6.1 Distribution of Bacterial Pathogens of Mosquitoes in Natural Habitats

Since the discovery of *B. thuringiensis var. israelensis* (Goldberg and Margalit, 1977), the search for new bacterial isolates from nature has been explored by several workers (Brownbridge and Margalit, 1986, 1987a; Delucca *et al.*, 1979, 1981, 1984; Martin and Travers, 1989; Padua, 1982; Padua *et al.*, 1980, 1984; Weiser, 1984; Weiser *et al.*, 1985 and Wickremesinghe and Mendis, 1980). An attempt has been made for the first time for assessing the application of *B. thuringiensis* or *B. sphaericus* from the samples collected from different habitats (soil, water, larval and aquatic root weeds) over a period of two years from Doon Valley, Rishikesh, Muni-ki-reti and Kotdwar, the Sub-montane region of Garhwal Himalaya. In all, 1942 samples yielded 312 mosquito larvicidal bacilli, belonging to different serotypes of *B. thuringiensis* or *B. sphaericus* indicating their wide distribution in nature. Comparatively, *B. thuringiensis* was more predominant than *B. sphaericus*. Several workers have earlier reported the wide distribution of *B. thuringiensis* (Aizawa *et al.*, 1961; Alfazairy, 1986; Goldberg and Margalit, 1977; Padua *et al.*, 1980, 1984; Knowles *et al.*, 1986; Ohba and Aizawa, 1978, 1986; Ohba *et al.*, 1979; Travers
DISCUSSION


The present contribution revealed more larvicidal bacilli isolated from soil followed by aquatic vegetation, water and immature specimens of mosquitoes. Further, B. thuringiensis isolates were comparatively more abundant than B. sphaericus. Lee and Seleena (1990) and Orduz et al. (1992) isolated various strains of B. thuringiensis and B. sphaericus from soil, water and debris samples collected from Malaysia and Columbia.

The present findings are in conformity with the results obtained by Manonmani et al. (1990) on larvicidal activity of indigenous isolates of B. sphaericus from natural breeding habitats like soil water and moribund or dead mosquito larvae and the studies made by Li et al. (1999); Ohba et al. (2000); Maeda et al. (2001); Quesada-Moraga et al. (2004) and Balaraman (2005) in isolating biolarvicidal strain from natural habitat and toxicity to mosquito larvae.

A. Distribution of Bacterial Pathogens of Mosquitoes in Soil

A total of 792 soil samples 131 yielded mosquito larvicidal bacilli, belonging to different serotypes of B. thuringiensis or B. sphaericus indicating their wide distribution in nature under the present study. It has been noted that B. thuringiensis is more predominant than B. sphaericus. The data also indicated that the B. thuringiensis is 13.13% and B. sphaericus is 3.4% of the total isolates collected from the soil. Four kinds
of serotypes of *B. thuringiensis* and three types of serotypes of *B. sphaericus* were reported under the present study.

Delucca *et al.* (1981) obtained 12 *B. thuringiensis* strains from 63 soil samples collected from cultivated land, grassy land, rock lands and virgin woods in USA and further more 4 different serotype were obtained. There was a significant difference between the present findings and the results of Delucca *et al.* (1981) in respect of types of serotypes. On the other hand, Weiser *et al.* (1984) collected 5 strains of *B. thuringiensis* obtained from 32 soil samples collected from mosquito breeding places of Nigeria. Manonmani *et al.* (1987), Ohba and Aizawa (1986) and Asimeng and Mutinga (1992) reported *B. thuringiensis* from soil samples of different regions.

Isolation of *B. sphaericus* isolates from the soil and mud has been carried out by a number of workers from different parts of the globe (Massie *et al*., 1985; Brownbridge and Margalit, 1987; Manonmani *et al*., 1990; Gupta *et al*., 1991 and Orduz *et al*., 1992; Monnerat *et al*., 2005). However, the workers like Lee (1988) and Lee and Seleena (1990) furnished information regarding the occurrence of both *B. thuringiensis* and *B. sphaericus* from soil samples of different habitats. Almost similar findings have been reported under the present study. The soil samples collected from habitats like ponds, paddy fields and streambed were found positive for both *B. thuringiensis* and *B. sphaericus*. None of the authors reported about the paddy fields from where the maximum isolates of bacilli were obtained. The present findings are also in accordance with the findings of Lee and Seleena (1990) in terms of wide spread use of *B. thuringiensis* and rare use of *B. sphaericus*. 
In the present study, more number of isolates of *B. thuringiensis* were obtained from the soil of paddy fields and transient pools, while, the less number of isolates were encountered from the soil of seepage pools, streambed and farmyard. With regard to *B. sphaericus* isolates, the maximum number of isolates was obtained from the soil of paddy fields followed by pond and streambeds. Thus it is clear that the soil from paddy fields contains more bacterial strains in the submontane region. Paddy fields were rich in *B. thuringiensis* strains yielding 36% of the strains from the soil habitats. The poorest sources were riverbed, grassland pools, farmyards and canals. For *B. sphaericus*, the paddy fields were also recorded as the better source. The death leaves and roots exudes of paddy may be the rich sources of nutrition for these strains. *B. thuringiensis* and H-14 among *B. sphaericus* H-5a5b serotypes were widely distributed in different habitats. The above two highly specific serotypes and their predominance reflected the ubiquitous nature of mosquitoes. Moreover, several strains of *B. thuringiensis* unknown serotypes showed different levels of toxicity ranging from extremely toxic to poorly toxic categories. Highly toxic strains of *B. thuringiensis* and *B. sphaericus* were confined to paddy fields and this may probably be due to the occurrence of epizootics in these habitats.

