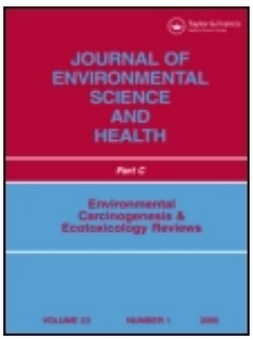

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Genotoxicity Testing and Biomarker Studies on Surface Waters: An Overview of the Techniques and Their Efficacies

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Rapid industrialization, use of modern agriculture practices, and fast urbanization vis-a-vis indiscriminate use of xenobiotics have led to the serious problems of water pollution in India and abroad. The complexicity of the pollutants in environmental samples demands a multitude of genotoxicity testing with increasing simplicity, sensitivity, and affordability. Moreover, various pollutants mutually affect their own toxic behavior, which complicates the problem of risk assessment. An overview, highlighting the genotoxicity testing system, such as Ames plate incorporation test, Ames fluctuation test, *E. coli* survival assay, *Allium cepa* toxicity/genotoxicity test, comet assay, and plasmid nicking assay, is presented in this article, and a comparison has been made to estimate the efficacy of these genotoxicity bioassays performed on some surface waters. Some work on toxicity biomarkers vis-a-vis studies on surface waters has also been included in the present review.

Keywords: Ames plate incorporation test; Ames fluctuation test; *Allium cepa* genotoxicity test; plasmid nicking assay; biomarkers; surface waters

INTRODUCTION

Human and industrial activities are at the origin of the discharge of multiple chemical substances in the environment and are the main causes of

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environmental pollution [1]. It is extremely difficult to quantify the risk associated with these chemical pollutants because of its complex nature [2, 3]. Although chemical analyses are the primary method by which industrial effluents are assessed for potential toxicity, such an approach contains inherent limitations. The chemical analyses are limited in their ability to characterize the chemical composition of wastewater and permit a subsequent chemical-specific genotoxicity or carcinogenicity assessment because of the complex chemical nature of these samples. In contrast to chemical analyses, bioassays provide a means of assessing complex mixture toxicity without a prior knowledge of toxicant identity and/or physicochemical properties. The utility and availability of bioassays has resulted in their use in the assessment of a wide range of industrial effluents and mixtures.

The extensive literature concerning genotoxicity of surface and wastewaters was discussed in previous reviews [1, 4–6]. However, continuous production of scientific data pertaining to surface water genotoxicity calls for frequent reviewing of the current body of information. In view of the remarkable number of publications on the mutagenicity of surface and wastewaters, there was a need for another review article on genotoxicity of surface and wastewaters.

There are a large number of bioassays available for the evaluation of genotoxicity of surface waters. These assays utilize a wide range of organisms and cell types and measure a variety of genetic changes. The genetic damage represents a range of DNA damages from point mutations to chromosomal alterations. However, there is no single test that adequately detects the types of genetic damage that may be induced by all chemical classes of genotoxic compounds and/or complex chemical mixtures. To the best of our knowledge, no previous review has been specifically aimed at evaluating the efficacy of various genotoxicity tests and biomarker studies on surface waters. The most commonly used genotoxicity bioassays for various waters have been discussed as follows.

Ames Test

The Ames Salmonella/microsome mutagenicity assay (Salmonella test; Ames test) is a short-term bacterial reverse mutation assay specifically designed to detect genotoxic activity in complex environmental mixtures such as river waters, lakes, industrial effluents, drinking water, and hospital wastewater [2–3, 5, 7–11]. The assay developed by Ames is the most commonly used mutagenicity test. It was modified and improved several times [12–15]. It is based on several histidine-dependent Salmonella strains each carrying different mutations in various genes in the histidine operon. These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms.

The reason for greater utility of the Salmonella mutagenicity assay among the known bioassays is likely due to the ease and cost-effectiveness of this test

compared to other assays. Hence, the Salmonella bioassay has been used more often than any other for evaluating the complex mixtures. The presence of mutation in *his* structural operon *genes* of these strains allows positive selection of his^+ revertants on minimal agar plates. A deletion of the *uvrB* gene in most of the tester strains results in higher efficiency of mutagenesis due to inactivation of one of the efficient bacterial DNA repair systems [12]. Bacterial strains used in the Ames test also bear the *rfa* mutation, which causes partial loss of the lipopolysaccharide (LPO) barrier that coats the surface of the bacteria and increases permeability to the large and non-polar molecules that do not penetrate the normal cell wall of *S. typhimurium* [12]. In addition, some of these strains harbor plasmid pKM101 that contains *mucA* and *mucB* markers responsible for enhancement of an error-prone DNA repair system [15–18].

It is imperative to use different strains of *S. typhimurium* because specific mutation at hot spots within the tester strains makes them more sensitive to respond to different mutagens [2–3, 10, 19]. In particular, his^- mutations of the TA102 strain can be reverted by mutagens that cause oxidative damage. Moreover, this strain can easily detect cross-linking agents [20]. TA97 and TA98 strains provide the information for the presence of frame-shift mutagens [2–3, 15, 21] whereas TA100 strain detects the base pair substitution mutagens [20].

