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Mitigation of Toxic Effects of 2 Naphthalene Sulfonate after its Treatment with Microbial Consortia

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Abstract

Present study aimed to treat 2 Napthalene Sulfonate (2NS), a dye intermediate with microbial consortia and assess its potential toxicological impact before and after treatment on the integrity of Deoxyribo-Nucleic Acid (DNA) in blood cells of Channa punctatus. Symbolic elevation in DNA damage with untreated 2NS administered fishes was observed as revealed by comet assay and micronucleus test. However, 2NS after being degraded using mixed bacterial population showed significant reduction in toxicological effects of 2NS. Thus, this study not only illustrated the adverse impact of such toxic contaminant of industrial waste but also suggested a highly efficient and eco-friendly way to remove the harmful xenobiotics from the environment which may help to reduce the exposure of aquatic fauna and flora to such lethal toxicants.

1. Introduction

The chemicals of man-made origin, specifically textile dyes and their toxic intermediates, are being released in the environment without treatment due to non-availability of any effective environmentally safe bio-treatment process. Different aromatic sulfonates such as amino benzene sulfonates, naphthalene sulfonates and anthraquinone sulfonates are the intermediates formed after hydrolysis of textile dyes during dyeing process or by non-specific transformations in the environment. The presence of $C\text{-}SO_3^-$ imparts polar nature to aromatic sulfonates[1,2] which obstruct their transport through cell membrane, rendering these compounds resistant to biodegradation by microbial species leading to their accumulation. Their release and accumulation in environment has public

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health concerns due to their carcinogenic, mutagenic and teratogenic attributes[3,4]. Thus there is a need to develop efficient and biologically safe treatment method for the removal of such toxic intermediates from wastewater generated by textile industries. In light of this there is an urgent need to isolate and screen the microbial diversity of unexplored sites polluted with molecules having similar functional groups and structure. The microbial diversity of such sites might have evolved exotic peripheral metabolic sequences to survive in presence of high concentrations of such contaminants. These sequences could transform xenobiotic molecules to less toxic or environmentally benign intermediates which could be further transformed by central metabolic pathways. Among aromatic sulfonates Naphthalene sulfonates are important products of industrial chemical processes and applied widely in concrete finishing, industrial textile processing and tanning of hides and in the manufacture of agrochemicals and pharmaceuticals. 2NS is mainly used as dye intermediate of azo dyes and pigments. Its salt is also used as a brightening and stabilization agent in the electroplating solution of printed wiring board industry[5]. 2NS get discharged to the environment through various points and non-point sources including azo dye producing plants, naphthalene refinery processing plants and disposal sites as well as Wastewater Treatment Plants (WWTPs). Several studies performed indicate its presence in the ground water as well as surface water present in the vicinity of an aromatic sulfonate and dye chemical plants. In the study performed by Hamann *et al.*[6], 2NS was found among 247 contaminants in the Lek River. The study also highlighted the first-order degradation half-life of 2NS which was reported to be <469 days showing its potential to remain in the environment for such a long time. Further studies also revealed the occurrence 2NS in 7 samples from the Bormida River[7]. Study performed by Pan *et al.*[8] also considered 2NS as the representative pollutant in his data. Since 2NS is being discharged into aquatic environment from several sources and having long half-life, it has become a serious issue of concern for the aquatic life. Thus, the present study aims to treat 2NS with microbial extract and assess the toxicity effects of 2NS before and after treatment on the integrity of DNA in blood cells of fresh water fish, *Channa punctatus* using comet assay and micronucleus assay.

2. Material and Methods

2NS was purchased from Himedia Laboratories. The components of different growth media and all chemicals/ solvents used in the studies were of the analytical grade and also purchased from Himedia laboratories.

2.1 Media Used

N/20 Luria-Bertani with the composition (g/L) of Casein enzymatic hydrolysate (0.5), Yeast extract (0.25) and Sodium chloride (0.25) was used for enrichment and isolation of cultures.

