

Humic Substances: Structure, Function, Effects and Applications

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Abstract: Humic substances (HS) are organic matters formed during the physical, chemical and microbiological transformation process of dead tissue of animal and plant. It is present ubiquitously in soil, sediments and aquatic environment. HS contain carbon, hydrogen, oxygen and nitrogen with small amount of sulfur. These elements are always present regardless of their origin and country. The macromolecular structures of HS are highly sensitive to different chemical conditions of solution. Their reactivity in an environment depends on the functional groups, macromolecular structure and concentration. HS exhibit dual aspects. The presence of Humic Acid (HA) in a water supply is undesirable as they raise total organic pollution level, colour intensity, distinct absorbing capacity and chemical activities. They have capability to induce cytotoxicity for many mammalian cells and are cause factor of many diseases. On the other hand specific properties of HA products enable their application in agriculture, industry, environment and biomedicine.

Key words: Humic substances, humic acid, fulvic acid, molecular spectroscopy, dual aspects.

Introduction

Humic substances (HS) are natural products, which constitute most important pool of transient refractory organic carbon in the geosphere. They are present ubiquitously in soil, sediments and aquatic environment. They are composed of carbon, hydrogen, oxygen, nitrogen and sulfur. These elements are always present in HS regardless of their origin and country or continent (Rocha et al., 1999; Davis et al., 2001; McDonnell et al., 2001). They are yellow to black in appearance, acidic, polydisperse (having different composition and molecular weights), polyelectrolyte, paramagnetic, with extremely complex structure and unique biological and physicochemical features, and finally hit a diagenic state where it is difficult to change them further. Both their dark colour and paramagnetism most likely result from the electronic structure of their core: aromatic subunits, an extended (π -electron) system in the semi-quinone

moieties and the electron donor-acceptor nature (Senesi et al., 1991; Cheng-Fang et al., 1999).

HS are most important as solar light absorbers. They contain chromophores that have capacity to absorb a wide spectrum of electromagnetic radiation in X-ray, UV, visible and IR range. This property enables them to radiate heat to the environment and also can act as a photoactive agent. A variety of methods in fluorescence spectroscopy have been applied as useful means to determine physicochemical characteristics of HS. Phenomena such as fluorescence, delayed photoluminescence and chemiluminescence of HS, as well as HS-photosensitized detoxication of xenobiotics in aquatic ecosystems, prove that electronic excited states and free radicals are generated in HS molecules or in their photoreaction products (Senesi et al., 1991; Etsu et al., 2000; Ceyda and Miray, 2005).

HS are known to be of high molecular mass polyhydroxycarboxylates which contain poly-aromatic

and aliphatic subunits. The degree of ionization of these macromolecules is governed by the amount of ionized phenolic and carboxylic groups, which is a function of pH of the solution (Arno, 2002). Their most common classification is based on the solubility at different pH values such as Humic Acid (HA), insoluble at pH < 2 (under acidic condition); Fulvic Acid (FA), soluble at all pH values and Humin (Hu) insoluble at all pH values (Davis et al., 2001; Karen et al., 2005).

Supra-molecular structure of HS has abundant spongy micro spaces with statistically average dimension (0.24–0.48 nm) which resemble molecular sieves that ensure efficient sorption of low molecular components like organic xenobiotics, herbicides, pesticides, metal ions, etc. Absorption of oxygen molecule, that has diameter of 0.127 nm, easily penetrates into polymer network. The interaction of light with HS produces reactive oxygen species (ROS), which are responsible for sensitized (photodynamic) processes in surface waters. On the other side HS reveal photo-reactive role and a high antioxidizing and free radical scavenging capacity (Danuta et al., 2002; Ceyda and Mİray, 2005).

HA is one of the most important forms of HS. HA plays an important role in various fields such as agriculture, industry, environment and biomedicine. On the other side they can raise aquatic environment pollution, induce cytotoxicity for many mammalian cells and induce growth retardation and apoptosis of fibroblasts (Danuta et al., 2002; Kuo-Jang et al., 2003; Mei-Ling et al., 2003). These important properties of HA have attracted attention of many investigators and the results over the years have brought new understanding on their structure and physicochemical properties. The aim of this article is to review the current state of understanding of HS and the awareness of their availability in the environment addressing human health. Although a lot of efforts have been made to study the formation process and the molecular structure of HS, the understanding of these processes is still the subject of long-standing and continued research.