In humans and lower animals, the cytochrome-based P450 metabolic oxidation system is present mainly in the liver and to a lesser extent in the lung and kidneys, which is capable of metabolizing a large number of chemicals to DNA-reactive or electrophilic forms. Some of the intermediate metabolites are potent mutagens in the Ames Salmonella assay. Since bacteria do not have this metabolic capability, an exogenous mammalian organ activation system (S9 fraction) needs to be added on to the petri plates together with the test samples and the bacteria. To increase the level of metabolizing enzymes, the animals are pretreated with the mixed-function oxidase inducer Aroclor 1254. Other inducers, such as phenobarbital and β-naphthoflavone, can also be used [22].

Ames Fluctuation Test

The fluctuation test is a statistical method for the detection of induced mutation in bacteria by test samples, carried out in liquid medium. Ames fluctuation test also makes use of the same Ames tester strains and S9 fraction. It was developed by Green and colleagues [23] and the growth of his^+ revertants in this case is detected by a drop in the pH of the medium responsive to color change of bromo-cresol purple indicator from blue to yellow. This test is also free from agar-associated problems. However, it could not gain such popularity compared with plate incorporation test, despite its high sensitivity [24–25], ability of automation [26], possibility of using hepatocytes for metabolic activation [23], and its better sensitivity for aqueous samples containing low levels of mutagens [27].

Allium cepa Test: A Sensitive Tool in Environmental Monitoring

Allium cepa roots are regarded as one of the pioneering organisms for use in cytogenetic studies and have been used by the US Environmental Protection Agency to evaluate toxicity and genotoxicity of industrial effluents [28–29]. The *A. cepa* test was introduced in 1938 by Levan [30]. Since then, it has been commonly used for environmental monitoring. This test has been widely used for the toxicity assessment of heavy metals, pesticides, and industrial wastewaters [2, 10, 31–35]. Our group [2, 35] has also used this test for the genotoxicity assessment of wastewater from Aligarh region. This test has also been used by other investigators for detecting the genotoxicity of surface waters [36–37]. This assay is cost-effective, easy to perform, and sensitive enough to respond to low concentrations of toxicants [38–39]. *Allium cepa* is an efficient test system routinely used to evaluate the genotoxic potential of chemicals in the environment due to its sensitivity and good correlation with mammalian test systems [31]. Among the tests carried out with *A. cepa*, chromosome aberrations provide important information and may be considered as an efficient test to investigate the genotoxic potential of the effluents.

SOS Defective *Escherichia coli* K-12 Survival Assay

E. coli DNA repair assay is based on the decreased survival of DNA repair defective mutants. The SOS repair in *E. coli* is a component of simultaneously induced expression of more than 40 genes called SOS response, which is especially triggered when the cells are treated with DNA-damaging agents [40]. It is well known that the SOS response is dependent on *recA* and *lexA* gene products as well as on the presence of single-stranded DNA [41–42]. The elevated expression of these genes increases the capacity of cells for DNA repair, damage tolerance, DNA replication, and mutagenesis [43]. Since mutant strains do not permit induction of the SOS response, the lack of SOS repair renders such strains extremely sensitive to DNA-damaging agents [44]. In this assay, SOS-defective *recA*, *lexA*, and *polA* mutants of *E. coli* K-12 along with their isogenic wild-type strain(s) are treated with the test samples for different time intervals, are suitably diluted, and are plated to assay the colony forming ability.

Comet assay or Single-Cell Gel Electrophoresis (SCGE)

The comet assay was first introduced by Ostling and Johanson [45] as a fast and effective way of measuring DNA damage in individual cells. In that assay, the lysis and electrophoresis was performed under neutral conditions, and staining was done with acridine orange. The image obtained looked like a "Comet" with a distinct head, comprising of intact DNA, and a tail, consisting of broken pieces of DNA; hence the name "Comet" Assay was given. The extent

of DNA liberated from the head of the comet was the function of the dose of test toxicants. However, in that procedure, only double strand breaks could be analyzed.

The neutral assay was modified by two groups: Singh and coworkers [46] and Olive and associates [47]. The former group used microgels, involving electrophoresis under highly alkaline conditions (pH > 13). This enabled the DNA supercoils to get relaxed and unwound, which were then pulled out during application of electric-current made possible the detection of single strand breaks in DNA and alkali labile sites expressed as frank single strand breaks in individual cells. This method was developed to measure low levels of strand breaks. Olive and coworkers conducted the electrophoresis under neutral or mild alkaline (pH = 12.3) to detect single strand breaks. This method was optimized to detect a subpopulation of cells with varying sensitivity to drug or radiation. The technique of Singh and associates [46] was found to be one or two orders of magnitude more sensitive than other techniques. Since then a number of advancements have greatly increased the utility of this technique for detecting various forms of DNA damage (e.g., single- and double-strand breaks, oxidative DNA base damage, and DNA-DNA/DNA-protein/DNA-Drug cross linking) and DNA repair in virtually any eukaryotic cell [48].

This assay has critically important applications in the fields of toxicology ranging from aging and clinical investigations to genetic toxicology and molecular epidemiology. The assay can be performed on a variety of samples, which can be obtained as a single cell population such as peripheral blood lymphocytes, nasal and buccal epithelium from clinically or occupationally exposed human population, and for in vitro studies on cell lines such as CHO, V79, mouse lymphoma, or cultured human lymphocytes and bone marrow cells [49].

Moreover, a variety of information related to genetic toxicology, human epidemiology, patients undergoing radio/chemo- therapy, ageing, and nutrition can be obtained. The assay has also been used for environmental biomonitoring utilizing earthworms, fishes, and mollusks exposed to polluted environments [50].