2.2 Experimental Model and Maintenance

The freshwater fish *C. punctatus* commonly called as snake head fish weighing 15 ± 2 g, having length 12 ± 2 cm were procured from local outlets and acclimatized in 200 liter capacity glass aquarium for 15 to 20 days under laboratory conditions. The water of aquarium was changed daily in order to avoid the accumulation of waste material and to reduce its ammonia content and fishes were fed with synthetic diet. The physiochemical parameters were checked using standard protocol[9]. The water temperature shifted from control to exposed 26.7 to 28.4 C, pH values ranged from 7.2 to 8.10, dissolved oxygen from 5.63 to 5.34, electrical conductivity 630µs/cm to 1234 µs/cm and Total Dissolved Solids (TDS) 307 mg/l to 612mg/l.

2.3 Experimental Design

Present experiment involves two parts. In the first part, 2NS was degraded with the help of mixed microbial species and the same was further verified by using analytical techniques such as UV VIS spectrum analysis and Thin Layer Chromatography (TLC). In the second part three groups of fishes were studied and the experiment was performed in triplicates. In the first group, ½ of LD_{50} i.e., 0.33mg/15g b.w. of 2NS[10] dissolved in N/20L.B medium was administered to fishes in the midline of the pelvic fins. Whereas in second group fishes were administered with 1.6ml of 200mg/l mixed microbial species treated 2NS (1.6ml of 200mg/l=0.33mg/15g b.w.). Fishes maintained in tap water were considered as control group. Finally, blood was collected by puncturing heart of fish after 24 h, 48 h, 72 h and 96h of intraperitoneal doses to study the

effect of untreated and treated samples on genotoxicity. Genotoxicity was evaluated using comet assay and micronucleus assay.

2.4 Enrichment and Isolation of 2NS Degrading Microorganisms

The microbial diversity present in the sample collected from the Effluent Treatment Plant (ETP), Buddha Nala, Ludhiana was enriched by inoculating 5% (v/v) of sludge sample in N/20 L.B supplemented with 30 mg/l of 2- NS in 100 ml medium in a 250 ml flask. The flask was incubated on a rotary shaker (Orbitek, Scigenics, Chennai, India) at 180 rpm at 30° C. After incubation for 2 days, 1.0 ml of cell free supernatant was read using UV–Visible spectrophotometer to determine transformation of 2NS. The cells from enrichment were further harvested by centrifugation at 10,000 rpm for 10 min at 4°C and were transferred to the fresh N/20 L.B supplemented with 50 mg/l of 2–NS. With 5 such successive transfers the concentration of 2NS was further increased upto 200mg/L in N/20 L.B.

2.5 Analytical Techniques were carried out to Validate Transformation of 2NS

2.5.1 UV Visible Spectrum Analysis

The cell free supernatant of abiotic control and biologically treated samples of 2NS was scanned in the range of 200–400 nm using UV–Visible spectrophotometer (Shimadzu, UV 1601, Japan) to compare the changes in absorption pattern in different spectra of abiotic and treated samples

2.6 Thin Layer Chromatography (TLC)

The liquid- liquid extraction of the transformed samples and their respective control was carried out with ethyl acetate for TLC analysis

The concentrated samples (were resolved on preformed silica gel plates (GF_{253}, Merck). The running solvent systems used for developing chromatogram were chloroform: methanol in the ratio of (7: 3) (v/v) for 2NS. The chromatograms were then observed under UV-light for the detection of aromatic compounds.

2.7 Genotoxicity

The alkaline SCGE was carried out using the method of Ahuja and Saran (1999)[11] with some modifications. Tissues were homogenized in PBS followed by centrifugation at 3000 rpm for 10 minutes. Standard procedures of 1% normal melting agarose coating followed by sample loading mixed with low melting agarose, then incubation, solidification and further lysis in electrophoresis buffer was followed. After lysis, the slides were incubated for 20 minutes in electrophoretic buffer followed by electrophoresis in the same buffer for 20 minutes. The slides were then neutralized with the neutralization buffer for 15 minutes and after overnight drying, were stained with ethidium bromide and analyzed under a fluorescence microscope by using excitation filter 515-560 nm with barrier filter of 590 nm at 40X magnification. 100 cells per treatment were scored.