Characterization of Humic Substances Using Various Techniques

It has been known that HS are a form of environmental organic matters that are formed during the physical, chemical and microbiological transformation process (humification) of dead tissue of animal and plant. It is not made up of discrete, well-defined molecules but is a class of substances that are produced and reside in terrestrial (soil organic matter (SOM)) and aquatic

(natural organic matter (NOM)), forming a major component of both the soil and aquatic carbon pools (Rocha et al., 1999; Davis et al., 2001). Approximately 80% of the total carbon in terrestrial media and 60% of the carbon dissolved in aquatic media are made up of HS (Eladia et al., 2005).

Some theories have been used to explain the origin of HS such as the sugar-amine condensation theory and the lignin theory or the polyphenol theory. Currently, most investigators suppose that HS have origin in lignin (Oglesby et al., 1967; Amber and Sonnenberg, 1999; Eladia et al., 2005). Polyphenols come mostly from lignin during its biodegradation and probably play a key role in the formation process. Various techniques are being used to characterize HS. Following is the review of these studies based on the origin of these substances such as aquatic and soil.

Aquatic Humic Substances

Aquatic HS are the large organic molecules formed by micro-biotic degradation of biopolymers and polymerization of small organic molecules that are derived from soil humus, terrestrial and aquatic plants (Rocha et al., 1999). Dissolved HS are the main constituents of the dissolved organic carbon (DOC) pool in surface water (fresh water and marine water), ground water and soil pore water.

HS in natural waters are polyelectrolyte with generally irregular structure and wide range of molecular weights. Many researchers have reported the properties of aquatic HS. The presence of carboxylic, phenolic and carbonyl groups give aquatic HS a high capability for the complexation of metal ions and other various compounds present in the water (Davis et al., 1999; Evangelou and Marsi, 2001; Ceyda and Mİray, 2005). This chemical behaviour significantly influences the transport, distribution and accumulation of metals in aquatic environments. Capillary electrophoretic methods (CZE, CIEF) are useful tools for the analyses of the electrophoretic behaviour of anionic polyelectrolyte of HS that correspond to the charge density distribution governed by their molecular size and acidities (Havel et al., 1998; Schmitt-Kopplin et al., 1999). Using high-pressure size exclusion chromatography method (HPSEC), with UV-Vis detector, in the range 220–280 nm, molecular weights of HS from variety of aquatic and terrestrial environments can be determined (Edward and Yu-Ping, 2001). Evaluation of the molecular size distribution data using UV-Vis and fluorescence spectroscopic analysis, photocatalytic (TiO₂/UV) degradation of HA revealed the formation of lower

molecular size (small fractions) and higher UV absorbing compounds (Danuta et al., 2002; Ceyda and Miray, 2005).

Stumm (1992) proposed that HS have both acidic functional groups (carboxyl and phenol) and hydrophobic moieties. Due to carboxyl groups, HS may participate in ligand exchange or electrostatic interaction with oxide surfaces. Hydrophobic properties allow HS to accumulate on surfaces because of their incompatibility with water, which is more polar than their surface (Earl and Ronald et al., 1981).

The molecular spectroscopy investigations (^1H -NMR, ^{13}C -NMR and UV-Vis) of aquatic HS indicate the presence of high aromaticity with small substitution and small amount of carboxylic carbon. The absorbance ratio, E_{350}/E_{450} , suggests the presence of relatively large proportions of aliphatic structures. Molecular size fractionation revealed that the metal traces have different size distributions, such as Mn, Cu, Cd and Ni, which were preferably found in the high molecular-weight fractions (10-100 kDa) (Rocha et al., 1999; Etsu et al., 2000). The humification degree of HS obtained from fluorescence spectra, decreases in the order of $\text{HA} > \text{FA} > \text{HS}$. The selective interaction between aromatic structures of HS and chlorine in the region of longer wavelength probably contribute for the formation of the intermediates as trihalomethanes (THM) (Earl and Ronald et al., 1981; Eduarda et al., 2004).

