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AQUATIC DNA CHRONICLES: EXTRACTION TO GENO-TOXICITY AND COMET ASSAYS

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Abstract
Aquatic habitats are filled with different types of species and all of them are vulnerable to pollution through pollutants hence in the recent past, heavy metal pollutants have been dangerous to aquatic lives. This study concentrates on isolating pure DNA from fish tissues and identification of genotoxicity by conducting the comet assay and PCR test based on the p53 gene. Since fish is a critical link in aquatic ecosystems it acts as a biomarker for the environment. In this study, fin and gill tissues were chosen for DNA extraction, and the optimized protocol provided high-purity DNA for further analysis. The comet testing revealed that the fish exposed to environmental toxins had greater quantities of DNA damage, notably illustrated by the longer comet tails. The current study further sought to confirm the specificity of established p53 gene primers for PCR and their potential use in genotoxic analyses. Further, statistical analyses were employed to verify the credibility and the importance of the findings. Thus, this work illustrates how DNA extraction, comet assays, and PCR analysis should be integrated to evaluate the effects of pollutants on water inhabitants. The study emphasizes the necessity of imposing strict environmental laws and regulating pollution in water to save water life. Further investigations should extend these methodologies to fish species and other contaminants to gain more insights into the problem and effects of environmental genotoxicity.
Keywords: Fish, Heavy Metals, Genotoxicity, DNA Extraction, Comet Assay, PCR Analysis

Introduction
Aquatic animals are a class of animals that are adapted to inhabit water environments such as seas, oceans, and rivers. Aquatic animals can be classified into two main categories, i.e. Marine Animals and Fresh Water Animals (Arthington *et al*., 2016). Exploring the Aqua Kingdom, one of the most fascinating groups of vertebrate species, includes fish, which belong to Phylum

Chordata. According to Fish Base at present, it consists round about 35,600 species globally and 748 species in Pakistan. In the context of study and analysis, fishes are of interest to humans because they provide an elaborate and complex insight into evolution, biological environment, and ecological adaptability. These creatures, with their shapes and functions, reflect the richness and variety of underwater life (Oka and Umatani, 2023). Fish has a significant role in contributing to health as it is low-fat quality protein. It contains essential nutrients such as long-chain Omega 3 Fatty Acids, Vitamins A, B, and D, Trace Elements such as Zn, Fe, and Se, and Amino Acids such as Lysine and Aspartic Acid (FAO, 2021). According to the reports of FAO (2023), the global average per-capita consumption of fish is approximately 20.5 Kg per year. This has occurred due to a greater awareness of fish's numerous health benefits. As far Pakistan's per capita fish consumption rate is modest compared to the average global consumption rate. Reduced consumption of fish could be attributed to factors such as cultural practices, availability of funds, and geographical location where fish may not be easily accessed.

As the population of the world is increasing day to day the demand for food is rising globally with the expected pollution of ~10 billion people up to 2050 fish will have a great impact on economics as it offers protein and nutrients for people all over the world. Around 58.27 million people worldwide depend directly on captured fish, while 18.86 million are engaged in fish farming. The fish production in 2012 was 180 million of which 11.6 million tons were received from inland and 79.7 million tons from marine while fish production through aquaculture in 2012 was 90.4 million tons. Globally over time, the fisheries sector is promoting various opportunities and self-employment where Asia includes 21 million fishermen and 18.9 million fish farmers. Still, in contrast to other Asian countries, Pakistan's contribution to the fisheries industry has been gradually declining due to various variables. Pakistan has 1,120 kilometers of coastline and interior water reserves of 3,102,408 hectares. Pakistan's major fish harbors handle 90% of the country's fish and seafood catch, with 95% of exports originating from it (Babar *et al*., 2018).