**B) Distribution of Bacterial Pathogens of Mosquitoes in Aquatic and Marshy plants**

In the present study out of 140 samples of aquatic and marshy plants collected from different natural habitats like ponds, canals, river, transient pools, cesspits yielded mosquito larvicidal bacilli belonging to different serotypes of *B. thuringiensis* and *B. sphaericus* indicating their wide distribution in nature. Comparatively, *B. sphaericus* was more predominant than *B. thuringiensis*. Several workers have earlier reported
the wide distribution of *B. thuringiensis* (Lee and Cheong, 1988; Gupta *et al.*, 1991, Grigarova *et al.*, 1988; Lysenko *et al.*, 1985; Singer, 1973; Singh, 1987). Further, the present findings are in accordance with Manonmani *et al.* (1991) based on the isolation of mosquito pathogenic bacilli from the roots of aquatic and marshy plants. However, the difference is that Manonmani *et al.* (1991) did screening on aquatic plants only, while under the present study, marshy plants have also been considered. There is a similarity between the present findings and Manonmani *et al.* (1991) in relation to abundance of maximum isolates of *B. sphaericus* than *B. thuringiensis*. Further, Manonmani *et al.* (1991) did not mention the details of sources of samples as in case of the present study. Nevertheless, majority of isolates of bacilli were recovered from ponds and canals while less number were recorded from the remaining habitats (riverbed, transient pools and cesspits). Aquatic plants had more larvicidal bacilli as compared to marshy plants. As far as the serotypes and toxicity groups of *B. thuringiensis* and *B. sphaericus* strains are concerned, there was a marked difference with the findings of Manonmani *et al.* (1991). Extremely toxic and highly toxic groups were relatively more than the toxic, moderately toxic and poorly toxic groups.

The rhizosphere region of hydrophytes is usually rich in sugars, amino acids, growth factors and other organic compounds because of the root exudates and hence is characterized by the high number and activity of microorganisms (Vancura, 1964; Rovira, 1965, 1969). Such a favourable environment might have attracted *B. thuringiensis* and *B. sphaericus* also, as is evident from the high incidence of these bacilli in root samples, in the present investigation. Between the two species, the incidence of *B. sphaericus* was more as compared to *B. thuringiensis*. The possible explanation is that *B. sphaericus* is more exacting carbohydrates and
vitamins, the nutrients readily available in the roots exudates favoring preferential colonization (Buchanan and Gibbons, 1975; Kuppusamy and Balaraman, 1990).

Ejiofor and Okafor (1988) and Lee and Seleena (1990) carried out a study on the isolation of larvicidal microbial control agent of mosquito from the vegetation. The strains as recorded by them are quite different and therefore in absence of any concrete evidences it is difficult to offer any explanation for these variations. Further, the data of the present study demonstrate that both *B. sphaericus* and *B. thuringiensis* colonized on the roots of aquatic and marshy plants and the chances of successful isolation of highly potent strains from them seems higher as compared to other sources.

C) **Distribution of Bacterial Pathogens of Mosquitoes in Water**

Under the present study, out of 399 water samples, 51 yielded mosquito larvicidal bacilli, belonging to different serotypes of *B. thuringiensis* or *B. sphaericus* indicating their wide distribution in nature. Earlier several workers have reported the wide distribution of *B. thuringiensis* in water (Ohba and Aizawa, 1978, Ohba et al. 1979, Wickremesinghe et al., 1980; Padua et al., 1981; Lee, 1988; Lee and Chong, 1988; Lee and Seleena, 1990; Gupta et al., 1991; Manonmani et al., 1991; Ohba et al., 1981 and Gunasekaran et al., 2002) or *Bacillus sphaericus* isolates (Grigarova et al., 1988; Lysenko et al., 1985; Singer, 1973; Singh, 1987; Brownbridge and Margalit, 1987; Manonmani et al., 1990 and Gupta et al., 1991).

Transient pools were found to be rich in *B. thuringiensis* strains yielding 60.78 % of the strains from the water samples. The poorest
sources were found to be lakes, seepage, pools and wells. On the contrary, for *B. sphaericus*, the canals and ponds were recorded as the better source. The serotype H-14, among *B. thuringiensis* and H-5a5b among *B. sphaericus* were widely distributed in different habitats. These two serotypes were highly specific to mosquitoes, since their predominance reflects the ubiquitous nature of mosquitoes. Several strains of *B. thuringiensis* unknown serotypes showed different levels of toxicity ranging from extremely toxic to poorly toxic categories.

The present findings were in conformity with the findings of Manonmani et al. (1987) who isolated mosquito pathogenic *B. thuringiensis* strains from breeding habitats in south India. They isolated 101 strains of *B. thuringiensis* (H-14) and 11 strains of *B. thuringiensis* non (H-14) serotypes. While in the present study, only 44 isolates of *B. thuringiensis* were recorded, of which 31 belonged to H-14. Further, the difference in respect of kind of habitats has also been found to be significant under the present study.

Subsequently in 1990, Manonmani et al. further isolated indigenous isolates of *B. sphaericus* from natural breeding habitats under three serogroups (H5a5b, H-6 and H-45). While in the present study, only H5a5b was isolated in addition to the nature of habitats. The present findings differs with the findings of Lee and Seleena (1990) and Lee (1988) in isolating the kind of strains of both *B. sphaericus* and *B. thuringiensis*. Similar observations was recorded in term of isolation of both *B. thuringiensis* and *B. sphaericus* from the water samples.

Highly toxic strains of *B. thuringiensis* and *B. sphaericus* were confined to ponds and transient pools. This probably may be due to the presence of epizootics in these habitats. De Lucca et al. (1981) opined that
B. thuringiensis initiates epizootics when the external environmental conditions aggravate the situation (the problem of survival of insect population during the drying up of habitats). The chances for such phenomenon are more frequent in confined temporary habitats such as ponds and transient pools, thus, leading to the occurrence of extremely toxic strains.

These results lead to conclude that *B. thuringiensis* and *B. sphaericus* are widely distributed in nature. Moreover, isolates of *B. thuringiensis* were more dominant than the *B. sphaericus*. This may be due to an alteration in the recycling process in nature caused by natural and anthropogenic activities.