Plasmid Nicking Assay

Plasmid nicking assay is a simple DNA damage assay in which single strand breaks in the DNA can be determined by electrophoresis via the differential mobility of the super coiled, open circle, and linear forms of the plasmid [51, 52].

In addition to conventional genotoxicity testing procedures, recently, the biomarker concept of toxicity has emerged and gained significant popularity in the scientific community. The following lines presents the essential features of this emerging field and its utility in water genotoxicology.

THE BIOMARKER CONCEPT

Typically, biomarkers are defined as quantitative measures of changes in the biological system that respond to either (or both) exposure and/or doses of xenobiotic substances that lead to biological effects. Although not explicitly contained in most definitions, the use of the term "biomarkers" or "biomarker response" is often restricted to cellular, biochemical, or molecular or physiological changes that are measured in cells, body fluids, tissues, or organs within an organism and are indicative of xenobiotic exposure and/or effect [53].

Changes that occur at the organismic, population, and assemblage levels are more usually referred to as "bioindicators." One possible reason for limiting the term biomarkers to sub-organismic changes is that one of the functions of biomarkers is supposedly to provide early warning signals of biological effects and that it is generally believed that sub-organismic (molecular, biochemical, and physiological) responses tend to precede those at the organismic or higher levels [54, 55].

According to the National Research Council of Canada [56] and World Health Organization [57] biomarkers can be subdivided into three classes:

1. Biomarkers of exposure, which cover the detection and measurement of an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism.
2. Biomarkers of effect, which include measurable biochemical, physiological, or other changes within tissues of body fluids of an organism that can be recognized as associated with an established or possible health hazard.
3. Biomarkers of susceptibility, which include the inherent or acquired ability of an organism to respond to the challenge of exposure to specific xenobiotic substances including genetic factors.

At a practical and operational level, there are 4 desirable characteristics of the biomarker assay: sensitivity, specificity, simplicity, and stability. The assay should be sensitive enough to detect early stage of the toxicity; specificity is desirable because it can provide evidence of the harmful effect of a particular type of pollutant. Simplicity is desirable to make an assay available to non-experts in a cost-effective way. Diagnostic kits such as enzyme linked immunosorbent assay (ELISA) could be developed if there were sufficient simplicity. Stability is important in the sense that unstable and short-lived responses are difficult to measure and interpret in field studies [58, 59].

In reality, no biomarker assay exists that has all these attributes, and it is unlikely that there ever will be such a biomarker. This limitation can be overcome by using a combination of biomarkers [60, 61].

Types of Biomarkers

Biomarkers have been distinguished as tier I and tier II according to their specificity [62]. Tier I biomarkers respond to specific contaminants and often involve enzyme inhibition. Tier II biomarkers respond to general sublethal stress and often involve enzyme induction [63].

Tier I Biomarkers

There are probably as many tier I biomarkers as there are toxicants. The inhibition of neural acetyl cholinesterase by organophosphate and carbamates compounds [63] and the degree of inhibition of the enzyme is closely related to tissue exposure [64], which makes them useful biomarkers for this type of pollution.

Metallothioneins are thought to serve a protective function during heavy metal exposure by sequestering most of the free metal ions [65]. The toxicity of heavy metals to animals can be correlated to the levels of metallothioneins in the liver [64, 66, 67].

Tier II Biomarkers

These are generalized stress responses, and their specificity to chemical insult and value as biomarker varies markedly. All organisms studied to date respond to adverse environmental conditions by synthesizing a highly conserved group of proteins known variously as heat shock proteins (HSP) or stress response proteins (SRP) [68]. Although little work has been done on their utility as biomarkers, they have been found to be produced in two mollusks (*Mytilus edulis* and *Collisella pelta*) in response to both heat shock and cadmium [69] and in a bacterium (*Alcaligens eutrophus*) in response to cadmium and the herbicide 2,4,5-T (2,4,5- trichlorophenoxyacetic acid) [70]. Many environmental contaminants enhance oxidative stress in animals and the specific lesions known to arise as a result of such stress are present in many aquatic animals [71]. A mechanism for the detoxification of oxidizing compounds involves ascorbic acid. Its levels have been found to fluctuate with a wide variety of toxicological and environmental factors [68, 72].

Genotoxicity vs Genotoxicity Biomarkers

Genotoxicity is the combined effect of xenobiotic(s) and its/their unique biomarker response in the test organism. S9 fraction is the most important biomarker-based machinery being used since beginning in the Ames testing.

1. While genotoxicity implies the structural and/or functional perturbation of DNA, the biomarkers are the biochemical and molecular changes in the cell leading to these perturbations.

2. The genotoxicity is the "final cellular manifestation" being the DNA as target whereas genotoxicity biomarkers are like "early warning signals."
3. The examples of biomarkers are "SOS response in bacteria," "ROS production," and "induction of MFOs within the cell," "induction of toxicity cum repair machinery," etc.

DISCUSSION

Although a lot of efforts have been made around the world on the development of new toxicity tests, very little attention seems to have been directed toward the development of a suitable battery of existing tests after desirable changes for evaluating genotoxicity of environmental samples as recommended in water tox workshop of International Development Research Centre (IDRC), Canada [26] and to fulfill the said criteria of sensitivity, simplicity, efficacy, and affordability.