Simultaneous determination of the concentration and molecular weight of HS by gel chromatography with fluorescence detector, obtained by the calibration curves, were linear in the concentration range of 0.1 to 10 ppm ($\text{SD} < 10\%$). The concentrations of aquatic HS in rivers were high in the summer and low in the winter and the ratio of the higher molecular weight was larger in the warmer season than in the cooler season (Etsu et al., 2000).

Soil Organic Matter

Compost is an end product of degradable compounds, intermediate breakdown products and dead microorganism cell walls that are classified as HS. The macromolecular structures of HS are highly sensitive to different chemical conditions of a solution. Their reactivity in the environment primarily depends on their functional groups, macromolecular structure (size, shape) and concentration induced by the composition of reacting media (solution, pH, interacting solid matrix) (Charles and Irwin, 1982; Evangelou and Marsi, 2001; Ladislau et al., 2001). The complexation of calcium by HA and FA was detected at slightly acidic, neutral and basic pH, and complexation power was favoured by increasing pH

(Quatmane et al., 1999). Strength of metal-ion humic complexes followed the order: $\text{Cu}^{2+} > \text{Cd}^{2+} > \text{Ca}^{2+}$ (Leenheer, 2004). The complexing ability of FA with Rare Earth Elements (REEs) is higher for Y^{3+} than other REEs (La^{3+} , Ce^{3+} , Sm^{3+} , Gd^{3+}) (Zhimang et al., 2001). The SEM analysis showed that the morphological structures of HA changes with pH variation, increase in aggregation, complexity of the structures, thickness, roughness and reduction of perforations as pH is reduced from 7.0 to 4.0 (Luciano et al., 2002).

In general, HA gives relatively featureless spectra without maxima or minima in the UV and visible region, with the absorbance decreasing with increasing wavelength. The specific absorption at 280 nm (ϵ_{280}) has been used as an index of the degree of aromaticity. Smaller values of ϵ_{280} indicate higher carbohydrate content (Sierra et al., 2004). This wavelength was chosen because a large number of chromatic compounds (phenolic, benzoic acids, aniline derivatives, polyenes and PAH with two or more rings) absorb in this region (270-280 nm). The ratio of the absorbance at 250 and 365 nm (E_2/E_3) has been used in some limnology studies as an indicator for humification, showing that this ratio decreased with increasing degree of humification (Chin et al., 1994). Peuravuori and Pihlaja (1997) have reported a moderate linear correlation between the ratio E_2/E_3 and ϵ_{280} and between E_2/E_3 and aromaticity of HS, e.g. the ϵ_{280} and aromaticity decreasing with increasing E_2/E_3 values. Chen et al. (1977) have reported the ratio E_4/E_6 (E_{465}/E_{665}) related to the molecular weight and the oxygen content of HS. Lowering in ratio E_4/E_6 reflects increase in molecular size and lower proportion of carboxylic acids.

IR spectra of HS were dominated by absorbance mainly due to aromatic, aliphatic, carboxylic and hydroxylic groups. Interpretation of IR spectra is based on the interpretations given by Schnitzer and Khan, Bellamy, Stevenson and Durig et al. (Quatmane et al., 2000). The strong band at $3400\text{-}3300\text{ cm}^{-1}$ reflects the OH-groups of alcohol, phenols and carboxylic acids. The absorption bands at around 2900 and 2800 cm^{-1} are caused by C-H stretching of methyl and methylene groups of aliphatic chains. The bands appear at around 1700 and 1600 cm^{-1} due to stretching frequencies of C=O bonds of carbonyl and carboxyl, and C=C aromatic bonds. A broad band at $1387\text{-}1400\text{ cm}^{-1}$ (COO^- , CH_3), a peak at around 1275 cm^{-1} (C-O stretching of aryl ethers) and broad peak at around $1100\text{-}1040\text{ cm}^{-1}$ (C-O stretch of polysaccharides).

