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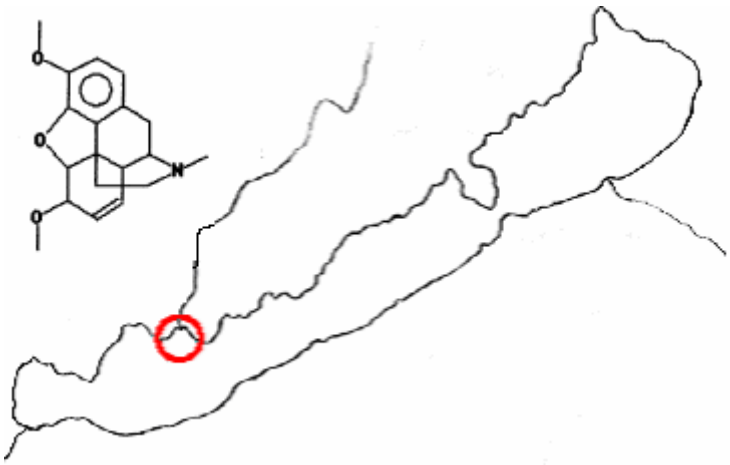

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# MICROBIAL ECOLOGY OF METHANE EMISSION IN RICE AGROECOSYSTEM: A REVIEW

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**Abstract.** Methane has profound impact on the physico-chemical properties in atmosphere leading to global climate change. Out of the various sources of $CH_4$, rice fields are the most significant contributors. The processes involved in the emission of $CH_4$ from rice fields to the atmosphere include $CH_4$ production (methanogenesis) in the soil by methanogens, methane oxidation (methanotrophy) by methanotrophs and vertical transfer of $CH_4$ via plant transport and diffusion or ebullition. In the overall methane dynamics rice plants act as : a) source of methanogenic substrate, b) conduit for $CH_4$ through well developed system of inter cellular air space (aerenchyma), and c) potential methane oxidizing micro-habitat in the rhizosphere by diffusing oxygen which favour the growth and multiplication of methanotrophs. Apart from mechanistic uncertainties, there are several other uncertainties in the estimation of $CH_4$ flux. Methane dynamics in the paddy field is controlled by a complex set of parameters linking the biological and physical characteristics of soil environment like temperature, carbon source, Eh, pH, soil microbes and properties of rice plants, etc. It has now become possible to isolate, detect and characterize the methanogens and methanotrophs by using molecular biological tools like PCR, FISH, etc. techniques. The apparent half saturation constant ($K_m$) and maximum oxidation rate ($V_{max}$) are distinctive parameters which determine the ability of bacteria to survive on atmospheric methane.
**Keywords.** *methane; methanotrophs; methanotrophy; methanogens; molecular tools*

## Introduction

Atmospheric methane ($CH_4$) is a potent greenhouse gas with high absorption potential for infrared radiation. Methane is present at about 1.8 ppmV in the atmosphere [135]. During the last 20 years, its concentration has been increasing, on an average at the rate of 0.8% $y^{-1}$ [125]. Due to this, $CH_4$ is of great concern as a greenhouse gas. Although the tropospheric $CH_4$ concentration is very low as compared to $CO_2$ (357 ppmV), methane accounts for 15 to 20% of global warming [71]. The total annual source strength of all methane emissions from anthropogenic origin is estimated to be 550 Tg [133]. Major sources of this input include natural wetlands, rice fields, enteric fermentation in animals, termites and land fills. The contribution from rice cultivation is estimated to range from 20-100 Tg $CH_4$ $y^{-1}$ with an average of 60 Tg $CH_4$ $y^{-1}$ [71]. The biogenic methane is mostly produced by methanogenic archaea (methanogens) in anaerobic environments i.e. sediments and flooded rice fields [59]. Each year methanogens produce about 400 million metric tons of $CH_4$ [49]. Recent studies have shown that methane is not only produced in anoxic rice fields soil but also directly from the roots of rice plants which are inhabited by a methanogenic community different from that in the rice field soil [92].


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According to a current estimate, rice production will need to expand by around 70% over the next 25 years to support the growing human population [39]. For this, intensified global fertilizer application will be essential, and this will exacerbate the methane problem. It is projected that the methane emission from rice cultivation may increase from the 1990 level of 97 Tg $y^{-1}$ to 145 Tg $y^{-1}$ by 2025 [5]. India is an important rice producing country, comprising 28.6% of world rice cultivated area [65]. During recent years, studies on methane emission from Indian rice fields have focused on the influence of soil type, season, water regime, organic inputs, fertilizers, rice cultivars and agrochemicals [111]. Using the baseline scenario, annual methane emissions for China, India, Indonesia, Philippines and Thailand were calculated to be 3.73, 2.14, 1.65, 0.14 and 0.18 Tg $CH_4$ $y^{-1}$, respectively [106].

Chemical and biological processes consume methane in the global methane cycle. The only known biological sink for atmospheric methane is its oxidation in aerobic soils by methanotrophic bacteria, this may contribute up to 10-20% to the total methane destruction [128], or between 15 and 45 Tg $CH_4$ $y^{-1}$ [71]. Methanotrophs oxidize $CH_4$ with the help of methane monooxygenase (MMO) enzyme. These bacteria are classified into three groups (Type-I, Type-II and Type –X) based on the pathways used for assimilation of formaldehyde and other physiological and morphological features [58]. An enormous effort is being made worldwide by microbial ecologists to isolate, detect and characterize methanotrophs and methanogens in different rice ecosystems by using molecular biological tools and techniques [18, 46, 57]. Methanotrophy is an aerobic process [52], but in marine sediments and in some saline inland waters it could be anaerobic [36]. The apparent half saturation constant ($K_m$), and maximum rate ($V_{max}$) of $CH_4$ oxidation are characteristic parameters, which determine the ability of methanotrophs to grow on atmospheric $CH_4$ [31]. Several workers have reported that methane oxidation occurs in rice microcosm [56], wetland rice [40, 158] and dryland rice fields [44, 45]. Methane oxidation in rhizospheric soil is considered as an important sink for $CH_4$ [44, 45, 56]. Therefore, the knowledge of several environmental factors (e.g. temperature, fertilizer inputs, crop phenology and soil moisture) that can provide feedback on the capacity of soil to oxidize atmospheric $CH_4$, may have significant consequences on the global atmospheric $CH_4$ budget.

This review presents an overview of the underlying microbial basis for production, oxidation and emission of methane in paddy fields under the influence of several environmental factors. Molecular ecological approaches for the isolation, detection and characterization of methanogens and methanotrophs are also described.

## Production of methane

### *Methanogens*

Methanogens are strictly anaerobic unicellular organisms originally thought to be bacteria but now recognized as belonging to a separate phylogenetic domain, the *archae* [53]. Phenotypic characteristics of methanogenic bacteria are listed in Table 1. 16S rRNA analysis suggested that methanogens can be categorized under three groups. Group I comprises *Methanobacterium* and *Methanobrevibacter,* Group II contains *Methanococcus,* and Group III comprises the genera including *Methanospirillum* and *Methanosarcina* [53]. They proliferate in anaerobic fresh water environments, such as sediments and the digestive tract of animals [147].


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***Table 1.*** *Characteristics of methanogenic and methanotrophic bacteria (Source: [53, 58, 74, 101]).*

| Characteristics | Methanogens | Methanotrophs |
|---|---|---|
| Cell form | rods, cocci, spirilla, filamentous, sarcina | rods, cocci, vibrios |
| Gram stain reaction | Gram +/– | Gram – |
| Classification | Archaebacteria | Eubacteria |
| Cell wall | pseudomureine, protein, heteropolysaccharide | peptidoglycon |
| Metabolism | anaerobic | aerobic |
| Energy and carbon source | $H_2$+$CO_2$; $H_2$+methanol; formate; methylamines; methanol, acetate | methane; methanol; dimethyl-ether, methyl formate, dimethyl carbonate |
| Catabolic products | $CH_4$ or $CH_4$+$CO_2$ | $CO_2$ |
| TCA cycle | Incomplete | Incomplete (Type-I) or complete (Type-II) |
| Carbon assimilation pathways | TCA cycle, gluconeogenesis | ribulose monophosphate pathways (Type-I) or serine pathways (Type-II) |
| Resting cells | - | cysts (Type-I) or exospore (Type-II) |
| GC content mol% | 26-60 | 50-62.5 |
| Typical species | *Methanobacterium bryanthii*<br>*Methanobrevibacter smithii*<br>*Methanomicrobium mobile*<br>*Methanogenium cariaci* | *Methylosinus trichosporium*<br>*Methylomonas methanica*<br>*Methylocystis minimus*<br>*Methylobacter albus* |

In these habitats, methanogens play an important role in the degradation of complex organic compounds. Most methanogens are mesophilic, able to function in temperature ranging from 20 to $40^0$C [147]. They are also found in extreme environments like hydrothermal vents where they thrive at temperatures above $100^0$C. Methanogens mainly use acetate (contributes about 80% to $CH_4$ production) as a carbon substrate but other substrate like $H_2/CO_2$ and formats also contribute 10-30% to $CH_4$ production [27]. All methanogens use $NH_4^+$ as a nitrogen source, although the ability to fix molecular nitrogen and the *nif* gene is present in all the three orders (*Methanobacteriales, Methanococcales and Methanomicrobiales*) of methanogens [120].

***Methanogenesis***

Methane is produced in the anaerobic layers of paddy soil by bacterial decomposition of organic matter [39]. The organic matter converted to $CH_4$ is derived mainly from plant-borne material, and organic manure [35], if applied. The anaerobic degradation of organic matter involves four main steps: a) hydrolysis of polymers by hydrolytic organisms, b) acid formation from simple organic compound by fermentative bacteria, c) acetate formation from metabolites of fermentations by homoacetogenic or syntrophic bacteria, and d) $CH_4$ formation from $H_2/CO_2$, acetate, simple methylated


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compounds or alcohols and $CO_2$ [163]. $CH_4$ is produced in rice fields after the sequential reduction of $O_2$, nitrate, manganese, iron and sulphate, which serve as electron acceptors for oxidation of organic matter to $CO_2$ [164]. Methanogenesis from all substrates requires a number of unique coenzymes, some of which are exclusively found in methanogens [98]. At least nine methanogen-specific enzymes are involved in the pathway of methane formation from $H_2$ and $CO_2$ [140]. In paddy soil, acetate and $H_2$ are the two main intermediate precursors for $CH_4$ formation [162].

***Methanogenesis from $H_2/CO_2$***

The first step of the pathway comprises the binding of $CO_2$ to methanofuran (MFR) and its $H_2$ dependent reduction to formyl-MF (first stable intermediate compound of the pathway). Formation of this complex is catalyzed by *formylmethanofuran dehydrogenase.* Further, the formyl moiety of the formyl-MF is subsequently transferred to tetrahydromethanopterin ($H_4$MPT), which with $H_4$MPT *formyl transferase,* formes methenyl-$H_4$MPT, while reduces to methylene-$H_4$MPT and then to methyl-$H_4$MPT. In both reactions, reduced coenzyme $F_{420}$ serves as the reductant. The $F_{420}$-dependent reduction of methenyl-$H_4$MPT is reversible and is catalyzed by *methylene* $H_4$MPT *dehydrogenase.* The reversible $F_{420}$ $H_2$-dependent reduction of methylene - $H_4$MPT to methyl-$H_4$MPT is catalyzed by *methylene-$H_4$MPT reductase.* In the next step of $CO_2$ reduction pathway, the methyl group is transferred from N-5-methyl $H_4$MPT to coenzyme M (2-mercaptoethane sulfonate) giving rise to methyl coenzyme M(methyl-CoM). The reduction of methyl CoM is catalysed by the methyl-CoM-reductase. This reaction involves two unique coenzymes. The first one is N-7-mercaptoheptanoylthreonine phosphate (HS-HTP) which acts as electron donor in the reduction of methyl CoM giving rise to $CH_4$ and mixed disulfide of HS-CoM and HS-HTP (CoM-S-S-HTP). The second coenzyme involved in this reaction is factor $F_{430}$ (a nickel porphinoid) which is the characteristic prosthetic group of the methyl reductase [16, 74, 140].

***Methanogenesis from acetate***

Methanogenesis from acetate starts with its activation to acetyl-CoA. *Methanosarcina* and *Methanothrix* use different ways of acetate activation. The former organism takes advantage of *acetate kinase* and *phosphotans acetylase* whereas the later makes use of *acetyl-CoA synthatase.* All three enzymes are soluble and oxygen insensitive. Further breakdown of *acetyl- CoA* catalyzes the cleavage of *acetyl-CoA,* giving rise to a methyl group, a carbonyl group and CoA, all of which are transiently bound to the enzyme. In a further step, the Co-*dehydrogenase* complex catalyzes the oxidation of the carbonyl group. The $CO_2$ is thereby formed and CoA is released from the enzyme, where the methyl group is transferred to a corrinoid-Fe-S protein. This complex catalyzes not only the cleavage of *acetyl-CoA* and oxidation of the carbonyl group but in addition, the transfer of the methyl moiety to $H_4$MPT. Further pathway from methyl $H_4$MPT to $CH_4$ takes advantage of the pattern similar to that discussed for the utilization of the $CO_2/H_2$ [16, 98].

***Thermodynamics of $CH_4$ production***

The process of methane production in paddy fields is thermodynamically exergonic [163]. The Gibbs free energy ($\Delta G$) for the process is mainly a function of the acetate concentration and $H_2$-partial pressure. A pre-requisite for early methane production


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seems to be a sufficiently high $H_2$-partial pressure that corresponds to $\Delta G$ of $H_2$ dependent methanogenesis (hydrogenotrophic) of less than about -23 kJ $mol^{-1}$ $CH_4$ [162]. The time until the onset of $CH_4$ production and the magnitude of production is a function of the quantity of easily degradable organic matter, reducible Fe(III) and sulfate [162]. Methanogens are energetically limited by availability of their substrates $H_2$ and acetate as long as iron or sulfate reducers are able to compete for them [1]. The methanogens have to compete for available substrates with other anaerobic bacteria, namely the nitrate, manganese, ferric iron and sulfate reducers. The competition for carbon substrates in general follows thermodynamic rules: nitrate reducers outcompete the other anaerobic bacteria for the substrates [149]. Several studies have reported different $\Delta G$ values for methanogenesis in various paddy fields. For Italian paddy fields the values of $\Delta G$ for methane production were found to be -31.6 to 34.8 kJ $mol^{-1}$ $CH_4$ [1] and -24 to -38 kJ $mol^{-1}$ $CH_4$ [27]. Peters and Conrad [123] found that $\Delta G$ ranged from -25 to -50 kJ $mol^{-1}$ $CH_4$ for German rice fields. Cultures of *Methanobacterium bryantii* required $\Delta G$ values of less than -30 kJ $mol^{-1}$ $CH_4$ for $CH_4$ production [31]. Kral et al. [86], reported that for cultures of other methanogens $\Delta G$ values varied between -32 and -60 kJ $mol^{-1}$ $CH_4$ for hydrogenotrophic methanogenesis. Yao and Conrad [162] calculated the $\Delta G$ for acetate dependent methanogenesis (acetoclastic) and concluded that unlike $H_2$-dependent methanogenesis acetate based process was apparently not under thermodynamic control. They found that $CH_4$ production was less at $\Delta G$ equal to -26 kJ $mol^{-1}$ $CH_4$ and was the average at $\Delta G$ equal to - 29 kJ $mol^{-1}$ $CH_4$. Roy et al. [130] have suggested that early $CH_4$ production is due to $H_2$-dependent methanogenesis and that acetate-dependent methanogenesis only starts later when sulfate and Fe(III) have been reduced in paddy soils. Further, methane production from $H_2/CO_2$ is not started before fermentation has increased the $H_2$ partial pressure to an amply high value to allow exergonic production of $CH_4$ at a $\Delta G$ of less than about - 26 kJ $mol^{-1}$ $CH_4$ [130, 162].

***Methanogenic population and $CH_4$ emission***

The mechanism of methanogenesis in paddy fields worldwide has been investigated in detail. However, information regarding methanogenic population size in rice fields is limited. Rajagopal et al. [126] were the first to carry out isolation and characterization of methanogens from Louisiana paddy fields and reported the presence of two *Methanobacterium*-like strains and two *Methanosarcina*-like strains. Joulian et al. [76] recorded the methanogenic populations from the paddy fields of France, the Philippines, and USA. Their results of the classic counts of methanogenes, and strains isolated and identified by l6S rRNA gene sequencing, suggested the dominance of *Methanobacterium spp*. and *Methano-sarcina spp*. among the culturable organisms. Reichardt et al. [129] revealed that methanogens were abundant in root extracts of mature rice plants. Methanogens are also exist on the rhizoplane of the rice plants [92]. Fetzer et al. [50] isolated four genera (*Methanobacterium, Methano-sarcin, Methanobrevibacter* and *Methanoculleus*) from Italian rice fields. According to Asakawa et al. [6] there are only two strains (*Methanobrevibacter arboriphilus* and *Methanosarcina mazeii*) of methanogens in rice fields which have been identified to the species level. Adachi [2], isolated *Methanobacterium* and *Methanobrevibacter spp.* from subtropical Japanese rice fields. Kudo et al. [87] reported the presence of *Methanosarcina, Methanogenium, Methanosaeta* and *Methanoculleus*-like organisms in rice paddy fields of Japan by using a molecular retrival approach with archael small


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subunit (SSU) rRNA encoding gene (rDNA) sequences. Results of investigations on methanogenic population size in various types of rice fields are summarized in Table 2.

***Table 2.*** *Population size of methanogens and methanotrophs in various rice fields.*

| **Ecozones/location** | **Methanogens** | **References** | **Methanotrophs** | **References** |
|---|---|---|---|---|
| Rice fields (Australia, France, Philippines, USA, Trinidad) | $<10^1$–$2.3x10^6$ cells $g^{-1}$ dw | [75] | $1.5x10^3$–$3.5x10^7$ cells $g^{-1}$ dw | [75] |
| Rice fields (Zhejiang, China) | $4.6x10^3$–$1.3x10^7$ CFU $g^{-1}$ dw | [108] | $3.0x10^6$-$2.3x10^8$ CFU $g^{-1}$ dw | [108] |
| Rice fields (Beijing, China) | $1.4x10^5$ – $2.3x10^5$ $g^{-1}$ dw | [64] | - | - |
| Rice fields (Germany) | - | - | $3.7x10^4$–$3.8x10^7$ bacteria $g^{-1}$ dw | [13] |
| Rice fields (Cuttack, India | $0.2 – 2.8x10^5$ MPN$g^{-1}$ soil | [90] | $4.2 x10^4$–$5.2x10^6$ CFU $g^{-1}$ dw | [89] |
| Rice fields (Cuttack, India) | 2.4–6.1 x $10^6$ MPN $g^{-1}$ dw | [88] | - | - |
| Rice fields (Cuttack, India) | $5.4 – 7.5x10^3$ cells $g^{-1}$ dw | [15] | - | - |
| Dryland Rice fields (Varanasi, India) | - | - | $5.3x10^6$–$7.4x10^7$ cells $g^{-1}$ soil | [42] |
| Wetland Rice fields (Varanasi, India) | - | - | $2.6x10^6$–$5.0x10^7$ cells $g^{-1}$ soil | [43] |
| Rice fields (Northern Italy) | - | - | $4.2x10^6$–$2.3x10^7$ cells g-$^{1}$ dw | [10] |
| Rice fields ( Narthern Italy) | - | - | $4.0x10^5$-$2.0x10^8$ cells $g^{-1}$ dw | [56] |
| Rice fields (Japan) | $5.2x10^4$–$1.1x10^6$ cells $g^{-1}$ dw | [151] | $1x10^4$–$1x10^7$ CFU$g^{-1}$ dw | [157] |

***Table 3.*** *Variation of estimates of global methane emission (Tg $CH_4$ $y^{-1}$) from rice fields during 1963-1998.*

| **$CH_4$ emission Tg $CH_4$ $y^{-1}$** | **References** |
|---|---|
| 190 | [85] |
| 280 | [48] |
| 59 | [28] |
| 95 | [78] |
| 35-59 | [138] |
| 120-200 | [32] |
| 70-170 | [62] |
| 142-190 | [14] |
| 47-145 | [136] |
| 25-60 | [116] |


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*Table 3. continued from page 6.*

| **$CH_4$ emission Tg $CH_4$ $y^{-1}$** | **References** |
|---|---|
| 20-100 | [68] |
| 66 | [79] |
| 50 | [114] |
| 20-150 | [124] |
| 60-105 | [70] |
| 20-100 | [71] |
| 25-54 | [132] |
| 30-50 | [117] |
| 50-80 | [93] |

The first measurements of $CH_4$ emission from paddy fields were conducted in California by Cicerone and Shetter [28].This was followed by extensive studies in Spain [138], China [26], USA [133], Japan [157], Philippines [115], and India [110, 139], but the estimates of methane emissions vary widely, e.g., from 18.0 to 27.1 mg $CH_4$ $m^{-2}$ $h^{-1}$ in Indonesia [119], 19.5 to 32.2 mg $CH_4$ $m^{-2}$ $h^{-1}$ in Thailand [160] and 19 to 79 mg $CH_4$ $m^{-2}$ $h^{-1}$ in Philippines; $CH_4$ emission from temperate rice fields is relatively lower (6.67 and 18.25 mg $CH_4$ $m^{-2}$ $h^{-1}$) [136]. These field experiments varied widely in the methodologies (closed chamber with automatic or manual sampling devices), sampling frequencies (continuous or sporadic sampling), observation periods (one season or several consecutive years) and sampling field designs (randomized plots or single fields). Recent estimates indicated that methane release ($m^{-2}y^{-1}$) from different rice ecosystems follows the order deepwater> irrigated> rainfed rice [115]. The distinction among irrigated, rainfed, and deepwater rice fields is a common feature of the available statistics of rice cultivated area. A specific assessment of these ecosystems will, therefore, directly improve the accuracy of regional and global estimates of the methane source strength [67]. The estimates on global $CH_4$ emission from paddy fields, however, have varied over time period, mainly depending on approach, technique and database used for extrapolation. Table 3 shows reported estimates of global methane emission rates from various rice fields during 1963-1998. These have varied from as low as 12 Tg $CH_4$ $y^{-1}$ to as high as 200 Tg $CH_4$ $y^{-1}$. This is largely due to the lack of uniformity in the methods used for collecting field data, location of experimental sites and sesional variations.This problem was addressed by Khalil and Shearer [79] who developed an inventory of direct flux measurements from a number of studies and modified the information from Matthews et al. [105] on the duration of growing seasons to estimate global and regional annual methane emission rates. They arrived at a figure for the global $CH_4$ emission rate of 66 Tg $CH_4$ $y^{-1}$.

***Pathways of methane emission***

The net amount of $CH_4$ emitted from soil to the atmosphere is the balance of two opposite processes - production and oxidation. Methane, the product of methanogenesis, escapes to the atmosphere from soil via aerobic interfaces where $CH_4$ oxidation takes place. There are three pathways of $CH_4$-transport into the atmosphere – molecular diffusion, ebullition and plant transport (Fig. 1).

In the temperate rice fields more than 90% of the $CH_4$ is emitted through plant transport [136] while in the tropical rice fields, significant amounts of $CH_4$ may evolve


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by ebullition (gas transport via gas bubbles) in particular during the early period of the season and in the case of high organic input [38].

Ebullition is also the common and significant mechanism of $CH_4$ flux in natural wetlands [155]. According to Sass et al. [131], ebullition can play significant role in $CH_4$ transport under high organic fertilization. If soil is unvegetated or plant aerenchyma is not yet well-developed, ebullition plays a major role in $CH_4$ emission [22] but it occurs only at surface layer and its rate is regulated by $CH_4$ concentration, temperature, soil porosity and plant aerenchyma [94].

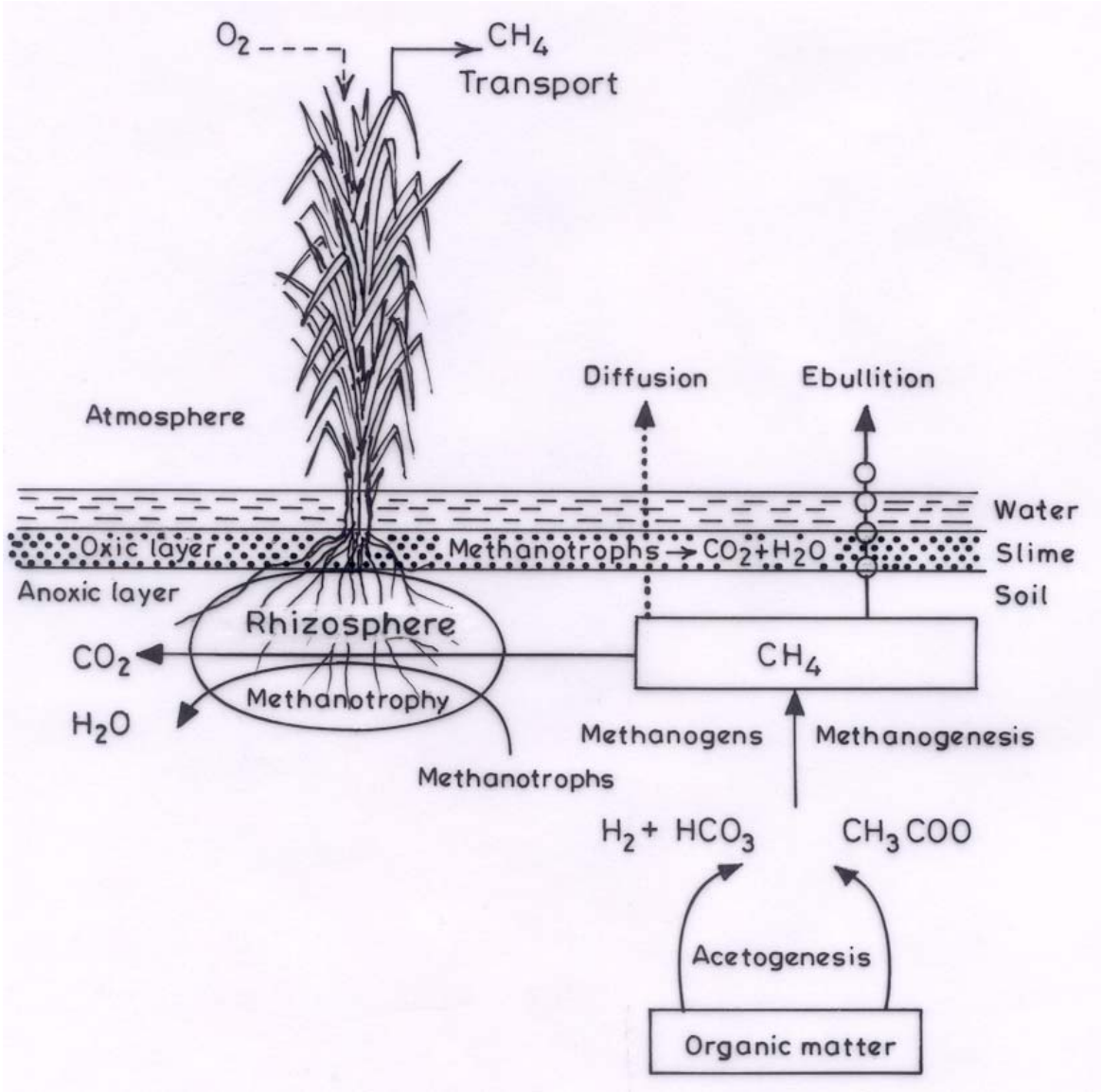


***Figure 1.*** *Conceptual schematic diagram of methane production, oxidation and emission from paddy field.*

Diffusion of $CH_4$ across the flooded soil and overlying water of the rice field to the atmosphere is a function of surface-water concentration of $CH_4$, wind speed and $CH_4$ supply to the surface water [137]. $CH_4$ diffusion through the soil is a very slow process because the 'diffusion rate' of gaseous $CH_4$ is very low in liquid phase (about 104 times


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slower than diffusion through the gas phase), therefore, it hardly contributes to the total $CH_4$ flux [8].

Plant mediated transport is the primary mechanism for the $CH_4$ emission from paddy fields, and contributes 60-90% to the total $CH_4$ flux [154]. Methane in the soil-water surrounding the roots dissolves into the surface-water of the roots, diffuses into the cell-wall water of root epidermis cells, and then diffuses through the cell-wall water of the root-cortex, depending upon the concentration gradient between the soil-water surrounding the roots and the lysigenous inter-cellular spaces in the roots [118]. Methane is then gasified in the root cortex and transported to the shoots via lysigenous intercellular spaces and aerenchyma. Eventually, $CH_4$ is released primarily through the micropores in the leaf sheath of the lower leaf position and also through the stomata in the leaf blade [118].

***Factors affecting methane emission***

Methane emission from paddy fields is controlled by a complex set of parameters linking the physical and biological characteristics of soil environments with specific agricultural practices. Methane production depends on the soil organic carbon content and quality, texture, Eh, pH, Fe content, sulfate content and salinity and application of fertilizers, etc.

*Soil pH. Eh and texture*

Methane production in flooded rice soils is very sensitive to pH with an optimum range between 6.7 and 7.1 [152]. Effect of soil pH on $CH_4$ production varied by about two orders of magnitude in four different Indian soils but was found to be maximum at pH around 8.2 [121].

Yagi and Minami [161] reported that values of redox potential (Eh) varied from -100 to -200 mV for the initiation of $CH_4$ production in paddy soils. Masscheleyn et al. [104] incubated rice soil under controlled redox levels ranging between -250 and +500 mV. They found the threshold for methane production to be -150 mV. Some suggested that soils containing greater amounts of readily decomposable organic substrates (acetate, formate, methanol, methylated amines, etc.) and low amounts of electron acceptors ($Fe^{3+}$, $Mn_4^{+}$, $NO_3^{-}$, $SO_4^{2-}$) are likely to show high production of $CH_4$. According to sequential oxidation -reduction order, molecular $O_2$ is the first to be reduced at an Eh of about +30 mV followed by $NO_{3-}$ and $Mn_4^{+}$ at 250 mV, $Fe^{3+}$ at + 125 mV and $SO_4^{2-}$ at -150 mV (Patrick, 1981). Subsequent to $SO_4^{2-}$ reduction, methanogens will start producing methane [8].

As texture determines various physico-chemical properties of soil, it could influence $CH_4$ production indirectly. Jackel et al. [72] found that rates of $CH_4$ production increased when the aggregate size of the soil increased. A negative co-relationship between $CH_4$ emission and clay content was reported by Sass and Fisher [133]. Seasonal $CH_4$ emissions indicated a negative relationship to clay content for Texan paddy soils [133].

*Temperature*

Methane emission is much more responsive to temperature. Temperature not only has an effect on methane production itself but also has an effect on the decomposition of


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organic materials from which the methanogenic substrates are produced [27]. The influence of temperature on $CH_4$ production rates has been reported for several rice ecosystems [121, 145]. Wassman et al. [156] observed a faster development of $CH_4$ production rate and higher maximum value with increasing temperatures between 25 and 35°C. Hattori et al. [59] recorded optimum temperature of 40°C for $CH_4$ production in Japanies paddy fields due to dominance of methanogenic population at this temperature.

*Growth period and crop phenology*

Wassmann et al. [154] recorded lower $CH_4$ fluxes in the early growth period of rice plant, which increased gradually during mid to late season and dropped to very low level before or after harvest. Jermsawatdipong et al. [73] found that more than 50% of $CH_4$ was emitted in the first half of the growth period in Thailand rice fields, while $CH_4$ emissions in Japanese rice fields occurred mainly in the second-half of the growth period [81]. Jermsawatdipong et al. [73] argued that the high temperatures from the beginning of rice growth in the tropics caused the main decomposition stage of soil and applied organic materials to shift to early growth stage which resulted in active $CH_4$ production from the very beginning of rice growth. Seiler [138] observed maximum $CH_4$ emission at the end of heading and flowering stage off rice plants in Spain.

Flowering period is generally considered as the peak period for methane emission. The peak emission value remains for a period of 10-15 days in the crop duration of 90-100 days. According to Holzapfel-Pschorn et al. [62] this period emits 90% of the total, methane during the whole crop season, because the biomass of rice crop increases gradually, reaching the maximum weight by flowering. Up to 50 % of the total methane emission from rice fields can be due to root exudation [35]. Methane emission decreases after flowering because the rate of photosynthesis declines after the commencement of grain development and hence the supply of available assimilates for methane production decreases [142].

*Diurnal and seasonal variations*

Emission rates of $CH_4$ generally increase rapidly after sunrise, reach a peak in the early afternoon then decline rapidly and level off at night. Methane emission rates during the early and late phase of plant growth varied with a distinct maximum in the early afternoon, while this variation pattern is less pronounced in the middle stage of plant growth [8]. Buendia et al. [21] reported that diurnal patterns of $CH_4$ fluxes are relatively similar across study sites in same climates and depend on crop phenology. Three seasonal maxima were found in Italy, the first shortly after flooding, the second during the vegetative growth stage and third during the grain filling and maturity stage of rice plants [136].

*Rice cultivars, organic manures and crop residues*

Rice cultivars have received high research priority because high yielding rice cultivars with low $CH_4$ emission rates can be easily extended to farmer's fields without any additional input and management practices [151]. Wang et. al. [153] argued that, cultivars influence the $CH_4$ emission by providing the soil with root exudates, decaying root tissues and leaf littre while Aulakh et al. [7] found significant variations in methane


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transport capacity of different rice cultivars. In Korean rice cultivars, the $CH_4$ flux among the rice varieties ranged from 36.9 g $CH_4$ $m^{-2}$ to 76.0 g $CH_4$ $m^{-2}$ [141]. The amendment of organic matter (cattle manure, pig manure, chicken manure, etc.) to a flooded rice field, increases $CH_4$, production. It reduces the soil Eh and provides carbon to methanogens. Organic materials influence the $CH_4$ formation through change in qualitative and quantitative properties of soil.

*Fertilizers*

Numerous studies have revealed the impact of chemical fertilizers on $CH_4$ emissions [3, 136, 139]. The effect of fertilizers on $CH_4$ emission depends on rate, type and mode of applications. Urea application enhances $CH_4$ fluxes over the growth season possibly by increasing soil pH following urea hydrolysis and the drop in redox potential, which stimulates methanogenic activities [152].

Lindau [95] reported decrease in $CH_4$ emission rate with ammonium nitrate application due to competitive inhibition of nitrate reduction in favour of methane production. Under field conditions, the application of sulphate based fertilizers such as $(NH_4)_2$ $SO_4$ and $CaSO_4$ have reduced $CH_4$ emission [24] and application of $K_2HPO_4$ enhances the $CH_4$ emission [4].

## Methane oxidation

### *Methanotrophs*

Methanotrophs (gram negative, aerobic bacteria belonging to the subset of a physiological group of bacteria known as methylotrophs) oxidize $CH_4$ via methanemonooxygenase (MMO) enzyme (Table 1). These bacteria are classified into three groups: Type-I, Type-II and Type-X. According to Conrad [29], all the methanotrophs that have so far been isolated and described belong to the Proteobacteria, of the $\gamma$ sub-class (Type I) or $\propto$ sub-class (Type II). The Type I group is represented by the *Methylomonas, Methylocaldum, Methylosphaera, Methylomicrobium* and *Methylobacter*. The Type-II comprises *Methylosystis* and *Methylosinus*. The members of the genus *Methylococcus* occupy an intermediate position and have been kept in to a separate group Type-X [58]. By using molecular ecology techniques, it has become clear that methanotrophs are ubiquitous in nature and well adopted to high or low temperature, pH and salanity [148]. Henckel et al. [61] found that both Type-I and Type-II methanotrophs were stimulated in rice fields with unsaturated water content. Bodelier et al. [18] reported that Type II methanotrophs dominated in unplanted, unfertilized soils and the presence of rice plant was an essential factor for Type-I methanotrophs to proliferate. Methanotrophic bacteria are present in the aerobic soil layer, rhizosphere [42, 56, 75] and on the roots and stem bases of flooded rice plants [158]. The physiology, biochemistry and ecology of methanotrophic bacteria have been recently reviewed [29, 41].

### *Methanotrophy*

The oxidation of methane by methanotrophs is initiated by methane monooxygenase (MMO) enzyme. The MMO occurs in two forms: as a membrane bound particulate form (sMMO) in all types of methanotrophs, and as a soluble form in Type-II and Type-


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X methanotrophs [102]. Results of comprehensive studies on the structure, function and regulation of the MMO have been recently presented by Murrell et al. [113].

According to Conrad [29], the biggest problem for the energy metabolism is the activation of the relatively inert $CH_4$ molecule. The activation is achieved in the initial step by the MMO which converts $CH_4$, $O_2$ and reducing equivalents to methanol and $H_2O$, i.e.:

$$CH_4 + O_2 + 2NAD(P) \xrightarrow{MMO} CH_3OH + H_2O + 2\ NAD(P)$$

The reducing equivalents are supplied by the subsequent dehydrogenation steps, e.g. the conversion of methanol to formaldehyde to formate to $CO_2$ in which a total of 6 electrons are liberated [29].

Two main $CH_4$ oxidation pathways, catabolic and anabolic are present in methanotrophs [34]. Catabolic pathway is purely enzymatic through which methanotrophs oxidize methane to $CO_2$ via methanol, formaldehyde, and formate catalyzed by the enzymes: methane monooxygenase, methanoldehydrogenase, formaldehyde dehydrogenase and formate dehydrogenase, respectively. In this pathway energy is released but carbon is not incorporated into cellular biomass. The anabolic pathway may be further divided into two sub-pathways, ribulose monophosphate pathway and serine pathway. In both these pathways carbon of methane is incorporated in cellular biomass at the level of formaldehyde. Carboxylic acids and amino acids are the intermediary products of the serine pathway. Serine transhydroxy-methylase is the initiater enzyme of this pathway. On the other hand, phosphoglycerated sugars are intermediary products in ribulose monophosphate pathway. RuMP pathway is a cyclic pathway in which fixation is followed by cleavage and rearrangement reactions. Type-I and Type-X methanotrophs follow RuMP pathway whereas serine pathway is followed by Type-II and Type-X methanotrophs [58].

***Methanotrophs population and methane oxidation in paddy soils***

In rice fields all type of methanotrophs have been detected [52]. From Italian paddy soils two strains of Type-II methanotrophs were isolated [56]. There is a large variation in number of methanotrophs on rice roots, e.g. < 0.1 MOB $mm^{-2}$ to > 120 MOB $mm^{-2}$ of root surface and in the soil (Table 2). It has been reported that the population size of methanotrophs depends upon location of experimental site, concentration of $CH_4$ in the soil [10] and concentration of $NH_4^+$-N [42, 75]. Rhizosphere has several orders of magnitude higher MOB population than bulk soil [42, 43, 56]. Population size of MOB in rice planted microcosm, rhisoplane, dryland and flooded rice soils as well as endorhizospheric population in rice roots increase with time [20].

Most quantitative data upon methanotrophic population size rely on MPN methods. The limitations of this method are well known. The medium may be selective for certain strains; cells may be in unculturable state; resting and active cells can not be differentiated and microcolonies may be counted instead of single cells [52]. Considering these limitations, the current state of knowledge about population size of methonotrophs from various paddy fields is given in Table 2.

Uptake of atmospheric $CH_4$ through biological oxidation has been reported in a variety of rice-agroecosystems (Table 4). First evidence of plant associated $CH_4$ oxidation came from studies with microcosms [52]. Several scientists estimated the amount of $CH_4$ that is oxidized in association with rice plants and compared with overall $CH_4$ oxidation [20, 56]. From the review of the available data of $CH_4$ uptake,


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Minami et al. [109] estimated the total terrestrial $CH_4$ consumption to be between 7 and 78 Tg $y^{-1}$. Although, rice field is an important source of $CH_4$, the data of $CH_4$ oxidation by unflooded paddy soil after harvest could be important for the $CH_4$ global budget [145].

***Table 4.*** *Methane oxidation rates in a variety of rice fields.*

| Site | $CH_4$ oxidation | Sources |
|---|---|---|
| Rice field (Panama) | 0.1-1.5 mg $CH_4$ $m^{-2}d^{-1}$ | [77] |
| Paddy field (Italy) | 0.1-7.6 nmol $CH_4$ $h^{-1}cm^{-2}$ | [30] |
| Paddy field (USA) | 170-460 mg $CH_4$ $m^{-2}d^{-1}$ | [54] |
| Paddy field (Italy) | 0.3-162.5 nmol $CH_4$ $h^{-1}g^{-1}dw$ | [12] |
| Paddy field (USA) | 126.2-348.5 ng $CH_4$ $h^{-1}g^{-1}$ | [153] |
| Paddy field (China) | 0.7-1.4 µmol $CH_4$ $h^{-1}g^{-1}dw$ | [17] |
| Paddy field (Japan) | 9.5-18.8 µg $CH_4$ $g^{-1}$ root $d^{-1}$ | [151] |
| Paddy field (China) | 54.0-892.0 ng $CH_4$ $g^{-1}h^{-1}$ | [23] |
| Paddy field (Italy) | 284-810 nmol $CH_4$ $h^{-1}gfw^{-1}$ | [72] |
| Paddy field (Italy) | 0.1-0.2 µmol $CH_4$ $g^{-1}$ $dwh^{-1}$ | [51] |

Gilbert and Frenzel [56] found that the greater part of the $CH_4$ produced in paddy soil is probably oxidized either in the surface layer of the paddy soil or in the rhizosphere of rice plants. Thurlow et al. [145] showed that unflooded paddy soils after drainage practices, are able to act as sink of $CH_4$ and vary in their ability to oxidize it depending on the soil temperature and atmospheric $CH_4$ concentrations. We have found that rhizospheric soil oxidized greater amount of $CH_4$ (dryland 64-86% ; flooded rice soil 46 to 64%) as compared to bulk and bare soils [40, 45]. Denier and Neue [37] reported that $CH_4$ emission from rice plants one week before panicle initiation increased by 40% if $CH_4$ oxidation in the rhizosphere was blocked.

### *Kinetics of methane oxidation*

The apparent half saturation constant ($K_m$), and maximum oxidation rate ($V_{max}$) of $CH_4$ oxidation are characteristic parameters which determine the ability of methanotrophs to grow on atmospheric methane. The $CH_4$ concentration is a key determinant of $K_{m(app)}$ but this could be mediated through the MMO enzyme, the methanotrophs or the bacterial community as a whole [47].

A model recently proposed by Koch [84] suggest that $K_{m(app)}$ may change in response to the dynamics of substrate utilization as determined by coupling between transport, growth and internal substrate pools. The general model may be useful when applied to methanotrophs, because the first product of methane oxidation, methanol, is sometime excreted and affects the kinetics of MMO [84]. Affinity of methanotrophs for $CH_4$ varies with growth conditions [45, 47]. According to King [83] the $V_{max}$ of root associated $CH_4$ oxidation varied largely with season, indicating quantitative and/or qualitative changes in methanotrophic communities.

Recent studies revealed that there are two types of $CH_4$ oxidizers present in the soil. One population, having a high affinity for $CH_4$, typically has $K_m$ in the range 1000 nM $CH_4$, and the other population, having a high affinity for $CH_4$, has $K_m$ in the range of 30 to 60 nM $CH_4$ [13]. These methanotrophs typically occur in upland soils that consume atmospheric methane [11], but can also be activated in the paddy soils [10]. However,


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the atmospheric $CH_4$ oxidation has hardly been studied in irrigated rice fields soil. Henckel and Conrad [61] found that moisturised air dried paddy soil does not oxidize $CH_4$ at atmospheric concentration unless it has been pre-incubated under elevated $CH_4$ concentration. A decreasing trend of $K_m$ and $V_{max}$ with decreasing $CH_4$ uptake rate along the soil depth was reported by Wang et al. [153].

Dubey et al. [44, 45] have found that $K_m$ and $V_{max}$ values for $CH_4$ oxidation in dryland/flooded rice fields decreased from rhizosphere to bulk to bare soil in confirmity with the decreasing $CH_4$ oxidation activity. Variations in kinetic parameters ($K_m$ and $V_{max}$) for different rice fields are shown in Table 5. Bender and Conrad [11] have stated that different $K_m$ values may indicate the existence of different types of methanotrophs in soils. According to Conrad [29] type II methanotrophs, which are frequently found in soils, are able to adopt to $CH_4$ concentration by changing their $K_m$. This difference could be due to differences in species composition and/or due to conditioning of methanotrophs under different soil microhabitats. According to King [82] all the methanotrophs that have been isolated from soil thus far do not possess the required kinetic properties. These methanotrophs have an ecological niche that is characterized not by atmospheric $CH_4$ oxidation but by oxidation of relatively high $CH_4$ concentration that emerge in the proximity of $CH_4$ production sites i.e. wetlands [128].

### *Factors affecting methane oxidation*

*Temperature*

Although microbial community is expected to respond to changes in temperature, there are contradictory reports regarding its effect on methane oxidation. Whalen et al. [159] showed that $CH_4$ consumption increased with increasing temperature (5-20°C) under high $CH_4$ (103ppm) amended atmosphere. Bender and Conrad [13] reported a linear response in the temperature range of 20-35°C, but 13-38% of the maximum activity remain even at 0°C. A weak relationship between $CH_4$ uptake and soil temperature suggests that an abiotic process, such as diffusion of $CH_4$ or $O_2$ can be a controlling factor for $CH_4$ uptake.

*Methane concentration and soil moisture*

Methanotrophs are highly sensitive to variation in $CH_4$ concentration in atmosphere [11]. $CH_4$ concentration affects the rate of consumption both directly or indirectly. Enhanced concentration of $CH_4$ increases the number of methanotrophs) which have a significant role in methanotrophy [103]. Bender and Conrad [13] reported that increase of microbial methane oxidation activity and number of methanotrophs at $CH_4$ mixing ratios exceeding about 100-1000 $\mu$1 $CH_4 1^{-1}$. The threshold value below which no $CH_4$ uptake occurs is much lower for soils than for sediments. For example, 2-3 ppm and < 0.1 to 0.4 ppm threshold values have been reported for sediments and soils, respectively [19]. The maximal $CH_4$ oxidation rates are probably determined by the magnitude of the supply of $CH_4$ to the zone of oxidation [82].

Early studies have shown that methane oxidation was sensitive to desiccation and dramatically decreased at soil moisture below at 20 % WHC [13, 72]. The low solubility of $CH_4$ in water enhances this effect, mainly at low limiting $CH_4$ concentrations.