In view of the complexity of water pollutants, neither the contaminant monitoring alone nor the effect monitoring with single test would serve the purpose because (i) the combined effect of different pollutants in the mixture may be synergistic, neutral, additive, or antagonistic; (ii) variable susceptibilities of different organisms would demand multiple bioassays; and (iii) a comprehensive test system would also need the organisms at different trophic levels.

Mutagenicity testing data of surface water performed using the Ames testing, *E. coli* survival assay, *Allium cepa* genotoxicity assay, comet assay, and plasmid nicking assays are summarized in Table 1.

Genotoxicity and toxicity studies of water samples collected from the Esta^ncia Velha stream of southern Brazil, a stream transporting both domestic sewage and effluents from the leather industry, exhibited high toxicity in the stream headwaters by *Allium cepa* phyototoxicity test. However, no evidence of chromosomal aberration/mutation was recorded by *A. cepa* genotoxicity test as well as by Ames plate incorporation test with or without metabolic activation [73]. The results suggest that the synergy among different compounds in domestic and industrial sewage discharges can make it harmful for river system biota.

A. cepa test performed on Sava river, Croatia over a three-month monitoring period exhibited an inhibition of root growth of more than 50%, a decrease in the mitotic index of more than 40%, and a significant increase in chromosomal aberrations compared with control water samples [74]. *Allium cepa* genotoxicity test conducted on Pitimbu River (Natal city, Brazil) showed an increase in the frequency of different forms of chromosomal abnormalities and/or micronuclei. Water samples collected from a site near an industrial area were found to be the most toxic and genotoxic as well [75]. The effects of

Table 1: Various Genotoxicity Bioassays Performed on Different Surface Waters

Sample Source	Assay Method/Strain	Chemical Analysis	Reference
Industrial wastewaters from Aligarh region (India)	Ames assay/TA97a, TA98, TA100, TA102, and TA104 Differential survival in *recA*, *lexA*, *polA* mutants of *E. coli* K-12	Heavy metals	Malik and Ahmad (92)
XAD concentrated Ganga river water at 3 different locations viz. Kachla, Fatehgarh, and Kannauj (U.P., India)	Ames assay/TA97a, TA98, TA100, TA102, and TA104	Organochlorines and Organophosphorus pesticides	Rehana et al. (93)
XAD concentrated Ganges river water at Narora (U.P., India)	Ames assay/TA97a, TA98, TA100, and TA102 Differential survival in *recA*, *lexA*, *polA* mutants of *E. coli* K-12	Organochlorines and Organophosphorus pesticides	Rehana et al. (94)
Noyyal river water Tamilnadu, India	Comet Assay	N.R	Rajaguru et al. (107)
Four different locations of Egypt	*Allium cepa* assay	Heavy metals	El-Shahaby et al. (77)
Composite soil from agricultural fields irrigated with industrial and domestic wastewaters (Aligarh City, India)	Ames assay/TA97a, TA102, and TA104 Differential survival in *recA*, *lexA*, *polA* mutants of *E. coli* K-12	Organochlorines and Organophosphorus pesticides	Aleem and Malik (88)
Industrial, surface, and ground waters from Aligarh (India)	Ames test and fluctuation test/ TA97a, TA98, TA100,TA102, and TA104	Heavy metals and pesticides	Siddiqui and Ahmad (10)
Industrial waste waters of Ghaziabad and Aligarh (India)	Ames test and fluctuation test/ TA97a, TA98, TA100,TA102, and TA104	Heavy metals and pesticides	Fatima and Ahmad (2)
Untreated industrial and municipal wastewaters from Drava river water, Solvenia	*Allium cepa* phytotoxicity/ genotoxicity test	N.R	Smaka-Kincl et al. (81)
Esta^ncia Velha stream of southern (Brazil)	*Allium cepa* phytotoxicity/ genotoxicity and Ames test	Chromium, Phenol and many physico-chemical analysis	Juánior et al. (73)

Industrial Wastewater from Ghaziabad (India)	Ames assay/ TA97a, TA98, TA100,TA102, and TA104 Differential survival in *recA*, *lexA*, *polA* mutants of *E. coli* K-12	Organochlorines and Organophosphorus pesticides	Ansari and Malik (90)
Textile effluents (Brazil)	*Allium cepa* genotoxicity test	Azo Dyes	Caritá and Marin-Morales (79)
Atibaia river water contaminated with petroleum refinery effluent (Brazil)	*Allium cepa* genotoxicity test	Heavy metals and nitrates	Hoshina and Marin-Morales (80)
Untreated wastewater in Manisa (Turkey)	*Allium cepa* genotoxicity test	Cr, Cu, Pb and Zn	Şık et al. (76)
Troy Wastewater Treatment Plant from Walnut Creek (USA)	Ames fluctuation test	N.R	Whatley and Cho (85)
Industrial effluents from textile and paint industries	*Allium cepa* genotoxicity test	N.R	Samuel et al. (78)
Sava river water (Croatia)	*Allium cepa* phytotoxicity/ genotoxicity test	Nitrate, Nitrite, Ammonium various physico-chemical analysis	Radić et al. (74)
Industrial waste waters of Saharanpur and Aligarh (India)	Ames test and fluctuation test/ TA97a, TA98, TA100,TA102, and TA104	Heavy metals and phenolics	Tabrez and Ahmad (3)
Industrial waste waters of Agra and Aligarh (India)	Differential survival in *recA*, *lexA*, *polA* mutants of *E. coli* K-12 and plasmid nicking assay	N.R	Siddiqui et al. (51)
Industrial waste waters of Aligarh and Yamuna river water (India)	*A. cepa* phytotoxicity and genotoxicity test	Heavy metals	Siddiqui et al. (35)

