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Detection of fish pathogens in freshwater aquaculture using eDNA methods: a review

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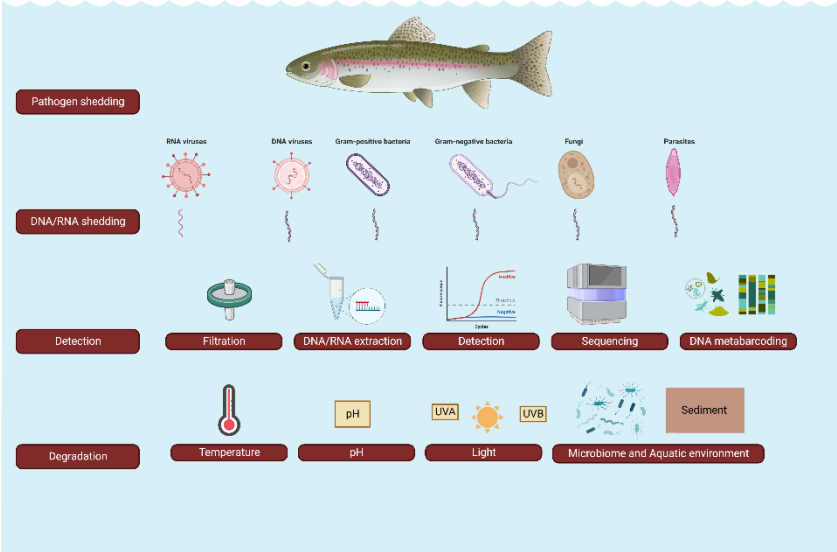
Abstract

Organisms release their nucleic acid in the environment including the DNA and RNA which can be used to detect their presence. eDNA/eRNA techniques are being used in different sectors to identify organisms from soil, water, air, and ice since long. The advancement in technology led to easier detection of different organisms without impacting the environment and the organism itself. These methods are being employed in different areas including surveillance, history, and conservation. eDNA and eRNA methods are being extensively used in aquaculture and fisheries setting to understand the presence of different fish species and pathogens in water. However, there are some challenges associated with the reliability of the results because of the degradation of nucleic acid by several factors. In aquaculture there are several diseases and parasites detected with these methods. In this review we discuss different aquaculture diseases and parasites detected with eDNA/eRNA approach and the fate of these nucleic acids when subjected to different water quality and environmental parameters. This review intends to help the researcher about the potential of eDNA/eRNA based detection of pathogens in aquaculture; this will be useful to predict the potential outbreak before it occurs. Along with that this paper intends to make people understand several factors that degrade and can hamper the detection of these nucleic acids.



Keywords : eDNA, eRNA, fish disease, surveillance, hydrolysis, degradation, qPCR

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Introduction

Environmental DNA (eDNA) analysis is a new scientific technique for identifying species from materials that contain cellular and extracellular DNA leached off all living organisms. The terminology for eDNA as extracellular DNA is noted by Pietramellara et al. 2008. However, researchers are using different terminology such as exDNA (extra cellular DNA) or cfDNA (cell-free DNA). The idea of obtaining DNA from the environmental sample was first demonstrated in 1986 by Ogram et al. 1987 and called environmental DNA (eDNA). The identification of various eDNA from macro-organisms validated the method as actually important in a conservation context, and it has been demonstrated in a wide range of ancient and modern habitats, both terrestrial and aquatic. (Haile et al. 2009; Epp et al. 2012; Bhadury et al. 2006; Andersen et al. 2012). Environmental DNA (eDNA) approaches are becoming more widely used in conservation biology, biodiversity research, and invasion ecology. One of the most significant benefits of eDNA sampling is the undemanding way of obtaining samples, as the target organism does not need to be isolated. The detection of parasites and diseases in water can also be done using environmental DNA methods. There is numerous evidence of the detection of several bacterial species from aquatic environments including *Aeromonas* and *Flavobacterium.*

