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AEROBIC DEGRADATION OF NAPHTHALENE, FLUORANTHENE, PYRENE AND CHRYSENE USING INDIGENOUS STRAINS OF BACTERIA ISOLATED FROM A FORMER INDUSTRIAL SITE

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ABSTRACT

Four bacterial strains were isolated from a former industrial site contaminated with organic and inorganic pollutants for decades. The isolation was done using naphthalene as sole source of carbon and energy during the enrichment. 16S rRNA gene sequence analyses of the four isolates (OC1, OC2, OC3, and OC4) assigned the strains to the genus, Enterobacter (OC1) and Pseudomonas (OC2, OC3, and OC4). The degradation and growth behavior of the four isolates was investigated on naphthalene, fluoranthene, pyrene and chrysene. All the strains utilized naphthalene, fluoranthene, chrysene but pyrene partially, as sole sources of carbon and energy. The time course studies using relative concentration > 100ppm, >115ppm, > 89ppm and > 12 ppm for naphthalene, fluoranthene, pyrene and chrysene respectively, resulted in rapid exponential increases in cell numbers and concomitant disappearance of the test substrates. Naphthalene was degraded between the range of 25 % and 99%, while chrysene degradation ranged between of 35 and 69%, pyrene 4 - 21% and fluoranthene 7 -19 %. Our results suggest that contaminated, former industrial sites contain a capable microbial community that may be used for bioremediation of the site.

Keywords: 16S rRNA, contaminated sites, bioremediation, napthalene, fluoranthene, pyrene, chrysene

INTRODUCTION

Soil contaminated with polycyclic aromatic hydrocarbons presents a considerable public health hazard (Cerniglia and Heitkamp, 1989; Samanta *et al*., 2002), particularly where other pollutants are also present. The problems are compounded when heavy metals are present in significant concentration (Springael *et al*., 1993; Sipila *et al*., 2010). Furthermore, the presence of pollutants could lead to temporal and spatial negative changes in the distribution of autochthonous microorganisms at the site of pollution. In this scenario, natural remediation of such sites by the native isolates could be hampered. Alternately, pollutants able to be used as microbial substrates might enrich populations that enhance degradation. From previous studies, PAHs contamination can significantly alter a region's ecology and present the greatest ecological challenge when streams, rivers and groundwater are at risk of contamination. Several studies have emphasized that the physico-chemical properties of PAHs and sorption to soil components over time reduce contaminant availability and degradability (Semple *et al*., 2007). Microbial degradation is one of the principal means of PAH removal from soils (Macleod and Semple, 2002; Doick *et al*., 2005; Macleod and Semple, 2006; Li *et al*., 2008) and is affected primarily by contaminant bioavailability and catabolic ability of indigenous microbial populations. Adaptation processes, that occur as a result of an increase in the hydrocarbon-oxidising potential of the microbial community (Macleod and Semple, 2006), encourage the development of microbial populations with the ability to degrade PAHs.

Naphthalene, is one of the 16 PAHs classified as priority pollutants by US Environmental Protection Agency (USEPA, 1994, 2004). This bicyclic aromatic hydrocarbon and its methylated derivatives are considered as some of the more problematic water-soluble fraction of petroleum (Heitkamp *et al*., 1987). With increasing aging times in contaminated soils, naphthalene shows a reduction in extractability (Ncibi *et al*., 2007), due hydrophobicity and resultant high solid-liquid distribution ratio, characteristics which also limit bioavailability and biodegradation. Chrysene, a high molecular weight PAH, is also of environmental concern due to its toxicity, carcinogenic and mutagenic nature (Nwanna *et al*., 2006). It is highly recalcitrant in soils under normal conditions due, at least in part, to limited bioavailability. Although aerobic PAH biodegradation has been studied for several decades, isolation and characterization of novel organisms

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remain of value because of the potential for discovery of microorganisms with unique and diverse substrate ranges and study of novel catabolic pathways. Successful enrichment and isolation of PAH-degrading isolates offer more intrinsic value than using molecular techniques alone (Hedlund and Staley, 2006). Isolation of new strains allows more detailed studies of biodegradation kinetics, enzymology, and genes encoding biodegradation enzymes. In addition, comparison of homologies between catabolic genes of microbes isolated from the same environment can allow assessment of horizontal gene transfer. Thus, from such knowledge, a deeper understanding of microbial-mediated mechanisms of catalysis of PAHs will provide new strategies for development of effective bioremediation of PAH-contaminated sites.

In the present study, several bacteria belonging to the γ-proteobacteria were isolated and screened for naphthalene, chrysene, fluoranthene and pyrene degradation. For the isolation, contaminated soils were collected and enriched in a PAH supplemented medium. Because preliminary investigation of these organisms revealed interesting substrate ranges and characteristics, we characterized them phylogenetically on the basis of 16S rRNA gene analysis. Although organisms belonging to other genera were also isolated, the scope of this work is limited to understanding the dynamics of degradation of naphthalene, chrysene, fluoranthene and pyrene by γ-proteobacteria enriched from naphthalene-fed enrich-ments under aerobic conditions.

MATERIALS AND METHODS

Chemicals

Analytical grades of high purity (>99%) naphthalene fluoranthene, pyrene and chrysene were procured from Sigma Aldrich Corp. (St. Louis, MO, USA). Sodium benzoate (99+ % purity), 2,2,4,4,6,8,8-heptamethylnonane (HMN), and organic solvents were obtained from Fisher Scientific Co. (Springfield, NJ, USA). Hexane, was purchased from EMD Chemicals Inc (Gibbstown, NJ 08027). PAH analytical standards used were purchased from Accustandard Inc (New Haven, CT 06513).

Stock solutions and media

All the enrichment and degradation experiments were performed using minimal salts (MS) medium as described by (Kim and Picardal, 2000; Nwinyi *et al*., 2008; Nwinyi, 2010, 2011) . The medium consisted of 0. 5g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.076 g $Ca(NO_3)_2.4H_2O$ and 1.0mL each of vitamin and trace mineral solutions per liter of 40mM phosphate buffer (pH 7.25). The naphthalene stock solution was prepared in HMN, a non-degradable carrier to provide an initial concentration of ca. 100ppm. This concentration represents the total mass in both the aqueous and HMN phases, divided by the aqueous volume. The appropriate stock solution was added using a gas-tight syringe in 250-µL aliquots to provide test compound concentration of ca.100ppm in the final medium. Concurrently, fluoranthene, chrysene and pyrene stock solution were prepared differently by dissolving the weighted test compounds in acetone respectively. Fluoranthene, chrysene and pyrene were added from the different stock solution of the test compound into the balch tubes using a gas-tight syringe in 250-µL aliquots, to provide test compound concentration of ca.116 ppm for fluoranthene, ca. 12ppm for chrysene, and pyrene ca 94ppm in the final medium. Solid MS medium was made by the addition of 1.8% Bacto-agar (Difco Laboratories, Detroit, MI, USA). MS medium was supplemented with the test compound, achieving an experiment dependent concentration of about 100ppm. The cultures were incubated at ambient temperature on a shaker table to aid mass transfer of the naphthalene into the aqueous phase. Preliminary investigations were carried out using MS medium supplemented with HMN as the sole carbon and energy sources to determine that HMN did not serve as growth substrate.

Enrichment of bacterial species

Enrichment of bacterial isolates were performed using soil samples collected from PAH- contaminated sites at the former McDoel Switchyard in Bloomington, Indiana, USA. The site had been previously contaminated with PAHs and other organic and inorganic pollutants. The soil samples were collected at three locations with indications of low to high level PAH-contamination based on a preliminary environmental audit. The first sample was collected from a soil thought to have moderate levels of PAH contamination. This sample and subsequent samples were placed in separate sterile jars and transported back to the lab at ambient temperatures. The second sample was collected underneath a creosote-coated log. The third sample was collected at an area of reported high PAH contamination from soils previously overlaid by a railroad ties. The soil was less sandy and darker in color than the other soils. PAH- degrading bacteria indigenous to the soils were enriched and isolated as follows: Five g of the different soil samples were weighed into 160-mL serum bottles containing 30mL of sterile MS medium. Due to the fact that existing PAHs in the contaminated soils may have limited bioavailability due to sorption and 'weathering', naphthalene (4mg) ca.133ppm was added to the enrichment bottles containing the MS medium as a supplemental carbon and energy source. All the 160-mL serum bottle bioreactors were set up in triplicates. The serum bottles were crimp-sealed with teflon-coated, butyl rubber stoppers to prevent losses due to volatilization and/or sorption. These were incubated horizontally on an orbital shaker table (Labline Instruments, Inc. Melrore Park, IL, USA) at ambient temperature. Air sparging was done weekly to re-aerate the headspace and biweekly

transfers were made using about 15% inoculum into new MS medium supplemented with the test PAHs. The procedure was repeated for seven successive times.

Isolation, purification and characterization of pure culture using 16S rDNA

Pure cultures from naphthalene-enriched media were isolated by directly plating aliquots (0.2mL) of highly-enriched cultures onto MS agar. Because we wished to prevent loss of catabolic plasmids from capable isolates, we used MS agar medium supplemented with naphthalene rather than nutrient agar to maintain selective pressure. The naphthalene was added to the medium using the spray plate technique as described by (Kiyohara *et al*., 1982). Immediately after spread-plating the 0.2mL aliquot of enrichment culture, an ethereal solution of naphthalene was uniformly sprayed onto the surface of the agar. The plates were sealed with parafilm film and incubated for 1 week at 30^{o}C. Naphthalene-degrading microorganisms were identified by cleared zones around an individual colony. The colonies were purified on MS agar sprayed with naphthalene and sustained on solid MS plates containing 2.5mM benzoate or 100ppm salicylic acid. For 16S rRNA gene analysis, genomic DNA was isolated from overnight cultures of isolates growing on 2.5mM benzoate using an UltraClean Microbial DNA Isolation kit (Mo Bio. Laboratories,Solana Beach CA,USA). Three eubacterial PCR primers; forward primer 8fm (AGAGTTTGATCMTGGTCAG) and reverse primers 926r (CCGTCAATTCCTTTRAGTTT) and 1387r (GGGCGGWGTGTACAAGGC) were used to amplify the 16S rRNA gene. The reaction mixtures were incubated at 95^{o}C for 2.5min and then cycled 33 times through the following temperature profile: 95^{o}C for 30s, 48^{o}C for 30s, and 72^{o}C for 1.5min, followed by a single 10min incubation at 72^{o}C. About 2µl of each amplification mixture was analyzed by agarose gel electrophoresis 10.0µg ml^{-1} (w/v) ethidium bromide to ascertain that amplicons were of the expected length. The PCR amplicons were subsequently cleaned using QIAquick Nucleotide Removal Kit from Qiagen Inc. (Turnberry lane, CA 91355). For the 16S rRNA sequencing, the PCR products were sequenced following an ABI Big Dye Terminator Cycle Sequencing reaction using an Applied Biosystems 3730 automated sequencing system (Applied Biosystems, Inc., Foster City, CA, USA). The resultant sequences were edited and aligned using CodonCode Aligner v. 2.0.6 (CodonCode Corporation, Dedham, MA, USA). Sequences were subsequently compared with deposited sequences in GenBank database using the BLAST algorithm available at URL http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1990).

Growth on different carbon and energy sources

Pure cultures were assessed for their potential to grow on naphthalene, fluoranthene, pyrene and chrysene. The tests were carried out in MS medium supplemented with each PAH test compound as sole carbon source. Although we examined aerobic PAH degradation, we conducted experiments in crimp-sealed tubes (Balch tubes) usually utilized for anaerobic studies. Prior to use in each experiment, tubes were baked in muffle furnace at 500^{o}C to remove organic contaminants. Growth and degradation studies were performed in Balch tubes containing 10ml of MS medium, the tested PAH, inoculum, and approximately 15mL air headspace to maintain aerobic conditions. . Different tubes were supplemented with different PAHs respectively. Naphthalene was added from an HMN stock solution at a concentration of ca.100ppm as described above and inoculated with 10^5 cells/ml of phosphate buffer (pH 7.25) washed cells pre-grown in 2.5mM benzoate. Fluoranthene, chrysene and pyrene were added from the stock solution into the balch tubes using a gas-tight syringe in 250-µL aliquots, to provide test compound concentration ca.116 ppm for fluoranthene, ca. 12 ppm for chrysene, and pyrene ca. 94ppm in the final medium. Balch tubes were crimp-sealed with teflon- coated, butyl rubber stoppers to prevent losses due to volatilization or sorption. The tubes were incubated horizontally on a shaker table at (120 rev/ min) at ambient temperature. All the stock solutions were aseptically prepared before use. Growth was monitored by counting the cells numbers using replicate tubes via epifluorescence microscopic examination. The cells were stained with acridine orange stain after fixation with 5µL of glutraldehyde. Visual examinations in concurrence with periodic GC analyses to measure the test compound disappearance was also done. In this study growth was positive when there is an increase in turbidity greater than the killed or abiotic control that was used. For statistical evaluation, at least 10 microscopic fields were randomly chosen and a minimum of 1000 cells were counted. Data are presented as the mean cell numbers ± the SEM.

Transformation of PAH compounds- naphthalene, fluoranthene, pyrene and chrysene experiments

Degradation study of naphthalene, fluoranthene, pyrene and chrysene were correspondingly conducted in the Balch tubes. The tubes were inoculated with respective bacterial cultures, crimp sealed and incubated horizontally on the shaker table at ambient temperature. The degradation reactions were stopped after 14days for naphthalene while experiments with fluoranthene, pyrene and chrysene were stopped after 21days. The degradation study was stopped by the addition of 5mL of hexane, vortexing for 1-2min and subsequently, mixed continuously on a tube rotator for 12hrs. The hexane extracts and aqueous phases were separated by centrifugation at 2190rpm for 20minutes using a Beckman GS-6 series centrifuge. The hexane and aqueous extracts were separated and the hexane extract collected for further analysis. Extracts were stored in target vials with a headspace of 1mL and crimp sealed using an

11mm Teflon rubber stopper from National scientific and stored at 4°C prior to analysis.

Analytical methods

GC analysis

Hexane extracts were analyzed on an HP 5890 Series II gas chromatography GC (Hewlett Packard Co., Palo Alto, CA, USA) fitted with an HP 3396 series II integrator and equipped with a flame ionization detector (FID). Hexane extracts (5µL injection volume) were injected using a 10-µl Hamilton gas-tight syringe through a 30m HP-5 megabore fused-silica capillary column (J & W Scientific, Folsom, CA, USA; 0.32mm id, 0.25µm film thickness). The GC utilized Helium (He) as the carrier gas and was programmed for naphthalene at an initial temperature of 50°C; this was held for 5min then ramped at 30°C/min to 180°C for 2min, then ramped to 300°C at 40°C/min for 4min. Analytical standards of PAHs were prepared in hexane. Typical coefficients of correlation for standard curves were 0.98-0.99.

STATISTICAL ANALYSIS

Statistical tests was performed using the Prism 4.0 computer software programme (Graph Pad Software, San Diego, CA, USA) and Statistical Package for Social Scientists (SPSS) 15.0.

RESULTS

Isolation and phylogenetic characterization of the PAH degrading strains

Eleven different microbial colonies were selected from the MS agar plates following initial enrichment on naphthalene. Upon screening individual isolates for growth on MS salicyclic acid and MS benzoate, we selected four isolates for further study. The colony morphology of some isolates observed under the fluorescent microscope showed non-spore-forming straight and slightly curved rods about 0.5-0.7µm x 1-2.5µm. Based on partial 16S rRNA sequencing (approximately 1300 bp), phylogenetic analysis placed our strains OC-2, OC-3 and OC-4 within the genus *Pseudomonas* (Table 1). The closest relative of strain OC-1 was an *Enterobacter* species with 99% similarity. Strain OC-2 had 99% homology as *Pseudomonas putida* (AB513735). Strain OC-3 had 99% identity as *Pseudomonas putida* (AB513735) and Strain OC-4 with 100% homology as *Pseudomonas putida* strain SP2 (GQ 200822). We have classified our isolates as Bacterium OC-1, Bacterium OC-2 , *Pseudomonas* sp.strain OC3 and *Pseudomonas* sp.strain OC4 (GenBank database accession numbers JN624749 through JN 624751 and JN983823).

Degradation of naphthalene by the bacterial species

Strains OC-1 OC-2, OC-3 and OC-4 ability to degrade naphthalene were examined using washed, benzoate grown cells. No carbon source other than naphthalene was provided. After 14days incubation, the different isolates ability to degrade the naphthalene was assessed by comparing the GC peak areas of the initial day time (0) and the final time (t). Growth on naphthalene was evidenced by intense turbidity of the culture media and significant reduction in the concentration of naphthalene. Strains OC-1, OC-3 and OC-4 were able to completely degrade almost all added naphthalene. (Figure 1a) shows the values of the net reduction (percent reduction in total naphthalene content) in naphthalene concentration. These were 99, 25, 99 and 99% respectively for strains OC-1, OC-2, OC-3 and OC-4. The initial concentration of

Table 1. Cloned fragments of 16S r RNA genomic DNA of PAH degrading bacterial species.

Bacterial strain	Tentative identity	Confirmed identity	Closest relative	Bacterial subdivision	% ID[a] with closest relative	Genbank accession no.	Length (nt)[1]
OC-1	*Enterobacter* species	Bacterium OC-1	*Enterobacter* species	γ-proteobacteria	99	JN624749	1309
OC-2	*Pseudomonas putida*	Bacterium OC-2	*Pseudomonas putida* (AB513735)	γ-proteobacteria	99	JN983823	1228
OC-3	*Pseudomonas putida*	*Pseudomonas* sp.strain OC3	*Pseudomonas putida* (AB513735)	γ-proteobacteria	99	JN624750	846
OC-4	*Pseudomonas putida*	*Pseudomonas putida* strain OC4	*Pseudomonas putida* strain SP2 (GQ 200822)	γ-proteobacteria	100	JN624751	1298

[a] *ID* identity, [1] *nt* nucleotides

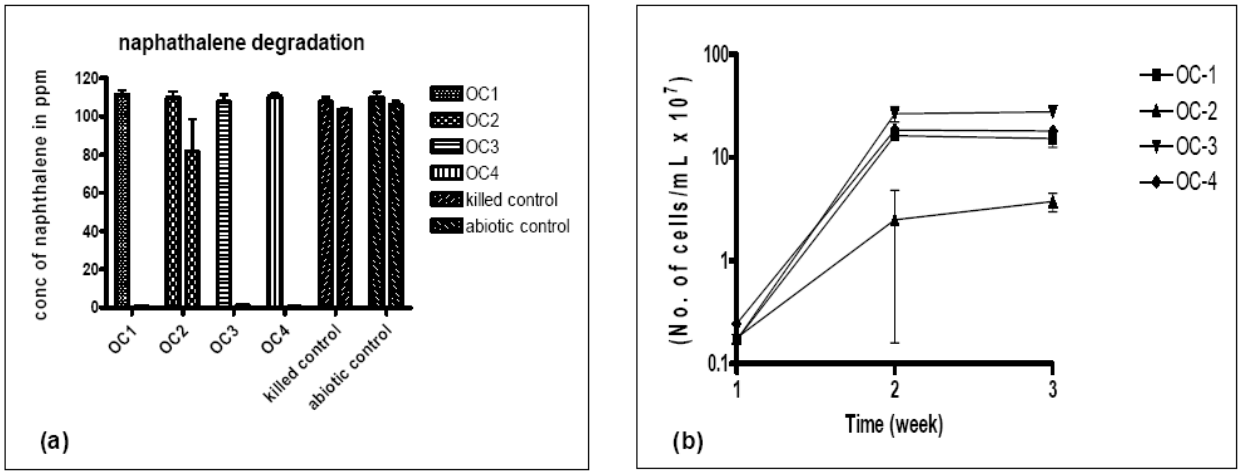

Fig. 1(a). Degradation of naphthalene by MS-benzoate grown cells of OC-1, OC-2, OC-3 and OC-4 incubated for 14 days. Data represent the mean and standard deviation of triplicate determination of initial and final concentration respectively. The large error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes. (b) Naphthlane-dependent growth and cell numbers distribution of Strains OC-1, OC-2, OC-3 and OC-4 in naphthalene incubated for 14 days. Data represent the mean of replicates tubes for initial time (0) cell density represented as (1) and final time (14 days) represented as (3) respectively. The x-axis value range was chosen as such to allow for even spread of the growth curve. The large error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes.

naphthalene at time zero was ca. 110 ppm while the final concentration ranged between 0.43–82 ppm. The mean biodegradation rate of naphthalene by strain OC-1 was 0.33 ± 0.01 mg L^{-1} h^{-1}. Strain OC-2 performance was less than that of strains OC-1, OC-3 and OC-4. The mean OC-2 biodegradation rate for naphthalene was 0.08 ± 0.06 mg L^{-1} h^{-1}. Strain OC-3 exhibited a biodegradation rate for naphthalene was 0.32 ± 0.01 mg L^{-1} h^{-1}. Strain OC-4 consumed naphthalene at the rate of 0.327 ± 0.01 mg L^{-1} h^{-1}. Following the end of the incubation period, a yellow colour was observed in some Balch tubes, suggesting that the strains may have incompletely degraded the naphthalene through a meta-cleavage pathway. Analyses were carried out by calibrating the HPLC with standards of intermediate products of naphthalene (salicylate, catechol and acetate) using an external standard method. When the aqueous extracts were analyzed for intermediate products, no product was detected, although the detection limit of the HPLC may have not allowed measurement of these limited intermediates. More likely, other intermediates may have been produced that were not detected with our HPLC method.

Growth of bacterial strains on naphthalene

We defined growth as an increase in cell numbers of at least one-order-of-magnitude and concomitant disappearance of the parent compound when compared to the abiotic and biotic control. Since naphthalene was dissolved in HMN, there was the need to determine that the observed growth was due to the presence of the test substrate naphthalene rather than the HMN. When HMN alone was added as the only carbon source in preliminary experiments, there was no appreciable growth observed for strains OC-1 OC-2, OC-3 and OC-4 over the time period of our experiments. A slight initial increase in cell numbers observed for some isolates fed HMN alone may possibly be due to continued cell division by the inocula or continued utilization of endogenous substrates. In all cases where growth occurred on the test substrates, cell numbers increased by a significant orders-of-magnitude more than tubes of the control (abiotic and biotic) HMN carrier. This clearly demonstrated growth on naphthalene. (Fig. 1b) shows the results of growth profiles of strains OC- 1-4. It shows a 1 to 2 orders-of-magnitude increase in cell numbers for all strains. Since cell numbers were counted after 7 days, it is not possible to ascertain if the benzoate-grown inoculum exhibited a lag period when presented with naphthalene as a substrate. Over the course of the experiments, strain OC-2 exhibited a smaller increase in cell numbers than did the other isolates. This is consistent with the lower naphthalene degradation rate of OC-2. Since naphthalene measurements were done at the end of the incubation, it is not clear if the cessation of growth after one week by OC-1, OC-2, and OC-4 was due to naphthalene depletion after 1 week.

Degradation of chrysene

All the strains OC-1-4, utilized chrysene as carbon and energy sources. However strain OC-4 was able to degrade more of the chrysene than other bacterial strains (Fig. 2a). The (mean and standard deviation values) of chrysene concentration used in this study was ca.12ppm. At the end

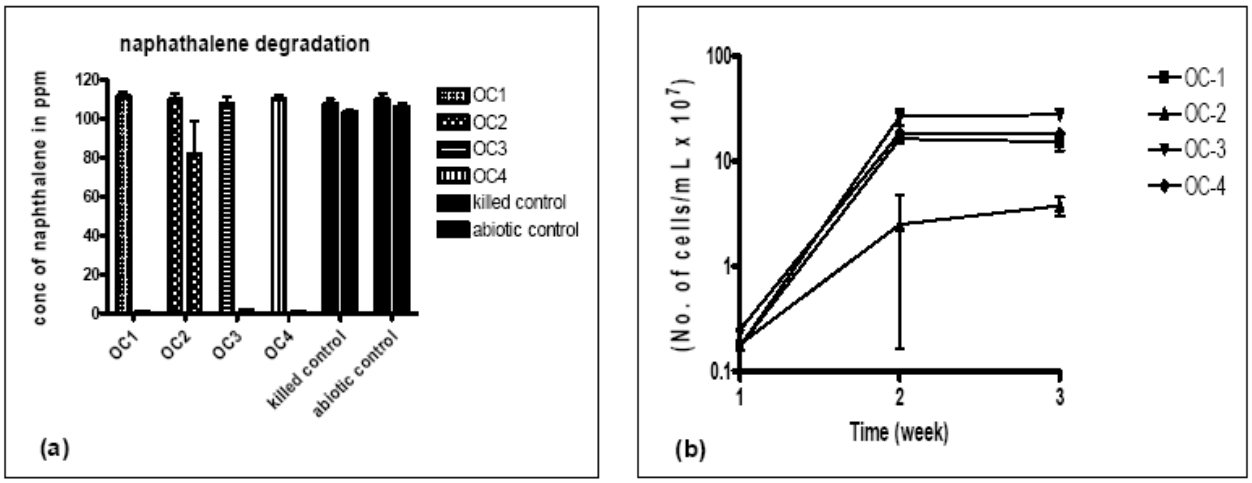

Fig. 2(a). Degradation of chrysene by MS-benzoate grown cells of OC-1, OC-2, OC-3 and OC-4 incubated for 21 days. Data represent the mean and standard deviation of triplicate determination of initial and final concentration respectively. The error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes. (b) Chrysene-dependent growth and cell numbers distribution of Strains OC-1, OC-2, OC-3 and OC-4 in chrysene incubated for 21 days. Data represent the mean of replicates tubes for initial time (0) cell density represented as (1) and final time (21 days) represented as (4) respectively. The x-axis value range was chosen as such to allow for even spread of the growth curve. The error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes.

of the incubation period, the strains degraded about 36 - 69% of the chrysene. The final concentration (mean and standard deviation) assayed was between 3-7 ppm. Strain OC-1 utilized the chrysene at 36% at volume biodegradation rate of 0.01 ± 0.0002 mg L^{-1} h^{-1}. Strain OC-2 used 52% of its chrysene as carbon and energy source at volume biodegradation rate of 0.014 ± 0.01 mg L^{-1} h^{-1}. Strain OC-3 utilized 68 % of chrysene at the rate of 0.02 ± 0.02 mg L^{-1} h^{-1}. Strain OC-4 consumed 69 % chrysene at the rate of 0.02 ± 0.001 mg L^{-1} h^{-1}. From growth profile study (Fig. 2b) the strains entered into log phase. Among the organisms evaluated strain OC-4 degraded about 69% of the chrysene.

Degradation of Fluoranthene

The degradation of fluoranthene was evaluated using strains OC-1,OC-2 ,OC-3 and OC-4. It appeared that there was a general lag period in the growth profile of the organisms (Fig. 3b). This may be because the organisms were pre-grown with benzoate as a substrate which does not apparently induce the requisite enzymes to degrade the fluoranthene. The percentage net reductions for fluoranthene are 13, 13, 19 and 7% for strains OC-1, OC-2,OC-3 and OC-4 respectively. Representing the concentration in ppm the intial concentration ca. 94ppm and the final concentration ca. 81ppm thus this represents, at best, a minor utilization of the fluoranthene (Fig. 3a). The mean biodegradation rate of fluoranthene utilized by strain OC-1 was 0.03 ± 0.01mg L^{-1} h^{-1}. Strain OC-2 utilized fluoranthene at rate of 0.03 ± 0.01mg L^{-1} h^{-1}. Strain OC-3 volume biodegradation rate utilized per hour was 0.05 ± 0.001mg L^{-1} h^{-1}. Strain OC-4 utilized fluoranthene at the rate of 0.02 ± 0.001mg L^{-1} h^{-1}. In the killed and abiotic controls there were minimal losses. On comparison between the different strains, OC-3 utilized the fluoranthene more than strains OC-1, OC-2 and OC-4. Statistical analysis performed at P- value (0.05) with SPSS 15.0 (Fig. 3c) showed that there was no significant difference ($P < 0.05$) in the data obtained for our initial and final readings among our strains and the controls. This further validates the consistency in our experimental setup and goes to show that our organisms may have similar behaviour in degrading fluoranthene. Nonetheless, it doesn't mean that the strains didn't degrade fluoranthene when there is no difference between cultures and controls. In (Fig. 3c), variance analysis showed that results obtained for each strain in the degradation study tubes analysed showed a significant difference ($P>0.05$) from those obtained for killed and abiotic control tubes. In comparison, fluoranthene analysis of tubes inoculated with strain OC-3 showed an enhanced significance over other strains and the controls. There was no apparent difference in degradation in cultures of OC-4 and the killed control. The variance analysis showed that the data obtained for OC-4 did not show significant difference ($P>0.05$) from the killed control.

The growth profile exhibited by strain OC-2 and OC-3 were similar (Fig. 3b). There are expectations that the organisms may have similar growth patterns in fluoranthene unlike the patterns exhibited by isolates OC-1 and OC-4 . With the exception of strain OC-4, the organisms, however, were marginally able to degrade fluoranthene, a tetracyclic aromatic hydrocarbon. All the

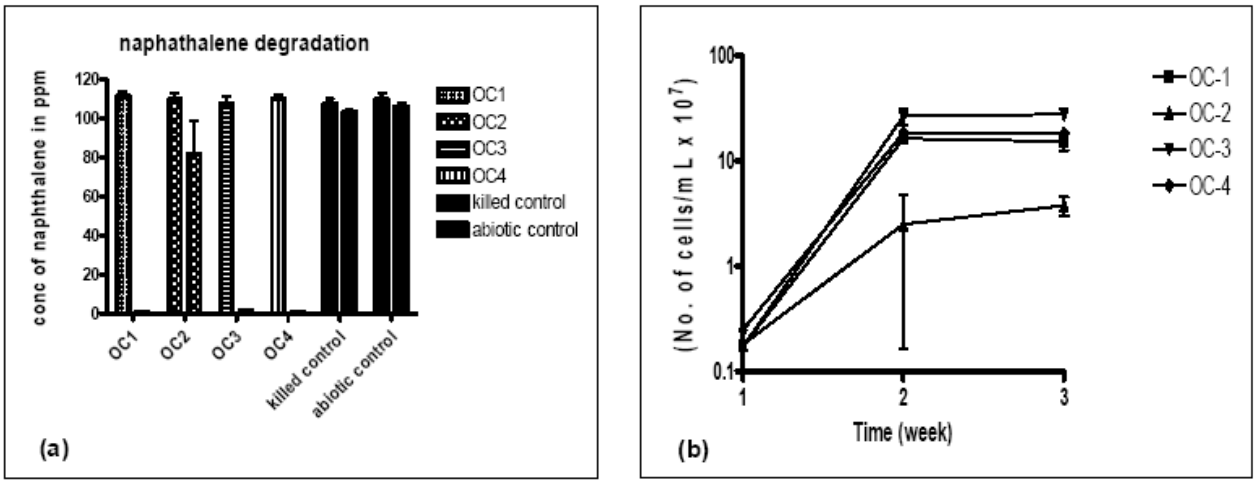

Fig. 3(a). Degradation of fluoranthene by MS-benzoate grown cells of strains OC-1, OC-2, OC-3 and OC-4 incubated for 21 days. Data represent the mean and standard deviation of triplicate determination of initial and final concentrations. The error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes. (b) Fluoranthene-dependent growth and cell numbers distribution of Strins OC-1, OC-2, OC-3 and OC-4 incubated for 21 days. Data represent the mean of triplicate tubes for initial time (0) cell density represented as (1) and final time (21 days) represented as (4) respectively. The x-axis value range was chosen as such to allow for even spread of the growth curve. The error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes.

organisms lag phase followed the same dynamics but however with different time periods for entry into the log phase. After the third week of observed increases, there was a decline in cell numbers. This may suggest accumulation of a toxic metabolite, although this hypothesis needs further verification. However, the rate of cell decline for strains OC-2 and OC-3 were different from OC-1 and OC-4.

Figure 3c. Statistical analyses of Strains OC-1, OC-2, OC-3, and OC-4 cultures incubated with fluoranthene for 21days ,compared with that of the controls (abiotic and killed) at p-value 0.05 Values presented in y-axis represent the difference in mean of obtained data from the initial and final concentrations, compared with that of the abiotic and killed controls. Values presented are from triplicate samples.

Degradation of Pyrene

The abilities of strains OC-1, OC-2, OC-3 and OC-4 were evaluated for the extent of pyrene degradation in (Fig. 4a) and their growth patterns (Fig. 4b). It was evident, that the strains exhibited similar trend of growth profile in pyrene (Fig. 4b). There was no observed lag phase until after about a week of incubation. Following that, there was general decline and a lag phase period. It suggests that the organisms may have been utilizing endogenous substrates due to previous enrichment in MS-benzoate. Of course, the decline in cell number suggests that there was period of adaptation and synthesis of the enzymes for the utilization of pyrene. Conversely none of the isolates exhibited even 1-order-of-magnitude increase in cell numbers after 4 weeks. This shows that no clear growth occurred. The initial concentration of pyrene was ca. 94ppm and after the incubation period of 21 days, the final concentration ca. 81ppm. Strains of OC-1 were able to consume about 21% of pyrene at the biodegradation rate of 0.04 ± 0.003 mg L^{-1} h^{-1} , while OC-2 used , 11 %, at the rate of 0.02 ± 0.001 mg L^{-1} h^{-1}; strain OC-3, consumed 4.71% at the rate of 0.009 ± 0.002 mg L^{-1} h^{-1} and Strain OC-4, consumed 17 % of the pyrene added as sole source of carbon and energy. The rate of the biodegradation 0.032 ± 0.0001 mg L^{-1} h^{-1} . This suggests, that these organisms can slowly utilize pyrene but however, over a long adaptation period. The long period of adaptation observed may be due to the concentration of the pyrene which was ca. 94ppm. Among these strains OC-1 exhibited more degradative ability. Variance analysis using SPSS 15.0 in (Fig. 4c) showed that results obtained for each strain in the degradation study tubes analysed showed a significant difference ($P>0.05$) from those obtained for killed and abiotic control tubes.

DISCUSSION

Enrichment using the target substrates has always proved to achieve novel metabolic capabilities from indigenous microbial strains that can be explored in dealing with xenobiotics in the environment. In this contribution, we report for the first time the isolation and characterization bacterial strains from the McDoel Switchyard site. The soils collected during our studies were contaminated with

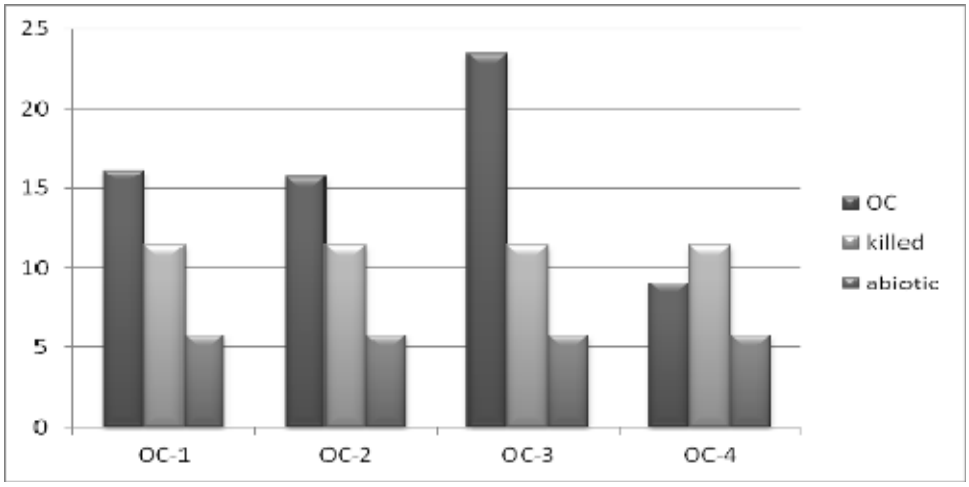

Fig. 3c. Statistical analyses of Strains OC-1, OC-2, OC-3, and OC-4 cultures incubated with fluoranthene for 21days, compared with that of the controls (abiotic and killed) at p-value 0.05. Values presented in y-axis represent the difference in mean of obtained data from the initial and final concentrations, compared with that of the abiotic and killed controls. Values presented are from triplicate samples.

inorganic and organic pollutants. The four bacterial strains that were isolated had the ability to degrade low molecular and high molecular weight PAHs – naphthalene chrysene, fluoranthene and pyrene partially. The identification of our strains to belong to the genus *Enterobacter* and *Pseudomonas* has continued to re-enforce the role the strain play in sustainable environmental management particularly in elucidation of naphthalene degradation pathways (Peng *et al*., 2008). The strains grew on MS salicylate and MS benzoate which are common intermediate products of naphthalene degradation.

According to reports of Ramos *et al*. (2002), most indigenous bacteria capable of utilizing aromatic hydrocarbons are challenged with securing carbon and energy sources from these compounds due to their potential toxicity and chemical stability. Thus this leads to persistence of such pollutants at sites of contamination. From previous studies, it is believed that toxicity of pollutants disrupts the cell membranes of most soil bacteria, influence production of toxic metabolites and can alter membrane fluidity, permeabilize the membrane and swelling of lipid bilayer (Sikkema *et al*., 1995; Park *et al*., 2004; Pepi *et al*., 2009). Furthermore, Heipieper *et al*., 1994; Sikkema *et al*., 1995 reported that with alteration of membrane structure, there could be a disruption of energy for transduction and the activity of membrane coupled proteins.

In this study, it was observed that naphthalene was volatile when dissolved in organic solvents such as dichloromethane and acetone. This however leads to inconsistency in the stock solution concentrations. This was investigated by measuring the stock solution in the GC FID chromatograph at different time intervals (data not shown). Thus with this anomaly, most workers using the aforementioned organic solvents may inadvertently be generating false positive results. Based on this we used 2,2,4,4,6,8,8 heptamethylnonane (HMN) to dissolve naphthalene and it yielded consistent values of the same concentration. HMN used as a carrier did not support significant or sustained increases in cell numbers in the abiotic and biotic controls. The changes in naphthalene concentrations without bacterial inoculation were very insignificant. It's believed that the HMN a highly branched alkane did not induce aromatic dioxygenase, thus functioned as intended i.e reduction in volatility and facilitated mass transfer of naphthalene into the medium. It may however, not be possible to discount that HMN may have influenced the result in an unknown manner.

Several workers have isolated bacterial species that can utilize naphthalene as a sole source of carbon and energy, most belong to the genera *Alcaligenes*, *Burkholderia*, *Mycobacterium*, *Polaromonas*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Sphingomonas*, and *Streptomyces* (Cerniglia, 1992; Auger *et al*., 1995; Story *et al*., 2001; Zhou *et al*., 2002; Kim *et al*., 2003; Pumphrey and Madsen, 2007). In the works of Pellizari *et al*. (1996), they reported of bacterial species isolated via naphthalene enrichment with ability to metabolize other organic pollutants. Thus the presence of naphthalene may be marginally effective in stimulating the cometabolism of other organic pollutants. This is due to the possibility of our strains to possess naphthalene dioxygenase that is known to be a versatile enzyme, able to catalyze a wide variety of other reactions. Our strains exhibited an ability to utilize a high molecular weight compound (chrysene) as carbon source. Chrysene, is a four condensed benzene

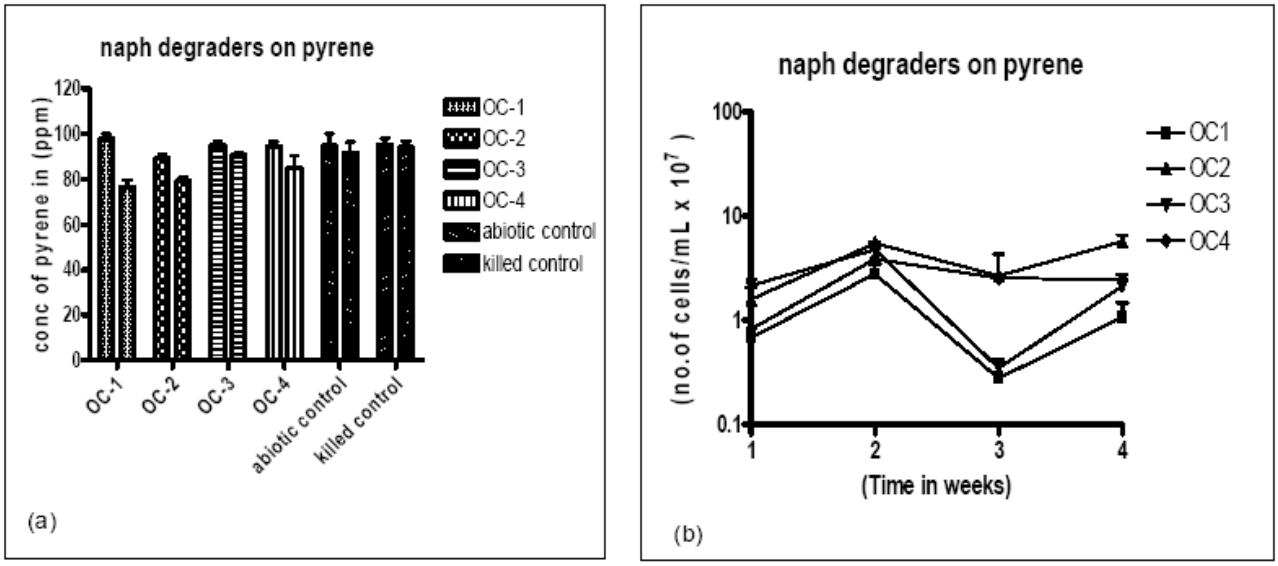

Fig. 4(a): Degradation of pyrene by MS-benzoate grown cells of strains OC-1, OC-2, OC-3 and OC-4 incubated for 21 days. Data represent the mean and standard deviation of triplicate determination of initial and final concentrations. The error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes. (b) Pyrene-dependent growth and cell numbers of Strins OC-1, OC-2, OC-3 and OC-4 incubated for 21 days. Data represent the mean of triplicate tubes for initial time (0) cell density represented as (1) and final time (21 days) represented as (4) respectively. The x-axis value range was chosen as such to allow for even spread of the growth curve. The large error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes.

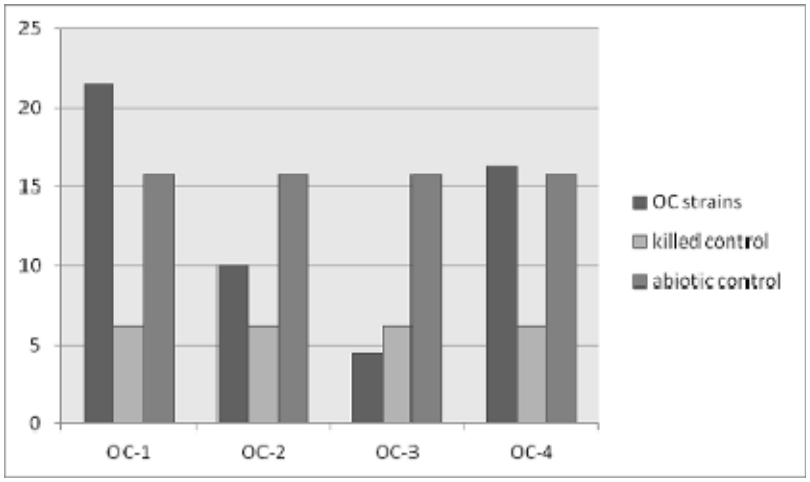

Fig. 4c. Statistical analyses of Strains OC-1, OC-2, OC-3, and OC-4 cultures incubated with pyrene for 21 days, compared with that of the controls (abiotic and killed) at p-value 0.05. Values presented in y-axis represent the difference in mean of obtained data from the initial and final concentrations, compared with that of the abiotic and killed controls. Values presented are from triplicate samples.

rings, produced via incomplete combustion of organic materials such as fossil fuels, other industrial processes, it's believed to be toxic, mutagenic and carcinogenic to humans. Microbial degradation is believed to be one of the major ways to clean up chrysene-contaminated environments. In the reports of (Tam *et al*., 2002; Hadibarata *et al*., 2009), microbial communities could have considerable potential to remedy aromatic hydrocarbon-contaminated sediment and remove chrysene from aqueous solution. High-molecular-weight PAHs (HMW PAHs) such as chrysene and benzo[*a*]pyrene are hard to be biodegraded (Massie *et al*., 1985; Heitkamp and Cerniglia, 1987; Yamada *et al*., 2003). However a number of bacterial species have been noted to degrade chrysene *Rhodococcus* sp.Strain UW1 (Walter *et al*., 1991), *Sphingomonas yanoikuyae* which oxidized

chrysene (Boyd *et al*., 1999) and *Pseudomonas fluorescens* that utilise chrysene and benz[a]anthracene as sole carbon sources (Caldini *et al*., 1995). Thus the efficiency in which PAH is biodegraded in different environment differs from another. Chrysene oxidation occurs by incorporation of an oxygen molecule in an aromatic ring. This is catalyzed by dioxygenase to a *cis*-dihydrodiol intermediate, which undergoes further metabolism via pyridine nucleotide dependent dehydrogenation reaction to produce catechols (Hinchee *et al*., 1994). In the reports of (Laor *et al*., 1999), the biodegradation of PAHs are reduced by sorption of the PAHs to sediments. Because PAHs are highly lipophilic, they tended to sorb tightly limiting their availability to microorganisms. Our bacterial strains have shown abilities to utilize chrysene, fluoranthene and pyrene significantly at low concentration. This further confirms the influence of toxicity, hydrophobicity to the biodegradation rate of PAHs. Obviously, the capacity of the bacterial strains OC-1, OC-2, OC-3 and OC-4 to utilize both low and high molecular weight PAHs is an indication of their possession of the ring fission enzymes (Ilori and Amund, 2000; Amund *et al*., 2006). Thus the isolation and phylogenetic characterization of our bacterial strains have provided a valuable resource for detailed examination of the PAH catabolic potential and identification of novel functional genes associated with the PAH degradation.

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ANTIBACTERIAL PROPERTIES AND PHYTOCHEMICAL ANALYSIS OF AQUEOUS EXTRACT OF OLEO-GUM RESINS OF *COMMIPHORA MYRRHA* AND *COMMIPHORA MOLMOL*

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ABSTRACT

The present study was performed to test the effectiveness of the aqueous extracts of *Commiphora myrrha and Commiphora molmol* in inhibition of four types of pathogenic bacteria; *Micrococcus luteus, Neisseria siccca, Proteus mirabilis* and *Pseudomonas aeruginosa*. The results showed that the aqueous extracts of *C. myrrha and C. molmol* have an inhibitive effect on the growth of each bacterium tested. The inhibition of bacterial growth decreased as the storage period of the myrrha was increased. *Commiphora myrrha* lost its inhibitive effect on *Proteus mirabilis* and *Micrococcus luteus* when myrrha was stored for one month, sex months and one year. The aqueous extract of *Commiphora molmol* lost its effect against *Proteus mirabilis* after one year of storage. *C. molmol* was seen when used as 50% of the concentration and stored only for a month as the inhibitive area decreased to 2.47 cm^2 for *Micrococcus luteus*, 2.43 cm^2 for *Neisseria sicca*, 2.17 cm^2 for *Pseudomonas aeruginosa* and 1.78 cm^2 for *Proteus mirabilis*. Chemical analysis of myrrha showed that it contains three components, 2-fluorodiphenylmethane, Tribenzo-1,2,3,4,5,6anthracene and 2-bromo-1-(4-bromophenyl)-Ethanone, known for their microbial inhibitive effect. In addition, antimicrobial activities of 12 pharmaceutical bacterial antibiotics were tested against the four bacterial strains used in the experiment. It was found that *Micrococcus luteus* is the most resistant, as it was only inhibited by five of the 12 antibiotics tested followed by *Proteus mirabilis* that was inhibited by six antibiotics. The growth of *Pseudomonas aeruginosa* was inhibited by eight antibiotics and *Neisseria sicca* was the one most sensitive to the common antibiotics as it was inhibited by nine antibiotics.

Keyworeds: *Micrococcus luteus, Neisseria sicca, Proteus mirabilis, Pseudomonas aeruginosa, Commiphora molmol, Commiphora myrrha,* inhibition zone.

INTRODUCTION

Antimicrobial drugs have proved effectiveness in control of bacterial infections but since pathogens evolve, and develop resistance there is a continous search for antimicrobial agents present in the plants (Cowan, 1999). Many studies have been carried out on natural substances for their antifungal/antibacterial activities and their effects compared to antibiotics present in the market (Sakagami *et al*., 2001; Velickovic *et al*., 2003).

Natural antibiotics' aqueous extract is easily obtained and they have little side effects compared to synthetic antibiotics (Adel and Mahasneh, 1999; DeBoer *et al*., 2005). *Commiphora myrrha* and *Commiphora molmol* belong to Bruseraceae family and are commonly nown as "Myrrh". Myrrh is one of the important medical plant. The resin of the plant is used in the treatment of wounds, intestinal disorders, diarrhea, coughing, chest pain (Ghazanfar, 1994) and gingivitis (Serfaty and Itid, 1988).

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Rahman *et al*. (2008) reported that resin of *C.molmol* is effective against many strains of *Staphylococcus aureus*. We find that the antimicrobial components extracted from the plants hinder the growth of the bacteria through mechanisms different from those used currently by antibacterial agents and they may have great remedial value in resisting the strains of germs (Harborne, 1998).

The minimum inhibitory concentration (MIC) of the alcoholic extract of the myrrh that affects the tested strains of *S. aureus* bacteria ranges from 31.25 to 250mg/ml (Abdullah *et al*., 2009). Al Ahmadi (2006) reported that the *Commiphora* resins oils are rich with vuoranossiscottrbin and a total of 20 different compounds have been identified from this genus. Vuoranossiscottrbin separated compounds or *Commiphora* resin extracts showed antibacterial, antifungal as well as anesthetic properties. This study aimed at finding natural plant-sources that can inhibit the growth of *Micrococcus luteus, Neisseria sicca, Poteus mirabilis* and *Pseudomonas aeruginosa.*

MATERIALS AND METHODS

Plant samples

The plant-samples, *Commiphora myrrha* and *Commiphora molmol*, were collected from retail-shops in Dammam city. The samples bought were one month, sex months and a year old i.e. they were in the shop for that period of time in room temperature. The bought samples were kept at 4°C until tested.

Microbial isolates source

Four bacterial isolates, *Micrococcus luteus, Neisseria siccca, Proteus mirabilis* and *Pseudomonas aeruginosa* were obtained from Department of Microbiology, Dammam University. The microbial samples were kept at 4°C until the test.

Preparing the extracts

Aqueous extract

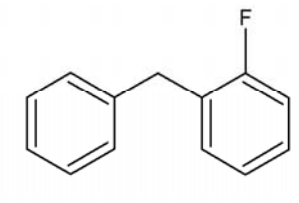
1, 2 and 5g powder of *C. myrrha and C. molmol* was dissolved in 10ml of sterilized distilled water. They were soaked at room temperature for 24hours, then filtered using layers of gauze (Boyraz and Ozcan, 2005).

Media preparation

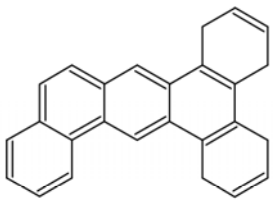
Nutrient Agar was prepared by dissolving 5g of meat extract, 2gm of yeast extract, 5gm of peptone extract, 5gm of sodium chloride extract and 15gm Agar in one liter of

Table 1. Inhibition zone (mm) of Myrrh extracts at various concentration on four microorganisms.

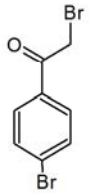
Type of myrrh	Storage period	Conc. of myrrh	Diameter of inhibition zone (mm)				Mean	
			Micrococcus luteus	*Neisseria siccca*	*Pseudomonas aeruginosa*	*Proteus mirabilis*		
C. myrrha	month	10	.68	1.66	1.17	0	.89	1.55
		30	1.59	2.05	2.08	.59	1.58	
		50	2.47	2.43	2.18	1.68	2.19	
	6month	10	0	1.08	1.07	.55	.68	1.10
		30	.78	1.32	1.09	1.65	1.21	
		50	.8	1.59	1.7	1.59	1.42	
	12month	10	0	1.25	.975	0	0	.41
		30	0	1.4	.98	0	.60	
		50	0	1.45	1.1	0	.64	
C. molmol	month	10	.53	.76	1.35	.67	.67	1.53
		30	1.48	2.28	1.93	1.81	1.89	
		50	1.69	2.45	1.98	1.99	2.03	
	6month	10	0	1.33	1.39	0	0	1.11
		30	.78	1.78	1.52	1.45	1.385	
		50	2.08	2.05	2.03	1.54	1.93	
	12month	10	0	.95	1.03	0	0	.76
		30	1.09	1.43	1.18	0	.930	
		50	2.35	1.68	1.34	0	1.34	
Mean			.91	1.61	1.45	.75		
# L.S.D.			.003	.019	.062	.003		



2-fluorodiphenylmethane



Tribenzo-1,2,3,4,5,6anthracene



2-bromo-1-(4-bromophenyl)-Ethanone

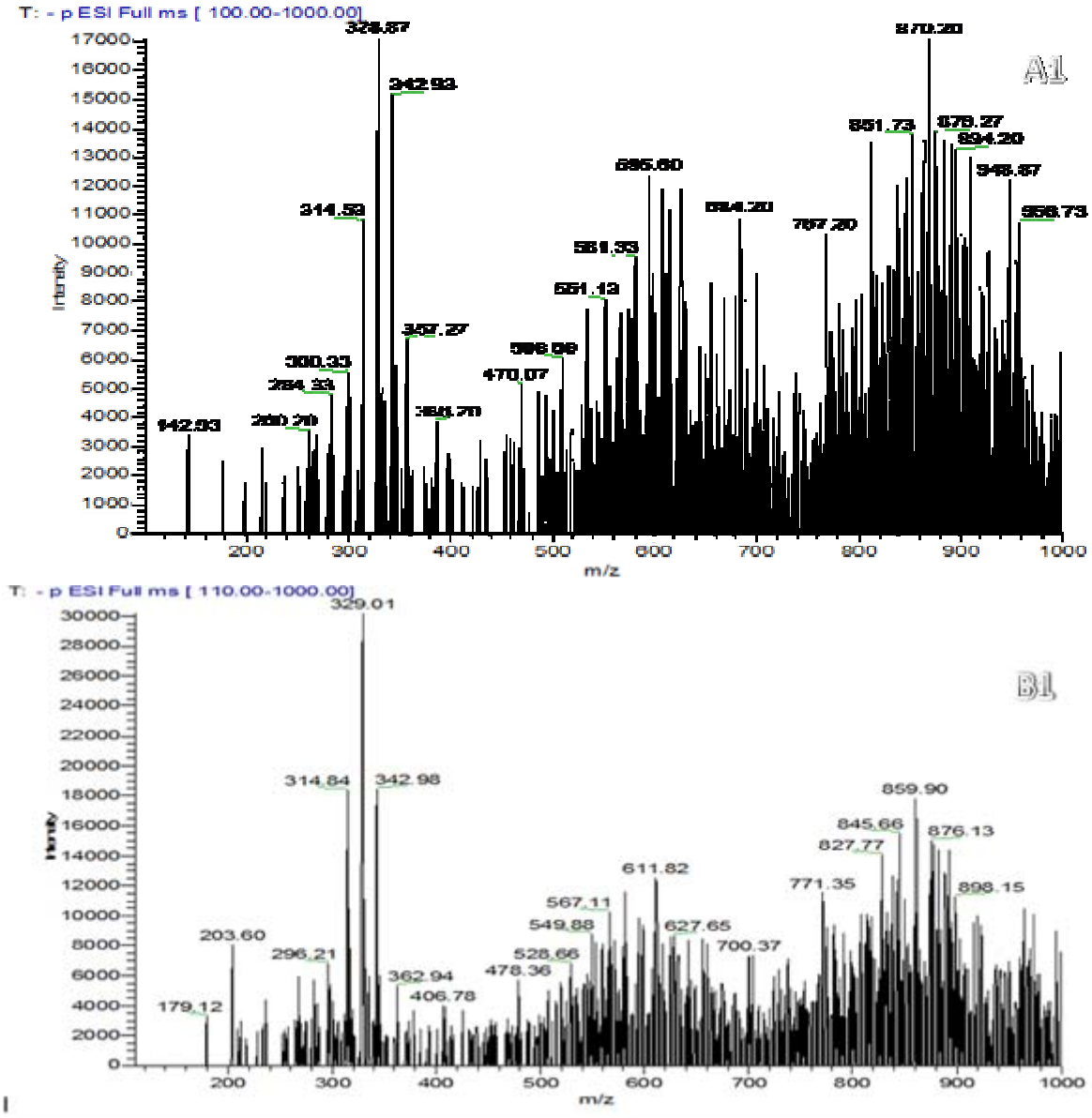


Fig. 1. Chromatograms of A1= Myrrh (*C. myrrha)* and B1=Myrrh (*C. molmol)* by mass spectrum after one month of storage.

water. Then transferred 250ml into flasks and autoclaved at 121°C for 15minutes.

Testing the effectiveness of aqueous extracts

The agar well diffusion method was used (Perez *et al*., 1990). 1ml of the Microbial inoculum was added in a sterile plastic petri dish and then 10ml of the medium was poured and left to harden. 1cm^2 holes were made and the extract was filled in holes, then incubated at temperature 23-30°C for 48hours. The results were recorded by calculating the inhibited area.

Detecting the composition of the chemical materials of Myrrha resin aqueous extract

Chemical composition analysis of myrrha was performed using mass spectrum to determine compounds responsible for antimicrobial activities.

STATISTICAL ANALYSIS

The statistical analyses was performed according to the fully randomized design and with three replicates for each treatment. The results were analyzed and compared at the 0.05 level of probability using the L.S.D. using the 16

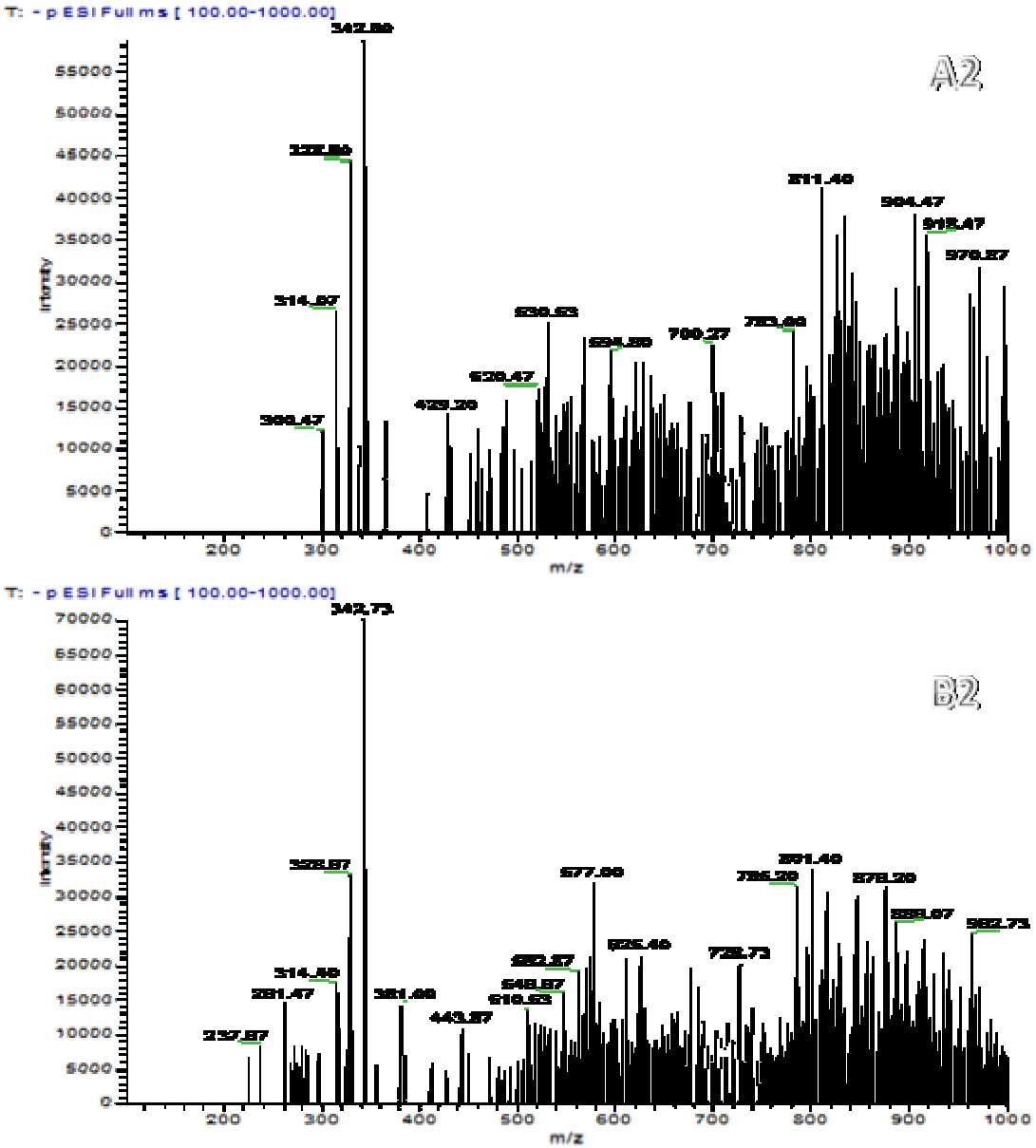


Fig. 2. Chromatograms of A2= Myrrh (*C. myrrha)* and B2=Myrrh (*C. molmol)* by mass spectrum after sex month of storage.

version of SPSS program according to the method of Norusis (1999).

RESULTS AND DISCUSSION

Effects of Myrrha aqueous extracts

Effects of Myrrha aqueous extracts were tested on four microbes using the agar well diffusion method, due to its quality, easiness of performing it and clarity of its results. The results were determined after 24-48hours by measuring the inhibition zones area. The results recorded in table 1 show that the increase in the storage period of myrrha reduced its inhibitive effect on most bacteria tested. It was also noticed that *C. myrrha* lost the inhibitive effect on the growth of *Proteus mirabilis* and *Micrococcus luteus* with all the different concentrations of the myrrha aqueous extracts stored for one month, sex months and one year.

The aqueous extracts of *C. molmol* lost its inhibitive activity against *Proteus mirabilis* only after storage for one year. Through the general averages, it is noted that the inhibition area was 1.55, 1.1 and 0.41cm^2 after storage for one month, six months and one year for *C. myrrha* respectively, while the inhibition are a reached 1.53, 1.11 and 0.76cm^2 for *C. molmol* during the storage periods respectively.