**D) Distribution of Bacterial Pathogens of Mosquitoes in Mosquito Larvae**

In the present investigation, out of 581 samples of mosquitoes larvae processed, only 28 isolates of *B. thuringiensis* and *B. sphaericus* were found positive. Although the prevalence of both *B. thuringiensis* and *B. sphaericus* was confined to mosquito larvae collected from ponds, canals, seepage pools, transient pools, drainage and laboratory infection (rearing colony). *B. thuringiensis* was recorded maximum from transient pools while, *B. sphaericus* from ponds. Mikhnovaska *et al.* (1972) explored bacterial flora of Culicid larvae *Culex pipiens molestus* and *Aedes aegypti* with special emphasis to their entomopathogenic properties. Later on, Vashanthi and Hoti (1992) made a study on the microbial flora (bacteria, fungi and actenomycetes) in the gut of *Cx. quinquefasciatus* found breeding in cesspits. Among bacteria, maximum number of isolates of *Bacillus* were recorded thus establishing a similarity with their present findings.
There are a number of contributions on the isolation of bacterial larvicides from the insect material (Aizawa et al., 1961; Bulla et al., 1975; Goldberg and Margalit, 1977; Grigarova et al., 1988; Weiser et al., 1985; Alfazairy, 1986; Lee, 1988 and Balaraman, 2005). The results of the present findings were similar in terms of kind of strains isolated and the sampling material collected.

Goldberg et al. (1977) isolated B. thuringiensis (H-14), the first strain from dead larvae of Culex pipiens collected from brackish water pools in Israel. Subsequently, Manonmani et al. (1987) isolated mosquito pathogenic B. thuringiensis strains from the mosquito larvae collected from various breeding habitats in South India. The present findings slightly differ from the findings of Manonmani (1987) in respect of the kind of habitats of mosquito larval sample collected and also in the kind of bacterial isolates.

Earlier Biswas et al. (1988) isolated biolarvicides from laboratory colonies of An. annularis. But in the present study, bacterial isolates were obtained from An. subpictus, An. culicifacies, Cx. quinquefasciatus, Cx. mimeticus and Ae. aegypti larvae.

Manonmani et al. (1990) isolated indigenous strains of B. sphaericus from dead mosquito larval samples collected from casurine plantation and rearing colonies of Cx. quinquefasciatus. However, in the present study, positive isolates for B. thuringiensis and B. sphaericus were recorded from ponds, canals, seepage pools, transient pools, drains and laboratory colonies. Hence, if appears a similarity in terms of laboratory infection of mosquito larvae. Another significant difference is concerned with isolation of bacterial strains from the following 5 species of mosquito
larvae - *An. subpictus, An. culicifacies, Cx. quinquefasciatus, Cx. mimeticus* and *Ae. aegypti*.

Gupta *et al.* (1991) isolated an indigenous serotype of *B. sphaericus* H-5a (9001) which possessed a high insecticidal property from the larvae of *Culex* species. But in the present investigation, no such strains were screened. Moreover, the authors didn’t mention the source of the strains of mosquito larvae.

Luxananil *et al.* (2001) isolated bacterial strains from mosquito larval guts of *Aedes aegypti, Anopheles dirus* and *Culex quinquefasciatus* collected from natural breeding habitats. They isolated strains of *B. cereus* and *B. thuringiensis* serovar *israelensis* c4Q2-72 and there exists a major difference with regard to the kind of strains isolated. However, there is a similarity, if the mosquito species like *Ae. aegypti* and *Cx. quinquefasciatus* are concerned. Further, the present findings also differ with the findings of Luxananil *et al.* (2001), as later didn’t mentioned any thing about the habitat source of mosquito larvae.

From the above statements, it is clear that the mosquito pathogenic strains of *B. thuringiensis* and *B. sphaericus* are obtained from a variety of habitats, indicating a wide natural distribution of larvicidal bacilli. Earlier, it has been opined that dead insects are better sources of pathogenic isolates of bacteria (*Goldberg et al.*, 1977), but, in the present study, entomotoxic isolates were obtained not only from dead larvae but also from soil and water samples. This indicates that apart from inset material, soil, water and aquatic and marshy plants in the submontane region of Garhwal Himalaya are also good sources for mosquito pathogenic *B. thuringiensis* and *B. sphaericus*. 
Transient pools were rich in *B. thuringiensis* strains yielding 27.5% of the strains from the soil habitats while in the water 16% of the strains were recorded. The poorest sources were lakes, rivers, farmyards, termitaria and cesspits. In the absence of any concrete evidence, it is difficult to offer any explanation for these variations.

For *B. sphaericus*, ponds were better sources accounting for 23.1% of the total strains obtained. This is because of the root samples of hydrophytes collected from these habitats which may have influenced *B. sphaericus* population as the root exudates of plants are known to be rich in nutrients.

Among different samples, the incidence of *B. thuringiensis* and / or *B. sphaericus* was higher in the roots of hydrophytes *i.e.*, 72% of them were positive whereas only 19.7% of soil, 13.6% of water and 4.6% of larvae were positive. Hitherto, dead insects have been reported as better sources of larvicidal bacilli (Kellen *et al.*, 1965; Singer, 1973; Weiser, 1984). On the contrary, in the present study, roots of aquatic plants were found to be the better sources followed by soil and water and larvae found to be the poorest sources.

The serotype H-14 among *B. thuringiensis* and H-5a5b among *B. sphaericus* was widely distributed in different habitats. Besides *B. thuringiensis* serotype H-14, strains belonging to other serotypes such as H-10, H-12, H-16, H-17, H-20 and unknown ones were also encountered. The strains of H-12 and H-17 serotypes were highly toxic to mosquito larvae. Till date no other report has claimed that these two serotypes are toxic to mosquitoes and this is the first report.
The strain belonging to the serotypes H-16 and H-10 were non-toxic. Padua et al. (1980) have reported 3 pathotypes of *B. thuringiensis* H-10 exhibiting different spectrum of activity. Of the 3 pathotypes, pathotype-1 is non-toxic to mosquito larvae but toxic to Lepidoptera, while, pathotype-3 is non-toxic to both. The non-toxic strain of *B. thuringiensis* H-10 obtained in the present study may belong to either of these pathotypes.