DNA is leached in the aquatic environment by different means such as mucosal secretion, bodily fluids, tissues, scales, skin, microbial cells, and cell ruptures. This gives the researcher the potential to isolate DNA from the different water sources without impacting the aquatic habitat. eDNA is not only being extracted from water samples but it is also being extracted from different substrates including soil, snow, and air as well. The extensive study of eDNA has led historians to identify new species and detect the presence of endangered species. Environmental nucleic acid including eRNA for one of the recent infective SARS-CoV-2 has been successfully isolated from hospital air sampling (Led icky et al. 2020). The advances in diagnostic techniques and instruments are one of the biggest reasons behind the success of environmental nucleic acid detection. Direct detection in water utilizing eDNA-based approaches eliminates the need to acquire and investigate diseased hosts, reducing disease monitoring effort and costs dramatically. Eukaryotic micro- and macrobial communities and populations have been effectively detected and monitored using eDNA analysis. The advances of eDNA analysis have resulted in efficient identification and quantification of these extracellular nucleic acids in different mediums. DNA metabarcoding, quantitative PCR and

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digital droplet PCR are some of the methods being used (Figure 1.). In this review paper we intend

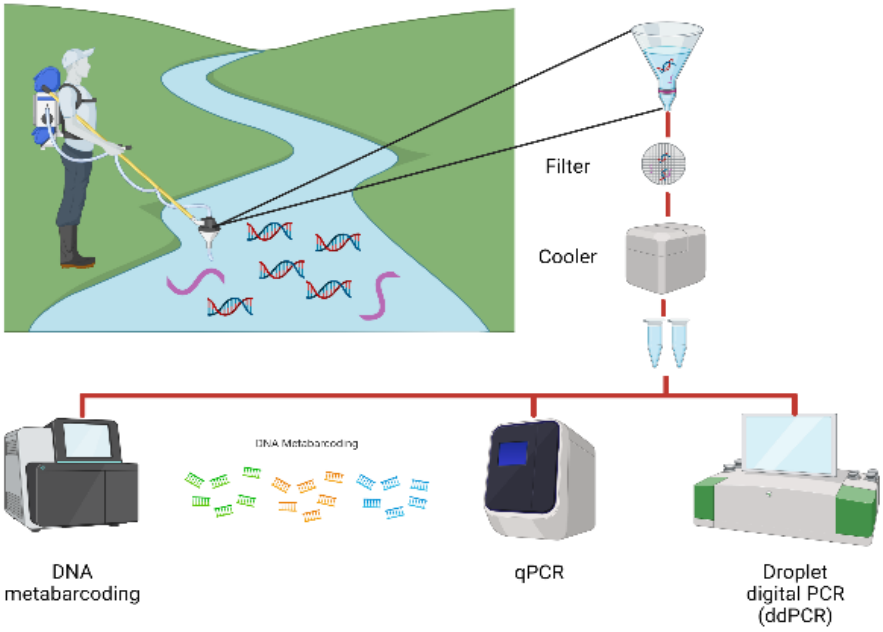

Figure 1:- Different methods of eDNA/eRNA quantification. qPCR, ddPCR, metabarcoding, RT-PCR are generally used to quantify the presence of different organisms from water samples.

to discuss the potential of eDNA in disease surveillance and fate of extracellular nucleic acids subjected to environmental conditions in water.