N.R.: Not reported.

different concentrations of untreated wastewater in Manisa, Turkey on the *A. cepa* root meristems exhibited various chromosomal aberrations such as high frequency of lagging chromosome, irregular distribution, polar slips, horizontal division, and sticky chromosome [76]. One study from Egypt reported a very high level of mutagenicity in the water samples collected from 4 different locations by *A. cepa* assay and that the presence of heavy metals in that test water samples might be the reason for the observed genotoxicity [77]. The two industrial effluents, derived from textiles and paints plants, induced chromosomal aberrations in root tip cells of *A. cepa* with vagrant chromosome, bridges, and fragments [78]. *Allium cepa* genotoxicity test performed on different concentrations of industrial effluents contaminated with azo dyes revealed a mutagenic effect of the effluent at concentration range 10% to 100%. However, no chromosomal aberrations were observed at concentrations lower than 10% [79]. *A. cepa* test performed on petroleum refinery effluents suggested that even after the treatments (physicochemical, biological, and stabilization pond), the final refinery effluent could induce chromosome aberrations and micronucleus in meristematic cells. Hence, the discharge of petroleum refinery effluents in the Atibaia river was suggested to interfere in the quality of river water [80]. The untreated industrial and municipal wastewaters from Drava River, Solvenia revealed sublethal and even lethal effects by *A. cepa* test [81]. In one study, water samples from 3 sites in the Córrego dos Bagres stream in the Franca municipality of the Brazilian state of São Paulo were subjected to the comet assay. The greatest DNA damage was recorded with water from a chromium-containing tannery effluent.

The mutagenicity of the water samples was also assessed by *A. cepa* genotoxicity assay, and the most frequent chromosomal abnormalities observed were c-metaphases, stick chromosome, chromosome breaks and losses, bridged anaphases, multipolar anaphases, and micronucleated and binucleated cells. Onion root-tip cell mutagenicity was also found to be the highest for water samples containing the highest levels of chromium [82]. Seven of 9 water samples collected along the river Rasina, Serbia were found to be significantly genotoxic using the *A. cepa* anaphase-telophase test by Vujošević and associates [83]. In one study, cytotoxicity and genotoxicity assays using the *Allium cepa* test-system were carried out to evaluate the effects of domestic and industrial effluents in the Monjolinho River, Brazil in different seasons of the year. In the summer and intermediate seasons, chromosome aberration, micronuclei, cell death, and inhibition of the mitotic index were observed in water samples collected at different sites. In the winter, either chromosome or cellular alterations was not observed. From chemical analysis of test water sample, it was inferred that the excessive concentration of heavy metals like Pb, Ni, and Cu were the culprit for the effects observed in *A. cepa* cells [84].

The sample from Walnut Creek from the Troy Wastewater Treatment Plant (TWWTP) was assayed for mutagenicity by Ames fluctuation test. Their

results indicate that Walnut Creek contains both mutagenic and promutagenic compounds, and TWWTP exhibit mutagenicity that may be refractory to or created by treatment processes [85]. Ames plate test performed on surface water samples from Taihu Lake, eastern China showed that some of the samples had the highest genotoxicity with or without S9 fraction. The mutagenic effect included, at least, two different molecular mechanisms: nucleotide point substitution on DNA molecules and frame shifting caused by nucleotide insertion or deletion [86]. Vargas and colleagues [87] reported that the highest mutagenic response on TA98 without metabolic activation incase of 100 nonconcentrated samples collected from Caıá River, Brazil, an area under the influence of a petrochemical industrial complex. However, the genotoxic potency of the test samples for both TA98 and TA100, in the presence and absence of S9 fraction, was found to be very high.

Aleem and Malik [88] performed different genotoxicity tests on Yamuna River at Mathura (UP), India. The results of *Salmonella* test demonstrated that the XAD-concentrated water samples had maximum mutagenicity with the TA98 strain, both with and without metabolic activation. In *E. coli* survival assay, all the test mutants invariably exhibited significant decline in their colony-forming units compared with their isogenic wild-type counterparts suggesting damage to the DNA of exposed cells as well as the role of functional *rec A*, *lex A,* and *polA* genes in coping with the hazardous effect of the pollutants present in Yamuna river water. Aleem and Malik [89] also performed different genotoxicity tests on Yamuna River at Okhla (Delhi), India. The results of Ames test revealed that the XAD-concentrated water samples had maximum mutagenicity with the TA98 strain in the absence and presence of S9 fraction. However, the liquid-liquid-extracted water samples were also found to be mutagenic but to a lesser extent compared with XAD extracts. The damage brought about in the DNA repair-defective mutants in the presence of XAD-concentrated water samples was also found to be markedly high compared with that of liquid-liquid-extracted water samples. Here again *E. coli* K-12 DNA repair defective mutants exhibited significant declines in survival compared with their isogenic wild-type counterparts. Gas chromatographic analysis of liquid-liquid extracted water samples showed the presence of the pesticides like DDT, BHC, dieldrin, endosulfan, aldrin, 2,4-D, dimethoate, methyl parathion, and malathion in the range of ng/L. Genotoxicity test performed on wastewaters of Ghaziabad City, India, also revealed similar results. Here the tested water samples exhibited significant mutagenicity with TA98, TA97a, and TA100 strains and survival of *polA* mutant of *E. coli* K-12 strain was found to be declined up to 81.7%. The probable role of contaminating pesticides in the wastewater was suggestive for the observed genotoxicity [90]. In the presence of XAD-concentrated tannery effluent collected from Kanpur, India, TA98 strain was reported to be the most sensitive strain in terms of mutagenic index followed by TA97a, whereas in terms of mutagenic potential,