Several strains of *B. thuringiensis* belonging to unknown serotypes showed different levels of toxicity ranging from extremely toxic to poorly toxic categories. Further studies on the host range of the *B. thuringiensis* strains belonging to serotypes H-12, H-17 and to unknown serotypes obtained in this investigation would be interesting and useful.

Besides the *B. sphaericus* strains belonging to H-5a5b serotype, strains belonging to H-16, H-45 serotype and unknown serotypes were also obtained. The toxicity of 4 strains of H-6 serotype ranged from non-toxic category to highly toxic category. De Barjac et al. (1988) reported the isolation of 10 strains of *B. sphaericus* H-6 from Ghana exhibiting same level of toxicity. The strain belonging to the serotype H-45 obtained in the present study has been assessed moderately toxic and several strains of unknown serotypes were assessed extremely toxic.

Highly toxic strains of *B. thuringiensis* and *B. sphaericus* were confined to ponds and transient pools and this may probably be due to the occurrence of epizootics in these habitats. *B. thuringiensis* has been isolates from a number of epizootics, mostly from confined habitats *viz.*, in silk worm rearing areas in Japan (Ishiwata, 1901; Ohba et al., 1979; Weiser et al., 1985) and in an epizootics of *Culex* larvae in Israel (Goldberg and Margalit, 1977). De Lucca et al. (1981) opined that *B. thuringiensis* initiates epizootics when the external environmental conditions aggravate
the situation during the drying up of for the survival of insect populations
and chances for such phenomenon are more frequent in confined temporary
habitats (ponds and transient pools) thus, leading to the occurrence of
extremely toxic strains.

Strangely, although a variety of samples from different habitats were
screened and many bacterial isolates were obtained, no other species other
than \textit{B. thuringiensis} and \textit{B. sphaericus} was found to be larvicidal. Even \textit{B.
brevis} and \textit{B. alvei}, the known pathogens of mosquitoes (Balaraman \textit{et al.},
1979) were not encountered in these samples.

It is interesting to note that processing of large number of samples
yielded larvicidal bacilli which differed widely in toxicity as well as
taxonomically. For example, out of 39, 70 and 80 samples collected from
paddy fields, transient pools and ponds respectively, \textit{B. thuringiensis} and \textit{B.
sphaericus} strains belonging to 2-3 serotypes were obtained and their
toxicity also varied widely.

These results lead to conclude that \textit{B. thuringiensis} and \textit{B. sphaericus}
are widely distributed in nature. The present study also suggests that
processing of more number of samples will yield strains with wide range of
toxicity. Roots of aquatic plants has been found to be better source of the
larvicidal bacilli. The study also reveals that the possibility of obtaining not
only the known but also unknown serotypes having extremely high
larvicidal activity through extensive surveys.

\textbf{6.2 Dynamics of Growth and Sporulation in Selected Bacterial Strains}

Significant variation in the growth, biomass yielded and sporulation were
exhibited by different strains of \textit{B. thuringiensis} H-14 and \textit{B. sphaericus} H-5a5b.
Among the \textit{B. thuringiensis} H-14 strains, growth was higher in strain B-88 followed by B-95, B-77 and B-38. Least growth was noted in the strains B-98 and B-113.

The stage of sporulation was not well demarcated in \textit{B. thuringiensis} H-14 strains, as sporulation was initiated well before the initiation of stationary phase. Similar observations were reported earlier (Balaraman and Hoti, 1987; Vinter, 1969) wherein sporulation was noticed in logarithmically growing cultures.

From the findings of the present investigation it can be concluded that among different \textit{B. thuringiensis} H-14 strains, B-113 and B-77 grow faster, sporulate well and thereby the chances are more to produce higher levels of toxin in short period of growth. Incidentally, these strains are the most toxic ones to a wide range of mosquitoes indicating that the early production of higher quantity of toxin is responsible for higher toxicity.

Among different \textit{B. sphaericus} H-5a5b strains, B-64 yielded maximum biomass followed by B-2, in a relatively shorter growth period than others. This was also reflected in the total cell counts. The other strains grew slowly and also produced less biomass. Thus, the dynamics of biomass production and sporulation in \textit{B. sphaericus} strains showed a great variation, though they belonged to the same serotype (H-5a5b). This may be due to the variations in their nutritional requirements as has been observed in \textit{B. sphaericus} strains belonging to different serotypes. As for the observations made by White and Lotay (1980), the strain 2297 (serotype H-25) required biotin, thiamine and nicotinamide, while, SSII-I, 1593 and 1881 (serotype H-5a5b) required only biotin and thiamine.

The \textit{B. sphaericus} strains have also been reported to differ in utilizing different carbon sources such as D-gluconate, acetate, butyrate, pyruvate,
glycerol, glutamate, histidine, L-proline, ethanolamine and acetamide (Baumann et al., 1984). While some strains used only one of these carbon sources, others used more than one. The inability of B. sphaericus to use sugars has also been noted (Baumann et al., 1984; Singer et al., 1966; Kuppusamy and Balaraman, 1990; Russell et al., 1989).

The results of the present study indicate that two B. sphaericus strains viz., B-42 and B-64 sporulated very well, producing $10^7$-$10^8$ spores/ml, with concomitant appearance of higher level of larvicidal factor, while others have produced less. Many workers (Singer et al., 1966; Singer, 1981; Kalfon et al., 1984) have reported problems in obtaining almost 100% sporulation of B. sphaericus. It has been speculated that nutrients (Kalfon et al., 1983), pH of the medium and oxygen supply (Yousten et al., 1984) may influence the rate of sporulation.