eDNA in fish disease

Bacteria

The ability of bacteria, archaea, and fungi cultures to release their genetic material into the extracellular medium has been reported, as well as in the context of multicellular microbial communities such as biofilms. Bacteria release their DNA in water by different methods including cell lysis and extrusion. The integrity of DNA released by cell lysis is usually more because the exonucleases cannot act fast to degrade the DNA. Extrusion is used as a survival strategy by certain bacteria such as Deinococcus radiodurans in which damaged DNA is released and new DNA is synthesized (Battista 1997). Many environmental bacteria including Micrococcus, Acinetobacter, Bacillus, Flavobacterium, Azotobacter, Pseudomonas, and Alcaligenes, release their genetic material while growing in the media (Paget and Simonet 1994; Lorenz and Wackernagel 1994). The amount of eDNA found depends on several factors such as temperature, salinity, turbidity, and vegetation. In freshwater systems the amount of DNA ranges from 1.74-7.77 µg/L (Deflaun et al. 1986). There are many fish bacterial diseases affecting freshwater aquaculture causing huge economic loss to the farmers. eDNA technique might help them to predict bacterial load in their farms. There are several research studies being carried out to find an efficient method to detect those pathogens directly from the water samples. In most cases of *Flavobacterium columnare* infection, a gram-negative bacterium affecting different fish species is found only externally in the skin, gills, and water samples before being systemic. Early and rigorous *F. columnare* diagnosis, as well as the implementation of practical preventive measures, are the only credible means of

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disease control. *F. psychrophilum* was found in different river water samples in Japan. They found a higher presence of *F. psychrophilum* during early summer and fall and the presence of this bacteria depends on the water temperature (Tenma et al. 2021). In addition to that *F. psychrophilum* and *Yersinia ruckeri* were also detected in the water of the salmon recirculatory aquaculture system (RAS). Similarly, 7 distinct species of *Aeromonas* were confirmed from coastal zones of the river basin in Bangladesh. Over the 2-year study period, they also found that the amount of *Aeromonas* changes with change in temperature using the eDNA method (Sadique et al. 2021). There are still many bacterial pathogens that are responsible for the loss which are yet to be studied.

Fungi

Fungi are one of the common fish pathogens in aquaculture settings. The most common fungal disease that affects fish species is saprolegniasis, branchiomycosis, and aspergillosis. Because farmed animals are typically held in high densities and exposed to constant stress and various types of pollutants, the risk of infection and disease spread is higher in fish farms than in wild environments. There are only a few studies on fish fungal disease identification using the eDNA method in aquaculture. Following high mortality outbreaks in the river Loue, for finding S. parasitica in water, a qPCR assay was designed. The pathogen was detected in river water but not in the tap water of surrounding villages. (Rochhi et al. 2017). There are other fungi identified by this approach from water that affects amphibians. *Batrachochytrium dendrobatidis* and *B. salamandrivorans*, two major fungal diseases of amphibians were found in water samples in Spain using a qPCR assay (González et al. 2021). DNA released from fungus cells has received less attention than DNA released from bacterial cells. Although fungi consist of more than 70% microbiome in soil due to the fast rate of DNA degradation in dead fungal cells, the contribution of fungal DNA to the eDNA pool in soil should be insignificant. The fungus can spread to ponds and rivers via rainwater flow and water infiltration. Adequate and efficient methods to detect the presence of these fungal pathogens using eDNA will allow the farmer to predict the fungal disease outbreak leading to timely management and control strategies.

Parasites

Environmental DNA (eDNA) sampling methods in conjunction with different molecular methods, is well suited to quickly detect presence of pathogens in different fish farms which helps the managers with valuable information that can be used to reduce the disease threats. Parasites are the most common group of fish pathogens that are being detected easily using eDNA method. Standard fish parasite surveillance entails capturing and euthanizing fish before manually inspecting for the presence of parasites. Using this conventional method is both expensive and time-consuming, and it necessitates the sacrifice of many fish species. eDNA/eRNA fragments of several species in water samples has recently been established as an accurate low-cost alternative in addition to the traditional monitoring techniques which require sampling the fish itself. Rusch et al. (2018) developed a ddPCR assay to detect eDNA in field samples, demonstrating the utility of eDNA detection in natural water systems for G. salaris. eDNA of Dactylogyrus species was detected in a consignment of ornamental fish water and confirmed by sanger sequencing. Although there are some limitations regarding the use of eDNA tool as a biosecurity and quarantine method. It detects eDNA from water and not directly from fish and this might create a false positive even though the fish might not have the targeted parasite, but the assay can show positive because of the source water used (Gonzalez et al. 2019). Chilodonella abundance was detected at varying levels across the year in the barramundi fish

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farm monitored in the study (Gomes et al. 2017). Jousson et al. 2005 developed an assay that can detect low concentrations of the parasite in tank water containing goldfish, presumably corresponding to an early stage of the disease. As a result, it could be a useful tool for monitoring and controlling ichthyophthiriasis in aquaculture.