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*Oxygen availability*

Oxygen availability depends upon soil porosity. As the porosity increases, a decreased volume of water is distributed in pore volume, decreasing the water film thickness. This increases the rate of substrate ($CH_4$) delivery to the methanotrophs for oxidation [102]. Methanotrophs in the rice rhizosphere do not have to compete for methane with microbial or chemical compounds, although there is a strong sink of methane by methane transport. However, intensive competition for oxygen occurs. The available values for $K_{(app)}$ for $O_2$ and $CH_4$ indicated that uptake of both substrates is saturated at concentrations of ≥ 10 μM [82].

***Table 5.*** *Observed kinetic parameters of $CH_4$ oxidation.*

| Ecozone/location | $K_m$ | $V_{max}$ | Reference |
|---|---|---|---|
| Rice field (Italy) | 4.0μM | 0.1μ mol $g^{-1}$ dw $h^{-1}$ | [20] |
| Rice field (Italy) | 4.1-165μg | 1.2-12.5μ g $h^{-1}$ $g^{-1}$dw | [153] |
| Rice field (Italy) | 56.0-186.0nM | - | [47] |
| Rice field (Italy) | 16.8nM | 839 n M g $dw^{-1}$ $h^{-1}$ | [72] |
| Dryland rice field (India) | 4.8-81.6μ g $g^{-1}$dw | 0.05-0.61μ g $h^{-1}$ $g^{-1}$dw | [45] |
| Flooded rice field (India) | 6.2-81.1μ g $g^{-1}$dw | 0.03-0.41μ g $h^{-1}$ $g^{-1}$dw | [40] |

*Nitrogenous compounds and soil pH*

Inorganic N influences $CH_4$ oxidation due to shifts in the population structure and the kinetics of methanotrophs [45]. This may affect the threshold value for $CH_4$ oxidation [82]. $NO_3^-$-N fertilization did not affect the $CH_4$ consumption but $NH_4^-$+N fertilization completely ceased $CH_4$ oxidation [66]. Nitrite was found to inhibit $CH_4$ oxidation in the cultures of *Methylomonas albus BG8* and *M. trichosperium OB3b* [83]. Recently it was shown that methane oxidation is stimulated by increased nitrogen availability due to unquantified nitrogen limitation of methanotrophs [18]. However, the most solidly substantiated explanation for ammonium inhibition of methane oxidation is competitive inhibition at the enzyme level. This occurs because, at the molecular scale, methane and ammonium are similar in size and structure [135]. As a result, the enzyme MMO can bind to ammonium ion and react with it. Because the possibility of competitive inhibition is fundamental to the biochemistry of methane oxidation, it was generally thought that inhibition should occur in paddy fields as well as in upland systems [135].


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In a pasture soil, $CH_4$ oxidation at pH 6.3 was greater than at pH 5.6 and was completely inhibited at pH 4.8-5.1 [66]. In certain other soils, oxidation has been reported at pH as low as 3.2 [143]. In general, low pH has an inhibitory effect on methane consumption although the mechanism responsible for this effect is not fully known [66].

## Inhibitors for $CH_4$ production and oxidation

An inhibitor specific to either methanogens or methanotrophs would be useful for distinguishing which of these organisms is responsible for $CH_4$ production and oxidation in environments in which such activities occur. A variety of chemicals used in agriculture such as pesticides and herbicides and nitrification inhibitors, are known to affect microbial processes. It is well established that $CH_4$ production is inhibited by acetylene, aminopurine, ammoniumthiosulphate, carbofurane, calcium carbide (capsulated), DDT, dicyandiamide, methyle chloride, methyle fluoride, nitrapyrine, pyridine, organochlorine and sodium azide and $CH_4$-acetylene, bromoxynil, dicyandiamide, DDT, 2,4,-D, ethylene, hexachlorocyclohexane, hydrazine, methomyle, nitrapyrine, phenylalanine, sodium thiosulphate, threonine and thiourea [9, 25, 147]. Nitrification inhibitors such as acetylene and nitrapyrin can inhibit the growth of nitrifiers, methanogens and methanotrophs [107]. Lindau et al. [96] found that $CH_4$ emissions from rice fields decreased by 35% and 14% following the application of encapsulated calcium carbide and dicyandiamide, respectively. Topp [146], found that the pesticides, bromoxynil, methomyl and nitrapyrin were inhibitory to $CH_4$ oxidation at 50 g $l^{-1}$. Sathpathy et al. [134] reported that the application of HCH (organochlorine insecticide) to flooded rice soils reduced the production and emission of $CH_4$. Kumarswamy et al. [89] have revealed that carbofuran inhibited net $CH_4$ production when applied at low rates (5-10 mg $g^{-1}$ soil), but stimulated it when applied at a rate of 100 mg $g^{-1}$ soil. Chan and Parkin [25] have recommended the use of acetylene and ethylene for inhibition of $CH_4$ oxidation and methyl chloride for inhibition of methanogenesis.

## Approach for Methanogens/Methanotrophs Detection at Molecular Ecological Level

Effective representation of the diversity towards desired functional gene is one of the major concerns of microbial ecology of any ecosystem. A preliminary attempt has been reported by Dubey et al. [46] where, by cluster analysis of ARDRA pattern, it has been shown that different types of methanotrophs dominate in rhizosphere and non-rhizosphere soils of tropical rice ecosystems. The growing demand of paddy with increasing population is an emerging issue and the scenario results in the over exposure of soil microflora with pesticide residues. As discussed earlier pesticides play a major role at the physiological level for the expression of key enzymes in methane production and its further utilization as a substrate. The question, what are the emerging criteria that decide the course of evolution for these bacteria, which play key role in maintaining the greenhouse gas methane in the ecosystem and for habitats exposed to such residues, is likely to trigger a lot of interest. Studies are required to compare the performance of diverse strains from such habitats followed by molecular analysis of key genes to understand which is the most susceptible genotype for such stresses. These studies are


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likely to generate the tracking probes for the same target genes but having substituted primary coding sequences that will help evaluating the impact on soil methane cycle and its relation to pesticide residues. Similarly, other parameters such as application of fertilizers and accumulation of inorganic compounds in soil will lead to modified soil chemistry which in turn will be changing the overall microbial community structure vis-à-vis their relation in density and survival of methane oxidizing bacteria. Therefore, parallel tracking tools would have to be designed to assess the existing microbial population using selected markers which could act as biomarker for associated biogeochemical cycles.

Several approaches have been adopted in molecular ecological studies for the detection and characterization of methanogens and methanotrophs in the various ecosystems [46, 112]. These are schematically shown in Figure 2. The first approach is indirect and relies on enrichment and/or isolation with subsequent characterization of methanogens and methanotrophs. The second approach is direct and relies on polymerase chain reaction (PCR) technique and use of phylogenetic and functional gene probes (Table 6) for the molecular analysis of these microbes in the environmental samples without the prerequisite of their cultivation. At each stage, in both approaches, molecular biological analysis of the key genes (*pmoA, mmoX, mxaF* for methanotrophs, *mcr, mtd, mth, mrt, frh* for methanogens and 16S r RNA ) can be carried out and comparison can be made to see if the same organism that grows in culture derived from the sample is representative of what is actually present in the environmental sample (as revealed by DNA analysis).

Several other approaches have also been used to study methanogens and methanotrophs. Phospholipid analysis has been used successfully for the detection of these microbes in the wetland ecosystem [144].

***Table 6.*** *Sequences of oligonucleotides commonly used as PCR primers and probes for molecular analysis of methanogens and methanotrophs.*

| Primers /Probes | Targets | Sequences ( 5′ → 3′ ) | Tm (ºC) | References |
|---|---|---|---|---|
| PRIMERS | | | | |
| A 189 f | pmoA ; pMMO/AMO | GGN GAC TGG GAC TTC TGG | 56 | [63] |
| A 682 r | pmoA ; pMMO/AMO | GAA SGC NGA GAA GAA SGC | 56 | [63] |
| 882 f | mmoX ; sMMO$^+$Methylotrophs | GGC TCC AAG TTC AAG GTC AG | 55 | [99 |
| 1403 r | mmoX ; sMMO$^+$Methylotrophs | TGG CAC TCG TAG CGC TCCGGCTCG | 55 | [99] |
| 1003 f | mxaF; All Methylotrophs | GCG GCA CCA ACT GGG GCT GGT | 55 | [100] |
| 1561 r | mxaF; All Methylotrophs | GGG CAG CAT GAA GGG CTC CC | 55 | [100] |
| ME1 f | mcrA; methanogens | GCM ATG CAR ATH GGW ATG TC | 50 | [60] |
| ME2 r | mcrA; methanogens | TCA TKG CRT AGT TDG GRT AGT | 50 | [60] |
| Probes | | | | |
| MSMX 860 | Methanosarcinaceae | GGC TCG CTT CAC GGC TTC CCT | 54 | [127] |
| MS821 | Methanosarcina spp. | CGC CAT GCC TGA CAC CTA GCG AGC | 54 | [127] |
| MC1109 | Methanococcaceae | GCA ACA TAG GGC ACC GG TCT | 47 | [127] |
| MB310 | Methanobacteriaceae | CTT GTC TCA GGT TCC ATC TCC G | 52 | [127] |
| MG1200 | Methanomicrobiales | CGG ATA ATT CGG GGC ATG CTG | 45 | [127] |

This technique relies on the fact that methanotrophs contain unusual fatty acids (16:1 and 18:1 derivaties). Phospholipid analysis is, however, limited due to small database


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on fatty acid profiles from methanotrophs [113] and the relatively high cost of equipment used in this analysis.

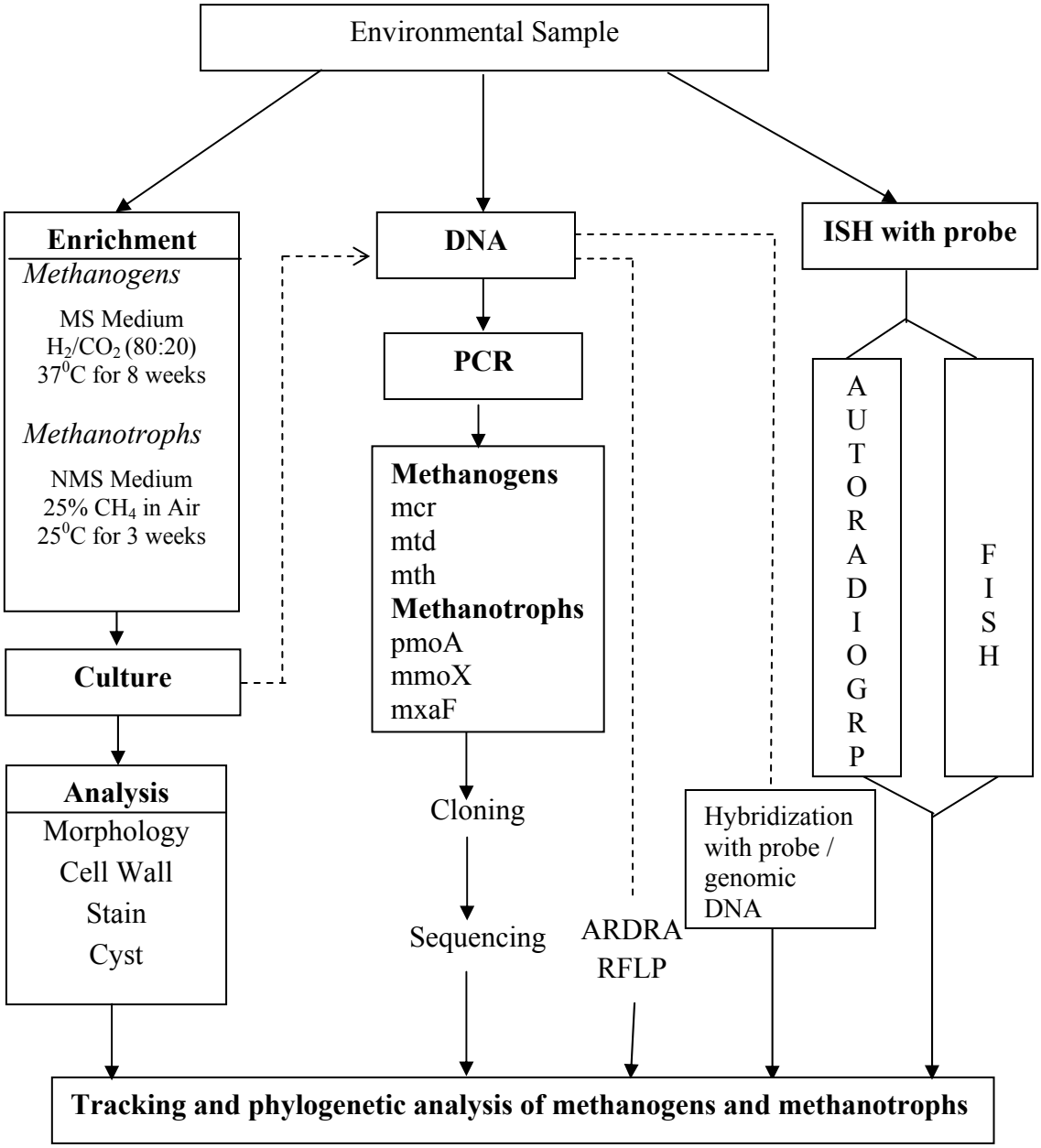


***Figure 2.*** *Generalized scheme of molecular anlysis for detection and phylogenetic study of methanogens and methanotrophs*

The detection and characterization of these microbes can also be attempted using antibodies either to whole cells of culturable methanogens/methanotrophs or to key enzymes such as MMO, MDH (methanotrophs) and MCR, MTD (methanogens). One of the most powerful tools in the molecular ecology is FISH (fluorescence in situ hybridization). FISH allows the specific detection and enumeration of methanogens/ methanotrophs directly in the natural habitats without cultivation. Grobkopf et al. [57] detected most of the archaeal group members (e.g. *Methanosarcinaceae,*


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*Methanosaetaceae, Methanomicrobiaceae, Methanobacteriaceae*, *RC-I* to *RC-IV* and *RC-VI*) on rice plant roots by using FISH.

## Conclusions

Methane is an important greenhouse gas and it affects the chemistry of the atmosphere. The ecological role of methanogens and methanotrophs in the methane dynamics in rice fields is still unclear. Current information is insufficient for the development of technology and strategy for reduction in methane emission from rice field at regional and global levels. Knowledge of comparative genomics and proteomics of methanogens and methanotrophs will contribute to the deciphering their population structure and existing mechanisms of methane emission in paddy fields.

It has now become possible to isolate, detect and characterize these microbes by using molecular biological tools like PCR, FISH, etc. techniques. Knowledge of structure and function of methanogens and methanotrophs communities will be beneficial for understanding the microbial ecology of methane to control the $CH_4$ turnover in rice soils.

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# WHY DO POLLINATORS BECOME “SLUGGISH”? NECTAR CHEMICAL CONSTITUENTS FROM *EPIPACTIS HELLEBORINE* (L.) CRANTZ (ORCHIDACEAE)

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**Abstract.** Eight populations of *Epipactis helleborine* (L.) Crantz originating from the area of Lower Silesia in Poland (Central Europe) were examined in respect to composition of their nectar and its influence on the insect attraction in field conditions. The chemical composition of *Epipactis helleborine* (L.) Crantz nectar was studied by means of GC/MS SIM. A number of compounds with potential narcotic properties were identified in the nectar, namely 3-{2-{3-{3-(benzyloxy)propyl}-3-indol, 7,8-didehydro-4,5-epoxy-3,6-d-morphinan and oxycodone. Pollinator and visitor insects were identified. The key role of ethanol in the process of alluring and stunning of the insects was discussed. A scheme of the influence of the identified compounds on the pollinators was proposed.
**Keywords.** *Epipactis helleborine, toxic nectar, morphinan derivatives, indol derivatives, ethanol*

## Introduction

Various species of plants attract their pollinators in a variety of ways, namely releasing specific attractants or offering the plant rewards as e.g. oils, stigmatic exudates, pollen [17] and nectar secretion [44]. Nectar does not perform any other role in the flowers of angiospermous plants apart from alluring the pollinators [20]. The main ingredient of the nectar in the majority of plants is sugar: glucose, fructose and saccharose [3, 47], which amount ranges from 50% to 75% [16]. Apart from it, it contains amino acids, lipids, organic acids, as well as various vitamins, enzymes, antioxidants, mineral ions and secondary metabolites [2, 17, 32].

Therefore, the aim of our research was to determine, which chemical compounds are responsible for alluring insects and testing the hypothesis, if the key role in the process of alluring the insects might be ascribed to ethyl alcohol or perhaps to other chemical compounds, that can have “narcotic” properties, as it was found for other plant-insects interactions for example in *Datura* species [8, 37, 42] and orchids such as *Cypripedium* sp. [5], *Cryptostylis* sp., *Dendrobium* sp., *Polyradicion* sp. that can have “narcotic” properties [1, 9].


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## Review of literature

So far, the research on the composition of sugars and amino acids in the nectar of orchids including *Epipactis* orchids was conducted by Pais & Chaves das Neves [39] and Pais *et al.* [40]. According to the literature, nectar produced by the *Epipactis helleborine* (L.) Crantz contains also ethanol [13, 31, 34]. Kevan *et al.* [27] and Ehlers & Olsen [13] found that ethanol is most likely of microbial origin, produced by microorganisms that expanded in the nutrient medium rich in sugars and amino acids, which is nectar. Probably these microorganisms reach the lip together with the pollinators which are visiting the flowers [13].

Literature data on the composition of secondary metabolites of the nectar of *Epipactis helleborine* (L.) Crantz are scarce and insufficient.

## Materials and methods

Eight populations of *Epipactis helleborine* (L.) Crantz growing under various habitat conditions in the area of Lower Silesia (South-Western Poland – Middle Europe) were chosen. Studies were performed at the sites of: Srebrna Góra, Karpacz, Kletno, Kotowice-Siechnice, Gozdnik, Krowiarki Mts., Orlickie Mts. and The Stolowe Mts. National Park. The size of the studied populations averaged from 69–428 individuals. The research was conducted during the years 2001–2004. Nectar was collected during the maximal secretion by the plants, namely between 11:00 a.m. and 15:00 p.m. Composition of the nectar was fairly stable amongst the eight populations.

Nectar was collected from flowers by using capillary and methylene chloride as eluent. Then the methylene chloride extract was dried over anhydrous $Na_2SO_4$ and evaporated up to volume 0.2 ml followed by analysis GC-FID and GC-MS. Thus, sample of extract was injected into ELWRO N504 gas chromatograph equipped with flame ionization detector (FID). The instrumental parameters and operational conditions were as follows: fused silica capillary column (60 m × 0,25 mm i.d.), a film thickness of 0.25 μm with a temperature program: 50 °C to 300 °C at rate of 3 °C/min, with nitrogen as carrier gas (about 1.2 $cm^3$/min).

Each analysis was performed in triplicate to assess the reproducibility of results.

For the purpose of definition of compounds present in the sample, extract was analysed by means of Hewlett Packard 5973 GC-MS system.

Nectar's influence on the behaviour and activity of the insects were examined under natural conditions. Microcapillary tubes containing collected nectar were placed in field conditions at a significant distance from *Epipactis helleborine* (L.) Crantz plants in order to observe insects interest in these capillary tubes. Observations were made from 11:00 a.m. to 15:00 p.m. from 15 July to 28 August 2001, 2002, 2003 and 2004. All possible pollinators and vectors were caught, identified by specialists and deposited in Department of Zoology, Wrocław University. The plant vouchers were kept in Herbarium, Department of Plant Systematics and Phytosociology, University of Wrocław.

## Results

Chromatographic analysis of methylene chloride extracts of nectar revealed the presence a set of well separated analytical signals (*Fig. 1*). Chemical formulas of compounds found in the nectar of *Epipactis helleborine* (L.) Crantz and discussed in this work are shown in *Fig. 3*.


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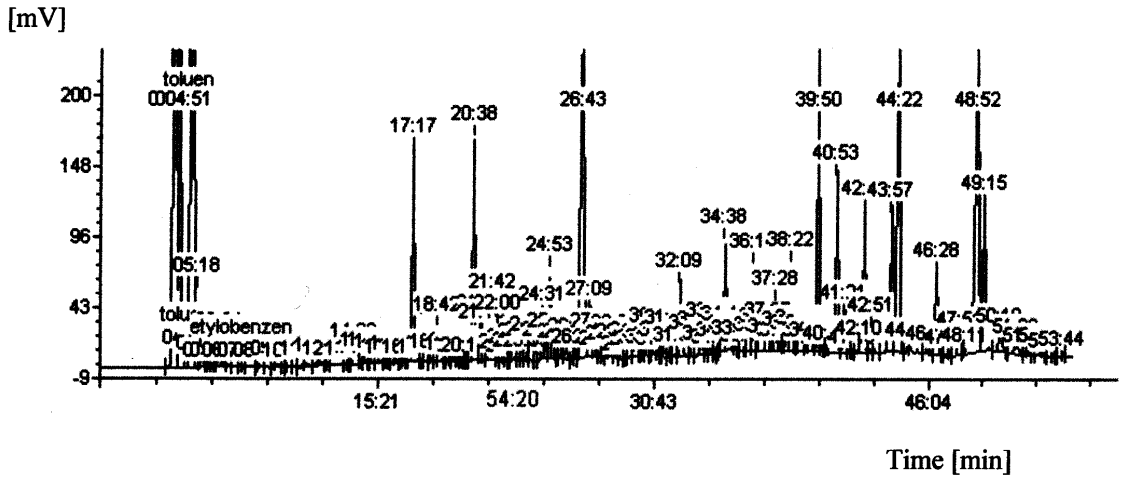


***Figure 1.*** *GC-FID chromatogram of the nectar components contained in Epipactis helleborine (L.) Crantz*

In order to identify individual substances the extract was examined by means of GC-MS. As seen from *Fig. 2*, the applied conditions resulting from the differences of the equipment used, gave different chromatograms. Methylene chloride extract of the nectar contains over 100 chemical species, which belong to the class of polar chemical substances. The identification of extract components was possible by searching for ions 72, 77, 91, 93, 95, 96, 103, 107, 108, 110, 121, 122, 126, 131, 134, 135, 136, 138, 150, 152, 153, 154, 168, 170, 180, 194 characteristic for indoles, alcohols, phenols, alkaloids, terpenes and individual substances were identified basing on NBS75K and NIST MS libraries.

Both chromatograms indicate a wide variety of compounds present in the nectar. Among them, set of derivatives of phenol, eugenol, indole and as well as derivatives of morphine were identified. Also occurrence of numerous of carboxylic acids was determined. Substances that occur in the nectar in higher amounts were identified and are shown in *Table 1*.

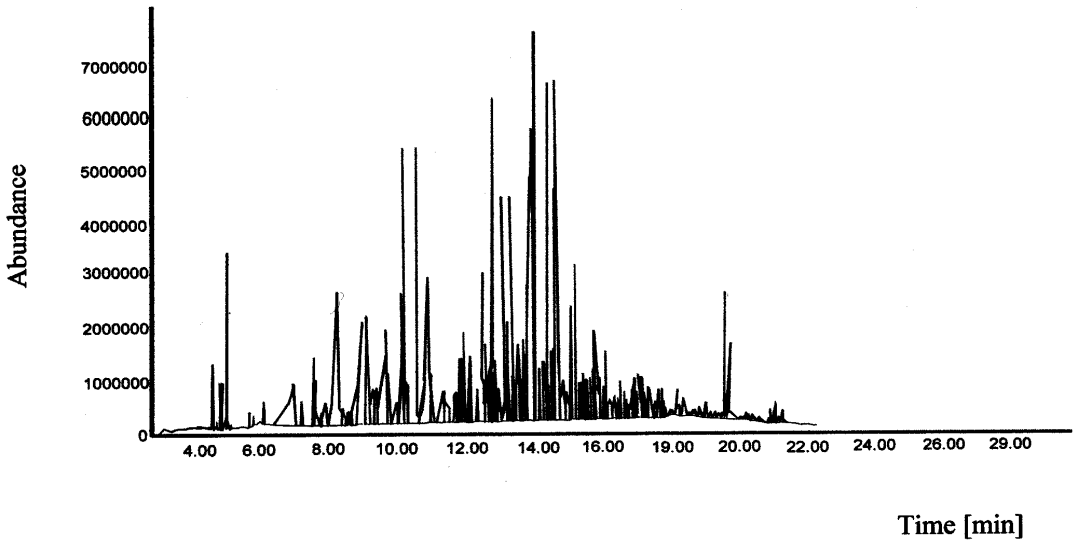


***Figure 2.*** *GC-MS chromatogram of the nectar components contained in Epipactis helleborine (L.) Crantz*


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***Table 1.*** *Nectar chemical composition of Epipactis helleborine (L.) Crantz*

| chemical | molecular formula | properties |
|---|---|---|
| 3-{2-{3-{3-(benzyloxy)propyl}-3-indole | $C_{26}H_{34}N_{2O}$ | antimicrobial [12], overpowering substance |
| xanthatin | $C_{15}H_{18}O_3$ | antimicrobial [18] |
| 7,8-didehydro-4,5-epoxy-3,6-D-morphinan | $C_{19}H_{23}NO_3$ | soporific, sedative, narcotic [4, 46] |
| 4,5*a*-epoxy-14-hydroxy-3-methoxy-17-methyl-morphinan-6-one (oxycodone) | $C_{18}H_{21}NO_4$ | narcotic [41, 51] |
| androstane-3,17-dione | $C_{19}H_{28}O_2$ | hormone |
| 2-furancarboxyaldehyde (furfural) | $C_5H_4O_2$ | toxic [22, 23], insect attractant, characteristic “almond/benz-aldehyde” odour |
| 2(5*H*)-furanone | $C_4H_4O_2$ | antibacterial [30] |
| 3-methyl-1,2-cyclopentanedione | $C_6H_8O_2$ | — |
| 2,6-dimethoxy-phenol (syringol) | $C_8H_{10}O_3$ | antimicrobial [24, 33] |
| 2-metoxy-4-(2-propenyl)-phenol (eugenol) | $C_{10}H_{12}O_2$ | antifungal [36, 48], antimicrobial [38], insect attractant [26, 43, 50] |
| 2,6-dimethoxy-4-(2-propenyl)-phenol (methoxyeugenol) | $C_{11}H_{14}O_3$ | antifungal, antimicrobial [19], insect attractant [26] |
| benzylalcohol | $C_7H_8O$ | — |
| 4-hydroxy-3-methoxy-benzylalcohol | $C_8H_{10}O_3$ | — |
| 4-hydroxy-3-methoxy-benzaldehyde (vanillin) | $C_8H_8O_3$ | characteristic odour |
| ethanol | $C_2H_6O$ | insect attractant [13, 31, 34] |
| 2,2-diethoxyethanol | $C_6H_{14}O_3$ | — |
| 2-hydroxy-benzene-methanol | $C_7H_8O_2$ | — |
| 4-hydroxy-benzene-methanol | $C_7H_8O_2$ | — |
| pentadecanol | $C_{15}H_{32}O$ | — |
| heptadecanol | $C_{17}H_{36}O$ | — |
| eicosanol | $C_{20}H_{42}O$ | — |
| benzoic acid | $C_7H_6O_2$ | — |
| tetraeicosanoic acid | $C_{20}H_{32}O_2$ | — |
| octadecenoic acid | $C_{18}H_{36}O_2$ | — |
| pentadecenoic acid | $C_{15}H_{30}O_2$ | — |
| heptadecenoic acid | $C_{17}H_{34}O_2$ | — |
| 9-hexadecenoic acid | $C_{16}H_{30}O_2$ | — |
| oleic acid | $C_{18}H_{34}O_2$ | — |
| eicosane | $C_{20}H_{42}$ | — |
| heneicosane | $C_{21}H_{44}$ | — |
| tricosane | $C_{23}H_{48}$ | — |
| pentacosane | $C_{25}H_{52}$ | — |
| hexacosane | $C_{26}H_{54}$ | — |
| heptacosane | $C_{27}H_{56}$ | — |
| octacosane | $C_{28}H_{58}$ | — |
| eicosanoic acid methyl ester | $C_{21}H_{42}O_2$ | — |
| tetracosanoic acid methyl ester | $C_{25}H_{50}O_2$ | — |
| pentadecenoic acid methyl ester | $C_{26}H_{52}O_2$ | — |
| hexadecenoic acid methyl ester | $C_{27}H_{54}O_2$ | — |
| heptanal | $C_7H_{14}O$ | characteristic odour |
| nonanal | $C_9H_{18}O$ | — |
| hexadecanal | $C_{16}H_{32}O$ | — |
| octadecanal | $C_{18}H_{36}O$ | — |
| nonadecanal | $C_{19}H_{38}O$ | — |


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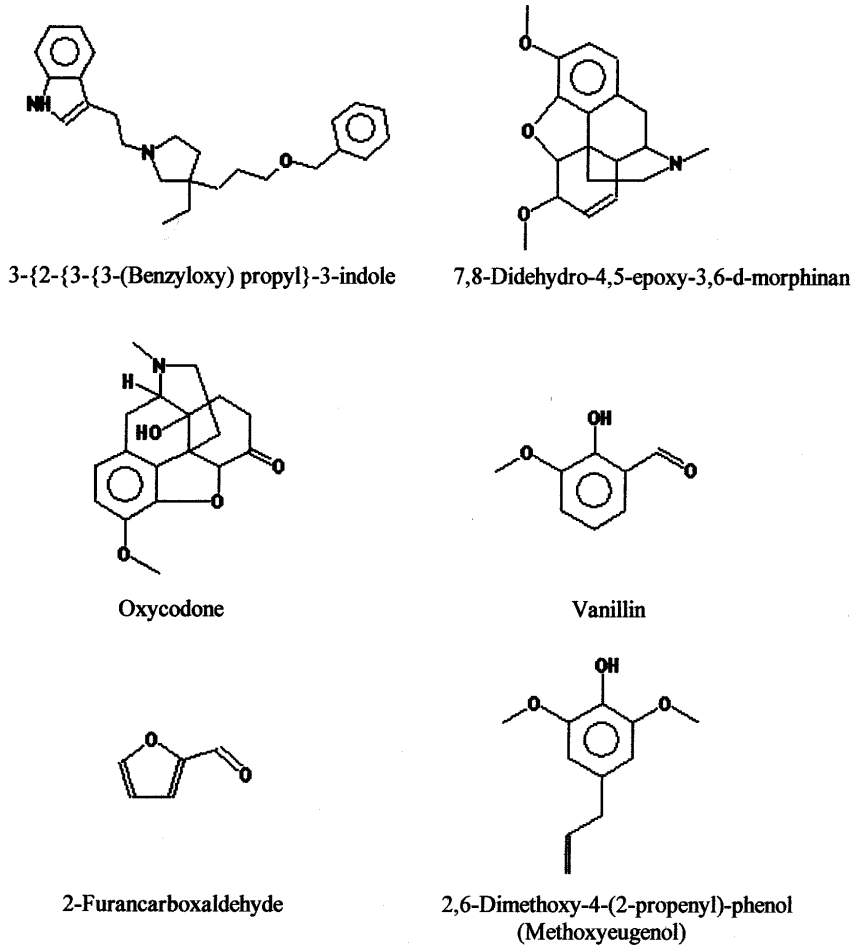


***Figure 3.*** *Molecular formulas of some compounds identified in nectar of Epipactis helleborine (L.) Crantz*

### *Attraction of insects*

We have observed attracting properties of the capillary tubes containing nectar extract towards some insects in field conditions. It is possible, that insects react mostly to eugenol derivatives and vanillin acting as attractants. It is well documented that these compounds have the greatest importance in the first stage of pollination, because of their well established role to have an ability to allure insects (*Fig. 4*). The process of pollinating in vivo was also a subject of our research. Nectar of Epipactis orchids is attractive to both group of insects: pollinators and visitors. We have been observing strong attraction of a big group of insects belonging to families such as: Syrphidae, Mycetophilidae (Diptera), Ichneumonidae, Formicidae, Vespidae, Apidae (Hymenoptera) and Anaspididae, Cantharidae, Coccinellidae, Nitidulidae, Lagriidae, Malachiidae (Coleoptera) as well Arctiidae (Lepidoptera).

## Discussion

The characteristic feature of the large number of species from Orchidaceae family is, that their nectar contains a group of alkaloids of various chemical origin. So far over 1500 different compounds with overpowering properties, coming from the orchids, have been described [1]. The majority of them belongs to two classes of compounds:


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pyrrolizidine alkaloids and dendrobine alkaloids [28, 29, 45]. Bell [7] suggests that some of the plants may produce hallucinogenic or narcotic substances, which addict the pollinators and cause visible disorientation of flight described by term „drunken insects”. Such behaviour of insects may also be caused by the action of ethanol, which is formed by fermentation process, as it was suggested by Løjtnant [31], Müller [34], Ehlers & Olsen [13] for *Epipactis* orchids. According to Kevan *et al.* [27], bumblebees became „drunken“ after visiting *Asclepias* flowers, sized by lot of yeast species.

In our opinion, the „drunken insects” effect might be also the result of the presence of other substances, such as: derivatives of indole, morphinans and derivatives of phenol, which we identified basing on GC-MS analysis (*Table 1).* The characteristic features of these compounds may have an impact on the behaviour of the insects. Furthermore co-occurrence of these compounds in the nectar may point at synergic effect. Such effect is not usual as seen from studies on eugenol, which is a substance that allures the insects, while methyl eugenol acts as a sex attractant of fruit fly *Daucus dorsalis* [50]. Methyl-eugenol is produced by many plant species, i.e. *Cassia fistulosa*, *Fagraea berteriana*, *Valeriana tuberosa*, and probably its role in the process of alluring pollinators is also important [15, 20, 43].

Identification of the presence of vanillin (4-hydroxy-3-methoxy-benzaldehyde), a compound which was for the first time isolated from *Vanilla planifolia* (Orchidaceae) confirms that plants which belong to one family are similar to each other not only in respect to genetics, but also in respect to presence of similar secondary metabolites. Interestingly, *Epipactis atrorubens* has an intensive vanilla scent [11].

Data presented in this paper show that *Epipactis helleborine* nectar is definitely not a scentless substance. It has not been known so far, which compounds or a group of compounds are responsible for its odour. The presence of compounds with strong scent properties such as vanillin, methyl eugenol, or carboxylic acid esters suggest that *Epipactis helleborine* scent comes from a mixture of several compounds. It also depends on the climate conditions in which the plants grow.

The results of GC/MS analysis of the nectar indicate, that probably its overpowering properties come not only from ethanol, but also from the other group of compounds, present in nectar, namely alcohols, phenols, organic acids and aldehydes. The effect of ethanol cannot be quantitatively estimated, because of its volatility and because that it is a product of metabolism of fungal species such as: *Cladosporium*, *Candida* and *Aspergillus* sp. There is no data on the correlation of the size and intesity of growth of fungal colonies with factual amount of ethanol in the nectar. It is also very probable, that the chemical decomposition of some of the compounds present in nectar might be also a source of ethanol and its derivatives. In Poland, the florescence period of *Epipactis* is the end of July and the beginning of August, so during the time when air temperatures during the day often exceed 28 °C. The quantity of ethanol that is produced by the microorganisms would have to be enormous, to counterbalance its evaporation speed and to maintain its overpowering properties.

The growth of microorganism colonies, which produce ethanol in natural conditions might be effectively restrained by the complex action of chemical compounds which have bactericidal and fungicidal properties. For example antifungal and antibacterial activity of furfural [22, 23], syringol [24, 33], indole derivatives, eugenol and methyl-eugenol which constituted major plant metabolites, is well documented [6, 21, 35]. Moreover, numerous literature data prove, that orchids belong to plants, which protect themselves very strongly from the attack of microorganisms, by producing various


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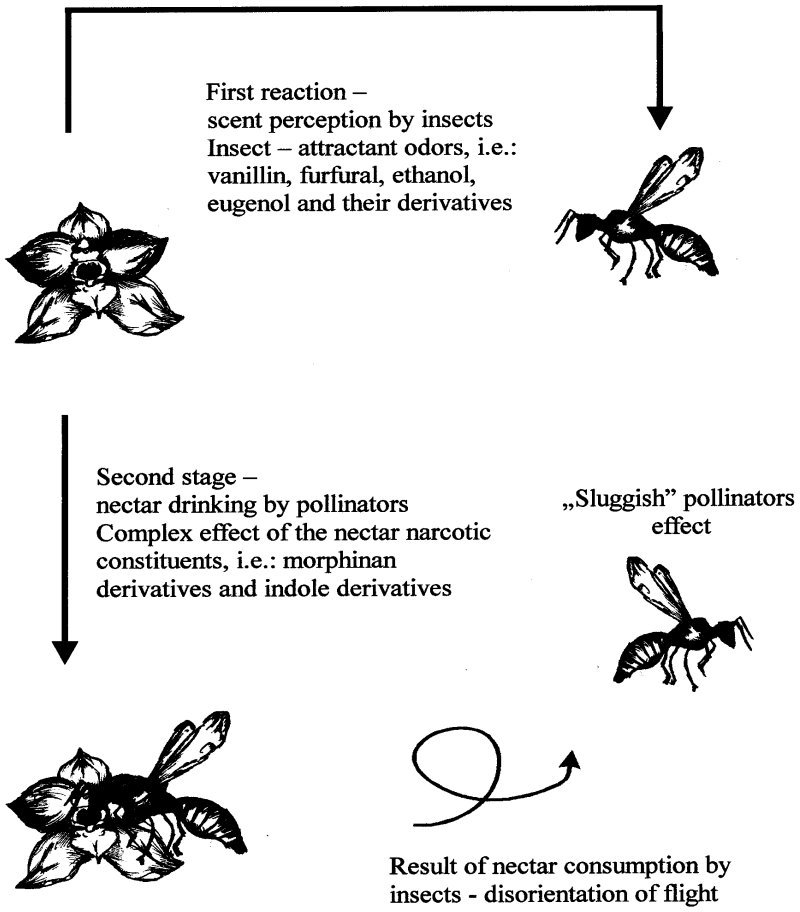


***Figure 4.*** *Hypothetical scheme of the chemical compounds' influence on the pollinators and visitors insects of Epipactis helleborine (L.) Crantz*

chemicals including phytoalexins [14]. The species of *Cladosporium* and *Candida* observed by Ehlers & Olsen [13] belong to these, which occur commonly in the nature, and therefore their role in the process of insect overpowering might not be as significant as the authors suggest. Finding these microorganisms in the laboratory conditions and therefore in artificial conditions, does not necessarily mean that they can reproduce in the nectar of *Epipactis helleborine* (L.) Crantz upon environmental conditions.

***Why do orchids produce „drunken” insects?***

Helleborine grow in various phytocoenoses. The majority of species grow in deciduous and mixed forests, but in Poland they may be seen on dunes, peat bogs and meadows, as well upon highly anthropogenic conditions, such as roadsides, cemeteries and municipal parks [10, 25, 49]. Such a diversity of habitats results in the diversity and size of the insect species visiting the plants.

Production of narcotic compounds such as 3-{2-{3-{3-(benzyloxy)propyl}-3-indol, 7,8-didehydro-4,5-epoxy-3,6-D-morphinan and oxycodone is one of the attempts to attract potential pollinators. The presence of oxycodone, which was reported to be a semisynthetic morphinan [41, 51], is of special interest here. These substances make the insects, which drink the nectar to become „sluggish”, what prolongs the time, which they spent on the inflorescence and therefore increases chance of pollinating larger


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number of flowers. Such a strategy is very effective if taking into consideration the fact, that *Epipactis helleborine* flowers are not morphologically attractive to insects and that attendance of potential pollinator is dependent on habitat.

Summing up, we propose two-stage reaction of insect to attractive components of orchids as shown in *Fig. 4*.

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# SPATIAL VARIABILITY OF PLANT FUNCTIONAL TYPES OF TREES ALONG NORTHEAST CHINA TRANSECT

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**Abstract.** Studying the spatial variability of plant functional types at large scale is important to understand the effects of environmental change on ecosystems. Here we classified the tree species in the forest area of Northeast China Transect (a middle-latitude transect and its environmental gradient was mainly driven by moisture) into three plant functional types (PFTs): drought tolerant, drought intolerant and middle type PFTs. We found that the average percentage of the drought intolerant and middle type of PFTs both increased significantly from 1986 to 1994. The drought tolerant and middle type of PFTs increased their covered areas at the western part of transect, but the covered area of the drought intolerant PFTs decreased about 48% at the western part. The dominance of the drought intolerant PFTs decreased while the dominance of the other PFTs increased. The net increments of these three PFTs were higher at 0–220 km than at 220–400 km. The negative net increments concentrated mainly at 150–350 km. The spatial autocorrelation of the drought intolerant and middle type of PFTs changed slightly from 1986 to 1994. The information entropy of the drought intolerant PFT increased across all scales and it indicated that impact from local disturbances was limited. All these indicate that the drought intolerant PFTs is vulnerable to the current environmental change. The spatial variations of different PFTs at large scales were mainly caused by the fluctuations of gradient of annual precipitation along this transect.
**Keywords.** *Northeast China Transect, plant functional type, precipitation gradient, spatial variation, tree species*

## Introduction

Plant functional types (PFTs) can be defined as a group of species sharing the same response to a perturbation [14]. Recent research indicated that the climate change will affect the spatial distribution of PFTs and their relative abundances [13, 25]. Comparison of the spatial distribution and its change for PFTs at a large area may help us to determine the general patterns of vegetation change as a consequence of environmental change.

Northeast China Transect (NECT) is identified as a middle-latitude transect for terrestrial ecosystem studies by the Global Change and Terrestrial Ecosystem (GCTE) (*Fig. 1*) [17]. Because this transect is parallel with latitude, its environmental gradient is mainly driven by moisture. In fact, the annual precipitation decreases from as high as 800 mm in the east to 100 mm in the west. Detecting the change of spatial characteristics of tree species under precipitation gradients would be helpful to study species dynamics in a large region under environmental change. Although some spatial characteristics and change of tree species, such as geographical distribution and frequency, have been studied [5, 7], the response of each individual species may severely restrict our ability to assess the possible vegetation change at large area. Aggregating species by shared traits into PFTs is a common methodology for linking properties of species level to environmental factors at large spatial scale [3, 11].


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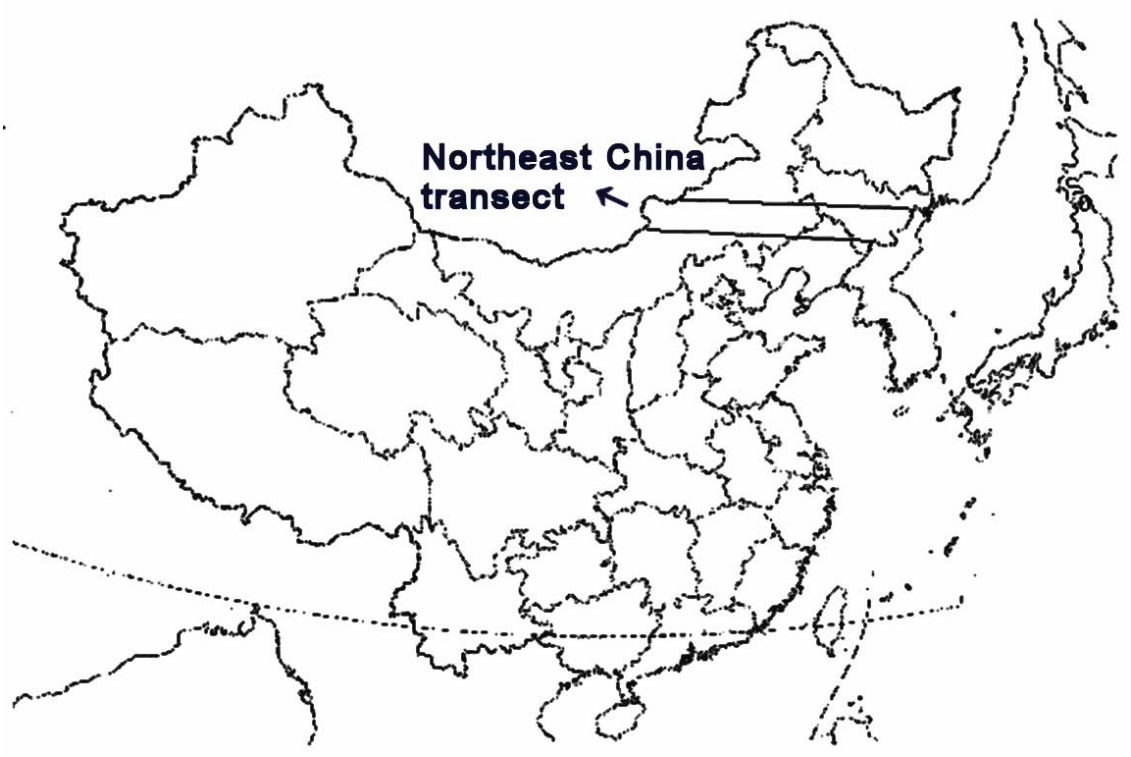


***Figure 1.*** *The location of Northeast China Transect (NECT).*

The change of PFTs in ecosystem would alter ecosystem functions (such as biogeochemical cycles, invasion resistance and stability in the face of disturbance) [20]. Prediction of the sensitivity of plant biogeography to climate dynamics and concurrent effects on ecosystem function is a pressing issue in global change science [26]. The aims of this research are (1) to compare the spatial distribution pattern and its change for each PFTs from 1986 to 1994 by using spatial analysis methods; (2) to study the relationships of different PFTs; (3) to find the possible causes for the change of vegetation along NECT; and (4) to assess the possible vegetation change along NECT under the environmental change.

## Materials and methods

### *Study area*

In this research, we chose the study area at approximately 125–130ºE and 43.55ºN. The data set was selected from the plot records conducted every 4 km in 1986 and 1994. The area of each plot was 30×30 $m^2$. These plots are permanent and are protected by local agencies. The information of each plot was used to represent the forest structure in this corresponding area. The total length is about 400 km. The main tree species in the forest area along NECT are *Betula platyphylla*, *Abies nephrolepis*, *Tilia* spp., *Betula costata*, *Betula dahurica*, *Juglans mandshurica*, *Phellodendron amurense*, *Fraxinus rhynchophylla*, *Populus davidiana*, *Ulmus pumila*, *Quercus mongolica*, *Pinus koraiensis*, *Acer mono*, *Fraxinus mandshurica*, *Picea* spp., and *Larix olgensis*. Other information about this research area can be found in [4, 5, 7].


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***Methods***

An integrated classification of PFTs was used in this study based on species' ecophysiological and morphological characters. This classification method proved successful to study the possible response of this ecosystem after the change of species composition [8, 9]. Three types of PFTs were classified in this research (*Table 1*), and they can be simply described as drought tolerant, drought intolerant and middle type PFTs. The relative percentage of each PFTs and the net increment ratio ($\lambda$) were calculated by their abundances and change, respectively. If one PFTs appeared in one plot, then this PFTs covered this corresponding area.