The most insecticidal B. sphaericus strains have been reported to produce maximum level of larvicidal factor only during sporulation (Myers et al., 1979; Bourgouin and De Barjac, 1980). In the present study also, maximum production of this factor was noticed when majority of the cells were in the tennis racket stage which coincide with the peak production of heat resistant spores. Interestingly, but for the strain B-43, all others have produced certain level of larvicidal factor during vegetative phase, which is in fair agreement with earlier reports, that B. sphaericus cells exhibited larvicidal activity well before the onset of sporulation (Myers et al., 1979; Jayachandran and Virmani, 1987; Broadwell and Baumann, 1986).

6.3 Susceptibility of Different species of Mosquitoes to Selected Bacterial Strain

The data on the comparative toxicity of B. thuringiensis H-14 and B. sphaericus H-5a5b strains to various species of mosquito larvae viz., Culex
**DISCUSSION**

Quinquefasciatus, Cx. mimeticus, Anopheles subpictus, Aedes aegypti and An. culicifacies have revealed that B-113 and B-77 were extremely toxic, B-88 and B-95 were highly toxic and B-38 and B-98 were moderately toxic against Cx. mimeticus. B-113 and B-77 were highly toxic and the other strains were moderately toxic against Cx. quinquefasciatus. B-113 was highly toxic, B-77 was toxic, B-38, B-88 and B-95 were moderately toxic while B-98 was poorly toxic against An. subpictus. B-113 and B-77 were moderately toxic and the other strains were poorly toxic against Ae. aegypti. All the strains were poorly toxic against An. culicifacies. The order of susceptibility of different mosquito species to different Bacillus thuringiensis H-14 strains is recorded as Cx. mimeticus > Cx. quinquefasciatus > An. subpictus > Ae. aegypti > An. culicifacies i.e., Cx. quinquefasciatus, An. subpictus, Ae. aegypti and An. culicifacies required 1.2-2, 1.4-2.1, 6-9.4 and 55.1-99.5 times higher dose compared to different B. thuringiensis H-14 strain as did Cx. mimeticus for 50% mortality.

Larvae of different mosquitoes showed great variations in their susceptibility to B. thuringiensis and B. sphaericus strains. Culicines were found to be more susceptible than Anophelines and Aedines. Striking differences in the susceptibility levels within Culicines were also noticed. This is in agreement with the findings of others (Faust, 1975; Goldberg, 1978; Balaraman et al., 1983; Mulla et al., 1984, 1985; Aly et al., 1988 and Davidson, 1989). Several factors such as, the gut pH (Faust, 1975), feeding behaviour, larval intrinsic factors (Sun et al., 1980) and / or the presence of specific toxin binding sites on the gut cells (Hofmann et al., 1986, 1988; Charles, 1987 and Davidson, 1989) have been attributed as reasons for the differential susceptibility of different species. In the present study, the involvement of such factors might have resulted in variations in susceptibility. Among different strains of B. sphaericus H-5a5b, B-42 and B-64 were moderately toxic to An. subpictus, toxic to Cx. mimeticus, moderately toxic to Cx. quinquefasciatus and poorly toxic to Ae.
aegypti and An. culicifacies. The order of susceptibility of the larvae of different species to Bacillus sphaericus strains was: An. subpictus > Cx. mimeticus > Cx. quinquefasciatus > An. culicifacies > Ae. aegypti i.e., Cx. mimeticus, Cx. quinquefasciatus, An. culicifacies and Ae. aegypti required 1.4-3.0, 1.9-4.8, 7.6-40.2 and 7.7-273.6 times higher dose compared to An. subpictus for inciting 50% mortality.

Another striking feature was that Aedes aegypti larvae were more susceptible to B. thuringiensis than to B. sphaericus strains. The susceptibility of Aedes aegypti was so poor that the B. sphaericus strains can be classified as non-toxic strains, and this observation is in agreement with that of the observations of Singer (1980). Pfannenstiel et al. (1990) have reported that an amino sugar (GlcNac) residue was necessary for B. thuringiensis H-14 toxin to express high level of toxicity in Aedes. Glycosylation of B. thuringiensis H-14 toxin with GlcMAc residues has been reported by Muthukumar and Nickerson (1987) and Pfannenstiel et al. (1990). The latter group speculated that B. sphaericus probably lacks glycosylating enzymes which attach the GlcNAc containing oligosaccharides to toxins. This follows that B. sphaericus toxins are not glycosylated and hence cannot express high levels of toxicity in Aedes aegypti larvae. Between different stains of the same species and serotype, reproductible differences existed in the toxicity levels. Possible reasons for these variations are the differences in the growth, sporulation rate and concentration of toxin/cell (Kuppusamy and Balaraman, 1990).

This study leads to conclude that different mosquito species differ significantly in their susceptibility to B. thuringiensis and B. sphaericus, irrespective of their toxicity level. Culicines were highly susceptible to B. thuringiensis and B. sphaericus strains. However, Ae. aegypti was more susceptible to B. thuringiensis H-14 than to B. sphaericus H-5a5b while vice-
versa was the case with *An. stephensi*. The reasons for the differences in the toxicity of the strains are due to differences in the sporulation pattern, per cell concentration of larvicidal factor(s), structural make up of larvicidal factor (Yamamoto *et al*., 1983) and complexity of the target-toxin relationship (Thiery and de Barjac, 1989). They were in agreement with the statement of Chowanadesai (1994) in considering a number of environmental factors such as temperature, pH, sewage effluents, sunlight and soil constituents which influences the larvicidal activity of bacterial toxin. Further, there is an agreement with Wirth *et al*. (2001) in considering the role of bacterial insecticides in mosquito control.