TA102 was recorded to be the most responsive. The liquid extracts were also genotoxic in terms of survival of *E. coli* K-12 mutants, suggesting the presence of DNA-damaging compounds in the tannery effluents [91]. The result was confirmed by the GC-MS analysis of the test water samples, which showed the presence of various well-known genotoxicants like diisooctylphthalate, phenyl methyl carbamate, dibutylphthalate, bis 2-methoxy ethylphthalate, and higher alkanes.

The mutagenicity testing study on industrial wastewaters from Aligarh region conducted by Malik and Ahmad [92] revealed the mutagenicity vis-à-vis Fe^{++}, Zn^{++}, Cu^{++}, Cr^{++}, and Ni^{++} contaminants present in the test samples. Moreover, *Salmonella typhirmuriurm* strains, TA102 and TA104, were found to be the most sensitive strains both in the absence and presence of S9 fraction. A significant decrease in the survival of DNA repair defective *Escherichia coli* mutants *recA, lexA*, and *polA* was also observed compared with their wild-type counterparts as a result of wastewater treatment. A genotoxicity study conducted by Rehana and associates [93] on the Ganga River at 3 different locations viz. Kachla, Fatehgarh, and Kannauj (U.P.) revealed the presence of some pesticides such as DDT, α-BHC, aldrin, dieldrin, dimethoate, and methyl parathion in the range of ppb. Ames test performed on XAD extracted test water samples exhibited a remarkable degree of mutagenicity with TA98, TA100, and TA97a strains with the probable role of contaminating pesticides in the river water. In another study performed on the Ganges River at Narora (U.P.) by Rehana and colleagues [94] XAD concentrated water sample exhibited a significant degree of mutagenicity with TA102, TA100, and TA98 strains of *S. typhimurium* both in the presence and absence of S9 fraction. A significant decrease in the survival of DNA repair defective *E. coli* mutants *recA, lexA,* and *polA* was also reported compared with their wild-type counterparts. The genotoxicity of 3 water bodies, viz. industrial wastewater of Aligarh city, ground water pumped out from the industrial area of Aligarh, and river water of Yamuna, downstream of Agra, India was evaluated by plate incorporation test and fluctuation test by Siddiqui and Ahmad [10]. All the test samples were found to be significantly mutagenic in both the testing systems wherein TA98, TA102, and TA104 strains were found to be maximally sensitive in plate incorporation assay while TA98 and TA100 strains were the most responsive strains in fluctuation test. The genotoxicity of industrial wastewater samples from Aligarh (AWW) and Ghaziabad (GWW) cities, India was confirmed and compared using Ames plate incorporation test, Ames fluctuation test, and *Allium cepa* test by Fatima and Ahmad [2]. The mutagenicity of the test samples was partly mediated by reactive oxygen species as evidenced by the use of various free radical scavengers. TA97a and TA98 strains were found to be the most responsive strain in both samples. Both the test water samples also induced various anaphase aberrations in the root cells of *Allium cepa*. Fragmentation of the chromosome was the predominant effect of the Aligarh water

sample, while the Ghaziabad sample induced chromosome stickiness. The crucial role of heavy metals and pesticides in the genotoxicity of AWW and GWW, respectively, was suggested in this study. Tabrez and Ahmad [3] compared the genotoxic potential of industrial wastewater collected from Aligarh (AWW) and Saharanpur (SWW) by Ames plate and Ames fluctuation test. They reported higher mutagenicity of SWW compared with AWW by both the testing system.

The bacterial mutagenicity on organic extracts from industrial effluents and Labe river water showed a dose-dependent increase in numbers of TA98 revertants. However, the cytogenetic effect in human peripheral lymphocytes in vitro was found to be insignificant [95]. In one study, water from the Porsuk River was investigated for their potential mutagenicity by Ames plate incorporation assay. A positive result in XAD extracts of water samples was obtained for TA98 at two stations. The presence of mutagens causing frameshift and base-pair substitution mutations in water of the Porsuk River was suggested [96].

Sanganer town, Rajasthan, India is famous worldwide for its dyeing and printing industries. There are about 400 industries involved in textile printing processes, which discharge effluents into nearby ponds and drains without any treatment. Therefore, to assess the possible genotoxic health risk and environmental genotoxicity due to the textile industry effluents, a study was carried by Mathur and associates [97] using the Ames mutagenicity assay. Their results clearly showed a high mutagenic potential of the tested effluents. A study conducted on Chao Phraya River, which is connected with canals of Bangkok, Thailand, and Japan, revealed the presence of some frameshift mutagens in this water sample [98]. Toxicology analysis on industrial discharge of Tucuman, Argentina revealed the phytotoxic and genotoxic nature of effluents in Allium roots. Micronucleus and anaphase aberrations were observed in *A. cepa* roots; however same test water sample did not exhibit mutagenic effects on *S. typhimurium* TA98 and TA100 strains with and without metabolic activation [99]. An evaluation of the genotoxic potential of different wastewaters viz. industrial, hospital, and domestic wastewater collected in the Rouen area was performed with Ames fluctuation test by Jolibois and Guerbet [100]. The results of their study showed that different types of wastewaters pose a different genotoxic risk. Organic fraction of drinking tap water from Seoul, Taejon, and Suwon was tested for mutagenicity in *S. typhimurium* strains TA98 and TA100 in the presence and absence of S9 mix. The extracts of the water from all 3 cities were reported to be mutagenic in TA 98 without S9 mix and in TA 100 with and without S9 mix. The chlorination process was attributed to the observed genotoxicity of the tap waters [101]. Grifoll and colleagues [102] performed a mutagenicity assessment of the dissolved and particulate phases of the Besos and Llobregat rivers, which flow along populated and industrialized basins near Barcelona, Spain. Both rivers share domestic, industrial, and agricultural uses and are recipients of a large amount of untreated effluents. The