***Table 1.*** *Classification of plant functional types (PFTs)* [8, 9, 12]

| PFT | included species | growth character | drought tolerance | shade tolerance | morphological character | occurring stage of succession |
|---|---|---|---|---|---|---|
| **drought tolerant** | *B. platyphylla*, *B. dahurica*, *P. davidiana*, *U. pumila*, *Q. mongolica*, *L. olgensis* | fast | higher | shade intolerant | mostly deciduous, broadleaved, only *L. olgensis* coniferous | early stage |
| **drought intolerant** | *A. nephrolepis*, *P. koraiensis*, *Picea* spp. | very slow | low | very shade tolerant | coniferous | late stage |
| **middle type** | *Tilia* spp., *B. costata*, *J. mandshurica*, *P. amurense*, *F. rhynchophylla*, *Acer mono*, *F. mandshurica* | middle | middle | middle shade tolerant | broadleaved | middle stage |

The spatial scale of autocorrelation was computed by GS$^{+TM}$5 (Gamma Design Software, USA) for each PFTs in 1986 and 1994 by Moran's I. The Moran's I statistic is a conventional measure of autocorrelation. With Moran's I, higher values indicate strong spatial correlation. The Moran's I in this study is defined as [24]:

$$I = \frac{\sum_i^n \sum_{j \neq 1}^n w_{ij}(x_i - \bar{x})(x_j - \bar{x})}{S^2 \sum_i^n \sum_{j \neq 1}^n w_{ij}}, \quad \text{(Eq. 1)}$$

where I is the measure of autocorrelation; n is the total number of samples; $x_i$ and $x_j$ are the observed values of the sample at site $i$ and $j$, respectively; $\bar{x}$ is the average of $x$; $S^2$ is variance. $w_{ij}$ is a symmetric weight matrix; in this study, $w_{ij}$ is 1 if location $j$ is within distance $d$ from $i$ or 0 otherwise.

The dominance of each PFTs, $D_i$, which was calculated at every location according to the following:

$$D_i = \sum n_i \frac{(n_i - 1)}{N(N - 1)}, \quad \text{(Eq. 2)}$$


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where $n_i$ is the abundance of $i$th PFTs, and $N$ is the total abundance of all PFTs at each location [23].

The data from each plot was combined into data set of scales in 4, 8, 16, 20, 40 and 80 km by pooling of contiguous quadrates along the transect. The Shannon entropy $H_\varepsilon(x)$ of each PFTs at different scales of $\varepsilon$ was calculated as the following [21]:

$$H_\varepsilon(x) = \sum p_\varepsilon(x) \log_{10} p_\varepsilon(x), \qquad \text{(Eq. 3.)}$$

where $p_\varepsilon(x)$ is the probability of observing PFTs $x$ at the $i$th patch element measured using samples of $\varepsilon$ units in size.

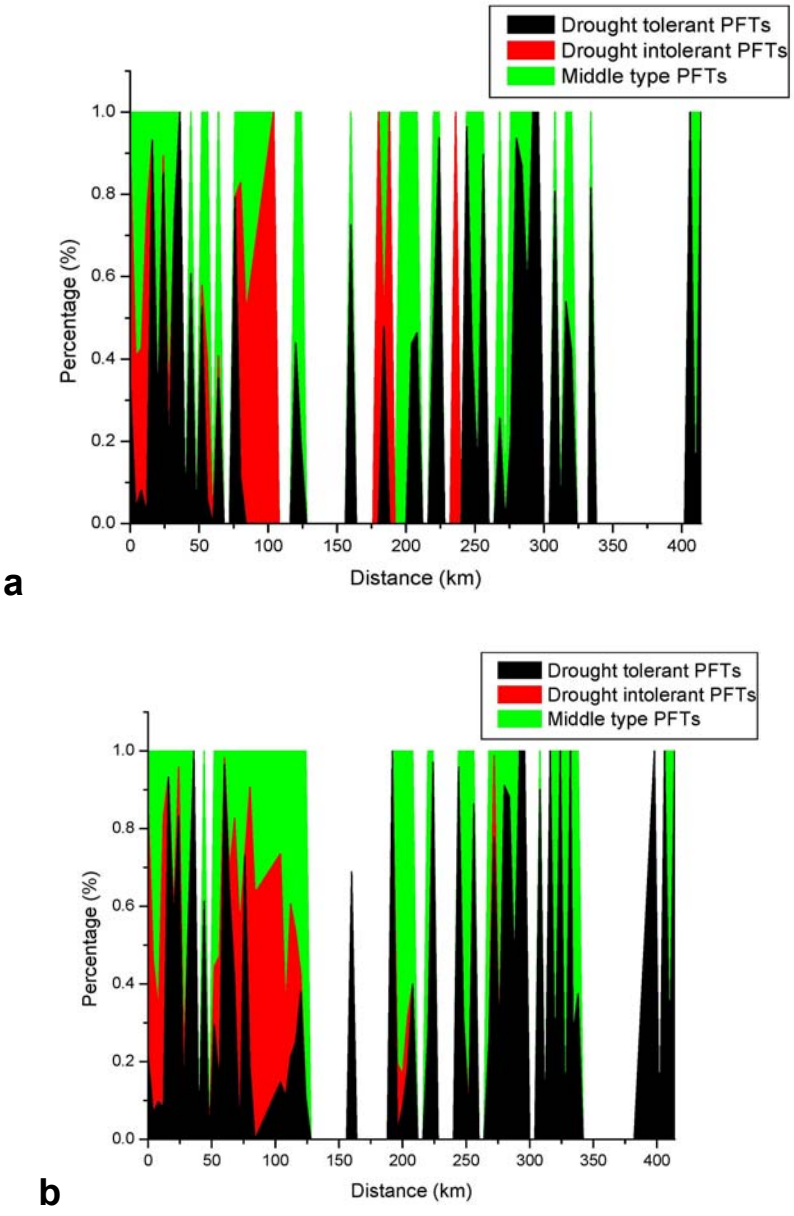


***Figure 2.*** *The percentage of covered area of three plant functional types (PFTs) along NECT in* a: *1986 and* b: *1994*

The interactions among PFTs were estimated by Taylor's power, which describes the PFTs-specific relationship between the temporal or spatial variance of PFTs and their


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mean abundances. The negative interactions among species in a community can decrease slopes of Taylor's power law [18]. In this research, the slope of log (mean abundance) and log (variance) of each PFTs in 1986 and 1994 was estimated along NECT. The results of each PFTs were compared between 1986 and 1994.

Climate information was collected from about 14 meteorological stations at or near this study area and was interpolated by Kriging of GS$^+$. The gradient ($G$) of annual precipitation at distance $i$ was estimated by $G_i = P_{i+n} - P_n$, where $P_n$ and $P_{i+n}$ are the annual precipitations at location $n$ and next location $i+n$.

## Results

### *Spatial distribution patterns of PFTs in 1986 and 1994*

On the whole research area the average percentages of the drought intolerant and middle type PFTs increased significantly ($p$<0.05) from 1986 to 1994, respectively, but the percentage of the drought tolerant PFTs changed not significantly ($p$>0.05) (*Fig. 2a* and *b*). For both the drought tolerant and middle type PFTs there was a higher covered area at the western part of transect (150–400 km) than the eastern part (0–150 km). For the drought intolerant PFT there was a higher covered area in the eastern part than that at the western part. This overall distribution pattern for each PFTs was not changed significantly during the time period from 1986 to 1994, but the covered area changed at different parts of NECT. For the drought tolerant PFTs the covered area increased about 25% and 27% at the eastern part and the western part, respectively. For the drought intolerant PFTs the covered area increased about 17% at the eastern part, but it decreased about 48% at the western part. For the middle type PFT the percentage increased about 6% and 23% at the eastern and western parts, respectively.

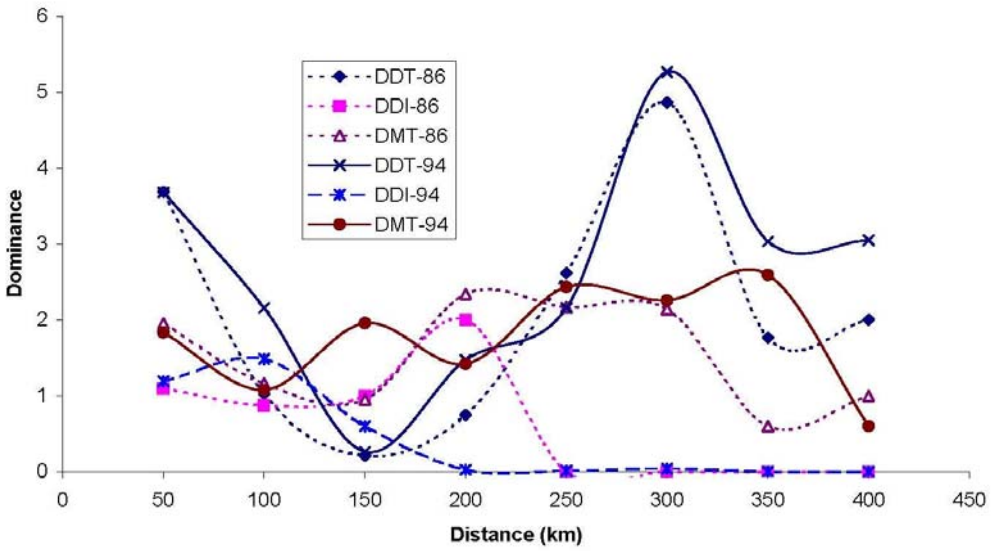


***Figure 3.*** *The dominance of three PFTs along NECT (DDT-86: dominance of the drought tolerant PFTs in 1986; DDI-86: dominance of the drought intolerant PFTs in 1986; DMT-86: dominance of the middle type PFTs in 1986; DDT-94: dominance of the drought tolerant PFTs in 1994; DDI-94: dominance of the drought intolerant PFTs in 1994; DMT-94: dominance of the middle type PFTs in 1994).*


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The total dominance along transect of the drought tolerant and middle type PFTs increased from 16.92 and 11.31 to 21.07 and 13.47, respectively, but the dominance of the drought intolerant PFTs decreased from 4.97 to 3.35.

The dominance of the drought tolerant and middle type PFTs increased at the most parts of this transect (*Fig. 3*) while the drought intolerant PFTs decreased from the distance about 150 km.

There was net increment ($\lambda$) of each PFT in the research area (*Fig. 4*). There were higher net increments for all PFTs at 0–220 km than those at 220–410 km. On average the drought tolerant PFT had a slight higher $\lambda$ ($\lambda = 1.52$) than that of the drought intolerant ($\lambda = 1.29$) and middle type PFTs ($\lambda = 1.15$). Negative $\lambda$ concentrated around 150–350 km.

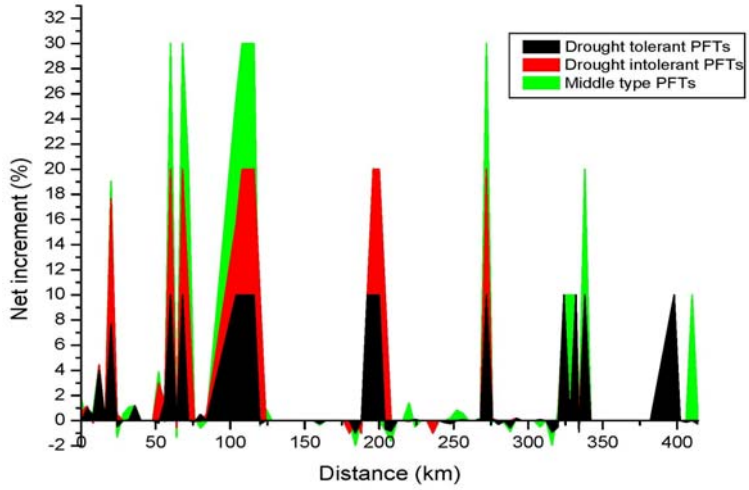


***Figure 4.*** *The net increment (%) of three PFTs along NECT from 1986 to 1994.*

### *Spatial autocorrelation of each PFT in 1986 and 1994*

The change of spatial autocorrelation for each PFTs from 1986 to 1994 was shown in *Fig. 5*. For the drought tolerant PFTs the pattern of spatial autocorrelation changed very little. For the drought intolerant PFTs there was a slight increment in the distance of spatial positive autocorrelation. For the middle type PFTs the positive autocorrelation decreased at 0–60 km, but it increased near 160 km.

The information entropy increased across scales for the drought intolerant PFTs from 1986 to 1994 while the other PFTs changed little (*Fig. 6*). The increase of information entropy of drought intolerant PFTs might be related its irregular spatial distribution change. This indicated that the spatial variability of drought intolerant PFTs was not mainly caused by local disturbances (such as windbreak) because information entropy increased at all scales. The local disturbances might cause change in PFTs at small scales. If change of land use occurred, then not only one of PFTs would change but all PFTs would be destroyed.

### *Interactions among three PFTs*

The interactions among three PFTs were not statistically significant (*Table 2*), which might mean that the spatial variation of each PFTs was mainly due to environmental factors.


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***Table 2.*** *The slope of log (means of abundance) and log (variances of abundance) for each PFT in 1986 and 1994*

| year | drought tolerant PFTs | drought intolerant PFTs | middle type PFTs |
|---|---|---|---|
| 1986 | 3.31±1.11 | 0.33±0.09 | 0.33±0.27 |
| 1994 | 2.29±0.96 | 0.47±0.13 | 0.24±0.13 |

## Discussion

### *Classification of plant functional types*

The complexity of each species' response to environmental change can be obviously reduced by treating a smaller number of PFTs. Experience indicates that this grouping can work well for specific ecosystems but that the groups often have characteristics unique to the ecosystem under consideration [28]. The classification of PFTs is case specific, and there is no classification of plant function types which can meet criterion for all different researches [19]. The assumption which it is now generally agreed is that functional types must be defined by reference to both demographic criteria and those features of life history, physiology and biochemistry that determine the responsiveness of plants to soils, land use and climatic factors [16]. In this study, the classification of PFTs is largely based on their integrated drought tolerance because the environment gradient of NECT is mainly driven by moisture. The ecophysiological and morphological characters and the life history of each tree species, such as the occurring times during the succession, were used to qualitatively classify its drought tolerance. This classification was successful to study the possible response of this ecosystem after deleting or adding different species [8, 9].

### *Spatial variations of PFTs along NECT*

The spatial variation of different PFTs at large scales can be used to indicate environmental change, such as soil moisture [1]. The local disturbances mainly occur at small scales and are impossible to change the spatial distribution of all PFTs across all scales. There were spatial variations for PFTs in the study area from 1986 to 1994 although the whole pattern was not totally changed from 1986 to 1994. There were higher covered areas in the eastern part than at the western part for the drought tolerant and middle type PFTs. The average percentage of the covered area increased significantly for the drought intolerant and middle type PFTs, also the covered areas changed at the different parts of the study area. In the eastern part the covered areas for all three PFTs increased, but at the western part the covered area of the drought intolerant PFT decreased while it increased for the drought intolerant and middle type of PFTs.

The dominance of the drought intolerant PFTs also decreased from the distance around 150 km, but the dominance of the other PFTs increased. Because the drought intolerant PFTs are tend to live at moisture area, and the drought tolerant PFTs are tend to grow at relative drought condition, the decreasing of the drought intolerant PFTs and increasing of the drought tolerant PFTs might indicate drought conditions at the research area. The drought intolerant PFTs may be vulnerable at the current climate change. The increase or decrease in the percentage of covered area and the dominance of each PFTs was mainly caused from its change in net increment. The negative net increment of three PFTs occurred mainly at 150–350 km. PFTs are strongly linked to climatic gradients [2]. The change of temperature in this area is not significant, but the change of precipitation has been dramatical in recent decades [7]. The spatial change of PFTs was mainly related with the high fluctuation of the gradient of annual precipitations in this


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area (*Fig. 7*). The change of precipitation gradient occurred mainly at the distance beyond 200 km. Because of the time delay in the response of tree species, the observed spatial variability of PFTs might be the result of the fluctuation of precipitation gradient along NECT for a long time. Changes in the frequency of very high temperature events may indirectly influence the relative abundances of different PFTs and for plant migration across the landscape at the regional scale [15]. Different PFTs in the arid area of NECT had different relationships with climate [22].

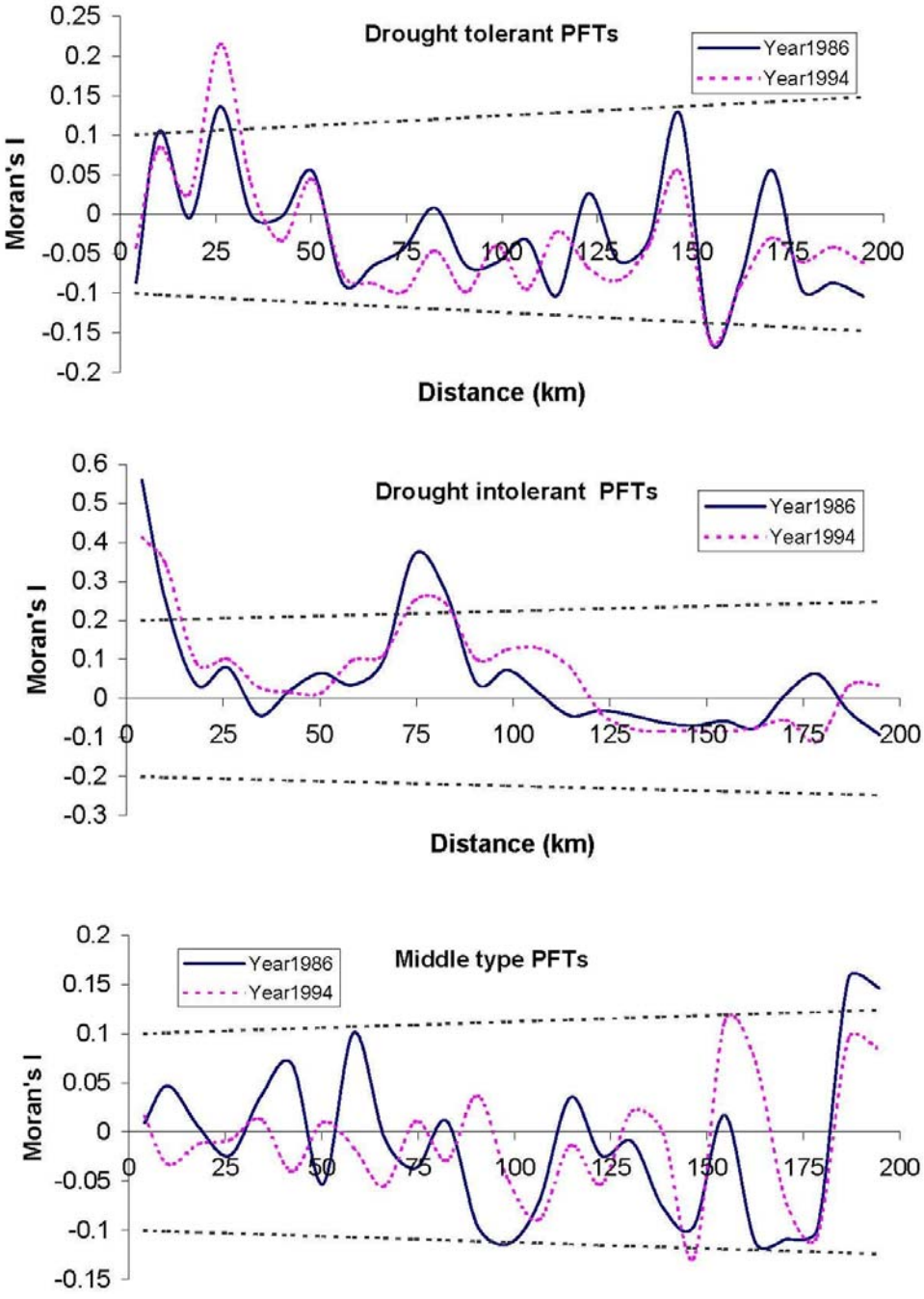


***Figure 5.*** *Comparison of the spatial autocorrelation of three PFTs in 1986 and 1994 (outside of the two straight lines is 95% confidence area).*


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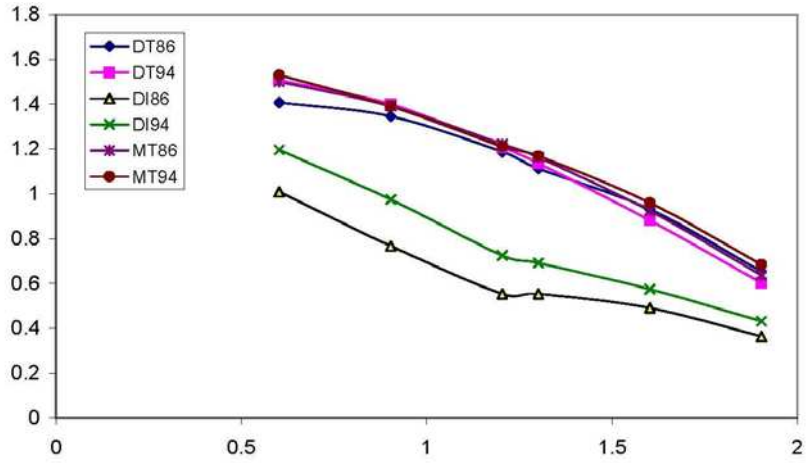


***Figure 6.*** *Change of the information entropy across scales for each PFTs (DT: drought tolerant PFTs; DI: drought intolerant PFTs; MT: middle type PFTs)*

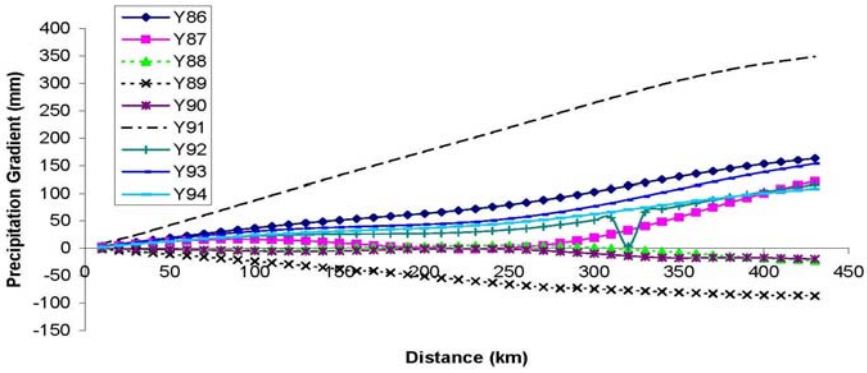


***Figure 7.*** *The precipitation gradient at different distances at NECT from 1986 to 1994 (Y86, Y87, Y88, Y89, Y90, Y91, Y92, Y93 and Y94: Year of 1986, 1987, 1988, 1989, 1990, 1991, 1992, 1993 and 1994)*

The spatial autocorrelation was used to study the spatial distribution pattern of each PFTs. Only the drought intolerant and middle type PFTs changed in their distances of spatial positive autocorrelation. The increase of information entropy of the drought intolerant PFTs across scales indicated that climate, especially the fluctuations of precipitation gradient, might be the main factors to impact the spatial distribution of PFTs. This result is consistent with that *P. koraiensis* would be replaced by *Q. mongolica* under climate change and increased concentration of $CO_2$ [5], and the current broadleaved Korean pine forest is not ecological safety [6]. In principle entropy method is applicable to ecological research at landscape [21].

***The possible causes for spatial variations of PFTs along NECT***

This study indicated that interaction change among three PFTs was not significant. The effect from change of biological competition or ecological succession might be limited. The local disturbances were not possible to cause the spatial distribution change of some PFTs at large scales; and the change in land use might destroy all PFTs at one area instead of just replacing some kinds of PFTs. The distance of spatial autocorrelation would be decreased if there were strong local disturbances. Therefore, the spatial variation of each PFTs was


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mainly due to the fluctuation in the gradient of annual precipitations although it might be complicated by time delay in the response of tree species. The global climate change would change the regional climate [10], and this change would enhance the gradient of environmental factors and eventually change vegetation structure and distribution.

## Conclusion

By analyzing the spatial pattern of PFTs of tree species along NECT with spatial analysis methods, we found the different spatial variation of three PFTs. From 1986 to 1994 the average percentages of the drought intolerant and middle type PFTs increased significantly along NECT. All three PFTs increased their covered area at eastern part of transect, and the drought tolerant and middle PFTs also increased their covered area at the western part of transect, but the covered area of the drought intolerant PFT decreased at 48% at the western part. The dominance of the drought intolerant PFT also decreased. Such decrease of the drought intolerant PFTs could be a signal of vulnerability to the occurring environmental change. There were higher net increments for all PFTs at 0–220 km than at 220–400 km and the negative net increments concentrated at 150–350 km. The spatial autocorrelation of the drought intolerant and the middle type PFTs changed slightly from 1986 to 1994. The impact from local disturbances was limited because of the increased spatial autocorrelation and the increased information entropy of drought intolerant PFT across large scales. The effect of interaction changes among three PFTs to their spatial variation was also limited. The spatial variation of PFTs was mainly caused by the fluctuation in the gradient of annual precipitation along this transect.

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# GROWTH AND REPRODUCTION OF *EISENIA FOETIDA* IN VARIOUS ANIMAL WASTES DURING VERMICOMPOSTING

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**Abstract.** The effect of various animal wastes on growth and reproduction of an epigeic earthworm *Eisenia foetida* was studied under identical laboratory conditions. For each waste, viz., cow, buffalo, horse, donkey, sheep, goat and camel, five hatchlings per 100 g of waste were inoculated and monitored for biomass gain, mortality, sexual maturity, cocoons production periodically for 15 weeks. No mortality was observed in any waste. The earthworms grew rapidly in cow, sheep, and goat wastes. Maximum weight gain and highest growth rate were attained in sheep waste. Net biomass gain/earthworm in different animal wastes was in the order of: sheep > donkey > buffalo > goat ≈ cow ≈ horse > camel. The number of cocoons produced per earthworm per day in different wastes was in the order: sheep > cow ≈ horse ≈ goat > camel > donkey > buffalo. Increase in the number of earthworms was 39.5-fold in horse waste and 26-fold in cow waste.
**Keywords.** *Eisenia foetida, animal waste, physicochemical characteristics, biomass, cocoon*

## Introduction

Animal wastes are considered as important resources that fertilize crop fields, supplement organic matters, and improve soil conditions, but are a source of environment pollution too. The US geological survey found that the increase in in-stream loads of nitrogen and phosphorus was strongly correlated with increased animal concentrations. Eutrophication from animal waste runoff has been linked to the outbreak of toxic micro-organisms such as *Pfiesteria piscicida*. The outbreak of *Pfiesteria* has been implicated in massive destruction and disease of fish and wild-life population. Animal wastes also significantly contribute to the excess bacteria and nitrates that are frequently found in groundwater. The nations which have large livestock operations experience local lagoon spills which lead to massive destruction of fish.

Currently the fertilizer values of animal wastes are not being fully utilized resulting in loss of potential nutrients. Animal wastes are also causing concern due to odour problems [29]. Organic wastes can be ingested by earthworms and egested as a peat-like material termed as vermicompost. As opposed to traditional microbial waste treatment, vermicomposting results in bioconversion of the waste streams into two useful products: the earthworm biomass and the vermicompost. The former product can further be processed into proteins (earthworm meal) or high-grade horticultural compost [30]. The latter product (vermicompost) is also considered as an excellent product since it is homogeneous, has desirable aesthetics, reduced levels of contaminants and tends to hold more nutrients over a longer period, without adversely impacting the environment. During this process, the important plant nutrients, such as nitrogen, potassium, phosphorous and calcium present in feed material are converted into forms that are much more soluble and


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available to plants than those in the parent compounds [25]. Vermicompost has also been reported to contain biologically active substances such as plant growth regulators [33].

The most promising earthworm species used for vermicomposting are *Eisenia foetida, Eisenia andrei, Eudrilus eugeniae* and *Perionyx excavatus. Eisenia foetida* is commonly used for cow dung vermicomposting in Northern India. Studies are not available on the use of *E. foetida* for vermicomposting of other animals' waste. In order to utilize this species successfully for outdoor vermicomposting of different animal wastes, its survival, growth and fecundity in different wastes should be known. The life cycle of *P. excavatus* for different animal wastes has been documented [11, 19]. But such data on life cycle of *E. foetida* in different animal wastes are not available.

The chemical composition of animal waste is known to be influenced by the feed of the animal, bedding material used and the way waste is collected, stored and handled before utilization [21]. This paper aims to investigate the effect of different animal wastes (cow, buffalo, horse, donkey, sheep, goat and camel) on the life cycle of *E. foetida*. It was hypothesized that waste of different animals would affect the life cycle of *E. foetida* due to differences in physico-chemical characteristics.

## Review of literature

Various organic wastes tested in past as feed material for different species of earthworms include sewage sludges [4, 9, 10], paper mill industry sludge [6], pig waste [7, 28], water hyacinth [14], paper waste [15], brewery yeast [6], crop residues [3], cow slurry [17], cattle manure [24], vine fruit industry sludge [2], rice stubbles, mango leaves [32] and activated sludge [18], textile mill sludge [20] etc.

Loh *et al.* [22] reported that biomass gain and cocoon production by *Eisenia foetida* was more in cattle waste than goat waste. Kale *et al.* [19] reported the potential of *Perionyx excavatus* to vermicompost different wastes (sheep dung, cow dung, biogas sludge and poultry manure and sand as control). The worms readily accepted cow and horse waste. Sheep waste was consumed 3 or 4 days after it was added. The growth, fecundity and mortality of *E. foetida* was studied by Gunadi & Edwards [16] in a range of different wastes (cattle manure solids, pig manure solids and super market waste) for more than one year. Worms could not survive in fresh cattle solids, pig solids, fruit wastes and vegetable wastes. The growth of *E. foetida* in pig wastes was faster than in cattle solid. The multiple additions of substrates prolonged the fecundity of worms, but there was a tendency of decreasing of the weight by worms after 60 weeks of the experiment. Singh *et al.* [31] studied the optimum moisture requirement during vermicomposting by *P. excavatus*. The study showed that a moisture content of 80% was optimum for stabilization of waste in minimum processing time.

Aquino *et al.* [1] studied earthworm fecundity in cattle dung and sugarcane bagasse mixed in different ratios. Maximum reproduction was observed in 1:1 and 3:1 ratios. The peels of bitter cassava root, a major source of food carbohydrate in tropics, form toxic waste which is lethal to the soil invertebrates and inhibit the root growth of the plants. Investigations by Mba [23] highlighted the ability of *Eudrilus eugeniae* to partially detoxify the wastes and convert the toxic cassava peels into valuable vermicompost. Vermicomposting of neem (*Azadirachta indica*) was studied by Gajalakshmi & Abbasi [13] in 'high-rate' reactors operated at the earthworm (*E. eugeniae*) densities of 62.5 and 75 animals per litre of reactor volume. Contrary to the fears that neem – a powerful nematicide – might not be palatable to the annelids, the earthworms fed voraciously on


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the neem compost, converting up to 7% of the feed into vermicompost per day. Indeed the worms grew faster and reproduced more rapidly in the neem-fed vermireactors than in the reactors fed with mango leaf litter earlier studied by the same authors.

## Materials and methods

Young non-clitellated specimens of *Eisenia foetida*, weighing 200–250 mg live weight were randomly picked from several stock cultures containing 500–2000 earthworms in each, maintained in the laboratory with cow dung as culturing material.

Fresh waste of seven different mammalian animals, viz., cow, buffalo, horse, donkey, sheep, goat and camel were collected from different animal farms located in Hisar city, India. The dung consisted of a mixture of faeces and urine without any bedding material. The main characteristics of animal wastes are given in *Table 1*.

All the samples were used on dry weight basis for biological studies and chemical analysis that was obtained by oven drying the known quantities of material at 110 °C. All the samples were analyzed in triplicate and results were averaged.

### *Experimental design*

Seven circular 1 l plastic containers (diameter 14 cm, depth 12 cm) were filled with 100 g (DW) of each dung material. The moisture content of wastes was adjusted to 70–80% during the study period by spraying adequate quantities of distilled water. The wastes were turned over manually everyday for 15 days in order to eliminate volatile toxic gases. After 15 days, 5 non-clitellated hatchlings, each weighing 200–250 mg (live weight), were introduced in each container. Three replicates for each waste were maintained. All containers were kept in dark at temperature 25±1 °C.

Biomass gain, clitellum development and cocoon production were recorded weekly for 15 weeks. The feed in the container was turned out, and earthworms and cocoons were separated from the feed by hand sorting, after which they were counted, examined for clitellum development and weighed after washing with water and drying them by paper towels. The worms were weighed without voiding their gut content. Corrections for gut content were not applied to any data in this study. Then all earthworms and feed (but no cocoons) were returned to the respective container. No additional feed was added at any stage during the study period. All experiments were carried out in triplicate and results were averaged.

The pH and electrical conductivity (EC) were determined using a double distilled water suspension of each waste in the ratio of 1:10 (w/v) that had been agitated mechanically for 30 min and filtered through Whatman No. 1 filter paper. Total organic carbon (TOC) was measured using the method of Nelson & Sommers [26]. Total

***Table 1.*** *Initial physico-chemical characteristics of various animal wastes*

| animal waste | moisture content (%) | pH (1 : 10) | EC (dS/m) | TOC (%) | TKN (%) | C : N ratio | TK (%) | TAP (%) |
|---|---|---|---|---|---|---|---|---|
| cow | 56.0 | 8.2 | 2.10 | 47.3 | 0.53 | 89.4 | 0.48 | 0.33 |
| buffalo | 72.3 | 8.4 | 2.60 | 51.9 | 0.56 | 93.0 | 1.07 | 0.50 |
| horse | 54.0 | 8.0 | 2.03 | 48.4 | 0.35 | 137.1 | 0.78 | 0.70 |
| donkey | 54.4 | 8.1 | 3.91 | 48.5 | 0.50 | 97.1 | 1.31 | 0.50 |
| sheep | 73.4 | 8.2 | 0.90 | 32.3 | 0.37 | 88.9 | 0.70 | 0.31 |
| goat | 21.8 | 7.6 | 2.56 | 43.8 | 0.47 | 93.5 | 0.72 | 0.37 |
| camel | 58.0 | 9.0 | 1.56 | 46.3 | 0.40 | 116.1 | 0.50 | 0.30 |


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Kjeldahl nitrogen (TKN) was determined after digesting the sample with concentrated $H_2SO_4$ and concentrated $HClO_4$ (9:1, v/v) according to Bremner & Mulvaney [5] procedure. Total available phosphorus (TAP) was analyzed using the colorimetric method with molybdenum in sulphuric acid. Total K (TK) was determined after digesting the sample in diacid mixture (cc $HNO_3$ : cc $HClO_4$ = 4 : 1, v/v), by flame photometer (Elico, CL 22 D, Hyderabad, India) [3].

## Results and discussion

### *Physico- chemical characteristics of the animal wastes*

*Table 1* summarizes the initial physico-chemical characteristics of animal wastes before use. The moisture content of the wastes varied between 21.8% and 73.4%. The pH values of the wastes were in alkaline range (9.0–7.6). The highest electrical conductivity 3.91 dS/m was in the donkey waste and minimum in sheep waste (0.90 dS/m). The TOC of different wastes were in the range of 32.3% in sheep waste to 51.9% in buffalo waste. The TKN content ranged from 0.35% in horse waste to 0.56% in buffalo waste. The minimum C:N ratio was 88.9 in the sheep waste; the maximum was 137.1 in horse waste. The potassium content ranged from 0.48% in cow dung to 1.31% in horse dung. Phosphorus content ranged from 0.30% in camel waste to 0.70% in horse waste. TKN content in our wastes was lower than that reported for cattle wastes in literature and hence, C:N ratios were higher that reported by other co-workers [3]

### *Growth of Eisenia foetida in various animal wastes*

No mortality was observed in any animal waste during the study period. Gunadi and Edwards [16] reported the death of *Eisenia foetida* after 2 weeks in the fresh cattle solids although all other growth parameters such as moisture content, pH, electrical conductivity, C:N ratio, $NH_4^+$ and $NO_3^-$ contents were suitable for the growth of the earthworms. They attributed the deaths of earthworms to the anaerobic conditions which developed after 2 weeks in fresh cattle solids. In our experiments, all the wastes were pre-composted for 2 weeks and during this period all the toxic gases produced might have been eliminated. It is established that pre-composting is very essential to avoid the mortality of worms.

The growth curves of *E. foetida* in studied animal wastes over the observation period are given in *Fig 1*. Maximum worm biomass was attained in sheep waste (1294±245 mg/earthworm) and minimum in horse waste (800±137 mg/earthworm). The maximum

***Table 2:*** *Growth of Eisenia foetida in different animal wastes*

| animal waste | mean initial weight / earthworm (mg) | maximum weight achieved / worm (mg) | maximum weight achieved on | net weight gain / worm (mg) | growth rate / worm /day (mg) | worm weight gained per unit dry animal waste (mg/g) |
|---|---|---|---|---|---|---|
| cow | 196±69 | 889±90 | 6$^{th}$ week | 686±22 | 16.3±0.52 | 34.0±1.9 |
| buffalo | 190±30 | 1026±210 | 13$^{th}$ week | 836±186 | 9.2±2.04 | 41.9±2.6 |
| horse | 138±48 | 800±137 | 10$^{th}$ week | 662±90 | 9.5±1.28 | 33.4±1.2 |
| donkey | 182±40 | 1116±208 | 9$^{th}$ week | 930±169 | 14.±2.71 | 46.6±1.1 |
| sheep | 192±67 | 1294±245 | 6$^{th}$ week | 1102±197 | 26.2±4.70 | 55.3±1.9 |
| goat | 210±29 | 904±174 | 6$^{th}$ week | 694±148 | 16.5±3.54 | 34.5±1.7 |
| camel | 212±46 | 806±47 | 6$^{th}$ week | 574±34 | 13.7±0.82 | 32.5±1.4 |


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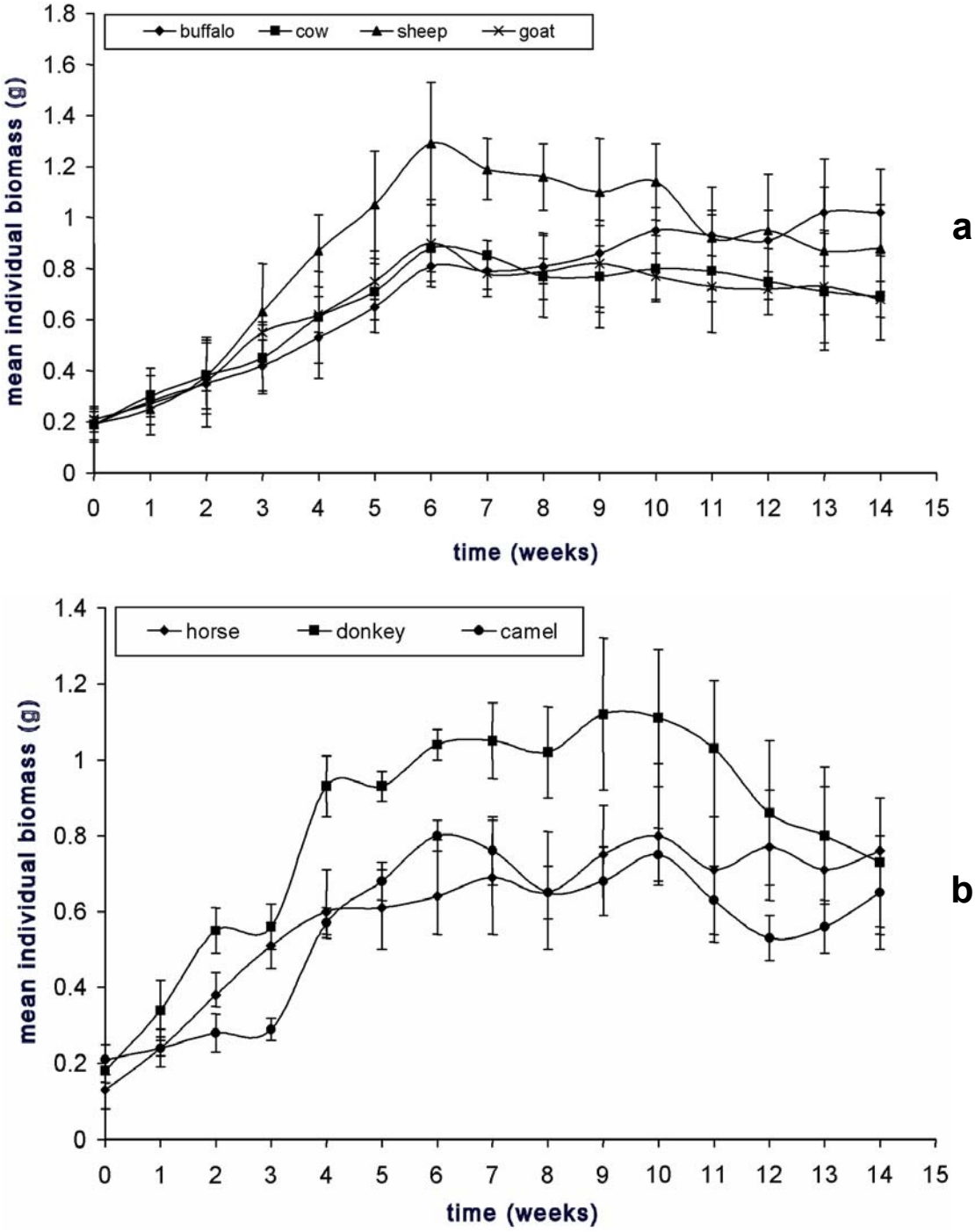


***Figure 1.*** *Growth pattern of Eisenia foetida on different animal wastes*

weight by earthworms was attained in the 6th week in cow, sheep, goat and camel wastes, where as it took 9, 10 and 13 weeks in donkey, horse and buffalo wastes respectively. Initially worms gained biomass but later after few weeks, weight loss by earthworms was observed in all the tested animal wastes. The loss in worm biomass can be attributed to the exhaustion of food. When *E. foetida* received food below a maintenance level, it lost weight at a rate which depended upon the quantity and nature of its ingestible substrates [27].


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The biomass gain for *E. foetida* per g dry weight of feed (DW) was highest in sheep waste (55.3±1.9 mg/g) and smallest in camel waste (32.5±1.40 mg/g). Edwards *et al.* [11] have reported a biomass gain of 292 mg/g cattle waste by *P. excavatus* at 25 °C. However, in our experiments, the biomass gain was only 34.0±1.90 mg/g by *E. foetida* species in cow dung at 27 °C. In horse waste, biomass gain by *P. excavatus* species was about 2 times higher than *E. foetida*. This difference could be due to the difference in species morphology and initial characteristics of the feed waste. Nauhauser *et al.* [27] reported that rate of biomass gain by *E. foetida* was dependent on population density and food type. Net biomass gain/earthworm per unit feed material in different feeds followed the order: sheep > donkey > buffalo > goat ≈ cow ≈ horse > camel. Net biomass gain by earthworms in sheep waste was 1.92 times higher than in camel waste (*Table 2*).

The growth rate (mg weight gained/day/earthworm) has been considered a good comparative index to compare the growth of earthworms in different wastes [11]. The buffalo (9.2± 2.04 mg/day/earthworm) and horse wastes (9.5± 1.28 mg/day/earthworm) supported the least growth of *Eisenia foetida*; camel and donkey wastes were marginally better than buffalo and horse wastes (Table 2). Lower growth rate in buffalo waste, in spite of attainment of more body weight than cow waste, was due to the fact that the time taken to achieve the maximum biomass was longer for buffalo waste than cow waste. Similar observations have been reported by Chaudhuri & Bhattacharjee [8] for vermicomposting of cow dung and kitchen waste by *Perionyx excavatus*. The worm growth rate was highest in sheep waste which was about twice than in camel waste. Earthworms grew at relatively similar rates in cow and goat wastes (Table 2).

***Sexual development and cocoon production***

*Table 3* summarizes the sexual development and cocoon production by *E. foetida* in different feeds. All individuals in all the feeds developed clitellum before day 21 except camel waste (day 28) after the start of the experiment. Cocoon production by earthworms was started by day 28 in horse, donkey, sheep and goat wastes; and by day 35 in cow, buffalo and camel wastes. *Fig. 2* shows the cumulative cocoon production by earthworm in different feeds. After 15 weeks maximum cocoons (155±18.36) were counted in sheep waste and minimum (62±23.57) in buffalo waste. The mean number of cocoons produced per worm per day of 0.44±0.052 in sheep waste was 231% greater than 0.19±0.072 cocoons produced per day in buffalo waste. The number of cocoons produced per earthworm per day in different wastes was in the order: sheep > cow ≈ horse ≈ goat > camel > donkey > buffalo. The difference between rates of cocoon

***Table 3.*** *Cocoon production by Eisenia foetida in different animal wastes*

| animal waste | clitellum development started in | cocoon production started in | total no. of cocoons produced after 15 weeks | no. of cocoons produced / worm | no. of cocoons produced / worm / day | cocoon production ceased after |
|---|---|---|---|---|---|---|
| cow | 3rd week | 5th week | 109±14.9 | 21.8±3.0 | 0.39±0.05 | 12th week |
| buffalo | 3rd week | 5th week | 62±23.6 | 12.3±4.6 | 0.19±0.07 | 13th week |
| horse | 3rd week | 4th week | 143±29.5 | 28.6±5.9 | 0.37±0.08 | 14th week |
| donkey | 3rd week | 4th week | 97±11.3 | 19.4±2.3 | 0.28±0.03 | 13th week |
| sheep | 3rd week | 4th week | 155±18.4 | 31.0±3.7 | 0.44±0.05 | 13th week |
| goat | 2nd week | 4th week | 124±28.4 | 25.4±5.7 | 0.36±0.08 | 13th week |
| camel | 4th week | 5th week | 89±15.7 | 17.8±3.1 | 0.32±0.06 | 12th week |


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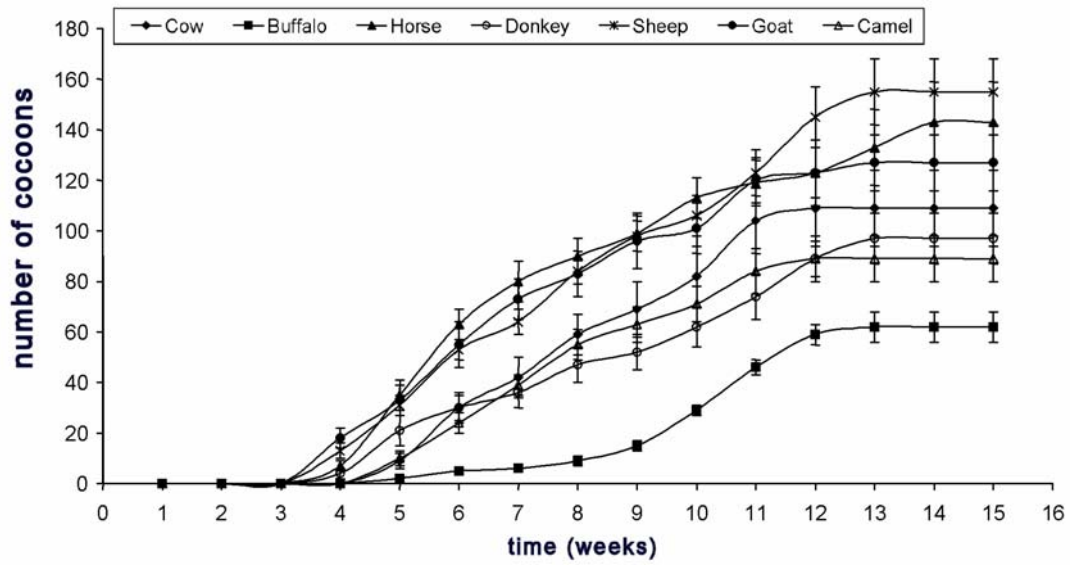


***Figure 2.*** *Cumulative cocoon production by Eisenia foetida on different animal wastes*

production could be related to the biochemical quality of the feeds, which is an important factor in determining the time taken to reach sexual maturity and onset of reproduction [11, 12]. Feeds which provide earthworms with sufficient amount of easily metabolizable organic matter and non-assimilated carbohydrates, favour growth and reproduction of earthworms [12]. But in our experiments, buffalo and donkey wastes were in contrast to this observation. The weight gain by earthworms was more in these feeds but cocoon production was lower than other feeds tested. It indicates that buffalo and donkey wastes are a good biomass supporting medium but not good for reproduction. A large proportion of the energy of mature worms is used in cocoon production. When cocoons are not produced the energy is utilized for tissue growth [8, 19]. The cocoon production was ceased by day 84 in cow and camel feed wastes; by day 91 in buffalo, donkey, sheep and goat wastes and by day 98 in horse waste.

