two parts are zoomed and to find out how the cells are there and how the cells are connected possibly connected.

Because from the co localization pattern like the yellow colour E. coli is you can see clearly the yellow is with the green and this the green is possibly from the nitro spider. So, there are there are a lot of information which could be obtained from this type of elegant technologies using the fish.

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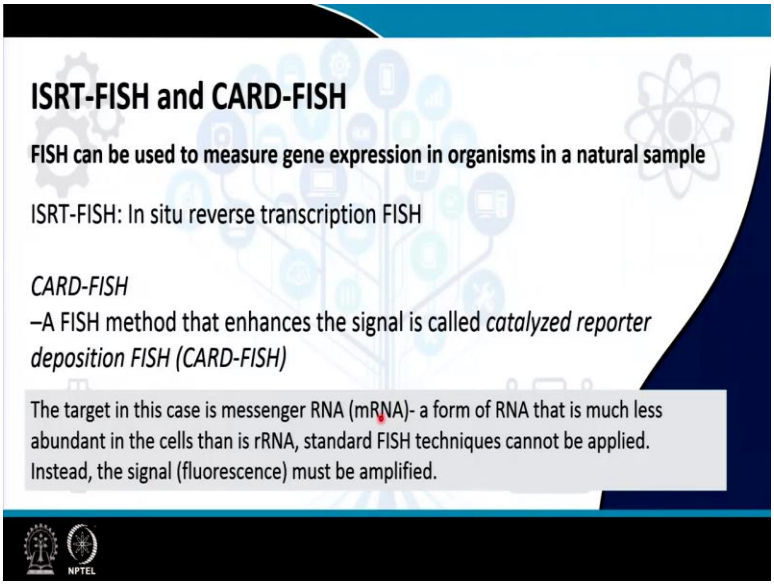

So, apart from this routine fish based method using a single probe or multiple probes capable of detecting multiple organisms. FISH can also be used to measure the gene expression in organisms in a natural sample two methods are found to be very relevant one is the ISRT or In Situ Reverse Transcription FISH and other is the card fish or the called catalyzed reporter deposition FISH or CARD FISH.

So, this CARD FISH method is very very effective found to be in order to enhance the signal which is there in in the cell. Now what we mean by enhancing the signal because the in many cases when we try to quantify the expression level of a particular gene or particular enzyme coding gene which are environmentally relevant the target in this case is a messenger RNA. So, it is the mRNA which is targeted and mRNA is a form of RNA that is much less abundant in the cell than the ribosomal RNA.

So, it is not a taxonomic delineation or taxonomic probe. So, the probes are targeted towards functional gene mRNAs. So, these mRNAs will be relatively lesser in number and based on the expression level of the particular gene they may be lower than others. So, there could be many mRNA but the mRNA for your interest of your gene of interest could be low. So, standard fish technique cannot be applied. So, instead this signal amplification kind of technique is used.

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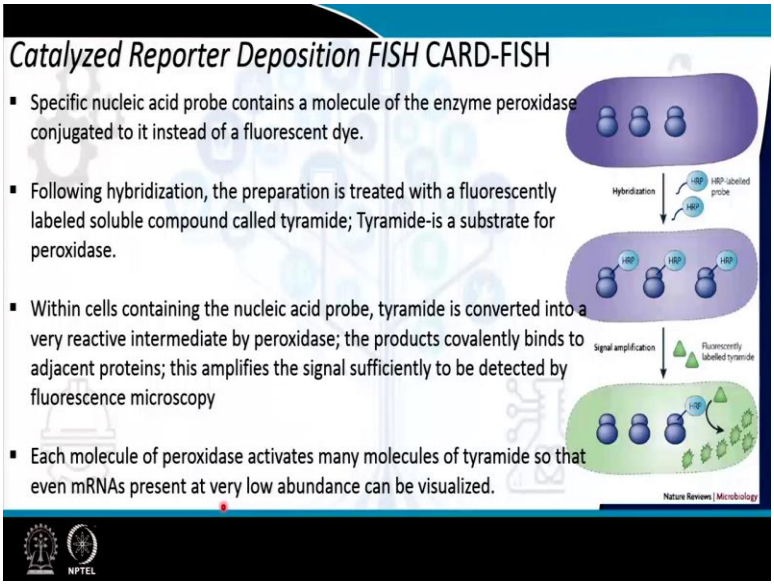

So, what we do here we use a probe but the probe is tagged to an enzyme which is a peroxidase enzyme. So, in this case is horseradish peroxidase. So, this is tagged with the probe instead of a fluorescent dye. So, fluorescent dye will come later. So, first this HRP coated or connected probe will be used and following hybridization the preparation is treated with the fluorescently labeled soluble labeled soluble compound called tyramide.