results indicated that both rivers were highly polluted by base substitution and frameshift mutagens and promutagens. Interestingly, Guzzella and associates [103] compared the mutagenicity of water samples from the Como Lake, Italy, and tried to find the source of water genotoxins. Highest mutagenic potency was found with TA98 in the presence of S9 mix in one water sample collected near the river mouth, and the authors concluded that river influent was an important source of mutagenic contamination. However, the lake water samples did not show any genotoxicity both with Allium root anaphase aberration assay and Allium root micronuclei assay.

In a study of the Rio Tercero River, Argentina by Alzuet and associates (104), the presence of S9-activated mutagens capable of causing base substitution and frameshift mutations was observed. The region is a heavily industrialized area and houses the main oil refinery in the country, several petrochemical industries, a rolling steel mill, and a sulfuric acid plant.

Comet assay is a powerful and highly sensitive method of evaluating primary DNA lesions. This assay was performed on planarians exposed to Diluvio's Basin (Porto Alegre, RS, Brazil) water for 13 days to screen possible DNA damages [105]. Their results indicated an increasing gradient of DNA damage toward basin's mouth. Such a gradient of DNA damage could be related to the gradual increase of pollutants among the different sample sites. Moreover, there was a correlation between the urbanization gradient that exists within the watershed and the genotoxicity. Comet assay performed on the leukocytes isolated from the healthy individual exposed with different concentration of lake water extract revealed seasonal variability in the genotoxicity. Moreover, the maximum genotoxicity was reported in the sample, which was collected in the winter season [106]. In one study, water samples from 3 sites in the Córrego dos Bagres stream in the Franca municipality of the Brazilian state of São Paulo was subjected to the comet assay using erythrocytes from the fish *Oreochromis niloticus*. A nuclear abnormality of the erythrocytes was reported that included blebbed, notched, and lobed nuclei, probably due to genotoxic chromium compounds present the water samples. The greatest DNA damage as observed by comet assay was recorded with water from a chromium-containing tannery effluent discharge site [82]. Rajaguru and colleagues [107] investigated the genotoxicity of water samples from the Noyyal River in Tamilnadu, India, using carp (*Cyprinus carpio*) by the comet assay. Immature carp was exposed to water samples collected from the river at 6 different locations. Extensive DNA damage was observed in cells from these organs exposed to polluted water samples, and the amount of damage increased with the duration of exposure. The highest level of DNA damage was obtained with samples taken from immediately downstream of urban centers. One study based on comet assay from Mahal, India reported a significant increase in DNA damage in peripheral blood lymphocytes of human population that consumed contaminated ground water [108].

Some Problems Encountered with the Conventional Tests of the Genotoxicity and Their Remedial Aspects

Sometimes epidemiological and other studies point toward a particular toxicant as the main culprit, thereby directing major efforts toward the toxicology of single substance and forgetting the complexicity of natural system and unconsciously ignoring the natural milieu of exposure. That necessitates the appropriate experiment design for extrapolation of the data under the natural condition. There are some technical problems related with the interpretation of data such as those that use Ames testing in large water bodies [10]. Similarly, sometimes the role of S9 fraction also becomes obscure because one substance may enhance while another reduce the number of his^+ revertants in its presence [3)]. Now imagine a situation in which these two samples get mixed up in the water body, as usually happens in riverine system. All these problems pose a challenge of a fundamental nature that this type of reductionist approach, at least in the field of environmental toxicology, is too simplistic and does not reflect what is happening in a real situation.

Conventionally, the sensitivity of the Ames tester strain in the plate incorporation assay is gauged on the basis of slope "m" of initial linear dose response curve [2, 3, 92–94, 109], and the sensitivity in the fluctuation test is based on the "Mi" values [3, 23]. Both the tests make use of common strains and the endpoints; hence a similar trend in sensitivity is expected for the same sample in both the tests. However, the pattern of sensitivity and thus the best responders were usually different in Ames plate incorporation and fluctuation tests. This disparity was reported by our group in earlier studies [3, 10]. We have suggested that the data in plate and fluctuation tests should be interpreted in terms of Mi values—Mi (p) and Mi (f) for the problem associated with the different mutagenicity testing systems but having the same strains and endpoints [10]. Moreover, in view of the data obtained with 3 samples of high, moderate, and mild mutagenicity, mild mutagenic samples are expected to be better assessed by means of fluctuation test whereas the samples containing potent mutagens are expected to be more precisely estimated by Ames plate incorporation assay [3, 27].