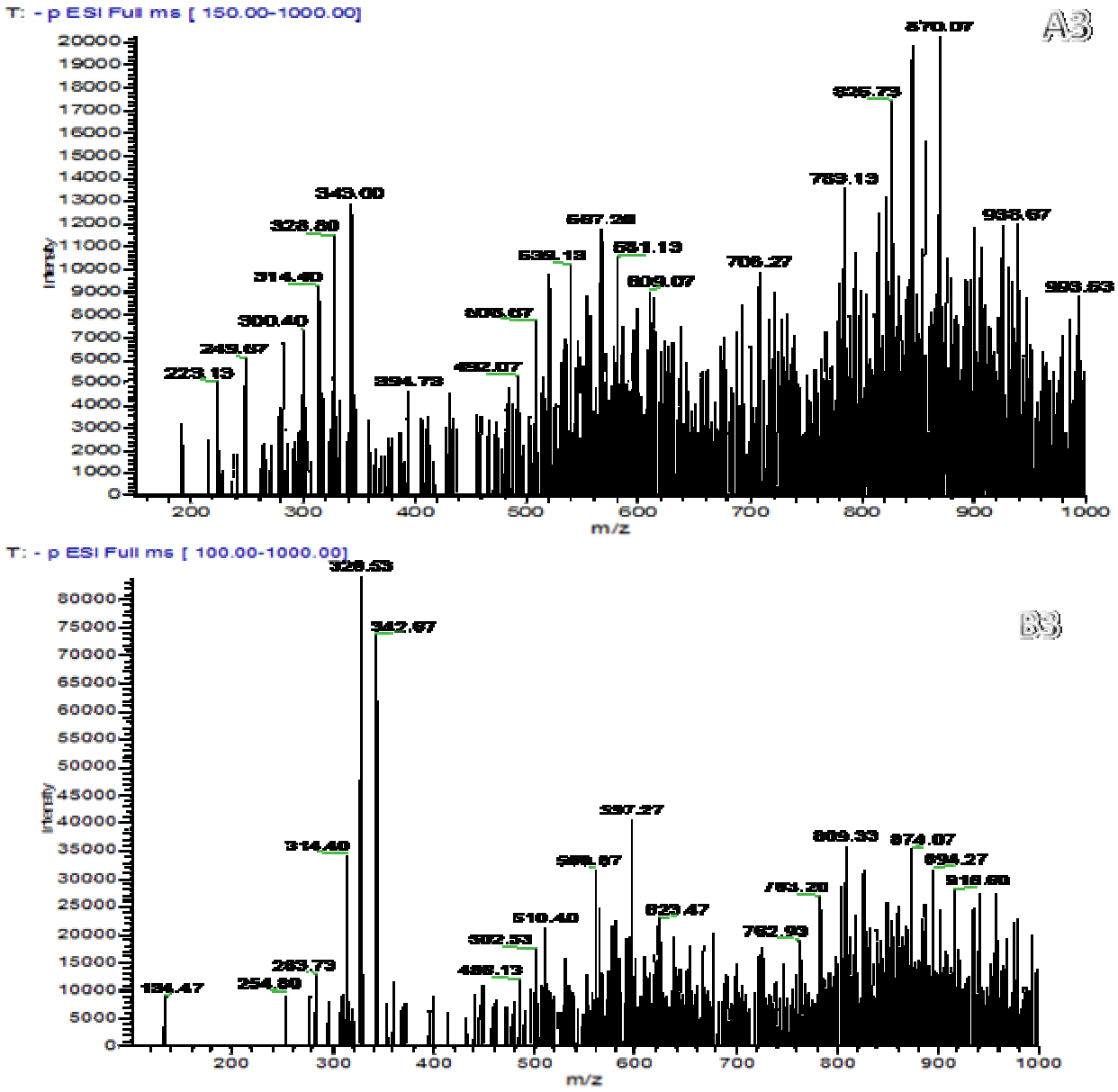


Fig. 3. Chromatograms of A3= Myrrh (*C. myrrha)* and B3=Myrrh(*C. molmol)* by mass spectrum after one year storage.

The greatest inhibition area were recorded when one month old myrrha samples were used with 50% concentration. The inhibited area were as follows: 2.47, 2.43, 2.18 and 1.68cm^2 for *Micrococcus luteus, Neisseria sicca, Pseudomonas aeruginosa* and *Proteus mirabilis* respectively with *C. myrrha*. While the inhibited area was 2.45, 1.99, 1.98 and 1.69cm^2 for *Proteus mirabilis*, *Neisseria sicca, Pseudomonas aeruginosa* and *Micrococcus luteus* respectively with *C. molmol*. The results obtained match the results obtained by other researches including Arora and Kaur (1999), Digraki *et al*. (1999), Okemo *et al*. (2001), Madamombe and Afolayan (2003), Al-Rashedi and Al-Habib (2011) and Akintobi *et al*. (2013).

The positive results show that these extracts contain some anti-microorganisms effective compounds such as the volatile oils, terpenes, phenols, flavonoids and Alsaboninat (Ellof, 1998; Ekwenye and Elegalam, 2005). The bacterial resistance to the tested extracts is due to the composition of the bacteria that resist the antibiotics, especially the thickness of the mucous layer surrounding the cell wall resulting from its adaptation with the excessive and wrong use of the antibiotics. This results in increase in the number of strains that resist antibiotics. These results matched with what is referred to by Ghareeb (2011) on the sensitivity of isolates to the *Staphylococcus aureus* that are distinguished with having mucous layer produced by the resisting isolates that were more

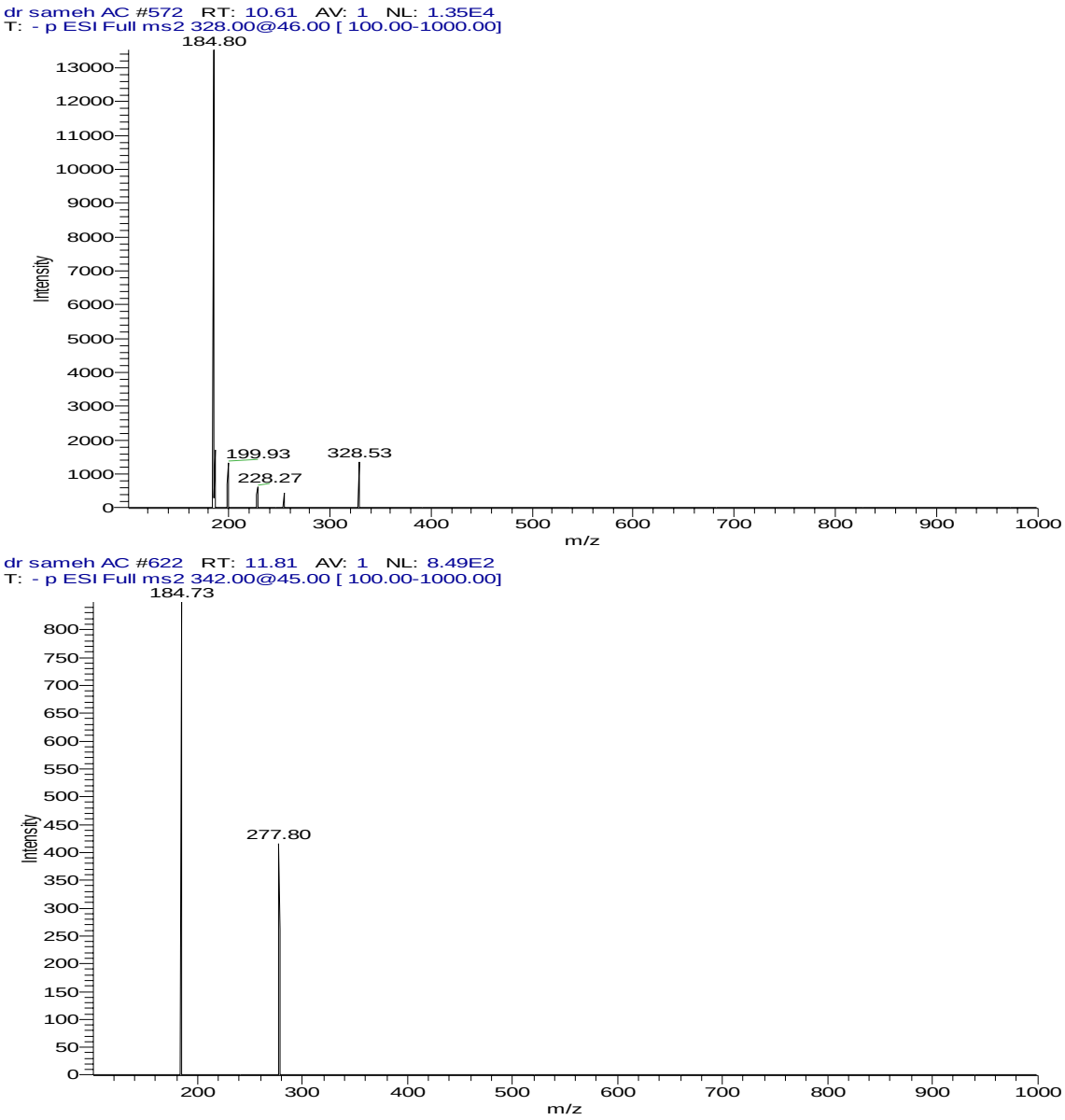


Fig. 4. Chromatograms Individualize the main compound of *C. myrrha and C. molmol* by mass spectrum.

thickness than the layer in the sensitive strains, the mucous layer covers the bacterial cell by some layers to form thin membranes called "Bio-film". Such membranes work as insulators and hinder the influence of the antibiotic thus increasing the resistance property (Kirisits *et al.,* 2007; Stapper *et al.,* 2004). Therefore, the mucous layer is an important factor which enables bacteria to produce resistance to antibiotics causing delay and difficulty in treatment of bacterial infections (Evans *et al.,* 1991). Bayer *et al*. (1992) founded that the mucous layer of *P. aeruginosa* plays an important role in the pathogenicity and the acquisition of resistance. It is one of the factors leading to the appearance of resistance to the antibiotics, and the mucous layer is distinguished with its viscosity and its soft gel composition of little inherence. This layer can surround any type of the bacteria giving it the ability to adhere to other materials. And they find that the bacteria that own capsule, purse or even mucous layer resists the macrophage cells in the human body that is considered as one of the defense lines in the human body.

Chemical composition of myrrha resin in the aqueous extract

The results obtained showed that the aqueous extract of *C. myrrha and C. molmol* contains compounds that inhibit the growth of the tested bacteria In order to know the

Table 2. Inhibition zone (mm) of antibiotic on four microorganisms.

Antibiotic	Diameter of inhibition zone (mm)			
	Micrococcus luteus	*Neisseria siccca*	*Pseudomonas aeruginosa*	*Proteus mirabilis*
Gentamicin	1.4	1.55	1.55	1.2
Neomycin	1.9	3.15	2.33	0
Cephalothin	0	2.25	0	0
Cotrimoxazole	0	3.1	1.58	0
Tobramycin	1.1	2.75	1.48	1.5
Carbenicillin	0	0	0	0
Chloramphenicol	0	0	0	0
Polymyxin B	0	1.75	1.26	1.4
Penicillin	0	0	0	1.9
Streptomycin	1.9	1.6	1.7	2.33
Oxytetracycline	1.3	1.75	1.75	1.3
Erythromycin	0	1.65	0,95	0
L.S.D.#	0.006	0.014	0.032	0.006

identity of these compounds, a chemical analysis has been performed for the composition of the myrrha using the method of mass spectrum (Figs. 1-4). It is proved that it contains three compounds known for their antibacterial effects as follows: 2-fluorodiphenylmethane, Tribenzo-1, 2, 3, 4, 5, 6 anthracene and 2-bromo-1-(4-bromophenyl)-Ethanone. The inhibitive effect of the extract may be referred to the existence of the volatile oils that are large terpene single compounds (Cowan, 1999). These oils have the ability to inhibit yeast and this is referred to the ability of the oil to analyze the cell wall. This leads also to the weakening the biological activities in the cell through overlapping with the cytoplasmic membrane function represented by the process of synthesis of protein and this inhibiting and stopping the process. This results also in hindering the process of active transfer of the ions and salts through this membrane (Al-Qaysia, 2008).

Test of examining the sensitivity to the antibiotics

12 types of pharmaceutical bacterial antibiotics were tested in order to know the sensitivity of the tested microbes to see their effectiveness towards these. The results in table 2 show the difference of sensitivity of bacteria tested to the different types of antibodies. The *Micrococcus luteus* was more resistant, and affected only by five of the antibiotics tested followed by *Proteus mirabilis* that affected by six antibiotics only. The growth of *Pseudomonas aeruginosa* inhibited only by eight antibiotics, while not affected by Cephalothin, Carbenicillin, Chloramphenicol and Polymyxin B. *Neisseria sicca* was the most sensitive one of the bacteria tested, resisted three thereof only; namely Carbenicillin, Chloramphenicol and Polymyxin B. the obtained results matched to some extent with the results obtained by Akintobi *et al.* (2013).

Ghareeb (2011) and Vasil (1986) found that the resistance of *P.aeruginosa* and *Staph. aureua* to the antibodies may have resulted by diferrent mechanisms including the production of enzymes able to break down the β-lactamase enzymes or change the permeability of the cell membrane in order to hinder the entrance of the antibiotic to the targeted area as well as its ability to change the metabolic pathways. Brown (1975) refers this to that some world hospital's is restricted to use one antibiotic to treat its patients resulting in appearance of mutant strains resistant to these antibiotics.

In general, the mechanisms followed by microorganisms for survival against microbial antibiotics is still ambiguous and debatable (Okemo *et al*., 2001). On the other hand, the chemical components of the plants play a role in protecting the plants from the microbial attack inside the plant. Some of these components may also be used by humans for protection against microorganisms (Kubo *et al*., 1995). This study recommends that more experiments be performed to test the natural plant components for antimicrobial performance.

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SUSCEPTIBILITY OF THREE *HIEROGLYPHUS* SPECIES (HEMIACRIDINAE: ACRIDIDAE: ORTHOPTERA) TO SOME STRAINS OF THE ENTOMOPATHOGENIC FUNGI FROM PAKISTAN

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ABSTRACT

The species of genus *Hieroglyphus* are a voracious and destructive pest of cash crops in Pakistan in order to decline their population three species of *Hieroglyphus* were treated with some strains of the entomopathogenic fungi under laboratory conditions. During the present study three pathogenic fungi species i-e *Metarthizium flavoviride* Gams and Roszypal, *Beauveria bassiana* (Bals.-Criv.) and *Aspergillus* sp. Micheliwere isolated and identified with infection the following incidence rates: (53%), (35%)and (12%) respectively on *Hieroglyphus* species. The proportional cumulative survival of *Hieroglyphus* in the different treatments of fungi is showed that insects treated with the pathogen began to die with full signs of mycosis on day 5th. All treated insects died by day 6th by application of *M. flavoviride* while other replicates of the *B. bassiana* and *Aspergillus* spp. all dying by day 7th. In contrast, control mortality was extremely low with only (6, 3, & 8) deaths of *H. perpolita, H. oryzivouous* and *H. nigrorepletus* respectively and with no signs of mycosis. This study recommended that *M.flavoviride*, *B. bassiana* and *Aspergillus* spp. among all the isolated entomopathogenic fungi are major factors of mortality in *Hieroglyphus* population and it might be used as bio-control agent to suppress the grasshopper's population in field.

Keywors: *Hieroglyphus,* pest, cash crops entomopathogenic fungi, bio-control agent, population.

INTRODUCTION

The species of genus *Hieroglyphus* is a voracious and destructive pest of cash crops in Pakistan and India (Roonwal, 1978; Riffat and Wagan, 2007, 2012). This genus is considered polyphagous and cause damage of millions of rupees annually. Control of these grasshoppers has generally involved "Knock off" chemical pesticides. On pesticides expenses reached in billions of rupees each year. However, because of increasing concern on its effect on non-target organism, human health and persistence in the environment, there is the need for environmental friendly alternative biological control that involves the use of natural enemies and pathogens to control pests among these; entomopathogenic fungi are very important to reduce the grasshoppers population in field (Gerson and Smiley, 1990; Moore *et al*., 1992; Seyoum *et al*., 1994; Shah *et al*., 1998). They are important biological control agents because of their observed capacity to cause spectacular epizootics. The fungi species affecting grasshoppers i-e *Entomopha gagrylli* (Fresenius), Batko attracted attention long ago as a potential bio-control agent and was briefly marketed in South Africa in 1898 as a bio-pesticide (Lomer *et al*., 2001).

Chapman and Page (1979) stated that entomopathogenic fungi are a group of fungi with complete and complex life cycles. They further urged that they could be used as classical biological control agents because of their observed capacity to cause spectacular epizootics. Furthermore, Prior *et al*. (1992) recommended the oil based formulation of entomopathogenic fungus *Metarhizum flavoviride* Gams and Rozsypal for control of locusts and grasshoppers in Africa. Douro-Kpindou *et al*. (1995) used *M. flavoviride* against the variegated grasshopper *Zonocerus variegatus* (L.) is the humid zones of southern Benin and get significant result. In addition to this, Thomas *et al*. (1997) treated rice grasshoppers *Hieroglyphus daganesis* Krauss with the fungi, and Kooyman *et al*. (1997) treated sencgalose grasshoppers *Oedaleus senagalensis* (Krauss) in optional scale application in the Sahelian zone of southern Niger. Steedman (1990) applied *M. flavoviride* against the *Z. variegatus*. Shah *et al*. (1994) recorded natural level of fungal infections in Acrididae and Pyrgomorphidae. Gunnarsson (1988) demonstrated an immune response in insects for (12hrs) after pathogen application and Gotz and Vey (1974) have shown rapid humoral encapsulation of *B. bassiana* hyphae, even within the cuticle, thus it is possible for an insect to be alerted for infection at a very early stage. There is bulk of information available on the use of entomopathogenic fungi against grasshoppers from abroad but unfortunately this area is completely neglected from Pakistan. It was therefore, felt necessary to undertake a laboratory examination, which involves

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evaluating the impact of various fungi species affecting grasshopper population from Pakistan. The basic aim of this study is to note susceptibility of *Hieroglyphus* species i-e *H. perpolita* (Uvarov), *H. oryzivorous* Carl and *H. nigrorepletus* Bolivar against different species of entomopathogenic fungi under controlled condition. The finding of this research will be helpful to determine the suitability of entomopathogenic fungi as agents for the biological control of grasshoppers. Moreover, with the help of these microbial components we can easily save our precious crops as well as huge amount expend on pesticide.

MATERIALS AND METHODS

Collection of samples

Hieroglyphus species were collected from agriculture fields of rice, maize, sugarcane, millets, fodder crops and their surrounding vegetation of grasses using sweep net (8.89cm in diameter and 50.8cm in length) as well as by hand picking. The collection was made during the year 2012 in the months of June to November from various provinces of Pakistan. Collected insects were taken to the laboratory and kept in cages (length 30.5cm, width 26.5cms). Grasshoppers fed maize leaves, and twigs surface, sterilized in 5% sodium hypochlorite solution as described by Prior *et al*. (1995).

Rearing of Insects

Insects were divided into groups of 50 to four replicates per treatment. No. discrimination was made between (age, class or sex) insects were then placed in cages (length 16.5 cm, width 13.5 cms) under laboratory (25^{o}-$23^{/}$N, 68^{o}-$24^{/}$E) conditions where the temperature fluctuated between 28 ± 2^{o}C to 39 ± 2^{o}C and relative humidity was 26 to 61%. A total of 4065 individual of *Hieroglyphus*, comprising a mix of final instars nymphs and immature, then mature adults were collected and maintained in the laboratory for up to 1 week prior to use.

Fungal isolation and sporulation test

Hieroglyphus cadavers removed from the cages, were surfaced sterilized in 5% Sodium hypochlorite and 75% ethanol solution and then rinsed in sterile distilled water. The cadavers were then left to dry for 48hrs as described by Dourou-Kpinduo *et al*. (1995). After drying these cadavers, they were humid incubated in clean desiccators at room temperature as described by Luz and Fargues (1998). Sporulation cadavers were regarded as being positive while non-sporulating cadavers were negative. The sporulating fungi on cadavers were isolated in pure culture on sabouraud dextrose agar (SDA), slopes and formulated in ground nut oil these fresh suspension were placed in both sonicator for 1minute to break up the conidial chains and conidial counts were made with a haemocytometer as described by Poinar and Thomas (1984).

Identification of fungal isolates

Identification of fungal isolates was carried by description given by International Mycological Institute (IMI), Manual of pathogenic fungi and bacteria (1983), the incidence of occurrence of the isolated fungi was recorded (Table 1).

Table 1. Collection of *Hieroglyphus* species from the different districts of the Pakistan during the year 2012.

a. (Sindh)

Districts	Species		
	H.perpolita *(n=591)*	*H.nigrorepletus* *(n=887)*	*H.oryzivorous* *(n=705)*
Karachi	5	13	0
Jamshoro	6	23	12
Thatta	16	67	17
Badin	17	101	0
Tharparkar	43	56	0
Umerkot	18	53	16
Mirpurkhas	23	134	13
Tando Allahyar	37	34	16
Tando M. Khan	41	55	0
Hyderabad	78	67	9
Khairpur	63	34	0
Shaheed Benaziabad	52	43	0
Dadu	66	123	179
Larkana	42	18	303
Jacoabad	41	43	117
Sukkur	43	23	23

Table continued…

Table 1 continued

b. (Punjab)

Districts	Species		
	H.perpolita (*n*=349)	*H.nigrorepletus* *(n=76)*	*H.oryzivorous* *(n=290)*
Bahawalpur	13	0	21
Chakwal	44	9	32
Faisalabad	24	6	18
Jhelum	19	5	11
Jhang	21	0	19
Kasur	13	3	10
Lahore	19	0	8
Mianwali	27	4	21
Multan	33	7	12
Rawalpindi	78	21	108
Rahim Yar Khan	35	13	12
Sahiwal	14	8	10
Sialkot	9	0	8

c. Khyber Pakhtunkhwa

Districts	Species		
	H.perpolita *n=(195)*	*H.nigrorepletus* *(n=91)*	*H.oryzivorous* *(n=617)*
Abbatabad	11	0	43
Battagrum	6	8	31
Charsadda	31	7	57
Dera Ismail Khan	10	4	18
Hairpur	8	3	127
Kohat	9	6	16
Mansehra	10	11	203
Mardan	31	9	11
Nowshehra	28	13	29
Peshawar	44	21	38
Swat	7	9	44

d. Balochistan

Districts	Species		
	H.perpolita *(n=72)*	*H.nigrorepletus* *(n=103)*	*H.oryzivorous* *(n=89)*
Barkhan	18	44	10
Kalat	21	13	12
Khuzdar	11	7	16
Lasbela	22	39	51

Pathogenicity Bioassay

Three fungal isolates used for the pathogenicity bioassay on *Hieroglyphus* the isolates include *Metarhizium flavoviride*, *Beavveria bassiana* and *Aspergillus* spp. The isolates were cultivated at 28^{o}C at photoperiod of 12hrs light and darkness 12h L:D) for 15 days as described by Balogun and Fagade (2004). After the incubation sterile spatula was used to harvest the conidia from the fungal culture. The harvested conidia were transferred into sterile McCartney bottles containing the ground oil. Then fungal spore's suspension in oil was prepared and the spore concentration determined using the Neuberger Haemocytometer as described by Lomer and Lomer (1996). Before the commencement of the bioassay insects were bred and conditioned to their cages for one week. Then 0.1ml of the spores' suspension was applied

Table 2. Identification of Entomopathogenic fungi isolated from *Hieroglyphus* species.

Isolates	Growth Morphology	Color	Phialidas	Spores	Probable organism
D1	Powdery mycelia	White or pale yellow	----	Clustered globular to flask shaped conidia	*Beauveria bassiana*
D5	Surface is powdery & finally crustose	Dark herbage green	Conidia in chain form	Globose conidia	*Metarthizium flavoviride*
D6	Fast growing & heavily sporing	Dirty green	Typically radiate	Typically globose to subglobose	*Asperillus* species

International Mycological Institute (IMI) Manual of pathogenic fungi and bacteria (1983)

Table 3. Mortality of *Hieroglyphus* population by treating with different pathogenic fungi species during the year 2012.

Treatments	Period days (Mean ±SE)						
	Ist	2nd	3rd	4th	5th	6th	7th
M. flavoviride	0.33±0.33[b]	0.00±0.00[d]	2.00±0.57[a]	7.00±1.15[a]	10.66±2.02[a]	27.00±2.30[a]	3.00±3.00[a]
B. bassiana	0.00±0.00[c]	2.00±1.00[a]	0.66±0.66[c]	4.33±1.45[b]	6.00±0.57[b]	10.33±1.20[c]	26.66±3.84[b]
Aspergillus Sp.	1.33±0.33[a]	1.00±0.57[b]	1.00±0.57[b]	4.33±0.66[b]	5.66±1.20[c]	13.33±2.02[b]	23.33±1.85[c]
Control	0.00±0.00[c]	0.66±0.33[c]	0.00±0.00[d]	2.00±0.57[c]	0.00±0.00[d]	1.00±0.57[d]	2.00±0.00[d]

Note: Mean in the same column followed by the same letters are not significantly different from one another at 5% level of probability.

carefully under the pronotal shield of the grasshoppers using sterile Pasteur pipette (Dourou-Kpindou *et al*., 1995; Thomas *et al*., 1997).

However, for the control experiment blank oil without spores was applied on the pronotal shield of the grasshoppers. In the last infected and uninfected grasshoppers were transferred into separate clean cages. Daily mortality was recorded, cadavers removed from the cages, were surface sterilized humid incubated and the causative fungi isolated in pure culture. This method was adopted for all the species studies viz: *Hieroglyphus perpolita, H. oryzivorous* and *H. nigrorepletus.*

RESULTS

Out of 4065 specimens of *Hieroglyphus* collected from field used for the study 90% of them died in the cages (Table 2) from which 74% of the cadavers recorded positive fungal sporulation results three fungi species were isolated and identified with infection the following incidence rates: *M. flavoviride* (53%), *B. bassiana* (35%) and *Aspergillus* sp (12%) (Fig. 1). The virulence bioassay involves the treatment of *Hieroglyphus* population with spore's suspension of *M. flavoviride*, *B. bassiana* and *Aspergillus* sp. The proportional cumulative survivals of *Hieroglyphus* in the different treatments of fungi are shown in (Table 3, Figs. 2-4). Insects treated with the pathogen began to die with full signs of mycosis on day 5th. All treated insects died by day 6th by application of *M. flavoviride* while other replicates of the *B. bassiana* and *Aspergillus* spp. all dying by day 7th. In contrast, control mortality was extremely low with only (6, 3, 8) deaths of *H. perpolita, H. oryzivorous* and *H. nigrorepletus* were recorded respectively with no signs of mycosis.

The highest lethal time of 6 days recorded for *Hieroglyphus* species by application of *M. flavoviride* suggested that its spores are lethal to *Hieroglyphus* species and could cause significantly high mortality in all treated species. This suggests that *M. flavoviride* might be a severe pathogen of *Hieroglyphus* species. The study provides further evidence that infection by *M. flavoviride*, *B. bassiana* and *Aspergillus* spp. causes a significant reduction in host feeding well before deaths. The average survival times of the treated insects in the present study were shorter than those typically observed in control trials. The high fungal infection incidence recorded on grasshopper's cadavers suggests that the fungi entomopathogens isolated are significantly important pathogens in the population of the *Hieroglyphus* (Fig. 4). This study provides further evidence that infection by *M. flavoviride* causes a significant reduction in host feeding well before death. The average survival times of the treated insects in the present study were shorter than those typically observed control treatment.

DISCUSSION

Orthoptera are attacked by many vertebrates and invertebrates natural enemies (Greathead, 1992) while recent review have emphasized the large number of pathogenic diseases being studied as possible biological control agents (Bidochka and Khatchatourians, 1991;

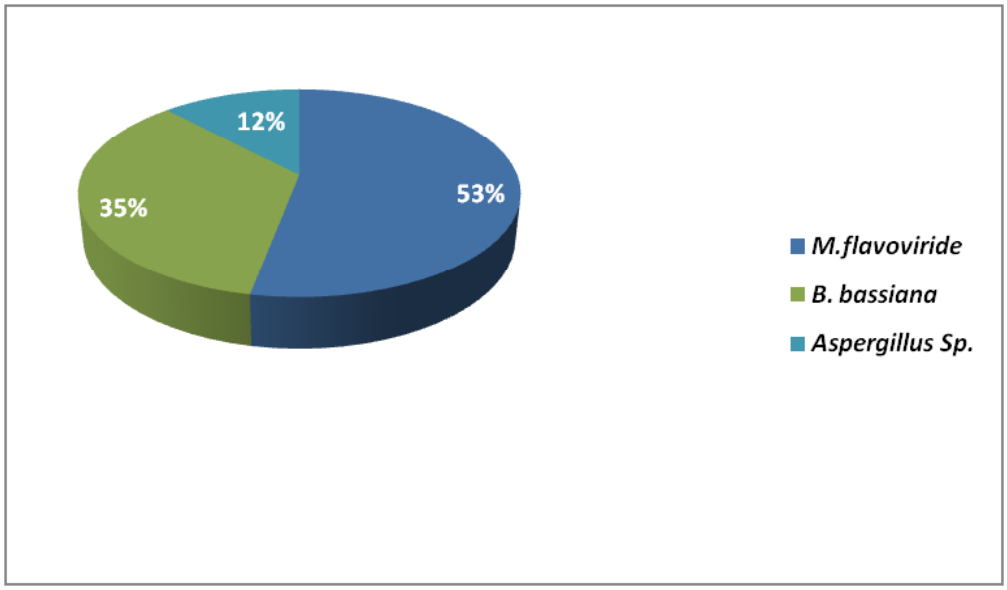

Fig. 1. Incidence of entomopathogenic fungi isolated from *Hieroglyphus* species.

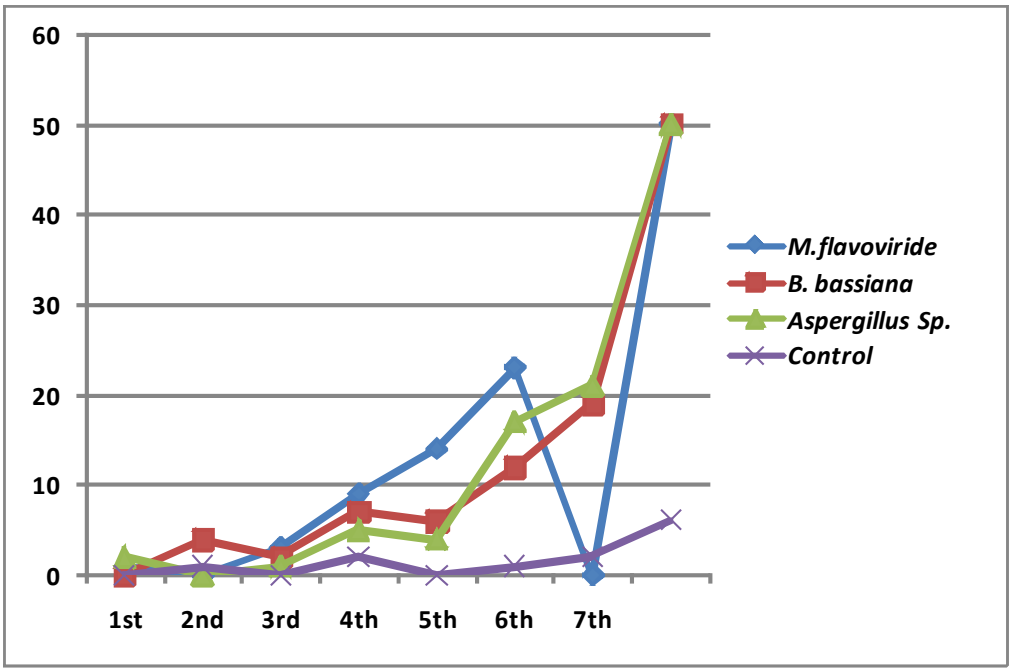

Fig. 2. Mortality of *Hieroglyphus perpolita* by treating with different species of fungi during the year 2012.

Streett and McGuire, 1990). The high fungal infection incidence recorded on *Hieroglyphus* populations suggests that the fungi entomopathogens isolated are important pathogens in the population of the *Hieroglyphus* are correlated with the finding of Hernardez-Crespo and Santiago Alvarez (1997). *B. Bassina* isolated in this study agree with the observation of Paraiso *et al*. (1992). Isolation of *Metarhizium* species from *Zonocerus variegates* (Linnaeus) Cadavers have been reported by Shah *et al*. (1994). Present study agreed on this account.

For the past century, entomopathogenic fungi have been known to come drastic decline among grasshoppers and locust populations. Consequently, most of the research has described fungal epizootics or attempted to utilized fungi as biological control agents. Presently we also did study under controlled condition and pathogen injected on the pronotum sheets of insects. It almost gave similar result as obtained by previous workers. A number of studies have shown both chemicals and pathogens to affect insect feeding rates. Haynes (1988) reviewed the

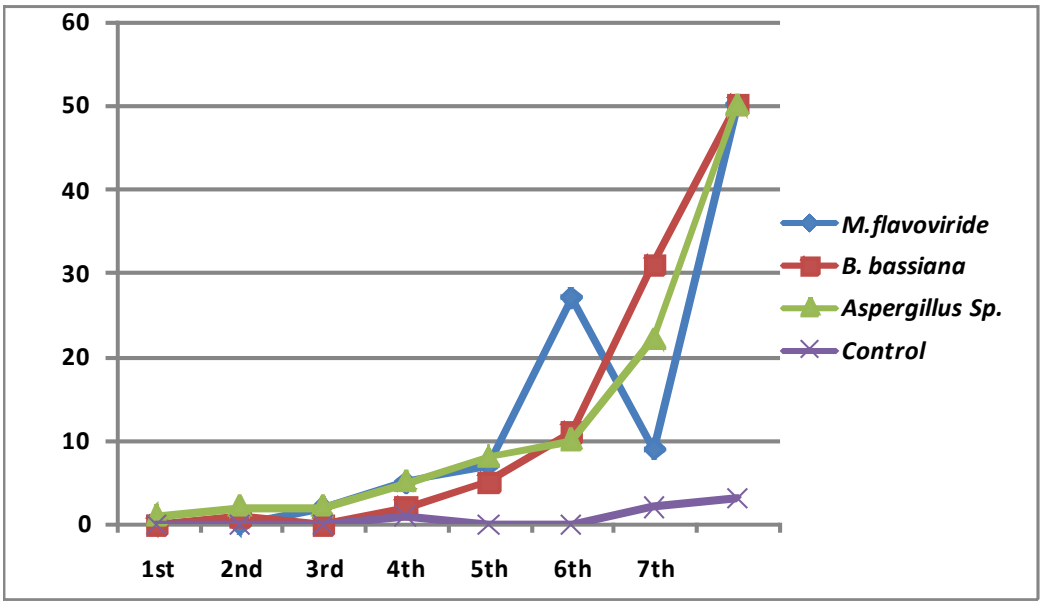

Fig. 3. Mortality of *Hieroglyphus oryzivorus* by treating with different species of fungi during the year 2012.

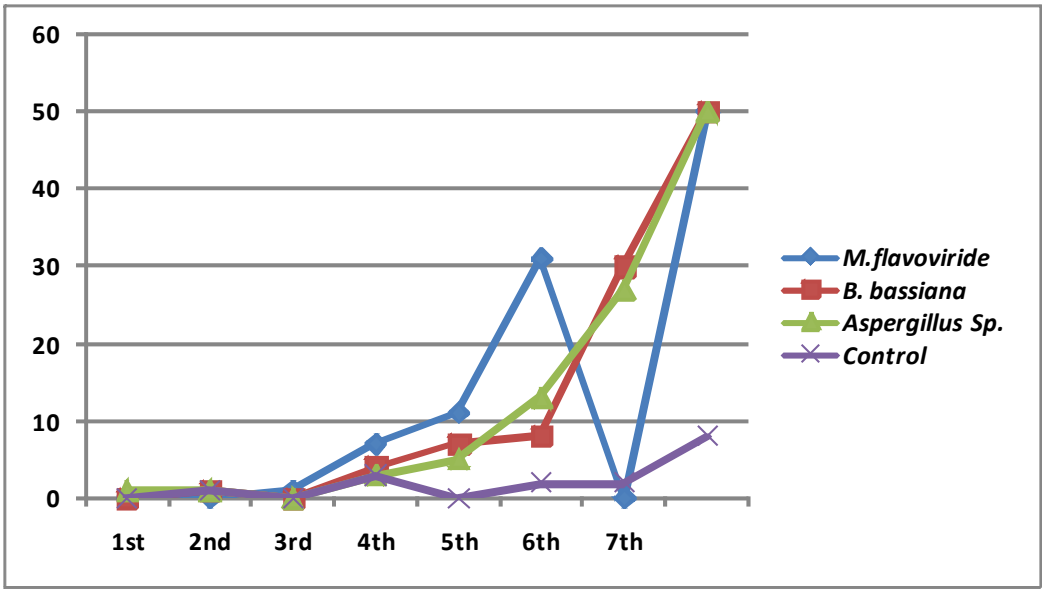

Fig. 4. Mortality of *Hieroglyphus nigrorepletus* by treating with different species of fungi during the year 2012.

sub-lethal effect of neuro-toxic insecticides on insect behavioral and found examples of both increases and reductions in food intake following exposure.

Moore *et al*. (1992) and Seyoum *et al*. (1994) both reported significant reductions in feeding by the Desert locust, *Shistocerca gregaria* (Forskal), following infections with *M. flavoviride*. Similar results have also been obtained from other host pathogen combinations including the North American rangeland grasshopper, *Melanoplus sanguinipes* (F.) infected with *Nosema locustae* Canning (Johnson and Pavlikova, 1986) and the armyworm, *Spodoptera exigua* Hubner, infected with *B. bassiana* (Balsamo) Vuillemine (Hung and Boucias, 1992). Evidence for depletion in nutrients in the host haemolymph has also been shown following fungal invasion (Zacharuk, 1971; Funk *et al*., 1993). All of these reported studies were carried out under very controlled, artificial laboratory conditions using insects inoculated with a high dose of pathogen and, in some cases, the

pathogen injected directly into the haemolymph. The aim of the current study was to improve on this by examining the effects of a range of pathogen doses on feeding rate and incubating insects under more natural conditions using cages maintained in the laboratory where the temperature range was optimum.

This study provides further evidence that infection by *M. flavoviride* causes a significant reduction in host feeding well before death. The average survival times of the treated insects in the present study were shorter than those typically observed in control. Thus, it is likely that even the lowest dose, at least when applied as a single source of inoculum under the pronotum, is higher then would be acquired in the field following spray application. That said, the reduction in feeding, as indicated by faeces production, was significant by the second or third day after inoculation for all doses. Even taking into account body size of the test insects, this is a faster and reduction at lower doses of pathogen than observed in other similar studies carried by (Moore *et al*., 1992; Seyoum *et al*., 1994).

Physical and biochemical events associated with the process of infection have been widely studied for *B. bassiana* (Balsamo) and *M. anisopliae* (Metsch.) Sorokin but much less so for *M. flavoviride*. Seyoum *et al*. (1994) suggested that significant colonization of the insect is necessary before feeding is reduced. Gunnarsson (1988) noted that hyphae of *M. anisopliae* did not reach the haemocoel of infected *S. gregaria* until 48 h post-inoculation. Significant colonization of tissues would not be expected to occur until sometime after this. In contrast, a study by Cheung and Grula (1981) in which *Heliothis zea* Boddie larvae were injected with *B. bassiana* revealed the gut walls were infiltered by long hyphae in just 48h. However, injection of the pathogen directly into the haemolymph by passes the processes and time associated with cuticular penetration. Thus, it is unlikely that sufficient colonization of tissues could have taken place within 48 h, it might give significant results.

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DEVELOPMENT AND EVALUATION A SIMULTANEOUS ASSAY METHOD OF METRONIDAZOLE AND DICLOFENAC POTASSIUM IN A PHARMACEUTICAL FORMULATION

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ABSTRACT

In this study, a simple, economic and selective HPLC method was developed and validated for the simultaneous estimation of Metronidazole (MET) and Diclofenac potassium (DIC-K). Reversed-Phase chromatography (RV-HPLC) was performed on a C18 column with methanol-Buffer 70:30%(V/V) as a mobile phase at a flow rate at 1ml/min. Metronidazole and Diclofenac Potassium have a maximum absorption at 254nm. The proposed method was successfully applied for determination of both drugs in one dosage form. Statistical analysis proved the method was precise, selective, specific and accurate for simultaneous analysis of Metronidazole and Diclofenac potassium.

Keywords: Metronidazole (MET), diclofenac potassium (DIC-K),HPLC, validation.

INTRODUCTION

Metronidazole (Fig. 1a), a pro-type nitroimidazole antibiotic used particularly for anaerobic bacteria and protozoa acts by disrupting the DNA helical structure, thus inhibiting nucleic acid synthesis (wikipedia.org, 2012; USP, 2012). The Diclofenac potassium 2(2,6 dichlorophenyl)amino benzeneacetic acid potassium salt (Fig. 1b) is a non steroidal anti-inflammatory drug (NSAID) taken to reduce inflammation and as an analgesic reduces pain in certain conditions such as arthritis, acute injury etc. Diclofenac works by inhibiting cyclooxygenase enzymes COX prostaglandin synthesis (wikipedia.org, 2012; USP, 2012). It was found that individually these drugs have been analyzed by many methods, RP-HPLC method has been used for determination of Diclofenac–K in combination with other drugs in one dosage form (Gowramma *et al*., 2010; Kasperek, 2008; Sunil *et al*., 2010). Many methods have been described in the literature for the determination of Metronidazole individually and in combination with other drugs in different pharmaceutical dosage forms (Rhaman *et al*., 2004; Mishal and Diana, 2005).

The combination of these two drugs is not official in any standard pharmacopoeia; hence no official method is available for the estimation of metronidazole and Diclofenac potassium in their combination dosage forms. The literature survey does not reveal any article related to the simultaneous HPLC or spectrophotometric determination of DICL-K-and MET in combination. To date there have been no published reports about the simultaneous quantitation of Metronidazole and Diclofenac potassium by HPLC. The present study describes simple, sensitive, precise, accurate, specific and repeatable HPLC method for the simultaneous estimation of Metronidazole (Fig. 1a) and Diclofenac potassium (Fig. 1b). The proposed method was validated as per ICH guidelines (ICH, 2005).

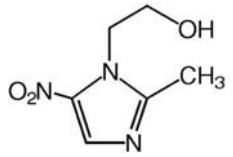

Fig. 1a. Metronidazole.

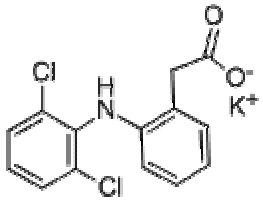

Fig. 1b. Diclofenac Potassium.

MATERIALS AND METHOD

Materials

Diclofenac potassium was provided by NPI (National Pharmaceutical Industries), Muscat, Oman and Metronidazole were obtained from Asia Pharmaceutical

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Labs, Syria. All chemicals and reagents used were of HPLC grade JT Baker, Netherland. All solvents used were of AR grade.

Instruments used were Reversed Phase RP- HPLC (Alliance) by Waters2469 with sampler programmed at 20µL capacity per injection. The instrument was controlled by using Empower software.
The wavelength used for detection was 254nm (USP, 2012).

The column used was C18 (250 mmx4.6, 5µ) obtained from Schirosorb ODS-2, Germany.

Preparation of mobile phase, buffer and standard, solution
Mobile phase used was a combination of methanol for HPLC grade and Buffer mixed in 70:30 ratio. The Buffer was prepared by mixing 0.01M phosphoric acid in equal volume with 0.01M Sod. Monophosphoric acid to obtain a buffer with pH 2.5 (pH was adjusted with phosphoric acid).

Standard stock solution containing 20µg/ml Diclofenac potassium and 20µg/ml MET was prepared using mobile phase. The stock solution was stored at 2-8^{o}C protected from light.

Optimization of HPLC method
The HPLC method was optimized and validated to develop a simultaneous assay method for DIC-K and MET. The individual standard and mixed stock solution of drugs were injected to HPLC (Fig. 2). For HPLC method optimization we used methanol and buffer in ratio70:30 at flow rate 1ml/min which gave acceptable retention time (t_R) and good resolution for Metronidazole and Diclofenac potassium drugs (Fig. 3).

Validation of the method
Validation of the optimized HPLC method was carried out with respect to the following ICH Guidelines.

Linearity and range
10.7mg of Diclofenac potassium was dissolved in 20ml of mobile phase. 2ml of this solution was diluted to 10ml resulting in a stock solution of Diclofenac potassium containing 0.107mg/ml of the drug. Six dilutions were prepared from 0.333µg/ml - 10.678µg/ml. Six dilutions for Metronidazole were also prepared ranging from 10.565µg/ ml-0.3301µg/ml. The linearity of the method was studied by injecting six concentrations of the drugs prepared in the mobile phase in five repeated injections in the LC system keeping the injection volume constant .The mobile phase was filtered through a 0.45µm membrane filter. The baseline was monitored continuously during this process. The detection wavelength was 254nm.The peak areas were plotted against the corresponding concentrations to obtain the Calibration graphs. The correlation coefficients, slope, y-intercept of the calibration curve were determined (Gowramma *et al*., 2010).

Precision
Precision was evaluated for inter-day (repeatability) and intra-day (Intermediate precision) variation. Repeatability studies were performed by analysis of three different concentrations 2.66, 5.33, 10.6µg /ml for Diclofenac potassium and 2.641, 5.28, 10.5µg/ml for metronidazole five times on the same day. The intermediate precision of the method was checked by repeating these studies on another HPLC, column on different days (Sunil *et al*., 2010; Baboota *et al*., 2007; Mahesh *et al*., 2010; Shaligrams *et al*., 2012).

Accuracy and Recovery
The accuracy method was carried out by applying the method to drug sample, Diclofenac potassium and Metronidazole on previously analyzed sample. The experiment was performed in triplicate. RSD (%), bias (%), and standard error of mean (SEM) were calculated for each concentration while the percent of recovery was found to be in the range of 101%---102% for both the drugs (Baboota *et al*., 2007; Shaligrams *et al*., 2012.

Limit of detection and limit of quantification
Limits of detection (LOD) and quantification (LOQ) represent the concentration of analyte that would yield signal – to – noise ratio of 3 for LOD and 10 for LOQ, respectively. The samples of Diclofenac potassium and Metronidazole were injected into LC system and measured signal from the samples was compared with those of blank samples (Baboota *et al*., 2007).

Robustness
To evaluate robustness of an HPLC method, few parameters were deliberately varied. The parameters included variation of HPLC system like Agilent HPLC and Water 2469 using different operators, different columns of similar type like HypersilC18, Shimadzu ODC, and at three different concentration levels 6,8,10µg/ml each for Diclofenac Potassium and Metronidazole (Gowramma *et al*., 2010; Baboota *et al*., 2007).

Specificity
The specificity of this method was ascertained by analyzing standard drug and sample. The spot for Diclofenac potassium and Metronidazole was confirmed by comparing the spectra with that of the standard. The peak purity of Diclofenac potassium and Metronidazole was assessed by comparing at three

different levels, i.e. peak start(S), peak apex (M) and peak end (E).

RESULTS AND DISCUSSION

The results of the validation of the simultaneous estimation method developed for Diclofenac Potassium and Metronidazole in the current study using Methanol: Buffer (70:30, v/v), are as discussed below:

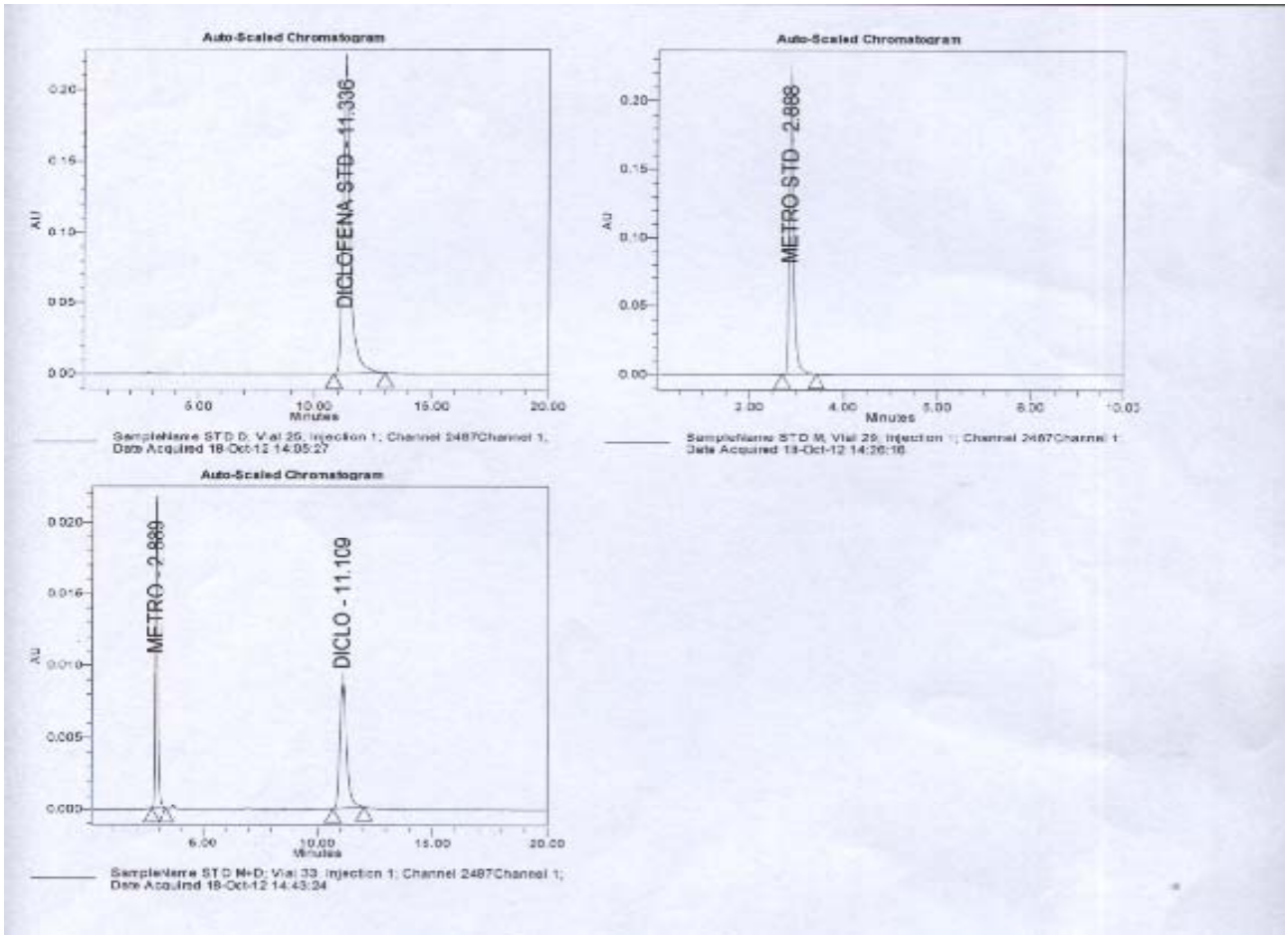
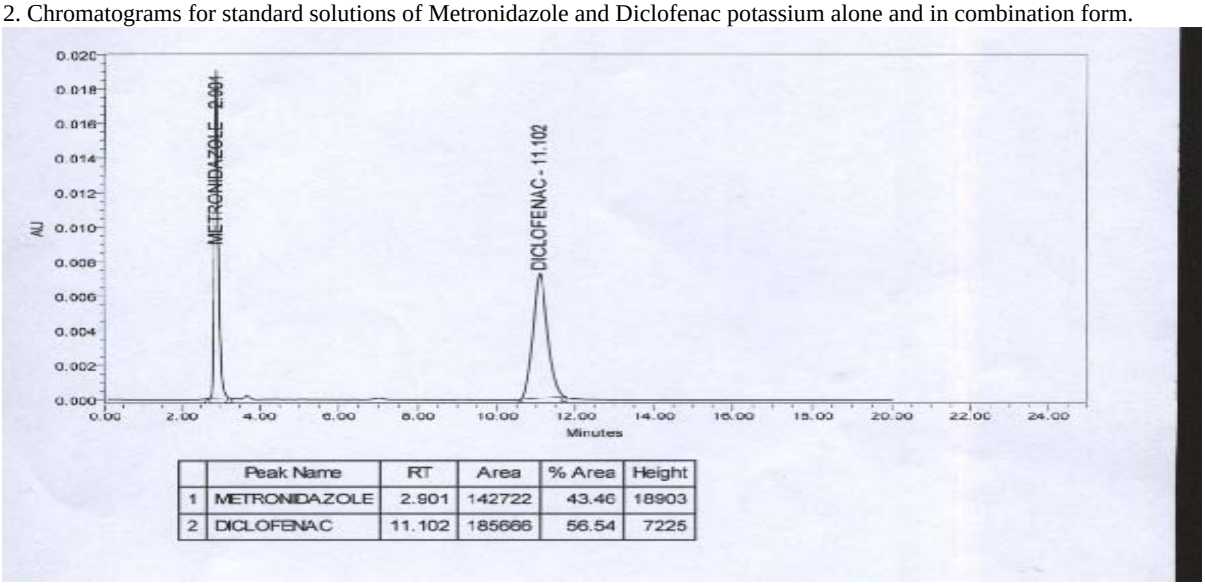


Fig. 2. Chromatograms for standard solutions of Metronidazole and Diclofenac potassium alone and in combination form.



	Peak Name	RT	Area	% Area	Height
1	METRONIDAZOLE	2.901	142722	43.46	18903
2	DICLOFENAC	11.102	185666	56.54	7225

Fig. 3. Chromatogram for both Diclofenac Potassium and Metronidazole drugs.

Linearity

The linear regression data for calibration plot are indicative of a good linear relationship between peak area and concentration of a wide range in a range of 0.33-10.67µg/ml and 0.33-10.678µg/ml for metronidazole and Diclofenac potassium respectively. The slope and intercept value for calibration was y= 9399 x (R^2=0.9996) for Diclofenac potassium and y=7097x(R^2=0.9988) for Metronidazole (Fig. 4). The percent RSD% was found to be less than 2% (Table 1). This performance shows a good correlation between response factor and concentrations of the drugs (Table 2).

Precision

Precision was evaluated by intra-day (Repeatability) and inter-day (Intermediate precision) variation, and different makes of the column. Repeatability (five replicates) was assessed independently for each of the different concentration. Results from the determination of repeatability and intermediate precision, expressed as RSD%, are listed in table 3. The low values of RSD indicated the repeatability of the method.

Recovery and Accuracy

The recovery of the method shows good recoveries of the Diclofenac potassium and Metronidazole in the range 100.41- 102.965 %. The value of recovery (%), RSD (%), Percentage bias, and SEM indicated that the method is accurate (Table 4).

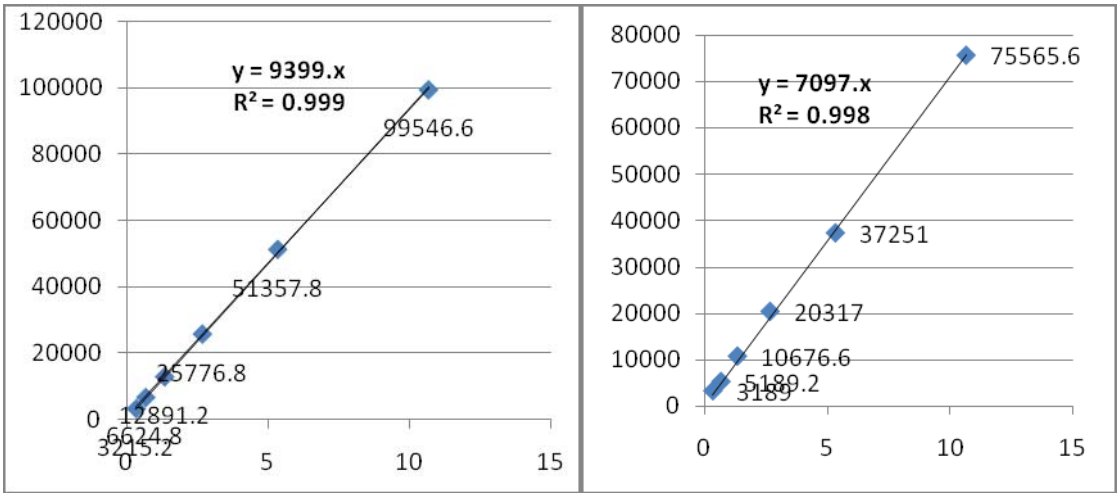


Fig. 4. Calibration curve of Diclofenac potassium and Metronidazole .

Table 1. Linear regression data for the calibration plot (n=5).

Conc.(ug/ml) DIC-K	Mean area	±SD	RSD(%)	95% confident interval	
				Low value	Upper value
2.66	25776.8	383.17	1.49	25184	25792
5.33	51357.8	395.23	0.77	50886	51779
10.676	99546.6	1284.44	1.29	98577	101116
106.6	1036417.250	9230.75	0.89	102316	1044187

Conc.(ug/ml) MET	Mean area	±SD	RSD (%)	95% confident interval	
				Low value	Upper value
2.66	20317	360.9	1.78	19924	20638
5.33	37251	460.7	1.24	36643	37658
10.676	75566	484.4	0.64	75001	76152
106.6	777373	4705.9	0.61	772088	783525

Table 2. Correlation between response factor and concentrations of the drugs.

Parameter	Diclofenac Potassium	Metronidazole
Linearity range	(0.33-10.67) µg/ml	(0.33-10.678)µg/ml
Correlation Co efficient	0.9996	0.9988
Slope	9399	70997

Table 3. Precision of the method.

Repeatability (Intra –day precision) *(n=5)*			
	Conc.µg/ml	Area under Curve AUC ±SD	RSD %
Diclofenac Potassium	2.66	25776.6 ±38.167	1.49
	5.33	51357±395.227	0.77
	10.67	99546.6±1284.4377	1.29
Metronidazole	2.641	20317±360.91	1.78
	5.28	37251± 460.72	1.24
	10.5	75565.6 ± 484.44	0.64

Intermediate precision (Inter-day)*(n=5)*			
	Conc.µg/ml	Area under Curve AUC ±SD	RSD %
Diclofenac Potassium	2	25356.1± 3993.84	1.58
	4	407547± 5917.254	1.45
	6	552918± 5660.394	1.02
Metronidazole	2	17792.33± 178.9814	1.005
	4	28872.33± 175.0467	0.61
	6	40114.67± 680.7983	1.70

Table 4. Accuracy of the method (*n=3*).

% of drug added to the analyte	Theoretical conc.µg/ml	Conc. mean	SD	Recovery	% RSD	% Bias	SEM
Diclofenac Potassium							
0	2.5	2.42	0.15272	101.76	0.64	-3.86	0.008
100	5	5.01	0.0608	101.47	1.22	+0.2	0.035
200	10	10.01	0.0666	100.41	0.67	+0.1	0.035
Metronidazole							
0	2.45	2.44	0.5	102.965	1.64	-0.4	0.030
100	4.9	4.85	0.0377	101.641	0.78	-1.04	0.028
200	9.8	9.96	0.0666	101.970	0.68	0	0.058

Limit of detection LOD and Limit of quantification LOQ

LOD and LOQ were determined by the standard deviation ($S_y/_x$) method. The LOD and LOQ was determined from the slope, S, of the calibration plot and standard deviation of the responses of the sample, by using the formula LOD=$3xS_{y/x}$ /S and LOQ=$10xS_{y/x}$/S. The LOD and LOQ were found to be 0.1345µg/ml and 0.4µg/ml for DIC-K and 0.1677µg/ml and 0,5507µg/ml for MET respectively which indicates the method can be used for detection and quantification of a quite wide range of concentrations (Table 5).

Table 5. LOD and LOQ of the method.

Drug	LOD $3xS_{y/x}$/slope	LOQ $10xS_{y/x}$/slope
Diclofenac Pot.	0.1345 µg/ml	0.4µg/ml
Metronidazole	0.1677 µg/ml	0.5507 µg/ml

Specificity

The peak purity of Metronidazole and Diclofenac potassium was assessed by comparing their respective spectra at peak start, apex and end position. It was found

that r(S, M) = 0.989071 and r (M, E) = 0.98669. A good correlation was obtained; the mobile and diluents did not show interference with the assay, indicating the specificity of the method.

Table 6. Specificity.

S (peak start)	M (Peak apex)	E (Peak end)
7.3	3.9	8.1
7	3.5	7.9
7.2	3.7	8
7.4	4	8.2
7.3	3.9	8.1

Robustness

It was observed that there were no marked changes in the chromatograms. There was no significant change in the retention time of Metronidazole and Diclofenac potassium when we changed the column and the HPLC. The low values of RSD, shown in table 7 and 8, indicated the robustness of the method.

Suitability

This included the column efficiency, resolution and peak asymmetry. The values obtained demonstrated the suitability of the system for the analysis of the drugs in combination calculated for the standard solutions (Table 9).

CONCLUSION

This HPLC method was developed and validated as per ICH guidelines which was accurate, precise and reproducible. The UV detection allowed an accurate

Table 7. Results from testing of the Robustness of the method by changing instruments (HPLC, Column).

DIC-K	Conc. µg/ml.	Area under the curve			Retention time			
		Mean AUC	±SD	RSD %	Mean t_R	±SD	RSD %	%Bias in t_R
	6	455538	6071.0301	1.332717	9.72	0.206	2.11	1.65
	8	691007.7	12211.235	1.767163	9.94	0.092	0.93	-0.62
	10	103006.3	2900.2181	2.815573	9.75	0.258	2.65	1.61

DIC-K	Conc. µg/ml.	Area under the curve			Retention time			
		Mean AUC	±SD	RSD %	Mean t_R	±SD	RSD %	%Bias in t_R
	6	46071.67	556.1747	1.2071	11.04	0.00493	0.04	-0.05
	8	68938.1	241.8077	0.3507	11.03	0.00057	0.005	0.003
	10	83142.33	499.1846	0.6003	10.98	0.0707	0.64	-0.39

Table 8. Results from testing of the Robustness of the method by changing instruments (HPLC, Column).

MET.	Conc. µg/ml.	Area under the curve			Retention time			
		Mean AUC	±SD	RSD %	Mean t_R	±SD	RSD %	%Bias in t_R
	6	76173.33	555.93195	0.73	3.04	0.0015	0.05	-0.04
	8	90581.01	389.49582	0.43	3.03	0.0010	0.03	0.03
	10	105100.3	1735.0015	1.65	3.02	0.0005	0.02	0.01

MET.	Conc. µg/ml.	Area under the curve			Retention time			
		Mean AUC	±SD	RSD %	Mean t_R	±SD	RSD %	%Bias in t_R
	6	40453	545.22564	1.3	2.841	0.001	0.04	0
	8	56501.67	482.90923	0.86	2.841	0.00057	0.02	-0.023
	10	67176	829.04282	1.23	2.406	0.00577	0.24	-0.138

Table. 9. Combination calculated for the standard solutions.

Drugs	Linearity range	Regression equation	R^2	LOD	LOQ
DIC-K	0.33-10.67	Y=9399.8x	0.9996	0.1345	0.4
MET	0.33-10-.67	Y=7097.8	0.9988	0.1677	0.55

quantification of Diclofenac potassium in combination with Metronidazole. The method was established by HPLC using ODS–C18 (250mmx4.6mm,5µ) with simple mobile phase containing alcohol: buffer (70:30) at a flow rate 1ml/min using 254nm. The HPLC method was validated shown satisfactory data for all the parameters tested. Statistical analysis proves that the method is suitable for the analysis of Diclofenac potassium and Metronidazole in combination. The reported method was found capable of giving faster analysis with good resolution and found to be reliable, economical and can be successfully employed for routine simultaneous estimation of Diclofenac potassium and Metronidazole in formulations. Study of the effects of exhaustive stress conditions is in progress.

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ANTIMICROBIAL EFFICACY OF *OCIMUM GRATISSIMUM* AND*VERNONIA AMYGDALINA* ON GASTROINTESTINAL BACTERIA

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ABSTRACT

This study was carried out to determine the antibacterial potency of *Ocimum gratissimum* and *Vernonia amygdalina* against isolates of *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis* and *Streptococcus faecalis* using sensitivity disc diffusion assay. Extraction was achieved by drying and blending into powdery form, which was separately mixed with distilled water and 70% ethanol in respective flasks. This was properly sieved and stored. The antibacterial properties of extracts of both samples were separately determined against the test isolates. The phytochemical test conducted revealed that the extracts of both samples possessed different biologically active constituents, namely: tannins, flavonoids, saponins, anthocyanins and phlobatannins, while betacyanins were found absent in both extracts. Physicochemical analyses of both extracts showed that a pH range of between 6.0 and 7.5 provided a broad spectrum of activities against a wide range of bacterial infections. Results of antibacterial screenings revealed that the water extracts of both samples were more efficacious than the ethanol extracts. However, both were found to be very active against the test organisms. It was further observed that water extracts were more active against *Bacillus subtilis, Staphylococcus aureus* and least against *Pseudomonas aeruginosa.* On the other hand, ethanol extracts were more efficacious against *Escherichia coli* and *Streptococcus faecalis.* It has been concluded in this study that *O. gratissimum* and *V. amygdalina* can be extensively used tradomedically in the Nigerian folk medicine to treat several bacterial infections, and are thus, recommended as a source of natural product for future use in the management and cure of multi-drug resistant bacterial infections in African continent and the world at large.

Keywords: Gastrointestinal, antimicrobials, bacteria, plants, medicinal.