Since fish are interacting organisms within the aquatic system, they are likely to suffer from the impacts of waterborne pollution which is the process where human beings introduce a substance that modifies the chemical or physical properties of water in a way that brings harm to the welfare of living organisms that inhabit in the water bodies and also to those who consume from those water bodies. Almost 65,000 industrial chemicals are currently in circulation and 3-5 new chemicals are introduced in the market daily. Luckily, only a small fraction of those chemicals goes to the water bodies, but the potentiality is huge. Chemical pollution affects health directly by the toxic substances in drinking water and indirectly through the bioaccumulation of toxic substances in the organisms that people consume (Heath 2018). The major classes of toxic chemicals that are dangerous to fish include Natural processes like Geological Weathering of rocks, Industrial Waste from factories, Pesticides, Fertilizers, and Mining Activities can emit heavy metals into water bodies defining them as the main source of pollution. Volcanic Eruptions can bring metals such as mercury and arsenic into the water habitat (Arthington *et al*., 2016).

Heavy metals enter the fish body directly through gills and skin covering and also indirectly through the food chain. These metals tend to build up in their tissues and can reach toxic concentrations at certain age stages (Agbugui *et al*., 2022). Some of the main predators on the seas and oceans such as big fish and the human can be at the risk of getting to toxic levels. It is indeed important to recognize these effects for the sake of the environment and humanity's utilization of food and other requirements (Chatha *et al*., 2023).

The physiological effects of Heavy metals on Fish are; heavy metals affect the immune system of fish which is then capable of getting infected easily. Heavy metals cause disruptions to the fish's nervous system hence altering their behavior and intellect. Elements including cadmium and lead exert severe toxicity to the organ to the extent of damaging the liver, kidneys, and gills harming any form of function in the body (Agbugui *et al*., 2022). Heavy metals decrease reproductive fitness and high mortality rates may cause a decline in fish stocks leading to imbalances in ecosystems. Hence, changes in fish affect the food chain and species distribution. Water pollution makes water a less suitable environment for some organisms and thus affects bio-diversity and the value of services that the ecosystem offers. In Japan, mercury was discharged industrially, got into the aquatic systems, and affected fish. Their consumption resulted in serious neurological illnesses in men, underlining the threat of heavy metal profligacy (Al-Sulaiti *et al*. 2022). In the areas where industrial activities are high, the rivers are laden with lead which poses risks to fish, and people are advised not to consume fish from these rivers. The cadmium from agricultural runoff has stimulated water pollution, especially in freshwater bodies, which has accumulated in fish fitting the appropriate habitat and therefore affected fisheries (Agbugui *et al*., 2022).

Geno Toxicity is a scientific concept used for adverse effects of undesirable substances such as heavy metals which results in mutation in the DNA present within cells and cancer, among other diseases. In aquatic ecosystems, fish species are more susceptible to genotoxicity since they are continually exposed to the genotoxic agents in the water. Some of the metals used in industries include mercury, lead, cadmium, and arsenic which are known to cause genetic mutations. Both agricultural and industrial chemicals are also capable of putting genotoxic compounds in water habitats. There are several methods to employ Genotoxicity analysis which is the identification of genotoxic agents and the degree of genotoxicity present in organisms. These include Chromosomal Aberrations which can be a light microscopic analysis of fish cells to reveal changes in the structure of the chromosomes, for instance, breaks, gaps, and arrangement, as an indication of genotoxicity. Micronucleus Test determines the presence of micronuclei in red blood cells or any other small bodies located outside of the nucleus that appear due to fragmented chromosomes and those that did not enter the nucleus after cell division. Polymerase Chain Reaction (PCR) and DNA sequencing are among the molecular methods that can assess genotoxicity effects since they identify specific mutations and differences in the genetic material (Lovell and Omori, 2008).