## Conclusions

Disposal of animal dung materials is a serious problem. Currently the fertilizer values of animal dung are not being fully utilized in India resulting in loss of potential nutrients. Our trials demonstrated vermicomposting as an alternate technology for the recycling of different animal dung materials using an epigeic earthworm *Eisenia foetida* under laboratory conditions. The dung materials strongly influenced the biology of *E. foetida.* Net biomass gain/earthworm in different feeds was in the order: sheep > donkey > buffalo > goat ≈ cow ≈ horse > camel. The biomass gain for *E. foetida* (live weight) per g dry weight of feed source (DW) was highest in sheep waste (55.3±1.9 mg/g) and smallest in camel waste (32.5±1.40 mg/g). The mean number of cocoons produced per worm per day of 0.44±0.052 in sheep waste was 231% greater than 0.19±0.072 cocoons produced per day in buffalo waste. The number of cocoons produced per earthworm per day in different dung materials was in the order: sheep > cow ≈ horse ≈ goat > camel > donkey > buffalo. Finally, cow, horse, sheep and goat wastes supported the growth and reproduction of *E. foetida,* hence can be used as feed materials in large scale vermicomposting facilities. Further studies are required to explore the potential of utilization of buffalo, donkey and camel wastes in mixture with cow or sheep or goat wastes.


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# MOULDS ASSOCIATED WITH MILK DEPENDING ON MACROCLIMATE AND GEOGRAPHICAL LOCATION

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**Abstract.** Moulds can be found in milk as contaminants from the environment. The specific qualities of climate, vegetation and land are the important factors affecting the quality of moulds and determinators of genus and species in connection with a certain geographical location. The study was carried out in 297 milk samples taken from different geographical location: A. lowlands, B. hilly-mountainous, C. alluvial plains by the river, D. submountainous and part of basin by the river in course of four seasons. The following media were used for growing moulds in laboratory conditions as: Sabouroud dextrosa agar, Czapek agar, Potato dextrosa agar. Moulds determination was carried out according to their micromorphological properties using moulds determination keys. According to the result of study it was conclude that moulds count in raw milk samples were as follows: A. region: *Fusarium* genus (44.1%) in spring, *Aspergillus* genus (30.8%) in summer, *Penicillium* genus (30.2%) in autumn and the same (32.1%) in winter; B. region: *Fusarium* genus (55%) in spring, *Penicillium* genus (34.8%) in summer, *Penicillium* genus (23.1%) in autumn and *Cladosporium* + *Penicillium* genera (28.6%) in winter; C. region: *Penicillium* genus (69.3%) in spring and the same (31.8%) in summer, *Geotrichum* genus (24.6%) in autumn and *Aspergillus* genus (20.9%) in winter; D. region: *Penicillium* genus (42.9%) in spring and the same (50.73%) in summer, *Cladosporium* genus (43.2%) in autumn and *Penicillium* genus (45.21%) in winter. Finally we concluded that different genus of moulds which were found, are in dependence of geographical locations and seasons.
**Keywords.** *moulds, raw milk, contamination, ecology*

## Introduction

The important influence of environmental factors on fungal growth has been demonstrated in a range of ecosystems. While there are some seasonal variations and certain peak periods most moulds have the capability of living year-round indoors as well as outdoors. Moulds spores established new colonies quickly making elimination difficult. Fungal spores are more abundant than any other airborne particles found in atmosphere including pollen grains. Moulds as inhalant allergen are of primary importance. One of the measures to avoid inhaling them is to remain indoors on windy days after a first frost when spores are abundant. But, it is almost impossible to escape mould spores so prevalent in and out-of-doors [12].

In a study of other authors [6] the seasonal variations had a significant effect on fungal growth. In the harvest period of the year or in such an environment where grain were stored, the *Fusarium* species was mostly found. The important factors for the growth of mould are fungal colonization of cereal grains before and after harvest. Variations in external conditions may not only affect the rate of growth of a mould but, in many cases, can bring about differences in way of growth.

The objective of the presented work was to determine the effect of temperature, moisture, geographical location and seasons of the year on population of moulds in milk samples.


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## Materials and methods

The study was carried out on 297 milk samples. Four different geographical locations where the milk samples taken from A. lowlands, B. hill-mountainous, C. alluvial plains by the river, D. submountainous and part by the river in course of four different seasons. For the recovery of moulds the following medium was used: Potato dextrosa agar (PDA) pH 5.6 which was prepared as described in the bacteriological Analytical Manual and supplement with filter sterilized chloramphenicol and chlortetracycline immediately before use. Other media which were used are as follows: Sabouroud dextrosa agar and Czapek agar prepared as described in the Official Method of Analysis. Three dilutions were prepared then they were transferred into sterile plates (1 ml/plate approximately) from samples and from three pepton diluent. Triplicate Sabouroud agar pour plating were made of appropriate dilutions as the percentage of samples contaminated with mould are listed in *Table 1*. In the end of the specified incubation period, the plates were analyzed for fungal population (CFU per gram). Growing colonies were observed at first macroscopically and then microscopically. At first we obtained cultures by sight and then with microscope. We used to isolate a particular mould in order to obtain a pure culture then we described the colony colour and colour changes in the medium texture of surface (described as loose or compact, plane, wrinkled or buckled, velvity, matted, flocose, hairy, ropy, gelatinous etc.) odour of any character of submerged hyphae, full details of spores (colour, shape, septation, size etc.). Mould growth on the surface on the Sabouroud medium was removed to the select medium like PDA or Czapek agar which were selective for some kinds of moulds. These pieces of information were sufficient to place the species in the correct class and order, and consideration of the rest of the date will lead to the family and then genus and species.

Mould was carried out according to their micromorphological properties using mould determination keys [3, 4, 9, 11, 12].

## Results and discussion

The results presented in *Table 1* showed in the region C isolation the higest percentage of contaminated samples was 91.30% in autumn. Minor contamination samples isolated in the region B. In the region A, the results showed variations from 64.00% in winter to 100% in autumn. In the region D, the results showed variations from 58.33% in winter, spring and autumn to 83.33% in summer. In the region C, the results showed variations from 57.14% in summer to 91.30% in autumn.

The isolated moulds were shown in the *Table 2*. The different genus of moulds isolated from milk samples during the year in the region A. Most frequently isolated moulds in winter were genus *Penicillium* (30.2%) and in winter were isolated seven different moulds colonies. In spring, most frequently isolated moulds were *Fusarium* (44.1%) beside four different moulds which were isolated. In summer, most frequently isolated mould were *Aspergillus* (30.8%) and eight other different moulds. Most frequently recovered in autumn were *Penicillium* genus (30.2%) and eight different genus of moulds. Most frequently isolated moulds in the region B were genera *Cladosporium* and *Penicillium* (28.6%) in winter, *Fusarium* (55.0%) in spring and *Penicillium* (23.1%) in summer. In the region C, most frequently isolated moulds in winter were members of the genera *Alternaria*, *Aspergillus* and *Geotrichum* (20.9%), in


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***Table 1.*** *The percentage of samples contaminated with moulds depends of macroclimate and geographical location in four seasons of the year.*

| location | season | percentage of samples with the colony of moulds |
|---|---|---|
| **A** (lowlands) | winter | 64.00 |
| | spring | 68.00 |
| | summer | 76.00 |
| | autumn | 100.00 |
| **B** (hilly-mountains) | winter | 46.67 |
| | spring | 75.00 |
| | summer | 57.89 |
| | autumn | 58.33 |
| **C** (alluvial plains by the river) | winter | 80.00 |
| | spring | 80.00 |
| | summer | 57.14 |
| | autumn | 91.30 |
| **D** (sub-mountain-ous and part by the river) | winter | 58.33 |
| | spring | 58.33 |
| | summer | 83.33 |
| | autumn | 58.33 |

spring, *Penicillium* and *Cladosporidium* (31.8%) and in autumn, *Geotrichum* (24.6%). In the region C, frequently isolated moulds were *Penicillium* genus (69.3%) in spring, and the same (31.8%) also in the summer, *Geotrichum* genus (24.6%) in autumn, and *Aspergillus* genus (20.9%) in winter. In the D region, frequently isolated mould were *Penicillium* genus (42.9%) in spring and the same (50.73%) in summer, *Cladosporium* genus (43.2%) in autumn and *Penicillium* genus (45.21%) also in winter. *Table 1* presents the percentage of samples contaminated with moulds depends of macroclimate and geographical location in four season of the year.

*Table 2* represents the spread and development in milk samples during the year in the four geographical locations.

The highest percentage of contaminated samples was on the alluvial plains by the river in the autumn or in the submountainous region and part by the river in the summer and spring too, but in the lowlands region in summer. The combined effect of medium water activity ($a_w$) modified atmosphere influenced on the conditional germination of different genus of moulds [3, 5]. A number of techniques for the enumeration and identification of viable mould propagules in the indoor air of houses were researched

***Table 2.*** *Spread and development of mould in milk samples during the year in the geographical location A, B, C and D.*

| genus of moulds | season | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | winter (%) | | | | spring (%) | | | | summer (%) | | | | autumn (%) | | | |
| | A | B | C | D | A | B | C | D | A | B | C | D | A | B | C | D |
| *Absidia* | — | — | — | — | — | — | — | — | 7.7 | 2.2 | — | — | 7.8 | 7.7 | 2.3 | 12 |
| *Alternaria* | 6.0 | 6.2 | 30 | — | — | — | — | 11 | 2.6 | 11 | — | — | 4.8 | 7.7 | 8.4 | 1.4 |
| *Aspergillus* | 11.7 | — | 21 | — | 30 | — | — | — | 31 | 6.5 | — | 2.8 | 8.5 | 6.0 | — | — |
| *Cladosporium* | 14.6 | 28 | 7.6 | 9.4 | — | — | 11 | 18 | 5.1 | 17 | 32 | — | 6.0 | 7.7 | 13 | 43 |
| *Fusarium* | 17.5 | — | — | — | 44 | 55 | 3.9 | 10 | 23 | 4.4 | 7.4 | — | 18 | 7.7 | 8.9 | — |
| *Geotrichum* | 3.6 | 15 | 21 | 38 | — | 15 | — | — | 10 | 10 | 8 | 32 | 6.0 | — | 24 | — |
| *Mucor* | 14.6 | 21 | 15 | 5.2 | — | — | 7.7 | 17 | — | 11 | 7.2 | 5.7 | 21 | 7 | 11 | 10 |
| *Penicillium* | 32.1 | 28 | 4 | 45 | 17 | 30 | 3 | 43 | 15 | 35 | 32 | 51 | 30 | 23 | 24 | 31 |
| *Rhizopus* | — | — | 3.0 | — | — | — | — | — | 5.1 | — | — | — | — | 15.4 | — | — |
| *Scopulariopsis* | — | — | — | 1.6 | 9 | — | — | — | — | 2.2 | — | 2.6 | — | 7.7 | 4.5 | 2.0 |
| *Trichoderma* | — | — | — | — | — | — | — | — | — | — | — | — | — | 7.4 | — | — |

A = lowlands; B = hilly mountains; C = alluvial plains by the river; D = submountainous and part by the river; — = not found


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by a number of authors [8, 7]. Some authors [1, 2] recommended incubation temperature of enumerations moulds at 25 °C. Incubation time between plating and counting colonies ranges from 5 days for determination of general populations of microflora to 4 weeks of more. The same conditions were used in this experimental work.

In our study, the most different genus of moulds were isolated during summer and autumn. Most frequently recovered moulds could be found in the cereal before harvest, this is why they are called "moulds of field". In our study, most frequently recovered moulds were: *Cladosporium*, *Alternaria* and *Fusarium* in spring and *Fusarium*, *Geotrichum* and *Cladosporidium* in autumn. Insects spread mould spores to great distances [10]. Spores of moulds can disperse in the air with the wind or in combination of wind and rain. Other research [8] analyzed contamination of air surroundings of Kembridge. This author found in the air most frequently spores of moulds from the genus *Penicillium* and *Cladosporium*, their number depended on seasonal variations. In the districts where the most frequent disease was endemic nephritis in the last two years, correlation was found between great mortality [2]. The harvest – according to the work of the some authors – is a process which changes the conditions of ecosystem, because the cereal from the open air are transferred in the indoors where it is stored. As our results presents the highest number of moulds were in summer, spring and autumn.

## Conclusion

On the received results we can conclude:

1. The percentage of moulds in the samples of raw milk varies depending on geographical location and season of the year. It begins with 46.64% in winter the region C and the highest number is in autumn 91.30% in the region C, too.
2. From the samples of milk most frequently isolated moulds were as follows: region A: genus *Fusarium* (44.1%), in spring, *Aspergillus* (30.8%) in summer, *Penicillium* (30.2%) in autumn and the same (32.1%) in winter; region B: genus *Fusarium* (55%) in spring, *Penicillium* (34.85%) in summer and the same (23.1%) in autumn, genera *Cladosporium* + *Penicillium* (28.6%) in winter; region C: genus *Penicillium* (69.3%) in spring and the same (31.8%) in summer, *Geotrichum* (27.0%) in autumn, *Aspergillus* (20.9%) in winter; region D: genus *Penicillium* (42.9%) in spring, and the same (50.73%) in summer, *Cladosporium* (43.2%) in autumn and *Penicillium* (45.2%) in winter.

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# COMPARATIVE UPTAKE AND PHYTOEXTRACTION STUDY OF SOIL INDUCED CHROMIUM BY ACCUMULATOR AND HIGH BIOMASS WEED SPECIES

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**Abstract.** Plant species have been recently used for heavy metal accumulation and most of the studies have been done on hyperaccumulator tolerant species. Metal hyperaccumulator plants though useful to phytoextract metal contaminant from soil, have many shortcomings such as low biomass, edible nature and difficult to harvest. This study is part of a series of studies that attempt to evaluate the phytoextraction potential of commonly found high biomass weed species that are harmless, non-edible in nature. We have investigated and compared five weed species (*Ipomoea carnea, Dhatura innoxia*, *Phragmytes karka Cassia tora and Lantana camara),* with two accumulator plants (*Brassica juncea* and *Brassica campestris*), in a pot study to assess Cr uptake in the range of 5 to 200 mg $kg^{-1}$ soil. The results indicated that *P. karka* showed much greater tolerance to metals than other plants, though the uptake was low. It was more effective at translocating Cr from soil to plant shoot. The order of Cr extraction was *I. carnea* > *D. innoxia* > *C. tora* > *P. karka* > *B. juncea* > *L. camara* > *B. campestris.* Among the studied plants *I.carnea* showed maximum chromium extraction and biomass growth, but the difference of shoot by root chromium concentration was least. Other than *Lantana camara,* all the tested weeds were better for chromium extraction than the accumulator *Brassica species.* To save the *Brassica species* infested by army moth, pesticide application was required, whereas weeds required no care.
**Keywords:** *phytoremediation, weeds, bioconcentration factor, transportation index*

## Introduction

India is one of the largest producers of leather and nearly 80 % of the tanneries are engaged in chrome tanning process [19]. Leather tanning, electroplating and stainless steel industries contribute to most of the chromium contamination, by disposal of wastewater directly to the streams and/or by over land disposal of sludge or solid waste [21, 24]. Non-biodegradability of chromium is responsible for its persistence in the environment; once mixed in soil, it undergoes transformation into various mobile forms before ending into environmental sink. The dominant forms of chromium in waste contaminants are dichromate ($Cr_2O_7^{2-}$) and/or chromate ($CrO_4^-$). Chromate, ($CrO_4^{2-}$), is the predominant form at pH > 6. It exists in pH-dependent equilibrium with other forms of Cr(VI), such as $HCrO_4^-$ and dichromate ($Cr_2O_7^{2-}$ ); these oxyanions are actively transported to cells by the sulfate transport system. Adsorption of Cr (VI) is considerably less; it is soluble at neutral to alkaline pH than at more acidic pH values [5, 6].

In view of the seriousness of Cr pollution, considerable efforts have been made to develop suitable methods for the remediation of chromium-contaminated soil. Phytoextraction; a part of phytoremediation technology, is considered for remediation of inorganic and organic contaminated sites because of its cost effectiveness, aesthetic advantages, and long-term applicability. It is well suited for large sites where other


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methods may prove impractical. For a country like India phytoremediation is best suited as it requires low investment, and relies on plants natural capability to take up metal ions from soil. After accumulation of contaminants, plants can be harvested and the biomass can be used as a source of energy along with recovery of metal from ash. This will complete a biogeochemical cycle and heavy metals can be isolated.

***Role of Chromium in Plants***

Chromium is not considered to be essential for plant growth and development; some studies have indicated that at low concentrations (1μM), Cr stimulates plant growth [8]. Chromium (Cr) exists predominantly in III and VI oxidation states. The hexavalent chromium Cr(VI) compounds are comparatively more toxic than Cr(III) due to their high solubility in water [12], rapid permeability through biological membranes and subsequent interaction with intracellular proteins and nucleic acids [7]. Chromium is toxic for agronomic plants at about 0.5 to 5.0 mg $l^{-1}$ in nutrient solution and 5 to 100 mg $kg^{-1}$ of available Cr in soil [11]. The species found to accumulate Cr are largely exotic; research into the mechanisms of Cr hyperaccumulation is scarce. The plant species *Leptospermum scoparium* (Myrtaceae) is an accumulator of Cr, it showed up to 20,000 mgCr $kg^{-1}$ in the foliage ash when grown on serpentine soils. Few Cr hyperaccumulator species have been identified to date, [3]. *Brassica juncea* has been found to be an excellent accumulator plant for Cr in soils, other metals accumulated by it are Cd, Ni, Zn and Cu [13, 22]. Hence it was considered as reference plant in our study, along with this *Brassica campestris was* also selected, as it belongs to the same family.

Looking at the environmental conditions of the polluted areas, hardy tolerant weed species were selected for phytoextraction study. Plants that can grow in both dry lands and marshy conditions were considered. These are *Ipomoea carnea*, *Dhatura innoxia*, *Cassia tora* and *Lantana camara* (all are local weeds). Another example of a plant that has shown much promise in the treatment of metal pollutant is *Phragmytes karka*, it has the ability to take up chromium from contaminated water [10, 22]. Though studies on *Phragmytes karka* were hydroponic, soil studies on chromium accumulation are scarce. Thus the study focuses on the ability of high biomass weed species to extract and decontaminate Cr spiked soil and discounts on the plants ability to accumulate large amounts of Cr in the tissues. All these plants naturally grow in the tropical climates and large parts of the Indian subcontinent. Cr (VI) was added to soil as Potassium dichromate solution at various concentrations, and chromium uptake and extraction were compared between accumulator and weed species.

## Materials and methods

As the pH of the soil is 8 ± 0.2, and Cr (VI) is more soluble than Cr (III), easily transported inside plant and is the predominant form at pH > 6; Cr (VI) was chosen as the study species. Topsoil from botanical garden was air-dried, sieved to (< 2 mm), and thoroughly mixed. Soil pH was measured in double distilled water using a solid: liquid ratio of 1: 2.5 after equilibrium for 2.5 hrs. The Organic Carbon is 5.5 ± 0.3 g $kg^{-1}$, $CaCO_3$ 70±6 g $kg^{-1}$, Clay 590 g $kg^{-1}$, semectite is the dominant mineral of the studied soil [20].


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***Soil Treatments and Sowing***

Pot culture experiments were conducted using soil treated (spiked) with Potassium dichromate solution. The final concentration of Cr added in soil was 5, 10, 20, 50, 100 and 200 mg $kg^{-1}$ respectively, and for comparison an unamended (control) was taken. Chromium solution was uniformly mixed with air-dried soil, kept for two weeks to stabilize and filled in pots (8 kg). Twenty seeds were sown in the soil to germinate; out of them only six uniform plants were allowed to grow in each pot, at a uniform distance. Pots were placed in net house shaded with transparent polythene sheet, to protect from rainwater leaching. Plants were grown under natural light and ambient temp in order to keep all plants under conditions as similar as possible. Fertilizers or soil amendments were not added to enhance growth or metal uptake.

***Plant Growth and Harvesting***

For growth studies individual plants were grown under similar conditions and at set time intervals 10 plants for each concentration and interval (i.e. 15, 30, 60 and 90 days) were harvested from the six replicate pots, without damaging the roots. Maximum recoverable portion of roots were procured and plants were rinsed in distilled water to remove dust and soil mineral particles. The plants were separated into leaves, stem and roots and oven dried at $85^{O}$ C for 36 hours and weighed. Shoot and root length (cm) and dry biomass (g) of different plant parts (leaves, stem, and roots) were taken for each treatment and interval. All the calculations of Cr uptake and extraction were done on dry weight basis.

***Analysis of Plant Mass***

Dried samples were homogenized using a wily mill before analysis. The samples were digested in acidic mixture of $HNO_3$: $HClO_4$ (APHA-1992), and chromium analysis was done in triplicate by Atomic Absorption Spectrophotometer (GVC 902).

Transportation index ($T_i$) gives the leaf/root chromium concentration and depicts the ability of the plant to translocate the metal species from roots to leaves at different concentrations. It was calculated, as:

$$T_i = \frac{\text{Cr content of the leaves mg kg}^{-1}}{\text{Cr content of root mg kg}^{-1}} \text{ x } 100$$

Metal uptake is also depicted as Bioconcentration Factor (BCF). It provides an index of the ability of the plant to accumulate a particular metal with respect to its concentration in the soil substrate [27]. It was calculated as follows:

$$\text{Bioconcentration Factor} = \frac{\text{Average chromium conc. in the plant tissue (mg kg}^{-1})}{\text{Chromium added in soil (mg kg}^{-1})}$$

It has limited application if one wishes to compare uptake of a plant species under different treatments. Since change in BCF is related to the individual plant biomass and soil elemental concentration, the efficiency of BCF is a better understood when compared between different harvests, plant species or elements.


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### *Data analysis*

Statistical significance of the observed differences between samples was determined by Student's *t*-test and ANOVA test. Differences were considered to be significant at $p \leq 0.05$ and highly significant at $p \leq 0.005$, level of significance.

## Results and Discussion

Chromium toxicity was evident in form of reduction of shoot and root length and total biomass of the plants. The growth of the plants was highly affected with increase in chromium concentration. Among all the tested plants *Phragmytes karka* was the only plant to grow above 20 mg Cr $kg^{-1}$ soil. Plants other than *P.karka* grew till maturity (90 days) only up to 20 $mgkg^{-1}$. At 50 mg $kg^{-1}$ Cr plants were able to germinate and grow up to 10 days, this clearly shows that conc. of 50 mg Cr $kg^{-1}$ soil is toxic to plants and this is in agreement with range mentioned earlier as the toxic range. Significant reduction ($p < 0.05$) in shoot length of plants was observed in comparison to control [18]. in their study concluded that Cr(VI) seems to act principally on plant roots, resulting in intense growth inhibition; this was evident in form of reduction of mass. As shown in Table 1, in comparison to control all the seven tested species exhibited sensitivity to Cr and all the plants showed highly significant ($p < 0.005$) reduction in dry biomass. *Brassica campestris* and *Brassica juncea* were the most sensitive showing 58% and 48% reduction in total dry mass respectively, *Dhatura* was least affected and showed a biomass reduction of only 21% at 20 mg Cr $kg^{-1}$ soil. *Phragmytes karka* was tolerant to 200 mg Cr $kg^{-1}$ soil, but the biomass showed 93 % reduction in comparison to control. The correlation of increase in biomass with respect to time was positive, whereas with increase in chromium conc. it was negative. Beyond 90 days only *I. carnea* and *L. camara* continued to grow, whereas other plants did not add biomass. The pattern of addition of biomass is important for phytoextraction studies because it is needed to estimate the best time to harvest the biomass, in this case harvesting after 90 days was best, as no mass was added beyond this period.

***Table 1.*** *Average Dry biomass (g) grown in chromium treated soils (n = 6) on 90th day.*

| Total Cr added in Soil (mg $kg^{-1}$) | *Brassica campestris* | *Brassica juncea* | *Dhatura innoxia* | *Ipomoea carnea* | *Phragmytes karka* | *Cassia tora* | *Lantana camara* |
|---|---|---|---|---|---|---|---|
| Control | 3.28 | 3.31 | 12.32 | 19.59 | 11.46 | 12.45 | 5.43 |
| 5 | 1.86** | 2.89* | 8.57** | 15.01** | 7.66** | 7.90** | 2.27** |
| 10 | 1.47** | 2.16** | 7.24** | 11.33** | 5.93** | 7.30** | 1.93** |
| 20 | 1.36** | 1.17** | 6.49** | 10.50** | 7.64** | 7.21** | 1.76** |
| 50 | NG | NG | NG | NG | 1.51** | NG | 1.09** |
| 100 | NG | NG | NG | NG | 1.06** | NG | NG |
| 200 | NG | NG | NG | NG | 0.78** | NG | NG |

Significantly different * ($p \leq 0.05$) & ** ($p \leq 0.005$) in comparison to control plant.

NG = No Growth observed
n = number of plants

### *Chromium uptake by plant tissues*

Chromium uptake by plants is mainly non-specific, probably as a result of plant uptake of essential nutrients and water. Plants can absorb both Cr(VI) and Cr(III);


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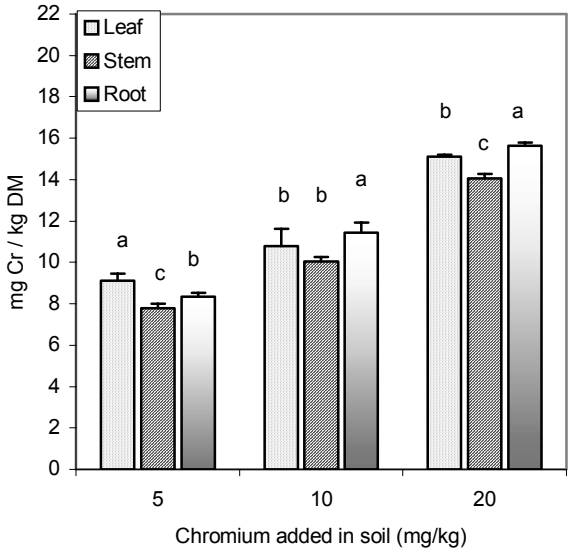
though Cr was added as hexavalent form it is expected that both the forms are simultaneously present in soil. At the end of 90 days, maximum Cr accumulation was in roots followed by leaves and stem in all the species, except *P.karka*. Statistically significant ($p < 0.05$) difference in accumulation of Cr in leaves, stem and roots has been shown in Figure 1 (a to g). At the earlier stage (15 days) concentration was maximum in leaves, but later roots accumulated most of the Cr followed by leaves and stem, this confirms the earlier results that metals get accumulated in leaves due to transpiration pull. Chromium (VI) is more easily transported inside the plant, as it has been reported to occur by an active mechanism [17].

***Figure 1a.*** *Chromium concentration (mg $kg^{-1}$ of dry matter) in leaves, stem, and roots of B. campestris. Different letters indicate significant difference between parts ($p < 0.05$).*




The initial symptoms of Cr toxicity appeared as severe wilting and chlorosis in Dhatura and Brassica species, as confirmed by [25]. They proposed that chlorosis appeared in the upper leaves of these plants, as an indirect effect of Cr, probably due to the retardation of Fe and Zn translocation. The primary toxic effect seemed to be membrane damage due to the high oxidative potential of Cr (VI), this was observed as necrosis in the lower leaves of D.innoxia. Two plants I.carnea and P.karka did not show any deformation except reduction in biomass. Most researchers [9, 23], using non-hyperaccumulator plants have reported that Cr is mainly accumulated in the roots, and relative lower in the leaves, this was observed in all the plant species except P.karka.

Maximum accumulation of Cr in plants was observed between 31 to 60 days, but the extraction was highest between 61 to 90 days. This shows that the increase in biomass was at a higher rate than accumulation of chromium between 31to 60 days, which got reduced at a later stage. The amount of chromium increased with both increase in time and soil Cr concentration, this depicts the accumulation of chromium was linear and showed a positive correlation. Only in case of *P.karka* the correlation was negative with increase in time. The average chromium uptake was significant ($p < 0.05$) with increase in metal concentration.


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***Figure 1b.*** *Chromium concentration (mg kg$^{-1}$ of dry matter) in leaves, stem, and roots of B. juncea. Different letters indicate significant difference between parts ($p < 0.05$).*

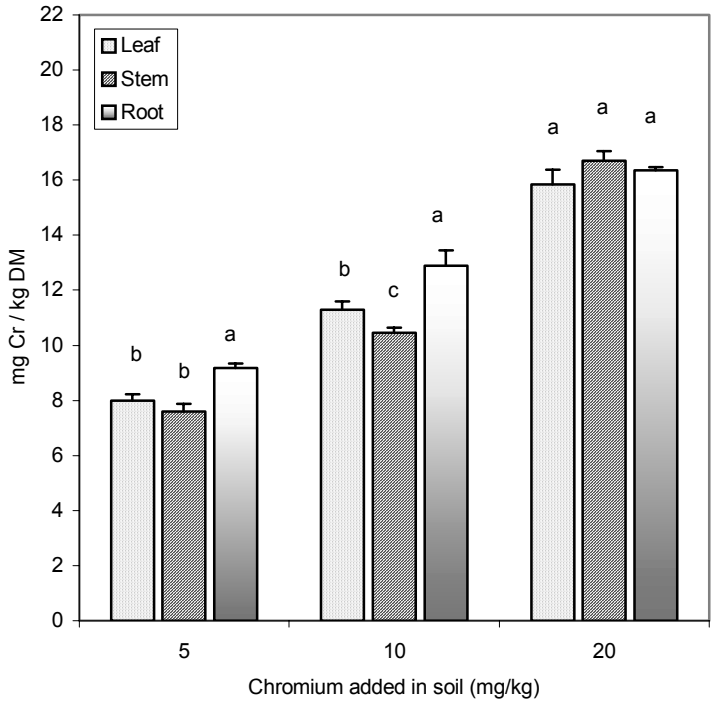


***Figure 1c.*** *Chromium concentration (mg kg$^{-1}$ of dry matter) in leaves, stem, and roots of D. innoxia. Different letters indicate significant difference between parts ($p < 0.05$).*

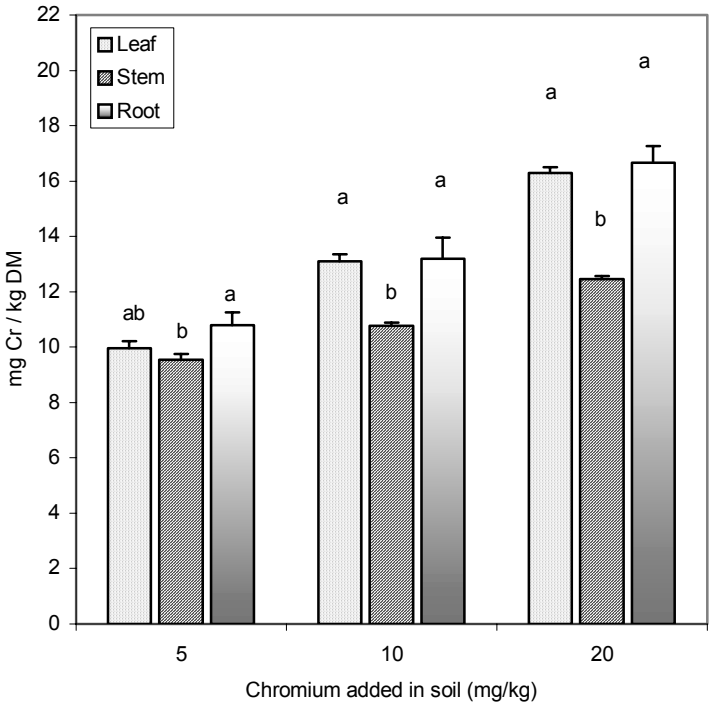



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***Figure 1d.*** *Chromium concentration (mg kg$^{-1}$ of dry matter) in leaves, stem, and roots of I. carnea. Different letters indicate significant difference between parts ($p < 0.05$).*

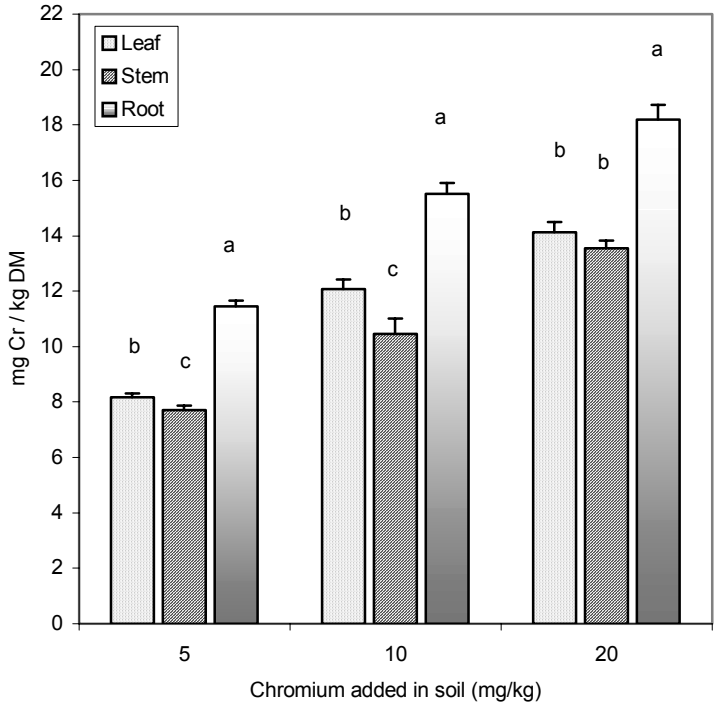


***Figure 1e.*** *Chromium concentration (mg kg$^{-1}$ of dry matter) in leaves, stem, and roots of P. karka. Different letters indicate significant difference between parts ($p < 0.05$).*

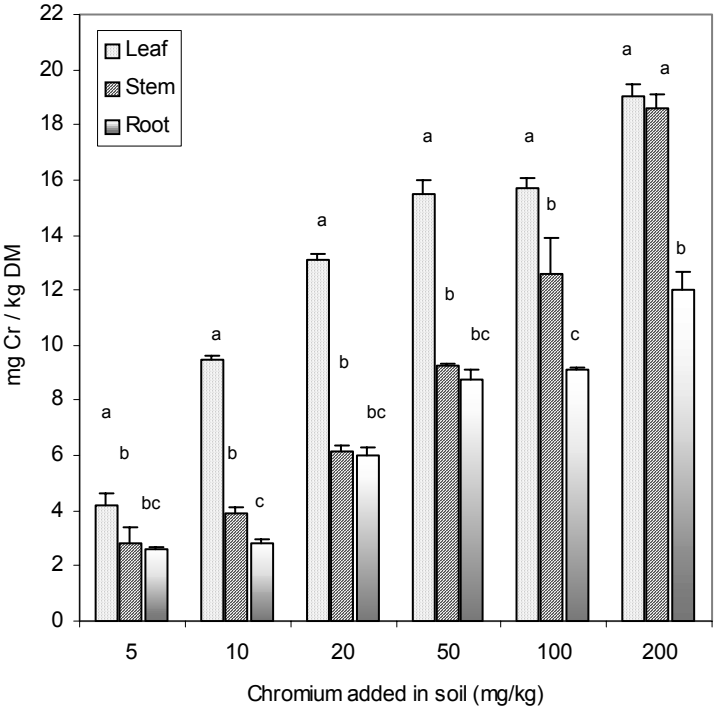



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***Figure 1f.*** *Chromium concentration (mg kg$^{-1}$ of dry matter) in leaves, stem, and roots of Cassia tora. Different letters indicate significant difference between parts ($p < 0.05$).*

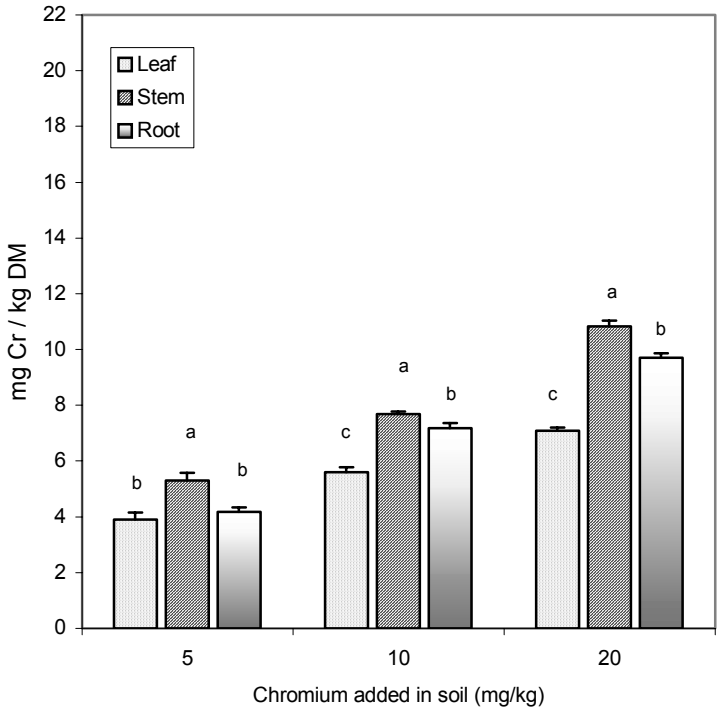
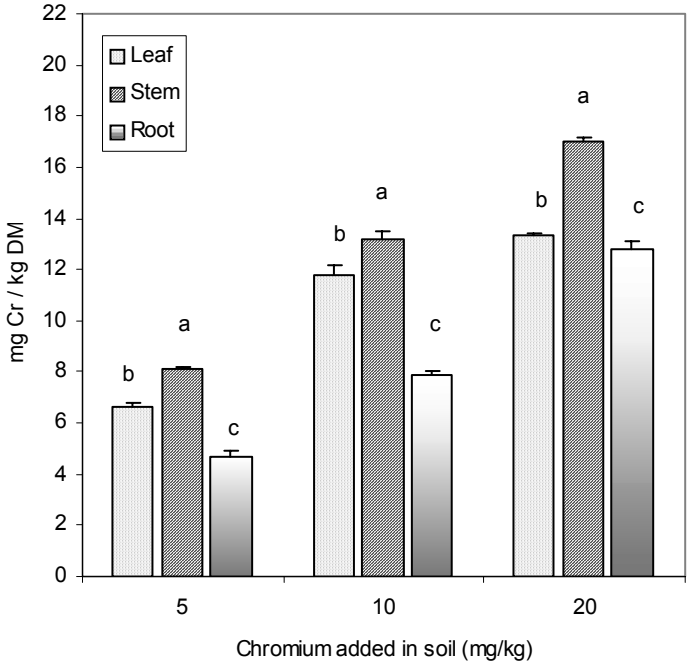



***Figure 1g.*** *Chromium concentration (mg kg$^{-1}$ of dry matter) in leaves, stem, and roots of Lantana camara. Different letters indicate significant difference between parts ($p < 0.05$).*





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Transportation index ($T_i$) - Plants must have the ability to translocate Cr from the root to the shoot, or to compartmentalize it, in order to continue absorption of Cr from the substrate. Better translocation is advantageous to phytoextraction; (i) it can reduce Cr concentration and thus reduce toxicity potential to the root, and (ii) translocation to the shoot is one of the mechanisms of resistance to high Cr concentration. *P.karka* showed maximum translocation but this was due to lower Cr accumulation in roots. Transportation index ($T_i$) was least and accumulation was lower in *I.carnea* but it had maximum shoot extraction. All the plants showed a general trend of fall in $T_i$ with increase in chromium concentration, thus showing inhibition of it translocation in plant body.

Bioconcentration factor (BCF) of Cr increased with increase in time, this means Cr accumulated up to maturity, as shown in Table 2. BCF is a useful comparison of metal accumulation power of different plants at same background metal concentration. It is bound to get reduced with increase in metal conc. in soil, but the trend in change of BCF at various intervals shows that the plant is extracting metal or not. In our study the value increased with increase in time.

***Table 2.*** *Bioconcentration Factor (BCF) of the studied plant species at different intervals.*

| Plant Species | Total Cr added in Soil (mg kg $^{-1}$) | 15 Days | 30 Days | 60 Days | 90 Days |
|---|---|---|---|---|---|
| *Brassica campestris* | 5 | 0.30 | 0.54 | 1.4 | 1.75 |
| | 10 | 0.33 | 0.41 | 0.87 | 1.07 |
| | 20 | 0.30 | 0.32 | 0.57 | 0.75 |
| *Brassica juncea* | 5 | 0.83 | 1.13 | 1.44 | 1.71 |
| | 10 | 0.53 | 1.03 | 1.11 | 1.20 |
| | 20 | 0.41 | 0.62 | 0.62 | 0.72 |
| *Dhatura innoxia* | 5 | 0.72 | 1.12 | 1.51 | 1.58 |
| | 10 | 0.88 | 1.01 | 1.15 | 1.12 |
| | 20 | 0.49 | 0.63 | 0.69 | 0.81 |
| *Ipomoea carnea* | 5 | 0.79 | 1.17 | 1.42 | 2 |
| | 10 | 0.56 | 0.72 | 1.18 | 1.21 |
| | 20 | 0.38 | 0.54 | 0.68 | 0.75 |
| *Phragmytes karka* | 5 | 0.48 | 0.65 | 0.77 | 0.66 |
| | 10 | 0.58 | 0.78 | 0.85 | 0.58 |
| | 20 | 0.41 | 0.72 | 0.63 | 0.416 |
| | 50 | 0.22 | 0.30 | 0.28 | 0.248 |
| | 100 | 0.12 | 0.16 | 0.15 | 0.136 |
| | 200 | 0.06 | 0.08 | 0.09 | 0.089 |
| | 5 | 0.37 | 0.67 | 0.77 | 1.145 |


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*Table 2. continued from previous page*

| Plant Species | Total Cr added in Soil (mg kg $^{-1}$) | 15 Days | 30 Days | 60 Days | 90 Days |
|---|---|---|---|---|---|
| *Cassia tora* | 10 | 0.35 | 0.68 | 0.67 | 0.83 |
| | 20 | 0.34 | 0.72 | 0.55 | 0.501 |
| *Lantana camara* | 5 | 0.28 | 0.41 | 0.45 | 1.37 |
| | 10 | 0.30 | 0.53 | 0.67 | 1.163 |
| | 20 | 0.31 | 0.54 | 0.51 | 0.726 |

### *Extraction of Chromium*

Chromium extraction is amount of metal accumulated by the whole plant mass or plant part. At 20 mg Cr kg$^{-1}$ soil and maturity, maximum extraction of Cr (151 µ g plant$^{-1}$) was observed in *I.carnea* followed by *D.innoxia* (97 µ g plant$^{-1}$) at maturity. The order of Cr extraction at above Cr conc. was *I.carnea* > *D.innoxia* > *C.tora* > *P.karka* > *B.juncea* > *L.camara* > *B. campestris.* Table 3, shows the Cr average extraction by a single plant at different treatments and intervals. *I.carnea* extracted more than five (5.2) times that of *B.juncea*, this was mainly because the biomass was more than five (5.8) times its dry biomass. *B.campestris, D. innoxia, P. karka and I. carnea* extracted maximum amount of Cr at 20 mg Cr kg$^{-1}$ soil. P.karka was able to grow above this conc. but the Cr extraction potential showed a sharp drop.