Virus

There are mainly two different forms of virus that infect fish species which are either DNA virus or the RNA virus. Several studies have shown that DNA and RNA from virus can be detected using eDNA or eRNA method (Minamoto et al. 2009; Haramoto et al. 2007; Kawato et al. 2021; Taengphu et al. 2021; Vilaca et al. 2020; Miaud et al. 2019; Weli et al. 2021; Bernhardt et al. 2020). Since DNA is more stable than RNA, detection of eDNA is more practical and easier than detecting RNA from an environmental source. There are many common forms of virus that are found in freshwater aquaculture including Herpesvirus, Viral hemorrhagic septicemia virus (VHSV), Infectious hematopoietic necrosis virus (IHNV), Golden shiner virus (GSV), Channel catfish virus (CCV), Red seabream virus, tilapia tilapine virus, and salmon alphavirus.

Disease identified by eDNA

Table 1:- Different fish diseases identified by eDNA/eRNA based detection system. The different environment the samples collected, and methods used to quantify are listed in the table.

Disease	Environment	Method	References
Virus			
Cyprinus Herpes Virus (CyHV-3)	Lake, Pond, River	PCR, Real time PCR	Minamoto et al. 2009; Haramoto et al. 2007; Honjo et al. 2012
Red seabream virus	Fish Farm (Seawater)	DNA metabarcoding	Kawato et al. 2021
Tilapia tilapinevirus	Pond water	Probe based RT-qPCR	Taengphu et al. 2021
Rana Virus	Lakes, Ponds	qPCR	Vilaca et al. 2020; Miaud et al. 2019;
Salmon aplhavirus	Seawater	RT-qPCR, RT-ddPCR	Weli et al. 2021; Bernhardt et al. 2020
Parasite			
Gyrodactylus salaris		qPCR, ddPCR	Rusch et al. 2018; Fossoy et al. 2020
Dactylogyrus spp.	Shipment water	qPCR	Trujillo-Gonzalez et al. 2019b
Chilodonella hexasticha	Pond water	qPCR	Bastos Gomes et al. 2017,2019
Ichthyophthirius multifiliis	Tank water	Real time PCR	Jousson et al. 2005
Myxobolus cerebralis	River water	Multiplex qPCR	Richey et al. 2018

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Ceratonova shasta	River water	qPCR	Richey et al. 2020
Parvicapsula minibicornis	River system	Real time PCR	Hallett and Bartholomew 2009
Tetracapsuloides bryosalmonae	River water	qPCR	Carraro et al. 2017,2018; Hutchins et al. 2018
Neoparamoeba perurans	Sea water	Real time PCR	Bridle et al. 2010
Schistosoma mansoni	Tank water, water bodies	qPCR	Alzaylaee et al. 2020
Fungi			
Saprolegnia parasitica	River water	Real time qPCR	Rochhi et al. 2017
Bacteria			
Aeromonas sp.	River, Pond	Real time PCR	Sadique et al. 2021; Fong et al. 2016
Flavobacterium psychrophilum	River, RAS	Real time PCR, ddPCR	Tenma et al. 2021; Lewin et al. 2020
Yersinia ruckeri	RAS	ddPCR	Lewin et al. 2020

eDNA production and degradation in water

The link between the production and its degradation of eDNA/eRNA is crucial for its detection and measurement (Thomsen, et al., 2012). When epithelial cells are shed or sloughed off through movement, excretion, and secretion eDNA/eRNA is released in the environment (Pilliod et al., 2014). Several studies have shown that physiological stress along with the size and number of individuals affects the DNA production rate (Takahara et al., 2012; Thompsen et al., 2012; Maruyama et al., 2014; Pilliod et al., 2014). There is evidence of both intra- and inter-specific heterogeneity in the creation of eDNA/eRNA, highlighting a need to better understand the process of eDNA/eRNA generation and degradation in different species and systems (Thomsen, et al., 2012; Maruyama, et al., 2014).