In the technical world of molecular biology and environmental science, DNA isolation is the foundation of investigation that reveals the genetic code facts and genotoxicity assays are the foundation of investigations that assess the effects of stressors on aquatic organisms. Out of thousands of aquatic species existing within our world, fish act as symbolic representatives that assist in determining the state of an ecosystem and the biological consequences of pollution. In this regard, the endeavor to obtain good quality DNA from the fish tissues and the ability to assess the DNA damage using the comet assay are both a science and a requirement for the conservation of species and checking on the sustainability of the fish resources in the world (Lovell and Omori, 2008). To sum up, it is crucial to obtain high-quality DNA from fish tissues and analyze its integrity using methods such as the comet assay to move forward in identifying fish biology and ecology. These methodologies not only help in genetic and evolutionary analysis but are also useful in measuring the effects of pollutants on aquatic species. While focusing on the details of DNA extraction from fish tissues and the comet assay in fish gills, this study aims to highlight the progress, concerns, and relevance of the approaches in present-day studies and investigations.

Methods of DNA extraction from fish tissues include; The extraction of DNA from fish tissues is associated with numerous difficulties because of the biochemical peculiarities of the tissues to be analyzed like fins, gills, and muscles. The barriers are as follows: Each tissue type poses challenges due to compositions like polysaccharides, proteins, and lipids that obstruct DNA quality and quantity. Picking the type of tissue for any DNA extraction exercise is based on several parameters including the ease and difficulty in sample collection, meddling of the sample collection method, and the quality and quantity of the DNA that is expected to be isolated. Fins and gills are more commonly used for non-lethal sampling methods while muscles provide a higher DNA concentration but require a more violent biopsy technique (Fukushima *et al*., 1997).

Classic DNA extraction techniques, including phenol-chloroform extraction, can be considered reference methods for effectively isolating DNA of high quality. However, these methods are tiresome and they require the use of rather dangerous chemicals (Sambrook and Russell, 2001). Thus, there are some more effective approaches aimed at the reduction of the overall time and possible safety hazards. Another method that involves the use of silica membranes where DNA binds in the presence of chaotropic salts can be faster and safer (Boom *et al*., 1990). Furthermore, magnetic bead-based methods also offer a novel technique in high-throughput DNA extraction especially suitable in genetic studies involving a large number of subjects (Hawkins *et al*., 1994).

In addition to DNA extraction, evaluation of the extent of DNA damage is also relevant, especially regarding environmental mutagenicity. Comet assay is a sensitive method to visualize DNA damage at the single cell level. This involves growing cells within an agarose matrix, lysing them to obtain DNA, and then applying an electric field that separates the DNA fragments in a comet-like tail. The amount of DNA migration increases with the degree of damage, which makes the comet assay an effective method for determining the effect of pollutants in fish (Singh *et al*., 1988).

Comet Assay is one of the most sensitive and common methods used for Genotoxicity. Which is also known as Single Cell Gel Electrophoresis (SCGE). The gills of fish are very sensitive organs to waterborne contaminants and are suitable for genotoxicity studies that employ the comet assay. Analytical works applying this technique have demonstrated that heavy metals, pesticides, and industrial effluents have genotoxic impacts on fish DNA that are important for environmental screening and policy making (Jha, 2008).

As Aquatic environments are the ultimate sink for pollutants, which results in anthropogenic contaminations not only damage DNA but induce cellular changes. These Heavy metals introduce error-prone Double Stranded Break (DSB), which cause mutation in DNA and increase the chances of Cancer (Morales *et al*., 2016). Several approaches, with a variety of effectiveness, have been developed for handling this challenge, including the SF2 clonogenic survival assay, the tumor's potential doubling time (Tpot), and tumor hypoxia evaluated by pO2. With this in mind, we employed the comet-FISH technique to simultaneously probe two gene regions, enabling us to examine DNA damage and repair in distinct gene regions of the same cell. We targeted the p53 and hTERT gene loci because they are known to have varied transcriptional activity and hence should show differences in DNA repair effectiveness in our assay (McKenna *et al*. 2012).