The results of present study slightly differ with the observations made by Mulla *et al*. (2003), Vilarinhos and Monnerat (2004) in the emergence of resistance and its management of field population of mosquitoes. Further, there is a significance difference between the present findings and the observations made by Chang *et al*. (1990), Barrauh and Das (1994), Mulla *et al*. (1999) and Zhakhongirov *et al*. (2004).

On the basis of above statements, it can be suggested that the bacterial agent should be chosen based on the local mosquito populations, so as to obtain optimum levels of reduction in the vector density.

### 6.4 Shelf life of Selected Bacterial Strains Formulations

Perusal of the literature reveals that there is almost no report on the comparative shelf life of different preparations. But, water dispersible powder of *B. thuringiensis* H-14 was found to be active for 9, 52 and 500 days when stored at 50, 30 and 5°C respectively (Ignoffo *et al*., 1982). In another study (Balaraman and Hoti, 1984), the WDP of *B. thuringiensis* H-14 (B-17) was
stored for 30 weeks at –40°C, 8°C and 30°C, without significant loss of activity. Lyophilised cells of IPS-80 were reported to have been stored for 22 months at refrigeration and room temperature without any reduction in activity, while, a slight reduction was noticed when stored at 50°C (Preserthphon, 1979; Anonymous, 1980a; Largent, 1981). Chen et al. (1984) stored IPS-82 (lyophilized powder) at 5°C for 2 years.

Similarly, contradicting reports for the preparations of *B. sphaericus* are available. While, Hertlein et al. (1980) reported the stability of a commercial powder of strain 1593 of *B. sphaericus* for 1 year at room temperature, Balaraman and Hoti (1984) reported significant loss of activity by 30 weeks when stored at –40°C, 8°C or 30°C. Lyophilized powder of *B. sphaericus* stored at 5°C retained complete activity after 10 weeks and lost 75% activity after 2 years. But, when stored at 50°C, the preparation was stable for 2 years (Bourgouin et al., 1984). On the other hand, the RB-80 (standard preparation of *B. sphaericus*) lost considerable activity within 5 weeks at 50°C (Balaraman and Manonmanii, 1986) and after 18 days of freeze-thaw operations (Zhang et al., 1986).

In the present study, the strain B-113, when stored as water dispersible powder at –10°C, it lost only 5% of its activity after 24 months and at 8°C it lost 18% of activity. At 30 and 40°C, it lost about 21% and 36% activity respectively at 24 months. When stored as lyophilised cells in hermetically sealed condition, it could maintain nearly 40% activity upto 15 and 17 months at –10 and 8°C respectively. However, 50% activity was observed upto 15 and 9 months at 30 and 40°C. And when stored in the unsealed condition, 50% of its activity was lost within 2-4 months, irrespective of the storage temperature.

LC$_{50}$ values of the lyophilized cells and water dispersible powder of *B. thuringiensis* H-14 strain B-77 at different temperatures (–10°C, 8°C, 30°C and
40°C). The strain B-77 when stored as water dispersible powder at −10°C and 30°C, it lost 18% of its activity after 24 months. However, it lost about 2% and 38°C activities respectively after 24 months at 8°C and 40°C. When stored as lyophilised powder under hermetically sealed condition at −10°C and 8°C, the activity was lost within the range 38-40% activity after 22 months, while at 30°C, 45% loss of activity was seen at 15 months. However, at 40°C, the activity lost as 50% at 14 months. And when it was stored in unsealed condition, 50% of its activity was lost within 1-4 months at −10°C, 8°C and 20°C. While at 40°C, above 50% lost activity was observed throughout the month of storage.

Guillet et al. (1979) and Dempah and Coz (1980) opined that the apparent decrease in the activity during storage in unsealed condition was due to absorption of water, as this material was highly hygroscopic. Probably the lyophilised biomass studied after hermetically sealing and the WDPs of different strains of B. thuringiensis H-14 and B. sphaericus H-5a5b used in the present study did not absorb moisture and hence retained the larvicidal activity for prolonged period.

The strain B-64 of B. sphaericus H-5a5b when stored as water dispersible powder, it lost only 15% of activity after 18 months at −10°C and within 32-35% activity after 24 months at 8 and 30°C and 50% activity after 11 months at 40°C. When stored as lyophilised powder under hermetically sealed condition, it lost 38-40% activity after 24 months at −10°C and 8°C. Also, 50% lost activity was seen after 11 and 3 months at 30°C and 40°C respectively. And when stored in unsealed condition, it lost 50% activity after 6 months at −10°C, 8°C and 30°C and after 2 months at 40°C.

LC50 values of the lyophilized cells and water dispersible powder of B. sphaericus H-5a5b strain B-42 at different temperatures (-10°C, 8°C, 30°C and
40°C). The strain B-42 when stored as water dispersible powder after 24 months, the loss of activity was 12%, 10% and 33% at −10°C, 8°C and 30°C respectively. At 40°C, about 50% loss of activity was observed after 12 months. When stored as lyophilized powder under hermetically sealed condition, it lost 40% activity at −10°C and 8°C after 15 and 20 months respectively. However, 50% lost activity was recorded after 12 months at 30°C and 40°C. And when stored in unsealed condition, 50% loss of activity was seen after 6 months at −10°C and 8°C while at 30°C and 40°C, it was after 3 months.

Prabhakaran et al. (2001) evaluated the self floating released formulation of *B. thuringiensis* and its larvicidal activities against *Cx. quinquefasciatus* in the laboratory. But in present study, its larvicidal activity against 5 species of mosquito have been assessed. The observations of the present study also differ from the observations of Vilarinhos and Monnerat (2004) and Suzuki et al. (2004) in respect of spore germination and multiplication of vegetative cells.

Conclusively, the data indicate that storage of *B. thuringiensis* H-14 and *B. sphaericus* H-5a5b strains as water dispersible powder presents deterioration of larvicidal activity for longer time as compared to lyophilised cells. Also, there was a slight increase in the activity of water dispersible powders during storage. If the strains are stored as lyophilised cells they have to be hermetically sealed and stored, as storage under unsealed condition leads to rapid deterioration. Regarding temperature, lower the temperature of storage, the longer the shelf life, irrespective of the storage material.