2.8 Results

2.8.1 Biodegradation of 2NS by Mixed Population

Results reveal that initially the microbial population of the enrichment was able to degrade 2NS (30mg/l) in N/20 L.B medium within 24h. Further the effect of increasing concentration of 2NS i.e. 50mg/L, 100mg/l, 150 mg/ and 200 mg/L in N/20 L.B on degradation efficiency of mixed microbial population was also evaluated. It was observed that with the increasing concentration of 2NS up to 200m/L, the time of degradation also increased up to 48h leading to 92% degradation of 200mg/L of 2NS. Figure 1. Figure 2 show the bacterial diversity isolated from enrichment in N/20 L.B supplemented with 200 mg/L of 2NS. The degradation was further confirmed with the help of thin chromatography layering technique. The concentrated samples were resolved on silica gel plates ($GF_{253,}$ Merck). The resolved chromatograms were observed under UV light for the detection of profiles of aromatic compounds. It is evident from the results presented in Figure 3 that the organic extract of abiotic control resolved into single spot at Rf value 0.63. The organic extract of samples treated (with different concentrations i.e., 100mg/l, 150mg/l and 200mg/l) indicates complete transformation of 2NS.

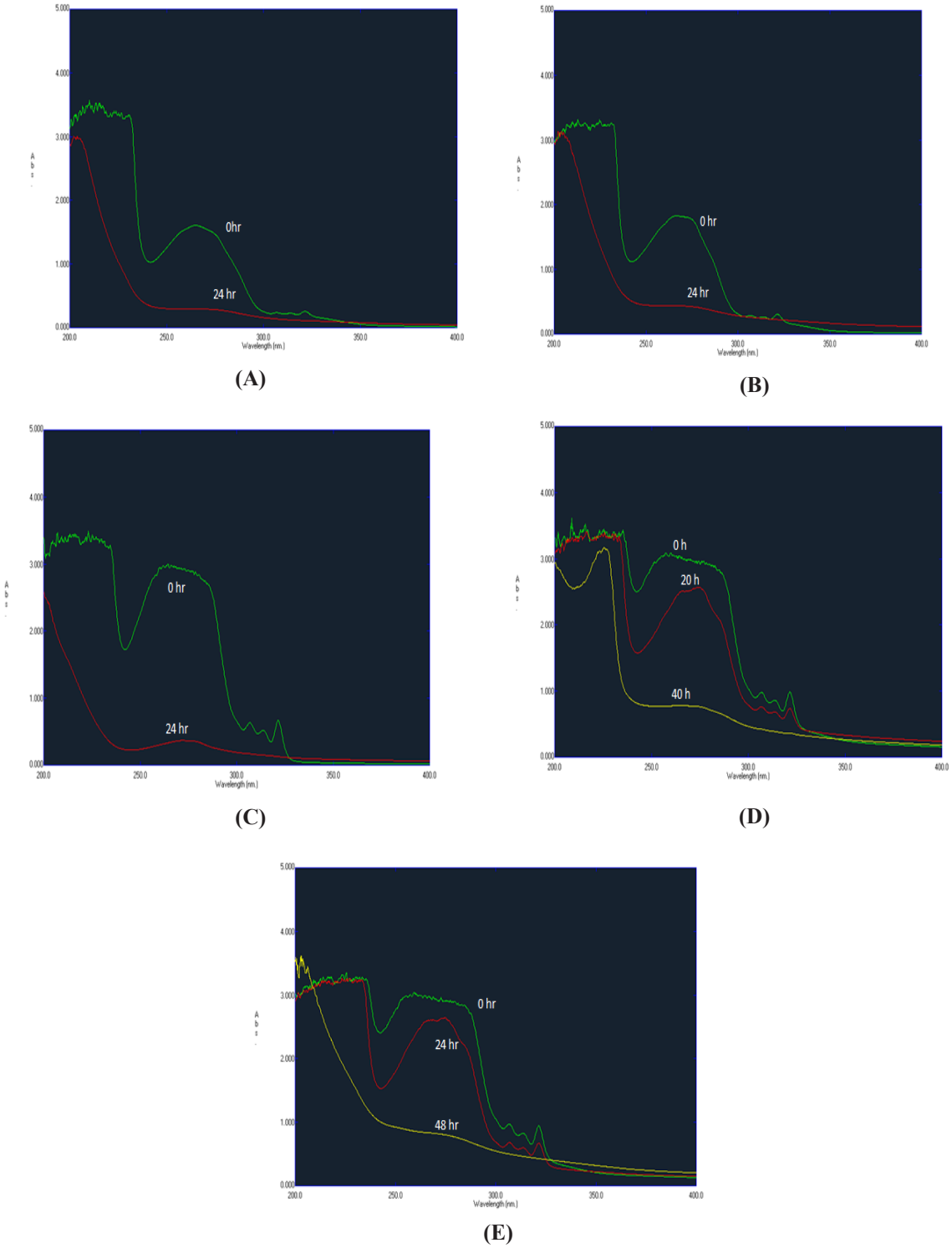


Figure 1. Degradation of 2 NS in enrichment **(A)** 30 mg/L, **(B)** 50 mg/L, **(C)** 100mg/L, **(D)** 150 mg/L, and **(E)** 200 mg/L by adapted mixed microbial population.

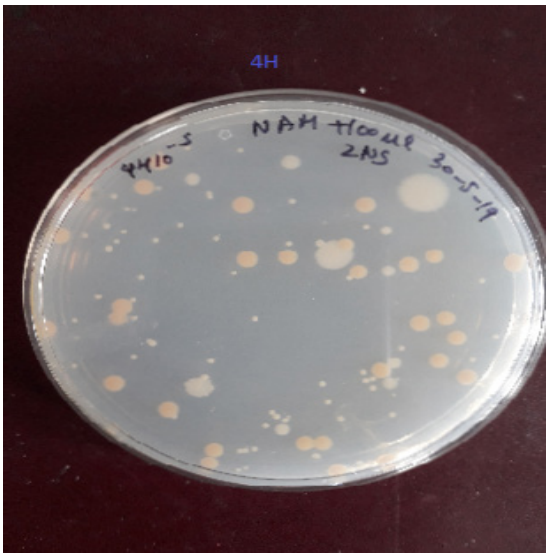


Figure 2. Bacterial diversity isolated from enrichment in N/20 L.B supplemented with 200 mg/L of 2NS.

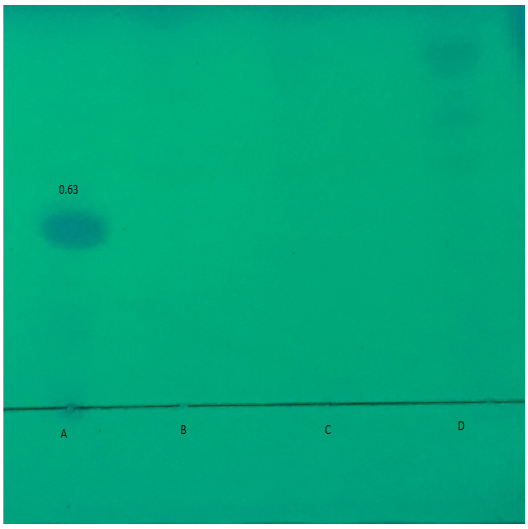
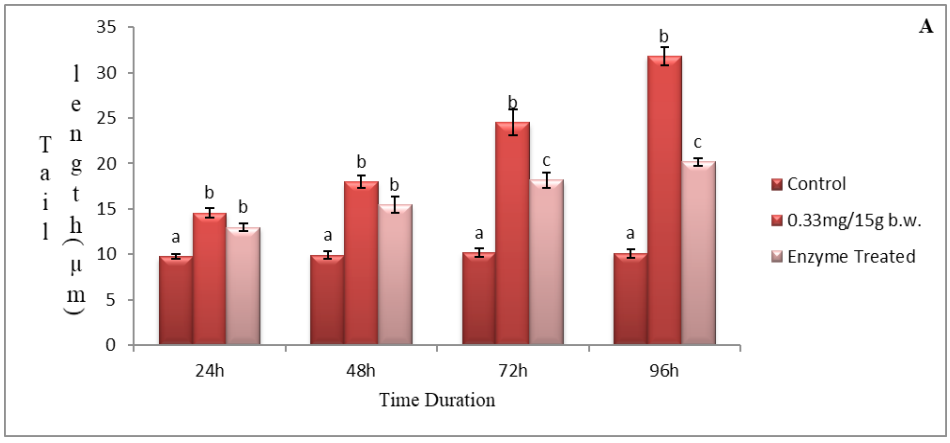


Figure 3. TLC of 2NS **(A)** abiotic control, biologically treated samples with concentration, **(B)** 100mg/L, **(C)** 150mg/L, and **(D)** 200mg/L.