^1H -NMR is also used to estimate the relative contents of aromatic and aliphatic protons in HS. The chemical

shift of 0-4.5 ppm is assigned to aliphatic protons and of 6-8 ppm to aromatic and hetero-aromatic hydrocarbons (Rocha et al., 1999). The peaks from 1.6 to 3.0 ppm can be explained as methyl and methylene groups. Peaks between 3.3 and 4.5 ppm are characterized by proton of carbon bonded to O or N heteroatom.

^{13}C -NMR is another technique used to examine complex organic structures of HS. The direct measurements of the carboxyl, aromatic and aliphatic carbon contents can be made by peak integration of their specific regions. Those are aliphatic (0-105 ppm); aromatic carbons, carbonyl (100-160 ppm) and carboxyl groups (160-180 ppm) (Rocha et al., 1999; Ghita et al., 2003).

The application of FTIR spectroscopy with an internal standard is similar to the current use of ^{13}C -NMR spectroscopy in the investigation of the functional groups composition of HS (Wilson et al., 1987). However, the sample size (5-10 mg) and instrumental analysis time (ten samples/hour) required to obtain an FTIR spectrum is significantly less than for a ^{13}C -NMR spectrum (>100 mg and 3-24 hours/sample) (Davis et al., 1999).

By sequential extraction, the decrease in the atomic ratio (C/H) and an increase in the atomic ratio (C/O) together represented increase in aliphaticity in conjunction with a decrease in polarity and aromaticity. Decreasing ratio (E_4/E_6) indicated an increase in molecular size with increasing number of extractions (Karen et al., 2005). Davis et al. (1999) have reported that E_4/E_6 has a strong relationship with 1600 cm^{-1} IR band that is assigned to the aromatic carbon content of HS.

Total acidity of HS coinciding with the ratio O/C indicates that O-containing functional group of HS contributed to their total acidity. Davis et al. (2001) used the ratio O/C of HS to suggest the origin of HS. He found that ratio O/C of soil HA and lake sediment HA was around 0.50, and those of soil FA and lake sediment FA were around 0.70 and 0.80, respectively. Steelink also reported that HS with lower ratios H/C and O/C have higher degree of polymerization and higher content of aromatic compound. On the contrary, HS have higher content of carbohydrate if the ratios H/C and O/C are higher (Gudrun et al., 2004; Karen et al., 2005). Carboxylic acid functional groups (1700 cm^{-1}) are important factors in determining the ratio O/C of the HS (Davis et al., 1999). Chen et al. (1977) have reported that with increasing composting time and the maturity of HA, there is loss of aliphatic groups and are richer in aromatic groups as more stable HA are formed.

Aliphatic structures in SOM contribute significantly to the increased sorption of organic pollutants (Karen et al., 2005). The higher content of carboxyl groups in HA increases the cation exchange capacity (CEC) in the soil, which is particularly important in process of heavy metal bonding (Debska et al., 2002). HA with hydroxyl-, phenoxyl- and carboxyl-reactive groups can form coordination compounds with metals. The ion-exchange equilibrium method using Dowex AG 50W-X8, 20-25 mesh Na(+) form was used to determine stability constants of complexes formed between HA and metal salts solution at pH 3.5. The stability constant ($\log K$) for different metal-HA complexes indicated the following order of the stabilities for complexes formed between HA and metal ions: $\text{Cu} > \text{Fe} > \text{Pb} > \text{Ni} > \text{Co} > \text{Ca} > \text{Cd} > \text{Zn} > \text{Mn} > \text{Mg}$ (Pandey et al., 2000).

In SOM, the insoluble Hu fraction is dominated. Total acidity and elemental compositions of HA are similar to FA. The elemental compositions, atomic ratios, FTIR and ^{13}C -CPMAS-NMR spectra of the HA and FA extracted from refuse compost were close to those of HA and FA from soil, sediment and water (Senesi, 1989). Using successive gel chromatography, HA with different molecular weight can be separated. The elemental and spectroscopic study showed that the low, medium and the large particle weight fractions of HA are, more or less, of the same quantitative and qualitative chemical composition that can be considered as associations of humic sub-units which are of the same nature (Quatman et al., 2000).