INTRODUCTION

Microorganisms have been found to be the causal agents of many diseases, a fact which has incited the interest of many scientists to detect substances which can be used to control the pathogens. These substances are known as antimicrobial agents. The term 'anti-microbial agent' is a general term for all substances that can be used systematically (introduced into the body) to inhibit or kill microbial pathogens regardless of their origin (Norton, 1991). Nature has endowed mankind with a rich store-house of natural antimicrobial agents – the plants. Plants are vital parts of man's existence, the most essential to his well being. Plants are able to synthesize a wide range of chemical substances which had been of tremendous value in the treatment and prevention of diseases. Studies carried out on them have revealed that the active ingredients in many of such are known to contain alkaloids, phenolics, saponins and tannins (Odebiyi and Sofowora, 1998). Medicinal plants are used in the treatments of diseases either alone or in combination with other plants parts. They are used as anti-infective agents, laxatives, cardiovascular and nervous remedies, proteolytic ferments, steroid sources, sweeteners, anti-tumour drugs, and a source of anti-malaria in dosage form (Sofowora, 1982; Gbile and Adesina, 1996; Owonubi, 1998). The research is therefore still on for a naturally occurring and readily available effective antibiotics of plant origin.

As a result of research into the active components contained in some plants extracts, considerable information has surfaced into the world of chemotherapy. For instance, Kusoje *et al.* (1968) obtained antifungal activity from "*rice bran ter*" highly potent in the treatment of eczema. Vegetable extracts of garlic, onion, turnip, green pepper and radishes were found to inhibit the growth of *Escherichia coli, Salmonella typhi, Shigella dysenteriae* and *Staphylococcus aureus* (Aldemy and Alli, 1970). A preparation from avocado pear has been shown to inhibit thirteen different species of bacteria (Neemen and Kashman, 1990).

Ocimum gratissimum; family Labiaceae is an herbaceous plant commonly found in tropical Asia, especially in India where it is used for aromatic baths of fumigations in the treatment of rheumatism and paralysis. It is widely distributed in tropical and temperate regions. The plant is also found in West Africa. In Nigeria, it is found in the savannah and coastal areas where it is used in the

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treatment of high fever (Oliver, 1980), epilepsy (Osifo, 1992) and diarrhoea (Oliver, 1980; Sofowora, 1993). Decoctions of the leaves have also been used in treatment of mental illness (Abdulrahman, 1992). The leaves of the plant are used as condiments in food. It is called "Efinrin" by Yoruba tribe of South-Western Nigeria , Ebavbokho in Benin (Delta State), Aai doya ta gida in Hausa (Northern Nigeria) and Nchonwu in Igbo (South Eastern Nigeria) (Owulade, 2004).

Lima *et al.* (1993) tested in vitro antifungal activity of thirteen essential oil obtained from some plants against dermatophytes in which that of *O. gratissimum* was found to be the most active, inhibiting 80% of the dermatophyte strains tested and producing zone greater than 10 mm in diameter. Nwosu and Okafor (1995) reported the antifungal activities of extracts of ten medicinal plants collected from southeastern Nigeria against seven pathogenic fungi. According to these authors, *O. gratissimum* inhibited the growth of *Trichophyton rubrum* and *Trichophyton mentagrophytes*. Ilori *et al.* (1996) reported the anti-diarrhoeal activities of leaf extracts of *O. gratissimum* that the extracts were active against *Aeromonas sobria, Escherichia coli, Salmonella typhi* and *Salmonella dysenteriae.* The authors have shown that the MIC for these organisms ranged from 8-50mg/ml while MBC were from 8-62mg/ml.

Vernonia amygdalina is a shrub or small tree of 2-5m with petiolate leaf of about 6 mm diameter and elliptic shape that grow predominantly in the tropical Africa. In Nigeria, the plant is locally called bitter leaf due to its very bitter taste. No seeds are produced and the three has to be distributed through cutting. They grow under a range of ecological zones in Africa, produce large mass of forage and are drought tolerant. There about 200 species of Vernonia (Bonsi *et al.*, 1995a).

These plants are qualified to be called medicinal plants by virtue of the fact that they have been in therapeutic use against various diseases like parasitic infection, URTI, Syphilis, other venereal diseases like gonorrhea, pneumonia and enteric fever respectively, for over one hundred years (Lewis and Elvin-Lewis, 1977). Just a few studies in Africa are available on the phytochemistry, dosage of administration and contraindication of medicinal plants compared to the array of available medicinal plants (Gundiza, 1985; Ebana *et al.*, 1991; Kola *et al.*, 2002). This explains why certain less potent toxic synthetic chemicals from the west are recognized and preferred to these more potent, less toxic medicinal plants. The potentials for multiple resistances by the isolates used in this research have been demonstrated by many researchers around the world (Diep *et al.*, 2008). Many isolates of *Escherichia coli* (and *Staphylococcus aureus*) for instance, are resistant to ampicillin, amoxicillin, tetracycline and trimethoprim-sulfamethoxazole (Aibinu *et al.*, 2004). In the year 2000, 7.1% cases of multiple drug resistant bacterial isolates to conventional antibiotics were reported (Sahm *et al.*, 2001). Umolu *et al.* (2006) reported that 67% of the resident isolates exhibited multiple drug resistance. The therapeutic failure of antibiotics in Nigeria, Africa and indeed all parts of the world buttresses the need to support the use of local medicinal plants.

In this study, five different genera of bacteria were employed against the antimicrobial efficacies of the plants' extracts. These are two gram-negative bacteria: *Escherichia coli* and *Pseudomonas aeruginosa*, and three gram-positive bacteria: *Staphylococcus aureus, Bacillus subtilis* and *Streptococcus faecalis*. These groups of bacteria are normal inhabitant of the gastrointestinal tract of man and animals. Therefore, since *O. gratissimum* and *V. amygdalina* are herbaceous plants and are known to have some medicinal efficacies, and results of studies on their medicinal potentials have not been extensively documented in the past. It is on this basis of the little available information on the antimicrobial significance of these plants that the present study was aimed at achieving the antibacterial efficacy of the water and ethanol extracts of *O. gratissimum* and *V. amygdalina* on selected bacteria species, phytochemical analysis on the plants extracts speculate on the significance of both extracts and hence, their chemotherapeutic functions in the treatment of diseases.

MATERIALS AND METHODS

Collection of plant samples

The medicinal plants used in this study were purchased on consultation of practitioners of ethno-medicine from Oshodi and Bariga Markets in Lagos State, and Oja-Oba and Bodija Markets in Oyo State, South- Western Nigeria. The samples were authenticated by experts in the School of Pharmacy/Botany, University of Lagos, Nigeria. They were kept in the refrigerator at 4^0C until used.

Test organisms

Two gram-negative bacteria: *Escherichia coli* and *Pseudomonas aeruginosa*, and three gram-positive bacteria: *Staphylococcus aureus, Bacillus subtilis* and *Streptococcus faecalis* were obtained as type strains from the culture collection unit of the Department of Biotechnology, Federal Institute of Industrial Research Oshodi (FIIRO), Lagos, Nigeria. The cultures were maintained on nutrient agar slants and sub-cultured fortnightly throughout the period of research.

Preparation of *Ocimum gratissimum* and *Vernonia amygdalina* leaves extracts

The leaves of the plants were dried in the oven at 50^0C for 48hrs. The dried samples were blended using milling

machine and later sieved to obtain the flour samples from both plants (Ugorji *et al.,* 2000). 200g of the flour samples of each plant was weighed and separately mixed with 1 litre (1000mls) of distilled water and 70% ethanol in respective flasks (Alade and Irobi, 1993; Ugorji *et al.*, 2000). Each flask was then allowed to stand for 72 hrs at room temperature with occasional agitation. The mixture was then filtered, and thereafter centrifuged at 5000 rpm for 20 minutes. The volume extracts obtained were then stored in screw cap bottles in the refrigerator at 4^oC (Ugorji *et al.,* 2000).

Phytochemical analysis of samples extracts

The preliminary phytochemical analysis was carried out employing the method of Culer (1982), Sofowora (1993), Odebiyi and Sofowora (1998) and Trease and Evans (2002). The extracts were screened for the presence of biological active constituents such as Tannins, Saponins, Flavonoids, Betacyanins, Anthocyanins and Phlobatannins.

Determination of the physicochemical parameters of samples' extracts

The pH of the extracts were determined using the pH meter (Unicam 9450) after its initial standard standardization using appropriate buffers. A graduated mercury bulb thermometer was employed to determine the temperatures of extracts. These were recorded accordingly.

Determination of antibacterial properties of extracts

Sensitivity disc method was employed as described by Kela and Kufeji (1995). The test isolates of bacteria were cultured in peptone water for 24hrs. 0.2ml of each suspension was taken and mixed with 10ml of nutrient agar in sterile petridishes. Discs of equal diameters were soaked in water-dissolved and ethanol-dissolved extracts' suspensions for 3hrs. The different discs from the different suspensions were removed and placed on the culture surface, and incubated at 37^0C for 24hrs. The culture media were examined for zones of inhibition.

RESULTS AND DISCUSSION

Table 1 showed the results of the phytochemical analyses of water extracts of *O. gratissimum* and *V. amygdalina*. These results revealed the presence of many biologically active constituents namely: tannins, flavonoids, saponnins, anthocyanins, phlobatanins, with the absence of betacyanins in the water extracts. These same active constituents were found present in the ethanol extracts of the samples but in different degrees (Table 2). The presence of these active constituents actually helped to confer the antibacterial properties on the extracts of both samples. The result is in line with the observation made by Kela and Kufeji (1995) while experimenting on the efficacy of *Moringa oleifera* and *Mitracarpus scaber*. They observed that the efficacy of these plant extracts was due to the presence of tannins, saponins and anthocyanins in the extracts. Alade and Irobi (1993) made similar observation when they carried out analyses on the antimicrobial activities of leaf extract of *Acalypha weikeisiana* against some selected organisms. They related the antimicrobial activities of the leaf extract to the presence of active constituents such as phenol and saponin. These bioactive compounds have been reported to possess antimicrobial potency (Sofowora, 1993).

The pH of water and ethanol extracts of *O. gratissimum* were 6.50 and 7.20 respectively with temperatures

Table 1. Phytochemical analyses of water extracts of *O. gratissimum* and *V. amygdalina.*

	Tannins	Flavonoids	Saponins	Anthocyanins	Phlobatannins	Betacyanins
O. gratissimum	+++	+++	+++	+++	++	-
V. amygdalina	++	++	+++	+++	+	-

Key: +++ = strongly positive; ++ = moderate positive + = trace positive; - = negative

Table 2. Phytochemical analyses of ethanol extracts of *O. gratissimum* and *V. amygdalina.*

	Tannins	Flavonoids	Saponins	Anthocyanins	Phlobatannins	Betacyanins
O. gratissimum	++	++	++	++	+	-
V. amygdalina	+	++	++	++	+	-

Key: ++ = moderate positive; + = trace positive; - = negative

Table 3. Physicochemical analyses of water and ethanol extracts of *O. gratissimum.*

Parameter	Extracts of *O. gratissimum*	
	Water Extract	Ethanol Extract
pH	6.50	7.20
Temperature	28 ± 2^0C	28 ± 2^oC

Table 4. Physicochemical analyses of water and ethanol extracts of *V. amygdalina*.

Parameter	Extracts of *V. amygdalina*	
	Water Extract	Ethanol Extract
pH	6.62	7.40
Temperature	28 ± 2^0C	28 ± 2^oC

Table 5. Antibacterial activities of water and ethanol extracts of *O. gratissimum* against some bacterial species.

Extract	Size of Zone of Inhibition (mm)				
	Escherichia coli	*Streptococcus faecalis*	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Staphylococcus aureus*
Water	8.0	9.0	6.0	10.0	10.0
Ethanol	6.0	6.0	8.0	9.0	7.0

ranging from 28 ± 2^0C (Table 3). Similarly, the pH of the water and ethanol extracts of *V. amygdalina* measured 6.62 and 7.40 respectively with the same temperature of 28 ± 2^oC (Table 4).

The antibacterial analyses of the extracts from both samples revealed that water extracts have higher efficacy in their inhibitory activities on the various test organisms than the ethanol extracts (Tables 5 and 6). The higher bactericidal effect of the water extract could be attributed to the concentration of the biologically active constituents following extraction. This confirmed water as a better solvent than ethanol thereby enhancing extraction, hence a higher concentration of the constituents. The passage of the active constituents across the bacterial membrane depends on the size of their particles, and it is probable that the active constituents extracted by water had finer particles which made them penetrate more easily through the membranes of the bacterial cells than the ethanol extract. It was, however, observed that the ethanol extract of *O. gratissimum* has greater efficacy on *Pseudomonas aeruginosa* than the water extract (Table 7). Also, ethanol extract of *O. gratissimum* was very efficacious against *Bacillus subtilis* but least effective against *Escherichia coli* and *Streptococcus faecalis* (Table 5). The results showed that the water extracts of *O. gratissimum* and *V. amygdalina* were more active against *Bacillus subtilis, Staphylococcus aureus* but least against P*seudomonas aeruginosa* (Tables 5 and 6). Furthermore, ethanol extract of *V. amygdalina* was also found to be highly active against *Staphylococcus aureus* and least in *Streptococcus faecalis* and *Pseudomonas aeruginosa* (Table 6). It was observed in this study that the antibacterial action of extracts of both samples against *Pseudomonas aeruginosa*, a bacterium well known for its constitutive resistance to many antibiotics, was pronounced. The degree of sensitivity of both extracts was expressed as measure of the diameter of zone of inhibition in millimeters. It is notable that this study is in line with several studies conducted on the antimicrobial properties of herbs and spices (Khan *et al.*, 1998; Dorman and Deans, 2000; Hsieh *et al.*, 2001). However, not many researchers put the use of such multi-drug resistant or beta-lactamase producers into consideration.

In conclusion, water extracts of both samples investigated were more efficacious than the ethanol extracts in their bactericidal activities. The bactericidal activities of both extracts could be attributed to the presence of the biologically active constituents namely: tannin, flavonoid, saponnin, phlobatannin, and anthocyanin in the extracts. Since the extracts of both samples were very potent against both gram-positive and gram-negative bacteria, they could be processed for use as broad-spectrum antibiotics. However, pharmacological standardization and clinical evaluation of these plant extracts, together with the determination of their Minimal Inhibitory Concentration, isolation and characterization of their active constituents, may lead to the commercialization of the extracts for use in the treatment of diseases, and for other important chemotherapeutic uses. Better therapy for many bacterial diseases could be detected in the barks and leaves of some neglected plants. Therefore, growing and use of *O. gratissimum* and *V. amygdalina* should be encouraged.

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CURRENT HABITAT, DISTRIBUTION AND STATUS OF THE MAMMALS OF KHIRTHAR PROTECTED AREA COMPLEX, SINDH

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ABSTRACT

Studies were undertaken in the Khirthar Protected Area Complex (KPAC) during 2010 and 2011 to record the species of mammals, their main habitats and distribution in this area. A total of 33 species of mammals were recorded and 22 main habitats of mammals were identified. The occurrence of 11 key species of the mammals was recorded in the different components of the KPAC. These species included Sind Ibex or Sind Wild Goat (*Capra aegagrus*), Urial (*Ovis vignei*), Chinkara (*Gazella bennettii*), Striped Hyaena (*Hyaena hyaena*), Caracal (*Felis caracal*), Honey Badger (*Mellivora capensis*), Indian or Asiatic Jackal (*Canis aureus)*, Indian Fox (*Vulpes bengalensis*), Red Fox (*Vulpes vulpes*), Desert Cat (*Felis silvestris*) and the Jungle Cat (*Felis chaus*). During the study, 1200 Sind Ibex, 1000 Urial and 150 Chinkara were sighted. Disturbance, human and wildlife conflicts, and degradation of habitats are the main threats to the wild animals particularly to Chinkara. These adverse environmental impacts need to be mitigated.

Keywords: Protected area, Khar Centre, Karchat Centre, distribution, status.

INTRODUCTION

The Protected Areas are recognized as an effective tool in conserving biodiversity and ecosystems. The Red List of Threatened Species (World Conservation Union-IUCN) documents that the loss of natural habitats is a predominant threat to biodiversity, and protected areas are widely regarded as one of the most successful measures implemented for the conservation of biodiversity. Currently, the World Database on Protected Areas has documented 148,000 protected areas worldwide (IUCN, 2013).

Pakistan has three categories of protected areas: National Parks, Wildlife Sanctuaries, and Game Reserves (Khan *et al*., 2010). The Khirthar Protected Areas Complex (KPAC) stretches over 4,350 km^2, encompassing the protected areas (PAs) that lie in Kohistan in the southwest of Sindh. The KPAC comprises of Khirthar National Park (KNP), the Mahal Kohistan Wildlife Sanctuary (MKS), Hab Dam Wildlife Sanctuary (HDS), the Surjan, Sumbak, Eri, and Hothiano Game Reserves (SGR). Khirthar National Park is listed as a Protected Category II area by the IUCN, and is the first of Pakistan's national parks to be included in the 1975 United Nation's list of National Parks and Equivalent Reserves.

The KNP, MKS, and HDS were officially declared as Protected Areas on January 31, 1974. The Park stretches over 3,087 km^2, while the MKS and the HDS cover 705.7 km^2 and 272 km^2 respectively. The SGR was established in June 1976 over an area of 285.3 km^2. The KPAC is significant for its sizable indigenous settlements, rugged terrain, valuable flora and mineral resources, and a number of rare wildlife species such as the Sindh Ibex, Urial, Chinkara, Houbara Bustard, Gray Partridge, See-see Partridge and Birds of Prey. Archeologically significant sites include the tombs in Taung, the Fort of Rannikot and the fossils and petrified forests of the Khirthar Range.

Some work has been previously done on the fauna of the KPAC. Initial benchmark studies were undertaken in 1979 - 80 and information was collected on the ecology, population and the status of the key species of the KNP particularly in respect of the ungulates (Haleem and Khan, 1975). The first Management Plan of KNP was prepared by Holloway and Khan (1973).

Afterwards, environmental baseline studies were initiated by the Sindh Wildlife Department and the University of Melbourne, Australia in 2000, and detailed report on the Wildlife of the area was prepared (Morgan and Harrington, 2001). Later on subsequent environmental

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monitoring studies were initiated under the Petroleum exploration activities in the area. Some useful data were collected (Qadir, 2000; Mirza, 2002; Hagler Bailly Pakistan, 2001, 2002, 2002a, 2002b; Halcrow Pakistan, 2002, 2002a; Akhtar, 2006). The second management plan of the KNP was prepared in 2005 (Hagler Bailly Pakistan, 2005). In a recent study, Khan *et al*. (2012), gave detailed information about the fauna and the environmental conditions of the Hub Dam Wildlife Sanctuary area of KPAC, and reported to have encountered 16 species of mammals, 160 species of birds, 23 species of reptiles, 03 species of amphibians, 29 species of fishes, and 25 species of plants.

The objective of the present study was to determine the habitat, current distribution and status of the mammals of Khirthar Protected Area Complex (KPAC).

MATERIALS AND METHODS

The present studies with particular reference to large mammals of the KPAC were undertaken during 2010 - 2011. Field observations were made using spotting cope 60x60 and binoculars 10x50 (Olympus). The animals were watched by walking along ridges and ravines in the early mornings and late afternoons or sitting quietly on cliffs and watching the aspects facing the observer. On spotting an Urial and Ibex herd, the number of individuals was counted. Information recorded included number, sex, age, date, time, latitude, and longitude and habitat type.

Counting procedure

All animals ahead of the observer were counted from 90 degree to the left of the path to straight-ahead to 90 degree to the right. Care was taken not to count an individual more than once; care was also taken in counting groups because, once groups are disturbed and started to move, additional animals might join the group. Binoculars were used only to check identifications and to assist counts of distant groups.

Point Surveys

Observation points were established in the study area and at each observation point, the observer recorded all sightings of the mammals at that site (Brower *et al.*, 1990).

Roadside Counts

This method (Khan *et al*., 2010) was applied mostly for the nocturnal mammals like Foxes, Jackals and Wild Cats as well as for the diurnal mammals like mongoose. For this purpose a 4x4 vehicle was used which was driven at a slow speed. These roadside counts were carried out during early mornings at dawn and during night by using search lights.

Line transects

The line transect or strip census method (Khan *et al*., 2010; Schemnitz, 1980) of population estimation involved counting the animals seen by traversing a predetermined transect line and recording the animal and distances on both side of the strip at which they were observed. The length of the strip multiplied by average total distance of both sides of the strip was taken as the sample area.

Pellet counts

Pellet counting in a specific area is a good technique (Khan and Siddiqui, 2011) for locating large mammals and determining their population. The technique involved removing all pellet groups from plots and then estimating from subsequent observations on those plots the number of groups per hectare to compare animal use of areas between sampling periods.

Baited Spotlighting

Most of the larger predatory mammals were difficult to detect using the normal spotlighting techniques; accordingly a technique was used to attract these animals close to the road. So fresh meat bait (e.g. the skin of a recently slaughtered Goat) was dragged behind the vehicles along a 10km long trail by each of the two vehicles. When the route was retraced in the reverse direction, often predatory animals were encountered following the scent trail along the road (Khan and Siddiqui, 2011). Species and numbers of animals were recorded and most of the times, their footprints were found imprinted on the loose ground. In addition to the above data, incidental sightings of all the large mammals, reptiles and any of the rarer bird species were also recorded.

RESULTS AND DISCUSSION

The overall animal habitats were classified into the following prime habitat types:

1. Mountain Ridges and Ravines
2. Mountain Escarpments
3. Stony Ground/ Rocky Areas
4. Sandy Plains
5. Wetlands
6. Riparian Areas
7. Village / Agriculture Area
8. Wasteland

Study Areas

The Sind Ibex is confined to the Khirthar range, Kambhu, Dumbar, Mungthar, Jahatang and Rannikot area and the Game Reserves; while the Urial is distributed in Dumbar, Jobo, Khar, Mehai, Jahatang, Mol, Benir and Molguy. Chinkara occurs in Khar area, Taung area, Karchat Valley and Bhaal Valley and also in the Game Reserves (Table 1).

Following are the main study areas for the present investigation which form most of the main components of the entire Protected Area Complex supporting the major populations of the key mammalian species:

Table 1. Important Sites for Mammals in KPAC.

S. No.	Study Areas	Co-ordinates
1.	Baran Naddi	N 25 42 25. 3 E 67 42 16. 3
2.	Behind Thonkri Hills	N 25 24 52. 2 E 67 16 46. 0
3.	Benir Ridge	N 25 29 19. 6 E 67 37 35. 9
4.	Dumbar Jabbal	N 25 40 17. 0 E 67 30 33. 1
5.	Dumbar Hills West	N 25 45 36. 6 E 67 31 10. 3
6.	Deedar Lak	N 25 31 18. 9 E 67 35 12. 0
7.	Gombok Area	N 25 26 51. 8 E 67 34 07. 4
8.	Halalo Pachran	N 25 15 29. 1 E 67 26 19. 4
9.	Karchat Flat Plain	N 25 47 40. 8 E 67 40 42. 1
10.	Kambho Hills	N 25 32 14. 9 E 67 45 58. 4
11.	Khirthar near Chamrhaywari	N 25 44 53. 9 E 67 41 38. 3
12.	Khar Centre	N 25 18 02. 4 E 67 11 24. 8
13.	Marri Hills	N 25 15 07. 7 E 67 13 15. 9
14.	Moidan Flat Plain Area	N 25 29 25. 5 E 67 16 21. 8
15.	Mol Area	N 25 27 15. 5 E 67 28 46. 9
16.	Rannikot Fort Area	N 25 53 56. 7 E 67 52 56. 3
17.	Sattani Bhor	N 25 51 42. 1 E 67 39 29. 9
18.	Sumbak Hills	N 25 24 42. 0 E 67 52 56. 4
19.	Sajjati Jabbal	N 25 39 09. 6 E 67 30 23. 6
20	Thonkri Hills	N 25 50 41. 4 E 67 32 08. 5
21.	Tikko Baran	N 26 02 06. 2 E 67 28 34. 5
22.	Uth Palan	N 25 11 58. 5 E 67 30 01. 1

Khirthar National Park (Karchat Area)

It forms the core area of the prime habitat of Sind Ibex and Urial. The key sites include the Kambhu Range, Dumbar area, Khirthar Range and the Rannikot Area (Fig. 1).

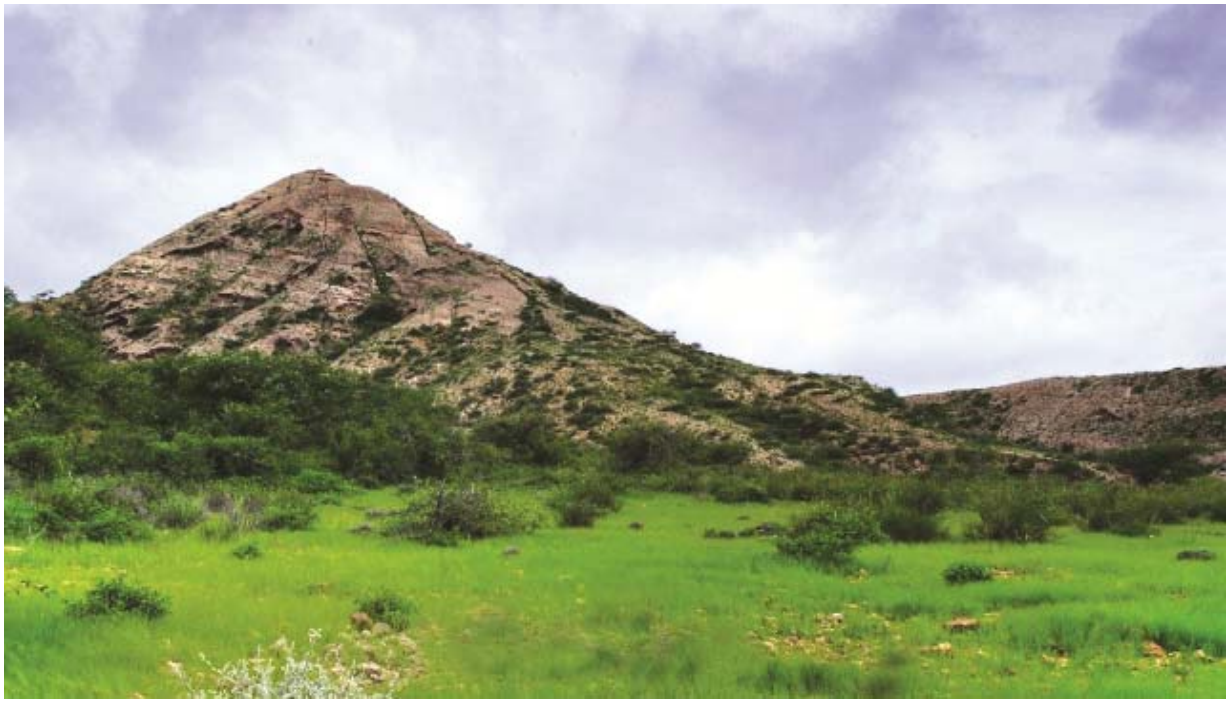

Fig. 1. Karchat, a core area of the Sind Ibex and Urial (source: pakwheels.com).

Khirthar National Park (Khar Centre Area)

It is also an important area for Urial and Sind Ibex. The important sites include the Marri Hills (Fig. 2), Munghthar Hills (Fig. 3), Lusar Hills, and the Thonkari Area. Munghthar and Lusar Hills are under the control of the Army. Moidan is an important area including sandy area (Fig. 5) for Chinkara.

Fig. 2. Marri Hills at Khar Centre.

Fig. 3. Munghthar Hills Area at Khar Centre.

Surjan Game Reserve

Surjan Game reserve has high population of Ibex particularly in Nimwari due to the presence of two water points and a good grazing ground (Fig. 4). It also has favorable plain area for Chinkara.

Fig. 4. Water point at Khirthar Protected Area Complex.

Fig. 5. Sandy Area at Khar Center.

Sumbak Game Reserve
It has sufficient grazing ground for Ibex, and also flat plain area for Chinkara. There is no disturbance to the animals.

Eri Game Reserve
It has ample grazing ground for Ibex and Chinkara but it is difficult to locate the animals due to lot of disturbance by the activities of the locals for digging the plant, *Commiphora mukul.* Hyaena sighting is reported mainly from Gujhri area. There is a wildlife observation post Turi Buthi on the top of mountain for making observations on Ibex. Foot prints of Hyaena were recorded here, while Honey Badger is also reported in the area.

Huthiano Game Reserve
It is a favorable area for Ibex and Hyaena. Caracal and Honey Badger have been reported from the area.

In the Khirthar Protected area Complex (KPAC), a total of 33 species of mammals have so far been recorded (Table 2). Out of which 11 species of large mammals were recorded during the present study (Table 3), which include the key species such as Sindh Ibex or Sind Wild Goat (*Capra aegagrus*) (Fig. 6), Urial (*Ovis vignei*) (Fig. 7), Chinkara (*Gazella bennettii*) (Fig. 8), alongwith Red Fox (*Vulpes vulpes*), Indian Fox (*Vulpes bengalensis*), Desert Cat (*Felis silvestris*), Jungle Cat (*Felis chaus*) and Indian Jackal (*Canis aureus)* which were sighted and counted during the present study. Hyaena, Caracal and Honey Badger were not sighted but have been recorded on the basis of presence of their footprints in the area, while Caracal was last seen in 1997. Three main species, though previously recorded from the area, were not recorded or reported during the present study period. Of these, Indian Pangolin and Wolf are very rare and Wolf was last reported in 1989, while the Leopard seems to be extirpated from the area. It was last reported in 1978. Indian Pangolin was last recorded during the Baseline Study in 2000.

Fig. 6. Group of Sind Ibex (*Capra aegagrus*) at Khirthar Protected Area Complex (source: CHAP).

Fig. 7. Urial (*Ovis vignei*) at KPAC (source: Sindh Wildlife Department).

Fig. 8. Chinkara (*Gazella bennettii*) at KPAC.

19 species of small mammals including the Bats, Hedgehogs, Mongooses, Hare, Palm Squirrel, Indian Porcupine and Rodents were recorded from the area (Table 2), sightings of 8 species of large mammals were made and the numbers of animals sighted were recorded (Table 3).

Table 2. A Checklist of the Mammals of Khirthar Protected Area Complex.

S. No.	Order	Family	Scientific Name	Common Name
01	Insectivore	Erinaceidae	*Hemiechinus collaris*	Longeared or Desert Hedgehog
02	Insectivora	Erinaceidae	*Paraechinus micropus*	Indian Hedgehog
03	Chiroptera	Vespertilionidae	*Scotophilus pallidus*	Yellow Desert Bat
04	Chiroptera	Vespertilionidae	*Scotophilus kuhlii*	Lesser House Bat
05	Carnivora	Hyaenidae	*Hyaena hyaena*	Striped Hyaena
06	Carnivora	Canidae	*Canis aureus*	Asiatic Jackal
07	Carnivora	Canidae	*Canis lupus*	Wolf
08	Carnivora	Canidae	*Vulpes bengalensis*	Indian Fox
09	Carnivora	Canidae	*Vulpes vulpes*	Red fox
10	Carnivora	Mustellidae	*Mellivora capensis*	Ratel or Honey Badger
11	Carnivora	Herpestidae	*Herpestes edwardsi*	Grey Mongoose
12	Carnivora	Herpestidae	*Herpestes javanicus*	Small Indian Mongoose
13	Carnivora	Felidae	*Felis silvestris*	Desert Cat
14	Carnivora	Felidae	*Felis chaus*	Jungle Cat
15	Carnivora	Felidae	*Felis caracal*	Caracal
16	Carnivora	Felidae	*Panthera pardus*	Leopard
17	Pholidota	Manidae	*Manis crassicaudata*	Indian Pangolin
18	Artiodactyla	Bovidae	*Capra aegagrus*	Sind Wild Goat or Sindh Ibex
19	Artiodactyla	Bovidae	*Ovis vignei*	Urial
20	Artiodactyla	Bovidae	*Gazella bennettii*	Chinkara
21	Lagomorpha	Leporidae	*Lepus migricollis*	Indian Hare
22	Rodentia	Sciuridae	*Funambulus pennantii*	Striped Palm Squirrel
23	Rodentia	Hystricidae	*Hystrix indica*	Indian Porcupine
24	Rodentia	Muridae	*Rattus rattus*	Roof Rat / House Rat
25	Rodentia	Muridae	*Mus musculus*	House Mouse
26	Rodentia	Muridae	*Mus booduga*	Little Indian Field Mouse
27	Rodentia	Muridae	*Mus saxicola*	Grey Spiny Mouse
28	Rodentia	Muridae	*Golunda ellioti*	Indian Bush Rat
29	Rodentia	Muridae	*Acomys cahirinus*	Cairo Spiny Mouse
30	Rodentia	Muridae	*Calomyscus bailwardi*	Mouse like Hamster
31	Rodentia	Muridae	*Gerbillus nanus*	Balochistan Gerbil
32	Rodentia	Muridae	*Tatera indica*	Indian Gerbil
33	Rodentia	Muridae	*Meriones hurrianae*	Indian Desert Jird

Table 3. List of Large mammals recorded at Khirthar Protected Area Complex

No.	Species	KNP (Khar)	KNP (Karchat)	Game Reserves
1	Urial	+	+	-
2	Chinkara	+	+	+
3	Ibex	+	+	+
4	Indian Fox	+	+	+
5	Red Fox	+	+	+
6	Jackal	+	+	+
7	Desert Cat	+	+	+
8	Jungle Cat	+	+	-
9	Hyaena	Foot Prints	Foot Prints	Foot Prints
10	Caracal	Foot Prints	Foot Prints	Foot Prints
11	Honey Badger	Foot Prints	Foot Prints	Foot Prints

*+ present, - absent

Large Mammals recorded at KPAC

The following eleven species of large mammals were documented in KPAC. These species were represented in the KNP (Karchat and Khar) and the game reserves except that Jungle Cat and Urial were not recorded from the Game Reserves.

Eight species of large mammals were sighted. Their numbers are given in table 4. (Estimation of the total population of these species was not attempted).

Species of Special Conservation Concern

The following species of mammals of the KPAC have been identified as species of special conservation interest being the indicator species viz. Sind Ibex, Urial, Chinkara, Striped Hyaena, Grey Wolf, Honey Badger and Caracal. Information about the current status of Indian Pangolin which is quiet rare in the area needs also to be collected.

Major Threats

Khirthar National Park (Khar Center Area)

There is lot of disturbance to the wild animals because of local communities using the same area for collection of drinking water. There is also competition for grazing between the domestic livestock and the wild animals. There is lot of disturbance to the wild animals due to the presence of villages and movement of local people in the area.

A very important habitat for Urial and Ibex viz. Munghthar and Luhsar Hills have been under the control of Army. Previously, herds of Urial and Ibex were usually sighted over the Munghthar Hills but now these are not seen as they have migrated from their main habitat to other areas. The two water springs in Marri Hills have gone dry, so there is shortage of water on water points for the wild animals which have to move to the distant water points in area.

Khirthar National Park (Karchat Area)

Like Khar Center, here in the Karchat area local communities have been using the same point for the collection of drinking water and due to the movement of people in the area, wild animals have been disturbed. There is competition for grazing between the domestic livestock and the wild animals. There is a lot of disturbance to the wild animals due to movement of local people in the area.

Game Reserves

There is lot of disturbance to the wild animals due to movement of local people in the area. There is also disturbance due to the road leading to Thana Bulla Khan passing through the Surjan Game Reserve. There is also shortage of water for animals in Eri and Huthiano and hence the animals tend to disperse to other areas. There are already two water points made in Huthiano but water is not available there.

CONCLUSION

Two of the main key species of the KPAC are surviving well, but Chinkara seems under threat due to the impact of increasing human population in the area and also expansion of agriculture practices in its core habitat. There is an urgent need for the proper management of the species and its habitat. There is big population of Ibex in Surjan and Sumbak Game Reserves areas, but the habitat in Eri and Huthiano needs to be improved by provision of water points in these areas.

Khar and Kharchat areas have been suggested for the development of ecotourism in Pakistan. Action in this regard may be taken. The boundaries of the different components of KPAC may be demarcated and maps and necessary information material may be prepared to highlight the importance of the area.

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Table 4. Total number of Large Mammals sighted in the Khirthar Protected Area Complex during 2010-2011.

No.	Species	KNP (Khar)	KNP (Karchat)	Game Reserves	Total
1	Urial	300	700	-	1000
2	Chinkara	30	100	20	150
3	Ibex	500	600	100	1200
4	Indian Fox	02	04	02	08
5	Red Fox	08	12	05	25
6	Jackal	15	20	05	40
7	Desert Cat	04	10	06	20
8	Jungle Cat	02	10	-	12

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PHYTOCHEMICAL COMPOSITION, ANTIOXIDANT PROPERTIES AND ANTIBACTERIAL ACTIVITIES OF FIVE WEST-AFRICAN GREEN LEAFY VEGETABLES

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ABSTRACT

Green leafy vegetables have been reported as sources of nutrients and folklore remedy for the treatment of infections in West Africa. The present study was designed to investigate the antioxidant, antibacterial and phytochemical components of the methanol extracts from five selected green leafy vegetables (*Telfaria occidentalis, Amaranthus viridis, Amaranthus hybridus, Lactuca taraxicofolia* and *Solanium aethiopium*). The *in vitro* antioxidant activities of the extracts were measured using 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity and the antibacterial activities were evaluated using the well agar diffusion assay. The antioxidant activity of the methanolic extracts of the selected vegetables revealed that the EC_{50} ranged from 1.8 to 9.0µg/ml. The EC_{50} values for free radical scavenging activity of *T. Occidentalis, L. taraxicofolia* and *A. viridis* extracts were significantly superior over the standard, gallic acid. The extracts also exhibited inhibitory effects against *Pseudomonas aeruginosa, Bacillus cereus, Enterobacter cloacae*, *Bacillus subtilis* and *Staphylococcus aureus* at the concentration of 100µg/ml. The present study scientifically validated the traditional use of the vegetables as possible therapeutic agents.

Keywords: West Africa, vegetables, antioxidant properties, antibacterial activity, phytochemicals.

INTRODUCTION

Africa is endowed with a variety of traditional vegetables and different types are consumed by the various ethnic groups for different reasons (Salawu *et al*., 2009). In some West-African countries, vegetables are considered as the cheapest and most available sources of important proteins, carbohydrates, vitamins, minerals and essential amino acids (Francis *et al*., 2012). Previous studies have shown that the consumption of vegetables is closely related with the decrease risks of diseases that resulted from oxidative stress, including cancer, diabetes and various infectious diseases (Doll, 1990; Liu, 2004). These findings have encouraged people to source for locally available vegetables for the treatment of various diseases (Akindahunsi and Mulinacci, 2009). Therefore, five vegetables (Table 1) were selected for this study because of their popular use as food additives and remedy for the treatment of infections in West Africa.

T. Occidentalis (fluted pumpkin) is one of the popular and widely grown vegetable crops in West Africa (Akoroda, 1990). Previous study revealed that long term feeding of *T. Occidentalis* supplemented diet caused a significant increase in weight of animals which may be due to its nutrients (Oboh *et al*., 2006). A study has shown that the ethanol root extracts of *T. occidentalis* possess antimicrobial potential (Odoemena and Essien, 1995; Okokon *et al*., 2007). *S. aethiopicum* is commonly known as 'African eggplant' and grown in West Africa for its immature fruit (garden egg) and leaves. *S. aethiopicum* is highly valued constituents of the Nigerian foods and indigenous medicines and commonly consumed almost on a daily basis by both rural and urban families (Akoroda, 1990). *L. taraxacifolia* has been domesticated as a leafy vegetable in West Africa (Burkill, 1985). *L. taraxacifolia* is used as a remedy for prevention and treatment of measles, diabetes mellitus. It is reported to possess hypolipidaemic effect (Ayensu, 1978; Adebisi, 2004; Obi *et al*., 2006). The leaves of *L. taraxacifolia* plant are fed to nursing cows to stimulate lactation and also to sheep and goat to induce multiple births (Wichtl, 1994).

A. viridis L. is also a widespread weed and occasionally cultivated in Nigeria, Gabon and DR Congo. *A. viridis* L is a traditional food which has potential to improve nutrition, boost food security, foster rural development and support sustainable land care (NRC, 2006). In Côte d'Ivoire, the leaf sap is used as an eye wash to treat eye infections and for treating convulsions and epilepsy in children. Furthermore, the plant possesses anti-proliferative, anti-fungal and anti-viral activities (Obi *et al*., 2006). *A. hybridus* is commonly called smooth amaranth, smooth pigweed and red amarantha in Nigeria. Leaves and young seedlings can be cooked as a spinach, added to soups or eaten raw (Tindall, 1983).

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So far, a great number of these vegetables have not been tested for their chemical composition, antimicrobial and antioxidant activities (Oboh *et al*., 2006). The aim of this study is to determine the phytochemical components, antioxidants and antibacterial potentials of five locally sourced green leafy vegetables. Our ultimate target is to give an insight on the health benefits of these vegetables in local dishes in order to sensitize the people on the need for their consumption.

MATERIALS AND METHODS

Selection of species

The botanical identity of the plant materials (Table 1) was confirmed by the Taxonomist at the Forest Research Institute of Nigeria (FRIN) Jericho Ibadan, Nigeria and the voucher specimens were stored in the herbarium.

Preparation of crude extract

The plant materials (leaves of *A. viridis, A. hybridus, L. taraxicofolia, S. aethiopicum, T. occidentalis*) were air-dried at room temperature for 3 weeks and then ground into powdered form. The powdered vegetable (100g) was macerated in 500ml of methanol for 72hours at room temperature. The extracts were then filtered through a filter paper (Whatman GF/C, England), concentrated using a rotary evaporator at 36^0C and stored at 4^0C until needed.

Phytochemical screening

The Folin-Ciocalteu assay using gallic acid as standard was used for the qualitative test of total phenolics from methanolic extracts of the selected vegetables (Makkar, 2000). Total phenolic concentrations were expressed as gallic acid equivalents (GAE) per gram dry matter. The test as described by Tadhani and Subhash (2006) was used to test for the presence of saponin in the vegetables. The presence of alkaloids was based on the quantitative method of Makkar and Goodchild (1996). The flavonoid content was determined as described by Hagerman (2002), with some modifications. 50µl of each MeOH extract were diluted with 950µl glacial acetic acid, followed by the addition of 2.5 ml of 4% HCl in methanol (v/v) and 2.5 ml vanillin reagent (4% vanillin in glacial acetic acid, w/v), after which the reaction mixture was incubated for 20 min at room temperature. After incubation, absorbance at 500nm was measured using a UV–Vis Spectrophotometer against water blank. Flavonoid content was expressed as catechin equivalents (CTE) per gram dry matter.

Condensed tannin content was evaluated using the butanol–HCl assay as described by Makkar (2000) and Ndhlala *et al*. (2007) and the percentage dry matter was calculated as equivalent amount of leucocyanidin (LCE) using the equation below:

$$\text{Condensed tannin}(\%) = \frac{(A_{550} \times 78.26 \times \text{dilution factor of extract})}{\%\ \text{dry matter}} \times 100$$

where A550 = absorbance of sample at 550nm. The formula assumes the effective E1%, 1cm, 550nm of leucocyanidin to be 460.

Table 1. Ethnobotanical data and percent (w/w) extraction yields of methanol extracts from selected Nigerian green leafy vegetables.

Plant species (family)	Voucher number	Traditional use	Extraction yield (%)
Telfairia occidentalis Hook.f. (Cucurbitaceae)	FHI. 107340	The leaves and vines are consumed as vegetables and the young seeds are eaten as food (Akoroda, 1990; Badifu and Ogunsua, 1991).	12.1
Lactuca taraxicofolia (Willd.) Schum. (Asteraceae)	FHI. 107399	The leaves are eaten fresh as a salad or cooked in soups and sauces (Burkill,1985).	10.7
Amaranthus viridis L. (Amaranthaceae)	FHI. 107395	The leaves are used as vegetables or as ingredients in sauces (Grubben and Denton, 2004).	14.2
Solanum aethiopicum L. (Solanaceae	FHI. 107394	The leaves are eaten raw and also when boiled or fried as ingredient of stews, soups and vegetable sauces.	11.8
Amaranthus hybridus L. (Amaranthaceae)	FHI. 107401	The leaves combined with condiments are used to prepare soup (Mepha *et al*., 2007). In Congo, the leaves are eaten as spinach or green vegetables (Dhellot *et al*., 2006). These leaves boiled and mixed with groundnut sauce are eaten as salad in Mozambique and in West Africa (Oliveria and DeCarvalho, 1975).	15.0

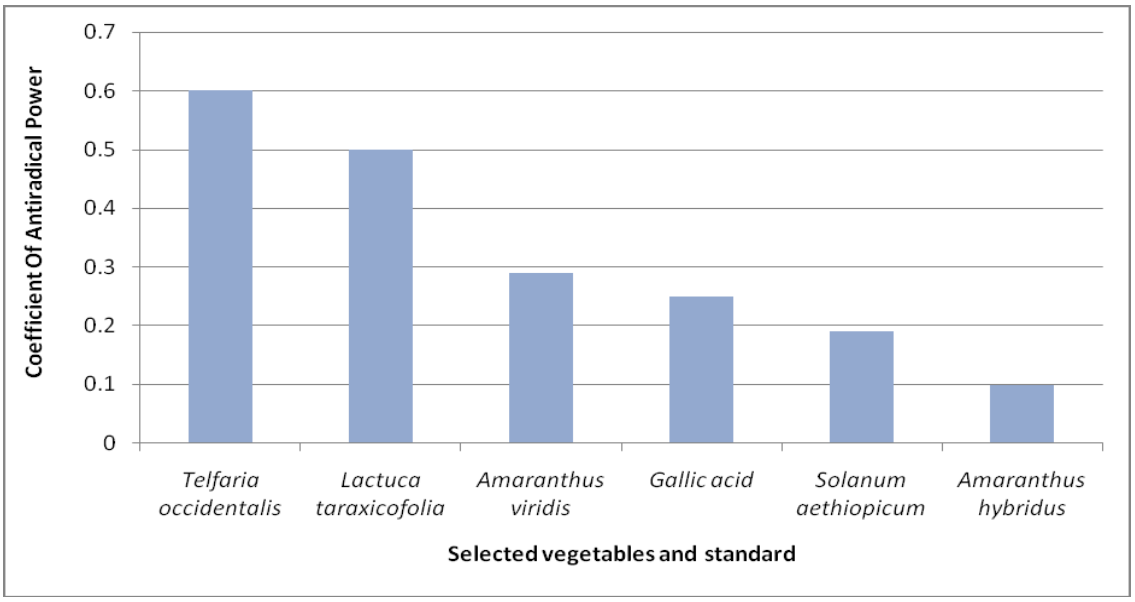

Fig. 1. Antiradical power of the selected vegetables and standard, gallic acid.

Radical scavenging activity of plant extract and gallic acid against DPPH radicals

Radical scavenging activity of the methanolic plant extracts and gallic acid against DPPH were determined using a modification of Hatano *et al*. (1988). Serial dilutions were made to obtain a concentration range of 10 to 0.0393µg/ml for the plant extracts and gallic acid. The reaction mixtures were made by adding 100 µl of the test sample to 900µl of 6.5 x 10^{-5} M DPPH solution in methanol. Then the absorbance was recorded at 515nm after 1minute and 5 minute interval up to 40minutes. The experiments were carried out in triplicate. However, the percentages of DPPH reduction of the test samples were calculated using the following formula:

$$\% \text{ Radical Scavenging Activity (RSA)} = \frac{Ab - As}{Ab} \times 100$$

Where Ab is absorbance of blank (DPPH solution) (t=0min)

As is absorbance of test sample ($t \neq 0$min)

The reaction kinetics for each concentration of the plant extract was plotted. From these graphs, the percentage of DPPH remaining at the steady state was determined. The antiradical activity (ARP) was then calculated from the equation: ARP = 1/EC50.

Antimicrobial assay

Bacterial cultures

The following bacteria were used: *Bacillus cereus, Bacillus subtilis, Enterobacter cloacae, Staphylococcus aureus* and *Pseudomonas aeruginosa*. All the cultures were isolated in our laboratory from fresh food samples and maintained on nutrient agars (Oxoid, England) at 4^0C (Ruijssenaars and Hartsmans, 2000). Selected isolates were plated on respective agar plates for subsequent work and maintained as pure cultures according to Bergey's Manual of Systemic Bacteriology (Sneath *et al*., 1984).

Agar well diffusion assay

The antibacterial activities of vegetable extracts were determined using an agar well diffusion assay (Lyudmila *et al*., 2003). The bacterial strains were cultured in a Mueller-Hinton broth (Oxoid, England) for 24h and diluted with sterilised distilled-deionised water. Then, 10ml of each culture (0.5 McFarland standards) was spread onto the surface of Mueller–Hinton agar (Oxoid, England) to create a bacterial lawn. The sterile cork borer (6mm) was used to make wells in the agar medium. These wells were then filled with 50 and 100mg/ml of the extracts prepared in 2% of DMSO. The plates were incubated at 37^0 C for 24 hrs. The antibacterial activity was compared with chloramphenicol (1mg/ml). Tests were carried out in triplicates.

Statistical analysis

All the data were expressed as mean ± standard deviation (S.D). Results were analyzed using one-way ANOVA followed by Student's t-test. Differences with p values less than 0.05 were considered to be significant.

RESULTS

Phytochemical screening

S. aethiopium and *A. hybridus* showed high contents of gallotannin, while *S. aethiopicum* and *L, taraxicofolia* presented high content of flavonoids (Table 2). In the condensed tannin content, higher levels of

phytochemicals were contained in the *T. occidentalis*. In addition, all the extract indicated the presence of alkaloids.

Antibacterial assays

All the extracts of the vegetables showed broad spectrum antibacterial activity, with zone of inhibition ranging from 10-25mm (Table 2). Besides the *T. occidentalis* extract, which had a zone of inhibition value of 25mm against *P. aeruginosa*, all the extracts showed low antimicrobial activity. With the exception of the extract of the *T. occidentalis* and *A. hybridus*, almost all the plants showed a low measure of the diameter of the zone of inhibition in (mm), against the gram-negative bacteria strains tested.

Scavenging effect of extracts and gallic acid on DPPH radicals

Table 4 shows the EC_{50} of the extracts in comparism with gallic acid (standard) relative to their ARP values. Figure 1 shows activity-relativity of the extracts in respect to the ratios of their ARP values, using gallic acid as a standard. Figure 1 indicates the antiradical power of the selected vegetable in relation to standard compound (gallic acids). *T. occidentalis* shows the highest activity-relativity, hence possessing the highest antioxidant activity. The order of increasing activity is *A. hydridus*>*S.aethiopicum* >gallic acid > *A.viridis*> *L.taraxicofolia*>*T. occidentalis.*

DISCUSSION

In some African countries keen interest has been committed to the commonly available green leafy vegetables which possess a remarkable potential to help people overcome the lethal diseases of modern society (Aletor and Adeogun, 1995; Aletor *et al*., 2002; Schonfeldt and Pretorius, 2011). In the present study, we have attempted to evaluate the antibacterial and antioxidants properties of five green leafy vegetables, namely *T. occidentalis, L. taraxicofolia, A. viridis, S. aethiopium and A. hybridus*. A preliminary phytochemical analysis was also carried out to determine the presence of different classes of secondary metabolites. Determination of antibacterial activity of the selected vegetable extracts tested against a panel of isolated pathogenic bacteria indicated that the concentration of the extracts directly influenced the antibacterial potentials of the vegetables (Table 3). Extracts with more than 14mm zone of inhibition were considered as having good antimicrobial activity (Aligiannis *et al*., 2001).

Table 2. Total phenolics, condensed tannin and flavonoid content of the selected vegetable extracts.

Plant secondary metabolites	*S. aethiopium*	*T. occidentalis*	*L. taraxicofolia*	*A. viridis*	*A. hybridus*
Total phenolics (mg GAE/g dry matter)	40.60±7.88	49.32± 4.07	28.38 ± 1.07	49.3±9.07	39.32± 6.07
Condensed tannin (% LCE/g dry matter)	0.46 ± 0.02	1.32 ± 0.12	0.96 ± 0.04	0.87± 0.05	0.90 ± 0.03
Gallotannin (µg GAE/g dry matter)	29.32±0.07	19.01±0.07	16.32 ± 0.07	23.2±0.07	31.32±4.12
Flavonoid (mg CTE/g dry matter)	0.96 ± 0.03	0.71 ± 0.07	0.97 ± 0.10	0.76± 0.05	0.34 ± 0.03

GAE, gallic acid equivalents; LCE, leucocyanidin equivalents; CTE, catechin equivalents. Data represented as means ± SD (n = 3).

Table 3. Inhibitory activities of plant extracts against five isolated bacteria (*B. cereus, B. subtilis, E. cloacae, S. aureus* and *P. aeruginosa*).

Bacterial strains	Diameter of zone of inhibition (mm)					
	Concentration (mg/ml)	*S. aethiopium*	*T. occidentalis*	*L. taraxicofolia*	*A. viridis*	*A. hybridus*
B. cereus	100	11±1.3	17±2.3	18±2.0	14±0.9	16±2.1
	50	12±1.1	13±0.5	12±0.9	12±1.0	14±1.2
B. subtilis	100	14±1.2	17±2.0	13±1.1	15±2.0	16±2.6
	50	12±1.2	14±1.1	NI	11±0.6	14±0.8
E. cloacae	100	14±0.9	20±2.8	NI	14±0.3	16±2.9
	50	NI	14±1.3	NI	11±0.9	13±1.2
S. aureus	100	14±1.0	20±3.2	13±0.9	11±0.8	15±0.9
	50	12±0.9	14±1.2	11±0.3	10±0.8	12±0.8
P. aeruginosa	100	11±0.8	42±5.9	NI	12±1.2	15±2.1
	50	NI	20±3.2	NI	NI	12±0.9

NI-No inhibition; Data represented as means ± SD (n = 3).

Among the studied vegetables, DPPH radical scavenging activity was found to be highly significant in *T. occidentalis* ($p<0.01$) while the remaining four vegetables showed good activity when compared with gallic acid (standard) (Table 4). The EC_{50} of *T. occidentalis* extract was about 3 times greater than standard antioxidant (gallic acid). The trend of antioxidant activity amongst the extracts in comparism with gallic acid is *T. occidentalis*<*L. taraxicofolia*<*A. viridis*<gallic acid < *S. aethiopicum*<*A.viridis* (Table 4). This revealed that *T. occidentalis, L. taraxicofolia* and *A. viridis* possess a higher antioxidant activity than gallic acid (positive control). In recent times restriction has been placed on synthetic antioxidant like butylated hydroxyl anisol, butylated hydroxyl toluene, tertiary butylated hydroquinone and gallic acid esters; which have been suspected to prompt negative health effects (Uusiku *et al*., 2010). Therefore, there is an urgent need to substitute these synthetic products with naturally occurring antioxidants. The selected vegetables may be considered as a good alternative for the synthetic antioxidants based on the high antioxidant activities exhibited by the selected vegetables.

Table 4. EC_{50} of the vegetable extracts and gallic acid (standard).

Vegetable extracts	EC_{50} (µg/ml)
T. occidentalis	1.8±0.2
L. taraxicofolia	2.0±0.3
A. viridis	3.4±0.25
S. aethiopicum	5.2±0.5
A. hybridus	9.0±2.1
Gallic acid	4.0±0.4

Data represented as means ± SD (n = 3)

Previous investigations have shown that the presence of phytochemicals in the selected vegetables showed an underlying contribution in respect to their medical and pharmaceutical relevance (Aletor and Adeogun, 1995; Aleto *et al*., 2002; Uusiku *et al*., 2010). Phenolic compounds including tannins, flavonoids, saponins and alkaloids have been implicated in pharmacological activities such as antioxidants, antimicrobial and anti-inflammatory activities (Wichtl, 1994). For instance, the use of *A. viridis* L. as an astringent (Francis *et al*., 2012) and the anti-venom properties of *L. taraxicofolia* were attributed to the presence of tannins.

Flavonoids, phenolics and tannins which are known antioxidants are found to be distinctively present amongst all the phytochemicals tested in all the selected vegetables (Table 2). The presence of these compounds in the extracts of these vegetables may also be the main cause of their high radical-scavenging activity (Table 4) and popularly reported high health beneficial properties (Oboh *et al*., 2006; Uusiku *et al*., 2010). Therefore, the use of the selected vegetable as a food supplement by the majority of people in West African may help in contributing to the total antioxidant defense system of the human body and this may account for the protection against diseases (Francis *et al*., 2012).

Consequently, the presence of phytochemicals such as tannins, phenolics as well as flavonoid in the selected vegetables (Table 2) may have crucial roles in the observed antioxidant and antibacterial potential of the leaves. Therefore, further work is being channelled towards the isolation and identification of the active ingredients in these vegetables.

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ECOLOGICAL IMPACTS ON THE POPULATION OF MARSH CROCODILES (*CROCODYLUS PALUSTRIS*) IN CHOTIARI WETLAND COMPLEX SANGHAR, SINDH: A SURVEY REPORT

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ABSTRACT

In this study, an ecological survey of the impacts on Marsh Crocodiles (*Crocodylus palustris*) in Chotiari Wetland Complex Sanghar was carried out during the month of January to December 2008. In order to assess, the microbial contamination, detection of pollutant indicator organisms in the water samples, using different physico-chemical parameters were performed. The level of different physico-chemical parameters like as temperature, electrical conductivity, total dissolved solids, calcium, magnesium, bi carbonate, chloride, sodium, potassium, sulphur, carbonate, biological oxygen demand and dissolved oxygen were monitored in water samples collected from Chotiari Wetland Complex to assess the impact of toxic pollutants. Toxic chemical contaminants were estimated below the detection limit, while another several chemicals were found within the range set by World Health Organization. The degree of contamination, proximity to pollution source and the metabolic ability of Marsh Crocodile suggest that the species are at threatened from the environmental contamination by the study of heavy metals. Marsh Crocodiles are considered endangered around the world due to the increase pollution and alteration of their habitat.

Keywords: Endangered species, pollution, metabolic, toxic, contamination.

INTRODUCTION

The Chotiari Wetland Complex (CWC) is an artificial water reservoir which is located at 20km North East of the Sanghar city, at 69'4E Longitudes and 26' 1"N Latitudes. It was constructed in December 2002 which covers an area of about 86km$_2$. The CWC is built upon small lakes, which are in many numbers. It has the total capacity of water storage is 0.75 million acre feet. The CWC received water from the Nara canal through Ranto canal escapes during the flood season. The depth of the reservoir is from 15' to 30' ft with sandy and salty bottom, which provides a suitable, surface for the growth of algae and aquatic plant species. The Chotiari Wetland Complex is a unique wetland complex and ecologically rich area. The Wetland complex is characterized by a mosaic of diverse habitats including forest, fresh and brackish water lakes, agricultural lands, rangelands, sand dunes scrub, reed beds, fish farms and swamps. Despite a very hot and arid climate zone the area is biologically most diverse and rare in the region. The reservoir has high ecological significance as it is home to many internationally important and endangered species listed in the IUCN Red List. The reservoir has support among important mammalian Endangered species of Hog Deer (*Axis porcinus*) and Fishing Cat (*Prionailurus viverrinus*) and two Vulnerable species of Chinkara (*Gazella bennettii*) and Smooth coated Otter, *Lutrogale viverrinus* (Sheikh *et al.,* 2004). There are two Vulnerable species, Marbled Teal (*Marmaronetta angustirostris*), a globally migratory birds, visit and breeds in this reservoir and also Pallas's Fish-Eagle (*Haliaeetusleucoryphus*), a Vulnerable bird resides on the site (Birdlife International, 2012; IUCN, 2012). The globally Endangered species of the wetland complex site is the Marsh Crocodile (*Crocodylus palustris*) (WWFP, 2007). Ecological studies on the lakes of Sindh like as CWC is very few. Ecological studies were carried out on Chotiari Wetland Complex by Leghari *et al.* (1995), Jafri (1997), Leghari *et al*. (1999) can be mentioned in this connection. Ecological changes in aquatic life depend upon the physico-chemical environmental characteristics of water bodies. The present study provides the information about the influence of physico-chemical factors on aquatic biodiversity as well as on population of Marsh Crocodiles. Before the construction of Chotiari reservoir, some biological and limnological studies on the Bakar lake complex were carried out by Jafri (1997), Leghari *et al.* (1997), Leghari *et al.* (1999) and Leghari and Khuhawar (1999). The present study was carried out on water quality and its impacts on natural productivity of Chotiari reservoir after its construction.

MATERIALS AND METHODS

Water samples and sediments were collected from the midstream. Eight sampling stations were selected from the entire lake for water sampling. Samples were

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collected from Fish farm 1, and 2, Jadupur, Simni, Nara Canal (right side), NWC (outlet), NWC (inlet) and Fauji Fish farm. Sampling was carried out 7:00am to 5:00pm at monthly intervals. Physical factors such as temperature of water, depth and transparency (secchi disk measurement was carried out at the sampling station). In the laboratory, chemical factors the mercury thermometer for reading of the temperature was dipped into the samples to a depth of 15 cm for 2-5 minutes, pH of water was measured with Orion Model 420 A pH meter, electrical conductivity, total dissolved solids and salinity of water was measured by WTW 320 conductivity meter, total alkalinity, total hardness, chlorides and phosphate were determined by using the standard methods for the examination of water and waste water (APHA, 1976), calcium, carbonate, bi-carbonate of water was measured with titration method (2310), magnesium was measured with Fluorometric determination method, potassium was measured with spectrometer and biological oxygen demand was measured with Winkler method and dissolved oxygen was measured with oxygen meter (Jenway Model 9071).

RESULTS

Values for physico-chemical parameters in water samples were collected from eight sampling stations from the entire CWC for water sampling and its associated areas. Water samples were collected from Fish farm 1, and 2, Jadupur, Simni, Nara Canal (right side), NWC (outlet), NWC (inlet) and Fauji Fish farm (CWC) in the month of January to December 2008 (Table 1-12).

Temperature of air/water °C: It is the major physical parameter which is directly related to chemical reaction in water bodies. The temperature of water bodies is an important parameter that directly affects the aquatic biodiversity and it also reduces the dissolved oxygen in water resources. It was observed that the highest temperature of air recorded during the month of June 47.37°C and the lowest value was found during the month of January 22.37°C (Table 13). Temperature of water was recorded highest in the month of July 44.37 and the lowest was found in the month of February 20.37 (Table 13).

pH: The lowest ranged of pH was recorded from the month of June and September. The higher level of pH increased in the month of January and February. The observed pH range of CWC water is 6.66 to 8.09, (Table 13).

Electrical Conductivity: The value of conductivity was recorded highest 978 us/cm in December and lowest 567 us/cm in October (Table 13). However, fluctuation observed in samples is due to water flow in the observed site. The standard level for electrical conductivity is 400 us/cm, as the water quality depends on TDS.

Table 1. Physico-chemical Analysis of water for the month of January 2008.

	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**Mean**
Date	11	11	16	16	20	20	25	25	
Time	11.00	12.30	10.3	12.40	2.00	5.00	11.00	1.00	
Temp: Air ◦C	21	22	18	21	22	20	19	22	24.37
Temp: H_2O ◦C	18	18	16	16	20	18	17	20	21.37
pH	7.97	7.43	8.32	8.68	7.40	7.90	8.71	8.32	8.09
EC mu/scm	751	131	1275	420	338	367	332	1187	600.12
TDS mg/l	405	7.99	688	226	148	212	193	568	404.87
Turbidity	13.5	160	42	10.5	91	17.8	7.5	7.2	416.4
Ca mg/l	32	35	20	12	20	31	29	42	27.62
Mg meq/l	34	11	53	32	18	19	16	73	32
Hardness mg/l	240	105	270	160	136	127	117	354	188.62
HCO_3ppm	140	70	335	135	132	162	93	209	159.5
Alkalinity mg/l	2.8	1.4	6.7	2.7	3.2	4.7	1.6	4.6	3.46
Cl mg/l	70	32	166	35	57	71	43	102	72
Na meq/l	73	6	158	19	7	28	17	113	52.62
K mg/l	4	2	21	5	3	5	7	8	6.87
SO_4 mg/l	131	6	65	25	6	15	6	208	57.75
As ppb	0	0	0	0	0	0	0	0	0
Co_3 ppm	0	0	10	0	0	0	0	0	1.25
BOD mg/l	3.5	3.1	3.2	3.8	3.0	3.3	3.6	3.7	3.4
DO mg/l	7.3	7.2	7.6	7.1	7.0	7.5	7.2	7.4	7.28

Table 2. Physico-chemical Analysis of water for the month of February 2008.