***Table 3.*** *Chromium extraction (µ g plant$^{-1}$) by whole plant, at given Cr treatments (mg Cr kg$^{-1}$) soils at different intervals.*

| Plant Species | Total Cr added in Soil (mg kg $^{-1}$) | 15 Days | 30 Days | 60 Days | 90 Days |
|---|---|---|---|---|---|
| *Brassica campestris* | 5 | 0.3 | 0.8 | 6.9 | 16.2 |
| | 10 | 0.5 | 0.9 | 7.3 | 15.5 |
| | 20 | 0.9 | 1.3 | 8.8 | 20.6 |
| *Brassica juncea* | 5 | 1.2 | 2.2 | 11.9 | 23.0 |
| | 10 | 2.6 | 3.8 | 16.4 | 30.4 |
| | 20 | 2.4 | 3.1 | 14.8 | 28.1 |
| *Dhatura innoxia* | 5 | 0.3 | 3.3 | 22.1 | 85.4 |
| | 10 | 0.5 | 3.7 | 25.7 | 87.9 |
| | 20 | 0.4 | 2.1 | 17.3 | 97.5 |
| *Ipomoea carnea* | 5 | 0.9 | 4.0 | 30.9 | 128.9 |
| | 10 | 1.2 | 6.0 | 40.7 | 136.2 |
| | 20 | 1.0 | 5.0 | 9.0 | 151.6 |
| | 5 | 0.6 | 4.0 | 11.5 | 25.3 |


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*Table 3. continued from previous page*

| | | | | | |
|---|---|---|---|---|---|
| *Phragmytes karka* | 10 | 1.2 | 7.4 | 18.2 | 33.2 |
| | 20 | 1.8 | 8.4 | 18.5 | 63.6 |
| | 50 | 3.9 | 8.8 | 12.2 | 18.9 |
| | 100 | 3.6 | 9.0 | 11.8 | 14.5 |
| | 200 | 3.6 | 5.8 | 12.0 | 14.1 |
| *Cassia tora* | 5 | 0.3 | 2.4 | 11.3 | 15.24 |
| | 10 | 0.5 | 3.1 | 13.2 | 18.41 |
| | 20 | 0.8 | 3.6 | 14.0 | 22.28 |
| *Lantana camara* | 5 | 0.1 | 1.6 | 6.8 | 15.59 |
| | 10 | 0.3 | 2.9 | 8.1 | 22.53 |
| | 20 | 0.4 | 3.6 | 10.4 | 25.56 |

In *B. juncea* and *P.karka* the extraction decreased with increase in Cr concentration. Relative percentage extraction in case of *B.campestris, B.juncea, D.innoxia* and *P.karka* was more by the foliage and in *I.carnea* the extraction was more by stem. The distribution of Cr between root and shoot in accumulator plants, however, indicated that the leaves also contained a much higher Cr concentration than that of non-hyperaccumulator plants, suggesting better translocation of Cr from root to shoot for hyperaccumulator plants.

## Conclusion

This study provides a promising start for biomass-based phytoextraction as it includes high biomass producing species, growing these species is practically easier than accumulators. The accumulator *Brassica species* infested by army moth, and pesticide was applied to save the plants, but for better results the both the *Brassica species* had to be replanted. Whereas weeds required no care to grow, this is a practical problem that will require care in field applications. Next research should focus on identifying the proper usage of the biomass produced, as it can be used as a source of energy. The results indicate that plant species differ significantly in Cr uptake capacity and distribution within the plant. *I.carnea* was much more effective than *B.juncea* for phytoextraction of Cr contaminated soils. This was due to two factors, (i) It was able to tolerate/ accumulate equivalent levels of Cr in soil. (ii) Though shoot/root ratio of Cr was much lower in *I.carnea*, its shoot biomass was much higher; a great proportion of total metal in plant is therefore sequestered in harvestable tissue. In the soil having more than 20 mg Cr $kg^{-1}$ soil, only *P.karka* showed the potential for phytoextraction.

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# EFFECT OF FARMING PRACTICES ON WETLANDS OF KISII DISTRICT, KENYA

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**Abstract.** Effect of farming practices on wetlands in Kisii District was determined through assessment of farmers' environmental awareness. Effective conservation of wetlands in the district cannot depend on prohibitions but should be based on users' knowledge and attitudes of wetlands. The present study examined farmers' knowledge of the environmental effect of agricultural expansion to wetlands; absence of knowledge of characteristics of farming activities and the attitudes of farmers with respect to planning mechanisms that might be used to support wetland protection in the area. The majority of farmers ignored the effect of agriculture on wetlands. Those who occupied wetland areas practiced intensive agriculture and were ignorant of the effect of this on water quality, soil and landscape. The government should implement training programmes for all wetland users in Kisii District to make them more environmentally aware of the impacts of farming practices on wetlands. This is meant to make them become more environmentally aware of the effects of farming practices on these ecosystems and eventually change their behavior. There is a need to build a conservation ethic among wetland users by educating them to sustainably utilize wetland resources and training them to practise sustainable agriculture.
**Keywords.** *farming, wetlands, agriculture, environmental awareness, attitudes*

## Introduction

Agricultural modernization has been taking place in Kenya over the last 40 years and intensified the agricultural production in the fertile farming areas. This process, while bringing production to record levels, has caused severe effects on the environment. Supporters of environmentally compatible agriculture emphasize the need to preserve the ecological balance of soil and water, secure food safety and maintain the health and quality of life for rural people and their communities.

Wetlands are fragile and valuable ecosystems supporting a diversity of species and habitats. They require environmentally compatible agricultural practice. Wetland degradation globally and appreciation of their ecological, economic and social values have generated a response from international and regional communities. In Kenya, drainage and conversion to arable cropping continue to degrade wetlands. By 1990, when Kenya ratified the Ramsar convention, most of the country's wetlands had been degraded. Drainage, land reclamation, overgrazing, eutrophication of inland waters caused by agricultural pollution are among the impacts of agriculture on Kenya's wetlands.

Responsibility for most of the above effects lies on the users of these areas, the farmers. These people, living in settlements close to the wetlands and earning their living from their resources remain alienated from the conservation policies, ignorant of the implications of their practices and uninformed of the new messages or the long-term benefits they could achieve. Even the few conservation projects in Kenya have not been adequately disseminated to the public.


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Current farming practices in Kisii district and the impact on wetlands depends to a great extent on the level of environmental awareness, knowledge and attitudes of farmers. In Kenya, decisions critical for the future of wetlands are taken with no concern of farmers' knowledge of the harmful effects of their practices and the ways they can overcome them. Furthermore, farmers' involvement in the formulation of environmental policies or implementation of management plans for wetlands has been totally ignored by the government. It has been assumed that rural people do not understand the issues relating to resource conservation and cannot, therefore be entrusted with this responsibility.

Conserving wetlands, while at the same time maintaining the agricultural resource base in Kisii district by practicing environmentally compatible farming, is a necessity. For this to be realized, it is important that conservation policy makers and agricultural officers should have sufficient information on farmers awareness of environmental issues, improve it if possible and make farmers in the district the key focus of a future wetland conservation programme.

Although wetlands have been widely studied in Kenya recently, quantitative analysis of the effect of agriculture on wetlands in the district is limited. Moreover, research on the relationship between farmers and wetlands is non-existent in Kisii district.

Wetlands commonly occur in human-dominated landscapes [11, 21]. Studies have shown that negative effects on wetland species and ecosystems functioning are due to human activities [10]. Farmers who own vulnerable ecosystems have a strong utilitarian attitude to the environment [31, 32]. Knowledge of the relationship between farming practices and wetland conservation is central to the development of sustainable farming and the formulation and implementation of effective management measures to conserve wetland resources in Kisii district.

The present study examined farmers' knowledge on the effect of agriculture practiced on wetlands in Kisii district; the attitude of farmers with respect to planning mechanisms that might be used to support wetland protection in the area and the need to build a conservation ethic among wetland farmers.

## Review of literature

The most commonly known wetlands are open coasts, flood plains, fresh water swamps, lakes, peat lands and swamp forests. Each one of these has a wide range of different wetland types. Irrespective of their types, sizes or locations, however, wetlands are of great and matchless ecological and socio-economic value. This is, for instance, true in the case of the valley bottom freshwater marshes which are central to this study. Wetlands play a key role in pollution assimilation and flood control, serve as breeding and nursery grounds for many species of fish and wildlife, and help maintain ground water supplies and water quality [8, 16, 24]. Africa has over 520 000 square kilometres of large standing water bodies and the possibility of sustainable development is vast, providing a reliable and profitable asset. So accurate delineation of wetlands where they occur is of great importance [13].

Wetlands commonly occur in human-dominated landscapes such as agricultural and urban regions [11, 21]. Studies have shown that negative effect on wetland species and ecosystem functioning can be expected in such areas due to human activities [1, 10, 11, 17, 18, 20]. A strong 'utilitarian' attitude to the environment has been found among farmers owning vulnerable ecosystems compared to other populations [31, 32]. Thus,


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assessment of the ecological status of wetlands in human-dominated landscapes is critical for their effective management and protection.

The values of wetlands are well documented [15], but the implications of their cumulative losses on national, regional and continental scales are not clearly understood. The following review indicates the kinds of impacts that could occur if we lose our wetland ecosystems. Wetlands are home to many plants and animals due to their temporal and spatial variability. They are rich in endemic, rare and endangered species. For example, more than half of Europe's most endangered birds depend on wetlands [6]. In Belgium, 97% of the 306 plants classified as rare, vulnerable, endangered or already extinct are wetland species. Such information is missing in our country and this study comes up with such statistics of the study area to help emphasis the need for conservation of wetlands.

One of the main ways in which mankind has been using the valley bottom wetland or fresh water marsh is cultivation. The importance of such wetlands in this regard lies mainly in their remarkably higher productivity compared to most upslope areas. In fact, as empirical findings indicate, some wetlands can produce eight times as much plant matter as an average wheat field. The cultivation of wetlands, which as a rule calls for some degree of drainage, however, can lead to their rapid degradation and loss of perennial supply of water unless it is done wisely. One of the well known consequences of the unwise cultivation of wetlands is not only the loss of the wetlands themselves but also the fast decline in the fertility of the soils. Wetland soils are formed under special chemical conditions of a waterlogged environment and tend to turn acidic under drained conditions. Thus, it is quite common for drained or severely degraded wetlands to become unsuitable for crop production or even for grazing. Drainage and other forms of disturbance associated with agriculture are widely identified as the main contributor to wetland loss. Williams [33] has suggested that globally, 160 600 $km^2$ of wetlands had been drained by 1995, primarily for agriculture and food production. For instance, it has been, estimated that about 90% of New Zealand's former wetlands have been absorbed by arable, pastoral and horticultural developments [22]. Wetlands are important elements in the global cycles of nitrogen and sulphur [9]. Inevitably therefore, the continuing loss of wetlands through drainage must have significant impacts which repercussions at present are not clearly understood.

Research on the relationships between farmers and wetlands is nearly non-existent in Kenya and rather limited internationally [32]. Similarly, quantitative analysis of the impact of agriculture on wetlands is limited [5] due to insufficient environmental monitoring.

Increased awareness about the adverse environmental and socio-economic consequences of the unwise exploitation of wetlands has resulted in worldwide calls for the sustainable management of fragile resources. However, the sustainable use of wetland resources has increasingly proven to be an extremely difficult and frustrating task in many developing countries. A substantial amount of literature that has appeared on this issue tends to carry the undertone that poverty is one of the major factors that make it very difficult to achieve the sustainable use of wetlands. Some writers boldly point out that the modern notions of natural resource conservation are simply at odds with the survival strategies of the poor of the Third World that are dependent on wetland resources.

There has been a growth of interest in wetlands and an accompanying change of attitude [33]. In some countries, rates of loss are now slowing [14]. At international


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level, the protection of wetlands is clearly reflected in the Ramsar convention. This convention plays an important role in facilitating the protection of wetlands of international significance. However, the full protection of the remaining wetlands in Kenya and in all other countries can only be achieved through implementation of management strategies at national or sub-national levels. This conclusion follows simply because most of the remnant wetlands do not qualify under the terms of the Ramsar convention, which is aimed at protecting wetlands of international significance.

Most authors contend that poverty contributes to wetland degradation in two closely related ways. First, they point out the fact that an intense competition exists between different categories of wetland users whose livelihood rests largely on their access to these resources. The lack of other means of survival makes the competition between them so uncompromising that they fail to reach a consensus on the sustainable exploitation of wetland resources. This, they say, is true in the case of the conflicts that often arise between upstream farmers who want more flooding, downstream farmers who want less, fishermen who are interested in early flooding and herdsmen who want greater access to wetland grazing. Secondly, they argue that the people who tend to exploit wetland resources in developing countries are in such a desperate economical situation that they cannot afford to use such resources judiciously. The main argument here is that since the poor „live within biomass-based subsistence economy"; their interests for short-time gains by far outweigh their willingness to treat wetlands caringly in anticipation of the long-term returns.

Although the literature tends to portray the poor as the primary users and abusers of wetlands in developing countries, there are sufficient indications that the poor may not always have access to these resources. For instance, some communities are known to have traditional resource management arrangements that regulate the ways in which and the extent to which wetlands could be exploited. Wetlands could either remain in near pristine conditions or the people may use them in an appreciably sustainable way where such resource management arrangements are strong enough to check their destructive exploitation by economically desperate and uncaring individuals. This is not to say that exploitation of wetlands by wealthier members of a community is necessarily sustainable. This simply means that such regulatory controls could significantly lessen the devastating pressures to which a substantially large and predominantly poor community could subject these fragile resources if they were open to unlimited access. To some extent, this appears to be true in the case of the wetlands of Kisii District of Western Kenya, which are presently being threatened by expansions of settlement and cultivation, brick making, urbanization and so forth.

Wetland losses are not easily reversible thus protection and conservation of the remaining ones is of paramount importance. There has been some progress toward protection of wetlands, but the pace has been slow. In the 1960's, the International Biological Programme (IBP) initiated project AQUA and the IUCN began project MAR. These were designed to increase protection of wetlands and to increase awareness of the importance of wetland and peatland ecosystems and the threats to which they were exposed [15]. In 1975 the Ramsar convention came into force and it was one of the world's first international conservation treaties with 45 signatory states, of which Kenya is one of them. Reports in 1987 suggested that the convention was short of funds, was breached by some of the signatories and did not include some crucial nations [25]. In practise, signatories also apparently tend to ignore the terms to which they are signatory a case the proposed study hopes to look into. There is also lack of an


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adequate database on wetlands. With inadequate information on extent, structure and function of our wetlands effective management is severely hindered. Despite much effort by IUCN to establish a database on wetlands [27] databases covering areas such as Kisii District are missing.

***Concepts of the study***

The population-environment debate during the 1980s incorporated a new dimension, the concept of sustainable development (WCED, 1987). The most important component of sustainable development is the protection of the environment. The carrying capacity of the earth is already under stress because of the expansion of human population.

According to Andrew [2], settlement pressure, wetland reclamation and destruction of forests combined with high rates of population growth and rapidly dwindling reserves of land for development of new agricultural areas, are all typical problems that face many developing countries.

Fog & Lampio [12] observed, „few of the world's major habitat type have suffered as drastically from man's abuses of the environment as wetlands". As evidence of this abuse, a recent report suggests that wetlands have been disappearing at a rate of about 1050 square kilometers per year in Kenya. At this rate wetlands have become one of Kenya's rarest ecosystems.

As Burns stated: „Individually, the loss or modification of small wetlands or corners of large wetlands may not seem important to developers keen to meet the challenge of bringing more land into use for agriculture, horticulture, housing or roads. The effects of these losses and modifications are cumulative and insidious, however." According to Williams [33] and Ehrenfeld & Schneider [11] drainage and other disturbances associated with agriculture are the main contributors to wetland loss and modification.

***Study area***

With a view to develop the knowledge of wetland users in Kenya about wetlands and their use, a survey of rural landholders in one district was carried out. Because the questionnaire addressed issues relating to planning mechanisms and approaches, the decision was taken to interview landholders within a single, and in this case a whole district planning unit. The aim was to standardize the survey in terms of the institutional context. The area that was eventually chosen was Kisii District, which is found in Nyanza Province, Kenya (*Fig. 1*)

This area lies between 0°30'–0°58'S and 34°42'–35°05'E. It occupies an area of 645 $km^2$ land and it is subdivided into five administrative divisions.

The district is predominantly rural and agricultural. About 77% of the land is sustainable for agriculture. The once abundant wetlands in the district now there are only remnants left. The extensive draining of wetlands for agricultural and settlement purposes accounts for most of the losses. The remnants contribute to the natural heritage of the district.

## Materials and methods

Data were collected in 2004 by interviewing 100 farmers from Keumbu division, 45 from Marani division, 50 from Suneka, 45 from Masaba division and 50 from Mosocho division.


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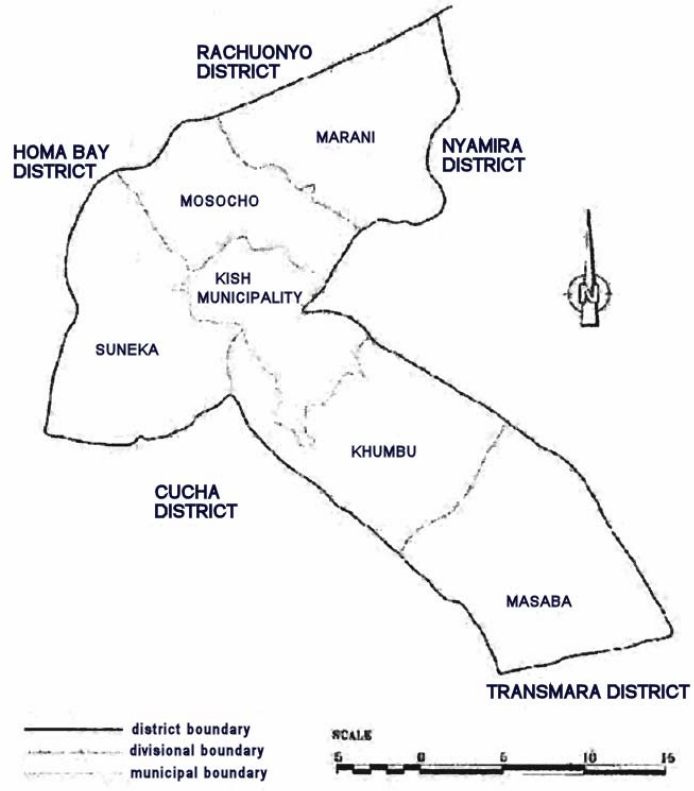


***Figure 1.*** *Map of Kisii District*

Information was collected on various demographic groups of farmers and their immediate family, characteristics of the farming practices, attitudes towards the wetlands, farming and the environment, knowledge of the effect of agriculture on the local wetlands. Environmental questions were dealt focused on local rural environment of the respondents.

A 9-grade scale was used to assess the level of knowledge respondents had about the environmental effect of agriculture. Each farmer was asked to say whether agriculture may impact on the local environment (water, soil, climatic conditions, wildlife and landscape beauty) and whether several farming practices such as application of chemical fertilizers, pesticides, land consolidation and drainage have any consequences on the environment. The knowledge score was computed by summing the response scores of the component items, using the following integral values: correct – 3, do not know – 2, and wrong – 1.

Correlation analysis was used to evaluate the ability of the individual questions to ascertain the attribute measured by the total scale (Likert scale). A high Cronbach coefficient (alpha) of 0.810 resulted indicating high validity of the test. Three items with low correlation coefficients were dropped from the analysis. Responses on the remaining eight items were subjected to factor analysis.

Statistical analysis was carried out with the help of SPSS (Statistical Package for the Social Sciences). Student's t-test for unpaired samples and one-way analysis of variance was used. The median value of knowledge on the environmental effect of agriculture, was used to the five samples into two groups: farmers who scored below the median (ignorant) and those who scored above the median (conscious).


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## Results

### *Characteristics of the landholdings*

The landholdings in the district ranged from 0.5 to 1.5 hectares. The respondents owned almost all of the land. Each farmer owned about 12 hectares of the wetland. Wetlands occupied 5% of the landholdings. Approximately 400 hectares (7%) of the land were covered by wetlands.

Farming was the primary land use and 80% of the respondents practised dairying and goat farming, 60% obtained all of their household income from land and 10% obtained more than 90% of their total income from farming. Only 5% of the farmers were found in the rural areas of the district

The average age of farmers was 40.5 years. They all had farming experience of 20 years and over two-thirds had more than 10 years of schooling. All the respondents said although they were cultivating wetlands farming was not a profitable business. Agriculture largely depends on family labour. Livestock such as cows and goats kept and grazed in the wetlands were reported to be bigger than those grazed elsewhere.

### *Wetlands values in Kisii District*

More than 60% of the respondents rated environmental issues as being important or very important (1 or 2 on a 4-point Likert scale)

In a question that went more directly to the subject of wetlands, the landowners were asked to rate (on a 4-point scale) several sources of value that might be recognized in wetlands (*Fig. 2*). The four values considered by the greatest number of respondents to be very important were the role of wetlands in maintaining water quality (rated as important by 90% of respondent). And as a habitat for species of socio-economic and cultural significance, 91% of the respondents recognized wetlands as offering habitat.

A chi-square contingency test revealed statistical relationships between several landholding and wetland values by the sample group. The proportion of income obtained from the landholding shows the value placed on wetlands as an area for grazing stock or brick making. For example of landowners obtaining 95% or more of their income from the landholding, 74% considered it to be important, while 33% landowners who earned less than 95% of their income from the landholdings considered it to be important. The proportion of income and the scenic value of wetlands were also important e.g. those earning less than 95% of their income from the landholdings, 87% considered the scenic value of importance, while only 50% of those earning 95% or more reported that it was important.

The proportion of income earned from the farm was linked to attitudes towards the importance and use of wetlands in Kisii district. These findings are consistent with other studies that established the relationships between affluence and the level of dependence upon the land, conservation attitude and behavior.

It was anticipated that the uses of wetlands for recreation contribute to positive attitude towards wetlands. In this study investigation of attitudes towards wetlands, recreation by the local community was defined to include hunting, bird watching, walking, fishing and any other leisure activity. Landowners (70%) in Kisii district who carried out recreational activities in wetlands considered them to be important for grazing. Of those landowners that did not use their wetland areas for recreation only 44% suggested that grazing was a valuable wetland activity. This result shows that landowners using the wetlands for recreational activities hold a more utilitarian view.


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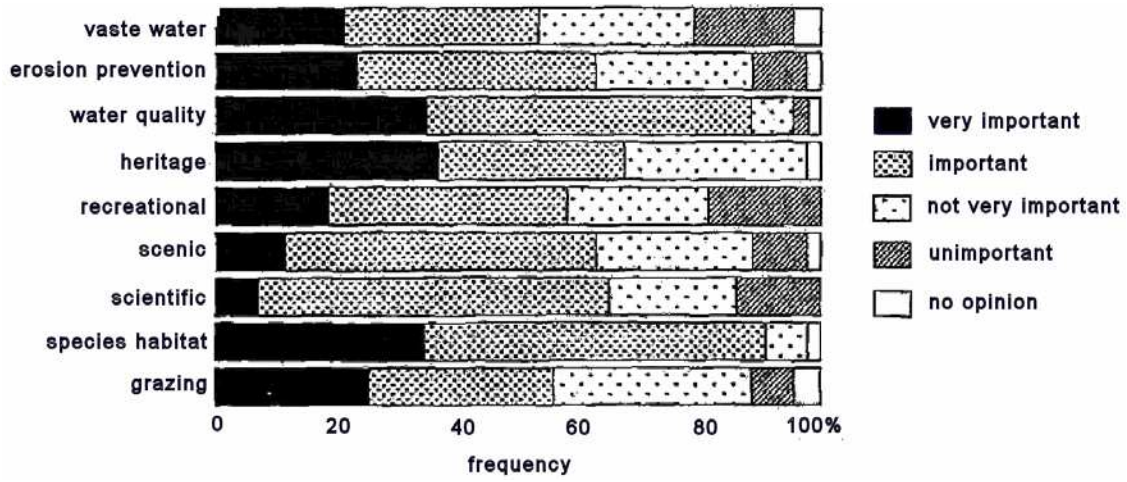


***Figure 2.*** *The values assigned to wetlands by landowners in Kisii District*

The drainage of wetlands represents one of the most significant and widespread threats to their effective preservation. Amongst the landowners surveyed in Kisii district, drainage of wetlands on the landholding had been carried out by 70% of the landowners. Drainage had been carried out for reasons relating to access, planting of exotic trees species, and reducing loss of stock, increased pasture and productivity, and the "conversion of wasteland". Of those landowners that had carried out drainage, only two had sought consent, as they are required to do by the Kisii district municipal council. In fact, the general awareness of the consent process was very low, only 31% of the respondents knew that consent was required for drainage works or other modification to wetlands. Another 60% affirmed that consent was not necessary, and 10% admitted they did not know whether it was necessary or not.

### *Levels of environmental awareness*

The level of awareness of the environmental effect of agriculture measured by the scale of 'knowledge' was lowest among farmers in the wetlands, followed by farmers away from the wetland areas (*Table 1*).

Investigating particular aspects of ignorance it was found that among 8 aspects presented, farmers in wetlands scored higher in six of them, while farmers not occupying wetlands scored higher in three providing a piece of evidence of the higher levels of ignorance among wetland farmers. Five of these differences (water quality, soil quality, wildlife, pesticide and chemical fertilizers) were statistically significant, indicating the magnitude of ignorance on the part of wetland farmers (*Table 2*).

Farmers occupying areas away from wetlands and scored higher on the knowledge scale were younger, less experienced in farming, had more formal education, practiced part-time farming, derived less income from farming and had more income from off-farm activities. Further it was discovered that farmers with mixed farms had significantly less knowledge about environmental effect of high input agriculture than crop farms.

## Conclusions and recommendations

The analysis has revealed that a large number of farmers in the wetland areas do not take the environmental effect of agriculture into account. Ignorance by farmers of wetland values and functions and of the magnitude of their effect on wetlands becomes


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***Table 1:*** *Level of knowledge of the environmental effect of agriculture among farmers of Kisii District based on Likert scale. N represents the values of responses*

| scale | farmers in the wetlands ($N$ = 100) | farmers away from the wetlands ($N$ = 140) |
|---|---|---|
| agriculture and environment | 20.4±0.4 | 22.9±0.3 |
| chemical usage | 8.2±0.1 | 8.9±0.1 |

***Table 2.:*** *Ignorance by farmers on the effect of farming activities in the wetlands of Kisii District based on Likert Scale*

| | farmers in wetlands | farmers away from wetlands |
|---|---|---|
| chemical fertilizers (%) | 45.5±0.08 | 34.2±0.04 |
| pesticide application (%) | 22.7±0.03 | 16.6±0.03 |
| land consolidation (%) | 96.6±0.01 | 96.2±0.01 |
| drainage (%) | 82.6±0.06 | 90.1±0.03 |
| water quality (%) | 65.9±0.04 | 43.7±0.04 |
| soil quality (%) | 53.0±0.04 | 54.0±004 |
| wildlife (%) | 68.4±0.07 | 32.0±0.04 |
| landscape (%) | 78.6±0.05 | 44.8±0.04 |

a major contributor to their degradation. Wetland farmers practiced a more intensive form of agriculture and were rated higher in their level of ignorance concerning the environmental effect of agriculture than farmers away from wetlands.

Both aspects (intensity of agriculture and ignorance) have to be seriously considered in any management plan to conserve the vulnerable resources of wetlands.

Farmers in wetlands have stronger utilitarian attitudes toward wetland resources than their counterparts away from wetland areas. Therefore, more effort should be placed in changing patterns of agricultural practices to a more sustainable manner. The revival of sustainable farming practices of the past, through policy measures securing income and providing proper training, would be more effective for the long-term viability of wetlands.

The knowledge gap concerning the environmental effect of modern agriculture among wetland farmers, along with the effect of training programmes, calls for immediate action to implement education / training programmes specifically designed to address the educational needs of wetland farmers in Kisii District. Providing farmers with relevant information and education regarding the environmental effects of agriculture and the effects of their practices upon the environment and resources must be placed higher in the conservation agenda. Farmers attending these training courses would be expected to become more environmentally knowledgeable and eventually change their behaviour in a responsible way. The role of public administration must also change from that of an implementing agency for specific conservation programmes to that of a facilitator, promoting, encouraging, guiding and making possible larger participation of rural people in developing and applying more sustainable forms of land use. Finally, development of off-farm employment opportunities, like eco-tourism and the introduction of permanent payments linked to agricultural practices that are compatible with the extensification of the production process (e.g. conversion to organic farming) are among the measures that can bring more sustainable use of agricultural resources in these wetlands.


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The overall outcome of this study is the need to build a conservation ethic among farmers, particularly in environmentally sensitive areas. This can only be achieved by:

1. educating farmers on the significance of conserving natural resources – especially those living in wetlands;
2. training them to practise sustainable forms of agriculture;
3. rewarding the most environmentally friendly agriculture.

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# VALIDATING SPECIES DIVERSITY OF BENTHIC ORGANISMS TO TRACE METAL POLLUTION IN KUWAIT BAY, OFF THE ARABIAN GULF

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**Abstract.** Benthic organisms diversity were observed in the sequence of Annelida > Mollusca > Crustacea > "Diversa" group. Levels of trace metals in benthic organisms were in the range 0.12–96.86 μg/g during winter and 0.98–54.13 μg/g in summer. Species diversity index (*H*'), evenness index (*J*) and index of dominance ($\lambda$) were in the range 0.951–1.368 bits/unit, 0.475–0.684, and 0.19–0.33 respectively, for benthic organisms sampled in Kuwait Bay sites. Evenness index (–) was found to increase with increasing *H*'. Seasonally, an inverse correlation was observed between species richness (*R*1 and *R*2). Comparative studies revealed low diversity indices correspondingly to the increase in trace metal level in benthic species collected from four sites except Doha wherein high abundance of certain benthic species and high trace metal levels due to manmade perturbations were observed altering the diversity indices. Furthermore, these indices will validate benthic organisms as an indicator to trace metal pollution in Kuwait marine ecosystem.
**Keywords.** *benthic organisms; trace metals; species diversity*

## Introduction

Rapid industrialization and recent 'Fish Kill' aftermath of the Gulf War I and II, has been causing concern over the stressed ecosystem in Kuwait Bay. Investigations on the harmful algal blooms (HABs), metal levels in marine organisms, bacteriological and toxicological studies and organic and inorganic pollutants supported evidences to marine pollution in this Bay [3–5, 9, 18, 23, 29, 31]. Several investigators observed metal concentrations in aquatic macro benthic organisms to be a better indicator than ambient (water) metal concentrations [2, 7, 11, 12]. Studies revealed oligochaetes as an indicator to organic and industrial pollution [6, 22]. They suggested that if benthos occurs in a density of 100–999 individuals/$m^2$, the water is unpolluted; 1000–5000 individuals/$m^2$, moderately polluted; and more than 5000 individuals/$m^2$ shows heavy pollution. Earlier observations showed benthic organisms to be highly sensitive to environmental stress due to trace metal pollution [13, 24, 25, 30]. They also noted anthropogenic pollution influenced by sedentary benthic organisms. In contrast to these findings, few investigators [15, 19, 26] observed low pollution levels coinciding with some oligochaetes and polychaetes densities in some geographical sites. However, in an overall view, they found the macro benthos to be an indicator of pollution. Meanwhile, studies on the concept of species diversity in community ecology intensified by ecologists over the past few decades. Observations revealed various diversity indices which responded differently to different environmental factors of biotic communities [21]. A combined index, which characterizes species abundance and evenness simultaneously, limits the dependent factors of sample size. Further, [14] observed species diversity indices are justifiable when subjected to comparative analysis. In Kuwait, no published data relating to the


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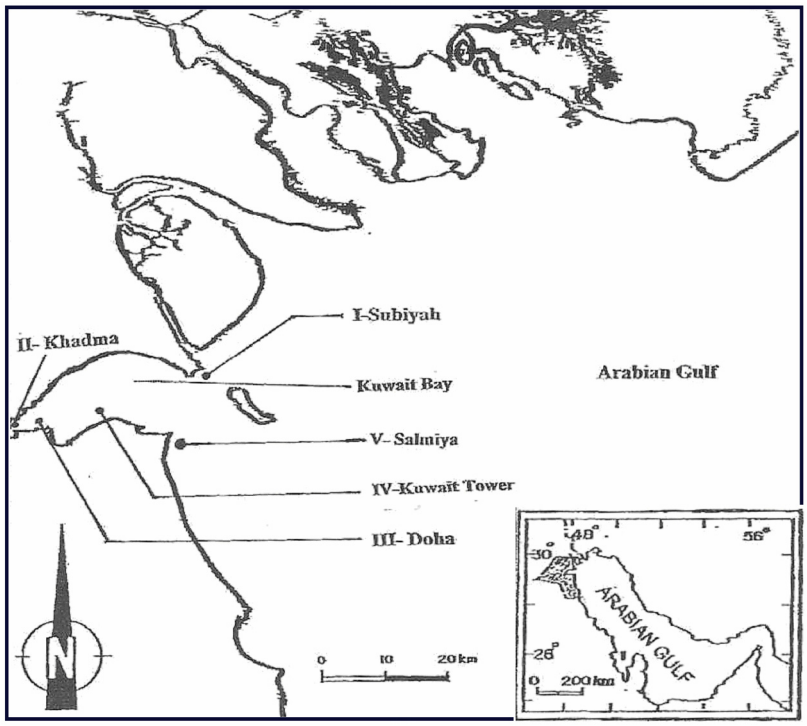


***Figure 1.*** *Map indicating the sampling sites*

species diversity of benthic organisms exist. Therefore, investigations were carried out to: (1) determine the abundance of benthic organisms in five sites of Kuwait Bay; (2) compare predominant trace metal levels in the major and minor benthic organisms (annelids, crustaceans, molluscs and grouped "Diversa" respectively) in Kuwait Bay sites; and (3) validate the diversity of benthic species as indicators to trace metal pollution with seasonal changes (summer and winter) in the Kuwait Bay.

## Materials and methods

Employing a 'Van-Veen' grab with a mouth opening of 0.1 $m^{-2}$, benthic organisms were collected from five Kuwait Bay sites. Further, from each site, five transect samples were collected to minimize sampling errors (*Fig. 1*).

The samples were separated from the sediment using a 0.5 mm sieve [13]. Following the classification [1, 8, 20] benthic samples were rinsed, weighed, dried at 50 °C in an oven (Gallencamp II) and homogenized. Benthic mollusc and crustacean species were removed from their shells during preparation but other organisms were analyzed without modification. Unidentified encrusting algae were scraped from mollusc shells. Dried benthic samples weighing 2 g were pre-treated in 3 ml nitric acid (Aristar grade, v/v) and 1 ml HCl (Aristar grade, v/v) in a Fischer brand sterile centrifuge disposable tube (50 ml capacity). After 48 hours, the samples were diluted to 50 ml in double distilled de-ionized water, digested in an automatic microwave digester (Spectro Prep-CEM) and the metal levels (μg/g) measured by atomic absorption spectrophotometer (AAS, Perkin Elmer 5100). The accuracy of the method was verified using standard material, DORM-1 for benthic samples from the National Research Council of Canada and average recoveries (95%) of all the trace metals achieved.

Species richness was calculated using the known indices [16, 17] as given below:


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$$R1 = S - \frac{1}{\ln n}, \quad \text{(Eq. 1)}$$

$$R2 = S - \frac{S}{\sqrt{n}}, \quad \text{(Eq. 2)}$$

wherein, $R1$ and $R2$ represent the species richness indices. The utility of $R2$ holds well, when a functional relationship exists between $S$ and $n$. $S$ is the total number of species and $n$ is the total number of individuals. If the assumption fails, the richness index will change with sample size in an unknown manner. Thus, $R1$ and $R2$ combination was worked out to draw better conclusions by another index [28]:

$$\lambda' = \sum_{i=1} n_i \frac{(n_i - 1)}{n(n - 1)}. \quad \text{(Eq. 3)}$$

For a finite community, Simpson's index ($\lambda$) gives the probability of two individuals drawn randomly from a population belonging to the same species. However, in the present study, infinite population was found and it was impossible to count all members and hence, the Simpson's unbiased estimator ($\lambda$') was used. $n_i$ is the number of individual of the $i$th species.

Diversity index [27] is:

$$H' = \sum_{i=1}^{s} [(n_i/n)\ln(n_i/n)]. \quad \text{(Eq. 4)}$$

This index ($H$') is a measure of the average degree of 'uncertainty' in predicting to what species an individual chosen at random from a collection of $S$ species and $N$ individuals will belong to. Thus, $H' = 0$ if there is one species in the sample and $H'$ is maximum only when all $S$ species are represented by the same number of individuals, that is a perfectly even distribution of abundances.

Evenness index [21]:

$$J = H' / H'_{\max}. \quad \text{(Eq. 5)}$$

This index represents $H'$ relative to the maximum value that $H'$ can obtain when all the species in the samples are even with one individual per species. In the present study,

$$H'_{\max} = \log_2 S$$

was used. (Eq. 6)

## Results and discussion

The abundance of benthic samples incorporated the mean counts/m$^2$ from the five transects collected from each site, off the Kuwait Bay (*Tables 1* and *2*).

Observations showed that the annelids increased in density during summer than in winter in Kuwait Bay, which supports the earlier study [2]. The overall counts were lower in Mollusca, Crustacea and "Diversa" groups when compared to Annelida, during summer and the reverse during winter. This could be attributed to (1) the high trace metal levels and other pollutants resulting in mortality of the shelled organisms and "Diversa" groups during summer, and (2) the high tolerance to environmental stress by the annelids in the Bay and supports the earlier findings [18, 30].


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Abundance of benthic annelids, molluscs, crustaceans and „Diversa" group at different sites were noted in the sequences of Doha > Kuwait-Tower > Salmiya > Subiyah > Khadma during summer (*Table 3*) and Doha > Kuwait-Tower > Khadma > Salmiya > Subiyah during winter (*Table 4*).

The Kuwait Bay sites revealed the total abundance of more than 1000 pollution indicator benthic species / $m^2$ irrespective of seasonal variation. Therefore, the classification of 'moderately polluted areas' with individuals >1000 species / $m^2$ in Kuwait Bay sites showed evidences to studies [6, 22].

The present study five trace metals such as Cu, Zn, Fe, Ni and Pb were chosen, and observed: (1) within the detectable limits in AAS, (2) to cause significant impact on marine organisms and (3) in high levels in Kuwait Bay waters. In general, trace metal

***Table 1***. *Composition and relative abundance (% frequency) of benthic organisms during summer in Kuwait Bay*

| species | site | | | | |
|---|---|---|---|---|---|
| | **Subiyah** | **Khadma** | **Doha** | **K. Tower** | **Salmiya** |
| **benthic Annelida** | | | | | |
| *Eulalia viridis* | 9 | 8 | 10 | 9 | 8 |
| *Polydontes melanoleis* | 3 | — | 4 | 2 | 6 |
| *Syllis gracillis* | — | 2 | 3 | 3 | 2 |
| *Ceratonereis erythroensis* | 9 | 6 | 15 | 11 | 7 |
| *Nephys tulerensis* | 5 | 3 | 5 | 4 | 3 |
| *Glycera convolute* | 2 | 3 | 3 | 2 | 2 |
| *Eunice indica* | 8 | 9 | 6 | 9 | 7 |
| *Marphysa sanguinea* | 1 | 4 | 1 | 1 | 2 |
| *Janua kayi* | 3 | 5 | 1 | 7 | 4 |
| *Megalomma quadrioculatum* | — | 3 | 1 | — | 4 |
| total | 40 | 43 | 49 | 48 | 45 |
| **benthic Mollusca** | | | | | |
| *Tellidora pellyana* | 6 | 5 | 5 | 5 | 5 |
| *Solen vagina* | 5 | 4 | 6 | 4 | 4 |
| *Donax scalpellum* | 5 | 6 | 7 | 7 | 5 |
| *Dentalium octangulatum* | 3 | 6 | 5 | 5 | 5 |
| *Cerithium scabridum* | 3 | 8 | 8 | 9 | 8 |
| total | 22 | 29 | 31 | 30 | 27 |
| **benthic Crustacea** | | | | | |
| *Platyischnopus longimanus* | 1 | 1 | 2 | 1 | 1 |
| *Balanus tintinnabulum* | 6 | 13 | 13 | 10 | 8 |
| *Diogenes avarus* | 1 | 1 | 1 | 4 | 1 |
| *Alpheus djeddensis* | 1 | 1 | 2 | 2 | 1 |
| *Pagarus perspicax* | 1 | 1 | 1 | 1 | 1 |
| total | 10 | 17 | 19 | 18 | 12 |
| **"Diversa" group** | | | | | |
| *Oligochaeta* sp. | 23 | 8 | 1 | 3 | 12 |
| *Nematode* sp. | 2 | 2 | — | 1 | 1 |
| *Nemertina* sp. | 1 | — | — | — | 1 |
| *Spongia* sp. | 1 | 1 | — | — | 1 |
| *Turbellaria* sp. | 1 | — | — | — | 1 |
| total | 28 | 11 | 1 | 4 | 16 |

levels were in the sequence of Mollusca (0.1–96.86 μg/g) > Crustacea (0.98–28.12 μg/g) > Annelida (0.08–4.71 μg/g) > and "Diversa" group (0.15–4.06 μg/g) irrespective of the


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two seasons which supports an earlier investigation [4] in Kuwait Bay. Trace metal levels were observed in the sequence of Zn > Fe > Cu > Ni > Pb in annelids and„Diversa" but Fe > Zn > Cu > Ni > Pb in molluscs and crustaceans, irrespective of seasons. Among the five metals, Zn was predominant in the bodies of annelids. The above-mentioned metals could be transferred or assimilated in their bodies from the sediment when compared with the other metals [11]. Meanwhile, observation showed a sequential change in Fe over Zn levels in molluscs and crustaceans, nevertheless the other metals observed in the sequence to that of the annelids. Doha consistently had the high trace metals level (0.93–96.86 μg/g) in all the samples than the other Kuwait Bay sites. This attributes to (1) stagnant water in the Bay causing accumulation of metals in these organisms, and (2) the discharge of domestic and wastewater into the Bay which supported earlier observation [5]. The

***Table 2***. *Composition and relative abundance (% frequency) of benthic organisms during winter in Kuwait Bay*

| species | site | | | | |
|---|---|---|---|---|---|
| | **Subiyah** | **Khadma** | **Doha** | **K. Tower** | **Salmiya** |
| **benthic Annelida** | | | | | |
| *Eulalia viridis* | 1 | — | 2 | 3 | 2 |
| *Polydontes melanoleis* | 1 | — | — | 1 | — |
| *Syllis gracillis* | — | — | 1 | 1 | 1 |
| *Ceratonereis erythroensis* | 3 | 4 | 6 | 5 | 4 |
| *Nephys tulerensis* | — | 1 | — | 1 | — |
| *Glycera convolute* | 1 | 1 | — | — | 1 |
| *Eunice indica* | 1 | — | — | — | 1 |
| *Marphysa sanguinea* | — | 2 | 3 | 2 | — |
| *Janua kayi* | 1 | 1 | 2 | 1 | 2 |
| *Megalomma quadrioculatum* | 1 | 1 | 2 | 1 | 2 |
| total | 9 | 10 | 16 | 15 | 13 |
| **benthic Mollusca** | | | | | |
| *Tellidora pellyana* | 1 | 1 | 4 | 1 | 1 |
| *Solen vagina* | 2 | 4 | 4 | 3 | 2 |
| *Donax scalpellum* | 1 | 3 | 3 | 1 | 1 |
| *Dentalium octangulatum* | 2 | 5 | 4 | 5 | 1 |
| *Cerithium scabridum* | 8 | 4 | 5 | 8 | 10 |
| total | 14 | 17 | 20 | 18 | 15 |
| **benthic Crustacea** | | | | | |
| *Platyischnopus longimanus* | 6 | 5 | 7 | 8 | 5 |
| *Balanus tintinnabulum* | 7 | 11 | 12 | 9 | 7 |
| *Diogenes avarus* | 6 | 8 | 7 | 6 | 6 |
| *Alpheus djeddensis* | 3 | 2 | 3 | 4 | 4 |
| *Pagarus perspicax* | 1 | 2 | 3 | 3 | 3 |
| total | 23 | 28 | 32 | 30 | 25 |
| **"Diversa" group** | | | | | |
| *Oligochaeta* sp. | 6 | 7 | 13 | 12 | 8 |
| *Nematode* sp. | 11 | 12 | 8 | 5 | 8 |
| *Nemertina* sp. | 12 | 9 | 6 | 6 | 12 |
| *Spongia* sp. | 13 | 9 | 2 | 8 | 9 |
| *Turbellaria* sp. | 12 | 8 | 3 | 6 | 10 |
| total | 54 | 45 | 32 | 37 | 47 |

***Table 3***. *Benthic organism count ($\times 10^2$ /$m^2$) and diversity indices during summer in Kuwait Bay*

| species | site |
|---|---|


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| | Subiyah | Khadma | Doha | K. Tower | Salmiya |
|---|---|---|---|---|---|
| Annelida | 4 | 5 | 15 | 12 | 8 |
| Mollusca | 2 | 4 | 13 | 10 | 3 |
| Crustacea | 1 | 3 | 10 | 9 | 2 |
| "Diversa" | 8 | 2 | 1 | 1 | 3 |
| total | 15 | 14 | 39 | 32 | 16 |
| *R*1 | 1.107 | 1.136 | 0.818 | 0.865 | 1.082 |
| *R*2 | 1.032 | 1.069 | 0.640 | 0.707 | 1.000 |
| $\lambda$ | 0.333 | 0.236 | 0.307 | 0.296 | 0.291 |
| *H*' | 1.136 | 1.333 | 1.176 | 1.196 | 1.234 |
| *J* | 0.568 | 0.665 | 0.588 | 0.598 | 0.617 |

*R*1: Margalef's index; *R*2: Menhinick's index; $\lambda$: Simpson's index; *H*': Shannon-Weaver's index; *J*: Pielou's index

***Table 4***. *Benthic organism count ($\times 10^2$ /m$^2$) and diversity indices during winter in Kuwait Bay*

| species | site | | | | |
|---|---|---|---|---|---|
| | Subiyah | Khadma | Doha | K. Tower | Salmiya |
| Annelida | 2 | 3 | 9 | 5 | 3 |
| Mollusca | 1 | 2 | 6 | 3 | 2 |
| Crustacea | 1 | 2 | 4 | 3 | 2 |
| "Diversa" | 3 | 2 | 1 | 1 | 1 |
| total | 7 | 9 | 20 | 12 | 8 |
| *R*1 | 1.541 | 1.365 | 1.001 | 1.207 | 1.442 |
| *R*2 | 1.133 | 1.000 | 0.670 | 0.866 | 1.060 |
| $\lambda$ | 0.190 | 0.250 | 0.300 | 0.240 | 0.250 |
| *H*' | 0.951 | 1.368 | 1.192 | 1.265 | 1.320 |
| *J* | 0.568 | 0.665 | 0.588 | 0.598 | 0.617 |

*R*1: Margalef's index; *R*2: Menhinick's index; $\lambda$: Simpson's index; *H*': Shannon-Weaver's index; *J*: Pielou's index

overall analyses revealed trace metal levels at 0.12–96.86 µg/g in benthic organisms during winter than in summer (0.98–54.13 µg/g). The reasons may be due to the trace metals transfer from sediment to the benthic organisms and supports the earlier findings [2, 5]. Comparative studies in most of the observations (*Table 5*) revealed higher trace metal levels in benthic molluscs and annelids in Kuwait Bay than in the earlier observations from other countries [3, 10, 24, 26]. Publications were recorded on the „Diversa" group in the light of abundance [30], but no studies related to their trace metal levels.