HS appear to be relatively stable up to about 300°C and all the spectral information is lost at 600°C . The metal forms of HS are less stable and decompose at a faster rate than HS. The thermal stability of metal forms is in the order: $\text{Fe(III)} < \text{Mn(II)} < \text{Ca(II)} < \text{Na(I)}$ (Janet and Francois, 1998). The resistance of humic material in soil depends on complex interaction of disordered cross-linked HA macromolecular structure in restricting the performance of soil enzymes. The phenolic structures of HA are most directly related to susceptibility to biodegradation. The molecular size of HA had the greatest influence in the early mineralization stages, whereas the C backbone (the concentration of O-alkyl and alkyl carbons) had a greater influence in the advanced transformation stages (Almendros and Dorado, 1999; Debska et al., 2002). HS inhibits the photolysis rate of 1-hydroxypyrene. The photolysis rate of 1-amonopyrene can be inhibited or enhanced by HS, depending on the type (the source) and concentration. The 1-hydroxypyrene and 1-amonopyrene are carcinogenic

and slightly water soluble polycyclic aromatic hydrocarbons (PAH). The photolysis rate of Atrazine can be enhanced by HA depending on the type and concentration (Kui et al., 2002).

Dual Aspects of Humic Substances

Harmful Effects

On one side the presence of HA in water supply is undesirable for several reasons as they raise the total organic pollution level, colour intensity, distinct absorbing capacity and chemical activities. They are prone to absorb organic and inorganic pollutants to stabilize mineral colloids. They increase photo-degradation of contaminants and decrease biological uptake and toxicity. Also it leads to the formation of biodegradable organic compounds during ozonation and enhance growth of microorganisms within the water distribution systems (Almendros and Dorado, 1999). HS are known to complex with trace metals (Davis et al., 1999; Rocha et al., 1999). They are implicated in the complexation/solubilization of pesticides and hydrocarbons in the aquatic environment (Davis et al., 1999; Kui et al., 2002). Besides these aspects, reactions between aquatic HS and halogen-based oxidants during water chlorination process can cause the formation of THM with potential carcinogenic effects (Philip, 1993; Yamada et al., 1998).

The water insoluble compounds, PAH, that are present in HA molecule, are a group of organic compounds which are toxic and many of them are known as carcinogenic and mutagens (Kui et al., 2002; Kuo-Jang et al., 2003). Also other water insoluble compounds such as amines, etc. could play a key role in the genotoxicity of the humic matter in the compost (Mei-Ling et al., 2003; Claudio et al., 2005). The US Environment Protection Agency (EPA) also lists them as priority pollutants (Campabell and Tessier, 1987).

It has been reported that HA has been shown to play a key role in the fate and transport of both organic and inorganic contaminants and strongly interact with toxic heavy metals to form partly water-soluble metal-organic complexes (Lu et al., 1997; Pinheiro et al., 2000; Evangelou and Marsi, 2001). Also it has been reported that HS absorb solar energy mostly between 300 and 500 nm. Absorption in this region can initiate a number of photochemical processes; producing ROS (e.g., hydrogen peroxide, singlet oxygen and super oxide) that are responsible for sensitized (photodynamic) processes in surface water (Danuta et al., 2002; Ceyda and Miray, 2005).

HA have been reported having capability to induce cytotoxicity for many mammalian cells. The redox properties are shown to be capable of reducing iron(III) to iron(II) in aqueous conditions over a broad range of pH (4.0-9.0). Also it is capable to reduce and release iron from ferritin storage to promote lipid peroxidation, then disturb the redox balance and elicit oxidative stress within a biological system (Kuo-Jang et al., 2003). HA has been implicated as a causal factor for goitre (Huang et al., 1994; Mei-Ling et al., 2002; Peter et al., 2003), bladder cancer, hepatoma, vascular disease and diabetes mellitus, Blackfoot disease and an endemic peripheral vascular disease in the southwest coast Taiwan (Lu et al., 1990; György, 2001) and Kashin-Beck disease, a chronic osteoarthritic disorder with necrosis of chondrocytes, prevailing in mainland China (Zhai et al., 1990; An et al., 1999).