	S1	S2	S3	S4	S5	S6	S7	S8	Mean
Date	12	12	16	16	20	20	27	27	
Time	11.00	12.35	1.40	5.20	2.30	4.00	11.30	2.30	
Temp: Air ◦C	20	21	22	19	22	21	21	22	23.37
Temp: H_2O ◦C	17	18	18	16	19	18	18	19	20.37
pH	7.80	8.40	8.12	8.60	7.50	7.62	8.80	8.35	8.14
EC mu/scm	755	1890	1255	435	352	370	335	1205	824.62
TDS mg/l	408	885	680	235	160	195	190	570	415.37
Turbidity	13.7	21.7	56	10.2	91	19.0	8.1	8.1	28.47
Ca mg/l	34	48	18	17	23	30	27	43	30
Mg meq/l	35	58	56	36	19	18	18	76	39.5
Hardness mg/l	238	305	258	170	148	116	122	372	216.12
HCO_3ppm	140	140	320	138	142	162	90	230	170.25
Alkalinity mg/l	2.7	7.1	6.2	2.5	3.7	4.6	2.1	4.7	4.2
Cl mg/l	72	137	160	31	59	80	41	93	84.12
Na meq/l	76	165	152	23	7	29	21	113	73.25
K mg/l	5	23	23	6	3	7	9	12	11
SO_4 mg/l	132	170	60	23	5	14	6	230	80
As ppb	0	0	0	0	0	0	0	0	0
Co_3 ppm	0	0	0	0	0	0	0	0	0
BOD mg/l	3.4	3.0	3.1	3.5	3.2	3.6	3.7	3.8	3.41
DO mg/l	7.2	7.0	7.1	7.4	7.1	7.3	7.5	7.6	7.27

Table 3. Physico-chemical Analysis of water for the month of March 2008.

	S1	S2	S3	S4	S5	S6	S7	S8	Mean
Date	06	06	13	13	20	20	26	26	
Time	11.20	12.50	12.00	1.00	10.45	2.00	2.30	4.30	
Temp: Air ◦C	30	31	32	32	30	32	33	32	23.37
Temp: H_2O ◦C	26	27	28	28	26	28	28	28	29.37
PH	7.51	7.15	7.09	7.78	6.78	7.23	8.85	8.12	7.56
EC mu/scm	896	474	457	1492	572	408	330	1190	727.37
TDS mg/l	483	255	247	804	308	220	158	560	379.37
Turbidity	58	53	222	12.0	102	10.8	7.3	7.3	59.05
Ca mg/l	56	44	60	24	67	67	28	43	48.62
Mg meq/l	36	19	12	74	13	8	17	73	31.5
Hardness mg/l	36	19	12	74	13	8	126	376	83
HCO_3ppm	200	145	170	430	150	140	94	228	194.62
Alkalinity mg/l	4.0	2.8	3.4	8.6	3.0	2.8	2.1	4.7	8.42
Cl mg/l	90	42	32	166	83	22	45	88	71
Na meq/l	65	20	13	164	26	29	21	118	57
K mg/l	4	3	2	17	4	2	5	8	5.62
SO_4 mg/l	116	36	12	78	18	32	7	207	63.25
As ppb	0	0	0	0	0	0	0	0	0
Co_3 ppm	0	0	0	0	0	0	0	0	0
BOD mg/l	3.0	3.2	3.4	3.7	3.1	3.6	3.8	3.3	3.38
DO mg/l	6.5	6.3	6.7	6.2	6.8	6.4	6.9	6.1	6.48

Table 4. Physico-chemical Analysis of water for the month of April 2008.

	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**Mean**
Date	02	02	07	07	15	15	23	23	
Time	2.20	4.15	11.00	1.45	11.15	2.30	1.00	2.00	
Temp: Air ◦C	35	34	33	35	34	35	36	36	38.37
Temp: H_2O ◦C	32	31	30	32	31	32	32.6	32.6	35.37
pH	7.87	7.92	7.89	8.64	7.50	7.74	8.45	8.30	8.03
EC mu/scm	745	1856	1216	413	326	367	332	1216	808.87
TDS mg/l	426	894	672	218	147	192	178	570	412.12
Turbidity	14.3	12.8	47	10.2	81	17.6	7.5	7.8	24.77
Ca mg/l	36	41	18	14	20	34	24	43	28.75
Mg meq/l	38	62	58	28	15	18	13	73	38.12
Hardness mg/l	247	320	260	156	132	113	102	358	211
HCO_3ppm	146	156	320	128	130	158	81	209	166
Alkalinity mg/l	2.9	7.3	6.3	2.4	3.2	4.1	1.5	4.1	3.97
Cl mg/l	78	152	158	31	48	80	34	89	83.75
Na meq/l	81	61	154	17	7	29	16	113	59.75
K mg/l	5	8	20	5	4	5	7	8	7.75
SO_4 mg/l	138	187	68	28	7	11	4	206	81.12
As ppb	0	0	0	0	0	0	0	0	0
Co_3 ppm	0	0	8	0	0	0	0	0	1
BOD mg/l	3.6	3.2	3.0	3.5	3.1	3.4	3.0	3.8	3.32
DO mg/l	7.5	7.1	7.0	7.2	7.3	7.1	7.4	7.6	7.27

Table 5. Physico-chemical Analysis of water for the month of May 2008.

	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**Mean**
Date	02	03	14	14	19	19	24	24	
Time	2.20	2.35	11.00	2.30	2.15	4.00	2.00	4.20	
Temp: Air ◦C	42	43	42	44	43	42	44	43	45.37
Temp: H_2O ◦C	39	40	39	41	40	39	41	40	42.37
pH	7.80	7.87	7.81	8.56	7.30	7.81	8.35	8.21	7.96
EC mu/scm	730	1800	1193	406	320	374	326	1205	794.25
TDS mg/l	421	840	654	209	140	196	170	564	399.25
Turbidity	14.6	12.4	45	10.0	76	17.9	7.1	7.2	23.77
Ca mg/l	32	37	17	16	21	31	22	40	27
Mg meq/l	37	57	52	29	17	17	12	70	36.37
Hardness mg/l	241	312	253	150	127	108	96	351	204.75
HCO_3ppm	142	149	305	120	122	151	89	203	160.12
Alkalinity mg/l	2.7	7.1	6.1	2.1	3.1	4.3	1.7	4.3	3.92
Cl mg/l	72	146	152	29	53	83	38	83	82
Na meq/l	84	63	149	19	6	26	14	107	58.5
K mg/l	6	7	18	5	4	6	6	7	7.37
SO_4 mg/l	130	180	62	26	6	10	4	200	77.25
As ppb	0	0	0	0	0	0	0	0	0
Co_3 ppm	0	0	13	0	0	0	0	0	1.62
BOD mg/l	3.2	3.1	3.0	3.4	3.1	3.5	3.2	3.0	3.18
DO mg/l	6.3	6.0	6.1	6.2	6.0	6.4	6.1	6.0	6.13

Table 6. Physico-chemical Analysis of water for the month of June 2008.

	S1	S2	S3	S4	S5	S6	S7	S8	Mean
Date	03	08	08	17	17	24	24	29	
Time	2.00	1.00	4.40	12.00	2.00	2.00	4.45	2.30	
Temp: Air ◦C	44	43	42	44	45	42	41	45	47.37
Temp: H_2O ◦C	41	40	39	41	41	39	38	41	44.37
pH	7.70	7.81	7.76	8.50	7.70	7.88	8.26	8.10	7.96
EC mu/scm	715	1780	1182	418	310	367	321	1195	786
TDS mg/l	416	825	645	200	133	186	178	552	391.87
Turbidity	14.0	12.1	41	10.3	71	17.3	7.0	7.4	22.51
Ca mg/l	30	34	16	19	20	36	24	52	28.87
Mg meq/l	32	53	50	30	15	15	10	81	35.75
Hardness mg/l	235	307	247	154	123	105	87	347	200.62
HCO_3ppm	136	140	298	116	120	143	82	195	153.75
Alkalinity mg/l	2.9	7.3	6.4	2.4	3.4	4.6	1.5	4.7	4.15
Cl mg/l	70	138	146	32	58	87	33	76	80
Na meq/l	80	58	141	17	7	25	12	102	55.25
K mg/l	5	6	16	4	5	8	7	6	7.12
SO_4 mg/l	125	172	60	23	5	13	5	190	74.12
As ppb	0	0	0	0	0	0	0	0	0
Co_3 ppm	0	0	10	0	0	0	0	0	1.25
BOD mg/l	2.2	2.0	2.3	2.6	2.1	2.4	2.5	2.8	2.36
DO mg/l	5.3	5.1	5.0	5.4	5.2	5.6	5.7	5.8	5.38

Table 7. Physico-chemical Analysis of water for the month of July 2008.

	S1	S2	S3	S4	S5	S6	S7	S8	Mean
Date	07	07	16	16	22	22	29	29	
Time	2.00	4.45	2.15	4.30	11.00	2.00	2.00	4.40	
Temp: Air ◦C	40	39	41	38	37	39	38	37	43.37
Temp: H_2O ◦C	37	36	37.5	35.5	34	36	35.5	34	40.37
pH	7.87	7.80	7.57	8.50	7.10	7.74	8.30	8.10	7.87
EC mu/scm	785	1850	1140	415	336	368	320	1186	800
TDS mg/l	415	820	640	217	144	186	160	556	392.25
Turbidity	14.2	12.6	40	10.4	71	16.9	7.5	7.8	22.55
Ca mg/l	27	35	14	19	20	28	25	45	26.62
Mg meq/l	31	50	46	31	18	19	16	80	36.37
Hardness mg/l	235	305	257	155	123	105	90	342	201.5
HCO_3ppm	136	142	297	126	120	145	80	200	155.75
Alkalinity mg/l	2.3	7.5	6.6	2.5	3.5	4.1	1.9	4.7	4.13
Cl mg/l	65	137	157	34	56	80	42	80	81.37
Na meq/l	74	57	154	21	5	23	16	100	56.25
K mg/l	5	6	16	8	5	9	7	6	7.75
SO_4 mg/l	123	172	60	35	7	14	5	190	75.75
As ppb	0	0	0	0	0	0	0	0	0
Co_3 ppm	0	0	10	0	0	0	0	0	1.25
BOD mg/l	2.9	2.6	3.0	2.8	2.7	3.3	3.0	2.8	2.88
DO mg/l	5.6	5.3	5.2	5.7	5.4	5.9	5.1	5.6	5.47

Table 8. Physico-chemical Analysis of water for the month of August 2008.

	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**Mean**
Date	02	02	10	10	18	18	25	25	
Time	2.20	4.25	11.00	2.00	2.00	4.40	12.00	2.30	
Temp: Air ◦C	36	34	35	37	38	34	35	37	39.37
Temp: H_2O ◦C	32	30	31	32	32	30	31	32	35.37
pH	6.7	6.8	6.5	6.2	7.5	6.6	6.1	6.9	6.66
EC mu/scm	720	1040	1136	390	300	350	315	1190	680.12
TDS mg/l	400	820	643	200	130	180	158	530	382.62
Turbidity	14.0	12.7	41	10.8	70	17.5	7.0	7.0	22.5
Ca mg/l	26	32	20	19	20	30	20	35	25.25
Mg meq/l	32	60	48	32	15	16	10	76	36.12
Hardness mg/l	235	308	243	158	123	103	90	340	200
HCO_3ppm	150	154	300	130	120	140	82	195	158.87
Alkalinity mg/l	2.9	7.4	6.6	2.8	3.5	4.0	1.8	4.1	4.13
Cl mg/l	78	153	158	35	50	87	40	78	84.87
Na meq/l	70	52	152	23	7	21	17	90	54
K mg/l	5	6	21	6	4	6	7	8	7.87
SO_4 mg/l	120	187	68	35	6	12	6	180	76.75
As ppb	0	0	0	0	0	0	0	0	0
Co_3 ppm	0	0	15	0	0	0	0	0	1.87
BOD mg/l	2.9	2.7	2.6	2.8	3.0	2.4	2.5	2.3	2.65
DO mg/l	6.80	6.63	6.50	6.45	6.30	6.70	6.56	6.72	6.58

Table 9. Physico-chemical Analysis of water for the month of September 2008.

	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**Mean**
Date	03	03	11	11	19	19	26	26	
Time	11.00	2.00	2.00	4.50	2.30	3.30	11.10	2.15	
Temp: Air ◦C	32	34	33	31	30	29	32	34	35.37
Temp: H_2O ◦C	28	30	30	27	26	26	28	30	31.37
pH	6.95	7.10	7.40	7.70	7.15	7.92	7.80	7.56	7.44
EC mu/scm	700	1700	1150	380	310	337	312	1175	758
TDS mg/l	390	810	620	206	135	208	180	543	386.5
Turbidity	14.0	12.0	39	11.0	72	17.3	7.9	7.1	22.53
Ca mg/l	36	33	61	20	20	37	28	36	33.87
Mg meq/l	40	50	46	31	22	20	16	78	37.87
Hardness mg/l	230	300	240	157	134	114	100	364	204.87
HCO_3ppm	136	141	320	130	132	160	96	193	163.5
Alkalinity mg/l	2.9	7.5	6.4	2.9	3.8	4.7	1.8	4.8	4.35
Cl mg/l	70	140	143	32	60	90	42	89	83.25
Na meq/l	75	60	140	20	7	30	18	103	56.62
K mg/l	10	9	21	6	5	8	8	8	9.37
SO_4 mg/l	123	185	67	30	8	13	5	190	77.62
As ppb	0	0	0	0	0	0	0	0	0
Co_3 ppm	0	0	0	0	0	0	0	0	0
BOD mg/l	3.8	3.9	3.8	3.4	3.2	3.5	3.0	3.3	3.48
DO mg/l	5.2	5.6	5.1	5.7	5.3	5.8	5.0	5.9	5.45

Table 10. Physico-chemical Analysis of water for the month of October 2008.

	S1	S2	S3	S4	S5	S6	S7	S8	Mean
Date	05	05	13	13	20	20	28	28	
Time	12.30	1.30	12.00	2.00	12.30	2.30	12.00	2.40	
Temp: Air ◦C	27	27	28	29	29	30	28	30	30.37
Temp: H_2O ◦C	24	24	25	26	26	26	25	26	27.37
pH	8.52	8.45	8.92	8.72	7.92	7.76	8.78	8.40	8.43
EC mu/scm	1196	283	688	282	283	347	279	1178	567
TDS mg/l	645	152	370	152	152	198	158	623	306.25
Turbidity	7.3	267	0.2	7.7	5.9	18.3	6.1	7.8	40.03
Ca mg/l	32	32	24	20	20	29	23	29	26.12
Mg meq/l	73	5	34	10	12	19	13	81	30.87
Hardness mg/l	380	100	200	90	100	118	107	392	186.87
HCO_3ppm	240	110	130	90	90	2.7	94	238	124.33
Alkalinity mg/l	4.8	2.2	2.6	1.8	1.8	42	1.6	4.3	7.63
Cl mg/l	100	37	83	32	32	23	37	116	57.5
Na meq/l	102	17	66	22	17	17	19	109	46.12
K mg/l	8	3	6	4	4	6	5	7	5.37
SO_4 mg/l	208	20	93	6	6	11	8	223	71.87
As ppb	0	0	0	0	0	0	0	0	0
Co_3 ppm	0	0	20	10	20	26	17	0	11.62
BOD mg/l	4.0	4.3	4.2	4.7	4.5	4.1	4.4	4.3	4.31
DO mg/l	4.0	4.4	4.2	4.3	4.1	4.1	4.5	4.4	4.76

Table 11. Physico-chemical Analysis of water for the month of November 2008.

	S1	S2	S3	S4	S5	S6	S7	S8	Mean
Date	04	04	12	12	23	23	30	30	
Time	2.00	4.00	2.30	4.00	2.00	4.15	1.40	4.30	
Temp: Air ◦C	32	30	31	29	30	28	26	24	35.37
Temp: H_2O ◦C	28	26	27	26	26	25	23	21	31.37
pH	8.62	7.99	8.45	7.98	7.85	7.85	7.15	8.42	8.03
EC mu/scm	1408	243	767	359	1930	300	310	1180	812.12
TDS mg/l	792	131	413	194	940	170	180	657	434.62
Turbidity	0.5	160	8.9	17.2	80	7.5	5.6	7.8	35.93
Ca mg/l	16	24	36	24	224	18	21	36	49.87
Mg meq/l	53	11	32	16	58	13	11	68	32.75
Hardness mg/l	260	105	220	125	799	98	95	368	258.75
HCO_3 ppm	400	70	140	120	360	87	87	232	187
Alkalinity mg/l	8.0	1.4	2.8	2.4	7.20	1.9	96	43	20.33
Cl mg/l	195	32	80	35	202	37	38	108	90.87
Na meq/l	192	6	96	18	66	28	16	109	66.37
K mg/l	22	2	6	5	2	5	4	9	6.87
SO_4 mg/l	28	6	124	10	288	7	6	196	83.12
As ppb	0	0	0	0	0	0	0	0	0
Co_3 ppm	0	0	0	30	0	12	22	0	8
BOD mg/l	4.3	4.7	4.0	3.9	3.7	4.1	4.2	4.5	4.17
DO mg/l	5.6	5.2	5.1	5.3	6.1	6.2	6.0	5.4	5.61

Total dissolved solids (TDS): The TDS level was observed highest 435.12mg/l in December and the lowest level was observed 306.25mg/l in August (Table 13).

Turbidity: It was observed that the level of turbidity highest 59.05 in March and lowest 17.93 was recorded in December (Table 13).

Calcium: The level of calcium was recorded highest 49.87mg/l in March while lowest level was recorded 26.1 mg/l in October (Table 13).

Magnesium: The level of magnesium in water samples were recorded highest of 39.5meq/l in February while lowest level was recorded 30.87meq/l in October (Table 13).

Hardness: The highest range of hardness were observed 266.12mg/l in December and lowest ranged were observed 8 mg/l in March (Table 13).

Bi Carbonate: The level of bi carbonate in water sample was recorded highest 216.12mg/l in December while the lowest level of bi carbonate was recorded 124.33mg/l in October (Table 13).

Alkalinity: The highest ranged were observed 20.33mg/l in November and the lowest level was observed 3.46mg/l in January (Table 13).

Chloride: The level of chloride in water samples were recorded highest 101.75mg/l in December while the lowest was recorded 57.5mg/l during the month of the October (Table 13).

Sodium: The level of sodium in water samples were recorded highest 73.25meq/l in February while lowest level was recorded 46.12meq/l in October (Table 13). The Sodium concentration increases during the winter while the level of sodium decreases during the summer season.

Potassium: The level of potassium in water samples were recorded highest 11mg/l in February while lowest level was recorded 5.62mg/l in March (Table 13).

Sulphate: The level of sulphate in water samples were recorded highest 83.12mg/l in November while lowest level was recorded 57.75mg/l in January (Table 13).

Arsenic: It was observed that the level of arsenic in water samples recorded zero in every month of the year (Table 13).

Table 12. Physico-chemical Analysis of water for the month of December 2008.

	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**Mean**
Date	06	06	14	14	20	20	29	29	
Time	2.00	3.30	12.00	2.00	12.30	2.30	2.00	4.00	
Temp: Air ◦C	22	21	21	22	20	21	22	20	24.62
Temp: H_2O ◦C	20	19	19	20	18	19	20	18	22.37
pH	8.50	8.16	8.25	7.58	7.50	7.71	7.50	8.56	7.97
EC mu/scm	1395	1890	752	530	340	368	315	1316	978.25
TDS mg/l	780	920	453	245	147	192	176	568	435.12
Turbidity	0.8	1.3	8.3	11.7	89	17.9	7.2	7.3	17.93
Ca mg/l	18	213	31	17	23	26	25	32	48.12
Mg meq/l	58	56	28	38	18	16	13	54	35.12
Hardness mg/l	270	715	236	186	132	135	113	342	266.12
HCO_3ppm	416	370	180	153	131	183	94	206	216.62
Alkalinity mg/l	8.3	7.46	2.6	3.2	3.4	4.8	1.5	4.5	4.47
Cl mg/l	191	213	87	56	49	84	43	91	101.75
Na meq/l	186	94	95	27	7	29	21	108	70.87
K mg/l	23	18	8	4	3	7	6	8	9.62
SO_4 mg/l	26	235	139	28	6	15	5	206	82.5
As ppb	0	0	0	0	0	0	0	0	0
Co_3 ppm	0	0	0	0	0	0	0	0	0
BOD mg/l	3.6	3.2	3.5	3.7	3.1	3.0	3.8	3.4	3.41
DO mg/l	6.2	6.0	6.4	6.1	5.9	5.7	6.3	6.1	6.08

Carbonate CO3: The highest value of carbonate was observed in October 11.62 and lowest value was observed in March 1 (Table 13).

Biological oxygen demand (BOD): The highest value of the BOD was observed 4.31mg/l in October and lowest ranged were observed 3.38mg/l in March (Table 13).

Dissolved Oxygen: The level of DO was recorded highest 7.28mg/l in January and lowest ranged was observed 4.76 mg/l in October 2008 (Table 13).

DISCUSSION

The Chotiari Wetland Complex is a shallow water lake, having a sandy and salty depth. The depth and area of CWC is variable depending upon the influx of water. The water level varies with the seasonal change in the quantity of water which enters into the lake. Present depth has been recorded from 15-30 ft and its level decrease to 8-18 ft in the dry season. Seasonal fluctuation of physico-chemical parameters, a similar rise in dissolved oxygen in winter season has been reported by different researchers (Singh *et al*., 1980). According to Rao (1986) due to causes of reduction in microbial decomposition of dead organic matter, low organismal respiration demand, increased growth of submerged macrophytes and solubility of atmospheric oxygen by reduction in temperature. The results of pH and alkalinity values indicated that the lake water remained slightly alkaline throughout the period of study due to the inflow of sufficient amount of water through the Ranto canal (Nara canal). The permissible limits of hardness by WHO is recognized 200mg/l. The hardness of lake water has measured little high from the given guideline of WHO (1984). This increase of hardness in the water could be due to the inflow of rain water. Salinity, conductivity and TDS were substantially higher; this probably indicates that there could be some contamination of domestic sewage and agricultural waste water supply from the Ranto canal (Nara Canal). The chloride is a pollution indicating parameters i.e. related to sewage contamination with the degradation products. In the reservoir amount of chloride was recorded in normal range. The WHO gives 250mg/l of chloride as an acceptable amount in the drinking water. However, the level of Ca, Mg, Na, K, SO_4, HCO_3, CO_3, COD and BOD was detected in elevated concentration compared to the maximum acceptable limits (Table 13). The salinity of water is the main factor which can be effected on the aquatic life of plants and animals (Khuhawar and Mastoi, 1995) have also reported higher salinity of water in the lake. The seedlings of commercial fish species in CWC like *Labeo rohita, Cirrhinus mirgla* and *cattla cattla* are very sensitive and cannot be tolerated at the higher range of salinity. The physico-chemical variables of CWC when compared with other lakes of Sindh, such as Keenjhar lake, (Chloride 38.9mg/l, salinity 0.05mg/l, alkalinity 20mg/l, Khuhawar *et al*., 1998), Haleji lake, (Alkalinity 525mg/l, chloride 75mg/l, TDS 338mg/l, Khuhawar *et al*., 1998), Hamal lake (Hardness 670mg/l, chloride 1750mg/l, alkalinity 275mg/l, (Khuhawar *et al*., 1998), Bakar lake [TDS 580mg/l, alkalinity 550 mg/l, hardeness 210mg/l (Jafri *et al*., 1997)] and Hub Dam [Transparency 2.1-3.3 m, pH 6.8-7.5, dissolved oxygen 3.1-5.3mg/l, salinity 0.15-25ppt., dissolved solids 50ppm (Iqbal and Kazmi, 1988)] indicated that all these lakes still retain the typical fresh water characteristics despite progressive eutrophication.

Table 13. Over all mean of Parameters of the Water Analysis 2008.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Temp: of air	22.37	23.37	23.37	38.37	45.37	47.37	43.37	39.37	35.37	30.37	35.37	24.62
Temp: of H_2O	21.37	20.37	29.37	35.37	42.37	44.37	40.37	35.37	31.37	27.37	31.37	22.37
pH	8.09	8.14	7.56	8.03	7.96	7.96	7.86	6.66	7.44	8.43	8.03	7.59
EC mu/scm	600.12	824.62	727.4	808.87	794.25	786	800	680.12	758	567	812.12	978.25
TDS mg/l	404.87	415.37	379.4	412.12	399.25	391.87	392.25	382.62	386.5	306.25	434.62	435.12
Turbidity	41.4	28.47	59.05	24.77	23.77	22.51	22.55	22.5	22.53	40.03	35.93	17.93
Ca mg/l	27.62	30	48.62	28.75	27	28.87	26.62	25.25	33.87	26.12	49.87	48.12
Mg meq/l	32	39.5	31.5	38.12	36.37	35.75	36.37	36.12	37.87	30.87	32.75	35.12
Hardness mg/l	188.62	216.12	83	211	204.75	200.62	201.5	200	204.87	186.87	258.75	266.12
HCO_3 ppm	159.5	170.25	194.6	166	160.12	153.75	155.75	158.87	163.5	124.33	187	216.62
Alkalinity mg/l	3.46	4.2	8.42	3.97	3.92	4.15	4.13	4.13	4.35	7.63	20.33	4.47
Cl mg/l	72	84.12	71	83.75	82	80	81.37	84.87	83.25	57.5	90.87	101.75
Na meq/l	52.62	73.25	57	59.75	58.5	55.25	56.25	54	56.62	46.12	66.37	70.87
K mg/l	6.87	11	5.62	7.75	7.37	7.12	7.75	7.87	9.37	5.37	6.87	9.62
SO_4 mg/l	57.75	80	63.25	81.12	77.25	74.12	75.75	76.75	77.62	71.87	83.12	82.5
As ppb	0	0	0	0	0	0	0	0	0	0	0	0
Co_3 ppm	1.25	0	0	1	1.62	1.25	1.25	1.87	0	11.62	8	0
BOD mg/l	3.4	3.41	3.38	3.32	3.18	2.36	2.88	2.65	3.48	4.31	4.17	3.41
DO mg/l	7.28	7.27	6.48	7.27	6.13	5.38	5.47	6.58	5.45	4.76	5.61	6.08

At CWC the process of eutrophication is high at its due to the shallowness of the basin, but the range of many chemical parameters has gone up, beyond the permissible limits, recommended by the WHO (1984). Irrigation system of Pakistan, which in one of the largest contagious system in the world, is now facing the enormous problems. The shortage of water is the major problem, along with water logging, water salinity, water quality, over exploration of ground water and sea water intrusion.

Recently a study conducted at the Chotiari Reservoir, and Rais *et al*. (2011) reported that 32 species of reptiles including three species of freshwater turtles, 15 snakes, 13 lizards and one crocodile. While Rock Python and Indian Marsh Crocodile were recorded as Threatened Species. Crocodile focuses its attention on of major issue is water pollution concerned directly with the human health to polluted water (Chang *et al*., 2012). The Crocodile receives legal protection, but poor enforcement of hunting rules, profitable foreign trade in skins, loss of habitat and food resources directly effects his population (Chang *et al*., 2012). A survey was conducted by the Zoological Survey of Pakistan, five hundred specimens were recorded at Makhi and Baqar Dhand of the Chotiiari reservoir (www.wildlifeofpakistan, 2013).

The quality of water is the major factor to determine the diversity and health of aquatic flora and fauna (Gachal *et al*., 2001). The indiscriminate uses of agricultural chemicals enhanced chemical pollution in aquatic resources (Gachal and Slater, 2003; Gachal *et al*., 2004, 2006). Excess fertilizers used in the field runoff into the courses causes' uncontrolled algal growth and eutrophification and pesticides in water may kill aquatic life including fish which is the feeding of Crocodiles.

CONCLUSION

Based on present study, the site is faced by many shortcomings in impact and prediction. The major threat is faced to crocodiles and other key species of the wetland complex, destruction of their habitats. The seasonal flooding of the reservoir can destroy the nesting and eggs of crocodiles (Santiapillai *et al.,* 2001). Increased water level in the wetland complex not only inundated the fertile land but also caused excessive water seepage to western and southern areas of the site and its adjoining agricultural lands became waterlogged, salinized and barren. In the reservoir the fish stocks are slowly depleting due to the practices of unsustainable and overfishing. The change in the quality of water was recorded that the hazardous chemicals were found during the analysis of water in the laboratory, which result the harmful effect on the Marsh Crocodiles. The various fishing camps or nets can also found in the Chotiari Reservoir and its surroundings. It is evidence that the remaining population of Marsh Crocodile in Chotiari Reservoir is disturbed with the interaction of a large number of people such as boats, fishing nets; forest clearing fires usually reduces the suitability of the habitat for the crocodiles. It was observed that after the construction of the reservoir the major income resources of local people the agriculture lands, fish stocks of lakes and rangelands have been adversely affected and also the livelihoods of the people. The reservoir has enhanced poverty in local communities and the local communities are struggling to generate their source of income on marginalized natural resources, due to these practices the negative impacts on the habitats and its associated biodiversity.

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ASSESSING THE ADOPTION OF ROLL BACK MALARIA PROGRAMME (RBMP) AMONG WOMEN FARMERS IN IKORODU LOCAL GOVERNMENT AREA OF LAGOS STATE

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ABSTRACT

Combating malaria globally, especially among women and infants, has become a public health, environmental and economic priority, as such special focus has been given to it in the millennium development goal. This has also led to the initiation of programmes such as the Roll Back Malaria Programme (RBMP). In view of this, this paper assessed the adoption of RBMP among women in Ikorodu local government area of Lagos state. Data were obtained from 150 women farmers who were purposively selected. The data gathered were analyzed using descriptive and logit regression analysis. About 62% of the respondents were between 31 and 50 years of age and 52% had more than 6 persons in their household, 60% had at least secondary education, 74% earned less than ₦20, 000 (122.6 USD) per month, 72% were full-time farmers, 82% had access to Primary Health Care (PHC), 55% were aware of the RBMP but only 46% have adopted the use of Insecticide-Treated Net (ITN). Furthermore, respondents' awareness of RBMP, income level, educational status, membership of cooperative association, frequency of malaria attack and patronage of the PHC centres positively influenced their adoption of RBMP and consequent use of ITN while age had negative influence on adoption of RBMP among respondents. To accelerate adoption of RBMP, there is yet the need to focus policy on collaborative efforts of health personnel, cooperatives, media houses and government agency in fashioning out awareness programmes that incorporate socio-economic characteristics of the audience especially at the local government level.

Keywords: Roll back malaria programme, insecticide treated net, logit regression, Ikorodu.

INTRODUCTION

Among the major diseases that are common in Africa, malaria is one of the greatest threats facing development in Africa today. It attacks an individual on an average of four times in a year with an average of 10 to 14 days of incapacitation (Alaba and Alaba, 2003). Studies have shown that about 350 to 500 million clinical disease episodes occur annually (Bawah and Binka, 2005). Over 75 percent of these mortality figures (especially children) are from African (MIM, 2001; Alaba, 2005). This statistics has serious implication for economic growth and welfare. The worry is even compounded by the fact that the disease is growing resistant to the cheap anti-malaria drugs and the poor households cannot afford the more expensive ACT combination therapy.

In Nigeria, malaria is the major cause of morbidity and mortality, especially among pregnant women and children below age five (Alaba, 2007). Malaria is a social and economic problem. The economic loss due to malaria in Nigeria is in excess of two million US dollars per year (WHO, 2005). Malaria is not only a health problem, it is also an economic problem. Malaria at the household level affects the productivity of the people and their assets acquisition capacity. Households also frequently spend substantial share of their income and time on malaria prevention and treatment as well as an effort to control mosquitoes (Coluzzi, 1999).

Because Malaria control is such a complex issue, its stakeholders are varied and therefore it requires well-coordinated international collaboration. The Roll Back Malaria (RBM) Partnership and Millennium Development Goals (MDG), which was launched in 1998 by the World Health Organization (WHO), the United Nations Children's Fund (UNICEF), the United Nation Development Programme (UNDP) and the World Bank, is one of this international collaboration that is aimed at achieving 80% use of insecticide treated net (ITN) among pregnant women and children below five years of age in Africa, especially in rural and semi-urban areas (WHO, 2008). Broadly speaking, the goal of the RBM programme is to half the malaria burden through interventions that are adapted to local needs via case management using Artemisin-based combination therapies, Insecticide - Treated Net (ITN) and other vector control measures, providing malaria treatment and Intermittent Preventive Therapy (IPT) for pregnant women and improving malaria epidemic preparedness and

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responses. These activities were to be facilitated by integration of malaria control activities in Primary Health Care (PHC) and other social activities and strengthening health information systems and research so as to strengthen community participation (FMOH, 2000; WHO, 2008; Salaudeen and Jimoh, 2009). Despite this laudable global effort at ameliorating the malaria menace, rural Africa still suffer gravely from malaria.

Rural and semi- urban Nigeria is largely agrarian, thus, the effects of malaria on agriculture, health, and development are widespread (Babalola *et al*., 2009). Women perform nearly all the tasks associated with farming and food marketing in Nigeria as well as most part of Africa (Todaro, 2000; FAO, 2010). It is against this backdrop that this study examined the factors influencing the adoption of the RBM programme among women farmers in Ikorodu local government area of Lagos state.

Malaria: the micro-macro linkage

Malaria has implications for the development of household members and the country through various mechanisms. One, malaria impairs the ability of people to work hard by losing productive time during care-giving activities, while adults with malaria severely compromise household/family resources, as their capacity to work, earn income and save for their families is reduced (WHO, 2000). Added to this, the illness generates new financial demands to cover medical treatment, threatening food supply and in extreme cases funeral expenses. Two, it affects child's development and compromise future productive capacity due to absenteeism from school associated with malaria attack. Also, malaria is known to be a main cause of anaemia, epileptic convulsions, growth faltering, and neurological squeal. These are all likely to affect children's performance at school. Three, in the agricultural sector or rural area, peak of malaria transmission has been found to coincide with the peak of planting and harvesting seasons when demand for labour is supposed to be highest. Thus, vast expanse of land goes uncultivated and substantial harvest are lost because workers are sick.

Current trends in malaria prevention

Progress, although limited, can be observed in malaria prevention and cure in Nigeria since 2000. The report of the Federal Ministry of Health (FMOH, 2005) identified the following progress over the period 2006-2010:

- The change and adoption of a new treatment policy from Chloroquine to Artemisinin based Combination Therapy (ACT) has been made.
- In spite of the inadequate human resources, a few recruitment was made in the National Malaria Control Programme (NMCP) and capacity building carried out to strengthen malaria programme management.
- Use of ITNs has increased from about 2.2% (2003) to about 6.8% (2005). This has provided a firm base for planned scaling up efforts.
- The malaria control programme has benefited from the strengthening of partners both in the public and private sectors.
- There has been more awareness created and more political commitment towards malaria control in the Country due to the Ministerial advocacy visits to States. This is also evidenced through the tax and tariffs waivers on ACTs, ITNS and insecticide treatment kits.

However, the FMOH report further revealed that a number factors still poses challenges (in form of weaknesses and threats) to the achievement of the success of malaria prevention programmes in Nigeria which include:

- Lack of knowledge on the interaction of the package of interventions and outcomes.
- A weak and constrained health system that may not cope with added pressures of a national programme expansion.
- Inadequate funding for effective programme management.
- Procurement and supply chain system that is in its infancy stages.
- Human resource gaps especially at sub-national level.
- Gaps in total required resources for meeting scaling up targets
- Low priority for malaria control by some policy makers at sub-national level

MATERIALS AND METHODS

The study was carried out in Ikenne Local Government Area of Ogun State. The Local Government is made up of five major towns viz: Ikenne, Ilishan, Irolu, Iperu and Ogere. The people of the local government engage in the planting of different food crops like maize, cassava, pineapples, various types of vegetables, e.t.c. They also engage in livestock rearing and trading. The climate is hot and humid which favours the proliferation of the mosquito vector. The vegetation and climate of Ikenne places it in the malaria belt (Babalola and Agbola, 2009).

Primary data were collected from 100 rural women farmers in the study area using purposive sampling procedure. The information collected from farmers (such as socio-economic, institutional, farm level etc) was based on one year production activities.

The logit regression model was employed to examine the factors that influence the respondents' adoption or non-adopting of the Roll Back Malaria programme. For the

purpose of this study, the use of ITN is conceived to reflect the adoption of RBMP. The model is specified as follows:

$Ln\ (P_i/(1-P_i)) = ß_0 + ß_1X_1 + \ldots. + ß_{14}X_{14} + e_i$ (Gujarati 1998)

Where

The dependent variables are the natural log of the probability of adoption of RBM programme (P_i) divided by the probability of not adopting ($1-P_i$), βo = the intercept, $ß_{1\ldots14}$ = regression coefficients, $X_{1\ldots14}$ = independent variables, and ei = error term.

The independent variables specified are factors affecting the adoption of RBM programme, and are defined below:

X_1 = Age (years)
X_2 = Income level per production season (₦)
X_3 = Educational status (years)
X_4 = Farming experience (years)
X_5 = Farm size (ha)
X_6 = Household size
X_7 = Extension services (Yes=1, No=0)
X_8 = Awareness of RBMP (Yes=1, No=0)
X_9 = Community Based Organization (CBO)/ Cooperative membership (Yes=1, No=0)
X_{10} = Frequencies of malaria attack per year
X_{11} = Cost of malaria treatment per year (₦)
X_{12} = Presence of vulnerable group (i.e. pregnant women and children under the age of five).
X_{13} = Patronage of Primary Health Care (PHC) centre when sick (Yes=1, No=0)
X_{14} = Number of days off farm as a result of malaria attack per month

RESULTS AND DISCUSSION

Descriptive statistics of the respondents

Results according to table 1 showed that the majority (46%) of the women interviewed were between 31 and 40 years old and 64 percent were married meaning that the majority of the respondents are still in their reproductive age, thus, the Roll-Back-Malaria programme (RBMP) is very relevant in the study area. Most of the respondents (60%) had at least secondary education. Most of the women (55%) claimed to be aware of the RBMP especially through the mass media (20%) followed by health centres (16%) and Community Based Organizations (13%) (CBO), however, 45 percent of the respondents were not aware of the RBMP. The nexus between respondents' educational level and awareness of the RBMP on adoption of the program is consistent with documented literature (Babalola *et al*., 2012). Monthly income for the majority (74%) of the respondents' households was less than ₦20,000 (US $ 122.6), with more than six members constituting the size for most (52%) households, a lot of the respondents live below a dollar a day, indicating presence of poverty in the study area. The majority (72%) of the respondents were full-time farmers and had access (82%) to Public Health Care (PHC). The use of mosquito repellant as a control measure for the prevention of malaria was mostly favoured by most of the households (26%) in the study area. the majority of the households interviewed (59%) had at least one pregnant woman, infant or both as members of the household.

Factors influencing the adoption of the RBMP

The regression result as presented in table 2 showed that out of all independent (explanatory) variables, the coefficient of educational level of the respondent ($p< 0.1$), income level ($p< 0.05$), awareness of the RBMP ($p< 0.1$), frequency of malaria attack ($p< 0.05$), and patronage of the PHC centre when sick ($p< 0.1$) were significant with a positive sign, indicating a direct relationship between these factors and the adoption of RBMP. This implies that increase in these independent variables would increase the probability of the respondents' adoption of RBMP. The coefficient for the age of the respondents ($p< 0.1$) was significant with a negative sign showing an inverse relationship between age and the probability of the adoption of RBMP. This implies that older women show less interest in the RBMP.

CONCLUSION AND RECOMMENDATIONS

This study assessed the adoption of Roll-Back-Malaria Programme (RBMP) among women farmers in Ikorodu Local Government Area of Lagos state. The findings demonstrated the importance of education, and awareness efforts to the adoption of RBMP. The nexus between income level, an important precursor of poverty status, and adoption of RBMP, was also established. The study also shows the impact the Public Health Care (PHC) delivery centres can have on the adoption of RBMP.

Based on the findings in this study, the following are being recommended:

1. Awareness campaign as regards the health and economic advantages of malaria prevention should be intensified especially via the mass media.
2. To accelerate adoption of RBMP, there is yet the need to focus policy on collaborative efforts of health personnel, cooperatives, media houses and government agency in fashioning out awareness programmes that incorporate socio-economic characteristics of the audience especially at the local government level. Finally,
3. There is need to intensify the focus on creating demand for Insecticide Treated Nets through all available health information channels.

Table 1. Basic Descriptive Statistics of Farmers' Specific Characteristics.

Variables	No. of Respondents (n= 150)	%
Education (years)		
None	27	18
Primary	33	22
Secondary	59	39
Post primary	31	21
Family Size		
< 3	8	5
4- 6	64	43
> 6	78	52
Farm Size		
< 1	48	32
1- 3	63	42
> 3	39	26
Age (years)		
< 30	45	30
31- 40	69	46
41- 50	24	16
> 50	12	8
Household monthly income (₦'000) *@ 1USD = ₦ 163.13*		
< 20	111	74
21- 50	27	18
51- 100	12	8
Employment Status		
Full-time farmers	108	72
Farming + clerical	15	10
Farming + artisan	27	18
Access to PHC (dummy)		
1: Yes	123	82
0: No	27	18
Awareness of RBMP (dummy)		
1: Yes	82	55
0: No	68	45
Main source of awareness		
CBO/Co-op	20	13
Mass media	30	20
Family/friend	9	6
Health centre	24	16
None (not aware)	68	45
Main method adopted for preventing malaria		
ITN	30	20
Drugs	32	21
Repellant	39	26
Herbs	27	18
Environ. Sanitation	16	11
None	6	4
Marital Status		
Married	96	64
Single	54	36
Membership of CBO/Co-op (dummy)		
1: Yes	95	63
0: No	55	37
Risk Group in the Household (i.e infant and/or pregnant woman)		
1: Present	89	59
0: Absent	61	41

Source: Field Survey, 2012

Table 2. Regression result for adoption of RBM program.

Independent variables	Beta coefficient	t-ratio
Constant	-.353	2.957
Age	-0.091*	1.856
Household size	0.425	1.335
Farm size	-0.598	0.714
Educational status	0.130*	1.908
Farming experience	-0.024	0.222
Income level	0.067**	2.220
Awareness of RBM	0.382*	1.871
Vulnerable groups	1.045	1.064
Frequency of attack	0.394**	2.297
CBO membership	0.010	0.955
Extension education	0.101	1.003
Patronage of PHC centre when sick	1.626*	1.887
Numbers of days off farm	-0.118	0.259

Significant at 10% level **significant at 5% level; Psuedo R^2 = 0.740; Log likelihood = -46.510*
Source: Computer from field survey data (2012).

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CARAPACE WIDTH WEIGHT RELATIONSHIPS OF MUD CRAB *SCYLLA SERRATA* (FORSKAL, 1775) FROM KARACHI COAST

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ABSTRACT

The length-weight and width-weight relationship study assumes an important prerequisite in fishery biological investigations. In this study, Carapace width weight relationship of both sexes of Mud Crab (*Scylla serrata*) were estimated. A total of 938 samples ranging from 40 to 200mm in width and weighing between 22g and 450g were collected during January 2009 to December 2010, and analyzed. The value of the regression coefficient for male (2.481), female (2.632) and combined sexes (2.571) in the present analysis are very much close to 3 and therefore *Scylla serrata* does follow the cube law.

Keywords: Karachi coast, carapace width, body weight, *Scylla serrata.*

INTRODUCTION

Crabs are widely distributed on mud, estuaries and mangrove areas. . The importance of crab is increases due to their consumption, therefore, the biological studies of crabs are become significant (Levent *et al*., 2009). The width/weight: relationship is regarded as more suitable for assessing not only fish, but also Crustacean (Sukumaran and Neelakantan, 1997; Tabash, 2001; Mohapatra *et al.,* 2010). The relationships between carapace length and weight of the crabs have many uses. They are often used to calculate the standing stock biomass, condition indices, analysis of ontogenetic changes and several other aspects of crustacean population dynamics (Atar and Seçer, 2003; Hortnoll, 1978, 1982; Olusoji *et al.,* 2009; Stickeny, 1972; Romaire *et al.*, 1997; Phinney, 1977). In addition they are used for the management of crab population. According to Lagler (1968) the relationship can be used to estimate the recovery of edible meat from crabs of various sizes.

The carapace width weight relation provides a means of converting measurements of width and weight. It can be an indication of some important events in the life history of fishes such as maturity and growth. The width weight relation, which is important information for fish and shellfisheries management has not been reported for *Scylla serrata* in Pakistan. As wild-harvested stock and a commercial aquaculture product *Scylla serrata* have an economic significance (Samonte and Agbayani, 1992; Perry, 2006).

MATERIALS AND METHODS

The sampling of Mud Crabs (Fig. 1) was done twice in a month for a period of two years from January 2009 to December 2010 from the commercial landings in Korangi fish Harbour (24°48′50″N; 67°13′45″E) Karachi Pakistan (Fig. 2). A total of 938 crabs (488 male and 450 female) was collected during the present study. Measuring all the crabs by width, length and weight to the nearest millimeter for the first two categories and to the nearest gram for the last. The Width weight relationships of all samples collected were determined by the expression $W = a L^b$, where W is the derived weight (g), L is the carapace length (mm) or width (mm), a, is the intercept of the regression curve and b the regression coefficient. The parameters a (intercept) and b (slope) are most easily estimated by linear regression based on logarithms; log (W) = log (a)+b log (L) (Lagler, 1968). The significance of regression was assessed by analysis of variance (ANOVA).

Equations expressing the width/length-weight relationships of mud crabs were calculated in relation to sex. For testing possible significant ($P < 0.01$) differences between the sexes Student's t-test was used for comparison of the two slopes.

RESULTS

The minimum, maximum and mean carapace widths (mm), carapace lengths (mm), and weights (g) (± SE) used in the analysis of width/length weight relationships are given in table 1. The parameters of width-weight relationship, length-weight relationship and width-length relationship estimated from the weight, length and width data are presented in tables 2 and 3 for male, female and overall mud crabs. The linear regressions between width

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Fig. 1. Mud Crab *Scylla serrata* (Source: Williams, 2002).

Table 1. Range and mean values of carapace length, width and body weight of Mud Crab.

Sex	N	Carapace	Width	Carapace	Length	Body	Weight
		Range	Mean S.D	Range	Mean S.D	Range	Mean SD
Female	450	40-200	101.32 +19.346	23-109	67.90 + 12.515	22-450	167.06+74.684
Male	488	50-170	103.55+18.012	38-149	68.26+11.507	39-500	197.32+89.752
Combine	938	40-200	102.48+18.687	23-149	68.09+11.996	22-500	182.80+84.192

and crab weight were highly significant ($P<0.01$). The carapace width-weight relationships were allometric for both sexes. There were no significant differences in slopes between males and females. Table 1 provides data on carapace length and width and on body weight. Statistical analysis showed that male and female do differ in their carapace width and individual weights ($P<0.001$) The mud crabs of male showed significantly wider carapace (103.55+18.012mm) range (50-170) mm and mean individual weight (197.32+89.752mm) range (39-500g) than female mean carapace width of (101.32+ 19.346mm) range; (40-200)mm and mean individual weight 167.06+74.684) range : (22-450g).

The parameter of Carapace width-weight relationship was allometric for both sexes.

The relationships described in relation to female and male mud crab population. In general males had steeper slopes (i.e larger b value) than female in a population which was attributed to the allometric enlargement of male chelae with sexual maturation. The difference in b between male and female were not remarkable when the data for the two species.

Plots of the male female and combine fit well with the regression line obtained for all individuals regardless of species, sex and population (see Figs. 1A, B, C). Notice that there were some plots that deviated below the regression line. These points correspond to post molt individuals with a soft body.

The width-weight relationships were calculated as:

Log W= -2.779 ± 2.481 log L, r = 0.974 (female)
Log W= -3.035 ± 2.632 log L, r = 0.986 (male)
Log W= -2.935 ± 2.571 log L, r = 0.967 (combined)

The values of regression coefficient for male (2.481), female (2.632) and combined sexes (2.571) in the present analysis are very much closed to 3.0 and therefore, *Scylla serrata* does follow the cube law (LeCren, 1951; Martin, 1949).

DISCUSSION

In various studies the width-weight regression equations and the exponent b often lies between 2.5 and 3.5, and is usually close to 3 (Petrakis and Stergiou, 1995; Stickney, 1972; Dulcic and Kraljavic, 1996; Jones *et al.*, 1999).

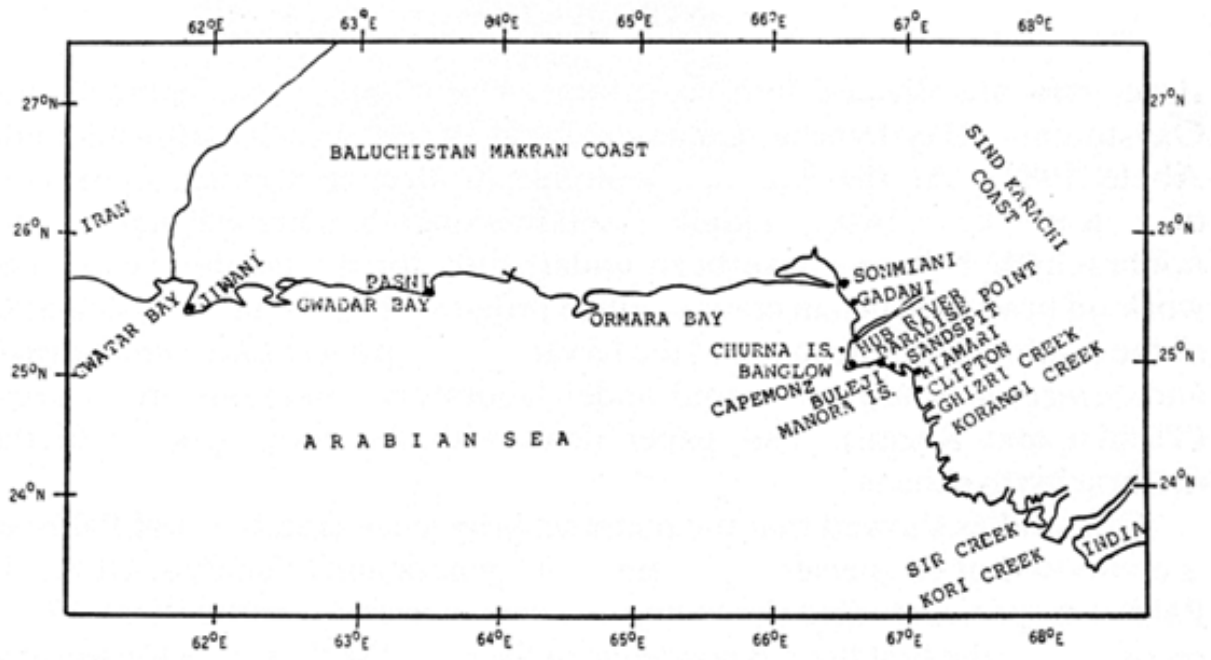

Fig. 2. Coastal region of Pakistan, showing study area Koangi Creek.

According to Pauly (1984), Miyasaka *et al.* (2007) and Mohapatra *et al.* (2010) from an extraordinarily large number of length-weight data taken from a wide variety of Crustacean, values of $b < 2.5$ or $b > 3.5$ are generally based on a very small range of sizes and/or such values of b are more likely to be an error. An exponent (b) value of 3 indicates symmetrical or isometric growth; values other than 3 indicate allometric growth. In the present study, the values for the exponent (b) remained below 3 and the calculated width/ -weight equation was allometric. The values of b ranged from 2.04 to 3.24 for *Callinectes sapidus* from Georgia, and this shows similarities with the b values of the present study (Stickney, 1972). In contrast, the values of b for two other marine portunid crabs (*Portunus sanguinolentus* and *P. pelagicus*) are larger in some cases. Even though the change of b values depends primarily on the shape and fatness of the species, various factors may be responsible for the differences in parameters of the width weight relationships among seasons and years, such as temperature, salinity, food (quantity, quality and size), sex, and time of year and stage of maturity (Pauly, 1984; Sparre, 1992). When the value b is different to 3 or weight growth.

The relationships between carapace width and weight and carapace length and weight have many applications. They are, for example, indicators of environmental condition, and are used to calculate biomass and to estimate the recovery of edible meat from crabs of various sizes (Lagler, 1968). They also have a practical value since they make it possible to convert length into weight and vice versa. On the other hand, body weight and carapace width are the most frequently used dimensions in the study of crustaceans (Sukumaran and Neelakantan, 1997).

The linear regressions between Crab width and weight were highly significant ($P<0.01$). The carapace width/length-weight relationships were allometric for both sexes. There were no significant differences in slopes between males and females.

CONCLUSION

The marketing of fishery products is an essential part of the success of all commercial fishing enterprises. The growing demand for the crab fishery product in the international market offers greater export opportunities for Pakistan Fishery products. Prudent resource exploitation and the production of the quality product according to European Union standards are demand vital to take advantage of the opportunities in fisheries.

The Carapace width weight relationship is important for biological study such as stock assessment and assessment of population parameters. *Scylla serrata* considering the total potential of fish stock in Sindh province waters, the rate of increase in the Crab production and the present catch these appear to be very good potential and scope for

Table 2. Carpace width and body weight of male, female and combine relationship in *Scylla serrata*.

			Regression Equation					t-value	
Realtionship Examined	Sex	N	A	b	R-sq	S.E "a"	S.E "b"	a	b
X:Carp.Wid	Female	450	-2.779	2.481	0.974	0.038	0.019	-73.265	130.771
	Male	488	-3.035	2.632	0.986	0.028	0.014	-107.049	186.596
Y:B.Wt	Combine	938	-2.935	2.571	0.967	0.031	0.015	-94.766	166.436

Table 3. Analysis of covariance (ANOVA) for comparison of regression line of Carp. Width weight relationship of male and female Mud Crab *Scylla serrata.*

Gender	S.S	df	Mean Square	F	Reg. coeff	Sig.
Female Regression	19.301	1	19.301	17101.00	.987	.000
Residual	.506	448	.001			.000
Total	19.807	449				
Male Regression	18.717	1	18.717	34818.153	.993	.000
Residual	.261	486	.001			.000
Total	18.978	487				
Combine Regress.	38.776	1	38.776	27700.938	.984	.000
Residual	1.310	936	.001			
Total	40.087	937				

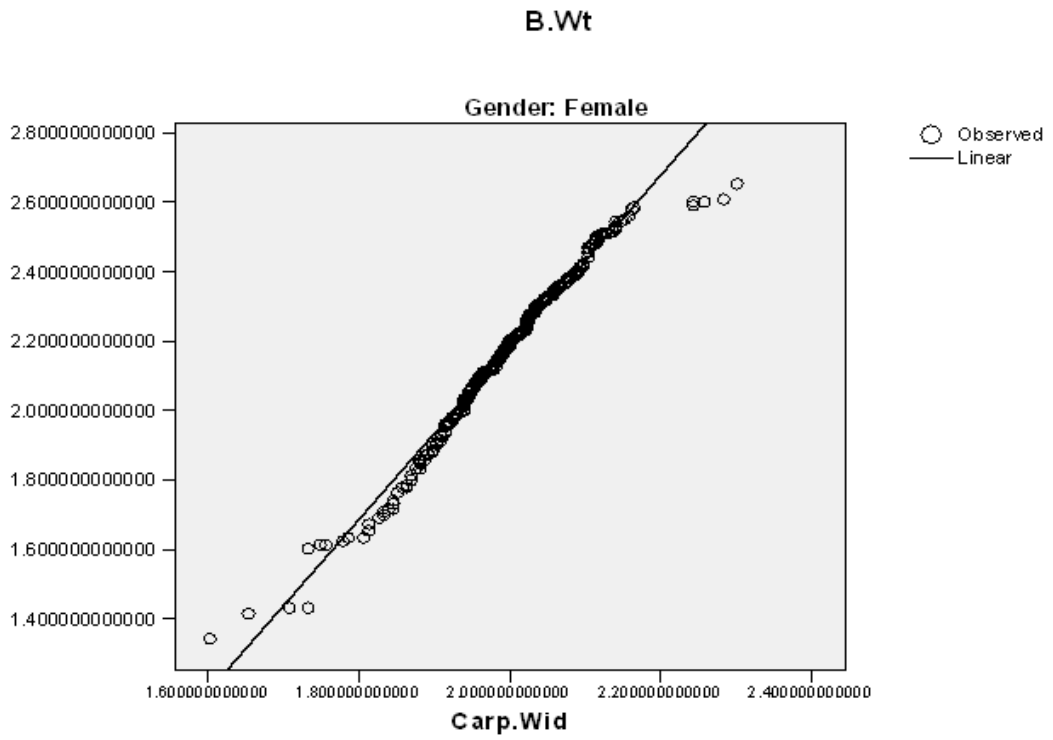

Fig. 1 (A). Carapace width weight relationship of female *Scylla serrata.*

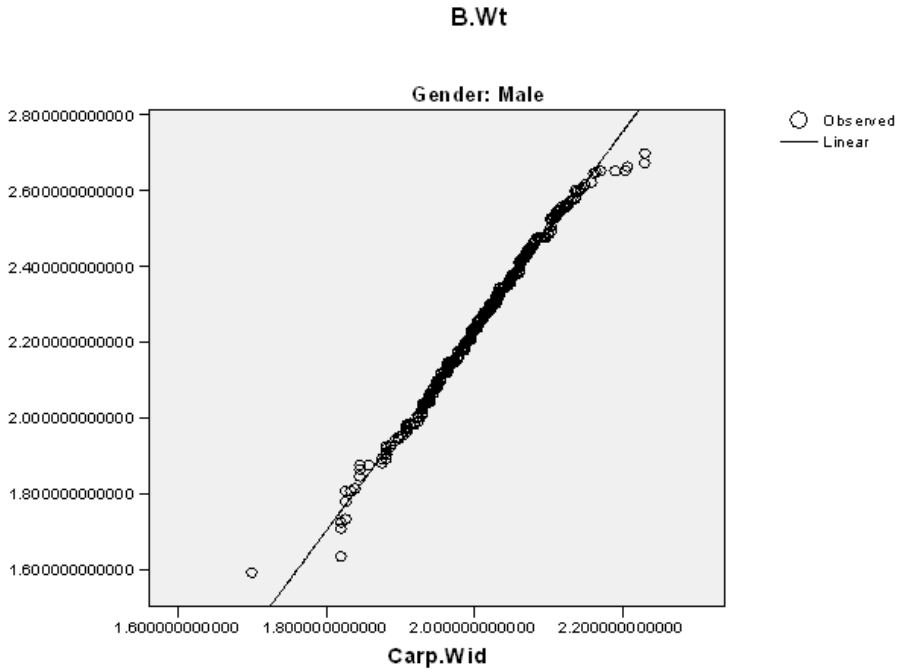

Fig. 1(B). Carapace width weight relation-ship of male *Scylla serrata.*

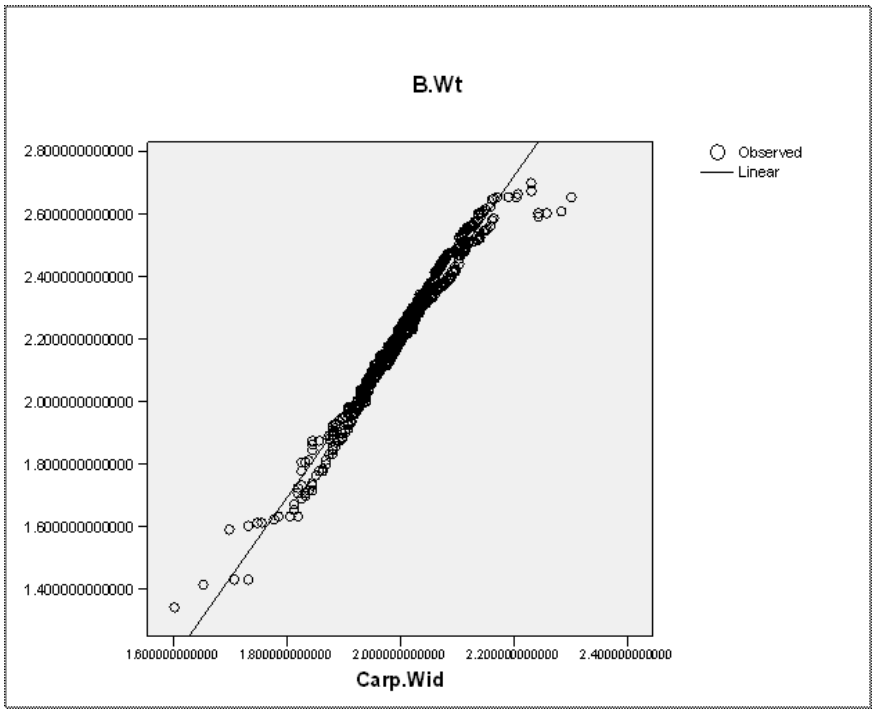

Fig. 1 (C). Carapace width weight relationship of combine (female: male) *Scylla serrata.*
Log W= -2.779 ± 2.481 log L, r = 0.974 (female) Fig 1A
Log W= -3.035 ± 2.632 log L, r = 0.986 (male) Fig.1B
Log W= -2.935 ± 2.571 log L, r = 0.967 (combined) Fig. 1C

further growth to study knowledge of its Carapace width weight relationships is necessary to provide adequate management of its fisheries and aquaculture.

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Short Communication

MICROBIAL CONTENT OF MANUFACTURED (FABRICATED) SOILS: 2002-2011

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ABSTRACT

The main goal of this study was to examine the effects of bacterial activity on soil health. Fabricated soils (FS) have been used in the study of AMD damaged soils in Pennsylvania, USA. This study was also evaluated the bacterial activity in soil as the indicator of soil health.

Keywords: Fabricated soils, acid mine drainage (AMD), microbial content, soil health and sustainability.

INTRODUCTION

The structure and function of the soil foodweb has been suggested as a prime indicator of ecosystem health by several authors (Kalevitch *et al*., 2004). Measurement of disrupted soil processes and decreased bacterial or fungal activity along with other parameters can serve to indicate a problem long before the natural vegetation is lost or human health problems occur. Estimates of the loss of U.S. soil resources due to erosion to range from 2 billion to 6.8 billion tons annually (Beasley, 1972; Pimentel *et al*., 1976; USDA, 1980; Harlin *et al*., 1987; Pimentel *et al*., 1995). Worldwide estimates indicate that between 10-15 million hectares of arable land are rendered unproductive annually due to soil losses (Pimentel *et al*., 1995). Many researchers are concerned with the damage that AMD does to the soil, water, and biological communities. Honey and Kagle (2008) evaluated the impact of acid mine drainage (AMD) on bacterial populations in the Upper Tioga River Watershed, PA, USA. Their study confirmed that pH levels certainly affected the microbiological activity in soil. Authors concluded that both biodiversity and population size has been impacted in AMD affected sites. In our prior study Kalevitch (2006) the proposed recipes of fabricated soils are based on the concept of the carbon-nitrogen balance in the soil as well as on the transformation of carbon products such as glucose, phenolics, and plant polymers. The main objective of this study was to examine the effects of bacterial activity on soil health, while using a FS substrate.

MATERIALS AND METHODS

Study Site

The study site is located in Butler County, Pennsylvania, USA. The site was strip mined in the 1950's and has experienced acid mine drainage in the subsequent years. The resulting site has remained sparsely vegetated since the original mining. To remediate this site, a fabricated soil amendment, a natural mixture of decaying substrates rich in aluminosilicate, carbon, nitrogen, phosphorus and potassium sources, was added to test plots. Soil samples were collected from the reclaimed mining site; some samples were collected from test plots with fabricated soil and one from abandoned mining soil.

Sample Analysis and Data Analysis

Microbial analytics were done by US-Microsolutions, Inc. Soil samples were obtained from different locations named by the presence of certain trees grown on these specific plots. The weighted soil sample was placed in beaker containing deionized water, was allowed to settle at room temperature for 30min, vortexed vigorously, and dilutions were streaked onto TSA (tryptic soy agar) plates. Plates were incubated at 27^{o}C for 10 days. The bacterial colonies were enumerated, identified and the number of CFU-colony-forming unit/gram of material calculated. All data are statistically significant. 95% of confidence intervals exist for all points, a= 0.05.