Furthermore, when we came across the problem associated with different compositions of samples but with the same pattern of mutagenicity in the two Ames testing systems, we recommended the analysis of specific reactive oxygen species (ROS) in the test water samples and use of specific ROS scavengers in plate as well as fluctuation test [2]. However, *Allium cepa* genotoxicity test was found to be capable of differentiating the two different samples. This might be due to the presence of a relatively much evolved detoxifying machinery. Moreover, this test targets the mitotic chromosomes and evaluates the chromosomal aberration.

As far as the biomarker studies on surface water were concerned, the studies conducted in our lab proposed some antioxidant enzymes (SOD and GR) and tissue injury marker enzymes (AST and ALT) as a biomarker of toxicity in a rat model [110]. In another study, we recommended EROD (CYP1A1) and PROD (CYP2B1/2) in rats as a biomarker of surface water pollution, which contains a high level of phenolics [111]. However, in the samples that contain a high concentration of metals these enzymes were found to be inhibited. *A. cepa* bulbs treated with a wastewater sample showed continuous increase in GST enzyme activity profiles with increasing concentration of wastewater. The chemical analysis of a test wastewater sample revealed the presence of heavy metals [112]. Another study conducted by Fatima and Ahmad [113] revealed a tremendous rise in EROD activity (up to 68-fold) in the presence of certain pesticides; on that basis they recommended the use of *Allium cepa* derived EROD as a potential biomarker for the presence of certain pesticides in surface water.

Pandey and associates [114] studying on *Wallagu attu* fish collected from Yamuna (India) river water reported that SOD, XOD, GR, and LPO can serve as a biomarker of water pollutants present in the river water. The industrial activity profile indicates vigorous industrial activity coupled with intensive use of chemicals in agricultural practices in the sampling area behind this oxidative stress. Biomarker study on the fish muscle and liver collected from the Kizilirmak River, Turkey contaminated with refinery wastewater strongly suggested SOD and GPx as the biomarkers of petrochemical wastewater toxicity [115]. In a study by Silva and associates [116] rats were orally fed with TieteÂ river water. Their results indicated the increase in LPO, creatinine, glucose, alanine transaminase, and amylase levels in the serum of animals that reflected the toxic effects of river-water contamination. From the available literature, it is quite clear that a lot of work on toxicity biomarkers had been performed on fish collected from polluted water samples. Ours was the first group who worked on a biomarker study on surface water especially in rat and/or *A. cepa* tissue models. Some biomarker studies on surface waters have been summarized in Table 2.

Based on our review, we recommend stricter water quality regulations, especially in India, which have already been promulgated in many countries throughout the world. Due to its biological significance, genotoxicity and biomarker study should be the main focus for pollution biomonitoring, mainly due to the increasing complexity of the chemical environment.

Best Way Out in Light of Current Knowledge

In light of our experience during the last two decades, we recommend the 3 bioassays in the following order for the estimation of surface water mutagenicity and one new approach—biomarker coupled genotoxicity test.

Table 2: Various Biomarker Studies Performed on Surface Water Samples

Source Sample	Proposed Biomarker	Tissue/Organ	Chemical Analysis	Reference
Tiete river water, Brazil	LPO	Serum of rats	Nitrogen, Phosphorus, and Iron	Silva et al. (116)
Industrial wastewater of Aligarh region (India)	SOD, CAT, GR and GST	*A. cepa* homogenates	Heavy metals	Fatima and Ahmad (112)
Industrial wastewater of Ghaziabad (India)	EROD	*A. cepa* homogenates	Pesticides	Fatima and Ahmad (113)
Industrial wastewater of Saharanpur and Aligarh (India)	SOD, GR and LPO	Liver and kidney of rats	Heavy metals and phenolics	Tabrez and Ahmad (110)
Industrial wastewater of Saharanpur and Aligarh (India)	EROD	Liver and kidney of rats	Heavy metals and phenolics	Tabrez and Ahmad (111)
Kizilirmak river contaminated with petroleum waste (Turkey)	SOD, GPx	Fish muscle and liver	N.R	Avci et al. (115)
Paper mill (India)	CAT	Fish liver	Metals	Ahmad et al. (67)
River water from Yamuna (India)	SOD, GR, XOD, and LPO	Fish gill, liver, and kidney	Various physico-chemical analysis	Pandey et al. (114)

N.R.: Not reported.

1. *Allium cepa* genotoxicity test
2. Ames plate incorporation test or Ames fluctuation test depending on the genotoxic potency of the test sample but using the "specific S9 fraction" obtained from the rat liver induced by the test sample itself.
3. *In vitro* human lymphocyte comet assay with validated protocol in the presence and absence of "specific S9 fraction."

Biomarker Coupled Genotoxicity Test: A New Approach

Aroclor-1254 induced rats are commonly used for preparation of S9 fraction. This xenobiotic metabolizing machinery is supplemented in the Ames testing for mutagenicity. However, S9 obtained from the test xenobiotics induced rats alone would reflect the real in vivo response by the test sample in the animal system, and this is the "largest set of modulated molecular machinery" and obviously the best candidate of toxicity biomarkers. If this "specific S9 fraction" is used for genotoxicity testing, it would be appropriately called a "biomarker coupled genotoxicity assay." It is well known that Aroclor-1254 does not induce the CYP2E family of oxidases, and several carcinogens including certain nitrosamines will not be evaluated as potent genotoxins in this case.

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