### 6.5 Environmental Factors Influencing the Activity of Bacterial Strains on Mosquito Larvae

The findings related to impact of abiotic and biotic factors influencing the activity of *B. thuringiensis* and *B. sphaericus* strains on the survival of mosquito larvae confirms the previous observations made by Wright *et al.*, 1981, 1987;
Mulla et al., 1982a; Igonuffo et al., 1983; Lacey and Oldacre, 1983; Becker et al., 1992 and Garcia and Desrochers, 1979. The tests to evaluate the effect of temperature and thereby distinct differences in the efficacy of *B. thuringiensis israelensis* in especially in II and IV instars larvae of *Ae. aegypti* and that too between 5-8°C, slightly resemble with the evaluation made by Becker et al. (1992). The only difference is that the findings of Becker et al. (1992) are based on *Ae. vexens*. Since, there were no significant differences in IV instars larvae at 8, 15 and 25°C, the findings are in accordance with Mulla et al. (1990) with a difference of some other species of the mosquito.

With regard to increased sunlight and its impact on the efficacy of *B. thuringiensis israelensis*, the findings resemble with Burke et al. (1983), Morris (1983) and Becker et al. (1992). There is a close similarity between the results of the present study related to the impact of sunlight on the activity of *B. thuringiensis israelensis* on *An. culicifacies* and *Ae. aegypti* larvae with those of Becker et al. Infact, the findings of Becker et al. (1992) are based on some other species of mosquitoes other than those selected in the present study.

In the present investigation, the results related to survival of *An. culicifacies* and *Ae. aegypti* under indoor and outdoor conditions resemble closely with those of Garcia and Desrochers (1979), but in different species of mosquitoes. The higher temperature of the outdoor tests during the 7 h period of Sun exposure may account for the observed differences. The study suggests that the mortality was zero and 10% under indoor conditions. Another explanation may be given on the basis that under outdoor conditions, some other factors have a major role in the survival of the mosquito larvae, than under indoor conditions.

Becker et al. (1992) emphasized the importance of competition among filter feeders, taking into considerations the LC$_{50}$ and LC$_{90}$ values in bioassay tests with *B. thuringiensis israelensis / B. sphaericus* formulations on the
survival of *Ae. aegypti* in different densities of Daphnia species. According to them, there was a decrease in the concentration of d-endotoxin in the water while conducting the experiments.

In the present study, *B. sphaericus* H-5a5b and *B. thuringiensis* H-14 were taken for the experimental purpose and the difference in the LC$_{50}$ and LC$_{90}$ values was recorded with or without *Daphnia* species population. Since there is less information on such experimental work, therefore the findings made under the present experimental design cannot be compared with the findings made by others.

The present findings showing a bit similarity with Nayar *et al.* (1993) in respect of biotic and abiotic factors that influences the larvicidal activity of *B. thuringiensis* against two mosquito species *viz.*, *Ae. taetrinorhynchus* and *Cx. nigripalpus*. However, observation have been made on *An. culicifacies* and *Ae. aegypti* under the present study. There is a major difference with Stevens *et al.* (2004) who worked on the toxicity of *B. thuringiensis* and *B. sphaericus* to IV instar larvae of *Chrinomus tepperi*. Others like Lacey *et al.* (1998), Chang *et al.* (1990), Mittal *et al.* (1995), Consoli *et al.* (1995), Kumar *et al.* (2000) also made significant contribution towards efficacy of biocides which are influenced by various biological and environmental factors including species specificity, feeding behaviour of larvae, temperature, exposure to UV radiation from sunlight and availability of different organisms in abiotic community. According to Mittal (2003) the temperature is an important factor which influences the toxicity of the bacterial preparation and the biocide is not effective in colder months. The present findings slightly resemble to Mittal (2003) in this context.

Based on the above statements, it can be concluded that the environmental factors particularly sunlight, temperature, indoor and outdoor conditions and
competition in food intake influence the effectiveness of microbial control agents in mosquito control programmes.

6.6 Field Evaluation to Test the Larvicidal Efficacy of Bacterial Strains

A. B. thuringiensis H-14:

When B-77 was applied to Litchi garden pits where breeding of Anophelines and Culicines was observed, it reduced the density initially to considerable extent. A relatively lower larvicidal effect was observed on early instars larvae, as compared with other larval stages. This may be explained on the basis that early instars do not normally feed for about 2-3 h after hatching and there has been daily egg laying in the pits. Laboratory studies (Sharma et al., 1983) and some field experiments (Balaraman et al., 1983) have shown that B. thuringiensis H-14 is more effective against early instars larvae as compared to late instars. But this study shows that it is not effective against the early first instars. The larval density gradually increased from the second day after treatment and late instars appeared by the fifth day. Pupae appeared by the sixth day after the first treatment in Ae. aegypti and this necessitated the application of B. thuringiensis H-14 once in 5 days, for effective suppression of pupal production. Reappearance of early instars larvae by 48h post-treatment and the resulting increase in their density thereafter indicated that the formulation tested did not have residual activity beyond 24h and has to be applied every 5th or 7th days depending on the duration of pupal development. These findings are in accordance with the findings of Balaraman et al. (1983), Schaefer and Kirnowardoyo (1980) and Sharma et al. (1983).
The rate of reduction of *Aedes aegypti* larvae varied in the days of post treatment, although the population was less, even then the reduction in the range of 50-100 % and 30-100 % for early and late instars, respectively was recorded. The pupae were recorded negligible. After the treatment, the pupae population was completely absent wherever their population was recorded before treatment.