RESULTS AND DISCUSSION

According to the soil legend, soils represented topsoil, manufactured soils, and mining soil plot. We placed a special emphasis on comparison of microbial composition of fabricated/manufactured soils in 2011, 2010, 2007 and 2002. Thus we compared analysis for manufactured soils that were fabricated this year, 1, 4 and 9 years ago. The bacterial and fungal composition of those soils is presented in table 1.

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Table 1. Microbial Presence in Studied Soils.

Sample	Year	Type	Bacterial Content	%	Fungal Content	%
FS	2011		*Stenotrophomonas maltophilia*	58%	*Rhizopus* spp.	N/A
			Gram-positive *Coryneform bacillus*	12%	*Penicillium* spp.	N/A
			Coagulase-negative *Staphylococcus* spp.	9%	Non-sporulating Derm. fungi	N/A
			Acinetobacter spp.	9%		
			Pseudomonas fluorescens	5%		
			Bacillus spp. 1	1%		
			Bacillus spp. 2	1%		
FS	2010		Gram-positive *Coryneform bacillus*	67%	*Hypodiscosia* spp.	88%
			Bacillus spp. 1	13%	Non-sporulating Derm. fungi	10%
			Pantoea spp.	13%	*Penicillium* spp.	1%
			Bacillus spp. 2	7%		
FS	2007	Chestnut	*Sphingomonas paucimobilis*	42%	Non-sporulating hyaline fungi	N/A
			Gram-positive *Coryneform bacillus* 1	15%	*Trichoderma* spp.	N/A
			Pseudomonas fluorescens	15%	*Penicillum* spp.	N/A
			Gram-positive *Coryneform bacillus* 2	12%	*Gliocladium* spp.	N/A
			Bacillus spp. 2	12%		
			Bacillus spp. 1	4%		
FS	2002	Poplar	*Stenotrophomonas maltophilia*	42%	*Fusarium* spp.	N/A
			Bacillus spp. 1	19%	Non-sporulating hyaline fungus	N/A
			Bacillus spp. 2	19%	*Cliocladium* spp.	N/A
			Burkholderia cepacia	19%	*Penicillium* spp.	N/A
FS	2002	RBW	Gram-positive *Coryneform bacillus*	55%	*Penicillium* spp.	N/A
			Bacillus spp. 1	14%	*Trichoderma* spp.	N/A
			Klebsiella pneumniae spp. Ozaenae	14%	*Alternaria* spp.	N/A
			Bacillus spp. 2	9%		
			Pantoea spp.	9%		
	2002	Pussy Willow	*Bacillus* spp. 1	43%	*Aspergillus versicolor*	40%
			Aeromonas spp.	30%	*Penicillium* spp.	33%
			Gram-positive *Coryneform bacillus* 2	19%	*Fusarium* spp.	13%
			Gram-positive *Coryneform bacillus* 1	4%	*Trichoderma* spp.	2%
			Bacillus spp. 2	1%	*Gliocladium* spp.	2%
			Pseudomonas fluorescens	1%		
Top Soil	2011		Gram-positive *Coryneform bacillus* 4	43%	*Penicillium* spp.	64%
			Gram-positive *Coryneform bacillus* 3	33%	*Fusarium* spp.	18%
			Gram-positive *Coryneform bacillus* 2	19%	*Papulaspora* spp.	18%
			Sphingomonas paucimobilis	3%		
			Bacillus spp.	1%		
			Gram-positive *Coryneform bacillus* 1	1%		
Mining Soil	N/A		Gram-positive *Coryneform bacillus* 1	73%	*Fusarium* spp.	63%
			Gram-positive *Coryneform bacillus* 2	12%	*Penicillium* spp.	23%
			Bacillus spp. 1	5%	*Trichoderma* spp.	5%
			Bacillus spp. 4	5%		
			Bacillus spp. 2	2%		
			Bacillus spp. 3	2%		

Fabricated soil, 2011 had 58% of *Stenotrophomonas maltophilia*, 12% of *gram-positive coryneform bacillus*, 9% of both coagulase-negative *Staphylococcus* spp and *Acinetobacter spp*, and 5% of *Pseudomanas fluorences. Bacillus* spp. was at 1%. Fungi population was represented by *Rhizopus* spp., *Penicillium* spp., and non-sporulating dematiaceous fungi. The percentage could not be calculated due to overgrowth of competing fungal flora.

S. maltophilia is the most abundant bacteria present in this sample. It is an aerobic, nonfermentative, Gram-negative bacterium. *S. maltophilia* are slightly smaller (0.7–1.8 × 0.4–0.7 micrometers) than other members of the genus. They are motile due to polar flagella and grow well on MacConkey agar producing pigmented colonies. It is catalase-positive, oxidase-negative (which distinguishes them from most other members of the genus) and positive for extracellular DNase. *S. maltophilia* is ubiquitous in aqueous environments, soil and plants; it has also been used in biotechnological application, Microwiki.

The one year old -2010 fabricated soil sample, had 67% of gram-positive *coryneform bacillus* vs 12% in fresh 2011 sample. It completely inhibited *S. maltophilia* that was present in fresh FS.

Bacillus spp increased to 7-13% vs only 1% in freshly prepared sample. Fungal count contained *Hypodiscosia* spp., *Penicillium* spp., and non-sporulating dematiaceous fungi. *Rhizopus* spp was not present as in fresh sample.

The 4 year old fabricated soil sample with American Chestnut growing on the plot had 42% of *Sphingomonas paucimobilis*. It is a gram-negative rod that exists in environmental niches such as water, including hospital water systems. It is not the part of normal human flora. This organism was not present in any of the recent samples, 2011 or 2010. The rest of the species were: gram-positive *Coryneform bacillus*-12%, *Pseudomanas fluorences*-15%, and different *bacillus* spp, 4-12%. Fungi population was represented by *Penicillium* spp., and non-sporulating hyaline fungi, including *Trichoderma* and *Cliocladium* spp. Total fungal count was 6.3x10.[6]

Fabricated soils manufactured 9 years ago had different types of trees grown on the plots: **Poplar, Red-Branched Willow and Pussy Willow.**

Poplar plot had 42% of *S. maltophilia*, 19% of *bacillus* spp. and *Burkholderia cepacia*-19%. *B. cepacia complex* (BCC) is a new group of organisms present in the soil sample. BCC is of catalase-producing, non-lactose-fermenting Gram-negative bacteria composed of at least seventeen different species. BCC organisms are typically found in water and soil and can survive for prolonged periods in moist environments. Fungi population was represented by *Fusarium* spp., *Penicillium* spp., and non-sporulating hyaline fungi. Also including *Gliocladium* spp. Total fungal count was 2.83x10.[6]

Red-Branched Willow Plot had gram-positive coryneform *Bacillus*-55%, *Bacillus* spp. 9-14%, *Pantoea* spp, 9% and *Klebsiella pneumonia* spp., *Ozaenae* at 14%. *Pantoea agglomerans* (formerly *Enterobacter agglomerans*) is a gram-negative aerobic bacillus in the family Enterobacteriaceae. All species of the genus *Pantoea* can be isolated from feculent material, plants, and soil, where they can be either pathogens or commensals.

The genus *Klebsiella* belongs to the tribe Klebsiellae, a member of the family Enterobacteriaceae. It is non-motile, rod-shaped, gram-negative bacteria with a prominent polysaccharide capsule. This capsule encases the entire cell surface, accounts for the large appearance of the organism on gram stain, and provides resistance against many host defense mechanisms.

Fungi population was represented by *Penicillium* spp., *Trichoderma* spp, and *Alternaria* spp. Total fungal count was 1.4x10.[6]

Pussy Willow Plot had 43% of *Bacillus* spp, 30% of *Aeromonas* spp, and 19% of gram-positive *coryneform bacillus.* ***Aeromonas*** are gram-negative facultative anaerobes that are straight rods or coccoid cells. They are inhabitants of aquatic ecosystems worldwide. These include groundwater and drinking water at treatment plants and in distribution systems and reservoirs as well as clean or polluted lakes and rivers.

Gram-positive *Coryneform bacillus* – Many species of corynebacteria are part of the normal flora of the skin & mucous membranes in human and mammals. Several species of *corynebacteria* have been found in the inanimate environment (e.g.) dairy products, plants, soil and activated sludge.

Fungi population was represented by *Aspergillus versicolor*-40%, *Penicillium* spp.-33***%***, and *Fusarium* spp.-13%, *Trichoderma* and C*liocladium* spp of 2% each.

Aspergillus versicolor is a widely distributed fungus being detected in very cold regions, unlike most other species of aspergilla which prefer warmer regions. It may be commonly found in soil, hay, cotton, dairy products, dried cereals, nuts, and especially spices. Total fungal count was 0.54x10.[6]

In case of mining soil, the majority of microflora were various *bacillus* spp. and gram-positive *Coryneform bacillus* that were present at 85%. The fungal species had *Fusarium*-63%, *Peniccilium*-23% and *Trichoderma*-5%.

In top soil 2011 the presence of Bacterial spp. was prevalent at 90+%, and only 3% of *S. paucimobilis* present. *S. paucimobilis* is an aerobic Gram-negative soil bacillus that has a single polar flagellum with slow motility.

Fungal content had *Penicillium* spp. at 64% and *Fusarium* spp., 18%. *Papulaspora* spp. also was at 18%.

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In top soil 2011 the presence of bacterial spp was prevalent at 90+%, and only 3% of *S. paucimobilis* present. Fungal content had *Penicillium* spp. at 64% and *Fusarium* spp., 18%. *Papulaspora* spp. also was at 18%.

Janzen *et al*. (2008), described the impact of AMD on diversity of microbial community and stream chemistry in the Shamokin Creek Watershed, PA. It is a well - known fact that diatoms as representatives of biodiversity indicate the health of a particular environment. Bacterial presence also indicates the level of ecological balance. The authors concluded that in AMD where the concentrations of iron are high, the predominant bacteria will be from phylum *Bacteroidetes*, and were closely related to known biofilm community members from acidic environments where they have been demonstrated to be involved in sulfur oxidation. Other bacterial species were closely related to *Sphingomonas* species. Soil ecology damage due to AMD has the potential to have a cascade of negative effects including the loss of vegetation leading to the loss of topsoil due to erosion.

As the environmental damage caused by AMD and surface mining operations increases, additional methods must be developed to repair or replace the topsoil in order to support normal ecological development. As soil is a necessary intermediate substrate in the regulation of the Biosphere activity, it is important to understand the long term effects of microbial change loss and to monitor attempts to amend soils to improve sustainability and viability of the soil.

As the research shown we have been able to maintain a healthy microbial presence in fabricated soils over 9 year period thus contributing to answer whether fabricated soils are a long-term or short-term solution. Future work will compare the mineral content of soil with microbial biomass and diversity.

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Short Communication

FECUNDITY OF *LABEO ROHITA* (TELEOSTEI: CYPRINIDAE) REARED IN EARTHEN POND IN LAHORE

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ABSTRACT

In this study, ripe and mature females *Labeo rohita* (Hamilton) were sampled from Punjab University Research Fish Farms Lahore, in June and July 2009. Mean body weight and mean total length of fish was 559.20±175.31g and 35.04±3.27cm respectively. The mean ovary weight was 88.76±30.71grams. The ovaries were extracted, dried and weighed individually. Mean number of eggs/g ovary and number of eggs/g fish were 891.50±126.32 and 142.47±37.22 respectively. The mean absolute fecundity was 80290±32955 and mean relative fecundity was 142466±37226. These values were higher in June than in July sample. Two ovaries from fishes of same body weight (460g) contained different number of eggs (78876 and 75850). The highest value of gonadosomatic index (22.68) was recorded in June, indicating breeding and spawning period of *L. rohita.* Fish weight was significantly correlated to total length (r = 0.780) and ovary weight (r = 0.710). The relationship between fecundity and fish total length, fish weight, ovary length and ovary weight showed positive linear correlation and the correlation coefficient values for these relationships were 0.543; 0.698; 0.536 and 0.914 respectively. The fecundity of *L. rohita* of present stock was significantly correlated to fish total length and body weight; ovary length and ovary weight. However, the ovary weight is the most reliable and better index of fecundity than total length and weight of the fish.

Keywords: *Labeo rohita,* fecundity, gonadosomatic index, culture condition.

INTRODUCTION

Fecundity is defined as the capacity of an individual fish to produce ripe eggs in one spawning season. This must be known to assess the reproductive and commercial potential of a fish stock (Das *et al*., 1989). For efficient fish culture and effective management it is important to know the fecundity of fish (Mian and Dewan, 1984). Studies on fecundity and its relationship with various body parameters viz. total body weight, total length, ovary length and ovary weight are very useful and important in increasing the fish production, stock management and assessment in any water body (Das *et al*., 1989).

Labeo rohita is a very popular food fish in Pakistan and has high consumers demand, because it is very delicious and nutritious and has high market value. It is extensively cultured in polyculture system under semi intensive conditions. This fish become adult at the age of 1.5 year and attain maturity at the end of 2nd year in ponds (Jhingran, 1986). Data on various aspects of biology and culture of *L. rohita* is available in literature. Several studies have been done on fecundity of warm water fishes by Khan (1972), Sinha (1972), Joshi and Khanna (1980), Nautiyal (1985), Somdutt and Kumar (2004), Joshi (2008), Bahuguna and Khatri (2009), Lone and Hussain (2009), Alam and Phathk (2010), Bhat (2011) and Lone *et al*. (2012).

Lone and Hussain (2009) reported 817094 eggs in 406g ovary of *L. rohita* (2012.25 eggs/g ovary) where the average body weight was 1738.76g. Alam and Pathak (2010) reported mean fecundity as 66823±4312.39 in *L. rohita* (mean body weight 315.64±16.59g). According to Bhat (2011) the correlation coefficient between weight and length of *L. rohita* was 0.98 and suggested that these two variables are highly correlated and significant (P <0.001). Most of these studies on fecundity of *L. rohita* have been done on wild fish populations. A recent study suggests that, the wild *L. rohita* is exhibiting decreasing trend in individual weight and population size in some water bodies in Punjab, Pakistan (Khan *et al*., 2011). It is important to know the expected number of eggs from brood fish, for proper planning to meet the fish seed production targets of hatcheries and nurseries. The aim of present study was to assess fecundity and its relation with various body parameters and calculate gonadosomatic index of *L. rohita* reared in earthen pond under semi intensive culture condition.

MATERIALS AND METHODS

The experimental fish, *L. rohita* was obtained from

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Punjab University Research Fish Farm Lahore in June and July 2009. Fishes were packed in icebox and brought to Fish Disease and Health Management laboratory. They were washed with clean tap water before examination within two hours after arrival in the laboratory. Total length (Tl) and total body weight (Wt) of each fish was recorded in fresh condition to the nearest 0.1cm and 0.1g. The fishes were dissected, ovaries were separated carefully and the moisture was dried with blotting paper. Ovaries were weighed and measured individually. The dry ovaries were preserved in 5% formalin solution for 24 hours (Bahuguna and Khatri, 2009). This helped to separate eggs from walls of ovary easily. Ten samples, of one gram portion of ovary from each lob were weighed on Electronic Scale. The egg samples were placed in Petri dish separately. Small amount of distilled water was added to each Petri dish containing eggs. This procedure hydrates and completely separates the eggs. The total number of eggs in each sample were counted carefully and recorded for further calculations. Absolute fecundity was calculated according to formula by Lone and Hussain (2009);

$$F = n\,G/g$$

F = fecundity; n = mean numbers of eggs in all sample; G=weight of ovary; g=weight of sample. The numbers of eggs/kg body weight of the fish (relative fecundity) and number of egg per fish (absolute fecundity) was also calculated by using simple algebraic formula. Gonadosomatic Index (GSI) was calculated according to formula by Singh and Srivastava (1991).

$$GSI = \text{Gonads weight (g)}/\text{Weight of fish (g)} \times 100.$$

The relationship of fecundity with fish weight, fish total length and ovary weight and ovary length was calculated by regression analysis with computer package Minitab.

RESULTS

Body weight, body length, body width, ovary weight and ovary length of *Labeo rohita*

In this study, 30 female *L. rohita* were studied. The mean body weight of fish was 559.20 ± 175.51g (Table 1). In June the ovaries were large and fully developed. In July the mean body weight was low, which is attributed to regression of the ovaries. The mean total body length was 35.040 ±3.272 cm, whereas, the mean standard length and mean width was 27.085±2.765cm and 8.7532±1.008cm respectively. The mean ovary weight was 88.767±30.71g. In June this value was 97.80 ± 32.759g and in July it was 79.733±26.58g. The higher ovary weight in June indicates the development of ovaries towards the peak breeding period of the fish. The mean ovary length was 16.138±1.504cm which was comparable to June value (16.917±1.421cm) but it was less in July 15.358±1.668cm (Table 1).

Gonadsomatic Index (GSI) and Fecundity of *Labeo rohita*

The mean gonadosomatic index was 15.940±3.33. GSI was higher in June (16.117±4.24) but low in July (15.762±2.22) (Table 1). The highest individual GSI value 22.68 was observed in June, which again points to peak breeding period of *L. rohita.* The number of eggs/g ovary was 891.50±126.32 and number of eggs/ g fish was 142.47±37.226. Absolute fecundity was 80290±32955 and relative fecundity was 142466±37226. Number of eggs/kg ovary, were assessed as 891500±126319 (Table 1). In June sample the number of eggs/g ovary was 965.40 ± 60.829; absolute fecundity 94316±33077 and relative fecundity 152087±22908. The number of eggs varied from 38584 (for 379.3g fish) to 180224 (for 1165.5g fish)

Table 1. Body parameters and fecundity of female *Labeo rohita.*

Month/sample size	June 2009 (n=15)		July 2009 (n=15)		Total
Parameters	Mean ± sd	Range	Mean ± sd	Range	Mean± sd
Body weight (g)	619.53±188.8	421.3-1165.5	498.87±142.15	379.3-942.0	559.20±175.51
Total length (cm)	36.847±2.921	33.9-43.0	33.23±2.579	29.9-40.2	35.040±3.272
*Sd. length (cm)	29.640±2.372	27.1 – 35.0	26.200±3.022	17.9-32.1	27.085±2.765
Body width (cm)	9.0867±1.109	7.5-11.0	8.4200±0.906	7.1-10.6	8.7532±1.008
Ovary weight (g)	97.800±32.759	66.6-176.6	79.733±26.58	56.2-120.4	88.767±30.71
Ovary length (cm)	16.917±1.4217	9.4 – 12.75	15.358±1.668	15.16-19.72	16.138±1.047
GSI	16.117±4.24	12.41-22.68	15.762±2.22	8.97-19.43	15.940±3.33
Eggs/g. ovary	965.40±60.829	830-1084	817.60±132.85	651-1009	891.50±126.32
Absolute fecundity	94316±33077	56724-180224	66265±27093	38584-117519	80290±32955
Eggs /g. fish	152.09±22.90	114.8-192.72	132.84±46.30	64.95-249.06	142.47±37.226
Relative fecundity	152087±22908	114860-192720	132845±46340	64950-249064	142466±37466
Eggs/kg ovary	965400±60829	830000-1084000	817600±132853	651000-1009000	891500±126319

*Sd. length. Standard length

per fish. However, in July all these fecundity values were less than June (Table 1). Interestingly, ovaries of two fishes with same body weight (460g), contained different number of eggs (78876 and 75850).

The relationships between various body parameters:

The relationship between fish body weight (Wt) and fish total length (Tl) can be expressed as:

$$Wt = -1112.1+4.77\ Tl\ (r=0.780)$$

The body weight of the fish was directly proportion to the fish length. The regression equation is linear. The correlation coefficient (0.780) correspond to significant positive correlation (P=0.000).

The relationship between ovary weight (OWt) and fish body weight (Wt) can be expressed as:

$$OWt = 6.2 +0.148Wt\ (r=0.710).$$

The ovary weight was directly proportion to the fish weight. The regression equation is linear and correlation coefficient (0.710) correspond to a positive significant correlation (P=0.000).

Relationship between fecundity (F) and fish total length and fish body weight

The relationship between fecundity and total length of fish can be expressed as:

$$F = -179668 +7419\ Tl\ (r = 0.543).$$

The fecundity of the fish was directly proportion to the total length of fish. The regression equation is linear and the correlation coefficient (0.543) indicate moderate positive significant correlation (P=0.000).

The relationship between fecundity and fish body weight can be expressed as:

$$F = -10735+158\ Wt\ (r = 0.698).$$

Fecundity is directly proportional to the fish body weight. The relationship between fecundity and fish body weight is linear and significant with 0.698 correlation coefficient (P=0.000).

The Relationship between fecundity and ovary length (Ol) and ovary weight (OWt)

The relationship between fecundity and ovary length can be expressed as:

$$F = -178454+16034\ Ol\ (r = 0.536).$$

A significant relationship between fecundity and the ovary length exist. This relationship is linear and significant with 'r' value 0.536 (P= 0.000).

The relationship between fecundity and ovary weight can be expressed as:

$$F = -5762+961\ OWt\ (r= 0.914).$$

A significant relationship between fecundity and the ovary weight exist. Fecundity is directly proportional to ovary weight. This relationship is linear and significant (r=0.914) (P= 0.000).

DISCUSSION

The correlation coefficient values between fish body weight and total length of *L. rohita* and ovary weight and fish body weight indicated that the fish is well maintained. Fecundity in *L. rohita* has been found to increase with increasing fish length, fish weight as well as ovary weight (Table 1). In the present study 965.40±60.829 eggs/g ovary and 152.09 ± 22.90 eggs/g fish were observed. Khan (1972) reported 1335 egg/g ovary and 535 eggs/g fish in two years old fish. Khan and Jhingran (1975) observed 1230 eggs/g ovary and 211 eggs/g fish. Jain and Mitra (1994) reported 307±29 eggs/g fish. Lone and Hussain (2009) observed 2012.55 eggs/g ovary and 469.93 eggs/g fish. The difference of fecundity observed by Lone and Hussain (2009) and in the present study may be associated with difference in mean body weight of fish in two studies. This difference in fecundity may also be attributed to the level of fish pond management at two sites. On the other hand, fish in pond show low fecundity and fail to spawn due to the stress of captivity, insufficient food and higher stocking density as stated by Billard (1995). It is reasonable to conclude that *L. rohita* reared under culture condition show variable fecundity as compared to natural population.

The ovaries of two fish with same body weight, contained different number of eggs. Alam and Pathak (2010) also reported that two same size *L. rohita*, contained different number of eggs. If these two studies are considered carefully, it can be concluded that *L. rohita* can produce eggs at a small size (Wt= 60g, OWt 2.4g and fecundity 25230, Alam and Pathak, (2010); and Wt=379g, OWt= 34g, fecundity 24616 present study). This variable fecundity may also be associated with genetic diversity in *L. rohita*, indicating that different strains mature and spawn at various body weight and size in its geographical range and is influenced by ecological factors. Lone and Hussain (2009) reported that, in fishes like *L. rohita*, water temperature, photoperiod and rainfall appear to affect growth and development of ovary.

GSI increases with maturation of fish and is highest during spawning season and after spawning it decline (Lone and Hussain, 2009; Alam and Pathak, 2010). Another study, Lone and Hussain (2009) reported maximum GSI value (22.73±0.94) in June in *L. rohita*. Alam and Pathak (2010) observed the highest GSI value 7.5 in August in *L. rohita.* In the present study the highest GSI value (22.68) was recorded in June. The variation in GSI values in *L. rohita* in these studies may be due to fish body weight and ovarian weight in respective fish.

Photoperiod and water temperature have been shown to correlate with gonadal weight and gonadosomatic index, and water temperature and long day length influence beginning and conclusion of spawning season in fish like major carps (Day *et al.*, 2004, 2005; Bhattacharyya and Maitra, 2006, Mylonas and Zohar, 2007).

Linear relationships exist between fecundity and fish length, fish weight, ovary length and ovary weight of *L. rohita*. Similar linear relationships have been reported in different freshwater fish species by various workers; Singh and Srivastava (1982), Sharaf *et al.* (1997), Somdutt and Kumar (2004), Joshi (2008) and Bahuguna and Khatri (2009). Fecundity of *L. rohita* was more closely related to the ovary weight (Alam and Pathak, 2010). Fecundity and ovary weight of *L. rohita* was strongly correlated than to weight of fish and length of fish and length of ovary as observed in this study. Hence, it is fair to conclude that ovary weight is a better index to estimate fecundity than total length and body weight. When fishes are kept in captivity for culture purpose they show some degree of reproductive dysfunction (Lone and Hussain, 2009) which may be due to low quality feed given to fish. Hence, the necessity of incorporating an optimum level of animal protein (up to 35%) in the diet of a fish *Beta splendens* (Regan) for maximum reproductive performance has been stressed by James and Sampath (2003). But one thing is very clear that with the onset of maturity, *L. rohita* with highest body weight and body length contain maximum number of eggs as observed in present study. In addition to this, ovary weight is the most reliable and the best index of fecundity than total length and weight of the fish. It is suggested that genetically more diverse brood stock may be used to produce good and high quality fish seed.

CONCLUSION

The present study has directed our attention to a point that the fish examined probably belonged to more than one genetic strains; where one strain matured at small size (<500g) and second strain matured at larger size (<1000g). This needs to be investigated.

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POTENTIAL ENVIRONMENTAL EFFECTS LINKED TO ELEMENTAL TOXICITY OF NEEM BIODIESEL AND ALTERNATIVE FUELS (B20/B100)

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ABSTRACT

Neem biodiesel is being developed as a future biofuel. Accompanying the growing global production of biofuels is the looming threat of environmental pollution. Such pollution can originate in two ways: firstly, combustion of biofuels containing elevated levels of toxic elements could contribute to atmospheric pollution; and secondly, poor quality biofuel (and waste products) that is returned to the environment could contaminate soil and aquatic resources. In this study the potential environmental toxicity of neem biodiesel was examined and compared with alternative fuels (B20/B100) using high performance ICP-MS. The neem biodiesel was prepared in our laboratory from neem feedstock (kernels and fruit) harvested in 2012. A dual acid-base catalyzed esterification process was employed to produce the biodiesel fraction. Prior to high resolution instrumental analysis all samples were digested in mild acidic media. The basic physical properties of the neem biodiesel were in agreement with those of regular petroleum diesel. Twelve elements (ranging from beryllium to uranium) considered to be environmentally toxic were detected in the neem biofuel and their levels displayed lower profiles of elemental toxicity compared to the B100 samples. B20, on the other hand, displayed reduced levels of toxicity in general. The study is of particular interest to environmental toxicology and sustainable development.

Keywords: ICP-MS, B20, B100, neem biodiesel, trace/toxic metals.

INTRODUCTION

Neem biodiesel is emerging as an alternative fuel (Pillay *et al*., 2012; Sekhar *et al*., 2009) and its characteristic toxic properties are relatively unexplored. In view of this, its elemental toxicity was studied instrumentally and compared with standard biofuels (B20 & B100). The rationale behind studying the toxicity of biofuels is their rising potential impact on the environment. A survey of the relevant literature revealed that, in general, toxic metal studies of biofuels have not been widely considered, and our research focused on this area of interest with particular emphasis on the comparative environmental effects of neem biodiesel. Recent media reports (Gonzales, 2013; Rubens, 2008) proclaim that dumping of poor quality biofuel could contaminate water supplies and create serious environmental pollution. With the growing production of biofuels, potential pollution of the environment by such alternative fuels has also grown and could lead to widespread organic and inorganic (toxic metal) pollution. Poor quality biodiesel from aborted biofuel processes and the accompanying waste products are often replete with noxious trace elements originating mainly from the soil and water used to cultivate the original biomass. These waste products include the wash water, organic material and catalysts. How are these unwanted substances disposed of? In some cases they are poured down the drain. In other cases they are returned to the environment, into landfills and other dumping sites. This creates a potential threat to the environment simply because their toxic elemental content could be transmitted to the soil and ultimately to the water table. It is well known that biodiesel can be derived from a variety of animal and vegetable oils (Sekhar *et al*., 2009). The biodiesels derived from different plant oils will have slightly different toxic metal contents due to the variation of cultivation methods, soil conditions, weather, plant parts used and processing technologies. A common method to generate biodiesel involves the transesterification of the triglycerides with the help of a catalyst to produce alkyl monoesters of chained fatty acids that have comparable properties to that of conventional diesel (Lin *et al*., 2009; Kalam, 2002; Muthu *et al*., 2010; Goering *et al*., 1982). Glycerol becomes a by-product of this chemical reaction that must be removed by separation processes (Schuchardta *et al*., 1998; Singh, 2010). Toxic and heavy elements are present in the biofuel, wash water and by-product. We found in an earlier study that retention of trace elements in the biodiesel fraction is appreciable (Pillay *et al*., 2012). Therefore, elemental analysis of the biodiesel component distinctly reflects the significant toxic metal content of the process.

Our biofuel was derived from indigenous non-edible terrestrial feedstock available in the region. The neem tree is prolific in the UAE and its fruit and seeds compared to most non-edible plant species have a higher concentration of oil (30% oil content), which is a major source of neem oil generally used as insecticides, lubricants and in

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medicines to treat various disorders. In this study toxic elements in neem biodiesel were measured by ICP-MS (inductively coupled plasma mass spectrometry) and compared with some commercial biofuels. ICP-MS has overtaken most modern analytical techniques for attaining ultra-low limits of detection (Ammann, 2007) especially for heavy metals, including the lanthanides and actinides. The multi-elemental capability of this hyphenated method to determine elemental levels in the ppm (mg/L), ppb (µg/L) and ppt range (ng/L) has certainly proved to be superior to contemporary methods such as neutron activation and ICP-OES.

The aim of this paper, therefore, was threefold: (i) to employ a high-performance instrument to characterize selected toxic elements in neem biodiesel (prepared in our laboratory); (ii) to compare our results with standard biofuels: B20; B100C [consumer grade]; and B100R [refinery grade]; and (iii) to evaluate the potential impact on the environment.

MATERIALS AND METHODS

Neem biodiesel preparation

Samples of neem fruit and seeds were harvested (in 2012) locally from surrounding areas and stored in a cool dry place. The vegetable oil was extracted in a blender with normal hexane. To prepare the biodiesel the transesterification process was applied with the help of a catalyst to generate a product with comparable properties to that of conventional diesel (Pillay *et al*., 2012). This transesterification process generally requires the use of methanol in a basic solution such as potassium or sodium hydroxide to produce the monoester and glycerol (by-product). However, the neem biofuel cannot be directly obtained in a one-step process because of the high content of free fatty acids (FFA) in the vegetable oil (Sekhar *et al*., 2009). Under basic conditions soap is produced and this reduces the yield of the reaction and also consumes excessive alkali. This also leads to a slower reaction time and the risk of incomplete conversion. Therefore, pre-treatment with methanol under acidic conditions is necessary to reduce the amount of FFA by converting them to fatty acid methyl esters (FAME). A dual process: acid-catalysed pre-treatment and base-catalysed transesterfication was thus necessary (Sekhar *et al*., 2009). The deprotonation step and catalyst restoration mechanism in the final stage are shown in figure 1.

Hyphenated ICP mass spectrometry

Samples (200µL) were digested in HNO_3/HF, 4:1 v/v in an industrial grade microwave oven and subsequently diluted and submitted for analysis using a Perkin Elmer SCIEX DRC-e ICP-MS (Fig. 2). The nebuliser gas flow in the instrument was 0.80 L/min. The analyte solution was aspirated into the instrument, converted by the nebuliser into a fine spray and mobilised to a plasma source where it was atomized and converted to ions, which were characteristic of the elements of the sample. These ions were subsequently transported to a mass spectrometer for detection. The technique is ultra-sensitive and can achieve limits of detection in the region of ng/L for most elements. The neem biodiesel and three standard biofuels (B20; biodiesel 100 consumer grade [B100C]; and biodiesel 100 refinery grade [B100R]) were prepared under identical conditions. The instrument was standardised with certified standards and a suitable internal standard was used to compensate for the possible drift in instrument measurements (Ammann, 2007). B20 biodiesel is a blend of 20% biodiesel and 80% petroleum diesel; and B100 is biodiesel in the neat form.

Instrumental performance

Prior to each run, the instrument was conditioned for linear calibration and background correction. An aqueous certified standard (Fluka 70007; 10.00µg/L per element) was employed to evaluate the performance of the instrument on homogeneous aqueous solutions. The sensitivity of the Perkin Elmer ICP-MS is linear over several orders of magnitude for aqueous samples, and can cover a wide range of concentrations in a single measurement. A measure of the repeatability in terms of the relative standard deviation (RSD) was computed and, in general, values less than 5% were attained demonstrating that the precision of the system for aqueous samples was satisfactory (Table 1).

RESULTS AND DISCUSSION

Elemental toxicity

Pollution of the environment by toxic elements in one form or the other has been the subject of extensive research. The properties of the biodiesel produced relies on the biomass used (Singh, 2010). The properties of the vegetable oil generated from the same plant species may fluctuate depending on fertilizers, soil conditions, weather, plant parts used and processing technologies. As

Table 1. Instrumental precision (µg/L) using a multielemental aqueous standard (Fluka 70007).

Measurement	Be	Co	In	Pb	Bi
1	10.7	9.66	9.98	10.3	9.62
2	11.6	9.87	9.68	10.2	9.63
3	10.4	9.74	9.94	10.7	10.1
Mean ± RSD	10.9 ± 5.7%	9.76 ± 1.1%	9.87 ± 1.7%	10.4 ± 2.5%	9.8± 2.8%

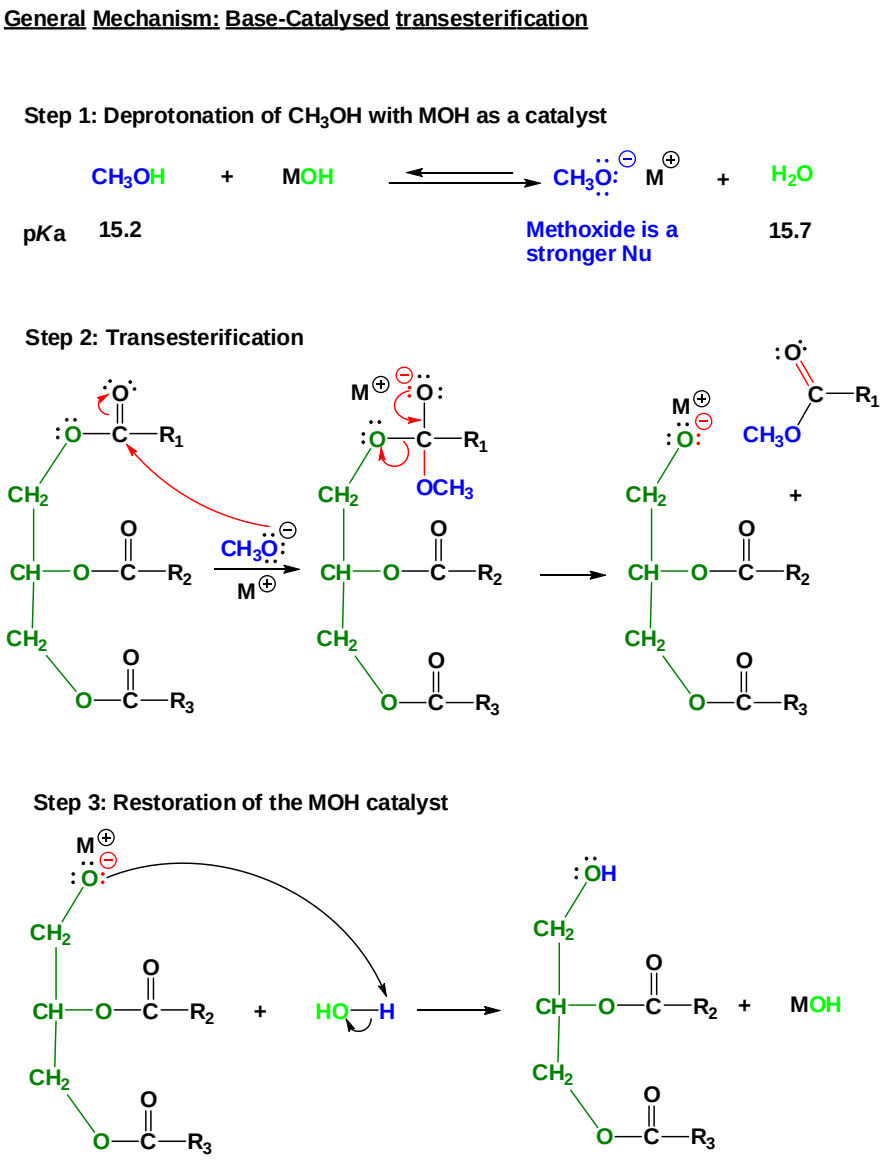

Fig. 1. Base catalysed mechanism showing deprotonation and transesterification steps.

aforementioned, the trace elements detected in neem biodiesel were studied against B20/B100 standard biofuels to compare levels of toxicity. The concentrations of neem biodiesel were also compared with drinking water standards to highlight the potential threat to the environment especially if discarded quantities of biodiesel find their way to drinking water supplies. The toxic elements investigated in this work have a maximum admissible drinking water level in the sub-ppm range, and many of them possess limits between 1-10µg/L (Kumar, 1994). Permissible atmospheric limits are within 10ng/m^3 (Kumar, 1994). Figures 3-5 display elemental trends (in the form of bar graphs) for the samples of interest. For convenience these elements are classified into three separate groups. The capability of ICP-MS for detecting 'exotic' toxic elements such as Be, Tl, Bi, Th and U is superior to other modern instrumental techniques. It is quite evident from the profiles shown in figures 3-5 (depicted in 3-D) that the range of elemental concentrations is fairly wide clearly depicting the comparative levels of toxicity, which are discussed below.

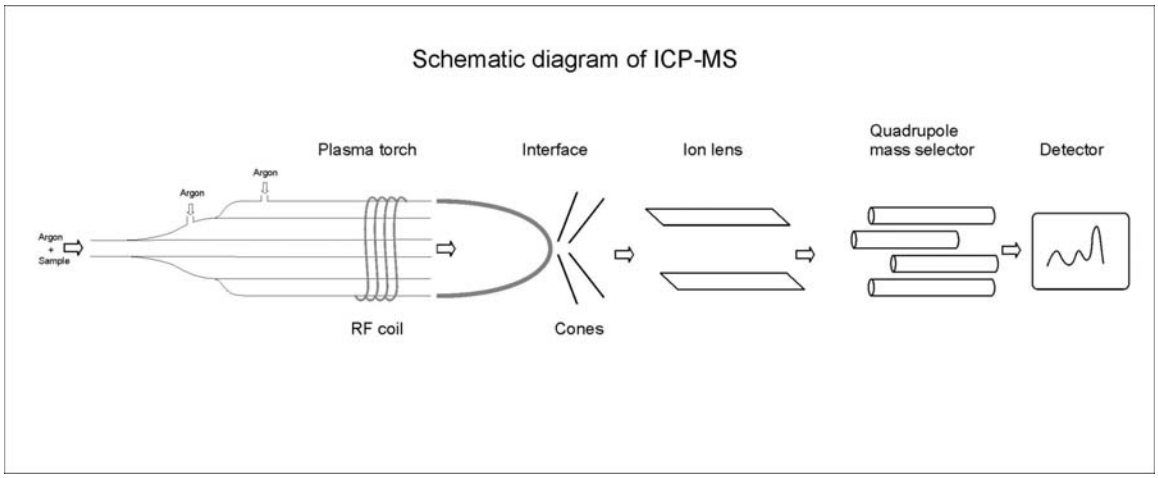

Fig. 2. A schematic of the ICP-MS instrument.

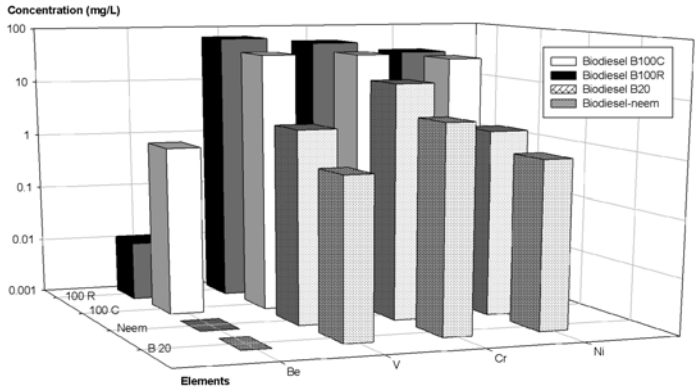

Fig. 3. Profiles for first group of elements in B20/B100C/B100R and neem biodiesel.

Characterisation study

Representative/transition metals: Figure 3 shows that a distinct pattern exists for B100C/B100R but similar trends are not apparent for B20 and neem biodiesel. In the case of Be, no detectable levels were observed for the B20, B100R and neem samples. The toxic effects of Be are still under considerable clinical research and it is known that elevated levels of Be in humans could lead to beryllosis and pulmonary disorders, including lung cancer (Kumar, 1994). The Be concentration in B100C was ~1mg/L, which was considered to be unusual and attributed to possible extraneous sources during the production of B100C. Vanadium, on the other hand, appeared in all samples. It is generally present in humans at ultra-trace levels (ng/L) and higher levels tend to affect the respiratory, circulatory and digestive organs. The levels in B100C/B100R were ~ 40 and ~ 66mg/L, respectively. Neem biodiesel and B20 displayed much lower levels: ~3 and ~1 mg/L, respectively. The elevated levels in B100C/B100R cannot be readily explained and could clearly create atmospheric pollution from combustion of these biofuels. Although the level of V in neem biodiesel is more than ten times lower than in B100C/B100R it is probably high enough to necessitate demetallisation procedures. Permissible drinking water levels of V are in the region of 15 µg/L so contamination from dumped biodiesel could have an impact on the environment. As regards chromium, the B100C/B100R levels were ~39 and ~53mg/L, respectively. B20 produced a level of ~5mg/L; and neem biodiesel ~15 mg/L. Here again the lowest levels were observed with B20. Compared to B100C/B100R the neem concentration was a factor of 2-3 lower, which could be explained by considering the differing factors associated with growth, cultivation and production of the feedstock related to these products. Chromium toxicity is usually linked to its oxidation states Cr^{3+} and Cr^{6+}, of which Cr^{6+} is considered to be more hazardous to human health. Speciation in neem biodiesel and commercial biofuels is a subject of future study but knowledge of the total levels of Cr gives an overall picture of the extent of its toxicity. In drinking water Cr permissible levels are <0.10mg/L. The detected levels reflected in figure 3 are much higher than this indicating that if these alternative fuels found a pathway to drinking water sources a hazardous situation would undoubtedly be created. The threshold level of atmospheric Cr is

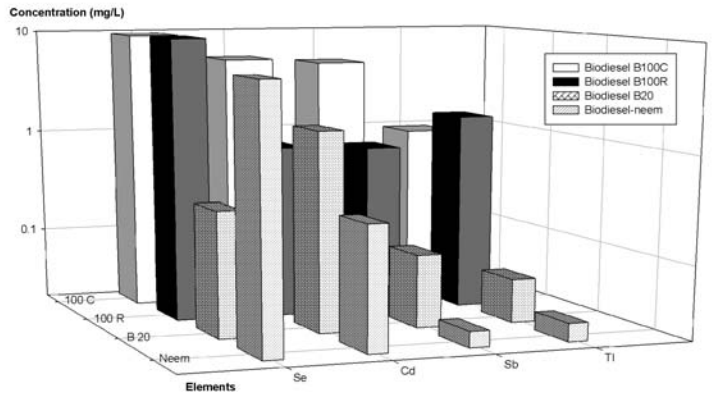

Fig. 4. Profiles for second group of elements in B20/B100C /B100R and neem biodiesel.

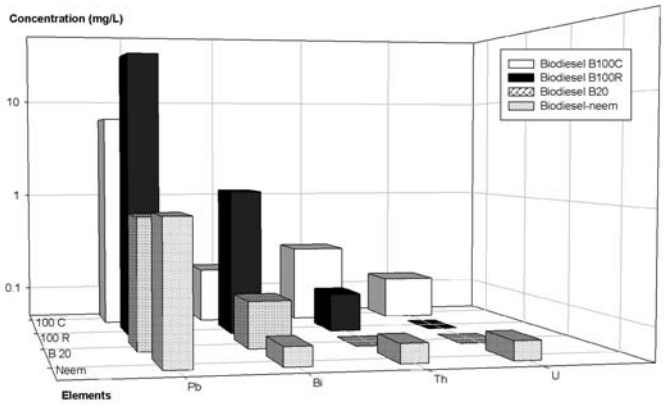

Fig. 5. Profiles for third group of elements in B20/B100C/B100R and neem biodiesel.

<0.5ng/m^3 purporting that combustion of the biofuels shown here could be a distinct threat to the environment. Nickel is another first-row transition metal that could lead to dermal and respiratory disorders in humans at elevated levels (Kumar, 1994). The threshold level of Ni in ambient air is 10ng/m^3. The admissible level of Ni in potable water is 20µg/L. Again we find that B100C/B100R display relatively elevated concentrations (~31 and ~34mg/L, respectively) compared to B20 (~1mg/L) and neem (~2 mg/L). Clearly the pronounced levels in B100C/B100R reflect the conditions and plant material from which they were derived and blended. As in the case of V, demetallisation processes to deplete Cr and Ni would be expedient prior to extensive use of these biofuels on machinery and in vehicles.

Intermediate/heavy elements: the concentrations of Se, Cd, Sb and Tl are shown in figure 4 for the biofuels of interest. A clear pattern of declining levels is seen for Se (B100C<B100R<neem<B20), but not for the other elements in this batch. The health effects of Se are still under wide clinical consideration. Its ingestion causes renal, cardiovascular and respiratory disorders (Kumar, 1994). The admissible level of Se in potable water is 10 ug/L; and its atmospheric limit is <1g/m^3. The experimentally recorded levels for Se for B100C/B100R were ~10mg/L in both cases. The neem biodiesel sample produced a concentration of ~5mg/L, and the B20 sample, ~ 0.5mg/L. Apart from the B20 sample the rest are considerably elevated when compared to the permissible drinking water level and if biofuels of this nature are returned to the environment it could penetrate the water table and affect aquatic resources. Unlike Se, cadmium is a common toxic metal. Its biological effects are well known. High levels of Cd can cause bone disease and kidney problems. In drinking water the maximum limit is 5ug/L. The levels of Cd detected in our experiments were: B100C/B100R: ~5 and ~1mg/L, respectively; B20: ~2mg/L; and neem: <0.5mg/L. Surprisingly, the B20 level for Cd is within the range of the B100C/B100R levels. It is possible that Cd could have been introduced into these biofuels via the blending and refining processes. Nevertheless, these levels in B20/B100C/B100R are far too elevated to be considered safe and use of these

biofuels could result in a potential hazard to the environment from either combustion or disposal. Antimony is known to be carcinogenic and elevated levels in the air and water could pose an environmental threat. The maximum permissible level in drinking water is 6µg/L (Kumar, 1994). The levels detected in our biofuels and neem biodiesel were far higher than this. The reported levels for the B100C/B100R are ~5mg/L and ~1 mg/L, respectively. The B20 and neem biodiesel samples produced results that were much diminished at <0.5mg/L. Thallium pollution is not common, and the fact that it exists at appreciable levels in the biofuels analysed indicates that it could be present in the feedstock itself. Thallium itself is a notable poison, and is seldom detected because of its highly diminished levels. Its maximum permissible dose in drinking water is 2µg/L and health effects associated with elevated Tl levels could be linked to renal and liver disorders. In our study Tl concentrations were ~1mg/L for B100C and B100R (comparatively high); and <0.05 mg/L for B20 and neem.

Post-transition metals/actinides: in figure 5 Pb is most pronounced and probably originated from the feedstock and processing methods. On the other hand, bismuth, thorium and uranium are rare toxic metals often escaping detection largely because their ultra-low concentrations in typical environmental samples are not within the reach of most contemporary analytical techniques. The clinical effects of these three metals (Bi, Th, U) are relatively underexplored and are the subject of extended research in toxicology. Bismuth poisoning constitutes ingestion of elevated levels of the metal in chemical form and could lead to multiple disorders including cardiovascular and respiratory problems (Kumar, 1994). The admissible level of Bi in potable water is 4µg/L. Figure 5 shows that all samples, B20/B100C/B100R/neem, reflect comparatively higher levels to within 1mg/L. Elevated levels of thorium and uranium levels in the human body are not desirable and could lead to multiple organ failure. B100C showed the highest level of Th and U at ~270 ~130ng/L, respectively. The sources of these elements are probably the soil and water used to cultivate the plants from which the biofuels are derived.

Potential environmental effects

Our research could provide useful information on the potential detrimental impact of these noxious elements on the environment. With the growing need for the development of alternative energy sources the study is of interest to sustainable living. The presence of abnormal levels of toxic elements in biofuels could create unwanted hazards especially if pathways exist for pollution of the biosphere and lithosphere. From this perspective the disposal of appreciable quantities of sub-standard biodiesel and waste products (glycerol) in landfills could lead to significant contamination of the water table. In arid countries this would be highly deleterious to aquatic resources because of the dependence of overhead streams and borehole water for watering livestock and arable areas. Due to the relatively slow movement of groundwater, contaminants from waste products could build-up, and thus pose a looming threat to ecosystems (Pillay *et al*., 2010). Atmospheric pollution is another potential hazard and combustion of biodiesel laden with undesirable trace metals is a daunting prospect. Of significance is that our research highlights the looming threat to the environment, especially if unwanted heavy metals from engine exhaust fumes pervade the atmosphere; and poor quality biofuel is discarded. Hence, the impact on the environment is a distinct cause for concern, and our work could make a useful contribution to continuing sustainability. With reference to the elements detected in this study, the source of Be is a matter of speculation and it can only be surmised that this element was present in the plant material from which the biodiesel was derived. The other toxic elements appearing in figure 3 (V, Cr, Ni) could have either been present in the feedstock or introduced via the relevant chemical processes (or both). All the four metals reflected in figure 3 possess their own particular toxic effects on the human body and could undoubtedly pose a threat if they infiltrated water supplies. Proceeding to figure 4 we find that the trend is not consistent and the highest concentration was observed for Se particularly in B100C/B100R and neem. The remaining metals (in Fig. 4), Cd, Sb and Tl also occur at appreciable levels and, here again, it is of interest to have some knowledge of their origins in biodiesel. Thallium, especially, is an "exotic" element escaping detection with less sensitive instruments and responsible for fatal disorders at elevated levels. It is unlikely to encounter Tl in the blending and refining processes leading to the inference that it could only occur in the original biomass. Lead (Fig. 5) is most pronounced and could commonly arise from numerous natural and technological sources. Bismuth on the other hand is uncommon and is of interest because Bi pollution is rare. It occurs at a maximum level of ~1mg/L and its effect on the environment and the human body is the subject of ongoing research. The actinides Th and U are equally exotic and occur at such low sensitivities in environmental samples that only the most sophisticated instruments can detect them. Largely because of this, their toxicology is not well defined and necessitates more profound clinical and environmental studies to obtain an insight into their roles in the human body and the biosphere.

CONCLUSIONS

Our work is of interest from the perspective of environmental pollution originating from discarded biofuels and accompanying waste products. The study attracts attention because the production of neem biodiesel is expected to increase, and it can be linked to

sustainable development. We found that the levels of several toxic elements in the samples investigated were appreciable and could cause serious contamination of drinking water supplies if dumped biodiesel pervaded such resources. In light of this possibility remedial measures to pre-empt such a prospect should be considered. One such measure to reduce trace metals in neem biodiesel is demetallisation. And to safeguard dumped waste biofuel, it would be practical to immobilize the waste biofuel by converting it to sludge (for example) using sand, gravel and waste oily sludge, and subsequently constructing solid blocks for storage in sub-surface caverns. This operation may be relatively inexpensive, because sand and oily sludge are plentiful especially in desert regions and any potential threat to the environment is thwarted.

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DATA-BASED MECHANISTIC MODELLING OF RAINFALL TO RIVERFLOW OF LARGE NESTED TROPICAL RAINFOREST CATCHMENTS IN GHANA

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ABSTRACT

Within the Data Based Mechanistic (DBM) Transfer Function rainfall to riverflow modelling approach a mathematical model in the form of a transfer function rainfall to riverflow is obtained by extracting information from the available time series data. The DBM methodology is able to use the data to identify the model structure in an objective statistical manner using the simplified recursive instrumental variable algorithm (SRIV). The approach requires few spatially-distributed data for the estimation of the models and is, therefore, suitable for data limited regions like West Africa. Within this paper we present a review of the application of the model in hydrological studies in different climatic conditions. The application of the approach to large nested catchments in the humid rainforest zone in Ghana have also been presented. The approach revealed an exponential form of non-linear behaviour for the catchments. The estimated model parameters and the associated dynamic response characteristics (DRCs) of time constant (TC) and steady state gain (SSG) indicates that riverflow generation within the catchments are not flashy. The model identified mathematical relationships which could be used to simulate flows in the catchments.

Keywords: Ghana, dbm model, rain forest, transfer function.

INTRODUCTION

Rainfall-riverflow modelling provides the means, for the investigation of the interaction between climate and riverflow. Understanding the dynamic link between rainfall and riverflow response can also give greater understanding of rainfall-riverflow processes through hydrologic interpretation of the Dynamic Response Characteristics (DRCs) of a catchment. This requires the use of extensive historical records which are generally lacking in Africa, as highlighted by Giles (2005) and Weston and Steven (2005).

In West Africa, the dearth of meteorological data is very common, as pointed out by van de Giessen *et al*. (2002). In Ghana, the situation is not far different from other African countries; hydrological and meteorological data of the country are inadequate and of poor quality, with the exception of a few stations (Adiku *et al*., 1997). Generally, rainfall and riverflow stations in the tropics are of low density and in some areas of interest; the requisite data is simply not available (Douglas, 1999). Some of the recording instruments are no more in existence, while the existing ones are deteriorating. This calls for the use of models that can handle few data inputs, and quantify the effects of sometimes poor data quality on model structures and parameters to be interpreted (Young *et al*., 1999; Young, 2001; Chappell *et al*., 2006). One of such approaches is the relatively new Data-Based Mechanistic (DBM) modelling routines (Young and Minchin, 1991; Young and Lees, 1993; Young and Beven, 1994; Chappell *et al*., 1999; Lees, 2000).

The Data-Based Mechanistic (DBM) modelling approach (Young and Lees, 1993; Young and Beven, 1994; Young, 2001; Chappell *et al*., 1999) involves three steps, which are a) extraction of information from the rainfall and riverflow records by fitting models to the data, b) identification of a range of transfer function models and their associated hydrological system parameters using objective statistical tests and c) selection of the model with the most plausible physical/hydrological explanation of the data. Unlike physics-based and conceptual modelling approaches, it is based on the concept whereby the data is allowed to suggest the type of model which is compatible with the input and output data in a stochastic manner. The mechanistic nature of the final stage of the approach allows the physical interpretation of the resulting model.

The DBM Transfer Function (TF) modelling approach is one of the routines within the DBM-CAPTAIN package (Taylor *et al*., 2007) used in hydrological modelling (e.g. see: Young and Beven, 1994; Young *et al*., 1997; Lees,

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2000; Mwakalila *et al*., 2001). The modelling routine is capable of revealing possible hydrological pathways within a catchment (Chappell *et al*., 1999; Young, 2001; Vongtanaboon and Chappell, 2004) and estimates the parameters or DRCs associated with the different flow pathways (Young *et al*., 1997; Chappell *et al*., 1999).

Within the DBM transfer function rainfall to riverflow modelling approach, an optimal mathematical relationship relating rainfall (input) to riverflow (output) in the form of transfer functions with its associated parameters is obtained. Generally, the identified model structure includes nonlinear components due to the effects of nonlinearity as a result of antecedent moisture in the sub-surface. The optimal model is selected from a range of model structures using objective statistical tests and the model structure's consistency with physical/hydrological theory. One of the major advantages of this modelling procedure over conceptual hydrological models is that the structure is objectively identified as part of the modelling procedure. The DBM model is robust and parsimonious as compared to the traditional modelling approaches (e.g. physics-based models), as it minimises the number of parameters while producing models with a high simulation efficiency (Chappell *et al*., 2006). It is, therefore, suitable for data limited region such as Africa in general and Ghana in particular.

The DBM concept has been applied successfully in rainfall-riverflow modelling (e.g. see: Young, 1993, 2001; Young and Beven, 1994; Young *et al*., 1997; Young, 1998; Beven, 2001; Lees, 2000) and flood forecasting (Lees *et al*., 1994; Lees, 2000; Young, 2002, 2006) in humid temperate conditions. In the tropics, Chappell *et al*. (1999, 2004a, 2004b, 2006) report of the application of the approach to the short-term behaviour of the rainfall-riverflow system and rainfall-suspended sediment system of the 0.44 km^2 Baru catchment in Borneo, Malaysia. The model has a 5 minutes resolution and explained 80% and 90% of the variance, respectively. In Thailand, the approach has been applied successfully to model rainfall and riverflow behaviour in large rainforest catchments (Vongtanaboon, 2004; Vongtanaboon and Chappell, 2004). Vongtanaboon and Chappell (2004) report that in the North Western Thailand, within the Mae Chaem catchment (3853 km^2), the output of the DBM model suggests that 97% of water flow to the river travels along with relatively little storage (time constant of 1.2 days). Again within Thailand, recently Vongtanaboon *et al*. (2008) have utilised the technique to model a large monsoon dominated catchment. Chappell *et al*. (2006) have applied the model to simulate the sensitivity of streamflow behaviour to different densities of skidder vehicle trails within a managed rainforest in Borneo, Malaysia.

In reservoir sedimentation analysis, the methodology has been applied successfully by Price *et al*. (2000) and Rowan *et al*. (2001). The maiden application of the model in Africa has been reported by Mwakalila *et al*. (2001). The model was used successfully to predict riverflow generation in a semi-arid environment in Tanzania, East Africa. Recently, in the same country, Vigiak *et al*. (2006) have reported of the successful application of the approach in a humid tropical rainforest catchment to simulate overland flow. The application of the approach in the Volta basin in West Africa has also been reported by Amisigo (2005).

The aim of this study is to apply DBM TF rainfall-riverflow modelling approach to study rainfall to riverflow behaviour within large nested forest catchments in Ghana. The specific objectives are a) to investigate the applicability of the DBM transfer function rainfall to riverflow modelling approach in large nested catchments in tropical rainforest within the River Pra basin in Ghana, using daily time-series, b) to identify the mathematical relationships between the catchment average rainfall and riverflow, and estimate their accompanying parameters with uncertainty and c) to give physical interpretation of the estimated parameters of the identified models in (b) and the accompanying Dynamic Response Characteristics (DRCs).

The study catchment and data series used in the analysis

The study was conducted using rainfall and riverflow data from the River Pra Basin which lies in the forest zone of Ghana within latitude 5º and 7º 30' N and longitude 0º and 2º 30' W, respectively (Fig. 1) with catchment area of 20778 km^2. It is the largest basin in the forest zone of Ghana and has enough water which is capable of generating hydropower (Dickson and Benneh, 1988). The basin lies in the Wet Semi-Equatorial climatic zone with climate that is influenced principally by the tropical maritime (monsoon) and continental (harmattan) air masses. Two distinct seasonal rainfall distributions (i.e. the bi-modal distribution) are normally experienced in the area which usually commences in March peaking around June with dry spell in August, peaking again in September and October. The rainfall pattern generally dictates the riverflow totals (Fig. 2) where most of the rivers in the basin are permanent; flowing throughout the year. The underlain geology of the basin is principally Birimian rocks with a section in the middle underlain by Tarkwain formation with soil cover which is predominantly Acrisols (Forest Ochrosols). The Pra basin is of national and global importance due to cocoa, timber and oil palm production in addition to food crops and mining industries.

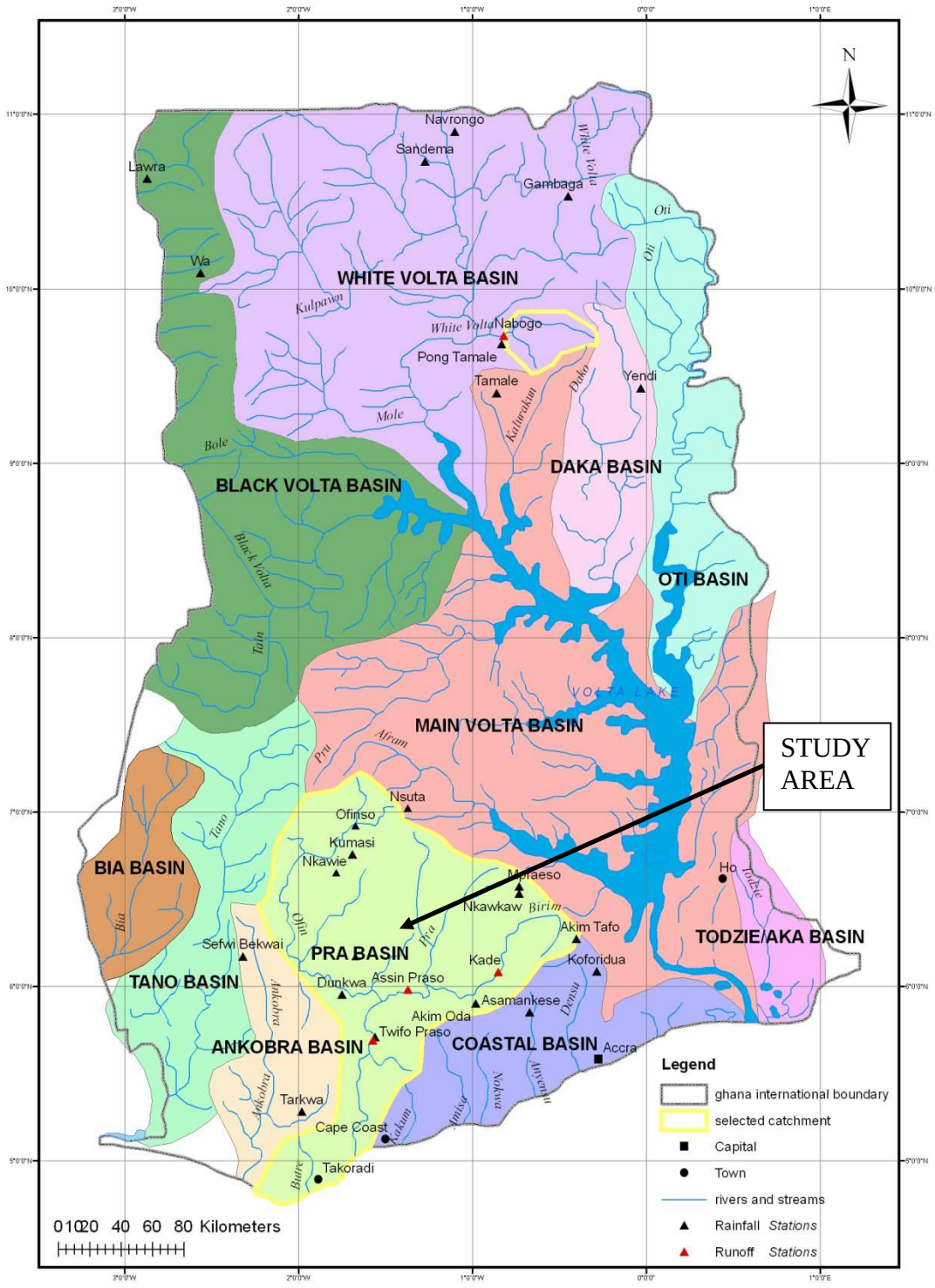


Fig. 1. Map of Ghana showing the location of the selected gauging stations (i.e. small red triangles) in the River Pra basin at Kade on River Birim, and Assin Praso and Twifo Praso on River Pra used in the DBM transfer function rainfall-riverflow modelling and the drainage basins in Ghana.