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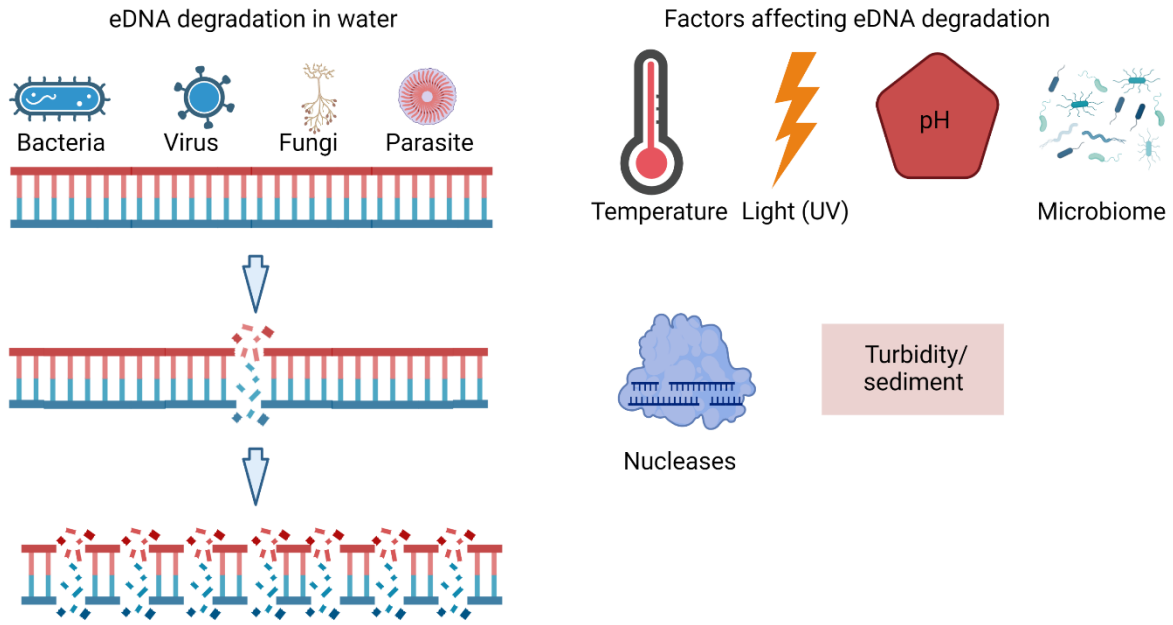

Figure 2:- Different factors affecting the nucleic acid degradation in aquatic environment. Nucleic acids are affected by temperature, light, p^H and the environment they are present in.

A range of factors, including light, temperature, enzymatic activity, and pH, impact the breakdown eDNA (Figure 2.). Addressing eDNA detection in an aquatic environment requires a full understanding of these factors' interactions and the effects they have on eDNA stability. Hence, Barnes et al. (2014) divided the factors influencing DNA persistence into three groups: the DNA molecule's properties, abiotic factors (light, substrates, pH, oxygen, salinity), and biotic factors (microbes and enzymes). The length, conformations, and whether a DNA molecule is membrane-enclosed, or free (also known as "naked") DNA are all properties of the DNA molecule that affect how quickly DNA breaks down in the environment (Romanowski et al., 1992; Alvarez et al., 1996; Zhu, 2006; Barnes, et al., 2014). Biotic and abiotic factors affecting DNA degradation in an aquatic environment are discussed below.