Now so first the hybridization is done with the probe containing the specific gene of interest sequence and tagged with the peroxidase enzyme. So, it will try to find out the complementarity through scanning the all the mRNAs present there and even if it is low in concentration this mRNA complementary probe will be able to bind because based on the hybridization condition which is set and once it is bound you allow the tyramide to be added there.

So, tyramide is a compound which will react with the peroxidase. So, rather peroxidase will react

with this thyramide and will produce a compound which is actually fluorescent. So, tyramide itself is not fluorescent but will produce a product which will be fluorescent and one peroxidase enzyme can keep on converting this dynamite to many many molecules of this fluorescence nature and these fluorescent molecules will bind to the adjacent a protein molecules and eventually the signal will be highly amplified now each molecule of peroxidase activates many molecule of tyramide.

So, that even mRNAs present at very low abundance can be visualized. So, you will be able to see if some cells are there who are expressing the gene of interest but the expression level may be low. So, even if it is very low you will be or you will be able to see the presence of that particular enzyme and in environmental biotechnology that gives us a big advantage because we are able to then confirm that the presence it is not merely present of a particular bacteria or particular archaea or fungi.

But it is the conformation it is coming that this organism or the process which is which is relevant for this particular event.

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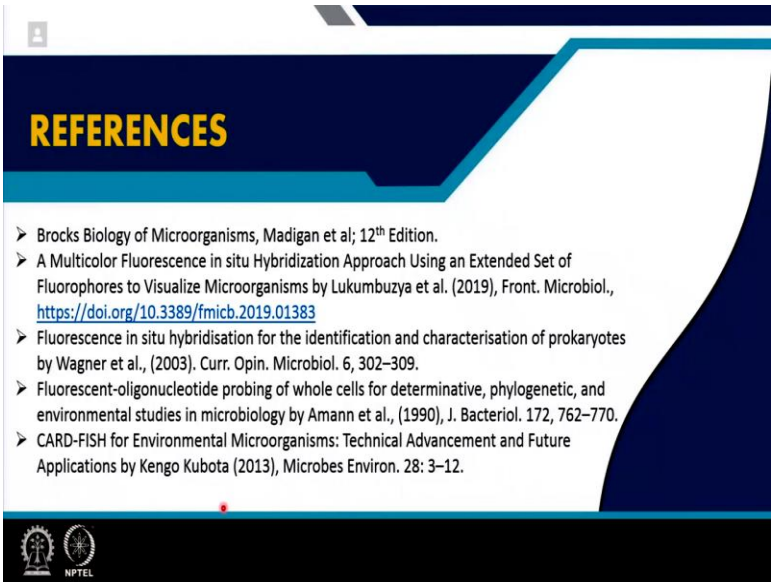

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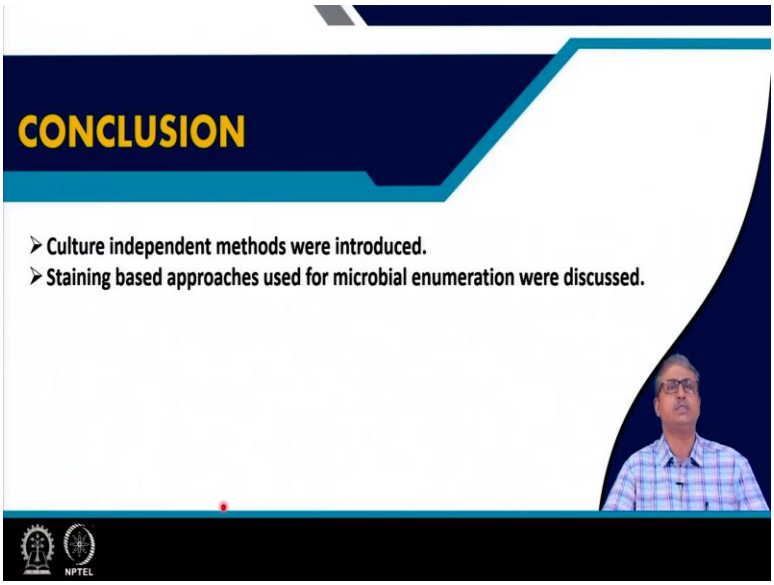

Now for this part of the lecture the following references will be helpful and in conclusion we just discuss about the brief scope of this environment environmental microbiology microbial community analysis through culture independent methods. And in particular we elaborated more on the different straining based approaches fluorescent dye based training based approach. Both the total count based method followed by the live debt detection method.

And then the advanced methods which are the fish and then FISH methods which are like CARD FISH which are capable of giving us information about the gene expression level even if when the when the copies of the mRNAs are or the expression levels are very low. So, with this we end this lecture and thank you so much.