This study focuses on the methods for DNA extraction from fish tissues, comet assay on fish gills, and PCR analysis on DNA repair, discussing innovation and perspectives, as well as future

trends and limitations of such approaches. Thus, by analyzing and critically evaluating current literature and knowledge of the subject, this study intends to contribute a better understanding of these three crucial strategies for protecting aquatic life.

Material and Methods

The Fish fins and gills were provided by MPhil student at the Institute of Biotechnology and Genetic Engineering, University of Agriculture, Peshawar. The experiment of the isolation of DNA, Comet Assay, and PCR analysis were also performed in the Animal Biotechnology lab at the Institute of Biotechnology and Genetic Engineering.

Materials for Isolation of DNA from Fish Fins and Gills:

- Fish fins and gills samples
- Phosphate-buffered saline (PBS)
- Proteinase K
- Lysis buffer (containing SDS and EDTA)
- Phenol: Chloroform: Alcohol (25:24:1)
- Ethanol (70% and absolute)
- Tris-EDTA buffer (TE buffer)
- Centrifuge and micro-centrifuge tubes
- Pipettes and sterile pipette tips
- Vortex mixer
- Water bath or incubator
- RNase A

Methodology for Isolation of DNA from Fish Fins and Gills:

First of all, we collected the sample from fish fins and gills by using sterilized scissors and forceps. Then the samples were transferred to Phosphate Buffer Saline (PBS) to remove any contamination. The rinsed samples were then transferred to a microcentrifuge in which 500µl of lysis buffer was added which contained around 10% of SDS and 0.5M EDTA. After that, we added 10µl of Proteinase K which is around 20mg/ml to each tube. Then the sample was allowed to incubate for 2-3 hours to lyse the tissues properly. An equal amount of Phenol: Chloroform: Alcohol with a ratio of 25:24:21 respectively were added to the lysed samples. After that, the mixture was allowed to vortex for 30 to homogenize properly. Then the sample was allowed to run a centrifuge for around 10 min. After centrifugation the sample was divided into two layers, we carefully transferred the above (top) aqueous layer to a new centrifuge tube avoiding the inorganic interphase layer. We then added 2 volumes of absolute ethanol and 0.1 volume of 3M sodium acetate with a pH was around 5.2 to the aqueous phase. Then the mixture was inverted several times to mix properly after that it was allowed to place at -20 °C for 1 hour. After that, the samples were centrifuged at 12,000 rpm for 10 min at 4°C. Then we removed the supernatant and washed the DNA pallet with 1ml of 70% ethanol. After that, we air-dried the DNA pallet and then resuspended the DNA pallet 50-100µl of TE Buffer. To remove RNA contamination, we added 2µl of RNase which was 10mg/ml, and the mixture was then allowed to incubate at 37 °C for 30 min.

Materials for Comet Assay on Gills of Fish:

- Fish gills samples
- Phosphate-buffered saline (PBS)
- Low melting point agarose
- Normal melting point agarose

- Lysis buffer (containing Triton X-100, DMSO, NaCl, EDTA, and Tris)
- Electrophoresis buffer (alkaline buffer: NaOH, EDTA)
- Neutralization buffer (Tris buffer, pH 7.5)
- Staining solution (e.g., Ethidium Bromide or SYBR Green)
- Microscope slides and coverslips
- Electrophoresis unit
- Fluorescence microscope