The extract of samples treated with the mixed microbial population after 48 h shows complete degradation of 2-NS (100,150 & 200mg/L).

2.9 Genotoxicity

Results of genotoxicity are summarized in Figure 4(A-B) and Figure 5(A-B). Two parameters including Tail length and Olive tail moment (µm) were considered to assess DNA damage while studying comet assay. Figure 4 revealed tail length and Olive tail moment (µm) in blood cells of *C. punctatus* after intraperitoneal administration of untreated and microbial treated 2NS till 96 hours at different time intervals. With increase in time duration, significant elevation in DNA damage was observed. After the acute exposure of untreated 2NS maximum DNA damage was observed at 96 hours showing 3.14 times folds increase in tail length as compared to control. Likewise the value of mean value of Olive tail moment increased from 0.91±0.07 to 3.01±0.67 (Mean± S.E.). Similar trend was observed for micronuclei and aberrant cell frequency (Figure 5 (A-B)) after acute exposure. The formation of micronuclei due to the subjection of untreated 2NS were higher significantly, as compared to control groups Thus significant effects ($P < 0.05$) of doses and time of exposure were revealed in the results. However, the fish groups administered with treated 2NS (showed a



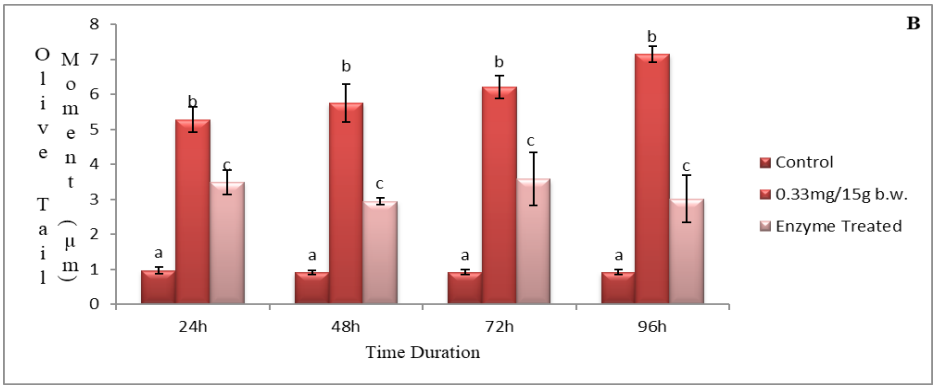
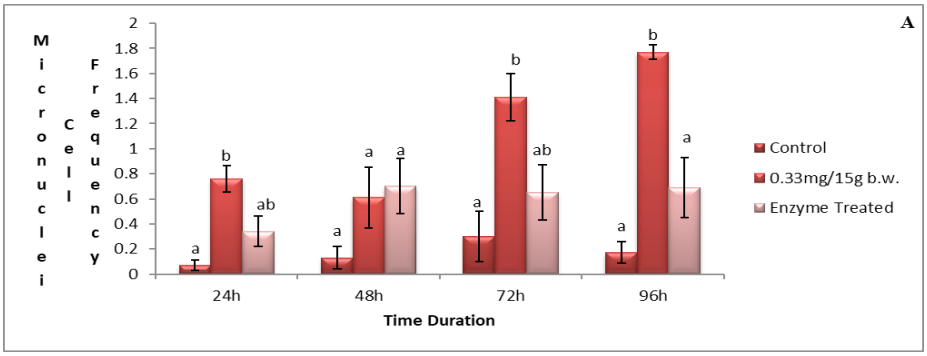


Figure 4. **(A-B).** Mean value of Tail Length (µm) and Olive tail moment (µm) in blood cells of *C. punctatus* after acute exposure of 2NS. Outcome is presented as mean and standard error value. Means that do not share a common letter are significantly different (p≤0.05).