Mei-Ling et al. (2003) have reported that HA induces oxidative DNA damage, growth retardation and apoptosis of fibroblasts in human. This suggests that HA-induced dysfunction of fibroblasts is implicated in the pathogenesis of Blackfoot disease.

Useful Applications

On the other side the specific properties of HS products enable their application in industry, agriculture, environmental and biomedicine. In agriculture field, HS play an important role in influencing significantly the quality and productivity of the soil. HA is one of the most important components of HS that helps to break up clay and compacted soils, assist in transferring micronutrients from soil to plants, enhance water retention, and increase seed germination rates. It slows down water evaporation from soils especially in soils with incapability to hold water such as soils in which clay is present at a very low concentration, in arid areas and in sandy soils (Eladia et al., 2005). The higher content of carboxyl functional groups in HA increases the cation exchange capacity in the soil, which is particularly important in process of heavy metal bonding (Evangelou and Marsi, 2001; Debska et al., 2002).

Many scientists have shown that beneficial microbes common to rich humus soils produce various anti-pathogenic substances for tuberculosis. They are dispersed throughout the interior of all raw HS, with the ratios, types and species consistent with the various types of humates (Mei-Ling et al., 2003). The indirect effects of HS are important as they integrate iron to the chelates and make it available to plants. Increasing the organic matter content of soils improves aeration, moisture retention, soil structure and has been found to reduce

plant diseases and control weeds more efficiently than commercial fertilizers and herbicides (Alexander, 1999; Debska et al., 2002).

HA enhance the root growth of maize (*Zea Mays*) seedlings in conjunction with a marked proliferation of sites of lateral root emergence and stimulate the plasma membrane H^+ -ATPase activity (Luciano et al., 2002). The presence of polyamines (plant regulators) may explain the hormone-like activity of HS (Young and Chen, 1997; Debska et al., 2002). Chi-Ming et al. (2004) have reported that HS stimulate the activities of both chlorophyllase a and b. FA stimulates more chlorophyllase a activity than either HA or Hu. HA stimulates more activity of chlorophyllase b than either FA or Hu.

Currently, humic materials are used as additives in fertilizers. Different salts of HS, such as calcium humate, sodium humate and ammonium humate were used to increase soil fertility (Mei-Ling et al., 2003; Eladia et al., 2005).

In industrial field, humic material has been used for various purposes such as additives to control the setting rate of concrete; to prepare a natural indigo to dye wood veneer; as additives to enhance the mechanical strength of ceramics; to colour clay tiles; as an agent for tanning leather and has numerous applications in the paper industry too (Eladia et al., 2005).

In environmental applications, the main task of HS in environmental chemistry is to remove toxic metals, anthropogenic organic chemicals and other pollutants such as herbicides, fungicides, insecticides, nematicides, dioxins and also some pharmaceutical products like estrogenic compounds from water, as they form water-soluble complexes with many metals including radionuclides (Lubal et al., 2000; Pacheco and Havel, 2001).

In biomedical applications, US Government report shows that there is a direct correlation between natural soil substances (humus, HA, FA) and the absence of tuberculosis in humans. This report and many other studies reveal that certain humic extracts, particularly FA, contain a magnificently powerful spectrum of natural micronutrients, phytochemicals, antiviral and antibiotic-like agents that directly inhibit and destroy disease pathogens, while fortifying and regulating the immune system. These extracts, although undetermined earlier, are considered potent as penicillin in equally small amounts (Martin et al., 1959; Gadzhieva et al., 1991).

In the present time increasing attention devoted to HA can be explained by their antiviral, profibrinolytic, anti-inflammatory, and estrogenic activities and heavy metal-binding activities of HS presents new possibilities

for their medical applications (Schmitt-Kopplin et al., 1999). Many medical studies show that HS, typically FA, has the power to provide protection against cancer and related cancer-causing viruses. Studies often show reversal of deadly cancers and tumours using special HS therapies (Gadzhieva et al., 1991; Joone et al., 2003).