Garcia *et al.* (1980) and Sudomo *et al.* (1981) reported that *B. thuringiensis* H-14 failed to cause cent per cent mortality among mosquito larvae present in polluted environments and in habitats with heavy vegetation and floating algae. Such findings have not been evaluated in the present investigation. The findings of present study also differs from Barrauh and Das (1994), Kumar *et al.* (1995), Sharma *et al.* (2003), Stevens *et al.* (2004), Zahiri *et al.* (2004) and Zahakhongerov *et al.* (2004) in conducting small scale field trial against the mosquito larvae using formulations of *B. thuringiensis* at different time interval.

**B. *B. sphaericus* H-5a5b:**

From the field tests, it is concluded that the *B. sphaericus* formulations cause significant reduction among larval populations of almost all the selected species within a period of 24 h after treatment. However, they do not show larvicidal action for prolonged periods as reported by many authors (Davidson *et al.*, 1984; Hertlien *et al.*, 1979; Hornby *et al.*, 1984; Jonathan *et al.*, 1981; Mulligan *et al.*, 1980; Nicolas *et al.*, 1987). In the present study, after the 1st treatment, there was 91% reduction in the early instars and 95% in late instars. However, cent per cent reduction in the immature population was recorded in subsequent treatments. This may be due to the fact that most of the *B. sphaericus* spores do not remain suspended in the larval feeding zone. Thus, there was
no evidence of any recycling by *B. sphaericus* in the true sense, in any of
the treated sites and these observations corroborate the earlier reports
(Davidson *et al.*, 1984; Hoti and Balaraman, 1984).

There is a significant difference with the observations as recorded by
Kumar *et al.* (1995) on the control of *An. stephensi* by applying *B.
thuringiensis* (H-14) at the rate of 1g/m² surface area. There is also a major
difference with the results of Kumar *et al.* (1998) who conducted field trails
of biolarvicides *B. thuringiensis* strains 164 and larvivorous fish *Aplochilus
blockai* against *An. stephensi* for malaria control in Goa. Similar studies
were conducted by Skovmand and Sanogo (1999). Further, in this regard
Barrauh and Das (1994) also carried out laboratory and field trails with two
formulations of *B. thuringiensis* and strains of *B. sphaericus* (B-42, 64, 87,
32) against mosquito larvae in different breeding habitats of Tejpur.
Subsequently, Haq *et al.* (2004) worked out experiments on the field
evaluation of biolarvicides against three species of mosquitoes (*An.
stephensi, Cx. quinquefasciatus* and *Ae. aegypti*) in Surat city. The present
findings with regard to day interval differs with the findings of Haq *et al.*
(2004) as they found that the application of these biolarvicide would be
required at 7-10 days interval.

### 6.7 Comparative Efficacy of Highly Potent Strains

The highly toxic strains of *B. thuringiensis* H-14 and *B. sphaericus* H-
5a5b were evaluated against *An. subpictus* in Litchi pits in comparison with
standard strains. There were 70%, 77.8% and 82.4 % reductions of early instars
treated with B-325, B-300 and ISP-80 respectively. However, cent percent
reduction of late instars of *An. subpictus* was observed for all the three bacterial
strains. The isolate B-325 and B-300 even when treated at 3.7 and 1.9 times
lesser doses respectively than IPS-80, caused similar level of reduction in the field larval population of the mosquitoes.

In the Litchi pits treated with B-325, the density of early instars increased from 6 on 2\textsuperscript{nd} day and 40 on the 10\textsuperscript{th} day and that of late instars from 0 to 23. Similar trend has been observed in the pits treated with B-300 and IPS-80, thus indicating a lack in the residual activity. As far as, the average percentage of reduction of both early and late instars against the efficacy of different bacterial strains is concerned, the maximum reduction was observed in B-300 followed IPS-80 and B-325.

As far the efficacy of \textit{B. sphaericus} (H-5a5b) is concerned, it was found that prior to application, there were respectively 29 and 5, 15 and 3 and 41 and 6 early and late instars per 5 dips in pits treated with B-288, B-381, 2362. After the 1\textsuperscript{st} treatment \textit{i.e.}, on the 2\textsuperscript{nd} day, there was a reduction in the density of early instars by 65.6, 100\% and 92.7\% respectively against the efficacy of B-288, B-381 and 2362. However, there was cent percent reduction in the density of late instars against all the selected strains of \textit{B. sphaericus} (H-5a5b). On the other hand, a slight change in the density of early instars was noted in the control pits, while, the density of late instars was comparatively more on the 5\textsuperscript{th} day than the 10\textsuperscript{th} day. However, a slight change has been observed in the efficacy of B-381. Similar findings have been observed in the reduction of late instars. Further, the results indicate that the higher toxic strains of \textit{B. sphaericus} (B-381 and 2362) brought about a considerable reduction in the immature density of \textit{An. subpictus} as compared to B-288.

Thus, the indigenous strains of \textit{B. thuringiensis} H-14 and \textit{B. sphaericus} H-5a5b were 2-3.5 times more efficient than the standard strains. This means that by using these highly toxic strains, there would be an overall reduction in the dose and hence volume of material required for application. While using
bacterial larvicides in operational programmes, one of the drawbacks is the large volume necessary for obtaining optimum level of control (Guillet et al., 1980; Mclaughlin and Billodeaux, 1983). The said problem can be solved to a greater extent by using highly active strains obtained from nature as reflected from the present study.

Zahiri et al. (2004) bioassayed 3 strains of B. thuringiensis and B. sphaericus against the larvicidal activity towards Cx. quinquefasciatus and Ae. aegypti at the University of California and their findings can be well compared to the present investigation. The only difference is with the species of mosquitoes and the kinds of strains taken under the present study. The vast variation observed in the toxicity of different strains may likely be due to the presence or partial presence or absence of cry and / or cyt gene(s) (Balaraman 2005). The present findings in respect of efficacy of highly potent strains are in a fair accordance with Poopathi and Tyagi (2006) that a newer and novel bacterial toxin with different structures and modes of action be identified to minimize the risk of developing insect resistance.