eDNA persistence-related factors in freshwater systems

Temperature

DNA deterioration in water occurs more quickly than in soil and sediments, possibly because of increased enzymatic and microbial activity at higher temperatures (Zhu, 2006). According to Matsui et al. (2001) who investigated the fate of dissolved DNA in a thermally stratified lake, DNA in the warmer epilimnion (upper layer) destroyed completely in 170 hours while degrading more slowly in the much cooler hypolimnion (lower layer). In contrast to samples exposed to the full sun for 18 days and those exposed to 20%shade for 18 days,

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respectively, Idaho giant salamander eDNA stored at 4°C and no light after 18 days contained 2030 and 733 times more eDNA (Pilliod et al., 2014). Similarly, the experiment carried out by Zulkefil et al. (2019) found higher degradation of eDNA at 35 ºC compared to the control at 5 ºC which was about 60% from the initial concentration. Likewise, the experiment carried out by Tsuji et al. (2017) to know about the eDNA degradation of two species i.e., ayu sweetfish (*Plecoglossus altivelis altivelis*) and common carp (*Cyprinus carpio*) at seven-time points, over a 48-h period, and at three different water temperatures (10 °C, 20°C, 30°C) found higher degradation at 30 °C. Some studies have found no effect of temperature on eDNA shedding rate (klymus et al., 2015; Takahara et al., 2016). Contrarily, Robson et al. (2016) discovered that Mozambique tilapia's DNA shedding rate was dramatically increased by tropical temperatures (23, 29, and 35 °C) (Oreochromis mossambicus). Inconsistencies in the research' findings could point to variations in eDNA production that are species-specific. Higher temperature often leads to degradation by double strand break of the DNA (Figure 3a).

Ultraviolet light (UV)

Since the stratospheric ozone layer is being destroyed, UV-B radiation can enter the water column and cause the destruction of eDNA by rupturing DNA base-pair bonds Figure 3b (Hader and Sinha. 2005). Contradictory findings have been found in studies that have investigated how UV affects eDNA degradation. Pilliod, et al. (2014) showed that after eight days in full sun exposure, eDNA was no longer detectable, however, after 11 days in partial shade and after 18 days in complete darkness, eDNA was still detectable in all samples. Given that eDNA decayed exponentially even in the absence of light and that temperature accounted for the bulk of the observed variation in eDNA degradation among samples, temperature may have a bigger effect on DNA degradation than light (Pilliod, et al., 2014). However, the study conducted by Zulkefli et al. (2019) with various levels (20, 50, and 100 µmol $m^{-2}s^{-1}$) of solar radiation had no observable effect on the degradation rate of eDNA. Interestingly, the same study indicated that UV light, regardless of whether it is UVA or UVB radiation, had no impact on the ability to identify DNA (Zulkefli et al., 2019; Machler et al., 2018). The notable significant differences were seen by co-varying light intensity and temperature (35ºC) at the end of the experiment compared to 5ºC treatment (Zulkefli et al., 2019).

Table 2:- Comparision of eDNA dacay rates among various types of eDNA, sources, and environmental factors. * *Indicates the significant effect on eDNA degradation in the corresponding study. (Table adopted from Zulkefli et al., 2019)*

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eDNA Type	Source	Environmental Factor	Decay Rate, (r) (day $^{-1}$)	Reference
Extracellular	Sediment sample Cyanobacterium *Anabaena variabilis*	Temperature*, microbial activity*, pH, light intensity	0.0931-3.2706	Zulkefli et al., 2019
Intracellular	Crustacean *Daphnia magna*	pH *, temperature, microbial activity, total dissolved nitrogen	Water derived 6.552–23.568	Seymour et al., 2018
	May fly *Ephemera Danica*		Biofilm derived 1.176–17.256	
	Eel *Anguila anguilla*			
Intracellular	Ayu sweetfish *Plecoglossus altivelis altivelis*	Temperature *, microbial abundance	0.48-7.2	Tsuji et al., 2017
	Common carp *Cyprinus carpio*			
Intracellular	Common carp *Cyprinus carpio*	Temperature*, trophic state*	0.35-2.42	Eichmiller et al., 2016
Intracellular	American bullfrog *Lithobates catesbeianus*	UV-B*, temperature*, pH	0.243	Strickler et al., 2015
Intracellular	Common carp *Cyprinus carpio*	Microbial community*, pH	2.52	Brnes et al., 2014
Extracellular	Sediment and water samples	Based on simplified OECD endurance test	0.009-0.133	Mao et al., 2014