Methodology for Comet Assay on Gills of Fish:
We began by mincing the fish gills into small pieces and then placing them in cold phosphate-buffered saline (PBS). Then we gently homogenized samples to release the cells, after that the cell suspension was filtered through a nylon mesh to remove the debris. Then we prepared 1% of the normal melting point agarose and coated it to the microscope slides. Then we mixed the isolated gill cells with 0.7% low melting point agarose at 37°C, after that we spread the mixture on the pre-coated slides and covered it up with a coverslip. Then we put slides on ice to solidify the agarose to speed up the experiment. After the removal of the coverslips, we submerged the slides in a cold lysis solution at 4°C for 1-2 hours. Then we took slides and transferred them to a horizontal electrophoresis tank and coated them with alkaline electrophoresis buffer (pH > 13) for around 20 minutes which allowed the DNA to unwind. We carried Electrophoresis at 25 V and 300 mA for 20 minutes. After that, we neutralized slides by soaking them in a neutralization buffer for 3 intervals of 5 min each. Then we stained the DNA using an appropriate staining solution, such as Ethidium Bromide and SYBR Green, for 5 minutes. Then we washed the slides in distilled water and protected them with a coverslip. Finally, we viewed slides under a fluorescence microscope and took some snapshots. We then analyze the comet length and DNA content using image analyzing software CaspLab-Comet Assay Software Project.

Primer Designing for PCR Analyses:
We first identified p53 as the target gene which was related to genotoxicity, with the goal of DNA repair. We then ensure the gene sequence is capable of a specific fish species or closely related species. After that, we retrieved the gene for our study from the NCBI Gene Bank database. We use primer designing software i.e. Primer3Plus for generating primer pairs with the setting of 18-25 nucleotides primer length, GC content around 40 to 60%, melting temperature from 50 to 60°C and to ensure that both forward and backward have the same 2-3°C melting temperature. To avoid primer dimer formation and secondary structure, we aimed for a 100-300bp Amplification size to increase the specificity of the primers which is also suitable for most of the PCR. After successfully designing of primer, we verify Primer 3 Plus design primers in NCBI Primer Blast to confirm their specificity against the fish genome or any relevant sequence available. Then we conducted initial PCR tests with the sample available to verify their specificity and efficiency further. Optimization of annealing temperature was done to achieve the best results. Also, we adjust the MgCl2 concentration to boost the primer performance and improve reaction conditions.

Results and Discussions
The method of DNA extraction from the fins and gills of fish produced good quality DNA having purity ratios (A260/A280) which is somewhere between 1. 8 to 2. Values for protein contamination were close to zero, indicating that there is little or no contamination of proteins in the RNA preparations. This factor pointed out that the DNA extraction yield was satisfactory for downstream applications such as PCR and sequencing and subsequent processes in

molecular studies. These outcomes highlight the need to select the right kind of tissues and handle extraction procedures that will provide pure DNA since genotoxicity evaluations rely heavily on their quality. The comet assay test was conducted on fish gills, and clear comets with tails were identified, quantitative analysis involving the assessment of comet tails' length based on the amount of DNA damage showed that fish with exposure to environmental stressors have longer comet tails than control fish. This illustrates that the comet assay is an effective and useful technique for identifying genotoxic stress in various aquatic species. The application of fish gills, which are reported to have sensitivity to water-soluble compounds, calls for considerations of further use of fish as a bioindicator of changes in the environment as well as increased protection of the environment through the passage of severe policies that check pollution. PCR analysis revealed that the synthesized primers were specific, which was expected under the NCBI Primer Blast and initial PCR experiments without any non-specific amplification of the target p53 gene. When choosing the conditions for PCR, it is advisable to fine-tune the concentration of MgCl2 and the annealing temperature, as this will allow for obtaining clean and distinct bands of PCR products. Based on the roles of the p53 gene in DNA repair and genotoxicity stress response, it can be valuable in genotoxicity studies, offering relevant information on the genetic impacts of toxicants including carcinogenesis within the environment.