Species diversity studies revealed (a) species richness (*R*1), (b) species richness (*R*2) to be the least but (c) Simpson index ($\lambda$) to be the highest in Doha when compared to the other sites during both seasons, (d) Shannon-Weaver's diversity index (*H*') between 0.951 and 1.368 bits/unit during both seasons and framed within the diversity of 2.000 bits/unit. In general, the species diversity indices were higher during winter than in summer. Quantitatively, benthic species were observed in high numbers in Doha site but, the *H*' and *J* indices were found moderate when compared to the other sites. This attributes to (1) the dominating mollusc and annelid species, and (2) those abundant species that could tolerate the metal polluted Doha site. Index (*J*) [21], revealed the highest value in Khadma with high *H*' but with low trace metal levels.


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***Table 5.*** *Comparative analyses on the mean trace metals levels in benthic organisms from different areas of the world*

| trace metals | benthic organisms | | | | location | references |
|---|---|---|---|---|---|---|
| (µg/g) | Annel. | Mollus. | Crusta. | "Div." | | |
| Cu | 3.14 | 51.01 | 5.21 | 2.18 | Kuwait Bay | present study |
| Zn | 4.71 | 68.01 | 22.93 | 4.06 | | |
| Fe | 4.38 | 96.86 | 28.12 | 3.13 | | |
| Ni | 1.01 | 1.27 | 2.48 | 0.90 | | |
| Pb | 0.98 | 1.21 | 2.05 | 0.87 | | |
| Cu | NS | 2.43 | 1.61 | NS | Taiwan | [10] |
| Cu | 4.13 | 2.16 | NS | NS | Cheaspeake Bay, U.S.A. | [24] |
| Cu | 2.40 | NS | NS | NS | Spain | [26] |
| Cu | 2.40 | NS | NS | NS | UK estuaries | [3] |
| Zn | 6.80 | NS | NS | NS | | |
| Fe | 4.92 | NS | NS | NS | | |
| Ni | 1.30 | NS | NS | NS | | |
| Pb | 0.30 | NS | NS | NS | | |
| Cu | 3.10 | 55.10 | 7.10 | NS | | |
| Zn | 25.00 | 82.50 | 35.12 | NS | | |
| Fe | NS | 148.12 | 45.80 | NS | | |
| Ni | NS | NS | 4.30 | NS | | |
| Pb | 2.50 | 1.20 | 2.30 | NS | | |

Annel. = Annelida; Mollus. = Mollusca; Crusta. = Crustacea; "Div." = "Diversa"; NS = not studied

## Conclusions

The present findings revealed the significance of trace metal levels in benthic organisms to the stressed ecosystem of Kuwait Bay. Observations showed relatively high (*H'*) species diversity index [27] in sites with low trace metal levels that justifies high or low abundance of benthic organisms, validating these benthic organisms as an indicator to metal pollution and enable environmentalists to take precautionary measures.

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# BIOLOGICAL ASPECTS OF MAIN MARINE MIGRATORY STURGEONS IN ROMANIAN DANUBE RIVER

MIGRATION OF FISHES IN ROMANIAN DANUBE RIVER, № 4

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**Abstract.** Marine migratory sturgeons form one of the most valuable fish population of the Lower Danube River ecosystem and are also extremely important for the economy of Romania and some other riverside countries and freshwater fisheries. Decreasing sturgeon presence in the last several decades has been a true concern either for commercial fishermen and biologists. This paper is trying to analyze some biological aspects of three sturgeon species: beluga (*Huso huso* Linnaeus, 1758), Russian sturgeon (*Acipenser guelden-staedti* Brand, 1833) and stellate sturgeon (*Acipenser stellatus*, Brandt, 1833) and to compare them with formerly registered data and scientific information. This paper also tries to reveal some ecological particularities concerning their migration and natural reproduction process that could help scientists and other people to find the most probable causes of the tendency why stocks are decreasing and suggesting some pertinent actions to be done on the necessary mitigation and protection activities concerning actual and further presence of these very interesting sturgeon species in Danube River.
**Keywords.** *Danube River, migratory sturgeons, biology, migration, biometric data*

## Introduction

Ones of the most interesting fish species in Europe from both scientific and commercial point of view are marine migratory sturgeons that swim up for reproduction to Danube River. Scientists are interested in them because sturgeons are considered among the most ancient fish species inhabiting the waters of the world. They appeared in the Upper Cretaceous, were very abundant in Devonian, but presently only two families exist: Acipenseridae and Polyodontidae, which altogether include 25 species. Because of the worldwide tendency of decreasing stocks some biologists believe that most of the sturgeon species are on their natural way to extinction [6] others consider the changes in their natural environment – especially in spawning places – and overfishing being the main causes of the evident decline of the populations of these fishes [5]. Fishing of sturgeons, from commercial aspects, is still a good business for interested investors, especially because of the high price of caviar and also a profitable activity for local communities of fishermen that depend mainly on fishing for their living. That could be one of the reasons of the actual tendency of accelerated decrease in the number of sturgeon populations which concerns both biologists, fishermen and other people involved in collateral activities in this matter. The present paper tries to bring up some bio-ecological aspects related to migration process of three marine sturgeon species in Lower Danube to compare them with formerly registered data and scientific information, to find the most probable causes of the decreasing of the stocks and suggesting some pertinent actions on the necessary mitigation activities and better management of this fishery.


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## Materials and methods

In 2000 and 2001, several expeditionary fishings have been performed, in order to collect samples from different locations on the Romanian sector of Lower Danube River. Most of them took place in the middle of migration seasons in the fall and spring, some also in extra-season, focused mainly on places known as preferred sturgeon spawning sites as km 102–103, km 155, km 186, km 231, km 399 and accidentally between km 772–779.

In order to obtain specific data for different locations, the following sectors have been pointed out on Danube River in which we have collected several samples: sector 0: Danube Delta to km 73; sector I: km 73 to km 155; sector II: km 155 to km 231; sector III: km 231 to km 309; sector IV: Borcea Danube Branch and Bala Danube Branch.

For collecting the samples specialized fishing gears as drift bottom gillnets and appropriate boats operated by two or three fishermen have been used.

The sample has been sexed by morphological characteristics such as presence or absence of carved linear hollow on the stellate sturgeon male abdomen and the general characteristics and size of the abdomen. Several individuals were dissected later for having exact evidence of sex and also to determine the stage of the maturation of gonads, gonad mass ratio and the absolute or theoretical prolificacy.

A total number of 117 sturgeon individuals have been caught and investigated (23 of beluga, 57 of stellate sturgeon and 37 of Russian sturgeon).

Other biometrical characteristics which have been registered: total weight with 0.1 kg accuracy, total length with 0.1 cm, and gonad mass with 0.001 kg accuracy. The age of fish was determined from the degreased fine cross-section of the first pectoral ray in a laboratory in case of every caught individual.

## Results

Marine migratory sturgeons have been caught in Danube River all around the year but the most abundant captures were registered during the periods of migration: fall and spring. It is supposed therefore, that water level and seasonality also have an important impact on the number of sturgeon spawners coming into Danube River from Black Sea.

Mainly, spring migration starts when water temperature is constantly higher than 4 °C and fall migration regularly begins when the temperature is less than 23 °C. Optimal intervals of temperature for spawning are a bit different according to each species' biology: beluga (10–17 °C), Russian sturgeon (15–21 °C) and stellate sturgeon (17–23 °C).

### *Beluga*

*Huso huso* Linnaeus, 1758 (beluga) is the migratory sturgeon that swims up into the Danube River just for reproduction. Older individuals prefer an earlier migration in spring season which actually starts in the winter months. Medium sized (middle-age) individuals migrate mainly in fall, while younger spawners at the end of spring.

Biometric data in *Table 1*. represent the measured characteristics of every single caught and investigated individual. Data show that beluga individuals weighted from 40–140 kg, with lengths of 178–286 cm and ages from 11–21 years old.

Females average gonad mass ratio has been generally no more than 9.5% by increasing to 11.5–13.0% for the individuals caught in the eve of the reproduction time and more than 15% to a maximum of 17.93% for the individuals investigated in the spawning season (regularly from mid-April to May).

The male : female ratio (M : F) was favorable for the males (average M : F = 0.44). In fact, for all species of sturgeons studied M : F has been significantly above this value.


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Absolute prolificacy has been extremely variable depending on the age and size of the females, from 0.24 to 3.2 million roes (frequently 0.3–0.9 million roes), the calculated average of absolute prolificacy for all caught female being 0.506 million roes. The evaluated theoretical prolificacy at the last stage of the maturation of gonads was between 29 and 32 roes per gram meanwhile the diameter of the roes were of 3.6–3.7 mm.

Theoretically the best reproduction sites should be in the deep area of Danube River (8–20 m deep) and hard bottom formed by gravel or sand such those of higher sector of Romanian Danube River. A few similar places could be found also upstream at Calarasi Town. Even when optimal conditions are missing, belugas are very likely reproducing also on different spots on the whole lower zone of Danube River, between 0 and 400 km.

***Russian sturgeon***

*Acipenser gueldenstaedti* Brand, 1833 (Russian sturgeon) migrates upstream Danube River in the fall and spring but the most important season takes place during the spring months (March–May) rather than fall (September–October). The spring migration may last till late June and because this species is well adapted to fresh water, few biologists consider that some biological forms of Russian sturgeon remain all year long in Danube River [3]. Russian sturgeon adult individuals use almost the same sites for spawning places as beluga.

Absolute fecundity has been between 0.04–0.4 million roes with the average of 0.14 million per female. Theoretical prolificacy for the last stage of the maturation was 40.5 roes per gram, the roe diameter being of 3.2–3.3 mm. Females gonad mass ratio differed by season: being 12–14% in October and more than 16% in April with a maximum value of 18.35%.

***Table 1.*** *Biometric data of all beluga individual caught in Romanian Danube River during the 2001 expeditionary fishing campaign*

| fish | fishing | sex | age | total length | total weight | gonad weight | gonad mass |
|---|---|---|---|---|---|---|---|
| 1 | | F | 23 | 286 | 145 | 21 | 14.48 |
| 2 | sector 0 | M | 11 | 175 | 40 | 0.5 | 1.25 |
| 3 | | M | 11 | 180 | 45 | 0.6 | 1.33 |
| 4 | | F | 16 | 214 | 80 | 12 | 15 |
| 5 | sector I | M | 15 | 220 | 75 | 2.5 | 3.34 |
| 6 | | M | 12 | 178 | 50 | 1.0 | 2 |
| 7 | sector II | M | 19 | 237 | 90 | 3.1 | 3.45 |
| 8 | | F | 21 | 250 | 120 | 19 | 15.83 |
| 9 | | F | 21 | 256 | 125 | 12.5 | 10 |
| 9 | | M | 15 | 220 | 75 | 1.7 | 2.26 |
| 10 | sector III | M | 19 | 240 | 80 | 3 | 3.75 |
| 11 | | M | 15 | 212 | 65 | 1.5 | 2.3 |
| 12 | | M | 18 | 235 | 82 | 3.2 | 3.9 |
| 13 | | M | 12 | 175 | 50 | 1.0 | 2 |
| 14 | | F | 21 | 250 | 117 | 19 | 16.2 |
| 15 | | F | 23 | 260 | 140 | 20 | 14.28 |
| 16 | | F | 17 | 202 | 115 | 18 | 15.65 |
| 17 | | M | 18 | 220 | 85 | 2.5 | 2.94 |
| 18 | sector IV | M | 11 | 175 | 45 | 0.6 | 1.33 |
| 19 | | M | 20 | 240 | 90 | 3.5 | 3.89 |
| 20 | | M | 13 | 215 | 68 | 2 | 2.94 |
| 21 | | M | 14 | 217 | 70 | 2 | 2.85 |
| 22 | | M | 19 | 245 | 98 | 3.5 | 3.57 |
| 23 | | M | 12 | 195 | 60 | 1.5 | 2.5 |


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***Table 2.*** *Average values of main biometric data calculated on beluga age classes*

| age (years) | total length (cm) | | total weight (kg) | | gonad weight (kg) | | gonad mass ratio (%) | |
|---|---|---|---|---|---|---|---|---|
| | males | females | males | females | males | females | males | females |
| 11 | 176.6 | NA | 43.3 | NA | 0.66 | NA | 1.30 | NA |
| 12 | 125.7 | NA | 53.3 | NA | 1.17 | NA | 2.17 | NA |
| 15 | 217.7 | NA | 71.2 | NA | 1.93 | NA | 2.69 | NA |
| 17 | NA | 214.0 | NA | 97.5 | NA | 15.00 | NA | 15.32 |
| 19 | 237.0 | NA | 90.4 | NA | 3.30 | NA | 3.63 | NA |
| 20 | 240.0 | NA | 90.5 | NA | 3.5 | NA | 3.89 | NA |
| 21 | NA | 250.5 | NA | 125.5 | NA | 17.87 | NA | 14.25 |
| 23 | NA | 260.0 | NA | 145.1 | NA | 25.8 | NA | 17.93 |

NA = not available

***Table 3.*** *Average values of some biometric data on stellate and Russian sturgeon (age in years, M = males, F = females)*

| age | *stellate sturgeon* | | | | | | | | *Russian sturgeon* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | total | | total | | gonad | | gonad | | total | | total | | gonad | | gonad | |
| | M | F | M | F | M | F | M | F | M | F | M | F | M | F | M | F |
| 4 | 70.0 | NA | 3.0 | NA | 0.08 | NA | 2.4 | NA | — | — | — | — | — | — | — | — |
| 5 | 78.0 | NA | 3.5 | NA | 0.15 | NA | 4.3 | NA | — | — | — | — | — | — | — | — |
| 6 | 85.5 | NA | 4.2 | NA | 0.12 | NA | 3.2 | NA | — | — | — | — | — | — | — | — |
| 7 | 90.2 | NA | 4.5 | NA | 0.15 | NA | 3.5 | NA | 67.5 | NA | 5.1 | NA | 0.15 | NA | 1.2 | NA |
| 8 | 92.7 | 97.3 | 5.2 | 6.2 | 0.18 | 0.98 | 3.0 | 16.5 | 107.2 | NA | 7.0 | NA | 0.15 | NA | 2.2 | NA |
| 9 | 113.0 | 125.8 | 5.4 | 7.1 | 0.12 | 1.16 | 2.4 | 16.4 | 109.1 | NA | 6.6 | NA | 0.15 | NA | 2.3 | NA |
| 10 | 118.7 | 127.0 | 6.1 | 7.0 | 0.27 | 1.23 | 3.7 | 16.0 | 113.3 | NA | 7.9 | NA | 0.27 | NA | 3.5 | NA |
| 11 | 119.0 | 138.2 | 6.7 | 9.5 | 0.33 | 1.55 | 5.2 | 15.4 | 117.5 | NA | 8.3 | NA | 0.31 | NA | 3.8 | NA |
| 12 | 126.7 | NA | 7.4 | NA | 0.36 | NA | 4.9 | NA | 119.0 | 123.0 | 7.9 | 12.4 | 0.44 | 2.1 | 4.3 | 16.7 |
| 13 | 145.0 | 148.0 | 8.0 | 11.2 | 0.41 | 1.81 | 5.14 | 16.0 | 126.5 | 133.0 | 10.7 | 13.8 | 0.46 | 2.2 | 4.6 | 15.6 |
| 14 | — | — | — | — | — | — | — | — | 135.7 | NA | 11.7 | NA | 0.35 | NA | 3.1 | NA |
| 15 | — | — | — | — | — | — | — | — | 136.0 | 132.5 | 12.9 | 14.8 | 0.95 | 2.3 | 4.2 | 15.5 |
| 16 | — | — | — | — | — | — | — | — | NA | 141.4 | NA | 16.5 | NA | 2.6 | NA | 15.7 |
| 17 | — | — | — | — | — | — | — | — | 138.0 | NA | 14.6 | NA | 0.72 | NA | 4.9 | NA |
| 18 | — | — | — | — | — | — | — | — | 140.0 | NA | 15 | NA | 0.76 | NA | 5.1 | NA |
| 19 | — | — | — | — | — | — | — | — | 138.0 | NA | 18.5 | NA | 0.98 | NA | 5.3 | NA |

NA = not available

The M : F ratio has been a little different in the case of Russian sturgeon than by beluga, females being more present in captures (M : F = 0.54) but yet less than males in number. Number of total male individuals was 25, with a relatively uniform distribution in all investigated sectors.

### *Stellate sturgeon*

*Acipenser stellatus*, Brandt, 1833 (stellate sturgeon) comes upstream for reproducing either in fall or in early spring. It is supposed that individuals that migrate in fall will spawn also in spring because their degree of gonad maturation is inferior to the one of the adults migrating in spring (usually at the beginning of March). The spawning usually takes place at the end of May in deep locations with fast current.

Study of the captured individuals shows that main capture have been represented mainly by small individuals that weighted no more than 6 kg as well as the number of bigger spawners were very reduced: just 9 from 57 (15.8%) individuals weighted more


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than 7 kg, with a maximum weight of 11.2 kg – a single individual caught on main branch of Danube River in Galati City neighborhood.

The estimated female gonad mass ratio was 6.5 to 7.0% for the younger individuals caught in fall and 14.15 to 16.48 for the biggest spawners caught in late May.

Absolute fecundity had a very large range (30 to 180 thousand roes) and theoretical prolificacy at the last stage of the maturation of the gonads was an average of 57.5 roes per gram, the diameter of the roes being no more than 2,4 mm (average: 2.2 mm). M : F ratio calculated for a number of 57 caught individuals was 0.50.

Long time observations demonstrate that stellate sturgeon prefers for spawning almost the same places as the other sturgeons: km 102–103, km 186, km 234–404 and km 772–779 of Danube River.

Collateral study done on 57 individuals in order to establish the nutritional spectrum of all these species of sturgeons has revealed an almost empty stomach to every single fish. This indicates that spawners do not feed themselves in Danube River before their reproduction time or they eat only accidentally. However, nutritional spectrum observations on the juvenile sturgeons (of 10 to 14 cm length) have indicated that they prefer worms and insect's larva (75%) and small crustaceans (25%).

## Discussion

The literature relating to sturgeons migration informs that all three studied species of sturgeons migrate for reproduction in the same seasons, but first the Russian sturgeon and stellate sturgeon enter Danube River in March, and beluga starts the migration in April. Also there is information that the minimum value of water temperature that triggers the beginning of the migration should be not less 6 °C [6]. In fact, we have found that the first species that entered Danube River was beluga, in January, when water temperature was 2.5 °C, followed in February, almost in same time by Russian sturgeon and stellate sturgeon. At higher water temperatures – such as registered in May and June – the increased intensity of migration is a common fact for all three species. However, the intensity and length of migration should also closely correlate with the change of water level during spring, summer and the beginning of fall. Higher water levels could be correlated with increased intensity of migration and also to a more successful reproduction and offspring development. It is quite difficult to build a credible estimation of migration intensity only on capture studies because some particularities of fishing sturgeons in Danube River [2]. High and long lasting water level induces an increased migration but also catching difficulties resulting a lower amount of captured individuals. Lower water levels usually mean lower intensity of migration but a relative increased capture due to the easiness of sturgeons fishing in Danube River in the low-water period. Even there is some information about a different biological type [4] that could lay spawn in fall (September–October). Beluga is reproducing certainly in the spring (April–May). Sexual maturity is accomplished at the age of 11–14 years old for the males and 16–18 years old for the females but sometimes it is supposed to be earlier, at about 14–16 years of age. During our expeditionary fishing campaigns we did not find any female younger than 17. Stellate surgeon reaches sexual maturity at different ages: 4–7 years for the males and 8–10 years for the females. There are also some other opinions about the age of reaching full sexual maturity such as 7–10 years for males and 10–14 years females [3] but we have found many females younger than 10 years that accomplished their final stage of gonad maturation and also a lot of male individuals younger than 7 years old. Russian sturgeons sexual maturity age is usually between 8–


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12 years for the males and of 13–15 years for the females. A single male individual that has been caught in Danube Delta zone was sexually mature at the age of 7 as well as two females at the age of 12.

Actual biometric measurements show that there is a very important difference between these data and the registered data of the early 20th century when most of the caught beluga weighted 200 kg to 600 kg with sizes of 3–5 m. That time it was not unusual to find in captures tremendous individuals like the huge beluga of 888 kg caught in 1980 near Sfântu Gheorghe Village [1]. Presently the medium weight of caught belugas were below 150 kg. The largest individual caught in our study was a female of 23 years old weighting just 145 kg. We do not have much very credible earlier data on Russian and stellate sturgeons to compare their average size with the present data, but it is important to show that for all sturgeon species the number of younger mature individuals were higher than older ones. The biological meaning of this interesting distribution on age classes is quite difficult to interprete. It could be a result of some successful reproductions in the last years and also a sign of an incipient but timid tendency of sturgeons stock increasing. Last data on dynamism of sturgeon captures seems to confirm that idea even there is not sure that the fishing effort has remained constant.

## Conclusions

The biology of beluga, stellate surgeon and Russian sturgeon that migrate for reproduction in Danube River, is not very well known. There are lacks of information on their behavior in the Black Sea where they grow up and became sexually mature and also not enough or enough credible data about some aspects related to the time spent for spawning in Danube River. There is indirect information about their preferred spots for laying their spawn that it is supposed to be the same area in which they used to crowd, and also about offspring biology either in Danube River or Black Sea. There are not too much biometric data and studies regarding their development and growing up process in their natural environment. We hope that our study could bring out some more aspects on this topic and will be a useful tool for further comparative studies. However, more specialized research including targeting and surveying of evolution of stocks, mortality evaluation and the effect of water pollution on roes and offspring need to be done. Further monitoring activities focused on biological, ecological and fishing aspects should be the key to find the appropriate solution to stop the general decreasing tendency of the stocks of these species.

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# ZOOCOENOLOGICAL STATE OF MICROHABITATS AND ITS SEASONAL DYNAMICS IN AN AQUATIC MACROINVERTEBRATE ASSEMBLY
## (HYDROBIOLOGICAL CASE STUDES ON LAKE BALATON, №. 1)

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**Abstract.** In the years 2002, 2003 and 2004 we collected samples of macroinvertebrates on a total of 36 occasions in Badacsony bay, in areas of open water (in the years 2003 and 2004 reed-grassy) as well as populated by reed (*Phragmites australis*) and cattail (*Typha angustifolia*). Samples were taken using a stiff hand net. The sampling site includes three microhabitats differentiated only by the aquatic plants inhabiting these areas. Our data was gathered from processing 208 individual samples. The quantity of macroinvertebrates is represented by biovolume value based on volume estimates. We can identify taxa in abundant numbers found in all water types and ooze; as well as groups associated with individual microhabitats with various aquatic plants. We can observe a notable difference between the years in the volume of invertebrate macrofauna caused by the drop of water level, and the multiplication of submerged macrophytes. There are smaller differences between the samples taken in reeds and cattail stands. In the second half of 2003 – which was a year of drought – the *Najas marina* appeared in open waters and allowed to support larger quantities of macroinvertebrates. In 2004 with higher water levels, the *Potamogeton perfoliatus* occurring in the same area has had an even more significant effect. This type of reed-grass may support the most macroinvertebrates during the summer. From the aspect of diversity relations we may suspect different characteristics. The reeds sampling site proved to be the richest, while the cattail microhabitat is close behind, open water (with submerged macrophytes) is the least diverse microhabitat.
**Keywords:** *biovolume, reed, cattail, macrophyte, macrofauna, bootstrap, Tukey-test*

## Introduction and aims

Lake Balaton, the largest lake in Central Europe has long been in the center of hydrobiological research. Thanks to more than a hundred years of scientific study, a massive body of knowledge has been gathered making it one of the most thoroughly researched shallow lake. Fresh water macroscopic invertebrates had been reseached for a long time in Hungary. At the end of the nineteenth century knowledge was very limited, only 207 invertebrate species of the lake were known. A hundred years later this number has gone up to 1300, but intensive research of fauna will likely push this number over 2000 [49]. The lakeshore is made up of diverse habitats. 58% of the shore is considered to be in natural state, 12% artificially scattered by rocks and the remaining 30% is paved. The natural parts of the north shore are characterized by belts of reeds. The deterioration of these areas covered by reeds is ever increasing. The stock forming dominant species is common reed [*Phragmites australis* (Cav.) Trin.] but the expansion of narrowleaf cattail (*Typha angustifolia* L.) to the determinant of reeds can be observed


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in some areas of lake Balaton [31]. This is one of the reasons why it is worth researching these two plant communities from the aspect of macroinvertebrate fauna.

Lake Balaton is characterized by different reed grass-stands reaching down to 2 meters in depth of which *Potamogeton perfoliatus* is the most common community forming species, next in line is *Myriophyllum spicatum* followed by *Ceratophyllum demersum* and *Najas marina* [25].

The most thorough research of macroinvertebrates living in water vegetations has been carried in reed-grass areas. A sound knowledge base is available in connection with the macrozoobenthon of rocky shores just like the invertebrate fauna of reeds. On the other hand there is very little available data about the characteristics of the different vegetational habitats – especially for narrowleaf cattail – in vicinity of lakeshore from the aspect of the macroinvertebrate fauna.

Previous works primarily concentrated on spatial patterns and are mainly of faunistic nature. From the temporal patterns only descriptive research has been carried out, moreover not enough attention was paid to the research of seasonal changes occurring on a shorter time scale. This is why it is appropriate to engage analysing zoocoenological spatial and temporal patterns, and to broaden our knowledge in this direction.

Today in the ecological literature the different schools of methodology are distinctly separated from each other.

- Pattern descriptions based on field work constitute one of the main directions e.g. [17, 29, 36, 46, 50, 51, 52, 53, 54, 56, 59].
- Modelling research either works with oversimplified situations or deals with purely theoretical questions e.g. [28, 34, 20, 35].
- Experimental research on the other hand often neglects the complexity of ecosystems e.g. [57].

For the elimination of the above mentioned problems recently new approaches emerged which try to merge the previous methods [21]. With our research at lake Balaton we wish to lay a foundation for future research conducted with a similar approach.

For the location of our research we chose a part of Badacsony Bay containing narrowleaf cattail stands, common reed stands as well as open water areas (in the process of being populated with reed-grass). Badacsony Bay is located on the north shore of Lake Balaton. More close up, the sampling site includes three different microhabitats differing from the aspect of the above mentioned vegetation. During 2002, 2003, and 2004 we collected samples on a total of 36 occasions from spring to last in the autumn. Our data was gathered from processing 208 individual samples. The first two years, especially 2002 proved exceptionally draughty with very low water levels.

In accordance with the above written facts, our aims in the current research were the following:

- to explore the zoocoenological patterns of macroinvertebrate assemblies;
- to explore the seasonal changes in zoocoenological conditions in the three characteristic microhabitats of Lake Balaton. As a first step we would only like have an idea of seasonal changes in quantitative conditions.

We would like the knowledge gathered about the seasonal dynamic patterns of different microhabitats to serve as a foundation for further

- ecological modelling research;
- designing of manipulative experimental setting;
- possible research of climate changes.


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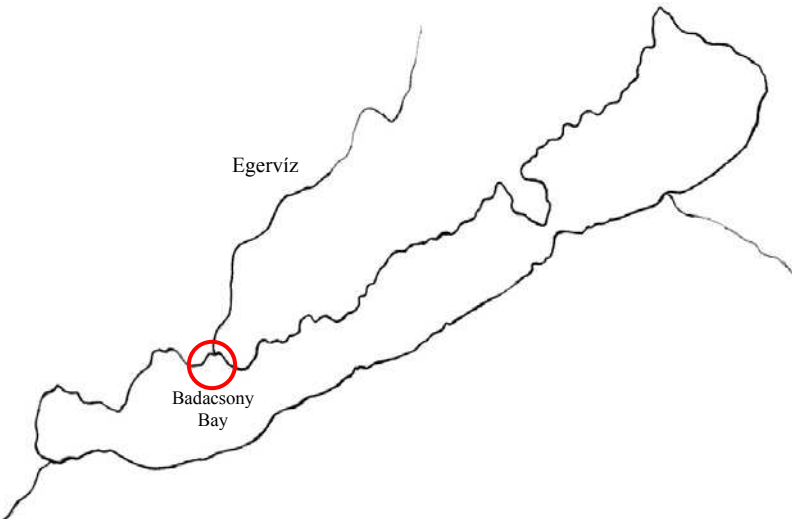


***Figure 1.*** *Location of Badacsony Bay within Lake Balaton.*

## Review of literature

### *Long-term changes of aquatic plant areas in Lake Balaton and other shallow lakes*

In the recent decades degradation of reeds in Lake Balaton can be observed. There are many studies published about the causes of the desolation of reeds [31] but the causes and reasons behind this phenomenon are not known in every case.

The replacement of common reed stands with narrowleaf cattail is a process present in almost all lakes across Europe including Lake Balaton, this process can be attributed the increasing eutrophization of waters and growth in halobity [31, 32].

Reed-grass plays a very important role in the life of lake Balaton. It is known that reed-grass is the antagonist of production of masses of algae, because in the spring the growth of reed grass stands takes away large quantities of nutrition from the ooze and from the water thus the algae population – whose development maximum is reached later on – cannot develop powerfully [63]. Production of algae in large quantities with its shading properties stunts the development of reed-grass. Balaton in its mesotrophic state rooted reed-grasses have a light compensation depth of about 2 meters. This explains why reed-grass was able to penetrate the lake to 2 meters in depth in the 1960's and why it was driven out to shallower areas in the period of vigorous algae production. [24]. Since 1995 oligotrophization can be observed in Lake Balaton, which can be attributed to decline in external phosphorus loads. In the Keszthely Basin the decline in the biomass of phytoplankton became detectable in 1995, thus following the decline of phosphorus loads with a 7–8 years delay, the change in the combination of plankton took a further 3 years delay [67]. The decrease of algae penetration – which can be observed since 1995 favours the advance of reed-grass. The decreased water levels observed since 2000 should also catalyse this process, however even by 1999 reed grass penetration has not reached the levels of the sixties, and in 2000 even less reed-grass was recorded [24, 25].

Many foreign studies deal with the decline of reed stands. The fragmentation of common reed stands was researched on Poygan lake (Wisconsin, USA) in connection with the changes in water levels and winter conditions [8] The higher stem densities corresponded to larger patch size, greater historical stability, and less fragmentation. In


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addition, larger patches tended to be deeper, and covered a greater range of water depths. Higher stem densities were associated with shallower water, though intermediate depths have experienced the greatest decline.

Although combinations of extreme water levels and winter temperatures did not significantly predict annual changes in area of all common reed stands, these factors explained most of the variance in stands with the greatest loss.

Considerable amount of work to be found about the spreading of reeds and other marsh vegetation in connection with different environmental conditions [10, 30, 33, 65].

***Study of macroinvertebrates in various stands of aquatic plant types***

There are many references in the literature that the quality and quantity of submerged and emergent macrophyte may play an important role in the spatial and quantitative patterns and the combination of species of the macroinvertebrate fauna [40], thus the relevance of our research is indisputable.

Works written about the flora and fauna tied to the reeds are summarized concisely in the book titled *Fauna of Reeds* [66].

Müller & *al*. researched parts of Lake Tisza with narrowleaf cattail and other aquatic plants [40]. According to their results areas with narrowleaf cattail stands contained the most species and this area also proved to be the richest from the aspect of spiders, insects and mayflies.

There are known results from the aspect of dragonfly and aquatic bug fauna of submerged and emergent macrophyte stands (amongst others *Typha* spp.) of many flatland lakes [43].

Nicolet & *al.* researched wetland plants, macroinvertebrate assemblages and the water's physico-chemical characteristics of 71 temporary ponds in England and Wales [42]. Their work primarily directs attention to the importance of temporary ponds from the point of view of nature conservation.

Dvorak researched the macroinvertebrates and their functional feeding groups in the narrowleaf cattail, common reed and *Nuphar lutea* colonies of a shallow eutrophic lake in the Netherlands [14].

Parson & Matthews' work [45] emphasizes the relation between the macroinvertebrates and the macrophytes, pointing out that this is an insufficiently researched subject in water systems. The authors examined the invertebrate macrofauna of emergent macrophytes (amongst others *Typha latifolia*) and submerged macrophytes (*Potamogeton* and *Ceratophyllum* species) in a small, shallow, eutrophic pond in the USA. They found significant differences in the density of the macroscopic invertebrates of the different aquatic plant types. The biggest differences were observed between the fauna of the emergent and the submerged plants, the causes of this can be traced back to the morphological difference between the plant types.

Olson & *al*. researched the connection of aquatic plants and macroinvertebrates in the USA [44]. The main plants of the area included common reed, *Scirpus acutus*, *Potamogeton spp.* and the narrowleaf cattail which they found to be the colony with most species and also to contain the biggest macroinvertebrate biomass.

Some macroinvertebrate colonies living in different microhabitats were researched in Lake Balaton as well [5]. In this case the different microhabitats were different reed-grass communities. Biró & Hufnagel's works contain important information about Balaton's aquatic and semi-aquatic bug fauna [4, 6], which is very important from our point of view because it includes information about Badacsony Bay as well.


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***Study of macroinvertebrates in Lake Balaton***

The detailed scientific study of Lake Balaton and its surroundings have started over a hundred years ago thanks to the activities of the Hungarian Geographical Society's Balaton Committee. The Committee has started its work on the initiative of Lajos Lóczy with the notion that the lake is endangered by becoming overpopulated with reed-grass. The research materials, the gathered and processed knowledge-base and the conclusions drew from them were published in the monograph titled *The Results of the Scientific Study of Balaton* (BTTE). Sixty writers took part in the completion of the very big, more than 6000 pages long BTTE. The work has also been published in German in its full length.

Zoological research on the other hand, had to be conducted under less fortunate circumstances. For this reason Géza Entz senior the leader of zoological research, emphasized that a well equipped shore side laboratory is needed. Despite this, much later, only in 1926 established the Hungarian National Museum a research base in Révfülöp, which one year later merged into the Hungarian Biological Research Institute (today: Hungarian Academy of Sciences Balaton Limnological Research Institute) at Tihany. The institute's designated mission was to research and get to know the life of Lake Balaton.

After the stagnation of post war years, the fauna research of lake Balaton almost completely stopped during the fifties and the first half of the sixties (due to science policy). The exceptions during these times were the National Museum's department of zoology and the Department of Systematic Zoology of Eötvös Loránd University. A sudden change was caused by the great fish dilapidation of 1965. Balaton was devastated by a number of biological disasters (cyanobacterial blooms, and fish dilapidation) after which studies of plankton and benthos began on the whole open water area of Balaton. Later a program was launched for the zootaxonomic study of the littoral zone. The scientific knowledge accumulated over the years made Lake Balaton one of the most thoroughly researched shallow lakes in the world. A large body of knowledge was gathered about Balaton's open water planktonic and benthonic invertebrates as well as invertebrates of the shore covered by aquatic plants (littoral region). The story of research of invertebrates of lake Balaton was summarised by Ponyi [49] and later by Berczik & Nosek [2]. The story and state of benthos research was reviewed by Dévai on the basis of the 250 existing works [12]. The recent research of invertebrate fauna of littoral zone is reported by G.-Tóth & *al.* [22, 23].

Even in the forties research was conducted by Entz about the macroinvertebrate fauna of different submerged macrophyte stands (*Myriophyllum spicatum* and *Potamogeton perfoliatus*) [15]. He carried out his research around Tihany in submerged macrophyte stands with different water depths. Béla Entz's work primarily concentrated on the description of composition of species, he placed no emphasis on the seasonal changes. Earlier works are characterised by the fact that they neglected seasonal dynamics, just like Ponyi's Crustacea study of Balaton [48]. The author studied beside the above mentioned two main reed-grass types the species *Ceratophyllum submersum* and designated many sampling points all over the Balaton. His work was oriented at the description of the crustacean fauna.

Quantitative research of the macroinvertebrate fauna of *Potamogeton perfoliatus* in Lake Balaton was first conducted by Bíró & Gulyás [7]. The authors took samples from five permanent sampling sites in the north shore of Lake Balaton in the summer months of three years. As they only took samples during two or three summer months, they were not concerned by seasonal changes. The merit of their work lies in quantitative data based on their particular sampling method.


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In recent years Muskó & *al.* took quantitative samples of macroinvertebrates for three years in the submerged macrophyte stands of the north shore of Lake Balaton [39] using the sampling method and device described by Bíró & Gulyás. During this research seasonal dynamics were also studied. During a year they took samples on a total of three (May–June, July and October) occasions and in another year they took samples on four occasions (May, July, September and October). Because of the methodology they used, their results are comparable with data gathered in much earlier years. They put special emphasis on the Ponto-Caspian invasive species.

In the scientific literature there are references to the seasonal changes of certain groups that make up the invertebrate macrofauna of Lake Balaton. Seasonal dynamics of certain groups of Balaton invertebrates on offshore bars have also been investigated by Dózsa-Farkas & *al.* [13]. Data about the seasonal fluctuation of certain invertebrates living in settlings and being a part of the fish nutrition were provided by Szító [62].

## Materials and methods

### *Sampling site*

The sampling site was designated in a part of Badacsony bay, where different emergent macrophyte stands intersect with open water. The examined plant communities are typical to the littoral zone in this part of Lake Balaton.

Various submerged macrophytes are tipical in front of the emergent macrophyte stands, but did not form continuous vegetation in the year 2002. However large quantities of reed-grass were found in territory of open water in the years 2003 and 2004. We designated three sampling points close to one another. These points were differentiated only by aquatic plant types, which formed the basis for the definition of the three microhabitats. The sampling points are situated 5–7 meters from each other. The sampling site can be reached by boat.

Microhabitats situated close to each other within the determined habitat were chosen in such a way, that differences between samples express exclusively the effect of microhabitats. The goal of our research is not describe the habitat types but to compare the type of microhabitats.

We describe the aquatic plant communities of examined area by Borhidi [9].

- **Reeds**: This type comprises such a community inside the „reed communities" (*Phragmition australis* Koch 1926) where the common reed [*Phragmites australis* (Cav.) Trin.] is dominant. It's called „reeds" (*Phragmitetum communis* Soó 1927 em. Schmal 1926). This community is typical where the littoral zone is in a natural state in Lake Balaton. The common reed is dominant among emergent macrophytes [25]. The lowest water level is noticed in reeds between the three microhabitats. (e.g. if the official water level of Lake Balaton is 60 cm (very shallow), the water level in this place is 70 cm) The stem density is considerable but some smaller inlets are found where the accumulation of vegetable debris may be significant. Samples were taken from marginal zones of reed stands, as well as from dense and from sparse parts. Reeds are never reaped that's why withered reeds provide some shadow in spring.
- **Cattail stands**: It's the "narrowleaf cattail stands" area (*Typhetum angustifoliae* Soó 1927, Pignatti 1953) inside the „reed communities". This species is typical in sublittoral zones of mesotrophic-eutrophic lakes, usually were ooze contains organic matter in large quantities. Cattail stands are sparse in their initial state


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but later become dense and high (2.5 m) [9]. In many cases in this part of Lake Balaton these plant communities make up the outer belt surrounding the reeds. Stem density is higher and narrowleaf cattail (*Typha angustifolia* L.) stands are high in this location.
This is where the biggest depth was observed out of the three sampling points probably because this vegetation showed significant advances towards the open water. (e.g. if the official water level of Lake Balaton is 60 cm, the water level in this place is 78 cm). Samples were taken from outer parts of cattail stands as well, from dense parts of cattail and from waters of smaller inlets.

- **Open water**: This expression refers to the place that isn't colonised by emergent macrophytes. In most cases reed-grass stands are found in this area but considerable continuous stands of submerged macrophyte were not observed in the year 2002. Actually all of 2002 may be considered poor in reed-grass because considerable submerged macrophytes wasn't found in even the broader surroundings of the sampling site, which is in contrast with what was observed in earlier years. Continuous spiny naiad (*Najas marina* L.) stands were appeared in the middle of summer in 2003. These reed-grass stands reached the surface by the second half of summer. Spiny naiad frequently constitutes continuous underwater fields in Lake Balaton that are hard to detect. [16]. According to Borhidi, spiny naiad stands (*Najadetum marinae* Fukarek, 1961) are typical in shore side areas with sandy and oozy bottomed shallow lakes that are in the process of salination. This halophyte reed grass is common in shallow waters not inclined to quick warming – areas usually shaded by reeds. According to Felföldy, this cosmopolitan species preferring warm places, with subtropical-mediterranean origin is more common in bays of mesotrophical lakes, where it is found in different reed grass communities, but almost always in a special, to some extent separated position. [18]. Here water depth has a transitional value compared to the shallower reeds and the deeper cattail areas. The claspingleaf pondweed (*Potamogeton perfoliatus* L.) – this submerged macrophyta is the typical reed grass in deeper water in Lake Balaton – appeared by early summer of the year 2004 and densed significantly by the second half of summer. Samples were taken from area of open water surrounded by two emergent macrophyte communities in obtuseangle. When the continuous reed-grass vegetation appeared we took samples from their areas too.
Actually the *Potamogeton perfoliatus* stands appeared in 2003 but these are found only in deeper waters far away from the sampling site. This reed-grass species constituted of larger continuous fields in the whole area in 2004. We often found some remains of reed-grass in a sampler, in most cases these were the mentioned species and the *Ceratophyllum demersum*. This plant was typical in internal inlets of emergent macrophyte stands farther away from examined locations.

### *Field work*

We strove to take samples frequently from the water body and ooze of the three microhabitats under the period of vegetation during three years. Taking semiquantitative samples by stiff hand net proved to be the most suitable method based on our previous research. The stiff hand net's form is symmetrical hemisphere. The maximum internal diameter is 14.8 cm, mesh size is 0.8 mm.

Taking quantitative samples would be effective but it would lead to difficult problems in area of emergent macrophytes. Nagy & *al* suggest a new sampler and


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***Figure 2.*** *Orthophoto of Badacsony Bay taken in the summer of 2002. The sampling site is marked. Areas of cattail form darker territories bordered by the reeds in the littoral zone. (Source: REGINFORM Kft).*

associated sampling procedure for quantitative analysis of the biota in macrophyte-covered water bodies by Aqualex [41]. The Aqualex is a cylinder, it was made of an aluminum plate, its base area is 0.5 $m^2$ and its height is 1 m. The bottom flange of sampler is sharp, this way it can cut the plants when it is thrown into the water body in vertical position. This method would have been unfortunate to use for two reasons. First this type of sampler is hard to obtain, it has to be manufactured in most cases, and on the other hand it is still unsuitable for collecting samples in an emerged macrophyte type vegetation. During the testing of Aqualex the conclusion was drawn that in case of researching high growth emerged macrophytes, it cannot be viewed as acceptable means of collecting samples [11]. Researchers conducting these tests in the case of this type of vegetation tried the so called "cutting method"(the part of the vegetation above the water level is cut.) This method did not prove to be representative enough, because there were significant differences in the number of taxa and species between samples taken at different times in the treated and untreated areas. The drastic change of the site (cutting of the parts above the water line which provide shade), but mostly the unevenness of the bottom (unevenness makes it impossible to suddenly close the sample taken), make this method unsuitable even on a theoretical basis.

Ten samples were taken from water body by stiff hand net. One drawing includes the whole water column (surface, medium and bottom region). We tried to achieve this objective with S-form drawing. Two samples were taken from upper layer of ooze in every case by the same sampler.

Macrofauna (with some vegetational rubbish were found in sampler) were preserved immediately in 6% formaldehyde. Samples of water body and ooze were handled separately.

Environmental factors concerning the sampling were registered in a notebook on every occasion. In some cases we measured the water depth. Data of official water level were picked up from the homepage of "Országos Vízjelző Szolgálat" (www.hydroinfo.hu). The average difference between official water level and measured water level was calculated.


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(This difference is in the case of microhabitat of cattail is: +18 cm, reed: +10 cm, open water: +14 cm.)

Photographs of sampling site were taken for the sake of more exact documentation. We took a photo on every occasion in 2003 (from the same angle). These photos were taken from a boat, standing in the place of open water facing the direction of emergent macrophytes. The Badacsony Hill can be seen in background. (e.g. *Fig. 3*).

***Figure 3.*** *Photograph of the sampling site taken on July 13th 2003.*

The period of field work lasted from spring to late autumn. Samples were taken in 2002 between 29 April and 16 November on 16 occasions. Unfortunately due to the extreme weather conditions (remarkably intensive waves) we had to finish the sampling in some cases, therefore some samples were left out. (These data are marked by a "?" in *Fig. 8*)

We took samples on 13 occasions from 31st of March to 9th of November 2003. The *Najas marina* stands were observed on 13th July for the first time. This plant constituted smaller underwater fields in a few places at this time. The spiny naiad reached the surface by the second half of July and this vegetation became dense during August. It sank to the bottom by October and formed an accumulated layer reaching to the borders of the emergent macrophyte stands.

Samples were taken altogether on seven occasions between 17th of April and 29th of November. At first we could observe the *Potamogeton perfoliatus* stands in June in the area of open water sampling site. In the months of June and July it formed a dense stand and in October there was a significant amount of reed grass accumulated in front of the emergent macrophyte stands.

The water level of Lake Balaton was extremely falling in consequence of the drought that begun in 2000. This process reached its negative peak in 2003 when low water levels not seen since the 1920's were recorded (this information is also available at www.hydroinfo.hu ) The water level of Lake Balaton was significantly higher in 2004.


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Of the sampling occasions the official water level was the highest on 30 June 2004 (82 cm) and the lowest on 19 October 2003 (24 cm). Both values, especially the minimum value in 2003, may be considered to be under the average water level corresponding to season, consequently the field-work was done under very droughty period.