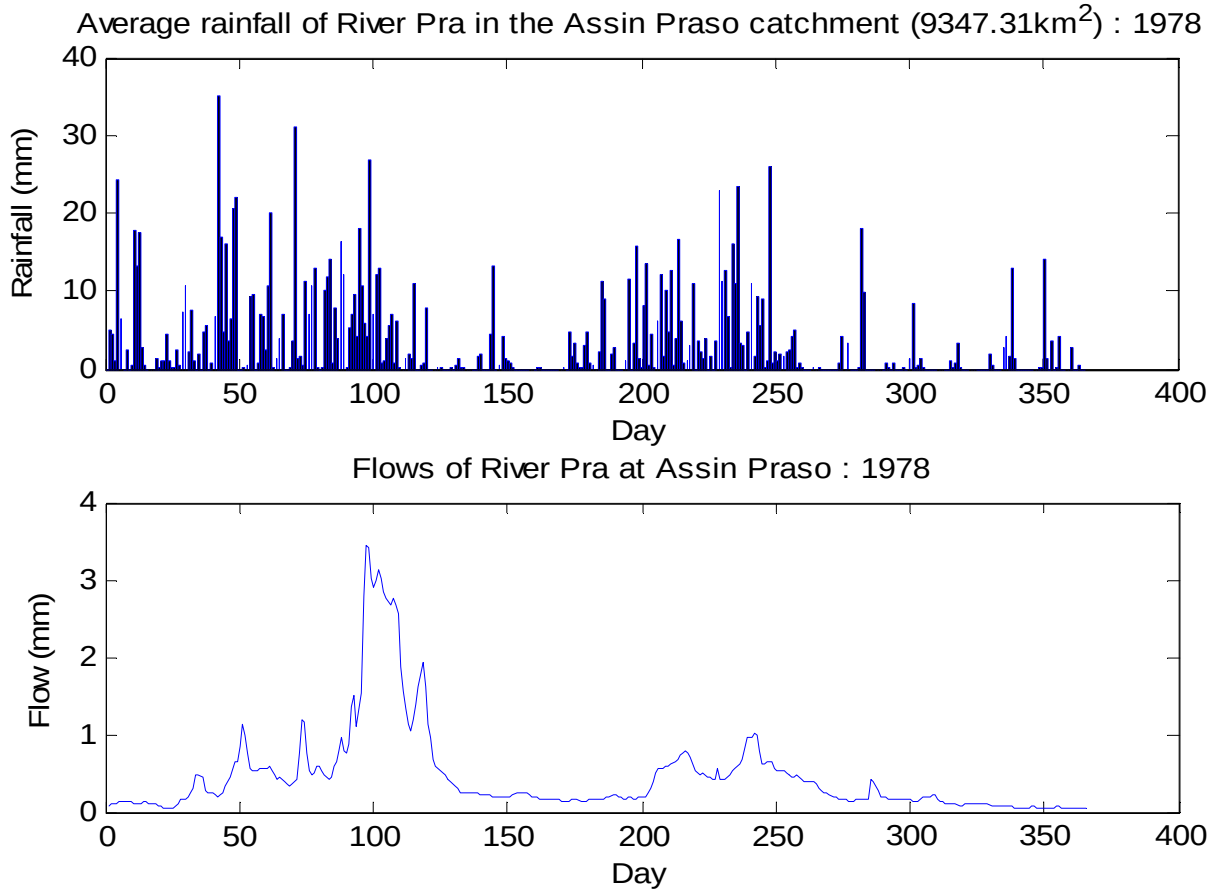


Fig. 2. Daily rainfall and flows of River Pra at Assin Praso for the 1978 water year (from March 1, 1978 to February 28, 1979) showing the bimodal regime of rainfall in the Forest zone which is followed by riverflow.

The data series used were daily riverflow (cumecs) and daily rainfall (mm) obtained from the Hydrological Services Department (HSD) and Meteorological Services Department (MSD), respectively in Accra, Ghana. Flows from River Birim gauged at Kade and River Pra gauged at Assin Praso and Twifo Praso were used (Fig. 1). The riverflow data in cumecs were converted to millimetres per day using the respective catchment areas of the selected gauging stations (Table 1).

Table 1. Catchment sizes for River Birim at Kade and River Pra at Assin Praso and Twifo Praso in the River Pra basin (see Fig. 1 for the location of the gauging stations)

River	Gauging Station	Catchment Area (km^2)
Birim	Kade	2126.67
Pra	Assin Praso	9347.31
Pra	Twifo Praso	20778.00

MATERIALS AND METHODS

Linear transfer function model (LTFM)

The general form of linear transfer function (single input-single output) which forms the basis of the transfer function model (TFM) package is given by

$$y_t = \frac{B\left(z^{-1}\right)}{A\left(z^{-1}\right)} U_{t-\delta} + \varepsilon_t \quad (1)$$

where the transfer function polynomials are defined as

$$A\left(z^{-1}\right) = 1 + a_1 z^{-1} + a_2 z^{-2} + ...a_N z^{-N} \quad (2)$$

$$B\left(z^{-1}\right) = b_0 + b_1 z^{-1} + b_2 z^{-2} + ...b_M z^{-M} \quad (3)$$

where y_t is the observed riverflow, U_t is 'effective rainfall', z is a backward shift operator (i.e. $z^{-q}u_t = u_{t-q}$) and δ is the pure time delay (i.e. delay between rainfall and initial river response). The *N* and *M* represent the number of *a* and *b* parameters, respectively. The residual ε_t is defined as

$$\varepsilon_t = y_t - Q_t \quad (4)$$

$$Q_t = \frac{\hat{B}\left(z^{-1}\right)}{\hat{A}\left(z^{-1}\right)} U_{t-\hat{\delta}} \quad (5)$$

where Q_t is the model output, $\hat{A}\left(z^{-1}\right)$ and $\hat{B}\left(z^{-1}\right)$ are the estimated TF polynomials in z^{-1} of Equation 2 and 3, respectively and $\hat{\delta}$ is the estimated pure time delay. In a first order model the estimates of the TF polynomials are $\hat{B}\left(z^{-1}\right) = \hat{b}_0$ and $\hat{A}\left(z^{-1}\right) = 1 + \hat{a}_1 z^{-1}$. Term $\hat{b}_0$ is the system production or gain parameter estimate (or 'water balance' term) which scales the difference in total volumes of input and output and $\hat{a}_1$ is the recession or lag parameter estimate which is linked to the 'residence time' of the response in the catchment. The derivation of Equation 1 can be found in Beven (2001a). According to Young (2003) the residual (ε_t; noise term) accounts for all the riverflow not explained by Q_t and includes factors such as the modelling error, noise in the data, the effects of unobserved inputs and spatial heterogeneity in the rainfall data. The order of the transfer function model is defined by the triad [*N, M,* δ]. Where *N* and *M* represent the number of *a* and *b* parameters in Equations 2 and 3, respectively and δ is the pure time delay.

Depending on the nature of the dominant pathways within a catchment, a first-order transfer function model (see: Young, 1992, 1993; Young and Beven, 1994; Chappell *et al*., 1999, 2004b) or a higher-order model (see: Young and Beven, 1994; Young, 1993; Young *et al*., 1997; Lees, 2000; Young, 2001; Vongtanaboon and Chappell, 2004) may best describe the rainfall-riverflow response. A typical first-order transfer function model is given by Young (1992, 2005), Young and Beven (1994) and Chappell *et al*. (1999, 2004b, 2006) as:

$$Q_t = \frac{P}{1 - \Re z^{-1}} U_{t-\delta} \quad (6)$$

where Q_t is the subsurface flow along the dominant flow pathway at time step *t*; *P* the production parameter; $\Re$ the recession parameter; *U* effective rainfall; δ the pure time delay between the effective rainfall and the initial riverflow response and z^{-1} is the backward shift operator. The DRCs which describes the rainfall-riverflow of a catchment are based on the parameters *P* and $\Re$ of Equation 6 and are given by Chappell *et al*. (1999, 2006) as:

$$SSG = \frac{P}{1 - \Re} \quad (7)$$

$$TC = \frac{-t_{base}}{\log_e(\Re)} \quad (8)$$

where *SSG* is the steady state gain (water balance term); *TC* is the time constant (residence time) and t_{base} is the sampling interval (in this study a day). The *SSG* indicates the amount of the rainfall which appears as riverflow following evapo-transpiration and other losses, while *TC* is a measure of the residence time of the rainfall in the catchment.

Modelling non-linearities in hydrological behaviour

The hydrological process of the translation of rainfall into riverflow is inherently nonlinear due to the effects of varying subsurface moisture (FAO, 1981; Young and Beven, 1994). To model this non-linearity, the effective rainfall *U* in Equation 1 is often related to the actual rainfall *R* and the observed flow y by a nonlinear function (e.g. a power law: see: Young and Beven, 1994; Chappell *et al.*, 1999; Beven, 2001).

Power law sub-model (SSSM)

In the power law application, the effective rainfall *U* in (Equation 1) is linked with the actual rainfall *R* and the observed flow y by a power law relationship which is referred to as the store–surrogate sub-model (SSSM) and is defined by Young and Beven (1994) as:

$$U_t = R_t y_t^{\alpha} \quad (9)$$

where α is the estimate of the power law exponent which is a measure of the sensitivity of the catchment to antecedent moisture conditions. The term α, usually ranges between zero and unity (Beven, 2001a) with the value of unity indicating higher sensitivity and zero no sensitivity, i.e. the linear model (Equation 1). Young and Beven (1994) estimated a value of 0.628 for a catchment in Mid-Wales in the U.K. and Young (1998) estimated a value of 0.770 for a catchment in the USA. Chappell *et al*. (1999) also estimated a value of 0.420 for equatorial catchment in East Malaysia. The catchment in the USA is clearly more sensitive to antecedent moisture conditions.

Bedford Ouse sub-model (BOSM)

Within the DBM methodology the Bedford Ouse Sub-Model (BOSM) (Young, 2001;
Chappell *et al*., 2004b, 2006) is also used to model the nonlinear component of the rainfall-riverflow process. The general form of the model is given in Chappell *et al*. (2004b, 2006) as:

$$U_t = R_t \theta_t \quad (10)$$

where

$$\theta_t = \theta_{t-1} + \frac{1}{\tau_u}\left\{R_t - \theta_{t-1}\right\} \quad (11)$$

where U_t is the effective rainfall (mm); R_t is the average (gross) rainfall (mm); θ_{t-1} is the unsaturated zone storage variable at the previous time step (mm); τ_u is the dimensionless nonlinearity term for the whole catchment

response. The nonlinearity term (τ_u) is obtained by an iterative process applied to the BOSM and transfer function expressions with the objective function set at a higher R_t^2 and a minimum YIC with θ initially set as zero. The IHACRES model (Jakeman *et al*., 1990; Jakeman and Hornberger, 1993) has also been used in the modelling of nonlinear behaviour in the rainfall-riverflow process (e.g. see: Post and Jakeman, 1996; Sefton and Howarth, 1998; Young, 2001). This model is an extension of the BOSM approach, which includes temperature effects.

Exponential function sub-model (EFSM)

An exponential function sub-model (EFSM) has also been used to quantify nonlinear component of the rainfall riverflow process (see: Young, 2006). The application of the EFSM within the DBM methodology of this study is probably, the first of its kind in the tropics. The general form of the model is given by

$$U_t = R_t\left(1 - e^{-\beta \cdot y_t}\right) \tag{12}$$

where U_t is the effective rainfall (mm); R_t is the average (gross) rainfall (mm); y_t is the observed riverflow (mm); β is the exponential parameter ($\beta \neq 0$). This approach was successfully used by Young (2006) to model the daily rainfall-flow data from the Leaf River catchment, with an estimated β parameter of 0.0124 and efficiency of 86.0%. This 1944 km^2 catchment is a humid watershed, located in Mississippi, USA.

Normalisation of 'effective rainfall' produced by nonlinear sub-model

In order to maintain mass balance, the effective rainfall from the EFSM and BOSM nonlinear rainfall filters are normalised in relation to the catchment average rainfall. The normalised effective rainfall Ue_t is given in Chappell *et al*. (1999) as:

$$Ue_t = U_t\left(\frac{\sum R_t}{\sum U_t}\right) \tag{13}$$

The nonlinearity term with these models can be incorporated into the triad to give [*N*, *M*, δ]^P where P is the nonlinear term (i.e. BOSM or EFSM filter), *N* and *M* represent the number of a and b parameters in Equations 2 and 3, respectively and δ is the pure time delay.

The EFSM model utilises past riverflows to derive the form of the nonlinearity while the BOSM filter requires only rainfall. In this study, the EFSM together with the BOSM are applied.

Model order (complexity) identification

The model identification may result in a range of models [*N*, *M*, δ] ^{P} giving a good fit to the data. A first-order model has one dominant mode water pathway describing the rainfall-riverflow response. A second-order model is normally explained by having two parallel water pathways, a fast pathway and a slow pathway (Young and Beven, 1994; Young, 1993, 2002). Example of fast pathways includes infiltration-excess overland flow (van Loon and Keesman, 2000) or shallow sub-surface flow (Chappell *et al*., 1998). Slow pathway includes flow deep within rock aquifers (e.g. Sefton and Howarth, 1998). The more pathways the model identifies as plausible, the higher the model order. The best of them is considered based around the coefficient of determination (R_t^2) (or Simplified Nash and Sutcliffe efficiency (1970) criteria in hydrological literature) and the heuristic Young Information Criterion (YIC) (Young and Beven, 1994; Lees, 2000; Young, 2001; Beven, 2001a) which are defined as follows:

$$R_t^2 = 1 - \frac{\sigma^2}{\sigma_0^2} \tag{14}$$

$$\text{YIC} = \ln\left(\frac{\sigma^2}{\sigma_0^2}\right) + \ln\left(NEVN\right);$$

$$NEVN = \frac{1}{np}\sum_{i=1}^{i=np}\frac{\sigma^2 P_{ii}}{\hat{\theta}_i^2} \tag{15}$$

where σ_0^2 is the variance in the observed data; σ^2 is variance of the model residuals; NEVN is the Normalised Error Variance Norm which is a measure of the model's parsimony (i.e. the degree of over-parameterisation in the model); $np = n + m + 1$ is the number of estimated parameters in the θ vector; $\sigma^2 P_{ii}$ is an estimate of the variance of the estimated uncertainty on the ith parameter estimate; and $\hat{\theta}_i^2$ is the square of the ith parameter in the θ vector.

The R_t^2 is a statistical measure of how well the model explains the variance of the data, if it is between zero and unity it is the proportion of output variance explained by the model: as the model fit improves its value approaches unity, thus when the variance of the residuals is low as compared to the variance of the data. If σ^2 and σ_0^2 are of similar magnitude then it tends towards zero and the model fits no better than the mean of the observed data. With particularly bad models (e.g. unstable), residual variance can be larger than that of the output data, which explains negative values of R_t^2.

The YIC is a more complex criterion that provides a measure of the balance between model fit and over-parameterisation (Lees, 2000). The first term of YIC is simply a relative measure of how well the model explains the data. Thus, when the model residuals get smaller and closer to zero the term becomes more negative. The second term quantifies the degree of over-

parameterisation in the model, and tends to become larger when the model is over-parameterised and the parameter estimates are poorly defined. Based on the above criteria the approach helps to identify a model which explains the data well with a minimum number of parameters which are statistically well defined.

DBM rainfall to riverflow modelling steps

The procedure for the building of DBM transfer function rainfall to riverflow model (see: Young and Beven, 1994; Lees, 2000; Young, 2001) is as follows:

1) Identify linear transfer function model for the time series data [y_t, U_t] using the Simplified Refined Instrumental Variable (SRIV) algorithm (Young, 1985, 1991). The SRIV method uses a recursive least square algorithm (Young, 1984) followed by the application of the instrumental variable (IV) method (Young, 1985) which removes the bias of the estimates.
2) Examine the model fit by visualisation and investigation of goodness of fit using R_t^2. If the model fit is satisfactory the analysis is complete, proceed to step 8. Otherwise, proceed to step 3.
3) Based on the analysis in steps 1 and 2 plus knowledge of the physical/hydrological system select the simplest transfer function model which appears capable of characterising the behaviour of the output variable (riverflow) in relation to the observed input (rainfall).
4) Obtain initial estimate of time variable parameters (TVPs) in a transfer function model by using fixed interval smoothing estimation (FIS) (Young, 1984; Young 1986; Young, 1998).
5) Investigate state dependent parameter (SDP) relations (e.g. gain versus riverflow) using scatter plots (Young and Beven, 1994; Young, 2001; 2003; 2006).
6) If a single relationship emerges, repeat the TVP estimation with the data processed in order of the ranked dependent state to improve the SDP relation.
7) In case of the presence of gain nonlinearities, reformulate the model as an input nonlinearity combined with a linear transfer function model and estimate the parameters using the SRIV method of system identification.
8) Investigate the physical interpretation of the different resultant models and select the one that explained the data well and has a sensible mechanistic (hydrological) interpretation of the data. This aspect of the approach is the 'heart and soul' of the DBM approach.

Application of the DBM TF model to the data

Initial visual analysis of the rainfall and riverflow data in the catchments revealed that the 1978 water year (i.e. from March 1, 1978 to February 28, 1979) was the only period where data was available at riverflow stations used in the study. The 1978 water year was, therefore, used as the period of analysis for the application of the DBM TF model.

The DBM TF model as outlined in above was applied to riverflows of River Birim at Kade and River Pra at Assin Praso and Twifo Praso within the River Pra Basin (see map: Fig. 1). Average rainfall over the catchments of Kade, Assin Praso and Twifo Praso at each time step of a day was used as input into the model. The averaging process was done by using the Thiessen Polygon approach (Mutreja, 1986; Linsley *et al*., 1988; Shaw, 1994). This approach allows area-weighted integration of rain gauge totals from gauges within and adjacent to the catchment to be used as input into the model.

Using the SRIV identification algorithm and YIC and R_t^2 as model order identification criteria a range of linear transfer function models relating the input (average rainfall) and the output (riverflow) were obtained for the above named riverflow stations. Due to catchment hydrological systems being inherently nonlinear, a time varying parameter (TVP) model was applied to investigate the form of the nonlinear behaviour in the data (e.g. see: Young and Beven, 1994: Chappell *et al*., 1999; Lees, 2000). The TVP model was estimated where the production parameter (P: see Equation 6) was allowed to vary whilst the recession parameter ($\Re$: see Equation 6) was kept constant, followed by State Dependent Parameter (SDP) modelling with the flow representing the dependent state. The SDP analysis quantifies any state dependency in the parameter variations which is associated with nonlinear behaviour in the catchments. More detailed discussion on SDP can be found in Young (2001, 2006).

To show the relationship between the production parameter and the flow, the sorted state (i.e. riverflow) and the sorted SDP estimates (from the SDP function) were plotted. Exponential relationship between them was

Table 2. Purely linear model identification of flows of River Birim at Kade, River Pra at Assin Praso and Twifo Praso in the River Pra basin.

Station	River	Model	YIC	R_t^2 (%)
Kade	Birim	[1 1 1]	-6.082	62.45
Assin Praso	Pra	[1 1 1]	-5.151	50.36
Twifo Praso	Pra	[1 1 2]	5.371	58.41

investigated using an optimisation routine fitted to the sorted SDP parameters (i.e. non-parametric estimate). Following that a separate optimisation routine was then used to estimate the exponential parameter β (Equation 12) for the data (i.e. parametric estimate).

The routine utilises the SRIV algorithm within an iterative procedure to optimise the exponential parameter while the SRIV model residual variance was minimised. The exponential parameter was then used to transform the catchment average rainfall, into catchment-average 'effective rainfall' using the EFSM (see: Equation 12) equation. To ensure mass balance, the catchment average 'effective rainfall' was normalised in relation to the catchment-average rainfall using Equation 13. After the normalisation, the SRIV algorithm was used again to identify a range of transfer function models relating the 'normalised catchment-average effective rainfall' to riverflow with their respective parameters. Using YIC and R_t^2, the model which explained the data well with good physical meaning of the estimated parameters was selected for each riverflow station.

RESULTS AND DISCUSSION

Purely linear TF modelling

The efficiencies (R_t^2) of purely linear transfer function modelling of the data at all the riverflow stations considered within the River Pra basin ranges between 50.36% and 62.45% (Table 2). These efficiencies are low, possibly due to the presence of nonlinearities as a result of variable antecedent moisture conditions (FAO, 1981; Young and Beven, 1994; Chappell *et al*., 2004a).

The model structures of all the catchments are first-order (see: Table 2). These structures only give preliminary indication of the likely model orders and time delays because the linear models only have low efficiencies.

TVP and SDP TF modelling

Investigation of the presence of nonlinearities using TVP and SDP modelling as explained in above resulted in an SDP fit of the observed riverflows, which describes the rainfall-riverflow response with efficiency (R_t^2) ranging from 98.11 to 98.78% for all the catchments. These indicate that the SDP model captured almost all of the nonlinearities in the rainfall-riverflow behaviour within the catchments. This suggests that the plot of the SDP parameter estimates would give the full nature of the nonlinear behaviour of the catchments. The plots of the SDP parameter estimates (namely gain or '*P*' in Equation 6) against the riverflows are shown in figure 3. The plots show that the gain parameter increases with increasing flow, which suggests that nonlinearities are present in the translation of rainfall to riverflow in the catchments.

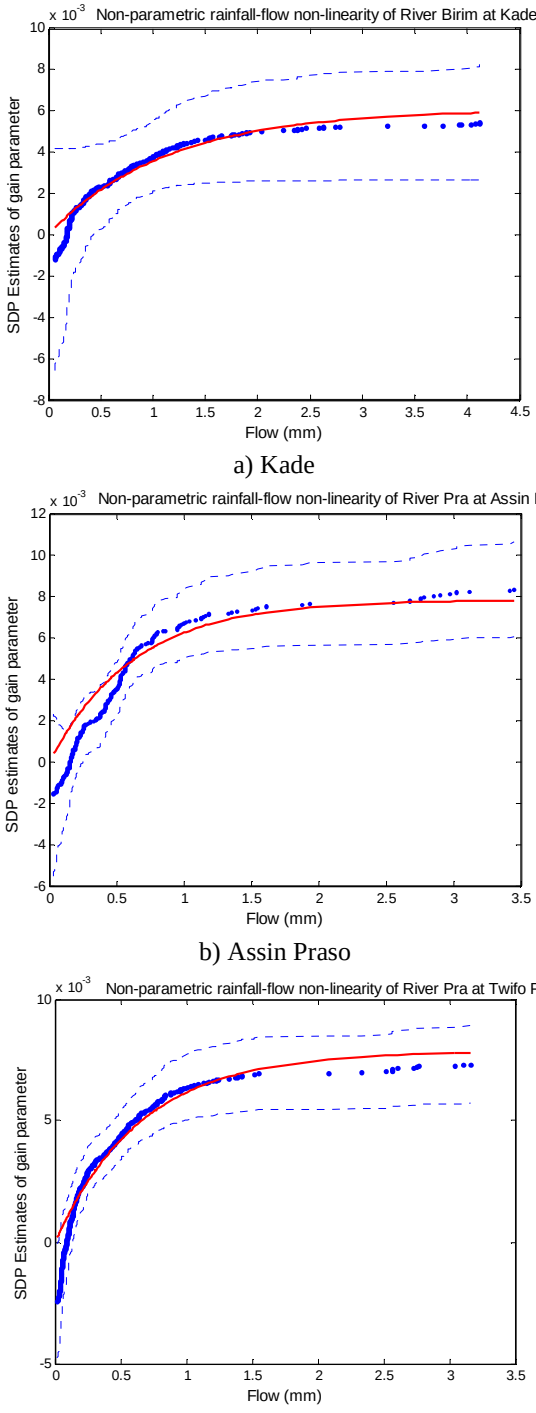


Fig. 3. Non-parametric estimate of the rainfall flow nonlinearity in gain parameter (see 'P' in Equation 6) as a function of flow (blue dots) and uncertainty (blue dashed lines). a) River Birim at Kade b) River Pra at Assin Praso and c) River Pra at Twifo Praso fitted with exponential curve (solid red line).

The plot of River Birim at Kade (Fig. 3a), River Pra at Assin Praso (Fig. 3b) and Twifo Praso (Fig. 3c) suggest that the nonlinear behaviour of the riverflows within the River Pra Basin follow an exponential relationship between the gain parameter and the riverflow. From the plots it could be seen that as the catchment wets up the instantaneous runoff coefficient (i.e. proportion of rainfall generating riverflow) keeps increasing but gets to a point where it does not change. This means that as the catchment wets up, the runoff coefficient increases, until it reaches a point where the runoff coefficient remains constant, effectively giving a linear relationship between rainfall and riverflow.

The estimated exponential function from the SDP modelling for the flows of River Birim at Kade and River Pra at Assin Praso and Twifo Praso are given as:

$$boKD_t = 0.006\left(1 - e^{-0.8812\, yKD_t}\right) \quad (16)$$

$$boAS_t = 0.0078\left(1 - e^{-1.5999\, yAS_t}\right) \quad (17)$$

$$boTW_t = 0.0078\left(1 - e^{-1.5433\, yTW_t}\right) \quad (18)$$

where *boKD*, *boAS* and *boTW* are the gain parameter estimates and *yKD*, *yAS*, and *yTW* are the riverflows of River Birim at Kade, River Pra at Assin Praso and Twifo Praso, respectively. Structurally, the exponential function is limited to be non-negative and this is a sensible solution in this case – also well contained within the uncertainty bounds of the SDP estimates.

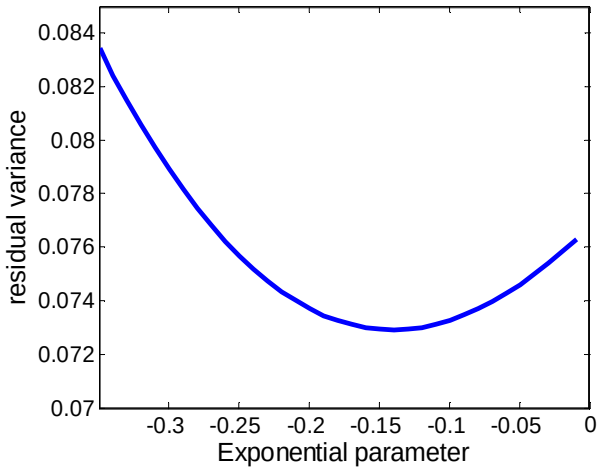
Nonlinear TF modelling

Final optimisation of the exponential parameter β (Equation 12) for the catchments, using iterative routines are shown in figure 4. The optimised values of β are for the estimation of 'effective rainfall' and subsequent modelling of nonlinear behaviour within the catchments. The plots suggest that, riverflow simulation is highly sensitive to β values.

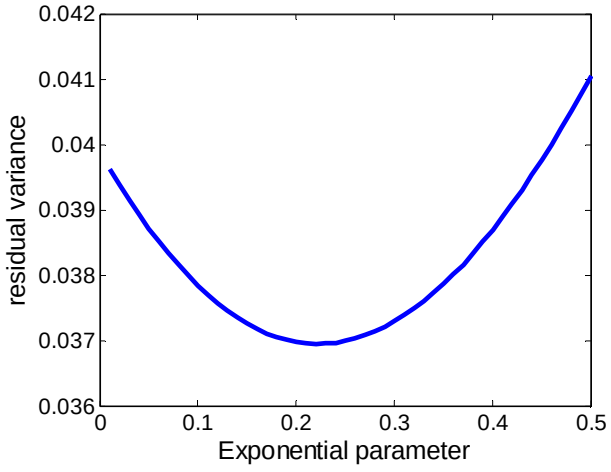
First-order modelling (Single water pathway)

The estimates of the exponential parameter for the catchments, model efficiencies and the resultant optimised first-order nonlinear transfer function model parameters and statistics optimised against YIC and R_t^2 as the objective functions are presented in table 3. The model parameters and statistics estimated for an optimised first-order transfer function model using the BOSM nonlinear filter are shown in table 4.

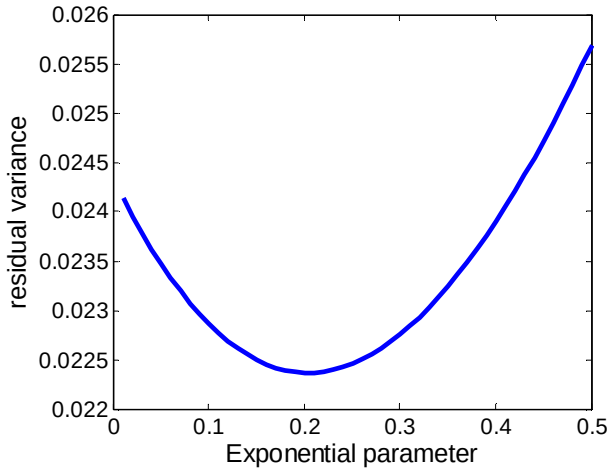
From table 3, the first-order EFSM model provides an excellent fit for riverflows of River Birim at Kade, River Pra at Assin Praso and Twifo Praso with efficiencies (R_t^2) of 88.26, 89.55 and 92.94%, respectively. The BOSM model (Table 4) gives efficiencies of 72.53, 69.71 and 77.94%, respectively, for the same stations in the basin.



a) Kade (EFSM)



b) Assin Praso (EFSM)



c) Twifo Praso (EFSM)

Fig. 4. Final optimisation of the exponential parameter of the EFSM (see 'β' in Equation 12) for the catchments for the estimation of effective rainfall and modelling of nonlinear behaviour (see: Fig. 1 for the location of the gauging stations).

Table 3. First-order nonlinear DBM model parameters identified for the catchments within the River Pra basin for the 1978 water year using EFSM as the nonlinearity filter.

Parameters and statistics	Catchments within the River Pra Basin		
	Kade	Assin Praso	Twifo Praso
Area (km^2)	2126.67	9347.31	20778.0
R_t^2 (%)	**88.26**	**89.55**	**92.95**
Model order	[1 1 0]	[1 1 0]	[1 1 0]
YIC	**-8.740**	**-9.021**	**-9.738**
β	-0.1409	0.2226	0.2037
$\Re$	-0.8947	-0.8860	-0.8868
σ($\Re$)	0.0043	0.0042	0.0035
P	0.0195	0.0132	0.0111
σ(P)	0.0007	0.0004	0.0003
TC (days)	8.9868	8.2618	8.3234
σ(TC)	0.3822	0.3275	0.2780
SSG	0.1853	0.1158	0.09828
σ(SSG)	0.0030	0.0019	0.0013

Note: R_t^2: Simplified Nash and Sutcliffe efficiency for model; Model order: [No. of denominators, numerators, pure time delays]; YIC: Young Information Criterion; β: exponential parameter; $\Re$: recession parameter; P: production parameter; TC: time constant; SSG: steady state gain of the transfer function; σ($\Re$), σ(P), σ(TC) and σ(SSG): standard deviation of parameter in the parenthesis. See Equation 12 (EFSM).

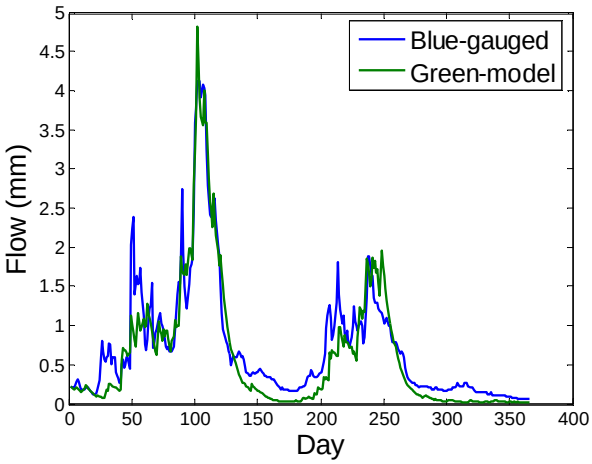
Table 4. First-order nonlinear DBM model parameters identified for the catchments within the River Pra basin using BOSM as nonlinearity filter to model rainfall to riverflow for 1978 water year.

Parameters and statistics	Catchments within the River Pra Basin		
	Kade	Assin Praso	Twifo Praso
Area (km^2)	2126.67	9347.31	20778.0
R_t^2 (%)	**72.53**	**69.71**	**77.94**
Model order	[1 1 1]	[1 1 1]	[1 1 1]
YIC	**-6.873**	**-6.315**	**-6.918**
τ_u	55	50	30
$\Re$	-0.9221	-0.9145	-0.9051
σ($\Re$)	0.0051	0.0069	0.0066
P	0.0171	0.0123	0.0114
σ(P)	0.0010	0.0009	0.0008
TC (days)	12.3271	11.191	10.0316
σ(TC)	0.8563	0.9827	0.7566
SSG	0.2197	0.1434	0.1201
σ(SSG)	0.0052	0.0040	0.0027

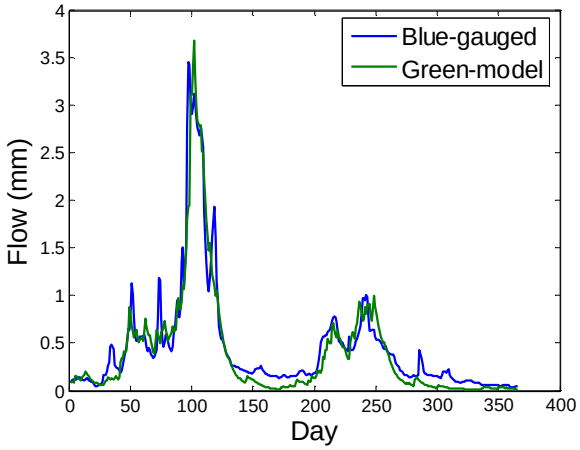
Note: τ_u : BOSM nonlinearity term. See Equation 11 (BOSM).

The EFSM is expected to perform better than the BOSM model, because in the evaluation of the nonlinear behaviour of the catchments the EFSM model uses riverflow as a surrogate of sub-surface moisture, unlike BOSM which *a priori* fixes the form of the non-linearity. Figure 5 and 6 shows the ability of the DBM model to capture the key dynamics inherent in the relationship between the incoming rainfall and the outgoing riverflow within the catchments, using EFSM and BOSM models as nonlinear filters, respectively.

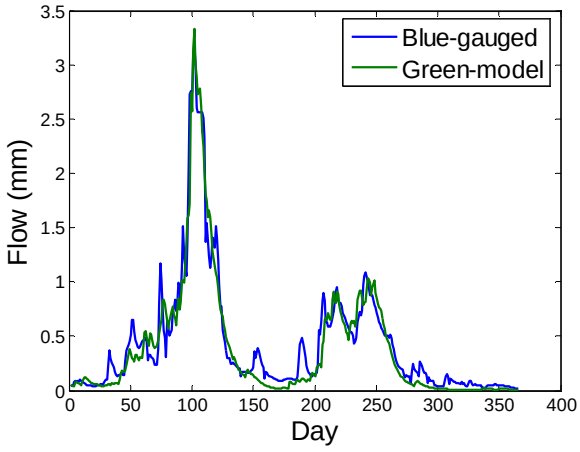
Generally, the performance of the models, in terms of explanation of the model output variance is excellent for all the models (Table 3 and 4) but the model fit shows that peak flows during the major rainfall season (i.e. May to June) were underestimated by the BOSM model (Fig. 6). However, the BOSM model predicted the recession flows very well as compared to the EFSM model at all the stations. (Figs. 6a, b and c). Thus, within the River Pra basin the BOSM and EFSM sub-models are recommended for low and high flow studies, respectively, based on their performance (Figs. 5 and 6).



a) Kade (EFSM)

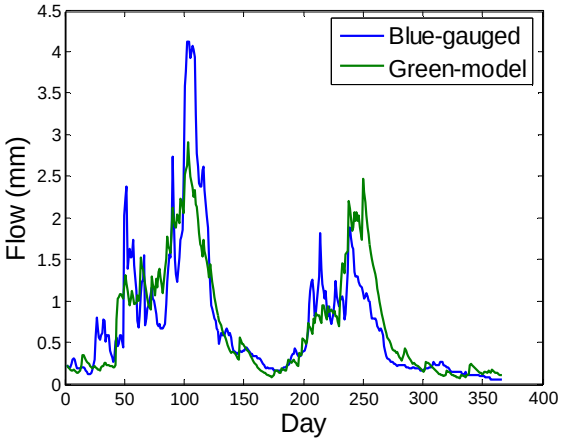


b) Assin Praso (EFSM)

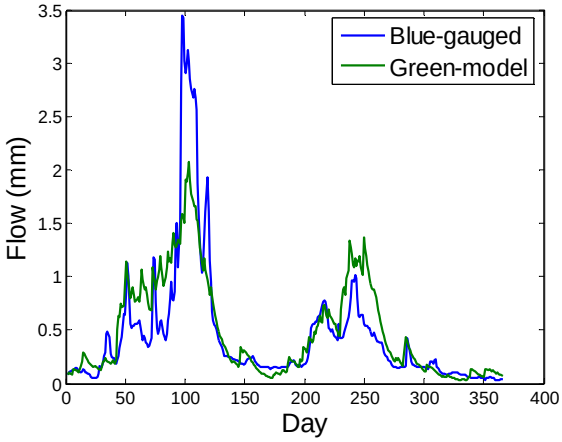


c) Twifo Praso (EFSM)

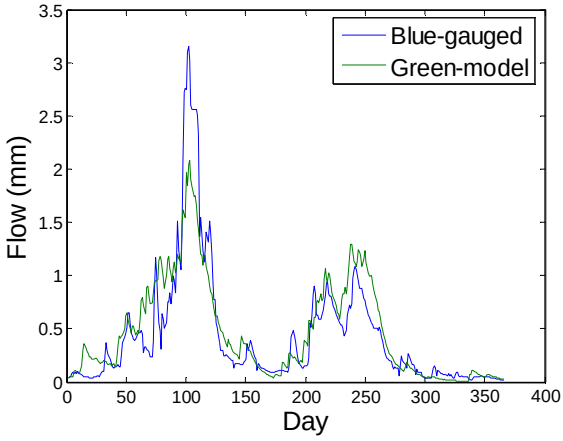
Fig. 5. Daily flows predicted by optimum first-order nonlinear transfer function EFSM model (green) against observed flows (blue) showing the DBM model's ability to capture the dynamics of the rainfall to riverflow generating mechanism in the catchments within the River Pra basin (i.e. from March 1, 1978 to February 28, 1979) (See: Table 3 for the models).



a) Kade (BOSM)



b) Assin Praso (BOSM)

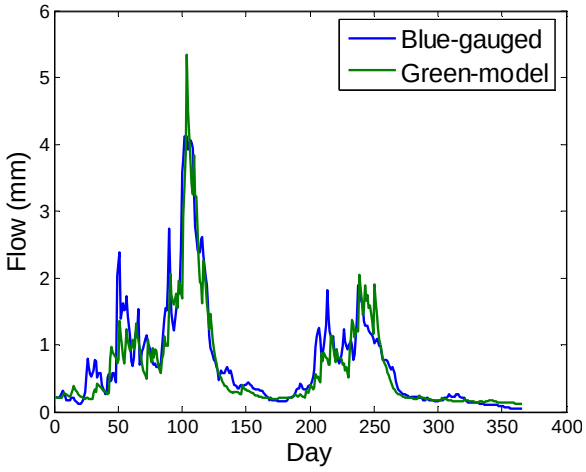


c) Twifo Praso (BOSM)

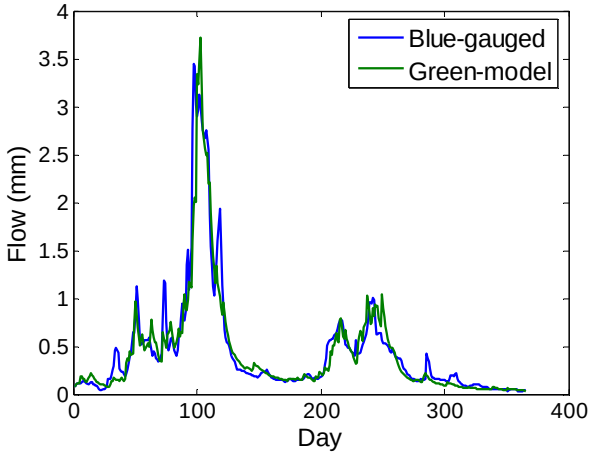
Fig. 6. Daily flows predicted by optimum first-order nonlinear transfer function BOSM model (green) against observed flows (blue) showing the DBM model's ability to capture the dynamics of the rainfall to riverflow generating mechanism in the catchments within the River Pra basin for the 1978 water year (i.e. from 1st March, 1978 to 28th February, 1979). (See: Table 4 for the models).

Higher-order modelling (multiple water pathways)

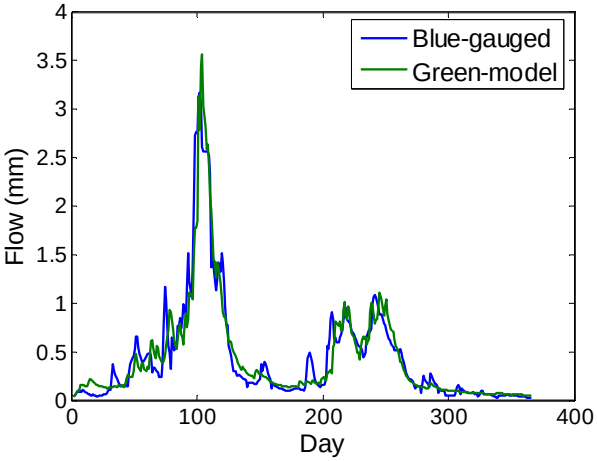
The presence of multiple runoff pathways in the catchments was also investigated using YIC and R_t^2 as the objective functions in higher-order modelling (up to fourth order). The results are shown in table 5 and 6 for the EFSM and BOSM models, respectively. From the tables, comparison of the R_t^2 and YIC of the higher-order models to those of the first-order models in the basin show reduced R_t^2 values and higher YIC values of the higher-order models. For instance, at Kade, R_t^2 and YIC of the first-order model reduced from 88.26% and -8.740 to 84.51% and -7.869, at Assin Praso from 89.55% and -9.021 to 88.45% and -7.302 and at Twifo Praso from 92.94% and -9.738 to 89.27% and -7.41, respectively, for the higher-order models. Similarly, the BOSM also shows reduction in R_t^2 and less negative YIC values at all the stations (Table 6). Thus, comparison of the model efficiencies and YICs of the first-order models to those of the higher-order models indicate that higher-order models could not be justified for the catchments despite the improvement in the fit of mid and late recessions (Fig. 7). Thus, a single pathway dominates the catchments behaviour in routing rainfall to riverflow in the basin.



a) Kade (EFSM)



b) Assin Praso (EFSM)



c) Twifo Praso (EFSM)

Fig. 7. Daily flows predicted by optimum second order nonlinear transfer function model (green) against observed flows (blue) showing the DBM model's ability to capture the dynamics of the rainfall riverflow generating mechanism in the catchments within the River Pra basin for the 1978 water year (i.e. from March 1, 1978 to February 28, 1979) (see: Table 5 for the models).

Final first-order models identified for the catchments

Based on the EFSM parameterisation of the nonlinearity (see: Table 3), mathematical relationships between rainfall input and riverflow output with no initial pure time delay were identified for the catchments i.e. Kade, Assin Praso and Twifo Praso. These are as follows:

Kade: $$Q_t = \frac{0.0195}{1-0.8947z^{-1}} UeKD_t \quad (19)$$

Assin Praso: $$Q_t = \frac{0.0132}{1-0.8860z^{-1}} UeAS_t \quad (20)$$

Twifo Praso: $$Q_t = \frac{0.0111}{1-0.8868z^{-1}} UeTW_t \quad (21)$$

where $UeKD_t$, $UeAS_t$, and $UeTW_t$ are 'normalised catchment effective rainfall' inputs for Kade, Assin Praso, and Twifo Praso, respectively and z^{-1} is the backward shift operator. The no pure time delay for the flows suggests that rainfall is more rapidly seen as riverflow in the basin.

Table 7 shows the performance of the DBM models compared with conceptual and physics-based models which have been applied in Ghana and the neighbouring countries. The Table shows that the performance of the simple first-order DBM TF models which require only four parameters, namely exponential parameter (β), recession parameter ($\Re$), production parameter (P), pure time delay (δ) gives efficiencies for similar African catchments (of a range of sizes) which are no smaller than those of complex conceptual or physics-based models.

Table 5. Comparison of YIC and R_t^2 of identified first-order and high-order nonlinear models using EFSM as the nonlinearity filter for the flows within the River Pra Basin.

Station	First-order model			Higher-order model		
	R_t^2 (%)	YIC	model order	R_t^2 (%)	YIC	model order
Kade	**88.26**	**-8.740**	**[1 1 0]**	84.51	-7.869	[2 2 2]
Assin Praso	**89.55**	**-9.021**	**[1 1 0]**	88.45	-7.302	[2 2 1]
Twifo Praso	**92.94**	**-9.738**	**[1 1 0]**	89.27	-7.41	[2 2 2]

Table 6. Comparison of YIC and R_t^2 of identified first-order and high-order nonlinear models using BOSM as the nonlinearity filter for all the gauging stations.

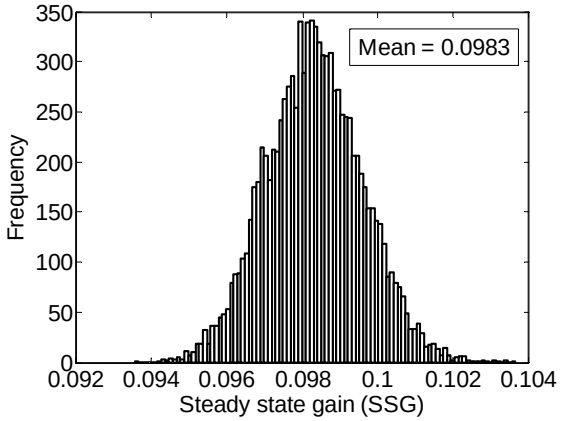
Station	First-order model			Higher-order model		
	R_t^2 (%)	YIC	model order	R_t^2 (%)	YIC	model order
Kade	**72.53**	**-6.875**	**[1 1 1]**	70.99	-5.956	[3 1 2]
Assin Praso	**69.71**	**-6.349**	**[1 1 1]**	61.65	-5.423	[3 1 0]
Twifo Praso	**78.44**	**-6.928**	**[1 1 2]**	76.83	-6.135	[3 1 0]

Other studies which demonstrates that the DBM TF rainfall-riverflow modelling technique performed efficiently with smaller number of parameters and data inputs can be found in Young and Beven (1994), Young *et al*. (1997), Chappell *et al*. (1999, 2004a, 2004b, 2006), Lees (2000), Young (1992, 1993, 1998, 2001, 2002, 2005), Mwakalila *et al*. (2001), Vongtanaboon (2004), Vongtanaboon and Chappell (2004), Romanowicz *et al*. (2006), among others.

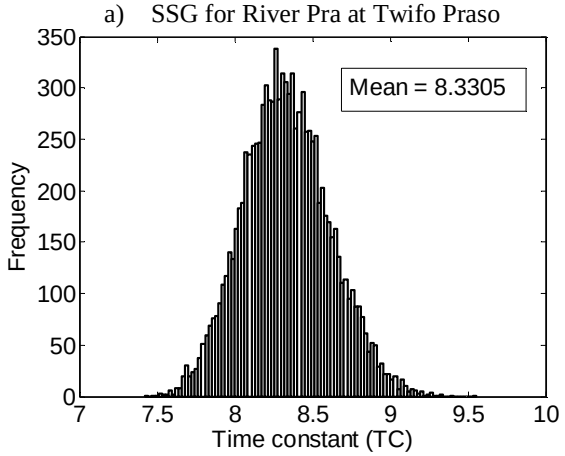
Results: Uncertainty analysis of derived parameters Time Constant (TC) and Steady State Gain (SSG)

In order to compare DRCs between the catchments (or with published data) the uncertainty in the estimated DRCs must first be investigated. The uncertainty on the DBM TF model parameters (i.e. recession parameter; $\Re$ and production parameter; *P*) was determined by assuming that the residuals follow a normal distribution (Young, 2003). It was necessary to quantify the uncertainty in the TC and SSG using Monte Carlo Simulation (MCS: see Young, 1998, 2001, 2003) analysis. MCS analysis is the simulation of a model where the model is run several times (in this study with 10,000 realisations) using different sets of parameters (here TC and SSG) which were selected randomly from the model-predicted standard error about the gain or production parameter (*P*) and the recession parameter ($\Re$).

Figure 8 shows the results of the analysis using 10,000 random realisations for River Pra at Twifo Praso. The distribution of the TC and SSG of the models for the other stations in the basin (not presented) were similar to that of Twifo Praso. The Figure suggests that the SSGs and TCs are symmetric about their means and so comparable with the mean value derived earlier. For instance, for River Pra at Twifo Praso mean SSG is 0.0983, estimated SSG = 0.0983, mean TC = 8.3501 days, estimated TC = 8.3234 days. The above indicates that the derived SSGs and TCs from the DBM TF parameter estimates follow normal distribution and are not highly uncertain.



a) SSG for River Pra at Twifo Praso



b) TC for River Pra at Twifo Praso

Fig. 8. Histogram of Monte Carlo analysis to evaluate the uncertainty associated with the derived parameters; steady state gain (see Equation 7) and time constant (days: see Equation 8) of the TF DBM model of River Pra at Twifo Praso showing well defined distribution about the means.

Table 7. Model efficiency (R_t^2: see Table 3) of identified DBM models compared with that of conceptual and physics-based models which have been applied to catchments in Ghana and neighbouring countries i.e. Ivory Coast, Burkina Faso and Benin based on model estimation.

Model	Type	Catchment	River	Area (km^2)	Country	R_t^2 (%)	Reference
GR2M	CCM	Samien	Sasandra	29,300.0	Ivory Coast	89.0	Paturel *et al*.(2003)
WBM	CCM	Samien	Sasandra	29,300.0	Ivory Coast	46.0	Paturel *et al*. (2003)
GR2M	CCM	Bada	Bandama	24,075.0	Ivory Coast	81.0	Paturel *et al*. (2003)
WBM	CCM	Bada	Bandama	24,075.0	Ivory Coast	69.0	Paturel *et al*. (2003)
GR2M	CCM	Samandeni	Moohoun	4,575.0	Burkina Faso	84.0	Paturel *et al*. (2003)
WBM	CCM	Samandeni	Moohoun	4,575.0	Burkina Faso	68.0	Paturel *et al*. (2003)
SAMULAT-H	PBM	Upper Aguima	Queme	3.2	Benin	82.0	Geirtz *et al*. (2006)
SAMULAT-H	PBM	Upper Niao	Queme	3.1	Benin	67.0	Geirtz *et al*. (2006)
ACRU	CCM	Manhia	Densu	2100.0	Ghana	82.0	Bekoe (2005)
EFSM	DBM	Kade	Birim	2126.67	Ghana	88.3	This study
EFSM	DBM	Assin Praso	Pra	9347.31	Ghana	89.6	This study
EFSM	DBM	Twifo Praso	Pra	20778.0	Ghana	93.0	This study

CCM: Conceptual model, PBM: Physics based distributed model, DBM: Data-based mechanistic, EFSM: Exponential function sub-model.

Results: Hydrological interpretation of estimated model parameters

Within the DBM methodology, hydrological/physical interpretation of the identified parameters associated with the model is very important and cannot be over-emphasised (Young, 2005). DBM models using the EFSM sub-model produced the most statistically sound models, and so it is these models that are interpreted physically/hydrologically. The parameters in the TF equations (Equations 19 - 21) to be considered are: exponential parameter: β, recession parameter: $\Re$, production parameter: *P*, pure time delay: δ, and the associated dynamic response characteristics (DRCs) i.e. the steady state gain: SSG, and time constant: TC.

Pure time delay (δ)

The pure time delay is defined as the response time for rainfall to be first seen as riverflow. The value for a catchment is large if a) the rainfall is disconnected from the water table, i.e. it includes large unsaturated zone storage or b) rainfall is located only in its headwater sub-catchments. No pure time delay was identified for all the catchments (Table 3). This means rainfall is rapidly seen as riverflow.

In Thailand, a humid tropical region like Ghana, within the X113, P47 and P14 catchments which are of size, 129, 521 and 3853 km^2, respectively, Vongtanaboon (2004) estimated no pure time delay (Table 8). Similarly, in the 0.133 km^2 C1 Bukit Berembum catchment in Malaysia, Chappell *et al*. (2004a) estimated zero pure time delay. Again in Malaysia, Chappell *et al*. (2006) obtained a value of 15 minutes, for the 0.44 km^2 Baru catchment. The data series were in 5 minutes time-step implying time delay of 3. Examples, of estimates of pure time delay of catchments in temperate conditions can be seen in table 8.

One would have expected that, the large catchments in the River Pra basin with long rivers would have had long pure time delays if rainfall fell only in the headwaters. Thus, perhaps the catchments have similar rainfall in the downstream areas. The small catchment of River Nabogo in the North of the same country (see: Fig. 1 for location) has a delay of a day (see: Table 8) possibly due to the relative dryness of the catchment (Acheampong, 1988; Kranjac-Berisavljevic, 1999; FAO, 2005; Ahenkorah *et al*., 1994) or disconnected deep groundwater storage (Bates, 1962b).

This study and the above observations suggest that, perhaps, pure time delay may not solely depend on catchment size.

Exponential parameter (β)

The exponential parameter β estimated for the catchments in the River Pra basin are River Birim at Kade: -0.1409 and River Pra at Assin Praso: 0.2226, and Twifo Praso: 0.2037 (Fig. 4). A value of 0.0124 was obtained for the Leaf River catchment located in Collins, Mississippi in the USA by Young (2006). Hydrological interpretation of this parameter is that the higher the β value, the more quickly the runoff coefficient increases with increasing storage, and the greater the resistance to antecedent moisture conditions.

Time constant (TC)

Time constant (TC) is a measure of the 'residence time' of rainfall in the catchments (Young, 2003, 2005; Chappell *et al.*, 2006), calculated by using Equation 8. TC for the catchments within the River Pra basin is River Birim at Kade, approximately: 9.0 days [7.78 – 10.74 days] and River Pra at Assin Praso: 8.26 days [7.22 – 9.75 days] and Twifo Praso: 8.32 days [7.42 – 9.55 days] with

Table 8. Comparison of time constants of first-order DBM models of catchments in different climatic regions ranked by size

Catchment	Area (km^2)	Climate regime	Geology	TFM	NLF	Time constant	Reference
Plot	0.000015	Temperate	Acid soil	[1 1 0]	SSSM	14.5 minutes	Fawcett *et al*. (1997)
C1	0.133	Humid tropical	Saprolite	[1 1 0]	SSSM	23 days	Chappell *et al*. (2004a)
Baru	0.44	Humid tropical	Mudstone	[1 1 3]	BOSM	37 minutes	Chappell *et al*. (2006)
Coalburn	1.50	Temperate	Mudstone	[1 1 0]	BOSM	8.6 hours	Chappell *et al*. (2006)
Bottoms	10.60	Temperate	Limestone	[1 1 1]	BOSM	8.3 hours	Chappell *et al*. (2006)
X113	129	Humid tropical	Sedimentary	[1 1 0]	SSSM	2.3 days	Vongtaboon (2004)
P47	521	Humid tropical	Metamorphic	[1 1 0]	SSSM	6.14 days	Vongtaboon (2004)
P14	3853	Humid tropical	Granite/Metamorphic	[1 1 0]	SSSM	7.28 days	Vongtaboon (2004)
Nabogo	1950.00	Tropical continental	Voltain	[1 1 1]	SSSM	10.13 days	Ampadu (2007)
Kade	2126.67	Humid tropical	Birimian	[1 1 0]	EFSM	8.99 days	This study
Koumangou	6070.00	Tropical continental	Voltain	[1 2 1]	SSSM	12.10 days	Amisigo (2005)
Assin Praso	9347.31	Humid tropical	Birimian	[1 1 0]	EFSM	8.26 days	This study
Twifo Praso	20778.00	Humid tropical	Birimian/Tarkwain	[1 1 0]	EFSM	8.32 days	This study
Porga	27197.00	Tropical continental	Voltain	[1 2 0]	SSSM	8.21 days	Amisigo (2005)

Note: TFM: Transfer function model structure; NLF: Non linear filter; SSSM: Store surrogate sub-model Equation 9; BOSM: Bedford Ouse sub-model Equation 10 and 11; EFSM: Exponential function sub-model Equation 12. [No. of denominators, numerators, pure time delays].

the uncertainty on the estimated values given in the brackets. These indicate that within the River Pra basin all the catchments (ranging in size from 2126 to 20778 km^2) have similar residence time for rainfall to appear as riverflow.

Within the Malaysian rainforest (i.e. in similar climatic conditions), using the same DBM methodology (Chappell *et al*., 2004a, 2006), entirely different time constants were obtained for the 0.44 km^2 Baru and the 0.133 km^2 Bukit Berembun C1 catchment (see: Table 8). Chappell *et al*. (2006) attributed the vast difference in the time constant to the different geological formation underlying each catchment. In Northern Thailand, in the P14 and P47 catchments, located in the same climatic conditions (Boochabun *et al*., 2004; Vongtanaboon, 2004) and underlain by similar geology (Table 8), Vongtanaboon (2004) estimated similar time constants for these catchments using the same DBM approach (Table 8).

The similar time constants identified for the catchments in the River Pra basin are possible, because the catchments lie within the same climatic condition (i.e. wet semi equatorial) and vegetational zone (i.e. forest zone) and have soil cover which is predominantly Acrisols (i.e. Forest Ochrosol). The whole basin is also principally underlain by the same geological formation (i.e. the Birimian formation) with a small section in the middle of the basin underlain by the Tarkwain formation (Bates, 1962a; Dickson and Benneh, 1988; Atta-Qauyson, 1999). The Birimian formation consists of mainly granitoids (Ahenkorah *et al*., 1994) whilst the Tarkwain formation consists of sandstones, schists, quartzite, and phyllites (Dickson and Benneh, 1988; Bates, 1962a). The catchments also have similar topography, which stretches through a sequence of gently rolling hills with general elevation of between 250m and 300 m above sea level (Dickson and Benneh, 1988).

The time constant of River Nabogo located in the northern part of the same country estimated by Ampadu (2007) using daily time series of rainfall and riverflow and that of the catchments estimated by Amisigo (2005) compared with that of the catchments in the River Pra basin (Table 8) indicates that they are similar in residence time. The climate, vegetation and geological formation underlying these catchments are different resulting in different types of soil cover through weathering. The soil cover in the River Pra basin is predominantly Acrisols (locally called 'Forest Ochrosol') which is deeply weathered and well drained (Brammer, 1962; Ahenkoral *et al*., 1994; Attah-Quayson, 1999). It is possible rainfall within the catchments percolates much deeper into and through the soil before it ends up as riverflow. Deep movement of water in regolith beneath Acrisol on granite and its impacts on rainfall-riverflow processes have been observed by Chappell *et al*. (2007), in catchments within the South East Asia.

The Nabogo, Koumangou and Porga catchment which are located in the northern part of the country are underlain by the Voltain formation which consists of sandstone, shale, mudstones, and limestone (Bates, 1962a; Boateng, 1966; Dickson and Benneh, 1988). The soil cover is predominantly Plinthosol (locally called 'Groundwater Laterites') which is poorly drained and shallow

Table 9. Comparison of steady state gain (SSG) and riverflow coefficient (RC) of the catchments in the River Pra basin for the 1978 water year (i.e. from March 1, 1978 to February 28, 1979)

Catchment	Area (km^2)	AR (mm)	AF (mm)	RC=AF/AR	SSG
Kade	2126.67	1187.40	263.97	0.2223	0.1853
Assin Praso	9347.30	1236.30	164.32	0.1329	0.1158
Twifo Praso	20778.00	1425.20	157.70	0.1107	0.0983

AR: Annual catchment average rainfall, AF: Annual riverflow leaving the catchment.

Table 10. Comparison of observed (ET_O**)** and DBM estimate (ET_M**)** of evapo-transpiration losses and possible catchment leakages of the catchments in the River Pra basin for the 1978 water year (i.e. from March1, 1978 to February 28, 1979).

Catchment	AR (mm)	AF (mm)	SSG	ET_O=AR-AF (mm)	ET_M= (1-SSG)×AR (mm)
Kade	1187.4	263.97	0.1853	923.43	967.37
Assin Praso	1236.3	164.32	0.1158	1071.98	1093.14
Twifo Praso	1425.2	157.70	0.0983	1267.50	1285.10

AR: Annual catchment average rainfall, AF: Annual riverflow leaving the catchment, SSG: DBM model estimate of steady state gain.

(Brammer, 1962; Attah-Quayson, 1999). One would have, therefore, expected that the shallow and poorly drained catchment in the North would be flashier (i.e. shorter residence time) than the catchments in the forest area but this is not the case. The catchments in the North does, have a rock aquifer beneath (Bate, 1962b) increasing the time constant to about 10 days. Declining groundwater levels attributed to the numerous (3000) abstraction boreholes drilled in the North have been reported by Gyau-Boakye and Tumbulto (2000) and FAO (2005). Over time, this might lead to a longer time constant.

The observations in Malaysia and Thailand coupled with the results in the River Pra Basin and the studies from other climatic regions which are shown in Table 8, suggest that time constant may be highly influenced by the nature of the geological formation and regolith underlying a catchment. Time constant may be used to predict the type of geological formation and regolith underlying a catchment, especially in catchments located in similar climatic conditions with similar topography and soil cover.

Steady state gain (SSG)

The steady state gain (SSG) calculated by using Equation 7, demonstrates the relationship between the equilibrium input (rainfall) and output (riverflow) of the DBM TF model and indicates physical losses (i.e. SSG < 1) or gain (SSG > 1) in the system (catchment) (Young, 2005). This DRC is analogous to runoff coefficient (RC) which is a measure of how much of the gross total rainfall (for example in a water year) appears as riverflow after evaporation and transpiration losses. For instance, RC of say 0.2 of a catchment over one year or more indicates that 80% of the rainfall has been lost through evaporation and transpiration with the remaining 20% appearing as riverflow.

The SSGs obtained for the catchments in the River Pra Basin (Table 9) are Kade: 0.1853; Assin Praso: 0.1158; and Twifo Praso: 0.0983 for the 1978 water year **(**i.e. from 1st March, 1978 to 28th February, 1979). These estimates are comparable to those estimated in the tropics, by Vongtaboon (2004), for the 3853 km^2 P14, 521 km^2 P47 and 129 km^2 X113 catchments which are 0.2447, 0.1211 and 0.1998, respectively. These indicate, as expected, that there are high losses within all the catchments which is typical of tropical conditions due to high rates of evaporation. For example, from table 10, SSG of 0.1853 of River Birim at Kade indicates that with average annual rainfall of 1187.4 mm, during the 1978 water year, about 81.47% which is 967.37 mm is lost through evaporation and transpiration leaving only 18.53% (i.e. 220 mm), to appear as riverflow.

The annual evapo-transpiration rates in the forest zone of Ivory Coast for the Tai II and Banco II catchments are 1363mm/year and 1195mm/year respectively, and that of the Guma catchment, in Sierra Leone is 1146 mm/year, all in West Africa (Bruijnzeel, 1990). In the forest zone of East Africa Bruijnzeel (1990) reports of similar evapo-transpiration rates of 1337mm/yr for Kericho catchment in Kenya and 1381mm/yr for Mbeya catchment in Tanzania and in South East Asia, 1170mm/yr for the Ciwidey catchment in Indonesia. These values are comparable to the observed and DBM estimates for the catchments in the forest zone of Ghana. This indicates

that the estimates are reasonable and probably, there are no leakages in the catchments.
In Ghana, according to Bates (1962b) generally, only about 1 to 10 per cent of rainfall ends up as riverflow in the rivers. This observation was based on few streams which were gauged around that time. However, the DBM model estimate of 18.53, 11.58 and 9.83% of the rainfall to appear as riverflow for Kade, Assin Praso and Twifo Praso catchments, respectively, are in agreement with the observation by Bates (1962b).

Table 9 reveals that, there is no significant difference between the catchments RCs (i.e. actual water balance term) and their SSGs (i.e. model water balance term). The slight difference between them might be due to modelling error. This suggests that the SSGs from the DBM TF model could be used as a sufficient representation of catchments actual water balance. However, recently, Chappell *et al.* (2006) have introduced a procedure where the effective rainfall is normalised to give SSG the same as the RC.