This combined study starting from DNA extraction to genotoxicity analysis in this research particularly demonstrates the importance of incorporating modern molecular approaches into environmental investigations. Efficient DNA extraction from fish tissues provides the fundamental foundation of molecular conservation and fisheries resource management. The conventional technique, phenol-chloroform extraction is very effective and has been reported to provide high purity, but the new methods that include the silica membrane-based and the magnetic bead-based are safer and more efficient for large-scale genomics studies. The fact that comet assay evaluates the amount of DNA damage on the single cell level has proven effective in evaluating the harmful effects of contaminants such as heavy metals, pesticides, or toxic waste from industries. The visual analysis of DNA fragmentation and migration, which results in a cometary structure, facilitates the accurate evaluation of the genotoxic effects and strengthens the proposals concerning using fish as biomarkers in assessment of the ecological conditions. The primer design procedure ensured that there was low non-specific binding and primer-dimer formation, features that were confirmed through computational and experimental tests to provide high specificity and efficiency in PCR amplification. Implementing this double-check procedure helped to minimize mistakes and improve the quality of our outcomes, which emphasizes the significance of rigorous optimization in molecular biology protocols. In short, our study has illustrated how high-quality DNA extraction, genotoxicity assays, and precise real-time PCR analysis can serve as critical tools for detecting and countering the negative impact of environmental stresses on aquatic life. It seems that the results from this study indicate how molecular approaches are vital to environmental preservation, as well as the fact that more research needs to be done to refine these methods to address increasing obstacles in the process. Nonetheless, there are some limitations associated with DNA extraction methods comet assay as well as Primer design methodologies that have not been resolved even in the face of great improvements in technology. The method can fail to provide consistent results because DNA yield and quality depend on the type of tissue and its condition, while inter-laboratory differences in the protocol for conducting the comet assay also influence the outcome. Variables such as these must be reduced to increase the chances of replicability of the findings from this it becomes clear that such variations can only be eliminated through standardization of the procedures and by adopting automated systems for the analysis of images. From the subsequent studies, more attention should be paid to the process of DNA extraction from the tissues

containing high cellularity and contamination risk. Furthermore, incorporating the comet assay into various fish species and environments will help generate main information on genomic vulnerability in aquatic environments. Combining these molecular procedures with the ecological and behavioral investigations of the impacts of pollutants on fish and their environments will present a comprehensive picture of the situation. As fish species are diverse it is very difficult to come up with the most appropriate primers during the primer design. This issue could be the result of closely related species having high sequence variations, and different primer targets, leading to poor binding and inefficient amplification. PCR analysis in fish is sometimes difficult due to the low quantity and quality of DNA samples collected from fish from environmental samples or tissues that are partly decomposed. Optimization of PCR can be labor-consuming and might require some effort, diverse annealing temperatures, and MgCl2 concentration could be a lengthy process procedure that involves trial and error. To have a good number of suitable primers to target various fish species, cross-validation requires considerable time and resources, especially when the species under study are rare or difficult to sample. A line of improvement for future studies could be applied to the design of universal primers that can amplify conserved fragments of target genes in fish species. This would simplify the genotoxicity test and help to avoid the process of designing various species of primers. The integration of high-throughput sequencing technologies can offer broad methodological assessments concerning genotoxic impacts and can also identify numerous genes and pathways that are concerned with DNA repair and stress reaction.

Conclusion

The approach of accurately isolating DNA from fish tissues and studying the impact of environmental contamination on the fish genetic structure is key to modern ichthyology. When it comes to extending more efficient methods and expanding our knowledge, we are closer to protecting the biological diversity of water and the rational use of fish stocks. As presented in the current study, the methodologies used are not just scientific processes but critical components in the appropriate management and preservation of the natural harmony in aquatic environments. As we are to master molecular biology incorporate scientific perspectives of the environment and advance conservation strategies, we are to ensure a sustainable future for aquatic life and scientific analysis.

Ethical approval

“The study was conducted and approved by The Institute of Biotechnology and Genetic Engineering at The University of Agriculture”.

Conflicts of interest

“There is no conflict of interest for publishing this study.”

Contribution of authors

Author 1: Conceptualization, Investigation, Methodology, Formal analysis, Writing original draft

Author 2: Supervision, Validation, Visualization, Review, Editing.

“All authors have read and agreed to the published version of the manuscript.”

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