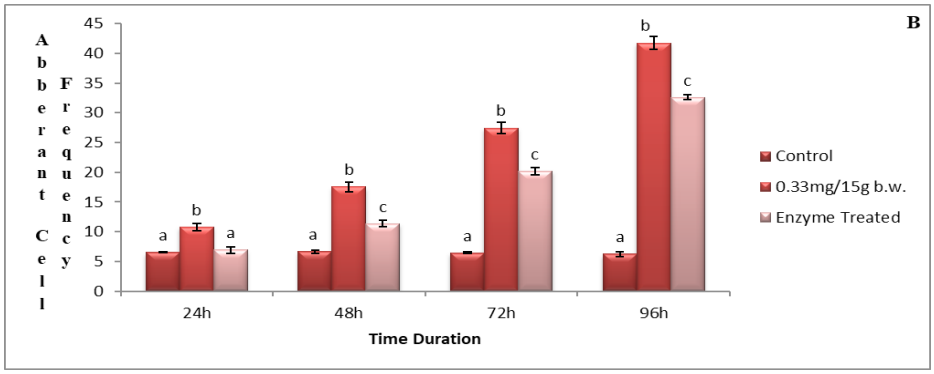


Figure 5. **(A-B):** Percent micronucleated cells and percent aberrant cells frequency in blood cells of *C. punctatus* after acute exposure of 2NS. Outcome is presented as mean and standard error value. Means that do not share a common letter are significantly different (p≤0.05).

decrease in damage to DNA as compared to untreated 2NS administered group of fishes as the value of tail length in blood cells exposed to treated 2NS drops from 31.8±1.03 to 20.15±0.4. Similarly, 2.37 fold reduction in the value of olive tail moment was also observed. 61.05% and 21.92% reduction in micronucleus cell frequency

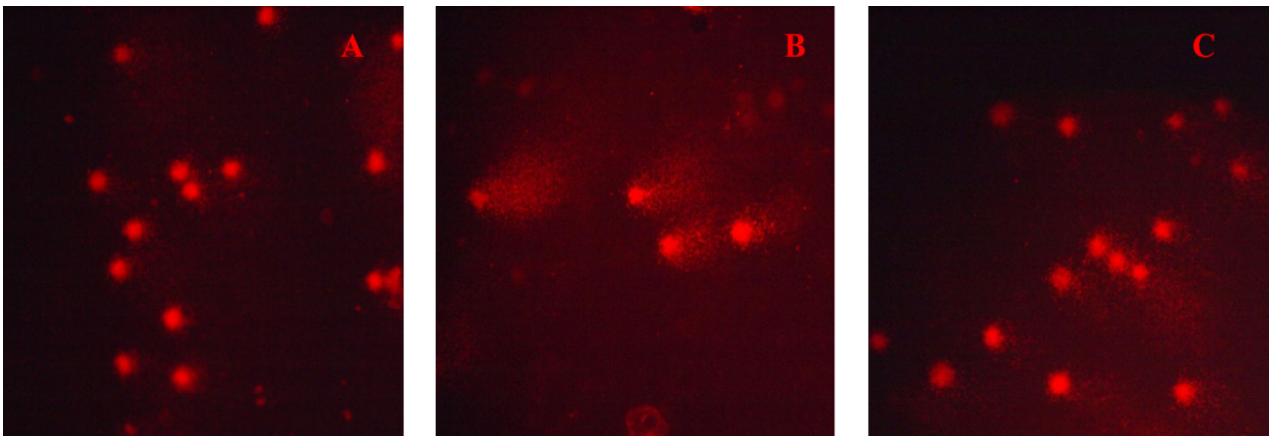


Figure 6. DNA damage observed by comet assay **A.** Blood Cells of control fish, **B.** Blood Cells of untreated 2NS intoxicated fish, **C.** Blood Cells of treated 2NS intoxicated fish.