pH

DNA hydrolysis is favored in acidic environments (Lindahl. 1993; Gates. 2009). In contrast, Stickler et al. (2015) noted that the increased rate of eDNA degradation at pH 4 was due to interactions with other factors and that the pH level itself had no impact on the degradation. In stream mesocosms with an acid-base gradient, the degradation rate of lotic multispecies eDNA was accelerated to undetectable levels in just two days (Seymour et al., 2018). Low pH will result in denaturing of the two strands of DNA while higher pH leads to the degradation of hydrogen bonds and separation of nitrogen bases in DNA (Figure 3c).

Environmental parameters

The consistent detection of eDNA in the aquatic environment depends on the flow rate (Ficetola et al., 2008; Nathan et al., 2015), solid materials, and dissolved substances in the water column and riverbed (Rees et al., 2014; Shogren et al., 2017). It is believed that the properties of sediments (suspended or benthic) might have an influence on eDNA degradation because sediments might adsorb DNA (Stewart et al., 1991; Lorenz et al., 1987), thus reducing the eDNA

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detection rates. However, eDNA can re-suspend from sediment (Graf et al., 1997), which can result in false positives or the identification of a species that is no longer present in the environment (Stoeckle et al., 2016). Additionally, dissolved materials in the water matrix can affect how quickly DNA is detected and potentially PCRs, such as humic acids (Albers et al., 2013). In this regard, an experiment conducted on environmental conditions on eDNA success in aquatic ecosystems suggested that the presence of sediments is responsible for lower eDNA detection in water samples regardless of flow-through or still water conditions. This was followed by the delayed release of eDNA in the presence of sediment. Additionally, humic substances had a higher inhibitory effect on eDNA detection followed by algae and siliceous sediment particles (Stoeckle et al., 2017). This study mentioned that application of eDNA methods in field survey conditions strongly depends on site-specific conditions like water flow conditions, sediment composition, and suspended particles.