CONCLUSION

The DBM transfer function rainfall and riverflow modelling approach is very robust (Young, 1998, 2001; Lees, 2000) and has been used effectively to model rainfall and riverflow behaviour of large catchments in the forest zone of southern Ghana. The approach was applied to catchments of size between 2126.67- 20778km^2 and the following conclusions can be drawn:

1. The DBM TF modelling process through SDP analysis has revealed the nature of nonlinear behaviour for the riverflow generation process in the forest zone of Ghana. Thus, exponential distribution which implies that within the forest zone, as the catchment wets up the instantaneous runoff coefficient (i.e. proportion of rainfall generating riverflow) increases up to a point and remain constant where riverflow generation becomes a linear relationship between rainfall.
2. The estimated parameters exponential parameter (β), recession parameter ($\Re$), production parameter (P), pure time delay (δ) and the associated dynamic response characteristics (DRC) of time constant (TC) and steady state gain (SSG) suggest that riverflow generation within the catchments were not flashy and that their response is dominated by single water pathway.
3. Analysis of the time constants suggests that the riverflow behaviour within the catchments is similar, with all of the catchments having high storages averaging about 8.5 days. The similar storages within the catchments have been linked to the similar geologies underlain them (Bates, 1962a; Boateng, 1966; Dickson and Benneh, 1988).
4. Comparison of the estimated SSG (i.e. the model water balance) with the RC (i.e. catchments actual water balance term) showed no significance difference between the two parameters thus, SSG could be used as a sufficient representation of the catchment water balance.
5. The analyses of the estimated TCs coupled with estimates from other climatic regions indicate that riverflow generation processes within a catchment is highly influenced by the geological formation underlying the catchment and that with a known time constant it may be possible to predict the nature of the geology underlain a catchment if the catchment is located within the same climatic conditions with similar vegetation, soil cover and topography.
6. The DBM modelling has led to development of mathematical relationships between rainfall and riverflow which could be used in simulating flows in the basin. The approach is recommended for the forecasting of riverflow in the country which would greatly improve the government's planning of water supply provision in the country.

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TURBULENT MODELING FOR NON-NEWTONIAN FLUID IN AN ECCENTRIC ANNULUS

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ABSTRACT

The aim of this study is the numerical simulation of non-Newtonian fluid in an eccentric annulus with rotation of the inner cylinder. To evaluate the modeling accuracy, the computational domain has been defined such that the results of this study can be compared with experimental results of Nouri *et al*. (1997). At first the governing equations were selected according to the follow physics. Then by selecting the appropriate network, the physical equations were solved using CFD technique and finite volume method. The turbulent flow modeling has been performed using K-ε, K-ω and K-εRNG methods. The obtained results of the study showed that the K-ω method is compatible with experimental data of Nouri *et al*. (1997) and thus is a more appropriate method for numerical analysis of this type of flow.

Keywords: CFD, turbulent modeling, non-Newtonian fluid, finite volume.

INTRODUCTION

Expect in areas very close to the wall, in turbulent flows, flow layer shape is not easily detectable due to the severe blending processes, and the fluid molecules follow a clear path. In other words, turbulent flow is a type of fluid flow in which the fluid is under fluctuation flow. In a turbulent flow, velocity in any point is always under fluctuations and variations, both in size and motion direction, so that detecting position of each particle inside the follow field and also at any time is difficult. The same permanent and non-specific fluctuations in velocity size can be observed in the size of pressure, temperature and density of each point. Generally, turbulent flows have the following features:

- Spatial and temporal irregularities
- Spatial and temporal continuous spectrum
- High Reynolds
- Increased dissipation of energy and momentum
- Being three-dimensional
- Being periodic

Given the unpredictable nature of turbulent flows, therefore, experimental relations are of special importance for their modeling. Regarding experimental simulation of turbulent flow, many studies have been conducted that differences between these studies depend on fluid type, flow geometry type, range of velocity: pressure and working temperature. Brighton and Jons (1964) experimentally investigated turbulent flow of Newtonian fluid in a fixed annulus. The examined fluid was air which is a compressible fluid. Escudier *et al*. (1994) and Escudier and Gouldson, (1995) examined laminar and turbulent flows of Newtonian and non-Newtonian fluid in a fixed annulus. In the following Nouri *et al*. (1993) fully investigated turbulent flows in an annulus. The external tube is coanstant and the internal tube is rotating. Both a Newtonian and a non-Newtonian fluid have been considered in the performed experiments (Nouri and Whitelaw, 1997).

However, experimental modeling of turbulent flow in real scale is a very costly and timely process and if by applying a suitable numerical model, the flow can be analyzed, certainly there will be enormous savings both in time and cost.

For example, in this study we seek to perform numerical modeling of fluid flow in an eccentric annulus, and the obvious example is in oil industry and drilling sector.

After that drilling bore which is often eccentric with the well, began drilling, mud of drilling which is considered a non-Newtonian fluid comes out of the bore and the well diameter. However, after drilling and in order to strengthen the well and provide an insulating membrane around the well, a metal shell with a diameter less than the well diameter is installed in the well with a distance from the well and eccentric, and mud of drilling is pumped out using a fluid pump inside the well and then cement slurry is pumped for the mentioned purposes.

This industrial example can be simulated with a numerical model. The aim of this study is to examine various numerical methods for modeling turbulent flow of non-

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Newtonian fluid in an eccentric annulus with rotation of inner cylinder. Finally, the appropriate model which has the most conformity with experimental data will be introduced as the appropriate model.

Computational domain

Computational domain has been considered as a periodic cylinder. The diameter of outer cylinder is 40.1 mm and the inner cylinder is 20mm (Fig. 1). The surface of cylinders is assumed as the wall, and the two ends as periodic boundary conditions and the space between the two cylinders as non-Newtonian fluid. Hexagonal cells the organization is used for networking. In figure 2 you can see Line coordinates of profile investigation of flow variables:

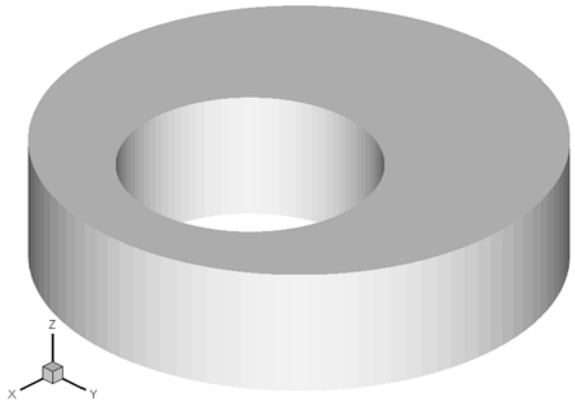

Fig. 1. Geometry of the solution domain.

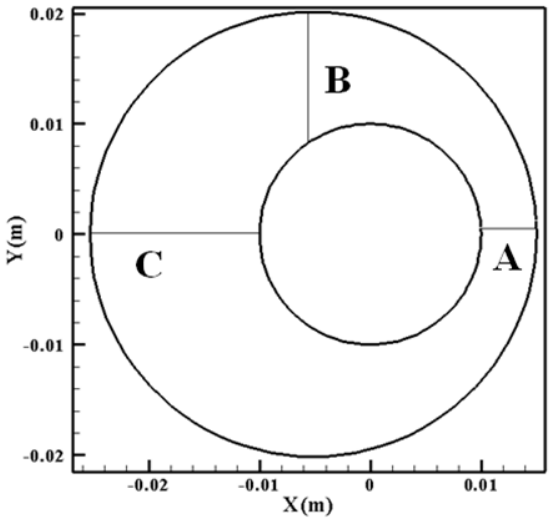

Fig. 2. Line coordinates of profile investigation of flow variables.

Computational domain was chosen such that results of this numerical simulation can be compared with experimental data of Nouri and Whitelaw (1997), in figures 3 and 4 you can see a look of the experimental device used in the study.

The governing equations of non-Newtonian fluid

The equations governing flow field resulting from motion of incompressible non-Newtonian fluid with constant density are as follows based on Navier Stocks equations.

$$\frac{\partial \rho}{\partial t} + \frac{\partial}{\partial x_i}(\rho u_i) = 0 \quad (1)$$

$$\frac{\partial}{\partial t}(\rho u_j) + \frac{\partial}{\partial x_i}\partial u_i \partial u_j = -\frac{\partial P}{\partial x_i} + \frac{\partial}{\partial x_i}(\mu + \mu_t)\left(\frac{\partial u_i}{\partial x_j} + \frac{\partial u_j}{\partial x_i}\right) + \rho g_i \quad (2)$$

Viscosity is defined based on the following exponential rule.

$$\mu = K \times \gamma^{(n-1)} \quad (3)$$

That γ shear stress rate and K concentration rate and n is the index of fluid behavior such that when n equals 1, the fluid is Newtonian and when n is smaller than 1, the fluid is non-Newtonian.

As seen in the above equation, besides non-Newtonian viscosity, a new coefficient has been added to the equation which is unknown and it's a turbulent viscosity coefficient that must be determined so that the problem would be closed and could be solved. Models based on turbulent viscosity are classified into various methods based on how the turbulent viscosity coefficient is calculated. Zero equation models, one equation models and two equation models are common models based on turbulent viscosity hypothesis. Unfortunately, there is no turbulent model that can be used for all conditions and different problems; and choosing the turbulent model depends on considerations such as flow physics, the obtained experiences of simulation for particular problems, the required accuracy, the power of computational resources (power of computer) and the available time for performing computations. Of course, capabilities and limitations of all models and conditions must be considered for selecting an appropriate turbulent model. In the following some of the two equation models used in the present study will be studied.

The simplest turbulent models that are relatively perfect are two equation models; because solving two transmission equations separately causes that turbulent velocity and the character length are determined separately. The standard K-ε model is in this group of turbulent models and is considered as one of the most powerful turbulent models for engineering problems. Being powerful, economic calculations and acceptable in a wide range of turbulent flows cause the popularity of this model in industrial and heat transfer issues. K-ε model is a semi-experimental model and its equations have been created based on experimental observations and phenomenological considerations.

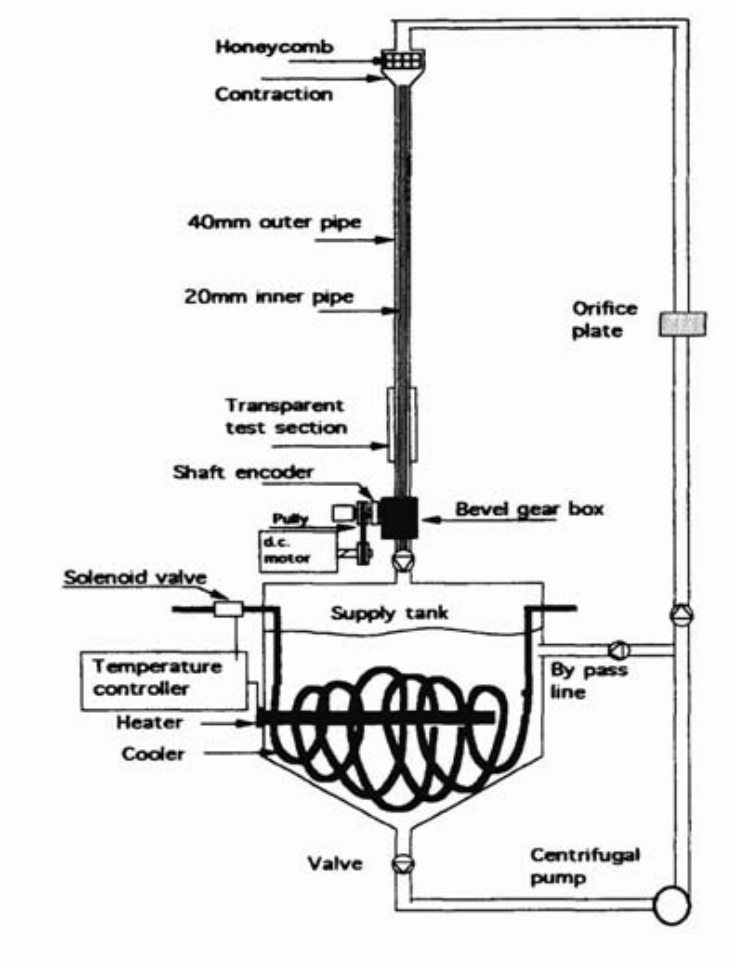

Fig. 3. Experimental setup in Nouri and Whitelaw (1997) .

Relations of turbulent modelK-ε are as follows:

$$\rho\frac{Dk}{Dt}=\frac{\partial}{\partial x_i}\left[\left(\mu+\frac{\mu_t}{\delta_k}\right)\frac{\partial k}{\partial x_i}\right]+G_k-\varepsilon \quad (4)$$

$$\rho\frac{D\varepsilon}{Dt}=\frac{\partial}{\partial x_i}\left[\left(\mu+\frac{\mu_t}{\delta_\varepsilon}\right)\frac{\partial \varepsilon}{\partial x_i}\right]+C_{\varepsilon 1}\frac{\varepsilon}{k}G_k-C_{\varepsilon 2}\frac{\varepsilon^2}{k} \quad (5)$$

And the vortex viscosity is expressed by the following relations:

$$\mu_t=\rho C_M\frac{k^2}{\varepsilon} \quad (6)$$

Constant coefficients of the model are expressed as follows:

$$C_\mu=0.09 \quad (7)$$

$$C_{\varepsilon 1}=1.92 \quad (8)$$

$$\delta_k=1 \quad (9)$$

$$\delta=1.3 \quad (10)$$

which K is kinetic energy of turbulent flow and e is dissipation rate of turbulent.

K-ε RNG model

This model has been obtained using statistical methods and is similar to the standard K-ε model. RNG model has higher accuracy in rapidly strained flows due to having additional terms in solving ε equation. Rotation effects on turbulence have been included in the RNG model which causes its higher accuracy in vortex flows. In the RNG model, an analytical formula is used for calculating a turbulent parentel number; while in the standard K-ε model, this number is entered the software by the user, and thus in all stages, it has a constant value. If the standard K-ε model is a model for flows with high Reynolds numbers, in the RNG mode, to determine the effect of viscosity on the flow an analytical differential formula has been used to model flows with low Reynolds number. Of course, the ability of the viscosity effect on the flow depends on the way of performing calculations

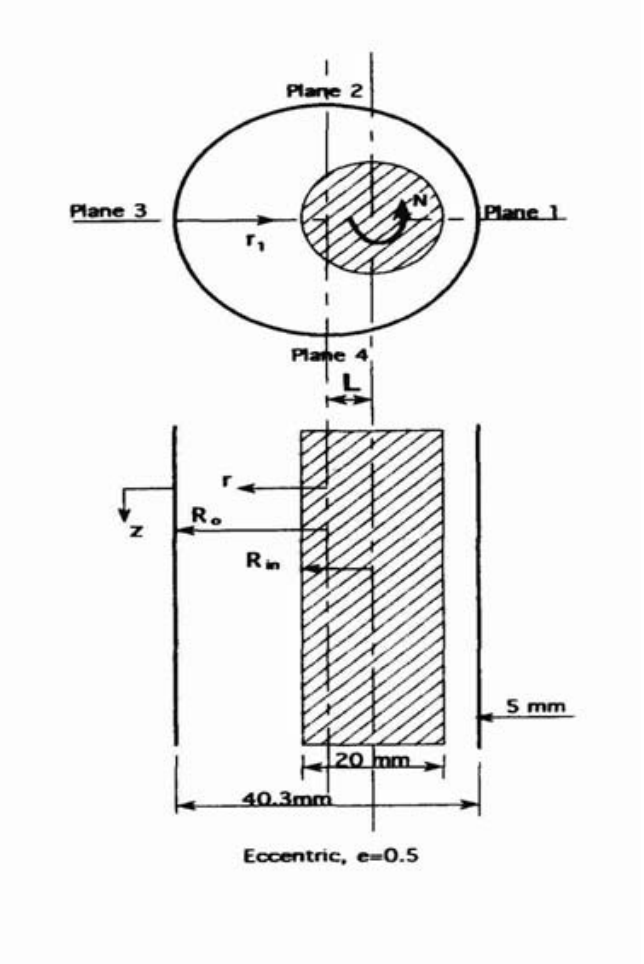

Fig. 4. Test section and co-ordinate system in Nouri and Whitelaw (1997).

and behavior near the wall area. These advantages have caused that RNG model has better accuracy and reliability in a broader range of flows than the standard K-ε model.

K-ω turbulent model

In this model by writing two transmission equations (one for turbulent kinetic energy and the other for dissipation rate of turbulent kinetic energy) and solving them, a longitudinal scale and a velocity scale are obtained that vortex viscosity is created from these values and using dimensional analysis. As K-ε model, k equation is solved for determining velocity scale, and ω equation is solved for determining longitudinal scale. Ω which is usually called specific dissipation has the following relation with K and ε:

$$\omega = \frac{\varepsilon}{\beta_* k} \quad (11)$$

Given the above ω definition, It is proved that applying boundary condition for ω equation is simpler that ε equation. High Re k-ω turbulent model equation is often called the standard k-ω model is as follows:

$$\frac{\partial}{\partial t}(\rho k) + \frac{\partial}{\partial x_j}(\rho u_j k) = \frac{\partial}{\partial x_j}\left[(\mu + \sigma_* \mu_t)\frac{\partial k}{\partial x_j}\right] + P_k - \beta_* \rho \omega k \quad (12)$$

$$\frac{\partial}{\partial t}(\rho \omega) + \frac{\partial}{\partial x_j}(\rho u_j \omega) = \frac{\partial}{\partial x_j}\left[(\mu + \sigma \mu_t)\frac{\partial \omega}{\partial x_j}\right] + \gamma \frac{\omega}{k} P_k - \beta \rho \omega^2 \quad (13)$$

In which P_k is:

$$P_k = \mu_t \left(\frac{\partial u_i}{\partial x_j} + \frac{\partial u_j}{\partial x_i}\right)\frac{\partial u_i}{\partial x_j} \quad (14)$$

And μ_f in the model is calculated as the following:

$$\mu_t = \gamma_* \frac{\rho k}{\omega} \quad (15)$$

the used constants in the model are as follows:

$$\beta = \frac{3}{40}, \beta_* = \frac{9}{100}, \gamma = \frac{5}{9}, \gamma_* = 1, \sigma = 0.5, \sigma_* = 0.5$$

Assumptions and equation solving method

Finite volume method is used for solving physical equations (energy momentum continuity,…). In the finite volume method, physical equations are used in the integral form. There are two pressure-base and density-base solvers for solving integral equations. Under this condition, the flow is incompressible and the heat transfer is also ignored. Given the incompressibility of the fluid flow, density variations due to the variations in pressure and temperature, do not affect the flow equations; therefore, solution type is selected pressure-base solution. In order to compare the obtained results of this study with the experimental data, characteristics of non-Newtonian fluid is defined based on the study of Nouri and Whitelaw (1997). Concentration index is defined 0.04 and exponential index 0.75. Walls have non-sliding condition. In periodic boundary condition, the constant mass flux for non-Newtonian fluid flow was considered 1.3, 2.6 and 5.2kg/s, and also the velocity of the inner cylinder rotation is considered 0, 300 and 600 rpm based on the prionary guess of periodic boundary. Then repetition is continued until the convergence of residues.

RESULTS AND DISCUSSION

In this study, numerical modeling of non-Newtonian fluid in an annulus with rotation of the inner cylinder has been performed. The computational domain, flow physics and fluid type were selected such that the results of this study can be compared with available experimental data. At first, the appropriate network is generated and independence of the solution of the computational network was also investigated for the three networks of 8000, 20000 and 50000. Eventually, the network with 20000 cells was selected as computational network. The maximum difference of results between the two 20000 and 50000 networks is about 4 percent. In figure 5 you can see a view of the produced network:

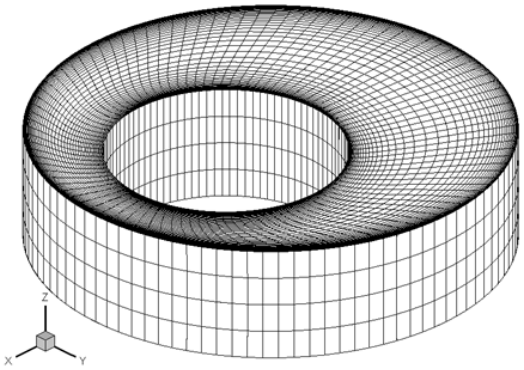

Fig. 5. Networking solution domain.

In figures 6 and 7, sensitivity of normalized horizontal and vertical velocity components to the number of computational network cells can be observed:

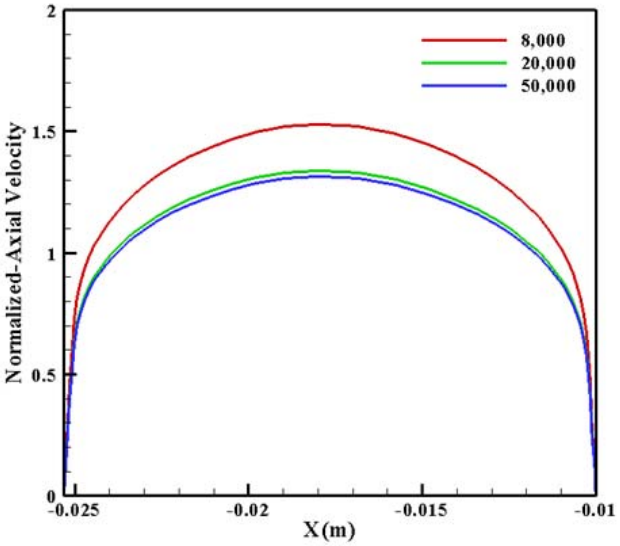

Fig. 6. Examining sensitivity of normalized flow of axial velocity to the number of computational network cells.

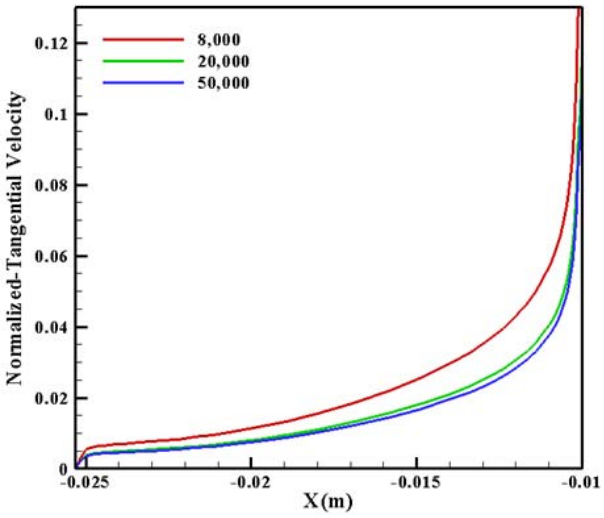

Fig. 7. Examining sensitivity of the normalized tangential velocity to the number of computational network cells.

For verifying the numerical modeling, experimental data of normalized flow of axial velocity and normal tangential velocity have been used.

In figure 8, you can see the investigation of sensitivity of the normalized axial velocity to the turbulent model along with the experimental data.

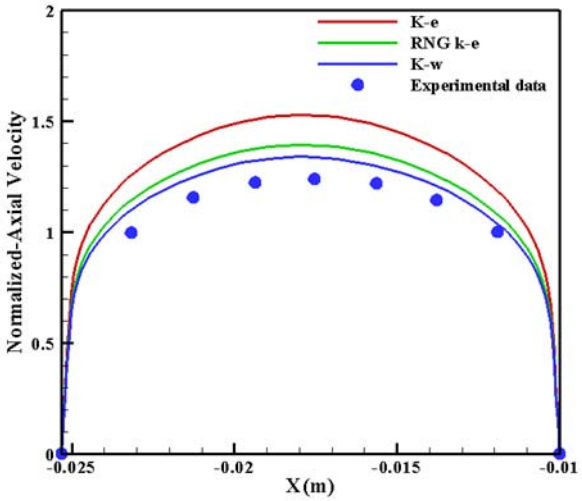

Fig. 8. Examining the sensitivity of normalized axial velocity to the turbulent model.

In figure 9, sensitivity of normalized tangential velocity to the turbulent model along with the experimental data is visible.

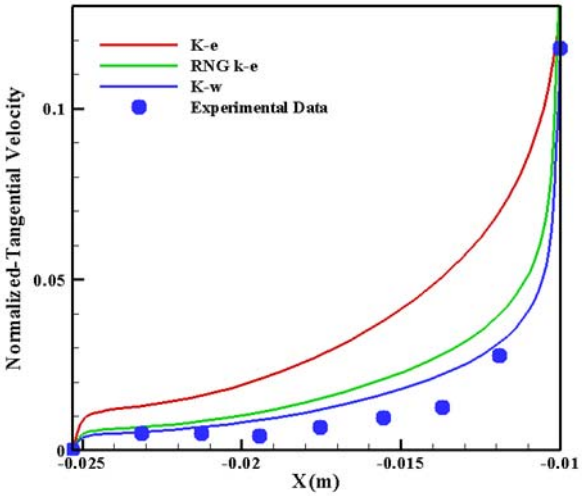

Fig. 9. Examining the sensitivity of normalized tangential velocity to the turbulent model.

As it can be observed in figures 8 and 9, k-w model is more compatible with experimental data of Nouri *et al.* (1993) and thus is a more appropriate model for analyzing this type of flow. The maximum difference of the results of this model and experimental data is about 8 percent.

CONCLUSION

The aim of the study is to select an appropriate numerical model for analyzing non-Newtonian fluid flow in an eccentric annulus. For verifying the turbulent flow model, experimental data of Nouri and Whitelaw (1997) has been used. Thus, computational domain, characteristics of the fluid and flow were selected such that to be compatible with experimental conditions of these researchers.

First, an appropriate model was generated for numerical solution of basic equations on the computational domain. Independence of solution of the computational network was also examined for the three 8000, 20000 and 50000 networks. Eventually, the network with 20000 cells was selected as computational network. The maximum difference of results of the two 20000 and 50000 networks is about 4 percent. In the following, by applying the three models, results of normalized axial and tangential velocities along with the experimental data have been drawn. The results of this study showed that k-w model is more compatible with experimental data and thus is an appropriate model for analyzing this type of flow.

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CHEMICAL ASSESSMENT OF NATURAL SPRINGS OF SINDH PAKISTAN

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ABSTRACT

The focus of the field study reported here are the water quality issues of twenty four natural springs located in Karachi, Thatta, Jamshoro and Tharparkar districts of the province of Sindh, Pakistan. The samples collected from these springs were analyzed for water temperature, electrical conductance, TDS, salinity and pH and major cations (Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}) and anions (Cl^{-}, HCO_3^{-}, SO_4^{2-}) by electric probe, flame atomic absorption spectroscopy, uv/visible spectrometric and volumetric methods. A paired t-test was used to assess 24 pre- and post-sampling data during the period 2000 - 2001. The order of relative abundance was detected for major cations was Na^{+}> Ca^{2+}> Mg^{2+}>K^{+} and anions Cl^{-}>HCO^{3-}>SO_4^{2-}. The values of positive correlation in the number of pairs showed the origin of transport from same lithology. The factor analysis (FA) was applied to water quality and the first two factors identified were responsible for approximately 80% of total variance. The hierarchical cluster analysis was made using the ward method for group relationship and Pearson correlation coefficient derived for parametric relationship, percent sodium (% Sodium), Langelier saturation index (LSI), sodium adsorption ratio (SAR), residual sodium carbonate (RSC), and permeability index (PI) were made for quality of data and identifying the suitability of water for drinking, industrial and agricultural purposes. The residual sodium carbonate and sodium adsorption ratio indicated for more than 75% springs, were suitable for irrigation purposes.
The spring water showed spatial variations among physico-chemical parameters and results were compared with WHO guidelines for drinking water.

Keywords: Sindh, springs, salinity, water quality, chemical assessment.

INTRODUCTON

The springs are natural escapade of water flow from ground to the earth's surface, instead of a dug well. It seems to be valuable but the quality is questioned. Water quality is important for health and economic development; chemical composition of water varies with reasons, anthropogenic influences and natural processes Simeonov *et al.* (2003). The spring water was classified in different types depending upon the composition of major ions HCO_3^{-} , Cl^{-}, SO_4^{2-}, SiO_2 , Na^{+}, K^{+}, Ca^{2+} and Mg^{2+}, mainly originated from dissolution or mineralization of rocks (Davisson *et al*., 1994; Afsin *et al*., 2006).

In the context of springs, these natural waters are more concentrated in areas where there have been volcanic activity in recent ages or the past. The quality determined was found rich in sodium by Risenhoover and Peterson (1986), the condition is often accompanied by high levels of calcium 3135 mg/L (Bechtold, 1996), chloride 5500 mg/L by Otero and Soler (2002), bicarbonate 570 mg/L from Howari *et al.* (2005) and certain levels of potassium 2200 mg/L reported by Marie and Vengosh (2001).

Natural mineral water is characterized by its salts content, trace elements or other constituents and where appropriate, by certain effects; also, by being in its original state; both the conditions have been preserved from all risk of pollution. The composition, temperature and other essential characteristics of water of underground origin must remain stable at source within the limits of natural fluctuation; and may not be affected by possible variations in the rate of flow. (Misund *et al.,* 1999; Baba *et al.,* 2008). Salts, sulphur compounds, and other compounds are among the substances that can be dissolved in the water. These minerals have various effects on the health of a person (Burton and Cornhill, 1977; Burton *et al.,* 1980; Epstein and Zavon, 1974). The management and conservation strategies for cold water springs has been reported by Jose Barquin and Mike Scarsbrook (2008). Reimann and de Caritat (1998) have reported natural concentrations in water from different parts of the world. The springs, other than in Sindh province of Pakistan have been studied e.g by Waring (1965) for general characteristics of temperature, flow and associated rocks. Manzoor *et al.* (2002) assessed reservoir

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temperature of thermal springs in northern areas of Pakistan by isotope geothermometry. Farida–Hewitt (1998) contributed her work on spring at Karakorum as a source of water in scarce and its therapeutics. The Shah and Danishwar (2003) studied fluoride in spring water in Naranji area. Whereas Hussain *et al.* (2010) determined springs water quality of Samahni valley for biological life, temperature and pH. However boron contaminations in spring water of Neelum valley was studied by Akram *et al*. (2011). Todaka *et al.* (1999) reported on geothermal characteristics of hot springs of Karachi. Javed *et al.* (2009) highlighted balneology considerations of mineral springs of Manghopir, Karachi. Physicochemical and biological assessment of some springs from Sindh have been reported by Jahangir *et al.* (2001) and Leghari *et al.* (2005, 2006). The objectives of present work is to report comparative studies of springs for chemical assessment and to highlight these valuable asset to academia, consumers, policy makers for better management to maintain quality of drinking, agriculture, tourism and therapeutics of springs in the region. Most of these springs were not reported earlier other than this laboratory. In this study water of 24 springs were collected at different interval during 2000-2001, the locations are showed in figure 1. The results obtained are compared with permissible limits for drinking, livestock management and irrigation purposes to evaluate the natural resources available.

MATERIALS AND METHODS

The representative sample of spring water was collected from each location in cleaned plastic or glass container. The sampling bottles were rinsed several times ≥3 with sampling water prior to sample collection (APHA, 2005). Temperatures of air 1 m above surface water and water were measured by immersing mercury thermometer in the source. The conductivity, salinity and total dissolved solids were estimated with Orion 115 conductivity meter in the field and pH was recorded with Orion 420A pH meter in the laboratory. In meantime the collected samples were stored in cool box, transported to laboratory and filtered if necessary through 0.45µm membrane and analyzed immediately following the protocol of standard

Fig. 1. Springs indicated in map of Sindh and province location in Pakistan along neighboring countries (Source: Google Earth).

methods (APHA, 1992). A portion of fifty milliliter of water was preserved by 2ml 10% HCl and was analyzed within 30 days for sodium, potassium, calcium and magnesium by air acetylene flame atomic absorption spectrometer at 589.0, 766.5, 422.7 and 285.2 nm respectively at the conditions recommended by the manufacturer (Varian Spectr AA-20, Australia). The analysis was carried out in triplicate (n=3) with integration time 3 sec and delay time 3 sec. Five different concentrations of elemental solution were used to establish a calibration curve from standard salts and earlier of this standardized with primary salts. All standard methods of titrimetric, spectrophotometric and atomic absorption spectrometry were applied and standardized for valid data collection. The total hardness was measured by titration with ethylenediamine tetraacetic acid disodium salt using erichrome black T as indicator at pH 10 (APHA, 2005). Total alkalinity was determined by hydrochloric acid titration using methyl-orange as indicator for endpoint and chloride was analyzed by silver nitrate ($AgNO_3$) titrant using potassium chromate (K_2CrO_4) solution as an indicator. Sulphate was determined at acidic pH by spectrophotometrically using barium sulphate turbiditimetric method (APHA, 1992).

Geography of the study area

Sindh is located at the western corner of south asia, geographically Sindh province is third largest in size and second most populated in Pakistan, stretching about 579 km from north to south and 442 km (extreme) or 281 km (average) from east to west, with an area of 140,915km^2. The province may be regarded as a low and flat, Most of springs exist in the hilly areas from south west of central to lower Sindh and ends within coastal areas of Thatta and Karachi. Although most of the springs are scattered in arid region, localities are benefited from these valued springs (Table 1). Sindh province is situated in a subtropical region; hot in the summer and cold in winter. Temperatures frequently rise above 46°C between May-August and minimum average temperature of 22°C occurs during December and January. The annual rainfall reported by Pakistan Meteorological Department (2011) averages 400 mm, mainly during July and August since 1971-2000. The southwest monsoon wind begins to blow in mid- February and continues until the end of September whereas the cool northerly wind blows during winter months from October to January. There are no surface water resources to recharge aquifers, other than annual precipitation within the study area. Due to

Table 1. Description of springs, location and discharge flow.

S. Id.	Sampling Stations	Location (GPS)	Description of sampling stations	Discharge flow
St.1	Manghopir I	24°59’16.5”N 67°02’34.4”E	Spring inside Hall, located at north west of Karachi city, Size of spring length 40, width 5 and depth 5 feet (altitude 259ft.),separate bath for men and women	The water receipt, discharge balances the amount withdrawn
St.2	Manghopir II	24°59’17.0”N 67°02’34.1”E	Hot spring open well bath of length 2, width 3 and depth 4 feet.	do
St.3	Manghopir III	24°59’14.9”N 67°02’36.7”E	Size of spring 4 ft each of width and depth (Altitude 288ft), located in south east of about 20-30 m from St.1 and St.2	do
St.4	Manghopir IV	24°58’47.9”N 67°02’05.6”E	A pond about 50feet long, 40feet wide and with the depth of 5feet. (Altitude 224ft.) The surface of earth is rocky and there are some trees inside. A number of crocodiles inhabit in the pond. Open view by grill and a gate.	The pond is perennial and water oozes from bottom.
St.5	Abdullah Shah Ghazi	24°48’36.9”N 67°01’49.6”E	It has dimensions of 10 x 10 feet with depth of 2 to 3feet. (Altitude - 47ft).	The water receipt, discharge balances the amount withdrawn.
St.6	Shree Ratneshwar	24°48’45.8”N 67°01’36.9”E	The spring is located in natural depression in a cave at the foot of tomb in south within a constructed area. It is 1Km far from sea beach Clifton and about 500m far from spring Abdullah Shah Ghazi, Karachi (St.5). (Altitude - 39ft)	do

Continued…

nonavailability of the surface water, groundwater is mostly used to meet all water requirements.

Geology of the study area

Pakistan is spread along in contact between the Indian and Eurasian plates and is situated in the north western corner of the Indian plate. To its south-east is indian plate and to the north the Asian/Karakorum continental plate. Tertiary units of the western fold belt comprises mainly Sulaiman, Khirthar and Pab and are variety of regional names (Hemphill and Kidwai, 1973; Shah, 1977) and correlation between regions and dating is at a reconnaissance level. The Sulaiman and Khirthar formations include early to mid eocene rocks of Ghazi and Khirthar marine shelf facies. These are overlain by marine facies of the oligocene Nari formation and miocene Gaj formation Smewing *et al.* (2002). The Khirthar mountains extend to the south east ward curving for about 240km from northwestern extremity of Sindh Mirjat *et al.* (2011). The northern half of the range is flanked by sharp hills

Table 1. continued…

S. Id.	Sampling Stations	Location (GPS)	Description of sampling stations	Discharge flow
St.7	Baba Bukhari	24°52’36.8”N 67°01’36.9”E	It is located on Shahrah–e– Faisal (Airport road), approximately 4km from Drigh road railway station. Bath for men and women. The water pool measured for length and width of 12 ft and depth 3feet. (Altitude 77ft.)	28L/ Sec
St.8	Dhabeji I	24°50’07.9”N 67°06’06.6”E	It is located near tomb of Baba Bukhari, Dhabeji, district Thatta	3-4L/min
St.9	Dhabeji II	-	This is located about 3 Km from the east of the Dhabeji railway station with Size of 4x6feet and water depth of 1 to 2feet	5-6L/Sec
St.10	Gharo I	24°25’07.9”N 67°31’20.3”E	Spring located eastern side of Bhambhore bridge, district Thatta	1L/Sec. (Apparent at low tide)
St.11	Gharo II	-	Spring located western side of Bhambhore bridge	5L/Sec. (Apparent at low tide)
St.12	Rannikot I	25°53’54.5”N 67°52’49.1”E	A main spring at Mohn gate, 30Km in the west from Indus highway from the town Sunn, district Jamshoro	70 L/Sec.
St.13	Rannikot II	-	A natural pool, near Merrikot lower.	The water receipt, discharge balances the amount withdrawn.
St.14	Rannikot III	-	Natural pool near Sunn gate, fort entrance.	do
St.15	Lal Bagh I	-	200-300m far from east side of Lal Bagh graveyard located 2 Km from Sehwen city and 0.5Km from Indus highway district Jamshoro	do
St.16	Lal Bagh II	-	The constructed pond in a cubical shape, about 50m far from spring I, used for drinking and bathing separately for men and women	do
St.17	Lakhi Shah Saddar I	26°16’50.98” N, 67°50’41.33”E	About 20 x 12 feet with depth o f 2 to 3 feet (Altitude 260ft), district Jamshoro	7 L/Sec.
St.18	Lakhi Shah Saddar II	-	About 200m north of main spring (St.17), at a small distance appears as pool of about 14 x 8 feet with depth about 1 to 1.5 feet (Altitude 258ft).	4 L/Sec.

Continued…

Table 1. continued…

S. Id.	Sampling Stations	Location (GPS)	Description of sampling stations	Discharge flow
St.19	Lakhi Shah Saddar III	-	Located of 2-3km from main hot spring (St.18) in the southwest within the same valley and appears as a small pool of about 2 x 2 feet with a depth of about 0 .5 ft. (Altitude 150 ft).	0.5 L/min.
St.20	Ghazi Shah	26.25N, 67.30E	Escape opening of about 7.5 feet wide and 9 feet height with water depth of 1.5 feet. (Altitude 500ft). Spring is about 32Km in south west of Johi town, 4Km from village Ghazi Shah, Taluka Johi, district Dadu	3 L/Sec.
St.21	Kai	26.25N, 67.42E	Size of 20 x 25 feet with water depth of about 6 to 8 ft About 40 Km west of Sehwan and 25 km from Jhanghara village, Taluka Sehwen, district Jamshoro	1 L /Sec.
St.22	Thoba	-	Natural pond at village Loni about 8km far from Jhangara, Taluka Sehwen	-
St.23	Khumb	-	Stream about 15km from Jhangara, Taluka Sehwen	-
St.24	Inchaile Sir	24.25N, 70.40E	Spring located to 1km from Nagarparkar town , district Mithi	2 L/min.

do =The water receipt discharge, balances the amount withdrawn
- = Not available

developed on the tertiary Nari series, overlying them to the east is the Gaj group. In geological map of Pakistan (Fig. 2) Sindh have been indicated and classified with four important geological characteristics e.g quaternary, tertiary recent, late mesozoic and tertiary marine shell sediments and are represented vertically. The geological time scale is divided in three main eras, they gradually become older in order of cenozoic, mesozoic and paleozoic. The quaternary and tertiary epochs are among of cenozoic era and the mesozoic eras are indicated in the figure 2 and the geology is explained following in three regions.

Karachi region: The two formations are continuously exposed from the western coast of Karachi to northern Sindh. These exposed rocks are Nari and Gaj formations of oligocene (tertiary) and miocene age (quaternary) respectively (Fig. 2). The Nari and Gaj formations consist of limestones, shales and sandstones which are divided into several smaller units Shahid *et al.* (1996). Nari formation predominantly consists of greenish gray, brown to buff colored, fine to coarse grained, massive, cross bedded sandstones. Occasionally conglomeratic and calcareous in nature. The division is widely exposed on the lower slopes of eastern Khirthar range in low grounds of Thana Bola Khan up to Jungshahi. The Gaj formation is mainly composed of calcareous sandstone and limestone interbeded with shale. The sandstone is soft, fine grained and yellowish brown to gray in color. Occasionally the limestone is cream colored when fresh but brown when weathered (Farshori, 1972). A number of springs were found in Karachi and Thatta districts from St.1to St-11. Some of the hot springs were found in faults and are shown in a conceptional model of Karachi geothermal system by Munawar (2009) (see Fig. 3).

Dadu and Jamshoro region: The lakhi springs of eocene within lakhi range (Table 2) is mainly composed of tertiary rocks, the north south thrust fault of lakhi range runs along the eastern flank for most of 35 miles from Lakhi Shah Saddar to Rannikot fort and seems to extend further southward (Farshori, 1972). The samples St.17-19 were collected from lakhi range (Fig.1). The rannikot group is named after the Rannikot fort in laki range and is in paleocene age with its lithological characteristics of yellowish brown sandstone and shale interbedded with limestone. The upper contact of the group with ghazij formation and lower contact with pab formation are

conformable Blandford (1876). The samples St.12-14 were collected from the rannikot fortress and St.19-23 from the same region are shown in figure 1.

Thar region: The whole area is covered with extensively thick cover of sands dunes, extending down to an average depth of 80m. Surface rock exposures are almost absent, except limited outcrops of granitic basement in Nagarparkar. A few scattered outcrops of mesozoic and tertiary strata are exposed across the Indo-Pakistan border. Thar desert rests upon a structural platform where granitic basement is at shallower depths Zaigham and Ahmed (1996). The granite basement has pre-jurassic rifting, which caused flexure and the ultimate development of the Thar basin. The basement shows rise towards southeast and deepening towards northwest, as a result of paleozoic-mesozoic divergent tectonics. The consistent trends of the stratigraphic sequences from mesozoic to tertiary periods indicate that the incipient rifting of the basement was pre-depositional. The younger formations

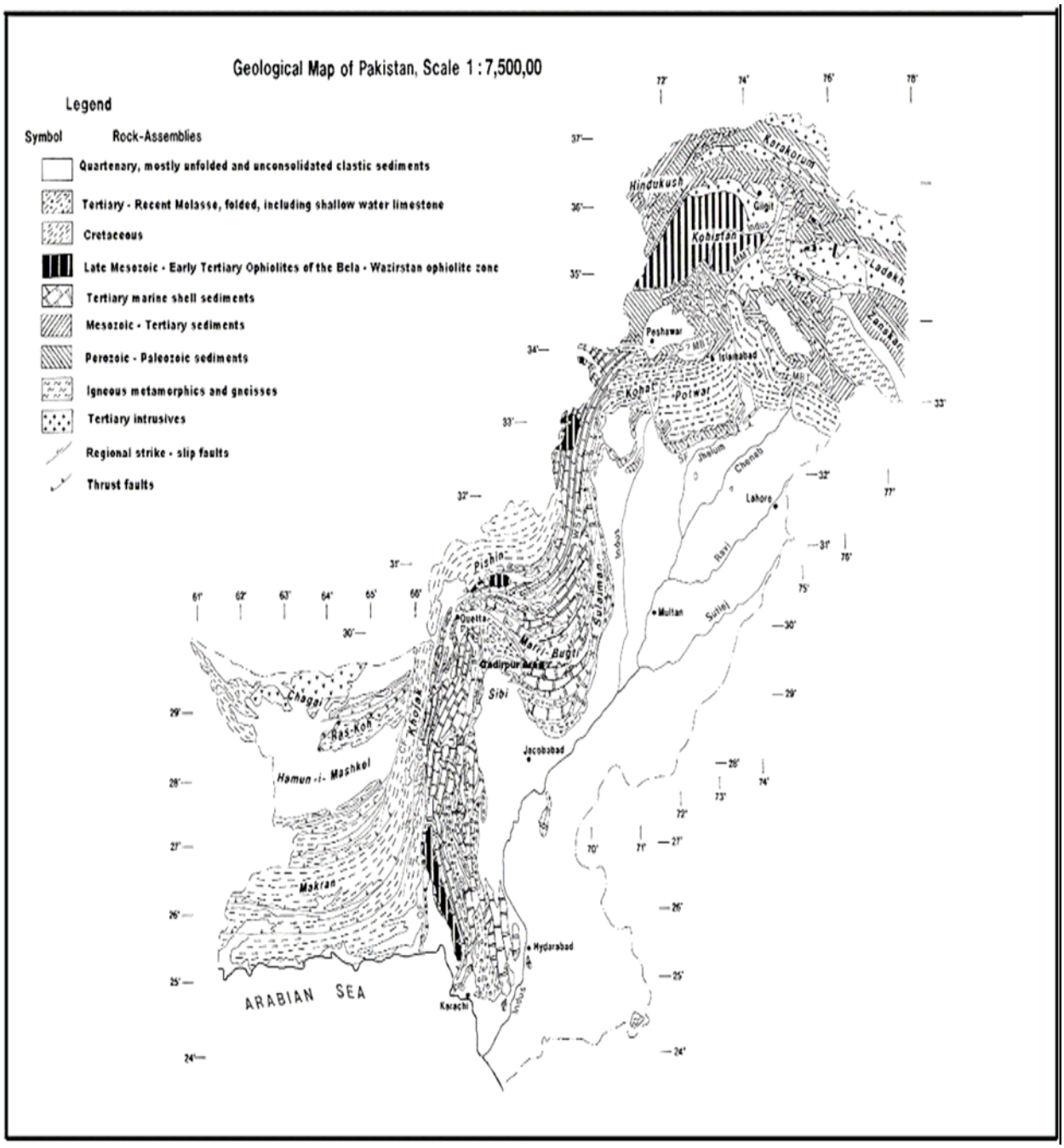

Fig. 2. Geological Map of Pakistan adopted from Bender and Raza, 1995.

Table 2. Stratigraphy of Southern Indus basin, Pakistan (adopted from Shah, 1977; Dolan, 1990).

Depth to Formation (m)	Stratigraphic unit	Age	Dominant Lithology
5	Lakhi Formation	Eocene	Limestone
95	Rannikot group (Lakhara F.)	Paleocene	Sandstone , gray shale
888	Rannikot group (Bara F.)	Paleocene	Sandstone, gray shale, basalt
888	Rannikot group (Kadro F.)	Paleocene	-
1035	Pab Sandstone	Late Cretaceous	Sandstone, brown shale

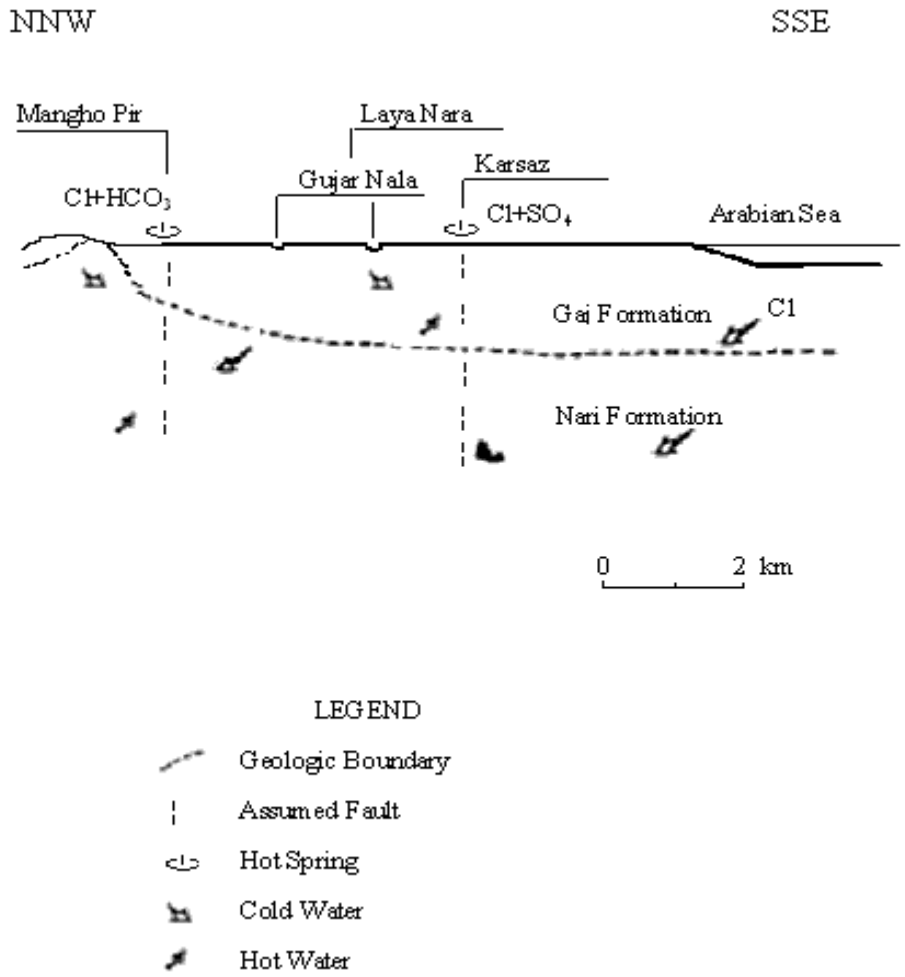

Fig. 3. Conceptional model of Karachi geothermal system by Munawar (2009).

are preserved and overlie the older in the northwestern part, where geological sequences are well developed. The older formations may be encountered at greater depths towards the basin and shallower on the continental shelf area towards southeast (Zaigham, 2002). The hydrogeology of Thar desert explained (Zaigham, 2002) for the ground water tapped by 83% of dug wells has an electrical conductivity (EC) value ranging from 2000µS/cm to more than 10,000µS/cm. In the central part, south and southeast of Chachro extending from Pakistan-India border to Islamkot, EC values are between 2,000 and 5,000µS/cm were particularly in the relatively deeper aquifer(s). The EC values ranging from less than 2,000 to 3,000µS/cm in and around Nagarparkar, where the basement units are exposed. The spring Inchile sir (St.24) is sampled 1 km far from Nagarparkar town.

RESULTS

Groundwater quality classification

The spring water is most probably a representative of groundwater quality a number of spring water samples (24) were collected and their results are given in table 5 along with the samples description provided in table 3. The geochemistry of ground can be represented by plotting the concentrations of major cations and anions in

Table 3. Physico chemical parameters of springs (in mg/L, pH unitless) of Sindh.

S. Id	Sampling Station	pH	EC µS/ cm	Sal. g/L	TDS	T Alk.	Har.	Ca	Mg	Sulphate	Water °C	Na	K	Cl
1	Manghopir I	7.74	2630	1.15	1683	250	175	34	28	206	45	350	13	387
2	Manghopir II	7.75	2670	1.15	1708	297	180	39	32	211	46	373	16	441
3	Manghopir III	7.45	3212	1.5	2105	307	265	97	60	118	47	440	24	556
4	Manghopir IV	7.1	4300	2.35	2752	435	335	196	41	332	26	340	15	905
5	Abdullah S Ghazi	7.5	4165	1.35	2666	467	326	117	78	322	30	491	58	554
6	Shree Ratneshwar	7.8	4185	2.25	2678	367	241	52	42	238	28	395	43	461
7	Baba Bukhari	7.4	8415	4.63	5474	331	596	283	141	342	35	693	105	1250
8	Dhabeji I	7.3	3101	1.5	1984	163	470	108	49	ND	30	342	16	702
9	Dhabeji II	7.5	4136	1.95	2646	110	580	77	56	ND	30	394	23	750
10	Gharo I	7.1	3897	2.1	2494	392	767	174	91	196	26	592	20	861
11	GharoII	7.1	3774	2	2415	413	717	177	65	196	26	446	21	835
12	Ranikot I	7.8	1217	0.5	779	205	292	95	39	112	28	132	18	296
13	Ranikot II	7.9	2500	1.1	1601	240	495	151	71	129	27	211	21	545
14	Ranikot III	7.7	3150	1.25	2015	243	690	176	88	146	26	315	31	782
15	Lal Bagh I	7.7	2301	1.35	1472	108	383	96	53	212	32	184	27	281
16	Lal Bagh II	7.34	2417	1.3	1545	125	390	105	55	225	31	194	32	310
17	Lakhi Shah Saddar I	6.78	13100	7.2	8384	242	612	304	139	871	40	1787	76	3560
18	Lakhi Shah Saddar II	6.6	14160	7.4	9062	236	672	315	177	969	42	1998	78	4266
19	Lakhi Shah Saddar III	7.2	1760	1.15	1126	112	126	76	23	127	32	158	58	150
20	Ghazi Shah	7.2	1079	0.25	690	115	207	43	25	ND	39	51	9	161
21	Kai	8.1	887	0.2	567	82	167	38	18	ND	35	54	7	111
22	Thoba	7.9	3515	1.65	2249	193	332	125	71	223	26	362	27	781
23	Khumb	7.5	3163	1.45	2025	157	472	91	43	127	29	301	22	593
24	Inchaile Sir	7.2	1452	0.9	946	234	277	78	42	128	29	112	17	273
	*WHO (2004)	6.5-8.5	-	-	100	120	200	75	30	250	-	200	24	250
	t-test paired	-2.03	0.87	2.41	0.93	-.94	0.79	1.80	1.81	2.86	2.56	1.52	1.83	0.68

ND= Not determined, - = Not decided

diagram from the Piper (1953). Aquachem software was used for developing the piper diagram (Fig. 4) and was used to portray principal hydrochemical compositional features in two parallel triangles along with diamond to top in a figure. The elements grouped in Na, K, Ca, Mg and HCO_3, SO_4, Cl developed in a trilinear diagram and showed the nature of springs water of sodium-potassium chloride (Na-KCl) and calcium-magnesium chloride (Ca-MgCl). The diagram showed predominance of cation evolved from Na+Ca to Mg-K and predominance of anion from Cl^- towards HCO_3 or SO_4.

The concentration of Na+K had indicated (triangle in left for cations) more than 50% for each in many of 24 springs, inversely most of samples showed lower concentration (50%) for Ca+Mg. The right side of triangle represented approximately all of springs were rich in chloride and found within 70-100%. The lower concentration of bicarbonate against the chloride, which did not exceeding greater than 30%. The $Cl+SO_4$ and Ca+Mg were found at both sides simultaneously, when judged the head of arrows raised upward within diamond shape (a blend of cations and anions). The $Cl+ SO_4$ indicated for almost all of samples lying within 75-100% with high concentration of anions. The scale for Ca+Mg indicated most of samples lower than 50% of concentration. Inverse to Ca+Mg the Na+ K had indicated more than half of springs rich in these concentrations and found more than 50%. The bicarbonate showed similar patterns as was found in triangle for the anions.

It can be readily seen (Table 4) that only two types of source springs were found, one of which is relatively rich in $NaCl/HCO_3$ and was indicated in many of samples St.1-6,10-14, 22, 23 and St.24. Whereas, the other type of source water was rich in $NaCl/SO_4$ for St.7, 15-18 and St. 19.

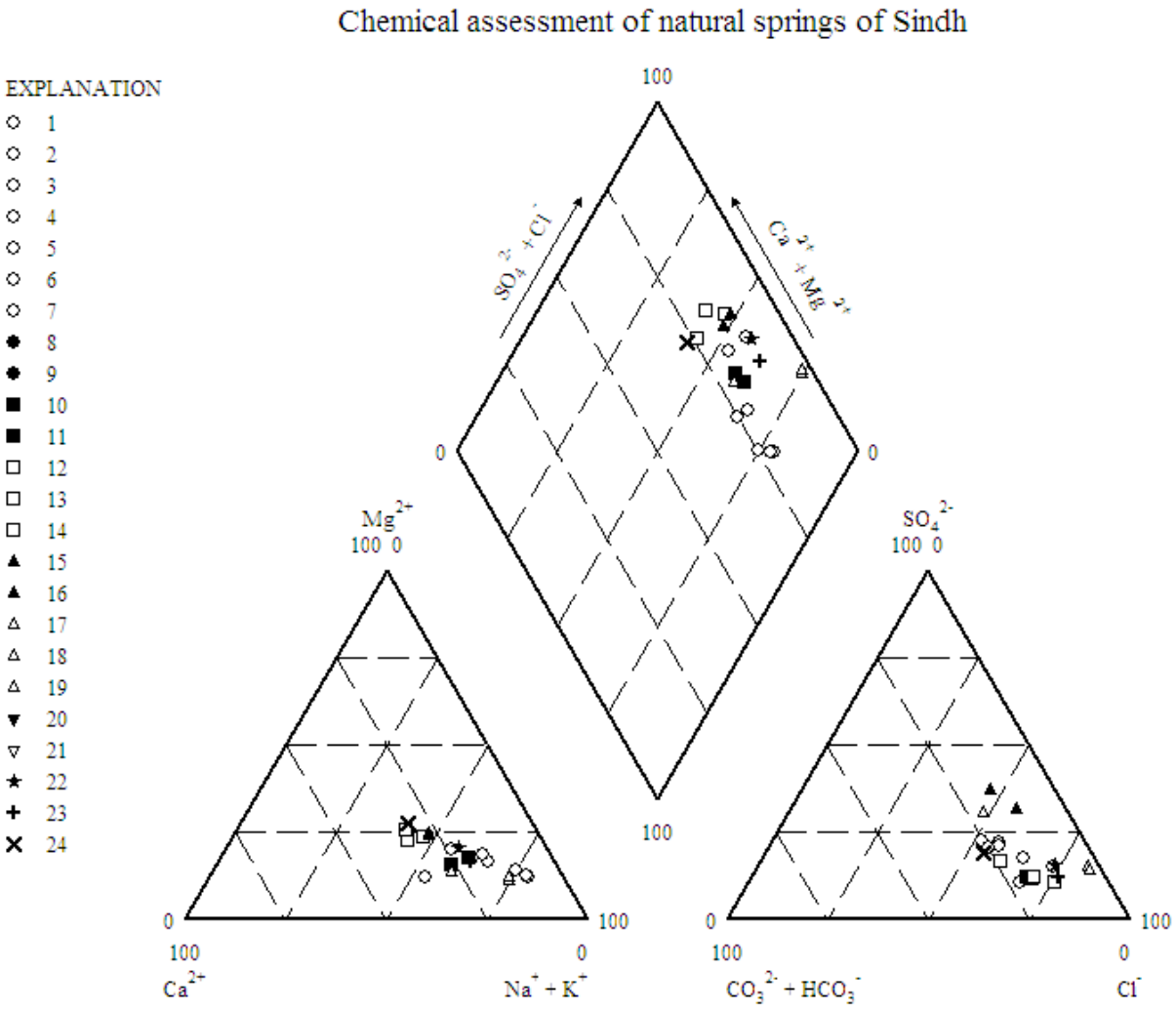

Fig. 4. Piper diagram showing water type of springs.

Statistical Analysis

The computer program Excel 2003 (Microsoft Office®) and SPSS 16 (SPSS Inc., Chicago, IL, USA) were used for results validation, data reduction and cluster analysis. Table 4 indicated each variable with standard deviation and standard error for thirteen physicochemical parameters and paired t test at degree of freedom 23 using t-distribution table and is indicated for most of variables below 1.7. The accuracy and precision of chemical data was validated from ionic charge balance error in milliequivalent adopted earlier by Lloyd and Heathcote (1985). After processing the data, all of the samples were found within acceptable limit maximum up to 10 percent (Fig. 8).

The ratio of total anions to that of cations ((anions)/ (cations)) was an indicator for completeness of measured parameters Mouli *et al.* (2005) and the scatter plot linear regression to sum of cations and anions in milli equivalent obtained r^2=0.9635 and indicated that the quality of data was good. Langelier saturation index (LSI) scale is commonly between -3 to 3 which was developed for predicting (Langelier, 1946) the degree of corrosion. The negative LSI values indicate unsaturation and potential to dissolve more salts. The number of springs distributed in three categories, the category 1 indicated samples St.18, St.19 and St.20 of negative LSI -0.00061, -0.084 and -0.18 respectively and these waters caused slight corrosion with non scaling. The five of springs St.8, St.9, St.10, St.23 and St.24 fell in corrosive nature, considered in category two and most of remaining (13 nos.) springs are comprised over in category three of range >0.5-2 and thus indicated to scale forming but non corrosive.

Residual sodium carbonate (WHO, 1989) is a measure of higher concentration of carbonate and bicarbonate in relation to calcium and magnesium and alternatively this influences suitability for irrigation. The excess quantities

of sodium bicarbonate and carbonate cause dissolution of organic matter in soil. Here we determined for 24 samples (Fig. 9), 18 samples indicated in safe rating (<1.25) with 75 percent of its credit, five springs (21%) were within marginal range (1.25-2.50) and only one spring was unsuitable (>2.50 for irrigation or plants).

Investigations of springs water for drinking and irrigation

The half of total spring discharges its water from source with flow rate of 0.5-7L/Sec, hence depending upon quality these waters are used for irrigation and drinking purposes. The two of springs from Rannikot and Baba Bukhari Karsaz having high flow of 70 and 28L/Sec respectively. The quality of spring in latter is not suitable for drinking and the flow disappears within the cracks of rock soil after exposure to some distance. The water of Rannikot is used for irrigation and drinking purposes by the population within hilly area. Two of springs with flow of 1-5L/Sec at the bank of Gharo creek discharges its water directly into the marine tides. The quality of this water is not suitable for drinking but could be used within salt tolerant irrigation. Among all the twenty four springs, only two samples of lakhi I and II showed high salinity and could not be recommended for drinking, livestock and poultry purposes (Ayers and Westcot, 1986). High concentrations of exchangeable (soluble) sodium and high levels of salts in soils negatively affect the plant growth. Soils with high exchangeable Na have poor tillage qualities and permeability. The sodium adsorption ratio (SAR) is more representative for the quality, which compares the concentrations of Na, Ca and Mg in irrigation waters. Since Ca and Mg bring moderate decline in the negative effects of Na, the salty taste of water is not that much as it should be. The SAR scale is adoptable for crop yield and nature of crop. The sodium adsorption ratio (SAR) is based on milliequivalent for classification of irrigation water. The spring water was classified on basis of sodium adsorption ratio (WHO, 1989) for detecting suitability of water for irrigation. It was admitted in majority of twenty springs with 92% share were in good category (<7) and only two of water samples found unacceptable (>7) for irrigation (Fig.10).

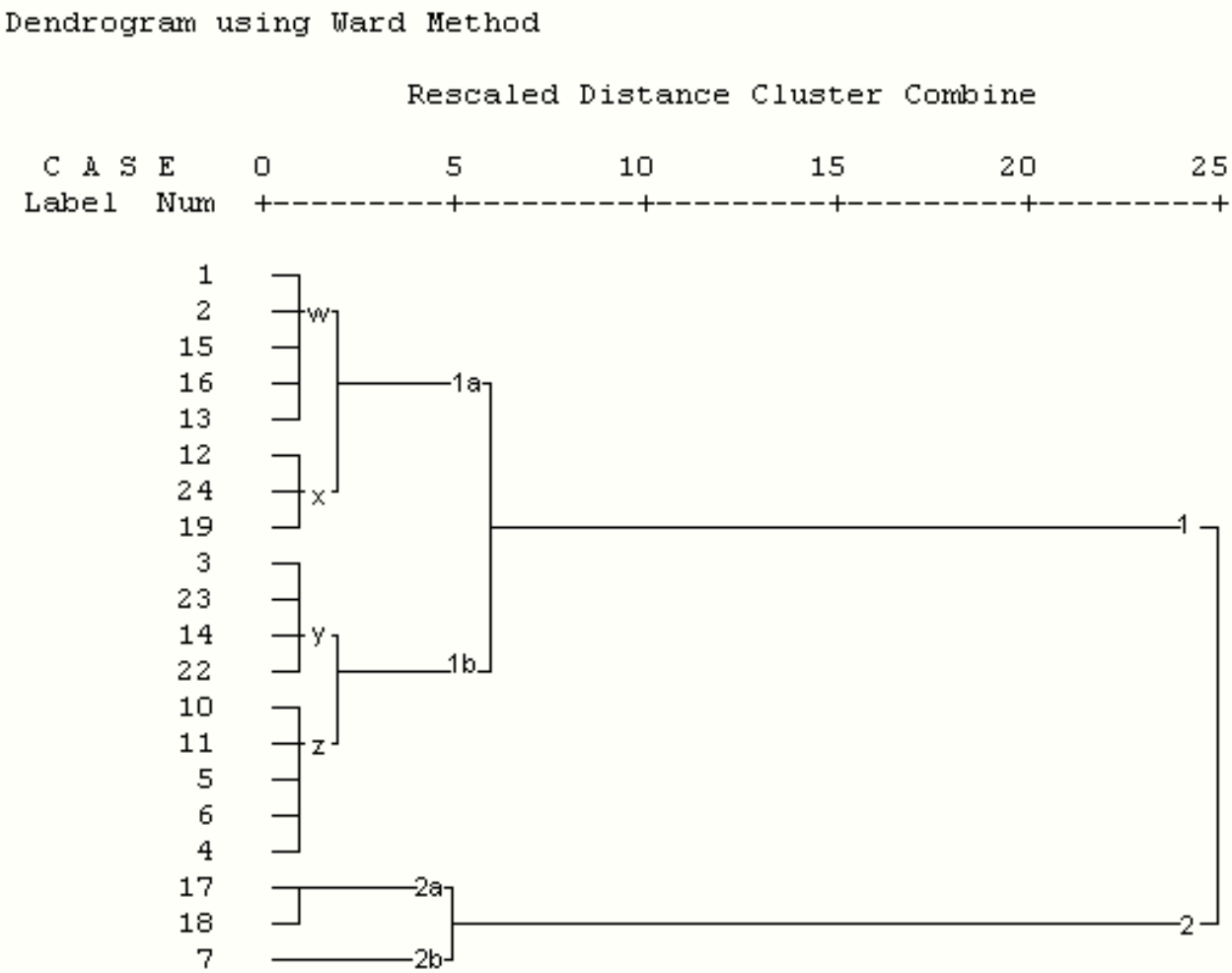

Fig. 5. Dendrogram showing Clusters among springs.