***Processing of samples***

Maroinvertebrates were selected on the basis of taxon and body size categories (morphon). Animals were classified as the most precise taxon category [3, 19, 37, 47, 58, 60, 61], in several cases identification to species was finished. Remains of animals (e.g. shell, exuvium) were also collected and categorised. Body size categories are important primarily from the aspect of calculating biovolume. We used five fundamental size categories (I–V) and some special size categories. The Ia. category was used for the case of Cladocera and Aphidinea, just over 1 mm size and in the case of Tubificidae, smaller (I–II–III) and larger (IV–V) categories were used. If some individuals proved to be particularly large, their length was registered (only some Tubificidae, on average 7 cm). For the $Chi^2$ test and the stochastic simulation (bootstrapping) we used the number of specimens, but in all other parts of our work we used the biovolume value of collected animals because both biomass and biovolume values represent quantity of macroinvertebrates rather than the number of individuals. Establishing dry mass is very troublesome it is worth to use biovolume instead. Calculation is based on comparing the form of animals to simple geometrical forms. The volume of geometrical forms may be calculated in a simple way. The invertebrates were compared to sphere ($V = \pi d^2$) or to cylinder ($V = \pi r^2 m$) ($V$ = volume, $d$ = diameter, $r$ = radius, $m$ = length, in millimeters.) The two subversion of cylinder geometrical form are the 'cylinder / 2' and 'thread' counted by cylinder's formula with smaller and smaller radius. Taxa with the corresponding geometrical forms are in *Fig. 2* and the values of length, diameter and radius can be seen in *Fig. 1*. Contracted tables were made for examination of spatial patterns, in which rows contain the microhabitats (samples of ooze and water body are separately) and the columns contain the morphons. Under the multivariate analysis we conducted the hierarchical clustering and ordination (non-metric multidimensional scaling) of morphons based on sampling sites (microhabitat–surface combination) and of sampling sites based on morphons. Based on these results, tables were rearranged. As the similarity measure we used the Morisita index, because this index is not sensitive to the variatons of morphon numbers. We made a new table in which the rows represented the higher taxon categories, the columns the sampling sites (microhabitat-surface combination), and the cells contained

***Table 1.*** *The meaning of size categories and the values of lenght (m), diameter (d) and radius (r) corresponding to the size intervals used during biovolume calculations (in mm).*

| size category | meaning | *m* | *d* (sphere) | *r* (cylinder) | *r* (cylinder2) | *r* (thread) |
|---|---|---|---|---|---|---|
| I | >1 mm | 1 | 1 | 0.15 | 0.075 | 0.05 |
| II | 1–5 mm | 5 | 5 | 0.75 | 0.375 | 0.25 |
| III | 5–10 mm | 10 | 10 | 1.5 | 0.75 | 0.50 |
| IV | 10–15 mm | 15 | 15 | 2.25 | 1.125 | 0.75 |
| V | 15 mm< | 20 | 20 | 3 | 1.5 | 1.00 |
| Ia | 2 mm | 2 | 2 | — | — | — |
| I, II, III | >10 mm | 8 | — | — | — | 0.40 |
| IV, V | 10 mm< | 20 | — | — | — | 1.00 |
| V (special case) | e.g. 70 mm | 70 | — | — | — | 3.50 |


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the numbers of the individuals. From every column random samples were taken with an Excel macro developed by ourselves. With the aim of this stochastic simulation (bootstrapping) we can generate arbitrary number of pseudo-replicates. In this case 10 new objects, each containing 1000 macroinvertebrate specimens, have been randomly generated. The comparisons of each year and microhabitats based on certain significant taxa were conducted with Turkey's pairwise comparisons.

For the examination of diversity the use of morphons proved to be the most suitable method – since the full list of species is not available – as any type of abundant object system's diversity, falling into any disjunct category may be in question [27].

We used the biovolume values for calculations instead of the number of species. Based on this we examined the morphon's biovolume diversity. The table used for the calculations included the value of biovolume in $mm^3$ in each microhabitat in each year. To compare diversity relations, Rényi's diversity ordering has been applied.

The seasonal dynamics of volume of macroinvertebrates collected during the three years are rendered in graphs.

The PAST program [55] has been used for multivariate data analysis.

## *Use of concepts*

*Macroinvertebrate assemblies*

The macroinvertebrate category categorises the invertebrate animals found in the particular habitat by their sizes. The bottom size category is defined by the mesh (0.8 mm) on the hand net serving as the sampling device. This term is used in a very wide sense, because according to the principles of zoocoenological sampling [1], every animal in the sample were accounted with. Thus the samples include animals under the mash size that got into the sample with debris (e.g. some Cladocera and Copepoda) and larval or juvenile stage of fishes. Separating the larval and juvenile stage of fishes and other groups from "real" macroinvertebrates would lead to great loss of information. Mostly because of the larval and juvenile fishes it would be worth to use a different term instead of macroinvertebrates but we feel it is more adequate to use and to interpret the term along with the known limitations. In the light of the above said: the term "macroinveretbrate assemblies" is to be interpreted along with the groups that otherwise would not fit into this category, either because of their size range (certain planktonic invertebrates) or taxonomical state (larval and juvenile fish).

*Microhabitat*

The smaller part of the researched water habitat which has externally well identifiable structural properties (such as water depth, bottom type, vegetation, water current characteristics) based on which it may be viewed as a homogenus habitat part from the aspect of the given research [26]. Our samples were taken from three microhabitats, which differ mostly in terms of vegetation, thus their naming was done accordingly: reeds, narrowleaf cattail stands and open water

*Morphon*

A category that takes into account the given animal's taxonomical position, ontological state and body size at the same time. The use of this category is justified for more reasons. The body size is important for the calculation of biovolume, on the other hand different sized individuals of certain species (in addition larvae and adults) may be typical to different microhabitats and/or different time intervals.


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## Results

### *Faunistic overview*

*Table 2* shows all the identified species and other taxa. The geometrical forms – needed for biovolume calculation – are shown in the second column. The next three columns contain average biovolume values ($mm^3$) of animals which were collected during the three years. Samples of three microhabitats differ significantly based on the results of $Chi^2$ test.

Based on *Table 2*, following statements can be made about macroinvertebrate fauna:

- Considerable quantities of Ponto-Caspian species were found in samples. *Limnomysis benedeni* has a particurarly large biovolume. This species was introduced in 1950's as a source of nutrition for fish [68]. Together with this species the *Dikerogammarus* species (Amphipoda) appeared and multiplied [48], these were collected too. Other typical representative of Amphipoda suborder is the *Corophium curvispinum*, it appeared together with zebra mussel (*Dreissena polymorpha*) in the lake in 1930's [38].
- Particurarly large quantities of Tubificidae and Chironomidae were found. These benthic animals primarly live in ooze.
- *Leptodora kindtii* considered to be typical in Lake Balaton are also found together with the other Cladocera.
- The most taxa are detectable in the reeds microhabitat.
- Bivalvia, Gastropoda, Hirudinea and larvae of Ephemeroptera and Zygoptera were found in significant quantities in reeds compared to other microhabitats.
- The most of Arachnoidea are spiders fallen to the water or semi-aquatic spiders, the smaller part is comprised of Hydrachnidae. Probably the reeds provide the most suitable habitat for these groups.
- Biovolume of Chironomidae larvae and Tubificidae are the most significant in cattail stands microhabitat.
- The most larval and juvenile stage of fishes are also found in cattail stands.
- *Dikerogammarus* spp. and *Argulus* spp. belong mostly to cattail stands among crustaceans.
- The least taxa are found in open water and usually in the smallest biovolume values too.
- The most planktonic crustaceans (Cladocera and Copepoda) belong to open water.

### *Spatial zoocoenological patterns*

Under the multivariate analysis we conducted the hierarchical clustering and ordination of morphons based on sampling sites (microhabitat-surface combination) and of sampling sites based on morphons. Samples of ooze and water body were separated based on results of classification of sampling sites. Samples of reeds and cattail stands were situated close to each other in 2002, on the other hand in other years samples of cattail stands and open water were close to each other. Ordination results of morphons (based on sampling sites) are depicted in *Figs. 4–6*, and for the list of morphons with the appropriate serial numbers see *Table 3*.


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***Table 2.*** *The identified species and other taxa with the corresponding geometrical forms (to which invertebrates were compared to during biovolume calculations) and the average biovolume values of taxa ($mm^3$) gathered during the three years in all three microhabitats.*

| taxa | form | cattail | reed | open water |
|---|---|---|---|---|
| **HYDROZOA** | | | | |
| *Hydra circumcincta* Shulze | cylinder | 0 | 0.9 | 0.5 |
| **OLIGOCHAETA** | | | | |
| (other) Tubificidae** | thread | 690.9 | 493.0 | 400.6 |
| *Branchiura sowerbyi* Beddard* | thread | 423.3 | 227.7 | 321.7 |
| *Pristina* sp. | thread | 3.4 | 3.9 | 3.6 |
| **HIRUDINEA** | | | | |
| *Piscicola geometra* L. | cylinder/2 | 3.9 | 11.3 | 0.4 |
| *Glossiphonia heteroclita* L. | cylinder | 1.0 | 2.7 | 0.1 |
| *Erpobdella octoculata* L. | cylinder | 8.1 | 12.7 | 0 |
| *Helobdella stagnalis* L. | cylinder | 0.5 | 2.2 | 0.1 |
| **BIVALVIA** | | | | |
| *Dreissena polymorpha* Pall. | sphere | 30.3 | 96.2 | 28.1 |
| *Pisidium* sp. | sphere | 0 | 1.1 | 0 |
| **GASTROPODA** | | | | |
| *Acroloxus lacustris* L. | cylinder | 0.1 | 0.6 | 0.1 |
| (other) Gastropoda | sphere | 85.3 | 157.6 | 57.4 |
| **ARACHNOIDEA** | | | | |
| Hydrachnidae | sphere | 1.5 | 6.1 | 1.9 |
| Araneidea | sphere | 7.9 | 18.8 | 1.2 |
| **CRUSTACEA** | | | | |
| *Limnomysis benedeni* Czern. | cylinder | 502.8 | 460.7 | 577.5 |
| *Dikerogammarus* sp. | cylinder | 48.0 | 15.2 | 2.1 |
| *Corophium curvispinum* G.O. Sars | cylinder | 5.3 | 6.7 | 1.1 |
| *Argulus* sp. | cylinder | 3.2 | 1.5 | 0.4 |
| *Leptodora kindtii* Focke | cylinder | 42.8 | 16.4 | 32.6 |
| (other) Cladocera | sphere | 21.9 | 13.1 | 100.8 |
| *Asellus aquaticus* L. | cylinder | 1.0 | 4.2 | 0 |
| Copepoda | cylinder | 0.04 | 0.02 | 0.04 |
| **COLLEMBOLA** | cylinder | 0 | 0.1 | 0 |
| **EPHEMEROPTERA** | | | | |
| Caenidae | cylinder | 0.3 | 10.0 | 0.3 |
| Baetidae | cylinder | 26.4 | 66.8 | 39.2 |
| **ODONATA** | | | | |
| *Ischnura* sp. | cylinder | 22.3 | 172.1 | 12.7 |
| Anisoptera | cylinder | 0 | 0 | 3.6 |
| **HOMOPTERA** (Aphidinea) | sphere | 0 | 5,9 | 0 |

**The particurarly big sized Tubificidae were separated. These were identified down to species level (Branchiura sowerbyi).*
***Other Tubificidae: Species identification was finished in some cases, where the big part of them were Pothamotrix sp.*
****We identified the larval and juvenile stage of fishes (Cyprinidae) [47] but species identification happened in only some of the cases. Three species are likely to be found in most cases: Rutilus rutilus L., Scardinius erythrophthalmus L. or Alburnus alburnus L. (one individual may be Rhodeus sericeus amarus Bloch).*


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*Table 2* ***(continued).***

| taxa | form | cattail | reed | open water |
|---|---|---|---|---|
| **HETEROPTERA** | | | | |
| *Aquarius paludum paludum* Fabr. | cylinder | 16.7 | 1.0 | 0 |
| (other) Gerridae | cylinder | 0.4 | 0.5 | 0 |
| *Micronecta meridionalis* Costa. | cylinder | 13.5 | 3.5 | 15.0 |
| *Sigara* sp. | cylinder | 0 | 1.0 | 3.4 |
| *Sigara striata L.* | cylinder | 1.0 | 3.0 | 0 |
| (other) Corixidae | cylinder | 0.1 | 0 | 0.4 |
| *Microvelia* sp. | cylinder | 0.3 | 0 | 0.1 |
| *Microvelia reticulata* Scholtz. | cylinder | 0 | 2.6 | 0 |
| *Mesovelia furcata* Mulsant & Rey | cylinder | 0.6 | 0.2 | 0 |
| *Ranatra linearis* L. | cylinder | 0 | 8.0 | 0 |
| **TRICHOPTERA** | | | | |
| Hydroptilidae | cylinder | 0.3 | 0 | 0 |
| Polycentropodidae | cylinder | 0.5 | 1.6 | 0.1 |
| Limnephilidae | cylinder | 0 | 0 | 0.1 |
| (other) Trichoptera | cylinder | 0.003 | 0.002 | 0.3 |
| **DIPTERA** | | | | |
| Chironomidae | cylinder/2 | 185.4 | 138.2 | 170.2 |
| Ceratopogonidae | cylinder/2 | 10.3 | 12.5 | 6.1 |
| Tipulidae | cylinder | 1.0 | 0 | 0 |
| Tabanidae | cylinder | 0.3 | 0.2 | 0 |
| Syrphidae | cylinder | 0.3 | 0 | 0 |
| „Diptera puparium” | cylinder | 8.1 | 2.9 | 16.0 |
| „Diptera imago” | cylinder | 5.8 | 4.2 | 1.6 |
| **PISCES** (Cyprinidae)*** | cylinder | 134.4 | 20.4 | 3.2 |

The groups that are typical of ooze and of the water body may be sharply isolated based on results of classification and ordination of morphons.

Making statements about observed macroinvertebrate groups is troublesome in most of the cases. Exact statements can be made about only abundant morphons.

The following groups are typical considering all three years at the same time:

- **Typical in ooze**. Tubificidae, Chironomidae and Ceratopogonidae have significant biovolume values in ooze of every examined microhabitats. It seems the snails are also more likely to come out of ooze, tied less to microhabitats.
- **Primarily living in ooze of reeds**. Mostly *Helobdella stagnalis* and *Glossiphonia heteroclita* leech species.
- **Primarily living in water body of cattail stands.** Bigger individuals of *Dikero-gammarus* species, *Argulus* sp., in general the *Aquarius paludum* (with the exception of 2004 when it hardly came into the samples.), *Mesovelia furcata* (primarily its adults) and certain larval and juvenile Cyprinidae
- **Primarily living in water body of reeds**. In general the *Dreissena polimorpha*, *Erpobdella octoculata* and Aphidinea taxa – if any – mostly come out of waters of reeds. The *Ischnura* sp. and *Sigara striata* adult in 2002 and 2003 were found in the reeds, while in 2004 it could be found in cattail stands just as much as in reeds. Caenidae proves to be more of a reeds type. *Hydra circumcincta* is found in the reeds but there are more found in the open water in 2004


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***Table 3.*** *The list of morphons with the appropriate serial number (S) derived from the taxonomic name, the category of body size, and in some cases from the state of ontogenesis (larva, pupa, adult).*

| S | morphon | |
|---|---|---|
| 1 | *Hydra circumcincta* | II |
| 2 | Tubificidae | I, II, III |
| 3 | Tubificidae | IV, V |
| 4 | *Branchiura sowerbyi* | V |
| 5 | *Pristina* sp. | I |
| 6 | *Pristina* sp. | II |
| 7 | *Piscicola geometra* | II |
| 8 | *Piscicola geometra* | III |
| 9 | *Piscicola geometra* | IV |
| 10 | *Glossiphonia heteroclita* | II: |
| 11 | *Erpobdella octoculata* | II |
| 12 | *Erpobdella octoculata* | III |
| 13 | *Erpobdella octoculata* | IV |
| 14 | *Erpobdella octoculata* | V |
| 15 | *Helobdella stagnalis* | II |
| 16 | *Dreissena polymorpha* | II |
| 17 | *Dreissena polymorpha* | III |
| 18 | *Dreissena polymorpha* | V |
| 19 | *Pisidium* sp. | II |
| 20 | other Gastropoda | Ia |
| 21 | other Gastropoda | II |
| 22 | other Gastropoda | III |
| 23 | *Acroloxus lacustris* | II |
| 24 | Hydrachnidae | Ia |
| 25 | Hydrachnidae | II |
| 26 | Araneidea | II |
| 27 | Araneidea | III |
| 28 | *Limnomysis benedeni* | I |
| 29 | *Limnomysis benedeni* | II |
| 30 | *Limnomysis benedeni* | III |
| 31 | *Dikerogammarus* sp. | I |
| 32 | *Dikerogammarus* sp. | II |
| 33 | *Dikerogammarus* sp. | III |
| 34 | *Dikerogammarus* sp. | IV |
| 35 | *Dikerogammarus* sp. | V |
| 36 | *Corophium curvispinum* | II |
| 37 | *Argulus* sp. | II |
| 38 | *Argulus* sp. | III |
| 39 | *Leptodora kindti* | II |
| 40 | other Cladocera | I |
| 41 | other Cladocera | Ia |
| 42 | *Asellus aquaticus* | II |
| 43 | *Asellus aquaticus* | III |
| 44 | Copepoda | I |
| 89 | Diptera pupa | II |
| 90 | Diptera pupa | IV |
| 91 | Diptera adult | II |
| 92 | Diptera adult | III |
| 93 | Diptera adult | IV |

| S | morphon | |
|---|---|---|
| 45 | Collembola | II |
| 46 | Caenidae larvae | II |
| 47 | Caenidae larvae | III |
| 48 | Baetidae larvae | II |
| 49 | Baetidae larvae | III |
| 50 | Ischnura sp. larvae | II |
| 51 | Ischnura sp. larvae | III |
| 52 | Ischnura sp. larvae | IV |
| 53 | Ischnura sp. larvae | V |
| 54 | Anisoptera larvae | IV |
| 55 | Gerridae larvae | II |
| 56 | *Aquarius paludum* larvae | III |
| 57 | *Aquarius paludum* | IV |
| 58 | *Microvelia* sp. larvae | II |
| 59 | *Microvelia reticulata* | II |
| 60 | *Mesovelia furcata* larvae | II |
| 61 | *Mesovelia furcata* | II |
| 62 | Corixidae larvae | II |
| 63 | *Micronecta meridionalis* larvae | II |
| 64 | *Micronecta meridionalis* | II |
| 65 | *Sigara* sp. larvae | II |
| 66 | *Sigara* sp. larvae | III |
| 67 | *Sigara striata* | III |
| 68 | *Ranatra linearis* larvae | V |
| 69 | Aphidinea | I |
| 70 | Aphidinea | Ia |
| 71 | Coleoptera | II |
| 72 | Coleoptera larvae | II |
| 73 | other Trichoptera larvae | I |
| 74 | Hydroptilidae larvae | II |
| 75 | Polycentropodidae larvae | II |
| 76 | Limnephilidae larvae | II |
| 77 | Chironomidae larvae | I |
| 78 | Chironomidae larvae | II |
| 79 | Chironomidae larvae | III |
| 80 | Chironomidae larvae | IV |
| 81 | Chironomidae larvae | V |
| 82 | Ceratopogonidae larvae | II |
| 83 | Ceratopogonidae larvae | III |
| 84 | Ceratopogonidae larvae | IV |
| 85 | Ceratopogonidae larvae | V |
| 86 | Tipulidae larvae | III |
| 87 | Tabanidae larvae | III |
| 88 | Syrphidae larvae | IV |
| 94 | Cyprinidae young larvae | III |
| 95 | Cyprinidae intermediate larvae | III |
| 96 | Cyprinidae older larvae | IV |
| 97 | Cyprinidae young juveniles | IV |
| 98 | Cyprinidae young juveniles | V |


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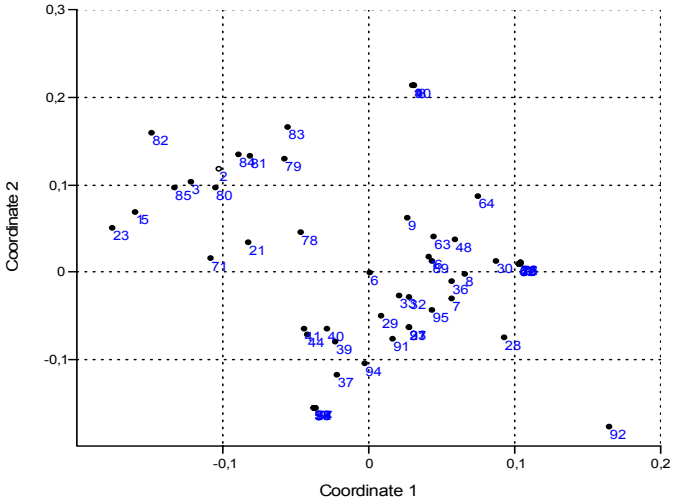


***Figure 4.*** *Ordination results of morphons based on sampling sites – 2002 (non-metric multidimensional scaling). For the meaning of the numbers see Table 3. Results of cluster analysis support the results of ordination.*

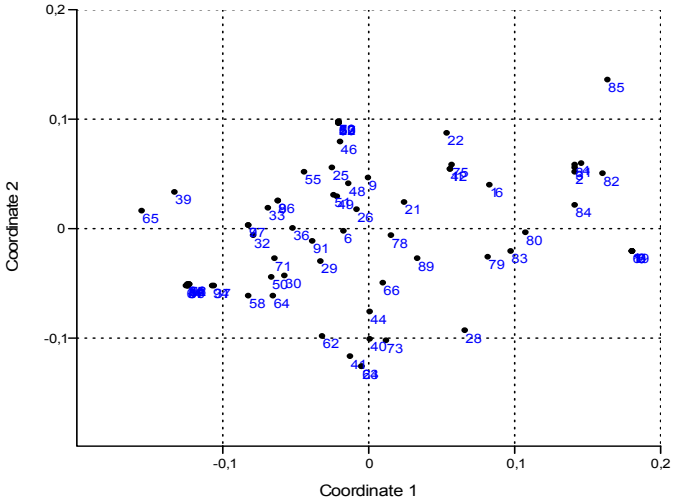


***Figure 5.*** *Ordination results of morphons based on sampling sites – 2003 (non-metric multidimensional scaling) For the meaning of the numbers see Table 3. Results of cluster analysis support the results of ordination.*

- **Typical in water body of reeds and cattail stands alike**. *Corophium curvispinum* definitely belongs to this category. Part of *Piscicola geometra* and smaller sized *Dikerogammarus* taxa also belong here. In 2004 *Ischnura* sp., most of *Acroloxus lacustris*, and smaller Caenidae and *Sigara striata* belonged here.
- **Primarily living in water body of open water**. There was no such group in 2002. Cladocera (including *Leptodora kindtii*) is definitely typical here. And in 2003 larvae of Corixidae.
- **Typical in water body (generally) and – in small quantities – in ooze**. Cladocera which, at other times is typical in open waters belonged to this category in 2002.


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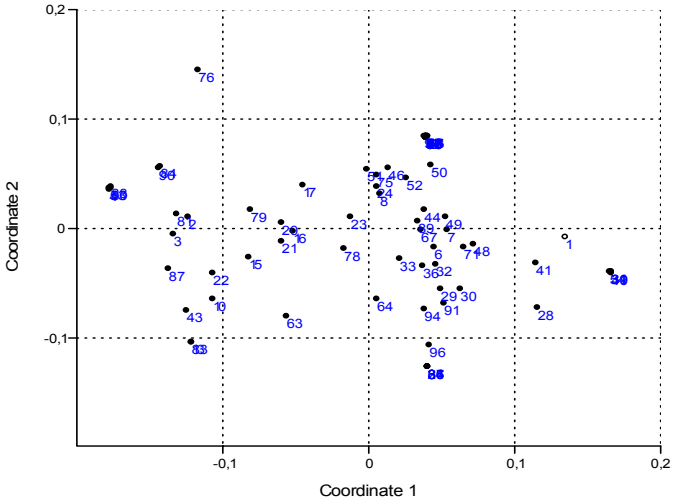


***Figure 6.*** *Ordination results of morphons based on sampling sites – 2003 (Non-metric Multidimensional Scaling) For the meaning of the numbers see Table 3. Results of cluster analysis support the results of ordination.*

Also belonging to this group are the planktonic crustacean: Copepoda. In general *Pristina sp*, *Baetidae*, an certain individuals of *Piscicola geometra* and most of the *Limnomysis benedeni* belonged here.

- In the years 2002 and 2003 *Argulus sp* and the young larvae of Cyprinidae together formed a separate group. In 2002 they were linked exclusively to reeds and in 2003 they were linked to the open water and the waters of cattail stands. They belonged into the same group in 2004 too amongst other species characterised by the waters of cattail stands.

***Table 4.*** *Value of higher level taxa (number of individuals) in th examined three years and the three microhabitats (open w. = open water). With the aim of stochastic simulation (boot-strapping) we generated pseudo-replicates based on this data matrix.*

| Taxa | 2002 | | | 2003 | | | 2004 | | |
|---|---|---|---|---|---|---|---|---|---|
| | cattail | reed | open w. | cattail | reed | open w. | cattail | reed | open w. |
| Hydrozoa | — | — | — | — | 6 | — | — | 1 | 4 |
| Oligochaeta | 649 | 658 | 261 | 1244 | 821 | 845 | 662 | 477 | 574 |
| Hirudinea | 6 | 2— | 2 | 5 | 17 | — | 19 | 49 | 4 |
| Bivalvia | 13 | 29 | 1 | 4 | 14 | 3 | 4 | 18 | 5 |
| Gastropoda | 8 | 2 | 1 | 17 | 51 | 25 | 13 | 58 | 21 |
| Arachnoidea | 1 | 4 | — | 3 | 7 | 6 | 3 | 5 | — |
| Crustacea | 1525 | 1318 | 88— | 27— | 115 | 1149 | 577 | 216 | 851 |
| Collembola | — | — | — | — | — | — | — | 1 | — |
| Ephemeroptera | 1 | 3 | — | 2— | 112 | 42 | 36 | 96 | 75 |
| Odonata | — | 2 | — | 11 | 46 | 11 | 7 | 33 | 4 |
| Heteroptera | 3 | 9 | 6 | 36 | 28 | 33 | 87 | 24 | 91 |
| Aphidinea | — | — | — | — | 26 | — | — | 8 | — |
| Coleoptera | 1 | 6 | — | 4 | 1 | 2 | 1 | 2 | 1 |
| Trichoptera | 2 | 1 | — | 6 | 9 | 2 | 2 | 5 | 2 |
| Chironomidae | 228 | 286 | 175 | 164 | 138 | 164 | 76 | 75 | 86 |
| (Other) Diptera | 12 | 19 | 9 | 19 | 29 | 14 | 15 | 9 | 8 |
| Pisces/Cyprinidae | 8 | 5 | — | 23 | 2 | 3 | 31 | 4 | — |


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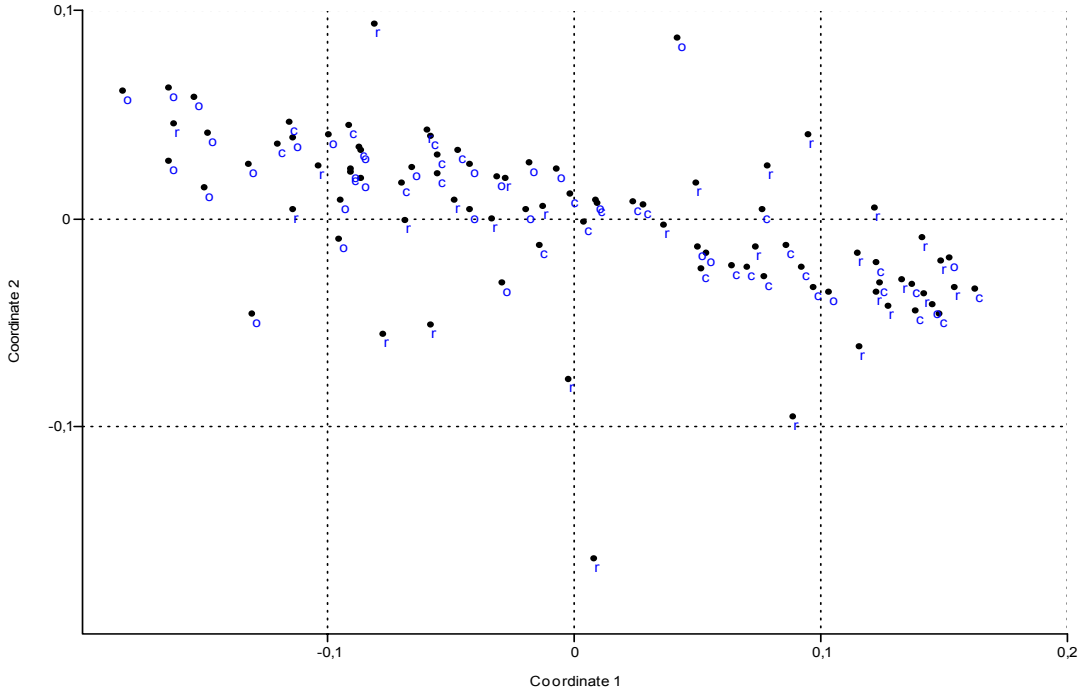


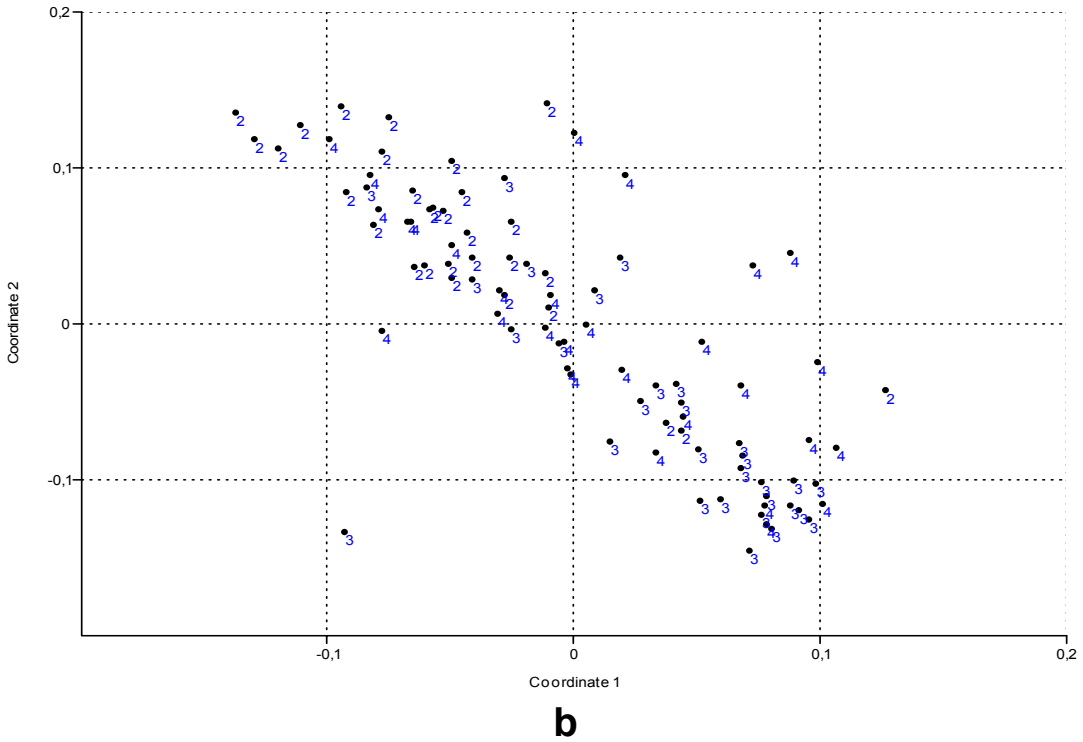


***Figure 7.*** *Similarity pattern of the objects (unfiltered data matrix; see Table 4) by stochastic simulation (bootstrapping).* **a.** *Objects mean the microhabitats (c = cattail, r = reed, o = open water).* **b.** *Objects mean the years (2 = 2002, 3 = 2003, 4 = 2004)*


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***Table 5.*** *The results of Turkey's pairwise comparisons of microhabitat-year combinations' stochastic simulated data (c = cattail, r = reed, o = open water) in case of Hirudinea (unfiltered data matrix; see Table 4).*

| | **c 2002** | **r 2002** | **o 2002** | **c 2003** | **r 2003** | **o 2003** | **c 2004** | **r 2004** | **o 2004** |
|---|---|---|---|---|---|---|---|---|---|
| **c 2002** | | 0.663 | **1.000** | **1.000** | 0.253 | **1.000** | 0.225 | *0.000* | **1.000** |
| **r 2002** | 2.587 | | 0.521 | 0.792 | **0.999** | 0.283 | **0.998** | *0.000* | 0.739 |
| **o 2002** | 0.297 | 2.884 | | **1.000** | 0.165 | **1.000** | 0.144 | *0.000* | **1.000** |
| **c 2003** | 0.297 | 2.290 | 0.594 | | 0.367 | **0.996** | 0.332 | *0.000* | **1.000** |
| **r 2003** | 3.520 | 0.933 | 3.817 | 3.223 | | 0.065 | **1.000** | *0.000* | 0.315 |
| **o 2003** | 0.848 | 3.435 | 0.551 | 1.145 | 4.368 | | 0.055 | *0.000* | **0.998** |
| **c 2004** | 3.605 | 1.018 | 3.902 | 3.308 | 0.085 | 4.453 | | *0.000* | 0.283 |
| **r 2004** | 11.490 | 8.907 | 11.790 | 11.200 | 7.974 | 12.340 | 7.889 | | *0.000* |
| **o 2004** | 0.170 | 2.417 | 0.467 | 0.127 | 3.351 | 1.018 | 3.435 | 11.320 | |

***Table 6.*** *The results of Turkey's pairwise comparisons of microhabitat-year combinations' stochastic simulated data (c = cattail, r = reed, o = open water) in case of Gastropoda (unfiltered data matrix; see Table 4).*

| | **c 2002** | **r 2002** | **o 2002** | **c 2003** | **r 2003** | **o 2003** | **c 2004** | **r 2004** | **o 2004** |
|---|---|---|---|---|---|---|---|---|---|
| **c 2002** | | **1.000** | **1.000** | **0.999** | *0.001* | **0.949** | **1.000** | *0.000* | **0.994** |
| **r 2002** | 0.619 | | **1.000** | **0.973** | *0.000* | 0.767 | **0.996** | *0.000* | **0.929** |
| **o 2002** | 0.707 | 0.088 | | **0.962** | *0.000* | 0.730 | **0.994** | *0.000* | **0.909** |
| **c 2003** | 0.937 | 1.555 | 1.644 | | 0.011 | **1.000** | **1.000** | *0.003* | **1.000** |
| **r 2003** | 6.186 | 6.804 | 6.893 | 5.249 | | 0.055 | *0.004* | **1.000** | 0.020 |
| **o 2003** | 1.732 | 2.351 | 2.439 | 0.795 | 4.454 | | **0.995** | 0.019 | **1.000** |
| **c 2004** | 0.530 | 1.149 | 1.237 | 0.407 | 5.655 | 1.202 | | *0.001* | **1.000** |
| **r 2004** | 6.716 | 7.334 | 7.423 | 5.779 | 0.530 | 4.984 | 6.186 | | *0.006* |
| **o 2004** | 1.219 | 1.838 | 1.926 | 0.283 | 4.966 | 0.513 | 0.689 | 5.496 | |

***Table 7.*** *The results of Turkey's pairwise comparisons of microhabitat-year combinations' stochastic simulated data (c = cattail, r = reed, o = open water) in case of Ephemeroptera (unfiltered data matrix; see Table 4).*

| | **c 2002** | **r 2002** | **o 2002** | **c 2003** | **r 2003** | **o 2003** | **c 2004** | **r 2004** | **o 2004** |
|---|---|---|---|---|---|---|---|---|---|
| **c 2002** | | **1.000** | **1.000** | **0.980** | *0.000* | 0.772 | 0.424 | *0.000* | *0.006* |
| **r 2002** | 0.089 | | **1.000** | **0.987** | *0.000* | 0.807 | 0.464 | *0.000* | *0.008* |
| **o 2002** | 0.022 | 0.111 | | **0.978** | *0.000* | 0.763 | 0.414 | *0.000* | *0.006* |
| **c 2003** | 1.475 | 1.386 | 1.497 | | *0.001* | **1.000** | **0.965** | *0.000* | 0.118 |
| **r 2003** | 7.762 | 7.673 | 7.784 | 6.287 | | *0.007* | 0.037 | 0.010 | 0.802 |
| **o 2003** | 2.340 | 2.251 | 2.362 | 0.865 | 5.422 | | **1.000** | *0.000* | 0.394 |
| **c 2004** | 3.094 | 3.005 | 3.116 | 1.619 | 4.668 | 0.754 | | *0.000* | 0.744 |
| **r 2004** | 13.040 | 12.950 | 13.060 | 11.570 | 5.278 | 10.700 | 9.947 | | *0.000* |
| **o 2004** | 5.500 | 5.411 | 5.522 | 4.025 | 2.262 | 3.160 | 2.406 | 7.540 | |

We generated pseudo-replicates based on stochastic simulation (bootstrapping). The unfiltered data matrix is shown in *Table 4*. We can make the following statements based on results of ordination of these groups (*Fig. 7*):

Strongly separated differences aren't observed between microhabitats because the points that symbolized microhabitats are situated closely to one another *Fig. 8a*. The years are separated better. (*Fig. 8b* 2002 more in the upper part of the figure, in the lower parts 2003, and finally 2004 mostly in the centre and lower parts.)


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***Table 8.*** *The results of Turkey's pairwise comparisons of microhabitat-year combinations' stochastic simulated data (c = cattail, r = reed, o = open water) in case of Odonata (unfiltered data matrix; see Table 4).*

| | **c 2002** | **r 2002** | **o 2002** | **c 2003** | **r 2003** | **o 2003** | **c 2004** | **r 2004** | **o 2004** |
|---|---|---|---|---|---|---|---|---|---|
| **c 2002** | | **1.000** | **1.000** | **0.951** | *0.000* | **0.961** | **0.997** | *0.000* | **0.987** |
| **r 2002** | 0.241 | | **1.000** | **0.980** | *0.000* | **0.985** | **1.000** | *0.000* | **0.997** |
| **o 2002** | 0.000 | 0.241 | | **0.951** | *0.000* | **0.961** | **0.997** | *0.000* | **0.987** |
| **c 2003** | 1.718 | 1.478 | 1.718 | | *0.001* | **1.000** | **1.000** | *0.000* | **1.000** |
| **r 2003** | 8.008 | 7.767 | 8.008 | 6.289 | | *0.001* | *0.000* | *0.003* | *0.000* |
| **o 2003** | 1.650 | 1.409 | 1.650 | 0.069 | 6.358 | | **1.000** | *0.000* | **1.000** |
| **c 2004** | 1.100 | 0.859 | 1.100 | 0.619 | 6.908 | 0.550 | | *0.000* | **1.000** |
| **r 2004** | 13.850 | 13.610 | 13.850 | 12.130 | 5.843 | 12.200 | 12.750 | | *0.000* |
| **o 2004** | 1.375 | 1.134 | 1.375 | 0.344 | 6.633 | 0.275 | 0.275 | 12.480 | |

***Table 9.*** *The results of Turkey's pairwise comparisons of microhabitat-year combinations' stochastic simulated data (c = cattail, r = reed, o = open water) in case of Heteroptera (unfiltered data matrix; see Table 4).*

| | **c 2002** | **r 2002** | **o 2002** | **c 2003** | **r 2003** | **o 2003** | **c 2004** | **r 2004** | **o 2004** |
|---|---|---|---|---|---|---|---|---|---|
| **c 2002** | | **1.000** | **1.000** | 0.609 | 0.045 | 0.429 | *0.000* | *0.004* | *0.000* |
| **r 2002** | 0.400 | | **1.000** | 0.787 | 0.093 | 0.618 | *0.001* | 0.010 | *0.000* |
| **o 2002** | 0.533 | 0.133 | | 0.836 | 0.117 | 0.680 | *0.001* | 0.013 | *0.000* |
| **c 2003** | 2.702 | 2.302 | 2.169 | | **0.923** | **1.000** | 0.087 | 0.473 | *0.001* |
| **r 2003** | 4.566 | 4.167 | 4.034 | 1.865 | | **0.980** | 0.772 | **0.997** | 0.081 |
| **o 2003** | 3.082 | 2.683 | 2.550 | 0.381 | 1.484 | | 0.163 | 0.654 | *0.004* |
| **c 2004** | 6.907 | 6.507 | 6.374 | 4.205 | 2.340 | 3.824 | | **0.994** | **0.914** |
| **r 2004** | 5.689 | 5.289 | 5.156 | 2.987 | 1.123 | 2.607 | 1.218 | | 0.412 |
| **o 2004** | 8.809 | 8.410 | 8.277 | 6.107 | 4.243 | 5.727 | 1.903 | 3.120 | |

***Table 10.*** *The results of Turkey's pairwise comparisons of microhabitat-year combinations' stochastic simulated data (c = cattail, r = reed, o = open water) in case of larval and juvenile fish (Cyprinidae) (unfiltered data matrix; see Table 4).*

| | **c 2002** | **r 2002** | **o 2002** | **c 2003** | **r 2003** | **o 2003** | **c 2004** | **r 2004** | **o 2004** |
|---|---|---|---|---|---|---|---|---|---|
| **c 2002** | | **0.955** | 0.541 | 0.445 | **0.944** | **0.931** | *0.000* | **1.000** | 0.541 |
| **r 2002** | 1.693 | | **0.996** | 0.032 | **1.000** | **1.000** | *0.000* | 0.880 | **0.996** |
| **o 2002** | 2.843 | 1.151 | | *0.002* | **0.998** | **0.999** | *0.000* | 0.385 | **1.000** |
| **c 2003** | 3.047 | 4.739 | 5.890 | | 0.028 | 0.024 | *0.000* | 0.606 | *0.002* |
| **r 2003** | 1.760 | 0.068 | 1.083 | 4.807 | | **1.000** | *0.000* | 0.860 | **0.998** |
| **o 2003** | 1.828 | 0.135 | 1.016 | 4.875 | 0.068 | | *0.000* | 0.837 | **0.999** |
| **c 2004** | 11.100 | 12.800 | 13.950 | 8.057 | 12.860 | 12.930 | | *0.000* | *0.000* |
| **r 2004** | 0.339 | 2.031 | 3.182 | 2.708 | 2.099 | 2.166 | 10.760 | | 0.385 |
| **o 2004** | 2.843 | 1.151 | 0.000 | 5.890 | 1.083 | 1.016 | 13.950 | 3.182 | |

Some characteristics observed in the similarity relation of certain years and certain microhabitats based on significant taxon categories (Turkey's pairwise comparisons; *Tables 5–10*).

- Generally there are only minimal differences between the microhabitats within the same year. This is especially true for the year 2002 and 2003 mostly in the case of Heteroptera. The same can be said about Gastropoda, Ephemeroptera


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and Odonata in the year 2002. In this case data corresponding to cattail in 2003 are completely identical to those of 2002.

***Table 11.*** *The morphon/biovolume diversity indexes belonging to the three microhabitats (c = cattail, r = reed, o = open water) during 2002, 2003 and 2004.*

| | 2002 c | 2002 r | 2002 o | 2003 c | 2003 r | 2003 o | 2004 c | 2004 r | 2004 o |
|---|---|---|---|---|---|---|---|---|---|
| **morphons** | 43 | 46 | 29 | 53 | 56 | 41 | 52 | 58 | 38 |
| **biovolume** | 30874 | 49733 | 32370 | 77059 | 58198 | 42792 | 54167 | 35176 | 45850 |
| **dominance** | 0.14 | 0.19 | 0.28 | 0.22 | 0.14 | 0.16 | 0.20 | 0.11 | 0.40 |
| **Shannon index** | 2.42 | 2.23 | 1.92 | 2.09 | 2.51 | 2.34 | 2.22 | 2.80 | 1.61 |
| **Simpson index** | 0.86 | 0.81 | 0.72 | 0.78 | 0.86 | 0.84 | 0.80 | 0.89 | 0.60 |
| **Menhinick** | 0.24 | 0.21 | 0.16 | 0.19 | 0.23 | 0.20 | 0.22 | 0.31 | 0.18 |
| **Margalef** | 4.06 | 4.16 | 2.70 | 4.62 | 5.01 | 3.75 | 4.68 | 5.45 | 3.45 |
| **equitability** | 0.64 | 0.58 | 0.57 | 0.53 | 0.62 | 0.63 | 0.56 | 0.69 | 0.44 |
| **Fisher alpha** | 4.92 | 5.00 | 3.14 | 5.56 | 6.11 | 4.47 | 5.67 | 6.78 | 4.07 |
| **Berger–Parker** | 0.25 | 0.35 | 0.50 | 0.35 | 0.28 | 0.34 | 0.37 | 0.21 | 0.61 |

- In 2004 data of reeds often differ completely from all other data in the same period. In the case of Hirudinea and Odonata the difference is complete and in the case of Gastropoda and Heteroptera there are only a few exceptions.
- Data of reeds in 2003 also differ in a lot of cases (Gastropoda and Ephemeroptera) from all others but mostly from those of 2002.
- In 2004 data of cattail and open water show great similarity with all the data of 2002 (Gastropoda, Odonata).
- However, in the case of Heteroptera samples taken 2004 differ in almost every case from the ones taken in 2002.
- In the case of Ephemeroptera in 2002 the data corresponding to open water differ from all others, and in the case of Cyprinidae data of cattail in 2004 differ.