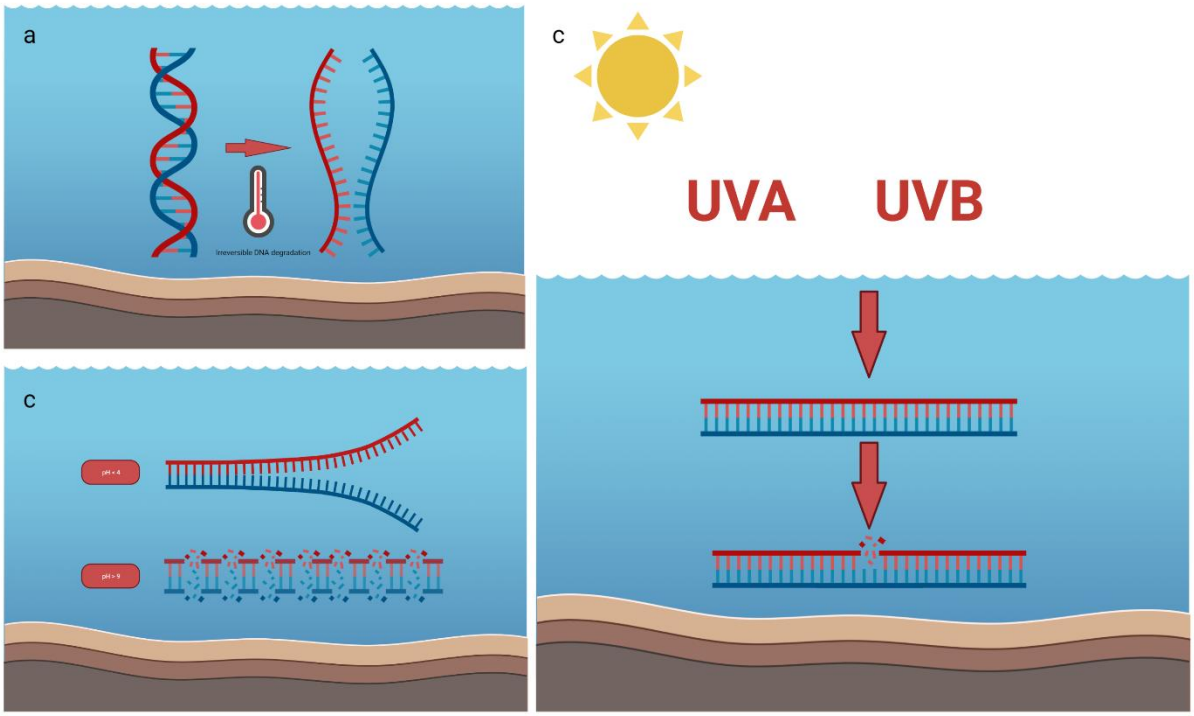

Figure 3:- Effect of temperature, light and P^H on DNA degradation process.

eDNA/eRNA have immense potential for disease risk monitoring, as they can improve our ability to determine the existence, diversity, and quantity of pathogenic organisms. Since traditional pathogen detection techniques frequently entail cultivating or necropsying host tissues, they are equally as resource intensive as those used to detect free-living pathogens. Detecting pathogens and parasites beforehand is a crucial step in aquaculture. Fish kills due to disease outbreak are common in aquaculture all around the world from warmwater to cold-water aquaculture systems. Management can be initiated to prevent the spread of disease and potential treatment of water timely. Parasites are often considered less significant in freshwater aquaculture, but they can decrease the final value of the product. In addition to that, there are several cases of co-infection with bacteria and viruses led by parasite infection (Xu et al. 2007; Zhang et al. 2015; Ogut et al. 2014). Certain challenges like accuracy, efficiency, and fate of eDNA/eRNA are of concern despite the efforts from many researchers around the globe.

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Sengupta et al. 2019 used eDNA method for detection of cercaria, one of the major parasites that affects both aquatic and human species. They used both field-based models and lab-based models to effectively detect the presence of this parasite. However, there are limitations regarding the methods such as the rate of decay and the life stage of the parasite identified using this technique cannot be determined. Thus, to increase the effectiveness of this method experiments and research need to be carried out at different conditions.
Our review provides comprehensive advances and detection of freshwater pathogens and parasites using eDNA techniques. In addition to that, our study reviews the effect of different environmental factors on eDNA degradation. The rate of degradation of DNA and RNA is different in marine environment than in freshwater system. The half-life of eDNA is found to be in a range of 7 h to 72h in marine water (Collins et al. 2018) which is faster than the freshwater system (Thomsen et al. 2012; Sassoubre et al. 2016). The degradation rate also varies with terrestrial environment and different seasons (Collins et al. 2018). Aside from abiotic factors such as oxidation and hydrolysis by depurination, biotic factors such as extracellular DNases produced by heterotrophic microbes are also likely to play a significant role in eDNA persistence dynamics (Barnes et al. 2016; Torti et al. 2015). eDNA analysis is changing the way we design and implement biodiversity monitoring programs, opening new opportunities for the future. This tool has a high potential for monitoring aquatic biodiversity including pathogens and parasites.

Author Contribution

All authors contributed equally to the manuscript. Conceptualization, K.B, A.K.Y; Writing-Original draft preparation, K.B, A.K.Y, P.J; Writing- Review and editing, A.Y.

Conflicts of Interest

Th authors declare no conflict of interest.

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