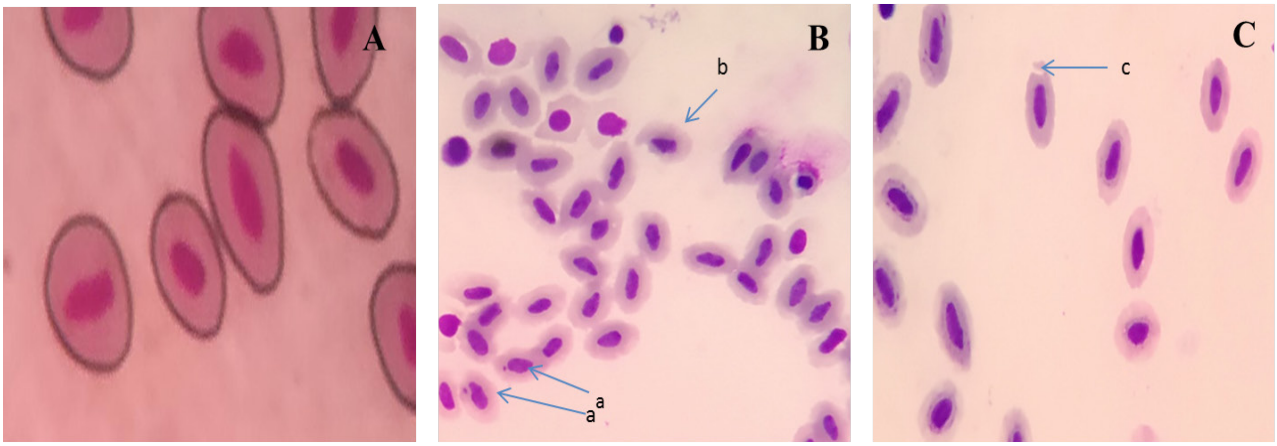


Figure 7. Cytoplasmic and nuclear abnormalities in blood cells of 2NS exposed fishes. **(A).** Blood Cells of control fish, **(B).** Blood Cells of untreated 2NS intoxicated fish showing, **(a).** Micronuclei, **(b).** disrupted cell wall, and **(C).** Blood Cells of treated 2NS intoxicated fish showing, and **(c).** lysed cell.

and aberrant cell frequency was also observed in blood cells extracted from fishes administered with microbial treated 2NS revealing the reduced genotoxic impact of microbial treated 2NS. Figure 6 shows microphotographs of DNA extracted from untreated (A) and treated 2NS (B) intoxicated blood cells. Figure 7 shows the cytoplasmic and nuclear abnormalities observed in the blood cells of *C. punctatus* after administration of untreated and treated 2NS.

3. Discussion

As DNA carries genetic information, its stability and integrity is crucial for life sustainability. So the damage associated with DNA caused by 2NS needs scientific approaches to combat the problems. Many parameters have been developed to study the toxic fate of contaminants and according to toxicity guidelines at least two assays should be carried out for *in vitro* and *in vivo* studies[12]. Thus the present study has used com*et al*ong with micronucleus assay for evaluating DNA damaging effect of 2NS. Comet assay is a relaible as well as authentic biomarker for percepting of DNA damage thus it is considered as the most propitious tool for assessing DNA damage. Beside comet assay micronucleus test is another promising and widely applied genotoxic biomarker[13,14]. Anitha *et al.*[15]

revealed that aneugenic agents and clastogenic process are the main reasons for the occurrence of micronucleus.

Various studies revealed that the tail length and olive tail moment are mainly taken as parameters for assessing genotoxicity[16-19]. In the present study of tail length and olive tail moment were used as markers for the evaluation of genotoxicity imposed by untreated as well as treated 2NS in blood cells of *C. punctatus*. Blood provides an ideal medium for toxicological analysis as blood is the most abundant and important body fluid and its composition reflect the total physiological condition of the body thus study was done on blood tissue. In micronucleus test, micronucleated cell frequency and aberrant cell frequency are taken as parameters for genotoxic and cytotoxic assessment. Studies performed suggested the consideration of nuclear and cytoplasmic abnormalities along with micronucleus[20-22]. Study performed by Osman *et al.*[23] also revealed the occurrence of other nuclear lesions along with MN in *Nile tilapia* and African catfish. The reason behind the occurrence of these nuclear deformities could be the detrimental effect of clastogenic pollutants which might further lead to chromosomal dysfunction[23,24]. The study performed by Fernandes *et al.*[25] revealed the toxicity on bone marrow of rats after giving textile dye C.I. disperse. Likewise, the elevation in micronucleus cell frequency was also observed in blood of gill and kidney of *C. punctatus* after the exposure of 4 nonylphenol[26]. This genotoxic potential of such toxicants might be due its interaction with DNA leading to breaks in strands of DNA, adduct and crosslink formation[27] which might further lead to incomplete transcription, cellular dysfunction and many other diseases in the organism[28].