Conductivity is another measure of irrigation water quality, and the measured conductivity in the field can be used as a measure of salinity. Lloyd and Heathcote (1985) suggest that a conductivity measurement of <250 µS/cm is of low salinity, with no detrimental effects on crops expected, 250 to 750 µS/cm represents medium salinity, with detrimental effects to sensitive crops expected, 750 to 2250 µS/cm represents high salinity, with adverse effects on many crops., and 2250 to 5000 µS/cm represents very high salinity, suitable only for salt-tolerant plants (Table 3). The nineteen springs out of twenty four indicated were with high salinity water.

Cluster analysis (CA)

The hierarchical cluster analysis was applied to detect site similarity in groups between sampling springs using ward method with euclidean distance as a measure of similarity (Fig. 5). Two of statistically significant clusters were formed; Cluster 1 comprised over sets 1a & 1b and these were further lined into subsets. w(St.1, St.2, St.15, St.16, St.13) and x(St.12, St.24, St.19); the y(St.3, St.23, St.14, St.22) and z (St.10, St.11, St.4, St.5). Cluster 2 was divided into two subsets 2a(St.17, St.18) and 2b(St.7). These waters represent to springs of Lakhi Shah Saddar and Baba Bukhari Karsaz. These showed similar characteristic features, natural background and were affected with similar concentrations or type.

Factor Analysis (FA)

The aspect of complexity arises from large number of parameters (Saffran, 2001), factor analysis was used to data reduction, A highly correlated variables were removed with smaller number of uncorrelated variables. It was found that first two eigen values were higher than 1 and these could be seen in the Scree plot (Fig. 6). The eigen values less than one observed for 3rd factor and so, were eliminated on statistical extraction. It was observed that a majority of the total variance of original data has been explained in first two factors. On either of factor rotation (varimax) or unrotated factor loadings (Johnson and Wichem, 2002) the proportion of total variance is explained by first two factors with contribution of variance 80% shown in table 6 and figure 7. The factor 1 is loaded with 68 percent and factor 2 with 12 percent; and remaining 10 components explained only 20%. The factor analysis of first two factors were indicated for water quality parameters of springs in tables 6 and 7.

Bivariate correlation coefficient

Correlation coefficient is bivariate analysis to measure relationship between pairs; it is a dimensionless index, ranging from -1 to +1 to reflect the linearity relationship between two data sets. The correlation matrix's for 13 variables were produced using SPSS, whereas values close to 1 proves strong relationship (Table 8). The EC indicated strong positive correlation with chloride (0.966),

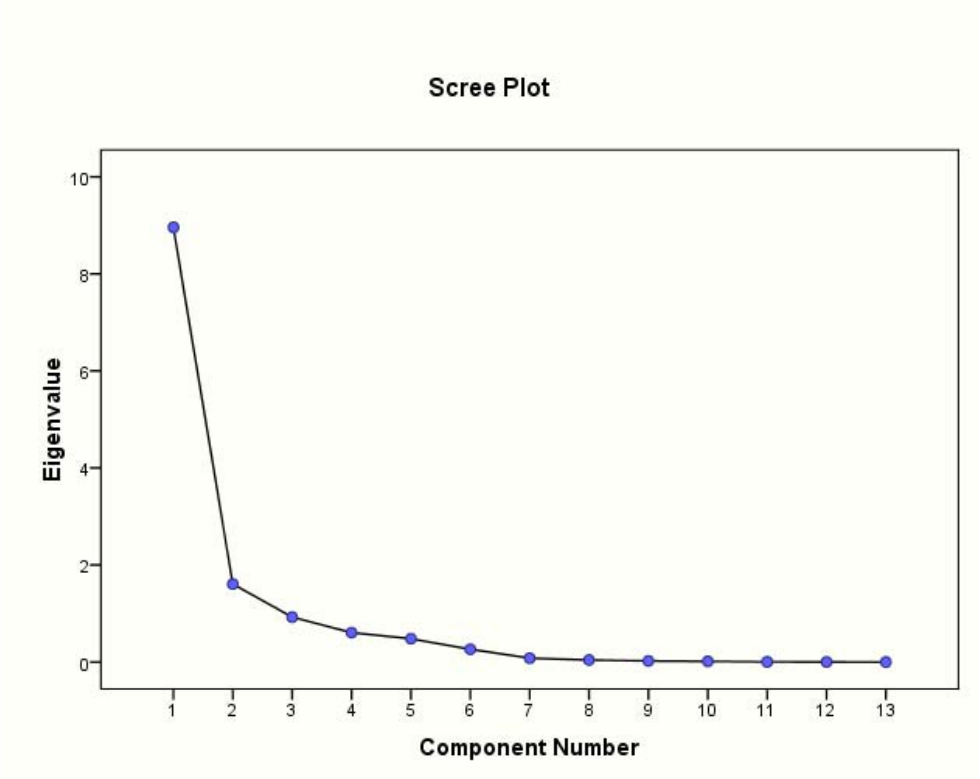

Fig. 6. Scree plot of eigen values against components for springs water quality data.

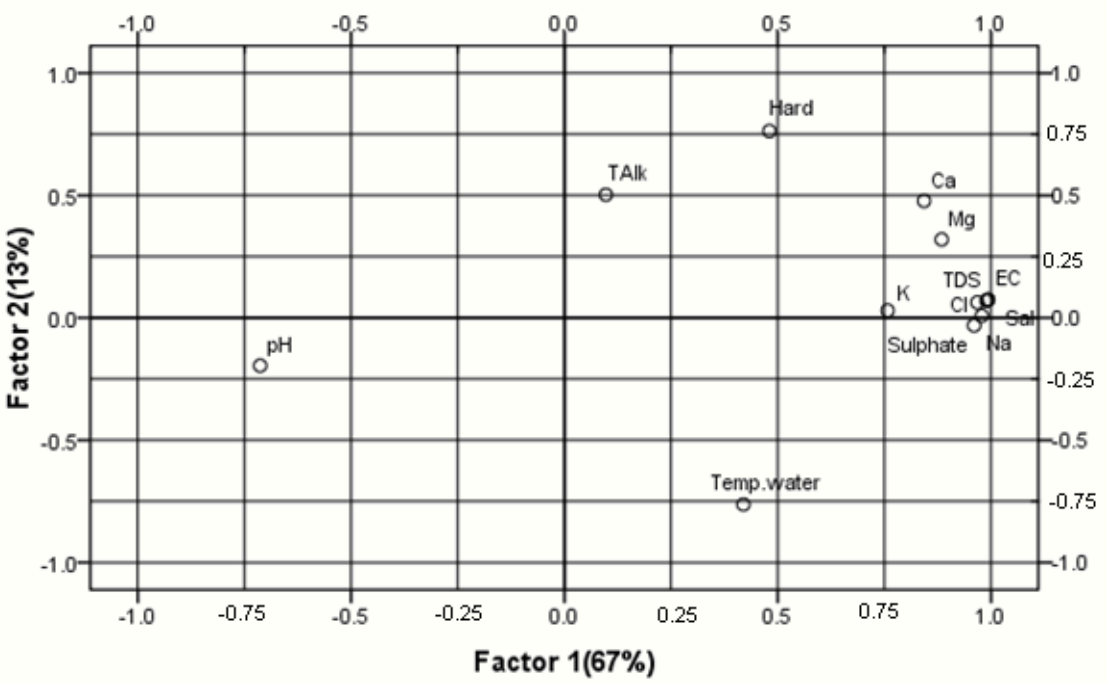

Fig. 7. Factor analysis of springs in Sindh.

magnesium (0.890), calcium (0.851), potassium (0.745) and moderate with hardness (0.516). The hardness represented moderate correlation with chloride (0.535) and sodium (0.496). The sodium indicated its significant positive relationship with EC (0.973), chloride (0.983), magnesium (0.849), calcium (0.781), potassium (0.638) and moderate with hardness (0.496). Chloride showed positive correlation with sodium (0.983), magnesium (0.856), calcium (0.828), potassium (0.602). The metal ions of calcium, magnesium and potassium showed its positive relation with EC 0.851, 0.890, 0.745 respectively. The calcium indicated positive correlation with magnesium (0.911), TDS (0.852), chloride (0.828), sodium (0.781) and potassium (0.664). Magnesium showed its strong positive correlation with calcium (0.911), sodium (0.849), potassium (0.742) and hardness (0.726). The potassium indicated strong positive correlation with magnesium (0.742), calcium (0.664), sodium (0.638) and moderate towards chloride (0.602) and hardness (0.288). It was noticed the pH indicated negative correlation with all chemical parameters and water temperature had proved negative correlation in such pairs.

Multivariate Elemental ratios

When Na and Cl values were plotted, most of samples fall on the 1:1 line with r^2=0.9599, the samples number St.1, St.2, St.3, St.5, St.6, St.10, St.15 and St.19 have slightly higher of Na values compared to chloride, and sample numbers St.4, St.7, St.8, St.9, St.11, St.12, St.13, St.14, St.16, St.17, St.18, St.20, St.21, St.22, St.23 and St.24 have lower Na values when compared to Cl. The Na/Cl ratios of springs were observed close to similar in case of dissolution of pure halite (0.65) which is maintained in solution unless significant cation exchange that reduces Na concentration (Leonard and Ward,1962; Richter and Kreitler, 1991). The series of some more ionic ratios of interest were selected Cl/SO_4, $(Na+K)Cl$, $(Na+K)/(Ca+Mg)$, Cl/HCO_3 and $(Cl+SO_4)/HCO_3$ and are presented with r^2 values 0.9015, 0.9599, -4.181, -2.466 and -0.652 respectively.

DISCUSSION

The twenty four springs from various locations of Sindh province were collected during study period 2000-2001. The samples descriptions are given in table 1 and the physicochemical parameters of water quality are indicated in table 3. The water temperature ranged between 26-47°C. The highest temperature was found at Manghopir III spring, manually which was entrapped in close constructed hall. In similar to another group of springs were lakhi that showed temperature of 42°C and is classified as "thermal spring". pH range of springs water was observed within 6.6 to 8.1 and fall in permissible limits of WHO (WHO, 2004) with an average value 7.4. The average minimum and maximum pH values were observed at Kai and Lakhi springs respectively.

The electrical conductivity (EC) and total dissolved salts (TDS) of the springs were within 887-14160 µS/cm and 567-9060 mg/L, with maximum for lakhi spring I and minimum for Kai spring, with an average of EC 3966µS/cm and TDS 2544mg/L (n=24). Only four springs

Table 4. Some quality analysis parameters of springs of Sindh

S. Id.	Sampling stations	LSI	SAR	PI	RSC	% Sodium	Ionic eq. balance percent error	Na/Cl meq./L	SO_4/Cl meq./L	Water-type
1.	Manghopir I	0.63	3.975	92.404	1.32	80.91	9.24	1.39	0.39	$NaCl/HCO_3^-$
2.	Manghopir II	0.80	3.914	90.968	1.65	79.49	9.99	1.30	0.35	$NaCl/HCO_3^-$
3.	Manghopir III	0.88	4.067	87.594	0.60	78.09	2.89	1.22	0.15	$NaCl/HCO_3^-$
4.	Manghopir IV	0.54	3.008	85.171	2.66	71.52	-0.36	0.57	0.27	$NaCl/HCO_3^-$
5.	Abdullah S Ghazi	0.83	3.800	83.442	1.44	74.31	-5.24	1.36	0.42	$NaCl/HCO_3^-$
6.	Shree Ratneshwar	0.63	3.70.3	88.099	1.94	77.22	0.34	1.32	-	$NaCl/HCO_3^-$
7.	Baba Bukhari	0.90	4.988	83.303	-2.51	78.24	1.61	0.85	0.20	$NaCl/SO_4^{2-}$
8.	Dhabeji I	0.2	3.893	90.059	-0.38	80.73	-2.27	0.75	-	-
9.	Dhabeji II	0.024	4.628	90.555	-1.22	83.81	4.09	0.81	-	-
10.	Gharo I	0.46	4.647	85.430	0.18	77.39	-5.36	1.06	0.16	$NaCl/HCO_3^-$
11.	GharoII	0.50	3.709	84.920	1.44	74.48	-5.76	0.82	0.17	$NaCl/HCO_3^-$
12.	Ranikot I	0.78	1.506	82.690	0.44	63.04	-1.89	0.68	0.28	$NaCl/HCO_3^-$
13.	Ranikot II	1.1	1.988	78.404	-0.52	64.63	-5.19	0.59	0.17	$NaCl/HCO_3^-$
14.	Ranikot III	0.90	2.782	80.513	-1.18	70.54	-8.84	0.62	0.13	$NaCl/HCO_3^-$
15.	Lal Bagh I	0.46	2.213	84.088	-1.09	72.71	3.46	1.00	0.55	$NaCl/SO_4^{2-}$
16.	Lal Bagh II	0.17	2.248	83.822	-1.08	72.45	6.74	0.96	0.53	$NaCl/SO_4^{2-}$
17.	Lakhi Shah Saddar I	0.16	13.61	93.079	-3.29	90.72	-2.34	0.77	0.18	$NaCl/SO_4^{2-}$
18.	Lakhi Shah Saddar II	0.00061	13.97	92.240	-9.94	90.19	-2.32	1.63	0.62	$NaCl/SO_4^{2-}$
19.	Lakhi Shah Saddar III	-0.084	2.382	93.411	0.15	80.03	-2.32	1.63	0.62	$NaCl/SO_4^{2-}$
20.	Ghazi Shah	-0.18	0.758	85.014	0.12	53.25	-0.76	0.49	-	-
21.	Kai	0.53	0.938	92.817	0.08	61.86	-4.21	0.74	-	-
22.	Thoba	0.83	3.563	85.886	-1.00	77.12	-3.51	0.71	0.21	$NaCl/HCO_3^-$
23.	Khumb	0.28	3.586	90.471	-0.19	80.36	-4.82	0.78	0.15	$NaCl/HCO_3^-$
24.	Inchaile Sir	0.21	1.209	78.563	0.59	56.63	-5.15	0.63	0.34	$NaCl/HCO_3^-$

- = Not determined

Table 5. Descriptive statistics of springs of Sindh

Parameters	No. of samples	Minimum	Maximum	Mean	Standard error	Standard deviation
pH	24	6.60	8.10	7.44	0.07	0.36
EC	24	887	14160	3966	683	3346
Sal.	24	0.20	7.40	1.98	0.37	1.85
TDS	24	567	9062	2544	438	2146
T.Alk.	24	82	467	242	23	113
Hardness	24	126	767	406	39	195
Ca	24	34	315	126	16	81
Mg	24	18	177	63	8	39
Sulphate	20	112	969	226	48	235
Water °C	24	26	47	32	1.39	6.84
Na	24	51	1998	446	96	474
K	24	7	105	32	5	24
Cl	24	111	4266	825	203	996

(16.3%) indicated TDS within maximum permissible limit for drinking water (WHO, 2004, 1000mg/L). High concentration of alkalinity imparts bitter taste and makes water unpalatable (Monique, 2003). This was within the range 82-467mg/L with maximum for Abdullah Shah Ghazi and minimum at Kai spring. The chloride ions as sodium salt indicate salty taste when present in amount more than 250 mg/L in water. The chloride content in the springs were observed within 111-4266mg/L with maximum at lakhi shah saddar spring II and minimum at Kai spring. Three springs (12.5%) indicated chloride within WHO permissible limits for drinking water (WHO 2004, 250mg/L). In drinking water the sulphate concentration over 250mg/L causes cathartic action especially in children and tarnishes bad taste to water. The amount of sulphate was observed in range between 112-

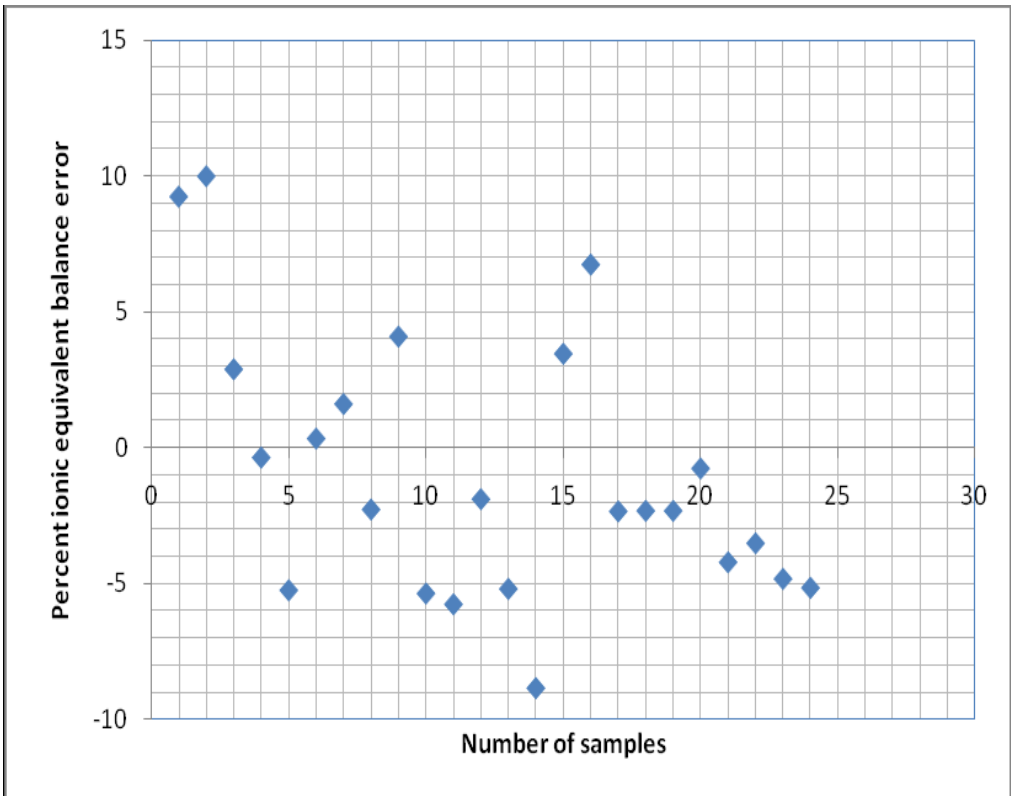

Fig. 8. Percent ionic equivalent balance error against springs

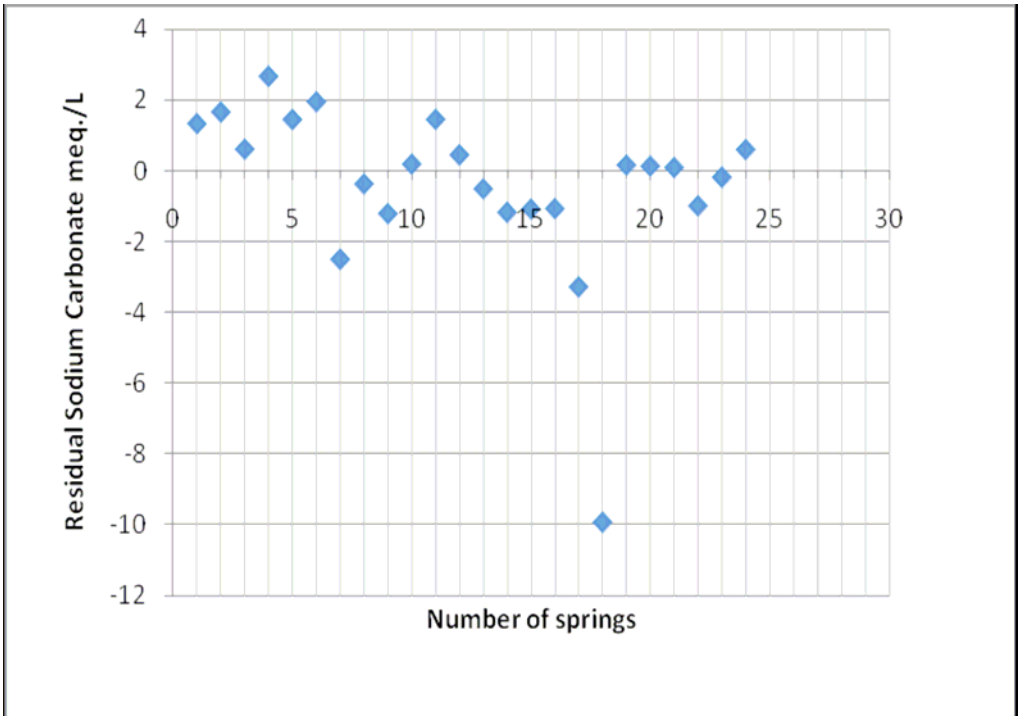

Fig. 9. Residual Sodium Carbonate against number of springs (values in milliequivalent/L)

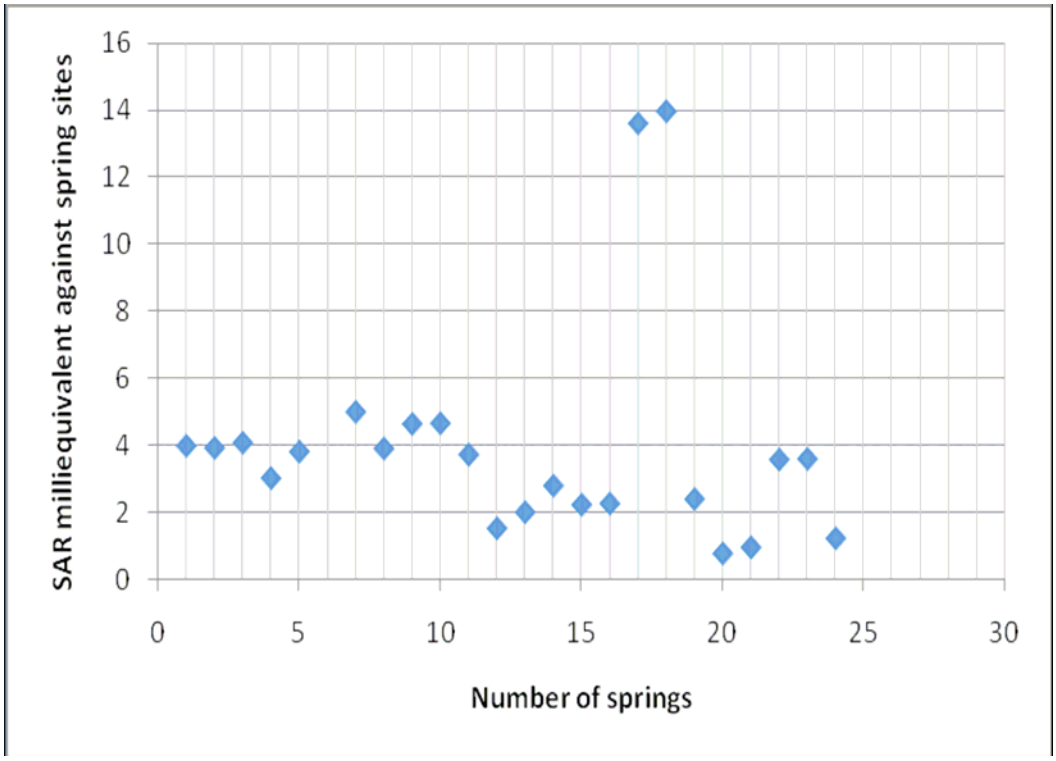

Fig. 10. SAR in milliequivalent against springs

Table 6. Factor analysis load of eigen values with cumulative percent of springs of Sindh

Total Variance Explained

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	8.896	68.433	68.433	8.896	68.433	68.433	8.898	68.433	68.433
2	1.554	11.685	80.386	1.554	11.953	80.386	1.554	11.953	80.386
3	0.916	7.048	87.434						
4	0.634	4.875	92.309						
5	0.473	3.640	95.949						
6	0.327	2.512	98.461						
7	0.094	0.720	99.180						
8	0.058	0.449	99.629						
9	0.025	0.189	99.818						
10	0.017	0.134	99.951						
11	0.004	0.033	99.984						

969mg/L with maximum for lakhi shah saddar spring II and minimum for lal bagh. At the extent of seventy percent of springs indicated sulphate concentration within permissible limits of WHO for drinking water (250 mg/L). It have been reported in areas where drinking water contains greater than 500 mg/L as $CaCO_3$, there is higher incidence of gallbladder disease, urinary stones, arthritis and arthropathies Muza-levskaya *et al.* (1993); high concentration of calcium carbonate in water generates no lather and creates scaling problem in boilers (Monique, 2003). The hardness in the spring water was observed within 126-767mg/L, with maximum in Gharo I and minimum for lakhi shah saddar III springs respectively. Seventeen percent of the springs indicated hardness within permissible limits of WHO (200mg/L). Sodium, potassium, calcium and magnesium were determined in spring waters and the results obtained were in the range of 51-1998mg/L, 7-105mg/L, 34-315mg/L and 18-177mg/L respectively (Table 3). All the springs indicated a similar trends in the concentration of metal

ions with the decreasing order Na>Ca>Mg>K, where the sodium was observed as dominant ion. A higher amount of Na intake may cause hypertension, congestive heart diseases and kidney problems (Singh *et al*., 2008). Its high content also decreases seed germination and agricultural productivity.

Table 7. Component Matrix, variable loadings on first two factors after varimax rotation

Parameters	Factor1	Factor 2
TDS	0.989	0.088
EC	0.989	0.088
Salinity	0.983	0.091
Na	0.966	0.149
Cl	0.966	0.092
Sulphate	0.942	0.185
Mg	0.942	-0.174
Ca	0.899	-0.330
K	0.742	0.093
pH	-0.732	0.080
Water ºC	0.300	0.815
Hardness	0.601	-0.666
T.Alkalinity	0.150	-0.451
Eigen value	8.896	1.554
% variance explained	68.43	11.95

A characteristic of potassium is similar to that of sodium and imparts salty taste. The important role in metabolic activity and maintaining osmotic pressure. Calcium and magnesium are essential elements, but their higher concentration converts the water to hard and may cause health problems Muza-levskaya *et al.* (1993). A significant concentration of Ca and Mg may develop sweet taste to the water. The concentration of Na, K, Ca, and Mg in the spring waters were compared with the permissible limits of WHO (2004) and was observed that 7 springs for Na, 14 springs for K, 5 springs for Ca and 4 springs for Mg indicated concentration levels within permissible limits of WHO.

CONCLUSION

The analysis of 24 natural springs located within Karachi city, district of Thatta, Jamshoro and Tharparkar Sindh, Pakistan were examined for 15 different parameters. pH of all springs were within acceptable limits for drinking water. The EC and chloride of four springs were within desired limits of WHO for drinking water. Seven springs reported were observed as thermal springs with their temperatures above than the surroundings. The sulphate and hardness of fourteen springs were observed within maximum permissible limits for drinking water. The sodium, potassium, calcium and magnesium showed acceptable concentrations for drinking in 7, 14, 5 and 4 springs respectively. The highest concentrations were found among two of distant springs located at Lakhi Shah Saddar, district Jamshoro and Baba Bukhari Karsaz springs of Karachi region. Most of springs are highly important for tourism. Almost all springs are located near to holy shrines of saints. Some of springs are used for therapeutic purposes, for sufferer's of skin diseases. Separate bath rooms are used for men and women. A few of springs are used for cultivation purposes. Nearly one third of springs are suitable for drinking and could be used for bottle mineral water industry. Some of thermal springs exercised to generate electricity to meet with increased requirement. The extraction of sulphur and valued minerals could be utilized for economic development.

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Table 8. Correlation Matrix of springs in Sindh

Parameters	pH	EC	TDS	T.Alk.	Hard.	Ca	Mg	Sulphate	Water °C	Na	K	Cl
pH	1.000											
EC	-0.671	1.000										
TDS	-0.670	1.00	1.000									
T.Alkalinity	-0.159	0.144	0.145	1.000								
Hardness	-0.455	0.516	0.516	0.214	1.000							
Ca	-0.662	0.851	0.852	0.203	0.761	1.000						
Mg	-0.551	0.890	0.891	0.143	0.726	0.911	1.000					
Sulphate	-0.687	0.957	0.955	0.078	0.393	0.761	0.794	1.000				
Water °C	-0.158	0.354	0.356	-0.096	-0.267	0.006	0.171	0.366	1.000			
Na	-0.694	0.973	0.972	0.119	0.496	0.781	0.849	0.963	0.421	1.000		
K	-0.434	0.745	0.749	0.036	0.288	0.664	0.742	0.644	0.214	0.638	1.000	
Cl	-0.701	0.966	0.964	0.054	0.535	0.828	0.856	0.963	0.334	0.983	0.602	1.000

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ESTIMATION OF BULK TRANSFER COEFFICIENTS OF MOMENTUM AND SENSIBLE HEAT FROM A HUMID TROPICAL BARE SURFACE

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ABSTRACT

The bulk transfer coefficients of momentum (C_D) and sensible heat (C_H) were determined from the field observation carried out on Nigerian Micrometeorological Experimental (Nimex_1) field, Ile-Ife, Nigeria. Direct determination of the transfer coefficients was done using the eddy correlation method. Analytical method was also used to compute the transfer coefficients with the use of bulk transfer algorithms. The C_D and C_H values obtained from eddy covariance method were higher than those from analytical method. Usage of the transfer coefficients obtained from analytical method will lead to slight underestimation of the fluxes. The atmospheric stability parameter z/L values were positive at nights (stability) but negative during the day (instability). The C_D and C_H values were higher during the day (instability) and lower at nights (stability). Whenever z/L was positive the sensible heat flux was negative.

Keywords: Bulk transfer coefficient of momentum, bulk transfer coefficient of sensible heat, stability, instability, atmospheric stability parameter.

INTRODUCTION

The bulk transfer coefficients of momentum and sensible heat over land surface controls the flux of sensible heat from the earth's surface. Atmospheric numerical models and energy budget studies require the bulk transfer coefficients of momentum (C_D) and sensible heat (C_H) to estimate the surface fluxes of momentum and sensible heat (H), (Jordan *et al*., 1999; Briegleb *et al*., 2004). The bulk transfer coefficients vary with regions and dynamics of the atmosphere (Stull, 1988) and accurate bulk transfer coefficients are necessary for numerical models. Hence the need for the validation of existing bulk transfer algorithm with values obtained from direct measurement of fluxes of momentum and sensible heat.

Values of C_D and C_H determined from other places are mostly employed in this part of the tropics since little or no work has been done in this area to determine the values. Errors set in when constant values are used throughout all the hours of the day, so diurnal values are needed.

Micrometeorological experiments are scarce in this part of the tropics and Nigerian Micrometeorological Experiment (Nimex_1) is one of the most comprehensive experiments in this area. The first phase of Nimex measurement started from15 February to 10 March 2004 at Ile-Ife Nigeria. The bulk transfer coefficients of momentum and sensible heat fluxes were not known in this part of the tropics, hence the need for this investigation.

Study Area

The data used in this investigation were collected at Nimex_1 site. The site is situated in southwestern Nigeria, at Obafemi Awolowo University, Ile-Ife ($7^0$33′N, $4^0$33′E). This is a tropical area where there are two main seasons viz: wet (April to October) and dry (November to March). The tropical climate is influenced by two air masses: the rain-bearing southwest monsoon originating from the Atlantic Ocean and the dry northeasterly continental air mass passing over the Sahara desert. The position of the Intertropical Convergence Zone (ITCZ) controls the amount, seasonal distribution and type of rainfall as well as the length of the wet season at any location in Nigeria. The region south of the ITCZ, depending on its distance from ITCZ, usually receives more rainfall while the region north of it experiences dryness. The ITCZ gets to its most northern position in July- August and its most southern position in December- February, at which time the dry season sets in (Balogun, 1981). The investigation at the Nimex_1 site was done during the transition period in-between the dry and wet season in 2004 to capture the dry and wet scenarios.

MATERIALS AND METHODS

Meteorological Measurement

The Nimex_1 field was located on an agricultural farm land at Teaching and Research farm, Obafemi Awolowo University Ile-Ife, Nigeria. The elevation of the field is 288 m.a.s.l. It is more or less a bare ground with canopy height less than 0.3 m as the bush on the experimental area was cleared just before the commencement of the

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project. Eddy covariance system comprising of a three dimensional ultrasonic anemometer (model USA-1) and a Krypton hygrometer (model KH2O) were deployed on the field. The ultrasonic anemometer was sampled at 16Hz giving the turbulent wind components, u, v, w and sonic temperature, T_s. The Krypton hygrometer was sampled at 8Hz giving the turbulent absolute humidity. The data were analysed and turbulent fluxes were calculated. The details of the analyses of turbulent fluxes were as presented in Mauder *et al*. (2007), Jegede *et al*. (2004a) and Jegede *et al*. (2004b).

The ground heat flux and net radiation were measured for the determination of the available energy. A self calibrating heat flux plate was used to measure ground heat flux at 0.02m, 0.05m, 0.1m and 0.3m depths. The Kipp and Zonen CNR1 net radiometer was used to measure net radiation.

Profile measurements of wet and dry bulb temperature at 0.9m, 4.9m and 10m height were done using Frakenberger Psychrometer; wind speed was measured at heights 0.7 m, 1.2 m, 2.2m, 3.3m, 5.2m, 7.2m, 10.2 m, and 14.8m with cup anemometer (A101M1/A100L2) and the wind direction was measured at 14.8m level using a rotating vane. Soil surface temperature was measured by Infrared Pyrometer KT 1582D. Soil temperature at depths 0.05 m, 0.1m, and 0.3m was measured by PT-100Ω Thermistor Thermocouple. Campbell Scientific CR10X data- loggers were used in storing the data. The details of the equipment used are stated in table 1.

Day of year (DOY)s 63, 66 and 67 were considered for this investigation. The profile and eddy covariance data were complete for DOYs 66 and 67 while there were data from 0700hr to 2400hour for DOY 63. The DOYs were chosen due to their relatively complete data for both eddy covariance and profile data.

Data analysis and quality control test

The effective fetch in percentage of the theoretical fetch from the field computed according to Gockede *et al*. (2006) were greater than 90 under unstable stratification for most wind directions, greater than 80 under neutral condition and the least was 62 for stable stratification with wind direction of 150^0 (SSE), Mauder *et al*. (2007). On daily basis the quality of profile of meteorological data from 15 m mast was checked by simple visual test according to (Foken, 2003). The quality of the eddy covariance data was checked using TK2, a software package written by Mauder and Foken (2004). It was developed at Obafemi Awolowo University, Ile-Ife, Nigeria and the University of Bareuth during the period of the experiment (Nimex_1). The guides for surface flux measurement and analysis given by Lee *et al*. (2004) were considered by Mauder and Foken (2004). The values that were not physically possible were removed before the calculation of the variances and covariances using the spike detection method of Vickers and Marhrt (1997) that was based on Hojstrup (1981). Cross correlation analysis was done for each averaging interval of the sonic anemometer and Krypton hygrometer that were sampled at different frequencies. This was done to determine the time delay between the two sensors. The method applied for cross wind correction for the correction of sonic temperature was that of Liu *et al*. (2001). Coordinate transformation was done using the planar fit method of Wilczak *et al*. (2001). Spectral models of Kaimal *et al*. (1972) and Hojstrup (1981) were employed for spectral corrections following Moore (1986). The buoyancy flux was converted into sensible heat flux following Schotanus *et al*. (1983). The latent heat flux was also corrected for fluctuations in density and mean vertical mass flow according to Webb *et al*. (1980). Test for steady state conditions and well- developed turbulence was carried out according to Foken and Wichura (1996) and Foken *et al*. (2004). All the above tests and analysis were incorporated

Table 1. Equipment deployed on Nimex_1 site.

Parameter	Sensor	Accuracy
Data acquisition	Campbell Scientific Data-logger CR10X	Not applicable
Wind direction	Vector Instruments Wind vane W200P	$\pm 2^0$
Wind speed	Vector Instruments Cup anemometer A101ML/A100L2	1^0
Wet and dry bulb air temperature	Theodor Friedrichs FrakenbergerPsychrometer	$\pm 0.05\ ^0$C
Soil surface temperature	Heitronics Infrared Pyrometer KT1582D	$\pm 0.5^0$C
Soil temperature	Campbell Scientific Thermistor Thermocouple	$\pm 1^0$C
Soil heat flux	Hukseflux HFP01SC self calibrating Heat flux plate	±3%
Net radiation	Kipp and Zonen CNR1 net radiometer	±10% of daily total
3D wind speed	Metek USA-1 3-D ultrasonic anemometer	$0.01 ms^{-1}$
Water vapour content	Campbell Scientific KH20 krypton hygrometer	$0.15 m^3 g^{-1} cm^{-1}$(sensitivity)

into (Mauder *et al*., 2007).

Parameterization of bulk transfer coefficient of momentum and sensible heat

The bulk momentum (τ) and sensible heat fluxes are given by:

$$\tau = \rho C_D U^2 \quad 1$$

and

$$H = \rho C_p C_H U(T_g - T_a) \quad 2$$

where ρ is the density of air

C_p is the specific heat of air at constant pressure

U is the mean horizontal velocity

T_g is the soil surface temperature, and

T_a is the air temperature

The bulk transfer coefficients are computed as: [3]

$$C_D = \tau / \rho U^2 \quad 3$$

$$C_H = H / \rho C_p U(T_g - T_a) \quad 4$$

$$C_H = (\overline{w'T'}) / U(T_g - T_a) \quad 4b$$

where w = vertical wind speed, and T = air temperature from sonic system .

The turbulent momentum flux and the friction velocity (u_*) are given by McPhee (2002) and Stull (1988) as:

$$\tau = \rho((\overline{u'w'})^2 + (\overline{v'w'})^2)^{1/2} \quad 5$$

$$u_*^2 = [(\overline{u'w'})^2 + (\overline{v'w'})^2]^{1/2} \quad 6$$

Bulk flux Algorithm

The bulk transfer coefficients of momentum and heat were computed from Garratt (1992) and Stull (1988) as:

$$C_D = k^2 / (\ln(z/z_0) - \psi_m)^2 \quad 7$$

$$C_H = k^2 / [(\ln(z/z_0) - \psi_m)(\ln(z/z_0) - \psi_h)] \quad 8$$

Where z is the height of measurement

z_0 is the momentum roughness length

ψ_m and ψ_h are the integral form of the Monin Obukhov similarity functions ϕ_m and ϕ_h which are dimensionless wind shear and temperature gradients respectively given by Zeng (1998) as:

$$\phi_m = \begin{cases} (1 - 16\zeta)^{-1/4} & \zeta < 0 \\ (1 + 5\zeta) & 0 < \zeta < 1 \end{cases} \quad 9$$

$$\phi_h = \begin{cases} (1 - 16\zeta)^{-1/2} & \zeta < 0 \\ (1 + 5\zeta) & 0 < \zeta < 1 \end{cases} \quad 10$$

The values of ϕ_m and ϕ_h under very unstable conditions were given by Kader and Yaglom (1990) as:

$$\phi_m = 0.7k^{2/3}(-\zeta)^{1/3} \quad 11$$

$$\phi_h = 0.9k^{4/3}(-\zeta)^{1/3} \quad 12$$

Under very unstable conditions, the similarity functions were described by Hostlag *et al*. (1990) as

$$\phi_m = \phi_h = 5 + \zeta \quad 13$$

Utilizing equations (9) to (13), ψ_m and ψ_h will take the form:

$$\psi_m = \begin{cases} 2\ln\left(\frac{1+x}{2}\right) + \ln\left(\frac{1+x^2}{2}\right) - 2\tan^{-1}x + \frac{\pi}{2}, & for\ -2 < \zeta < 0 \\ -5\zeta, & for\ 0 \le \zeta \end{cases} \quad 14$$

$$\psi_h = \begin{cases} 2\ln\left(\frac{1+x^2}{2}\right), & for\ -2 < \zeta < 0 \\ -5\zeta, & for\ 0 \le \zeta \end{cases} \quad 15$$

with

$$x = (1 - 16\zeta)^{1/4} \quad 16$$

Equations (14) to (16) were substituted into equations (7) and (8) to calculate C_D and C_H for the parametric determinations of the respective coefficients.

RESULTS AND DISCUSSION

Diurnal variation of C_D values

The wind direction recorded during the period of this investigation was north easterly which conforms to the expected prevailing wind at the transition period from dry to wet season. The wind speed was low (< 3ms^{-1}) throughout with night values lower (< 2 ms^{-1}). Half hourly meteorological and eddy covariance data were used; the resulting half hourly C_D values obtained from the eddy covariance and analytical methods were different. The mean daily values obtained from the analytical method were one order lower than those obtained from the eddy covariance method. The maximum diurnal values obtained from the eddy covariance method were higher, higher C_D values from eddy covariance method has been reported (Zhang *et al*., 2002). The analytical method gave the daily mean of C_D of 1.66 x 10^{-3} with standard deviation of 8.62 x 10^{-3} while the eddy covariance method gave a mean value of 2.28 x 10^{-2} with standard deviation of 1.71 x 10^{-2} for DOY 63. On DOY 66 analytically obtained C_D had a daily mean of 1.54 x 10^{-3} with standard deviation of 4.75 x 10^{-4} while the mean daily C_D value obtained from eddy covariance method was 1.24 x 10^{-2} with standard deviation 5.86 x 10 $^{-3}$. On DOY 67 the mean daily values for analytical and eddy covariance methods were respectively 2.70 x 10^{-3} and 2.52 x 10 $^{-2}$. The standard deviations were 6.78 x 10 $^{-3}$ and 6.33 x 10 $^{-2}$ for analytical and eddy covariance methods respectively, showing that eddy covariance computed values were

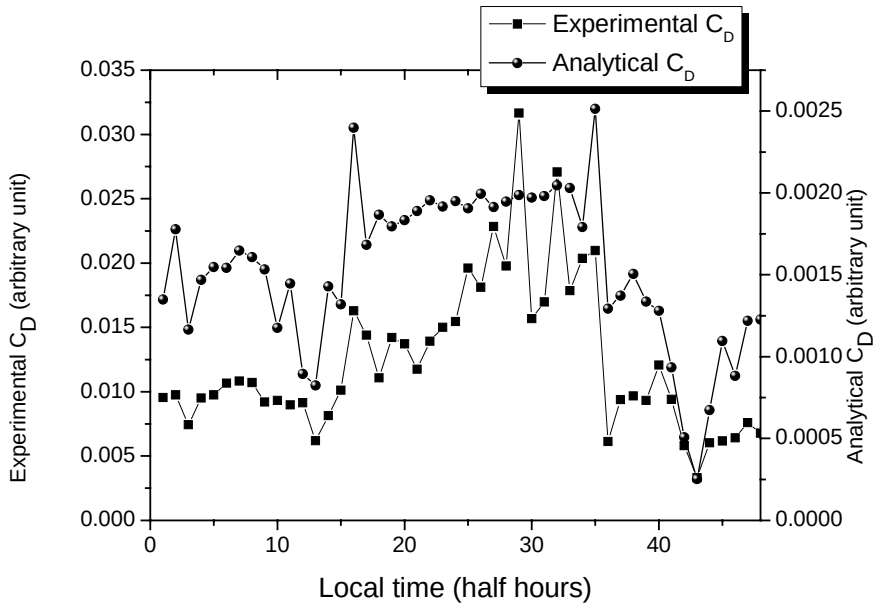

Fig. 1. Diurnal variation of bulk transfer coefficient of momentum, C_D for DOY 66.

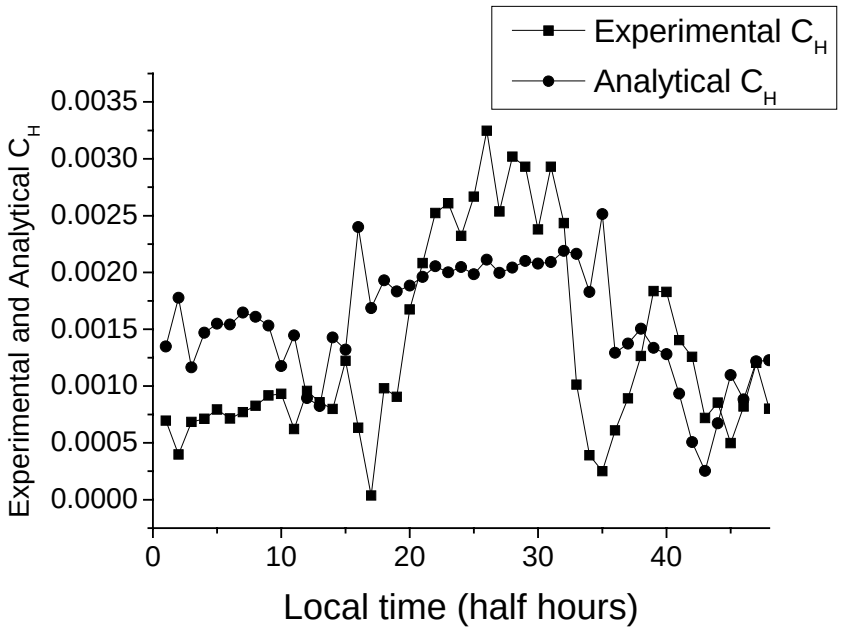

Fig. 2. Diurnal variation of bulk transfer coefficient of sensible heat (C_H) for DOY 66 (in arbitrary unit).

more scattered than the analytically computed ones. Diurnal variation is clearly seen in C_D values with maximum around noon and low values at night and morning hours figure 1.

Diurnal variation of C_H values

Diurnal variation of C_H was similar to that of C_D with maximum at noon and low values at evening, night and early in the morning, figure 2. The C_H values obtained from analytical and eddy covariance methods were comparable, the values were in the same order. Higher daily maximum and mean C_H values were obtained from eddy covariance method. On DOY 66 daily mean 1.57 x 10^{-3} and 1.32 x 10^{-3}, maximum 2.50 x 10^{-3} and 3.25 x 10^{-3} and standard deviation 5.05 x 10^{-4} and 8.56 x 10^{-4} were found respectively for analytical and eddy covariance methods. The maximum C_H was higher than the value obtained by Ishikawa and Kodama (1994) on snowmelt

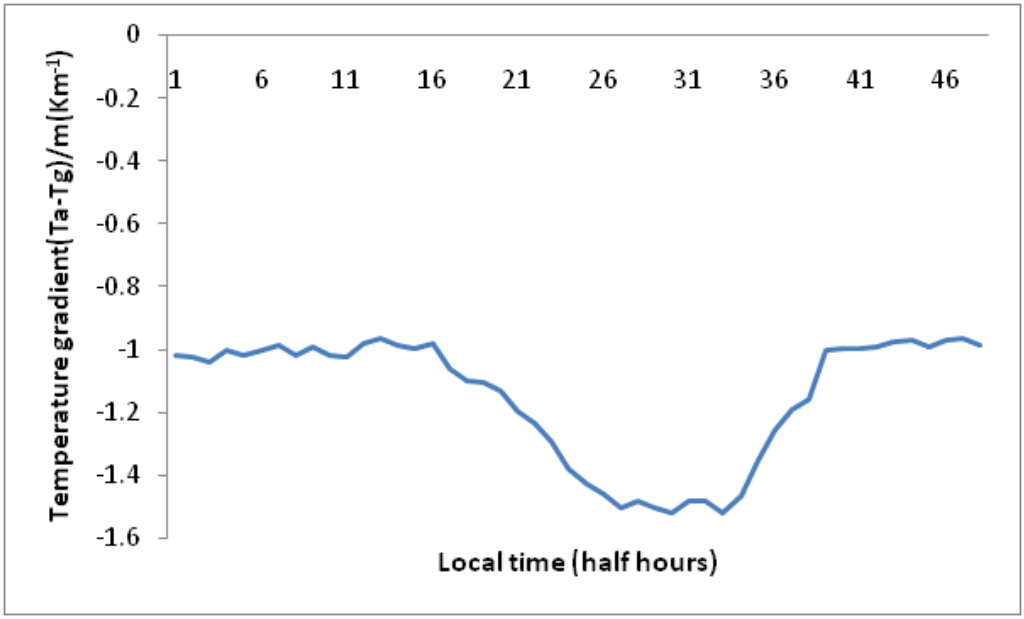

Fig. 3. Diurnal variation of temperature gradient for DOY 66.

surface (2.30 x 10^{-3}). The values obtained fitted well into the typical range of values for C_H (Stull, 1988).

On DOY 67 the analytical and eddy covariance methods respectively gave mean daily C_H values of 1.70 x 10^{-3} and 2.52 x 10^{-3}, maximum C_H values of 7.65 x 10^{-3} and 1.31 x 10^{-2} and standard deviations of 1.36 x 10^{-3} and 2.50 x 10^{-3}.The eddy covariance computed C_H values had more scatter than the analytically computed values. The maximum C_H values obtained on DOY 63were 3.78 x10^{-3} and 6.22 x 10^{-3} for analytical and eddy covariance methods respectively.

Comparison of the C_D and C_H values obtained from eddy covariance and analytical methods

The C_D and C_H values obtained from the eddy covariance method were taken to be more reliable since they were from directly measured friction velocity and covariance of vertical wind and temperature. Assumptions were made in the derivation of equations (9) to (15); variable meteorological conditions were not included in the analytical equations. The values obtained from eddy covariance method were generally higher than those from the analytical method since variable stability conditions were assumed uniform in the analytical method. (Zhang *et al*., 2002) also obtained higher C_D and C_H values from eddy covariance method than from the other methods.

The temperature gradient (T_a-T_g) m^{-1} was found to be

Diurnal variation of temperature gradient

negative both during the day and at night during the transition period contrary to the expectation of positive values at nights. The negative gradient implies a positive difference between soil surface temperature and the air temperature at any height up to 10 m (T_g-T_a) (Fig. 3); possibly due to the strong stability at night and high value of temperature in this area. It has been reported that (T_g-T_a) was positive at night when the temperature was high but negative when the temperature was low, in 82% of the stations considered in Alpine areas; only in few of the stations did thermal inversion occur, at night Colombi *et al*. (2007). The night time values of T_g were also found to be close to or lower than T_a, (Prigent *et al*., 2003). Temperature is always high in this part of the tropics so soil surface temperature may be higher than the air temperature even at night when there is strong stability. In the early hours of the day, evening and nights T_g-T_a reduced greatly since there was no input from the sun but it never went below zero, as the case may be in cold regions. The C_H was positive throughout all the hours of the day and night in most published literature. The sign of the temperature difference between the ground and the air (T_g-T_a); which is positive during the day while it is negative at night, controls the sign of the sensible heat flux in most places. Generally, in this part of the tropics, during the transition period from dry to wet season, the ground temperature was always higher than the air temperature making the temperature difference (Tg-Ta) to be positive throughout the day and night. This makes the sign of C_H to change to negative whenever the value of sensible heat flux is negative. This occurs when the stability parameter z/L is positive, when the atmosphere is stable, this is so at nights and before sunrise. To keep the sign of C_H positive throughout, negative sign was used to multiply the value of sensible heat flux. When the profile of air temperature was considered from 1m to 10 m; there

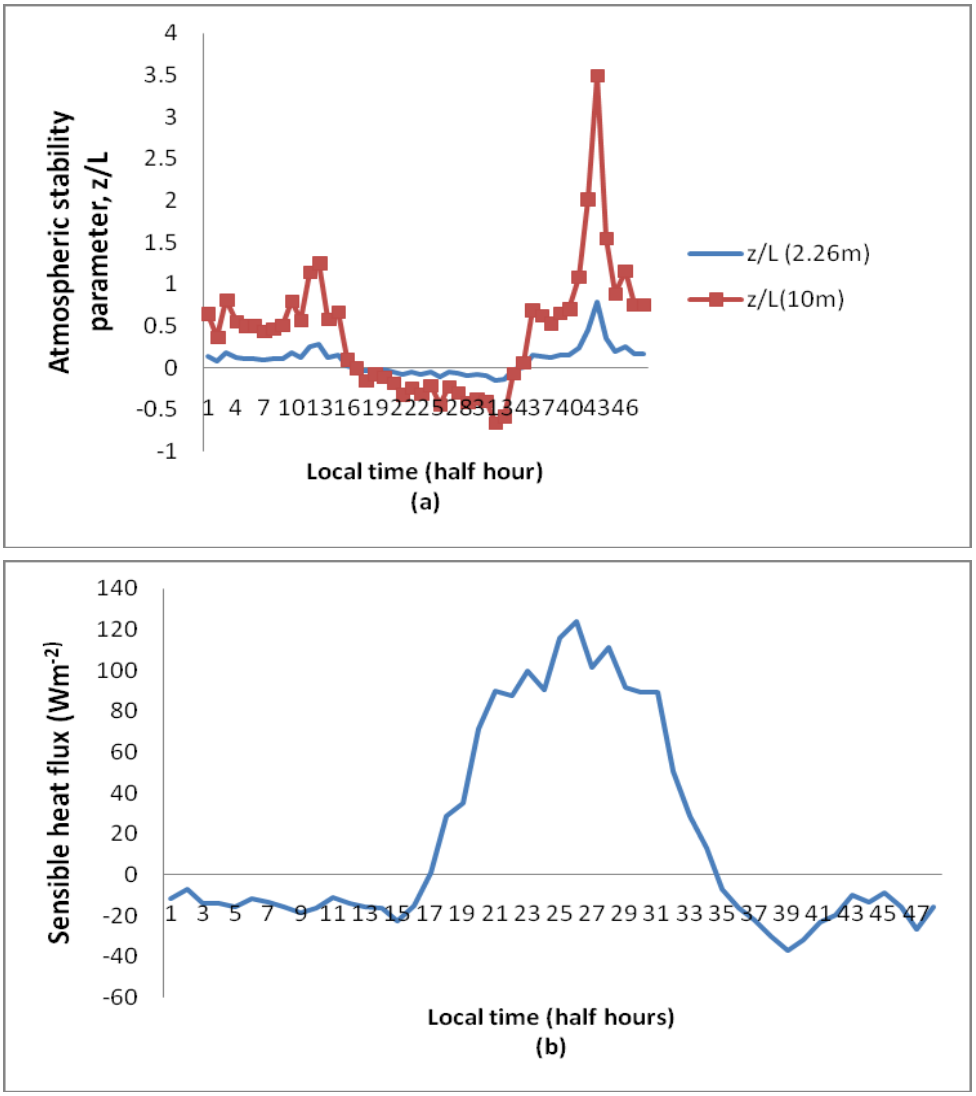

Fig. 4. Diurnal variation of (a) stability parameter, z/L at z= 2.26 m and 10 m and (b) sensible heat flux on DOY 66.

was always inversion at nights with temperature at 1m being the minimum, this is in agreement with existing observation that the minimum temperature on calm, clear and strongly stable night on grass, snow and bare surfaces was between 1 and 50 cm, (Oke, 1970). The minimum was not on the surface.

Diurnal variation of sensible heat flux and stability parameter, z/L

The sign of the sensible heat flux was opposite to the sign of z/L. When z/L was positive, the value of sensible heat flux was negative. The z/L is generally positive at nights, in the evening and early in the morning, at this time the atmosphere is stable so transfer coefficient is reduced. The positive sign of the temperature difference necessitates adding a negative sign to the sensible heat flux whenever z/L is positive to maintain the positive value of the turbulent bulk heat transfer coefficient. Sensible heat flux can be underestimated when the C_H values obtained from analytical method are used to compute it. The sensible heat flux will also lack diurnal variation when a constant bulk transfer coefficient is used for all the hours of the day (Fig. 4).

CONCLUSION

The importance of diurnal C_H and C_D values in modeling the energy budget of the earth and their unavailability in this part of the tropics led to their determination using both analytical and experimental methods. The experimental method was used to validate the analytical method. The experimental method gave lower diurnal C_H and C_D values with higher standard deviations. The obtained values were in the same range as those obtained

for other areas, Stull (1998), Zhang *et al*. (2002) and Ishikawa and Kodama (1994). These results can serve as a data base for this area.

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Short Communication

PECULIARITIES STUDY OF ACOUSTIC WAVES' PROPAGATION IN PIEZOELECTROMAGNETIC (COMPOSITE) MATERIALS

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ABSTRACT

This short paper has the purpose to discuss different coupling mechanisms that can be revealed in the coefficient of the magnetoelectromechanical coupling (CMEMC). Concerning the propagation problems of the shear-horizontal acoustic waves in the piezoelectromagnetics such as bulk homogeneous materials, inhomogeneous composites, and homogeneous plates, these CMEMC coupling mechanisms must be accounted to obtain wave characteristics in various configurations exploiting the smart piezoelectromagnetic materials. Indeed, many wave characteristics are already known for the shear-horizontal waves such as the surface, interfacial, and plate acoustic waves. It is obvious that they can have potential applications in the physical, biological, and chemical sensors, non-destructive testing and evaluation, etc.

PACS: 51.40.+p, 62.65.+k, 68.35.Gy, 68.35.Iv, 68.60.Bs, 74.25.Ld, 74.25.Ha, 75.20.En, 75.80.+q, 81.70.Cv
Keywords: CMEMC and coupling mechanisms, hexagonal (6*mm*) and cubic piezoelectromagnetics, magnetoelectric effect.

INTRODUCTION AND DISCUSSION

Piezoelectromagnetic (composite) materials, also frequently called the magnetoelectroelastic materials, are the well-known smart materials because the electrical subsystem of such materials can influence on the magnetic subsystem (and vice versa) via the mechanical subsystem. The interest in investigations of such smart materials is continuously growing that is seen in a large number of published review works. Using review paper (Zakharenko, 2013), the reader can find that already by about fifty reviews exist to this time. Today, piezoelectromagnetics can be utilized in various smart technical devices such as (biological and chemical) sensors, filters, delay lines, lab-on-a-chip, etc. Therefore, acoustic properties of such materials must be known.

The transversely isotropic (6 *mm*) piezoelectromagnetic materials are the most investigated and exploited because their characteristics can be disclosed in explicit analytical forms. This is extremely important for better understanding of their acoustic properties. However, even in the case of the employment of the quasi-static approximation (Auld, 1990; Dieulesaint and Royer, 1980) for the propagation of the shear-horizontal acoustic waves coupled with both the electrical and magnetic potentials, one can account a lot of material parameters of such smart materials. One of the very important characteristics of the smart materials is the coefficient of the magnetoelectromechanical coupling K_{em}^2 (CMEMC) that couples the material constants of the piezoelectromagnetics in the following formula:

$$K_{em}^2 = \frac{e(e\mu - h\alpha) - h(e\alpha - h\varepsilon)}{C(\varepsilon\mu - \alpha^2)} \tag{1}$$

Equality (1) contains the following independent nonzero material constants: the stiffness constant C, piezomagnetic coefficient h, piezoelectric constant e, dielectric permittivity coefficient ε, magnetic permeability coefficient μ, and electromagnetic constant α.

It is also clearly seen in equality (1) that the following coupling mechanisms containing the electromagnetic constant α can be apportioned in the CMEMC K_{em}^2:

$$e\mu - h\alpha \tag{2}$$
$$e\alpha - h\varepsilon \tag{3}$$
$$\varepsilon\mu - \alpha^2 \tag{4}$$

It is also indispensable to state that the values of $e(e\mu - h\alpha)$, $h(e\alpha - h\varepsilon)$, and $C(\varepsilon\mu - \alpha^2)$ in the CMEMC defined by expression (1) have the dimension of the mass density and can be therefore called the

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magnetoelectroelastic densities of coupling (MEEDC).

In dependence on the coupling mechanism which can play a major role, different wave characteristics of the propagation of the shear-horizontal acoustic waves in the piezoelectromagnetics can be obtained in explicit analytical forms. It is necessary to mention first theoretical works (Melkumyan, 2007; Liu *et al*., 2007; Wang *et al*., 2007; Wei *et al*., 2009) on the propagation of the shear-horizontal acoustic waves in such smart materials. In these recent works, the propagation of such acoustic waves along the boundary between the transversely isotropic (6*mm*) piezoelectromagnetic material and a vacuum was considered, i.e. the propagation of surface acoustic waves (SAWs) was treated. In these works, explicit analytical forms for the propagation of the piezoelectromagnetic SAWs localized at the boundary between two continuous media such as the piezoelectromagnetic half-space and a vacuum were obtained. Considering various mechanical, electrical, and magnetic boundary conditions at the boundary between the transversely isotropic piezoelectromagnetics and a vacuum, the author of theoretical work Melkumyan (2007) has demonstrated that twelve independent solutions can exist for the problem of the SAWs' propagation.

Following the results obtained in theoretical paper (Melkumyan, 2007), theoretical works Zakharenko (2010, 2011) have also studied peculiarities of the SAW propagation at the boundary between the piezoelectromagnetics and a vacuum exploiting different boundary conditions that are divided into mechanical, electrical, and magnetic ones. In book (Zakharenko, 2010), the SAW propagation in the transversely isotropic piezoelectromagnetics was also investigated and it was found that seven different SAWs can exist in addition to already discovered solutions (Melkumyan, 2007; Liu *et al*., 2007; Wang *et al*., 2007; Wei *et al*., 2009). The existence of these additional solutions discovered in Zakharenko (2010) is the consequence of the different coupling mechanisms (2), (3), and (4) clearly shown in the CMEMC (1). It is essential to state an analytical study of the propagation of the shear-horizontal SAWs in the transversely isotropic piezoelectromagnetic materials is significantly simpler in comparison with a study of the SAW propagation in the piezoelectromagnetics possessing the cubic symmetry. For the cubic piezoelectromagnetics, explicit analytical forms for the propagation velocities of the shear-horizontal SAWs cannot be revealed. As a result, the SAW velocities were calculated with numerical methods in Zakharenko (2011) that represents the single original work in this direction of the investigations.

It is worth mentioning theoretical works (Zakharenko, 2012 a,b) in which the shear-horizontal acoustic waves coupled with both the electrical and magnetic potentials propagating in the transversely isotropic piezoelectromagnetic materials were also studied. In a book Zakharenko (2012a), it was treated the propagation of such acoustic waves in non-homogeneous media consisting of two dissimilar piezoelectromagnetics with different mechanical, electrical, and magnetic properties and possessing the common interface, along which such non-dispersive acoustic waves can propagate. The propagation problems of dispersive shear-horizontal acoustic waves were considered in Zakharenko (2012b). This book studies the wave characteristics of the transversely isotropic piezoelectromagnetic plates representing the two-dimensional case. In this case, knowledge of the plate wave characteristics can allow the further miniaturization of various technical devices based on such smart (composite) materials. Also, the plate waves are widely used for non-destructive testing and evaluation of thin films. Concerning the applications in the aerospace industry, the plate waves can be used as one of the useful tools for inspecting of various defects of mechanical components with complex shapes.

CONCLUSION

These discussions introduced in this short paper can be useful for theoreticians and experimentalists working in the research arena of the propagation problems of the shear-horizontal acoustic waves in the smart piezoelectromagnetic materials such as the bulk homogeneous materials, inhomogeneous composites, and homogeneous plates. Indeed, one can account the coupling mechanisms discussed in this work in order to describe the propagation of the shear-horizontal acoustic waves in the piezoelectromagnetics. These acoustics waves can be useful, for instance, for applications in the non-destructive testing and evaluation.

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