Using the pharmaco-biological tests and modelling of the diseases it has been shown that sodium humate has a high antitoxic effect. A possibility to use it in medicine, veterinary science and animal husbandry as nonspecific pharmaceutical raising the organism resistance to the action of different unfavourable factors has also been proposed. The chemical properties of certain defined HA products enable their application in diseases of the digestive system of mammals when combined with metabolic disorders, especially in rearing age. The simple administration (via feed), their exceptional safety and the absence of side effects (e.g. allergy, resistance), as well as no residue formation in animal-derived products, allow a broad application of these substances in veterinary medicine and ecotoxicological aspects (Kuhnert et al., 1989; Lotosh, 1991; Ferrara et al., 2004; Trckova et al., 2005).

Conclusions

The humic substances (HS) exist in three forms such as Humic Acid (HA), Fulvic Acid (FA) and Humin (Hu). The study of humic substances forms an important component in relation to environment and health. The proper understanding of the structure, function and applications of humic substances will be helpful in controlling the pollution as well as benefit the agriculture development and production of new drugs.

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Contents

| | |
|--|-----|
| Monthly Frequency Distribution of Severe Tropical Cyclones—A Statistical Approach <i>Sutapa Chaudhuri and Anindita De Sarkar</i> | 1 |
| Preliminary Study for the Arsenate Removal in a Synthetic Wastewater by Acclimated Cultures <i>Ming-Cheng Shih and William A. Weigand</i> | 7 |
| Water Supply and Sanitation Condition of Slum Areas in Dhaka City <i>Md. Shahjahan Kaisar Alam Sarkar and Md. Mafizur Rahman</i> | 13 |
| Electrical Resistivity Survey to Delineate Groundwater Potential Zones in Granitic Terrain, Nalgonda District, India <i>Ratnakar Dhakate, B.C. Negi and V.S. Singh</i> | 17 |
| Transition Metals in Decomposing Macrophytes in a Wetland System <i>P.A. Azeez and B. Anjan Kumar Prusty</i> | 27 |
| Field Laboratory Studies on Short-term Paddy Crop in Semi-arid Region <i>G. Venkatesan, N. Venkat Kumar, M. Tamil Selvam, G. Swaminathan and S. Krishnamoorthi</i> | 37 |
| Interaction of Polychlorinated Biphenyls with Dissolved Humic Acid from Azraq, Jordan <i>Mahmoud A. Alawi, Fawwaz Khalili and Jafar Abd Elgani</i> | 45 |
| Assessing Radiometric Parameters at a Continental Global Atmosphere Watch (GAW) Station, Nagpur, in India <i>Jayanta Sarkar</i> | 49 |
| Use of Back-propagation Artificial Neural Networks for Groundwater Level Simulation <i>Azhar K. Affandi, Kunio Watanabe and Haryadi Tirtomihardjo</i> | 57 |
| Self Sufficiency of Water in Mainstream Housing - An Australian Experience <i>D.A. Luxmoore, M.T.R. Jayasinghe and M. Mahendran</i> | 67 |
| Environmental Problems Associated with the Paint Sector in Pakistan and their Assessment <i>Muhammad Abid and J.A. Chattha</i> | 75 |
| Concentration and Enrichment Factor of Trace Metals in the Coal Electric Power Station Sediments of Kapar, Selangor, Malaysia <i>Azlina Shafie, Zaharuddin Ahmad, Masni Mohd Ali and Che Abd Rahim Mohamed</i> | 81 |
| ❑ <i>Research Notes</i> | |
| Determination of Persistent Pesticide Residues in Ground Water of Agra Region Using Solid Phase Extraction and Gas Chromatography <i>Niti Sharma and Alka Prakash</i> | 91 |
| Standardization of Sampling Method for Physical Characterization of Municipal Solid Waste <i>J.A. John Paul and Thilagavathy Daniel</i> | 95 |
| ❑ <i>Scientific Note</i> | |
| Mumbai's Natural Calamity: Rain and Suffering - 2005 <i>M.H. Fulekar and M. Geetha</i> | 99 |
| <i>Letters to the Editor</i> | 103 |
| ❑ <i>Book Review</i> | |
| Air Pollution – A Global Problem: Some basic facts | 105 |
| <i>Calendar of Events</i> | 106 |
| <i>Environment News Futures</i> | 107 |