Over the last few decades, diverse organic pollutants including petroleum fuels, pesticides, solvents, pharmaceuticals, and other organic chemicals have been produced industrially and released intentionally or unintentionally during their transports or storages[29]. These organic pollutants are generally highly persistent, have low degradability, and can become trapped for long periods of time in the soil minerals of contaminated sites, and subsequently bioaccumulated, due to their hydrophobic and stable chemical properties. Because they have potentially adverse effects on human health and multiple environments (aquatic, terrestrial, and atmospheric), the contamination of these organic pollutants is a cause of great environmental concerns, which necessitates the need to restore contaminated sites. 2NS belonging to aromatic sulfonates is highly used dye intermediate. But being non-biodegradable the concern regarding its toxicity has rised. The results obtained in the present study reveals the significant increase in genotoxicity in blood cells of *C. punctatus* when exposed to untreated 2NS as compared to control illustrating the dire need of converting 2NS into harmless compound before being discharged into the aquatic bodies. Bioremediation, a technique that employs microorganisms to remove organic pollutants, has been proven to be sustainable, eco-friendly, and cost-effective among remediation technologies for contaminated sites[30]. Many diverse microorganisms that have good pollutant-degrading ability have been previously isolated through enrichment processes using synthetic basal media, which has led to extensive genomic and metabolic studies on pollutant biodegradation[31]. However, bioremediation of contaminated sites by these microorganisms often results in failure due to unfavorable several biotic and abiotic factors including low degradability, low viability, low pollutant availability, depletion of nutrients, and unfavorable pH, oxygen, temperature and moisture at contaminated sites[32]. Therefore, it has been suggested that the use of microorganisms mainly responsible for pollutant degradation at contaminated sites is requisite for successful bioremediation of contaminated sites[31,34,35]. Thus in the light of above statement an approach of degrading 2NS was made in the present investigation using mixed microbial diversity present in the sample collected from the effluent treatment plant. Studies performed by Brilon *et al.*[33] proposed that naphthalene sulfonates are degraded with the enzymes having gentisate as major metabolite. Degradation of these xenobiotics is initiated by oxygenlytic displacement of the sulphonic acid groups by breaking C-SO_3^- bond. Based upon the above study biodegradation of 2NS was performed. In the present investigation initially the microbial population of the enrichment was able to degrade 2NS (30mg/l) in N/10 L.B medium in 24h. Further the enriched microbial population was able to degrade 92% of the 2NS (200mg/l) in N/20 L.B within 48h revealing that with the increase in concentration of the enriched microbial population, enhancement in pace of degradation was obtained. Furthermore the use of TLC indicates the complete biodegradation this compound. After degradation the genotoxic effect of biodegraded 2NS study was performed

to evaluate the status of toxic potential of 2NS. Drastic reduction in parameters studied for DNA damage reveals the reduced genotoxic potential of 2NS in blood cells of *C. punctatus*. However further isolation and characterization of microbial species is necessary for implying this study as pure benefit for environment.

4. Conclusion

In this study, potential of enriched microbial populations was evaluated for biodegradation of Naphthalene sulphonate (2NS). The enriched microbial population was able to degrade up to 200 mg/L of 2NS within 48 h. The potential of this mixed microbial population can be further explored for the degradation of naphthalene and other complex poly aromatic compounds. This investigation also highlights that 2NS intoxication leads to genotoxicity in the blood cells of *C. punctatus*. The repeated exposure of human/animals to such toxicants and their accumulation in the food chain may pose serious public health risk. Thus, use of microbes or their efficient bio-enzymatic system–based treatment strategies for hydrolyzing 2NS may suggest an environmental friendly way to remove the harmful xenobiotics from the environment.

5. Acknowledgement

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6. Conflicts of Interest

The authors declare that they have no conflicts of interest.

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