***Diversity***

*Table 11* contains the values of the diversity indexes. The diversity profiles are shown in pairs on *Figs. 8a–f.* The most morphons belong to the reeds in each year and the least to the open water. In 2003 and 2004 the total biovolume values show a different picture. The dominance index is highest in the open water except of 2003 when it is highest in the cattail stands. In case of equitability similar incidents can be observed with the opposite signs. Based on the Shannon and Simpson indices and Menhinick index the reeds proved to be the most diverse, except 2002 when the cattail stands were the most diverse. According to the Margalef index the highest value belongs to the reeds in each year. The Berger-Parker index yields a different value for each year. Based on the diversity ordering the following characteristics may be observed:

- In 2002 cattail and reed cannot be put in order because the profiles intersect. Based on the position and shape of the profiles we may make the assumption that in case of dominant morphons the cattail stands are richer but in the case of rare morphons there is no significant difference on the side of reeds. In the same year the open water may be considered less diverse than the cattail and common reed stands in every domain of the scale parameter.
- In 2003 reeds proves to be more diverse than open water and cattail in every domain of the scale parameter. In 2003 cattail and open water cannot be put in order because the profiles intersect. Based on the position and shape of the profiles we may assume


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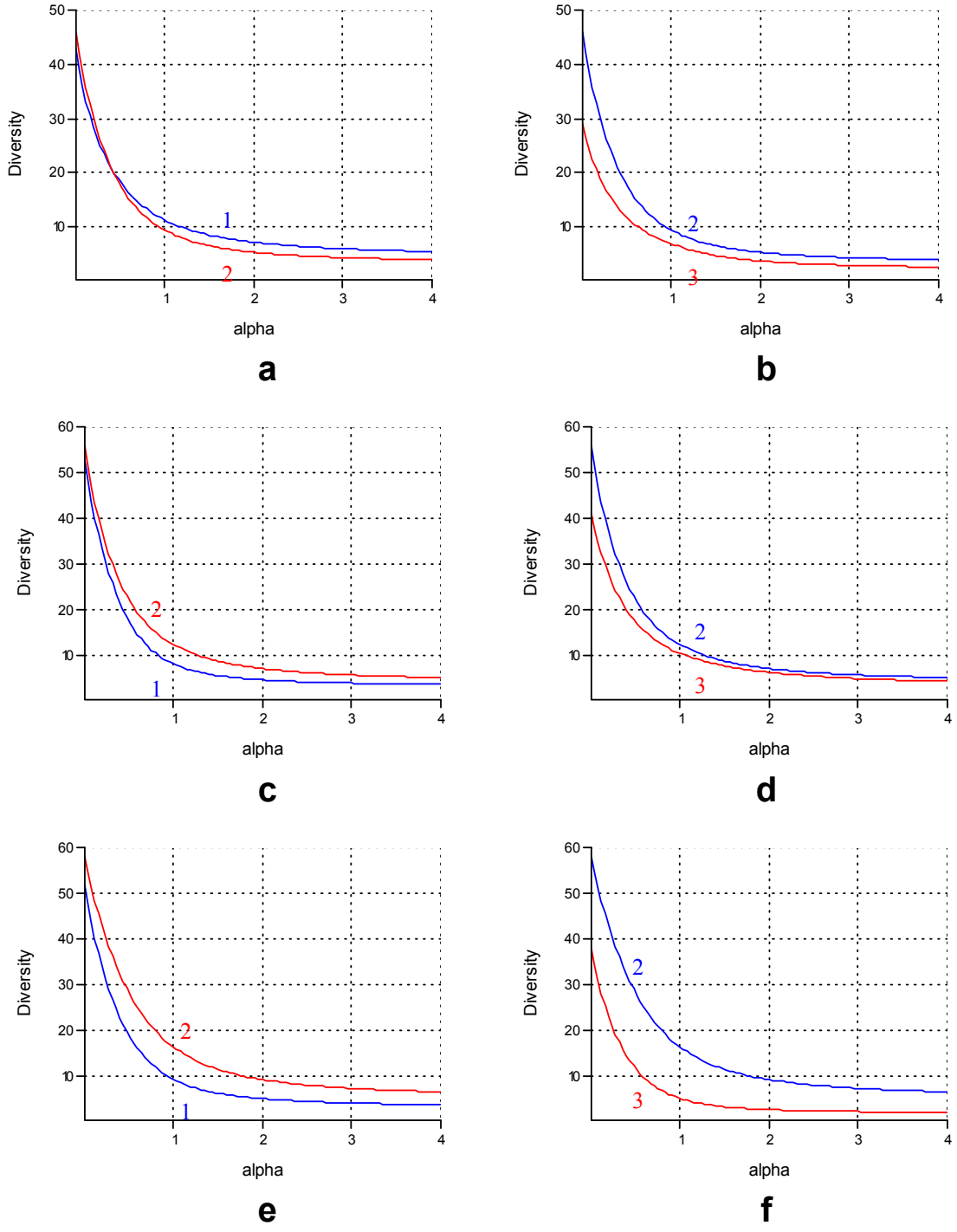


***Figure 8.*** *Diversity ordering of microhabitats in the years 2002 (***a** *and* **b***), 2003 (***c** *and* **d***) and 2004 (***e** *and* **f***). 1 = cattail, 2 = reed, 3 = open water. „Alpha" means the scale parameter (Rényi-diversity).*

that in case of rare morphons the cattail stands are richer but in the case of dominant morphons open water may be a richer environment.

- In 2004 reed stands are the most diverse followed by cattail, and last the open water (in every domain of the scale parameter).


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### *Seasonal dynamics of macroinvertebrates in the three microhabitats*

The examination was based on a table in which the sum of the value of the biovolume of the macroinvertebrates collected that day belongs to the sampling date, moreover the serial numbers corresponding to the dates are also included (*Table 12*). In each year the number of the first sampling day is 1.

Based on the *Figs. 9*, *10* and *11*, we can make the following statements:

- In total the most macroinvertebrate matter was observed in 2003 in all three microhabitats, and the year 2002 was the lowest compared to the other two years.
- Looking at the three years at the same time we can state that the low biovolume values in the spring were followed by a slight increase only to fall back at the beginning of summer (around June). From here during the whole summer a steady increase follows and we can record the highest values in the autumn (beginning of October). Late in the autumn decreasing starts.

***Table 12.*** *The data of sampling, their serial numbers (S), and the total biovolume quantities of macroinvertebrates ($mm^3$) corresponding to the three microhabitats.*

| date | S | cattail | reed | open water |
|---|---|---|---|---|
| 29 April 2002 | 1 | 1863,2 | 2197,3 | |
| 10 May 2002 | 12 | 2198,7 | 1733,4 | 770,45 |
| 26 May 2002 | 28 | 1099,7 | 1190,2 | 1745,1 |
| 09 June 2002 | 42 | 1879,9 | 1037,3 | 1120,3 |
| 23 June 2002 | 56 | 713 | 1494,2 | 290,58 |
| 01 July 2002 | 64 | 678,91 | 519,06 | |
| 16 July 2002 | 79 | 2307 | 1029,6 | 345,62 |
| 28 July 2002 | 91 | 3161,7 | 3387,2 | 1684,4 |
| 08 August 2002 | 102 | 1604,4 | 3220,6 | 1108,5 |
| 15 August 2002 | 109 | 2088 | 1661,9 | 159,18 |
| 220August 2002 | 116 | 1075 | 3075,8 | 390,83 |
| 04 September 2002 | 129 | 2543,3 | 5287,8 | 15682 |
| 22 September 2002 | 147 | 2077,2 | 7611,6 | 671,47 |
| 06 October 2002 | 161 | 2904,1 | 7363,4 | 3817,3 |
| 26 October 2002 | 181 | 2043,4 | 3715,9 | 3629 |
| 16 November 2002 | 202 | 2639,2 | 5205,1 | 953,22 |
| 31 March 2003 | 1 | 2373,4 | 1717,7 | 729,41 |
| 22 April 2003 | 23 | 4071,4 | 6802 | 3547,9 |
| 03 May 2003 | 33 | 12799 | 423,84 | 1861,1 |
| 25 May 2003 | 55 | 2073,8 | 675,28 | 904,99 |
| 09 June 2003 | 66 | 1236 | 730,94 | 3129,3 |
| 26 July 2003 | 83 | 2079,3 | 2250,1 | 1330,7 |
| 13 July 2003 | 100 | 1565,6 | 5298,3 | 30,034 |
| 26 July 2003 | 113 | 3561,6 | 5599,5 | 2223,9 |
| 12 August 2003 | 131 | 4274,5 | 7006,9 | 2019,5 |
| 31 August 2003 | 150 | 2323,7 | 4290,6 | 3435,4 |
| 17 September 2003 | 167 | 2419,8 | 2749,6 | 6931,2 |
| 19 October 2003 | 199 | 30219 | 13179 | 5184,5 |
| 09 November 2003 | 220 | 8061,1 | 7470,7 | 11461 |
| 17 April 2004 | 1 | 7191,6 | 5869,7 | 3965,5 |
| 30 May 2004 | 44 | 9844 | 2233,3 | 596,18 |
| 30 June 2004 | 75 | 2477,6 | 4308,7 | 1478,6 |
| 30 July 2004 | 105 | 5670,1 | 3133,2 | 7475,6 |
| 23 August 2004 | 129 | 8431 | 4603,6 | 22929 |
| 5 October 2004 | 172 | 12371 | 12155 | 7859,4 |
| 29 November 2004 | 227 | 8179 | 2868,3 | 1542,1 |


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- In 2002 the following statements may be made about the distribution of the macroinvertebrate biovolume between the microhabitats: From the second half of summer to the end of the year the biggest volume was found in the reeds. In the spring and early summer very steady values were recorded just about the same as in the other microhabitats. Cattail stands have an even value throughout the whole year. Open water shows the lowest values and the highest fluctuations. (the peak of September is very hard to interpret and may be an error).

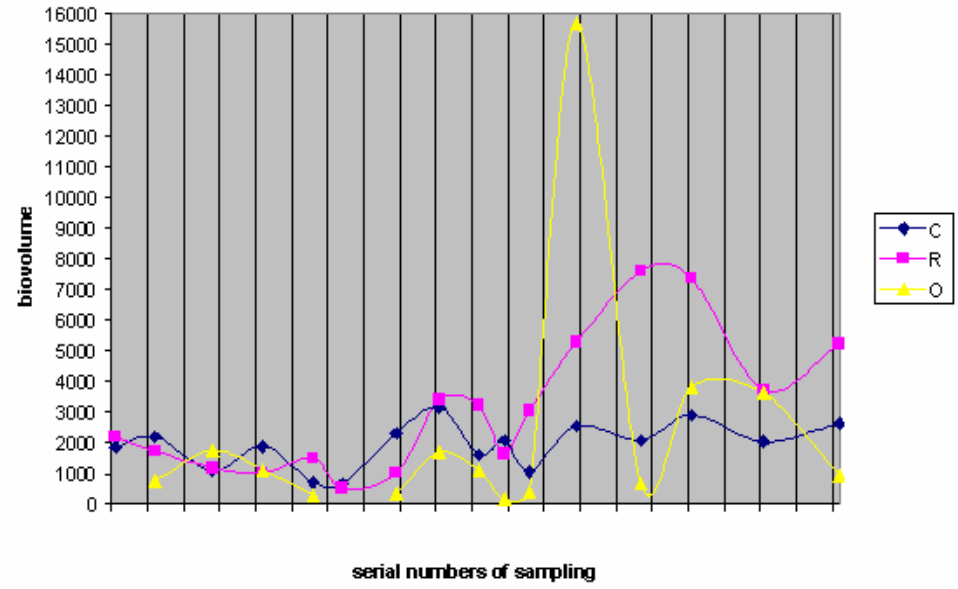


***Figure 9.*** *The seasonal changes in biovolume values ($mm^3$) of macroinvertebrates in the three microhabitats during 2002. (C = cattail stands, R = reeds, O = open water) Due to extreme weather conditions samples were not taken on the 28th of April on the open water sampling site, on the 10th of May (?) samples were only taken from the ooze and on the 1st of July (?) samples were not taken from the open water and from the water of cattail.*

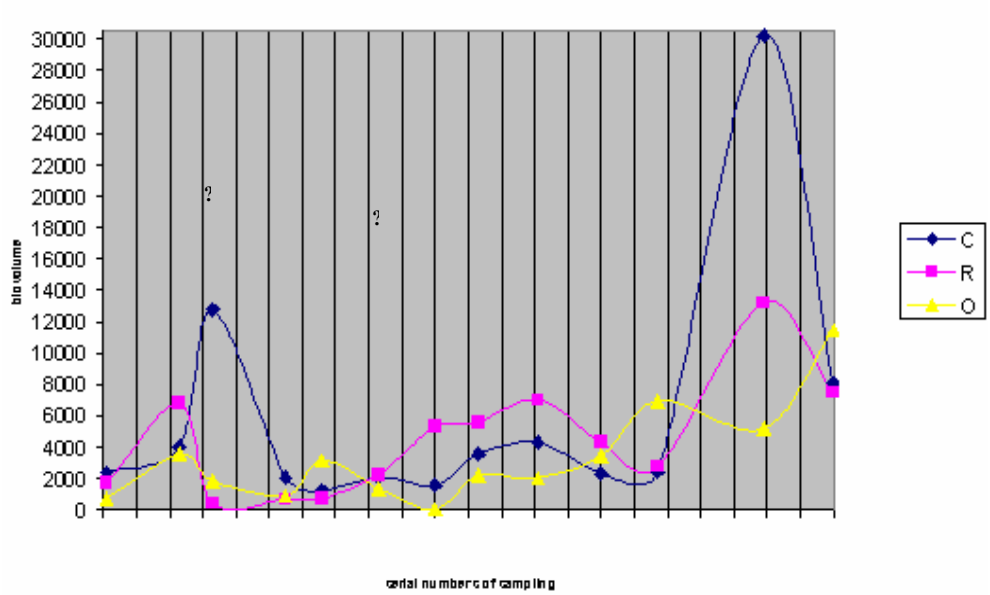


***Figure 10.*** *The seasonal changes in biovolume values ($mm^3$) of macroinvertebrates in the three microhabitats during 2003 (C = cattail stands, R = reeds, O = open water).*


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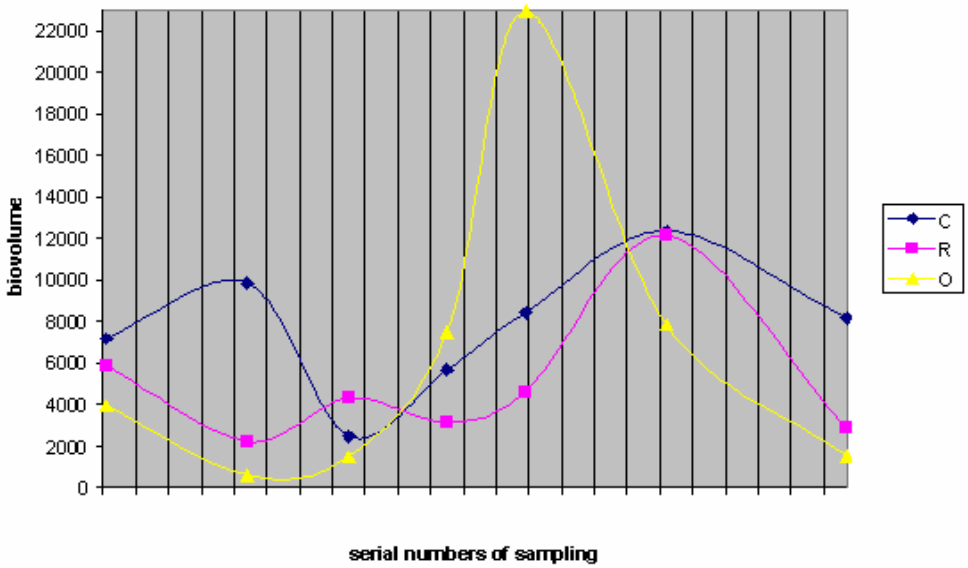


***Figure 11.*** *The seasonal changes in biovolume values ($mm^3$) of macroinvertebrates in the three microhabitats during 2004 (C = cattail stands, R = reeds, O = open water).*

- In 2002 and 2003 after the peak of August a slight decline can be observed until the end of the month followed by the rise in the autumn. This decline was not observed in 2004 but in this year only one sample was taken during August and there was no sample taken in September.
- In 2003 during the summer the highest biovolume values are linked with the reeds followed by cattail and open water. However during the spring and especially during the autumn the highest increase belongs to the cattail stands. The highest biovolume values were recorded during October of this year. In the case of open water a steady increase of biovolume was recorded from the middle of summer to the end of the year.
- In the spring of 2004 the biggest biovolume may be observed in the case of cattail stands. In the increase that lasts from the summer to autumn reeds is right behind the cattail stands and in the end catches up. In the second half of summer open water shows an ever stronger increase – overtaking the other microhabitats - , it reaches its peak in August and then decreases gradually.

## Discussion

### *Faunistical features*

Generally determinable characteristics do not include anything surprising about the current situation of fauna in Lake Balaton. According to the scientific literature, currently in Lake Balaton the *Dikerogammarus* and the *Corophium curvispinum* species are found of the Amphipoda crustaceans. This only differs from what we found in the aspect that the *C. curvispinum* dominates every habitat [38]. On ther contrary in our samples the *Dikerogammarus* sp. was represented with a larger biovolume value (in terms of the number of individuals the *C. curvispinumis* almost as high as the *Dikerogammarus* species).

Based on the total biovolume amount and the number of taxa it may be suspected that most macroinvertebrates live in the area of the common reed stands. This may be explained in a number of ways:


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- The lowest water depth is recorded in common reed areas, this is where the shore effects have the biggest impact. According to this, it is not the common reed as a species, but other environmental conditions may be decisive.
- The observation may be explained with the characteristics of reed stands. For example the harder stem that may provide a better surface for certain creatures forming biotecton that the vegetation found in the other two microhabitats.

Certain conclusions may be drawn from the cattail sampling site, based on the average volume taxons observed in the sampling area:

- In this area the loose ooze serves as an important substance because more of the species can be found here – living in the ooze in large masses – than in the other two microhabitats (Chironomidae larva and Tubificidae).
- In the cattail stands there are many *Argulus* sp. furthermore there are many larval and juvenile fishes. According to this, the presence of fish is most perceptible in this microhabitat. If this statement is acceptable then cattail stands may be considered the most important microhabitat from the aspect of fish.

It is obvious even from the taxonlist and the average volume of biomass that the fewest macroinvertebrates may be found in the sampling site called open water, this emphasizes the importance of emergent macrophyte stands. This more open area with submerged macrophytes may be most suitable for large volumes of planktonic crustaceans.

***Spatial zoocoenological patterns***

Up to this point our results did not show strongly separated differences in the three microhabitats. The cause of this is considered to be the marginal effect since the three sampling sites are next to each other, so that samples were taken from the sites' border zones. Therefore it may be worth to set more sampling points within the observed vegetations (inside the stands as well), if we would like to see what differences there are in terms of the macroinvertebrate fauna.

The groups that do not prefer one particular microhabitat are primarily masses of taxa (Tubificidae, Chironomidae, Ceratopogonidae) associated with ooze or in the open water – also found in masses – are the groups of *Limnomysis benedeni*, the *Pristina* sp. and the Baetidae group.

In connection with the water and ooze of reeds, we can find the not fish-parasite leeches. We can also find the most zebra mussel and occurrences of *Hydra circumcincta* too. These groups without a doubt require the surface provided by reeds. The leaf louse (Aphidinea) found presumably only damage the reeds but not other vegetation.

Of the groups belonging to cattail stands we would point out *Argulus* sp. and juvenile fish that emphasize the presence of fish.

There are more than one significant groups that appear in the water body of emergent macrophyte stands or in the ooze beside these areas. This indicates the importance of emergent macrophyte areas, regardless of the actual species that forms the main colony in the area.

The significance of reed-grass is highlighted by the fact that in 2002 a group typical of open water hasn't been separated. With the occurrence of submerged macrophyte the planktonic crustaceans that was distributed evenly in all areas was now concentrated in reed-grass. It seems that the Corixidae larvae prefer the *Najas marina*, because in 2003 they were a characteristics species in this area. It is presumable that for the *Hydra*


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*circumcincta* the *Potamogeton perfoliatus* is a more ideal microhabitat, in lack of such environment it can only be found in the reeds.

Results based on bootstrapping show significant differences between the different years rather than between the microhabitats. This is backed by characteristics observed in the case of certain taxa. Reeds seems to be the most separated microhabitat based on the defining water macro invertebrate taxa (Hirudinea, Gastropoda, Ephemeroptera, Odonata).

***Diversity***

The Rényi's diversity ordering method includes more diversity functions by special cases [64] this is why we primarily focus on discussing characteristics based on diversity ordering.

In 2002 the two kinds of emerged macrophytes hardly differed from one another, and the open water areas may definitely be considered poorer. This could mean that emergent macrophyte type habitat is the important factor for the macroinvertebrates and not the actual plant species forming the vegetation.

In 2003 the richest microhabitat in every domain of the scale parameter are the reeds, the open water and cattail areas display only smaller differences. This latter statement may be explained by the open water being populated by reed-grass.

In 2004 – with higher water levels and a more considerable reed-grass population – the unambiguous order in the diversity profiles (reeds – cattail stands – open water) directs attention again to the importance of emergent macrophytes. Altogether, we can declare that diversity is greatest in the reeds microhabitat.

***Seasonal dynamics of macroinvertebrates in the three microhabitats***

In 2003 higher biovolume values could be observed than in previous years. The highest biovolume values were recorded in October 2003 when the official water level of lake Balaton reached its lowest level at 24 cm. (At this time the water level at the sampling site was 34–42 cm) The observed characteristics indicate the effects of the drop in water levels, because shallower water warms up more powerfully, and the level of available light also changes. This influences significantly the littoral zone's flora and fauna. In our case this meant the increase in macroinvertebrates in the sampling site.

The strong increase in biomass in the open water area in the second half of 2003 coincides with the appearance and densing of *Najas marina* stands which stayed in its place falling to the bottom even in the autumn. The more "reed-grassy" quality of 2003 is due to the appearance of *Najas marina* in the open water area and because in the broader surroundings of the sapmpling site patches of reed-grass was appeared (mainly *Potamogeton perfoliatus*). It is important to know about the *Najas marina* that in Lake Balaton (as elswhere) it forms mainly underwater colonies, during its life cycle not one part of this plant emerges above the water level [16]. Therefore it is presumable that the extremely low water level is the reason for it reaching the surface of the water in 2003. This plant may have been present underwater in 2002 as well, but definitely not in such abundant quantities.

While reeds may be considered the most diverse, this may not be started for the whole of the macroinvertebrates. In general the most macroinvertebrates emerge from reeds compared to the other microhabitats, only in the summer period, and in 2004 even this was not true. In 2004 (more or less) during the whole year cattail attracted larger


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quantities than reeds. When the *Potamogeton perfoliatus* fields became significant this reed-grass area supports the largest quantity of macroinvertebrates.

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# ASSESSMENT OF MICROBIAL (BACTERIA) CONCENTRATIONS OF AMBIENT AIR AT SEMI-ARID URBAN REGION: INFLUENCE OF METEOROLOGICAL FACTORS

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**Abstract.** In the present study, outdoor airborne microflora (bacteria) at different locations, viz; institutional, health care, commercial, traffic, industrial and agricultural areas of Tirupati — a semi-arid urban region, southern peninsular India was investigated during winter season, 2004. Concentrations of airborne viable bacteria averaged between 19 ± 5 CFU/$m^3$ (IE) and 3 ± 5 CFU/$m^3$ (SVU) and observed the following trend among the locations: IE > TG > CBS > TUDA > RUYA > SVU. Airborne Gram-positive bacteria were most abundant, with more than 60 to 90% of the measured population at each location. Developed regression models have been explained about 50% (or greater) variation in bacteria concentration at each location (except RUYA), due to the effect of meteorological factors – temperature, RH, and wind speed. Among these factors, wind speed had the most pronounced influence on bacterial concentration, with the regression coefficient (β) varied between 0.225 and 2.092, followed by the temperature. The overall air quality index ($A_BQI$) with respect to bacterial composition of aerosol is found to be 22.33 which signifies that the quality of air is good. The results reveal that the airborne bacteria are contributed from terrestrial (soil) sources greatly followed by the little contribution from point sources.
***Keywords.*** *Bioaerosol, airborne bacteria, meteorological factors, multiple linear regression, quality index, India.*

## Introduction

Earth's atmosphere is known to team with airborne microorganisms, though the high light intensities, extreme temperature variations, low concentrations of organic matter, and a scarcity of water, make the atmosphere as unsuitable environment habitat for microbial growth [1]. Biological material may contribute about 20%, 22% and 10% to the total airborne particulate by volume in remote continental, populated continental and remote maritime environments respectively [2]. Most of them originate from natural sources such as soil, lakes, animals and humans [3]. Moreover, agricultural practices, healthcare units and industrial operations such as sewage treatment, animal rendering, fermentation processes, and food processing plants also emit viable microorganisms into the air [4].

Exposure to bioaerosol pollution is now an almost inescapable feature of urban living throughout the world, which associated with a wide range of adverse health effects including contagious infectious diseases, acute toxic effects, allergies and cancer [4,5]. Inhalation is of the predominant route of exposure resulting in adverse health effects. Other types of exposure, namely ingestion and skin contact may also be present besides inhalation [6]. Although the relationship is still poorly defined, increased mortality and


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morbidity believed to be caused by urban air microbial (bioaerosol) pollution are of great concern. Among the microorganisms present in the atmosphere, bacteria are often the highest in number, despite their high death rate due to environmental factors producing stress of various kinds, of the major being dehydration stress [7]. Though, most of the bacteria, or bacterial agents are not very potent allergens with the exception of spore forming actinomycetes; bacterial cell wall components, such as endotoxin (most prevalent in gram negative bacteria) and peptidoglycens (most prevalent in gram positive bacteria) are crucial agents with important pro-inflamatory properties that may induce respiratory symptoms [8].

It is important to note that geography and climate play an important role in determining the outdoor air microbial concentrations because the transport of bioaerosol is primarily governed by hydrodynamic and kinetic factors, while their fate is dependent on their specific chemical makeup and the meteorological factors to which they are exposed. The most significant environmental factors influencing the viability of microorganisms are temperature, relative humidity (RH), and wind velocity [9]. Also, the additional influences are exerted through oxygen, air ions, solar irradiance, and open-air factors. sHence, the monitoring of outdoor airborne microorganisms is necessary to evaluate the risk on human health and to study its evolution, and the interest in bioaerosol characterization has increased over the last few decades [10]. Most of these studies were carried through airborne fungi [11]. But up to now there are the limitations of the data available from monitoring for the bacteria found in the atmosphere [12].

The present study aim to investigate the current atmospheric load of airborne bacteria at different locations in Tirupati, a world famous pilgrim centre, where no survey of airborne bacteria has been attempted till now. Moreover, to estimate the influence of meteorological factors on bioaerosol along with bioaerosol pollution through modelling and quality indexing approach respectively.

## Materials and Methods

### *Study area*

Tirupati, a holy pilgrimage town for devotees of Lord Sri Venkateswara is situated in Chittoor district of southern Andhra Pradesh state in India at an altitude of 182.9 m (13.05 0 N latitude; 79.05 0E longitude). Tirupati — a semi-arid region prevailing the continental type of climate with three distinct seasons: winter, summer and monsoon, represents an urban area surrounded by major industrial and agricultural activities [13]. The meteorological data (weekly average) for the study period are given in Table 1. Sampling points were selected for the collection of airborne bacteria, based on the specific activity of the area in different parts of Tirupati. Sri Venkateswara University (SVU) campus, which represents the institutional area encompassed with educational institutions and good plantation. Tirupati urban development authority (TUDA) premises, represents the higher commercial prone with constant automobile traffic, vegetable market and residential density beside the site. Government hospital (RUYA) located towards north of the town with dense of forest represents the health care zone. Sri Venkateswara dairy firm (SVDF), is surrounded with cultivating fields located towards the west of the town represents the agricultural area.


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***Table 1*** *Weekly average level of meteorological factors during the study period (January to February, 2004) at Tirupati.*

| Week No. | Temperature ($^0$C) | | Relative humidity (%) | | Wind vel. (km/hr) |
|---|---|---|---|---|---|
| | Min. | Max. | I | II | |
| 1 | 17.90 | 28.66 | 79.00 | 49.29 | 8.10 |
| 2 | 15.30 | 28.43 | 70.86 | 36.43 | 8.60 |
| 3 | 14.01 | 30.00 | 78.86 | 35.71 | 6.28 |
| 4 | 19.98 | 30.89 | 77.57 | 49.57 | 6.03 |
| 5 | 19.50 | 31.46 | 78.71 | 41.57 | 7.01 |
| 6 | 18.61 | 31.53 | 73.00 | 42.71 | 7.44 |
| 7 | 15.96 | 31.16 | 64.43 | 33.71 | 6.88 |
| 8 | 15.53 | 33.37 | 51.57 | 23.71 | 7.07 |
| 9 | 16.60 | 34.47 | 56.00 | 20.00 | 6.48 |

While central bus station (CBS) located at center of the town, passing national high way and railway track beside the site, represents the severe traffic prone, whereas industrial estate (IE) located towards the east of the town is encompassed with different types of major and small scale industries along with food processing units and sewage treatment plants.

### *Sampling and Analysis*

Airborne bacteria were collected by impaction onto an agar medium, using a portable Mini-Patrisol Air Sampler PM10 (Model 2100, Ruppricht & Patashnik, USA) operating at about 10 l/m3 for 2 to 8 minutes, with a frequency of about once in a week during winter season (i.e. from January to Februay, 2004) placing at about 1.5 m above the surface, to simulate the human breathing zone. Before or after each sampling, the sampler surface was disinfected with a 70% ethonal solution. Sampling has been carried out for all the six sampling points on each sampling day during daylight hours, usually between 09.00 and 17.00 hrs. A modified version of the National air monitoring schedule (MNMS), excluding weekends, was used to assure that the sampling events were uniformly distributed among days during the workweek.

Sampled Nutrient agar plates were incubated at 30 0C and the aerobic bacteria were enumerated after 48 hours [14]. Cycloheximide, final concentration of 0.5 mg/ml, which has been previously shown not to affect bacterial counts [15], was added to the media to inhibit growth of fungi. Further on each plate, approximately 50% of the entire colonies were isolated for partial identification by light microscopy based on Gram reaction and bacterial morphology.


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### *Modelling approach*

Modelling technique in environmental research is gaining popularity because of the possibility that exists for the representation of complex problems in one model, which can be used to analyse and forecast the real problems [16,17). In the present study, multiple stepwise linear regression procedure was used to estimate the impact of meteorological factors (temperature, RH and wind velocity) on airborne bacteria concentration and the form of this linear regression model is:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \varepsilon \qquad \text{Eq. 1.}$$

where Y is bacteria concentration, X1 is temperature, X2 is relative humidity, and X3 is wind velocity. β0 is a constant representing the general level of bacteria irrespective of the effect of meteorological factors. β1 is regression coefficient indicating the marginal effect of temperature, β2 is regression coefficient indicating the marginal effect of RH and β3 is regression coefficient indicating the marginal effect of wind velocity.

## Results and Discussion

### *Variation in ambient air levels of bacteria*

Table 2 presents the average levels of total culturable bacteria along with statistical results for each location. Concentration of airborne bacteria varied in the range of 1 to 32 CFU/m3 (Fig. 1) for different locations in Tirupati during the study period (January to February, 2004); the obtained bacterial counts were consistent with previous data where airborne bacteria varied between 10 to 100 CFU/m3 [18]. Among the locations studied, IE showed the highest level (19 CFU/m3) where the food processing units and sewage treatment plants existing might have contributed greatly. Also, the bare soil surrounding the location may have contributed significantly, which is further evident from the higher percentage of Gram positive bacteria [19]. Subsequently SVDF (16 CFU/m3) and CBS (15 CFU/m3) showed the greater levels of airborne bacteria. High background level of bacteria at SVDF might be due to constant input from the vegetation along with soil [20]. Abundance of bacteria at CBS may be due to many factors such as vehicle traffic, turbulent airflow, and amount of suspended dust and density of people carrying germs. It is further evident from the strong correlation with atmospheric pollutants (NO, CO, Hydrocarbons), which may have a protective effect on microorganisms, depending on microbial species [21]. While TUDA is with moderate concentration, both SVU and RUYA showed the lowest levels of bacteria. It represents the better cleanliness along with the maintenance of sterile conditions at respective sites, whereas the moderate concentration at TUDA is due to the rottening of wastage vegetables and the density of people carrying germs and also the urban air is constantly stirred up by a steady stream of automobile traffic. But the level of bacteria at each location are well below the threshold value (TLV), 50 CFU/m3 (WHO).


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***Table 2*** *Average ambient air levels of bacteria (CFU/m3) along with statistical results at each location of Tirupati.*

| Location | Mean ± SD | Variance | Kurtosis | Skewness | Minimum | Maximum |
|---|---|---|---|---|---|---|
| SVU | 3 ± 1 | 1.50 | -0.2857 | 8.37E-17 | 01 | 05 |
| RUYA | 4 ± 2 | 2.50 | -0.5143 | 0.2711 | 02 | 07 |
| TUDA | 10 ± 2 | 5.19 | -0.5948 | 0.3827 | 07 | 14 |
| CBS | 15 ± 1 | 5.36 | -0.7002 | -0.26 | 13 | 17 |
| IE | 19 ± 5 | 24.50 | 0.4301 | 0.8172 | 13 | 29 |
| SVDF | 16 ± 3 | 11.11 | -0.0517 | 0.2663 | 11 | 22 |

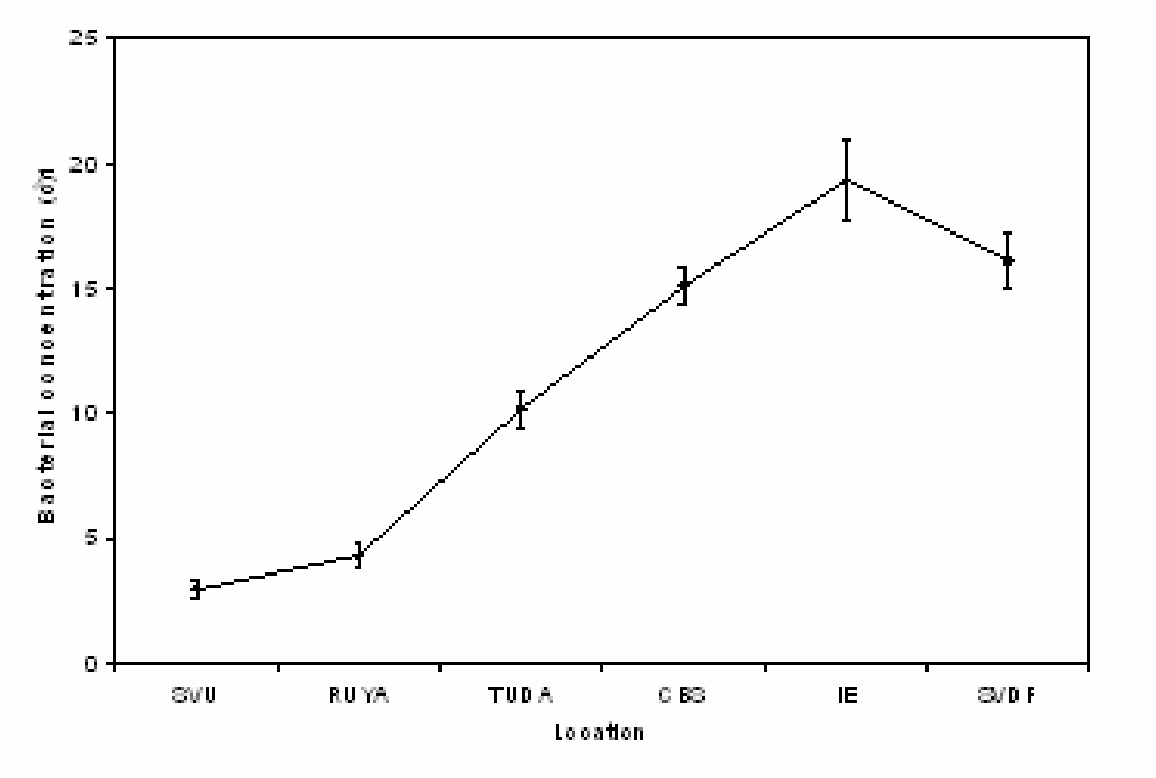


***Figure 1*** *Mean level of airborne bacteria showing the standard errors at each location of Tirupati*


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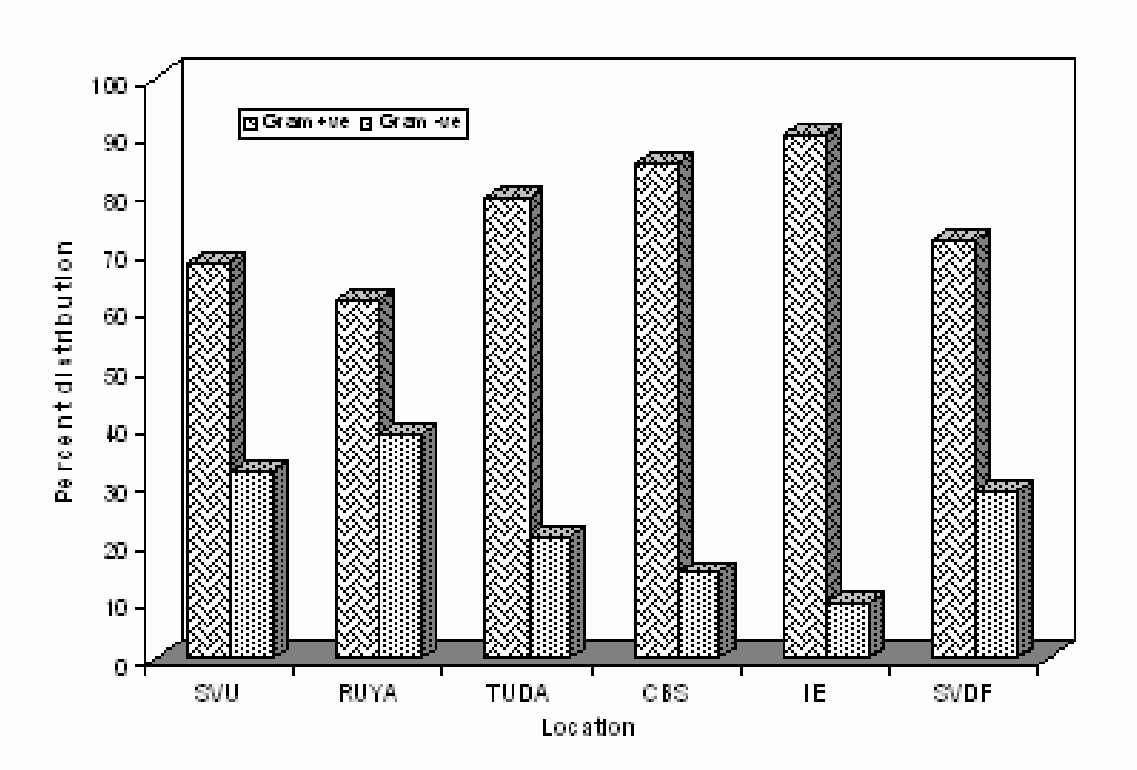


***Figure 2*** *Frequency distribution of Gram positive and Gram negative bacteria at each location of Tirupati*

Kurtosis calculations on the data set have given a negative value at each location except industrial estate (Table 2). This characterises a flatness of the distribution of the bacteria compared with the normal distribution. Whereas the positive kurtosis at industrial estate indicates a relatively peaked distribution, which signifies that there are sporadic high emissions of bacteria from certain sources causing a peaked distribution. Skewness calculations in a data set characterize the degree of asymmetry of a distribution around the mean. Positive and negative skewness indicate the distribution with an asymmetric tail extending toward values that are more positive and negative respectively. As for as asymmetry in the data set is concerned this study has shown a wide variability (Table 2), which is mainly the result of the meteorological effects, and further of the widely changeable source emission pattern. Standard deviation calculations on the data set show a lower dispersion of the values of the bacteria while the variance measure the extent to which the actual observations vary from the central value during the study period.

The different forms of airborne bacteria, as well as their Gram reaction showed un similar pattern of percentage distribution at each and every location of Tirupati (Fig. 2). The concentration of airborne Gram positive bacteria were significantly larger than airborne Gram negative bacteria (which was less than 10%) and is in agreement with other reports [22]. It was previously demonstrated that Gram positive bacteria have greater resistance and survival ability outdoors than Gram negative bacteria under strong sunlight [23]; that characterize the troposphere of Tirupati; can only be found in the spore form. From the results (Fig. 2) it is evident that Gram negative bacteria has been occurred in high concentration at RUYA (38.46%) and SVU (32%) followed by


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SVDF (28.58%) where the richest plantation is available, when compared with other locations. Increase in Gram negative bacteria in urban areas has been reported in previous studies [18] suggesting that sampling environment had a qualitative influence on airborne contamination and the additional sources of contamination such as hospital incinerator which has previously been reported to produce bacteria in the airborne state may have increased the Gram negative bacteria in respective locations. Apart from this, plants may release the Gram negative bacteria heavily. Abdel Hameed and Khoudr [20] reported that the suspended particulate in agricultural fields may contain about 103 CFU/gm of Gram negative bacteria and contribute about 2/3 to the total airborne bacteria. At each site, the greater contribution of gram positive bacteria has been observed, which indicates that the bacteria are highly contributed from the soil at Tirupati.

### *Influence of Meteorological Factors*

The impact of meteorological factors such as temperature, RH and wind velocity on airborne bacteria concentration has been examined through multiple linear regression modelling approach, which provides the effect of each meteorological factor on bacteria concentration. Apart from the point sources, the environmental factors can affect the level of bioaerosol in atmosphere. The data for the study period has been used to fit the regression models for each location separately through stepwise regression procedure with SPSS software version 11.5, and the resulted models along with R2 and F-value are presented in Table 3.

***Table 3*** *Regression models developed for each location at Tirupati*

| Location | Regrssion Model | $R^2$-value | F-value |
|---|---|---|---|
| SVU | -4.920 + 0.241 (temp) - 0.051 (RH) + 0.746 (wind vel.) | 0.572 | 2.232 |
| RUYA | 9.910 - 0.312 (temp) + 0.0095 (RH) + 0.225 (wind vel.) | 0.275 | 0.831 |
| TUDA | -1.450 + 0.181 (temp) + 0.0909 (RH) + 0.346 (wind vel.) | 0.466 | 1.453 |
| CBS | 3.291 + 0.0419 (temp) - 0.0008 (RH) + 1.659 (wind vel.) | 0.798 | 6.579 |
| IE | 11.205 - 0.745 (temp) + 0.204 (RH) + 2.092 (wind vel.) | 0.769 | 5.556 |
| SVDF | -10.269 + 0.740 (temp) - 0.0684 (RH) + 1.865 (wind vel.) | 0.504 | 1.695 |

Regression model showed that the meteorological factors have significant influence on airborne bacteria concentration. The models obtained can explain about 27.5, 46.6, 50.4, 57.2, 76.9 and 79.8 percent of variation in bacteria concentration in terms of wind velocity, temperature and RH at different locations; RUYA, TUDA, SVDF, SVU, IE and CBS respectively. The regression model developed for both CBS and IE are well fitted and were statistically significant ($p = 0.048$ and 0.000 respectively) where as other models do not show the significant value ($p = <0.05$) but the model at each site explained the significant variation in bacteria, which signifies that the bacteria at each site might have contributed greatly from similar source, probably terrestrial sources along with few more point sources with respect to the site.


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Wind velocity showed a very good positive correlation at each location (Table 3) signifying that the bacteria concentration will increase with increasing wind velocity. Wind velocity has showed the highest regression coefficient (β=2.092) at IE and lowest at RUYA (β=0.225), means the increase in 1 km/hr leads to a marginal increase of 2.092 CFU/m3 at IE and 0.225 CFU/m3 at RUYA respectively. The influence of wind velocity as a dilution and survival factor of airborne bacteria has been largely demonstrated in dispersion models and environmental reports [24], and the results are in good agreement with previous reports [25].

Temperature is also a significant variation factor for airborne bacteria, which governs the rate of change of water vapor and the rate of change of heat between the surface and environment. It also affects the viability of airborne bacteria through the evaporation of their cellular water. In the present study temperature showed the positive correlation at TG (β=0.740), SVU (β=0.241), TUDA (β=0.181) and CBS (β=0419) and also the negative correlation at RUYA (β=0.312) and IE (β=0.745) respectively. Moreover, most environmental studies have reported the significant effect of temperature on bacterial counts depending on species and sampling environments [26]. The highest positive correlation (β=0.740) was observed at SVDF where the activity is agricultural and most of the bacteria might have contributed from vegetation. It shows that the release of bacteria will be increased with increasing temperature by reducing the binding force [27]. The highest negative correlation (β=0.745) was observed at IE where the bacteria might have contributed from terrestrial (bare soil) along with point sources, due to damaging effects of UV radiation [28].

Relative humidity (RH) showed a relatively low significant effect on airborne bacteria. It showed a positive correlation at RUYA (β=0.0095), TUDA (β=0.0909) and IE (β=0.204) and also the negative correlation at SVDF (β=0.0684), SVU (β=0.051) and CBS (β=0.0008) respectively. Among all the locations a significant correlation was observed only at IE (β=0.204) i.e. the bacteria concentration increases with increasing RH which is in good agreement with other reports [29]. Though the negative correlation at SVDF and SVU is not significant which also coincides with other previous reports [30], and there is no obvious relationship between RH and airborne bacteria at CBS (β=0.0008). The results reveal that the concentration of bacteria may increase either with the increase or decrease in RH, because the higher percent of RH favors the viability where as the lower percent of RH favors the spore release in greater number.

### *Assessment of Bioaersol Pollution*

Air quality index (AQI) shows how clean or polluted air is, and what associated health affects might be a concern for human beings (EPA). Air quality indices (ABQI's) obtained for each site along with percentage of deviation with mean value were presented in Table 4.


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***Table 4*** *Air quality index (ABQI) with respect to ambient air levels of bacteria at Tirupati.*

| Location | $A_BQI$ | % of Deviation with mean value |
|---|---|---|
| SVU | 06.00 | -73.13 |
| RUYA | 08.00 | -64.17 |
| TUDA | 20.00 | -10.43 |
| CBS | 30.00 | 34.35 |
| IE | 38.00 | 70.17 |
| SVDF | 32.00 | 43.41 |
| Overall (Mean) | 22.33 | |

The indicies enable us to assess the quality of air at each location and can also be used to compare with each other. The higher the ABQI value the greater the level of bioaerosol pollution and greater the health concern. An ABQI value of 100 generally corresponds to the National Air Quality Standard for the pollutant, which is the level, set to protect public health. ABQI values below 100 are generally thought of as satisfactory. When ABQI values are above 100, air quality is considered to be unhealthy at first for certain sensitive groups of people, thus for every one as ABQI values get higher. The ABQI for each location individually and the overall ABQI for Tirupati are found to be well below the permissible value (Table 4), which indicates that the overall air quality of Tirupati with respect to ambient air levels of bacteria is very good. Among the different locations studied, IE showed the good air quality (ABQI > 33) and all other locations showed very good quality of air (ABQI < 33) which reveal that the region is free from bioaerosol pollution due to the contamination airborne bacteria. The trend of air quality for different locations in Tirupati is as follows: SVU > RUYA > TUDA > CBS > SVDF > IE.

## Conclusion

A study on ambient air levels of bacteria and the influence of meteorological factors on airborne bacteria concentration have been carried out at different locations of Tirupat, a world famous pilgrim centre. Highest bacterial levels were observed at IE (19 CFU/m3) followed by SVDF (16 CFU/m3) and CBS (15 CFU/m3) and the lowest at SVU (3 CFU/m3). Airborne Gram-positive bacteria contributed highly, with more than 60 to 90% of measured population at each location, which signifies that the bacteria might have released from terrestrial (soil) sources greatly. Among the meteorological factors – temperature, RH and wind velocity, the wind velocity had showed the pronounced effect on bacterial concentrations with positive correlation ($\beta$=0.225 to 2.092). The resulted regression models have explained about 27.5 to 79.8% of variation of the airborne bacterial concentration at different locations. The overall BAQI was found to be 22.33, which reveals that the quality of air at Tirupati is good in terms of bioaerosol (airborne bacteria) pollution.


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