

Seasonal prevalence & resting behaviour of *Anopheles minimus* Theobald & *An. fluviatilis* James (Diptera: Culicidae) in east-central India

S.S. Sahu, K. Gunasekaran, P. Vanamail & P. Jambulingam

Vector Control Research Centre (ICMR), Puducherry, India

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Background & objectives: *Anopheles minimus* has recently been reported to have re-appeared in Keonjhar district of Orissa after a period of about 45 years of launching the malaria eradication programme. *An. minimus* and *An. fluviatilis* were the incriminated major malaria vectors in the district, endemic for falciparum malaria. The information on seasonal prevalence and resting behaviour of the vectors is crucial for implementing appropriate malaria control measures. Therefore, a study was undertaken on seasonal prevalence and resting behaviour of *An. minimus* and *An. fluviatilis* in this district.

Methods: Seven randomly selected villages of Keonjhar district, Orissa, were studied during August 2005 to November 2007. Daytime resting collections indoors and outdoors were made covering three seasons of the year. The *Anopheles* mosquitoes obtained from different habitats were identified. Collections were maintained separately according to different sites as well as heights of the walls in human dwellings.

Results: Among the indoor collections, the densities of *An. minimus* and *An. fluviatilis* were higher in human dwellings than cattle sheds. *An. fluviatilis* was the predominant (41.5%) species followed by *An. minimus* (26.3%) in human dwellings. The density of both the vector species in human dwellings peaked during rainy and winter seasons followed by summer. Walls were the most preferred site by these vectors for resting and the maximum number was collected at a height of 3 to 4 ft.

Interpretation & conclusions: The resting behaviour of the vector species increases their contact with the sprayed walls and therefore, a quality residual spraying of human dwellings focusing indoor walls could interrupt the malaria transmission in this area.

Key words *Anopheles fluviatilis* - *An. minimus* - Keonjhar district - resting behaviour – seasonal prevalence

Malaria continues to be a major public health problem in India. The hilly tracts of Indian subcontinent are generally malarious. The worst affected regions are the hilly-forested areas of Orissa State. Among these, Keonjhar district (part of east-central India) is one such area, which has been hyperendemic for falciparum

malaria since many decades, recording deaths due to malaria every year (327 reported deaths during 2000 to 2005)¹⁻³. Majority of the malaria cases (>95%) are caused by *Plasmodium falciparum*¹. Considering persistence transmission of malaria with high records of malaria deaths in the district, studies were conducted

to identify the vectors and their relative importance¹⁻³. *Anopheles minimus* was recorded in this district after a period of about 45 years of launching the malaria eradication programme¹. Subsequently, *An. minimus* and *An. fluviatilis* were identified as the major malaria vectors in the district^{2,3}.

Vector control is one of the essential components of any malaria control programme. An understanding of the biology and ecology of the vectors is crucial for disease-threat analysis and for the development and implementation of vector control strategies. Important among these are seasonal prevalence and resting behaviour of the vectors. In spite of the well-known vectorial status of *An. minimus* and *An. fluviatilis*, no adequate information on abundance in relation to time and resting pattern is available for these vector species in this part of the country. Therefore, a study was undertaken from August 2005 to November 2007 in Keonjhar district, Orissa State.

Material & Methods

Study area: The study was carried out in seven randomly selected villages viz., Mamulipusi, Puradihi, Dhankuniasahi, Boitarani, Natokotha, Bayakumutia, and Mundla of Baunspal Primary Health Centre (PHC), Keonjhar district. All these villages are situated on hilltops and are characterized by presence of perennial streams and rivulets which are the major breeding habitats. Majority of the villagers live in huts made of mud walls and either thatched or tiled roofs. Each hut in the front has a verandah, where people sleep during night. The walls of the hut are usually of 7 feet in height. There was a gap of 2 to 3 feet between the two side walls and roof. The cattle sheds are kept close by human dwellings. Three seasons, summer (March-June), rainy (July-October) and winter (November-February) are pronounced in this area. The minimum temperature ranged from 8°C in December to 32°C in May and the maximum from 19.0°C in December to 42°C in May¹.

The district has been endemic for malaria, having incidence recorded throughout the year. During 2001-2005, the annual parasite incidence (API) ranged from 13.9 to 25.8 per thousand population, with 208 reported malaria deaths (source: Office of the Chief District Medical Officer, Keonjhar). More than 95 per cent of the malaria cases were caused by *P. falciparum*³. DDT had been in use for indoor residual spraying from 1958 to 2000 under the National Malaria Control Programme and DDT was replaced by synthetic pyrethroids during

2001-2004. Subsequently, in 2005, DDT was brought back and from then onwards, two rounds (1st round in May-June, 2nd round in September-October) of indoor residual spraying using this insecticide was carried out in both human dwellings and cattle sheds. In the study villages, insecticide was last sprayed during September- October 2007.

Resting collections indoors: Daytime resting mosquitoes were collected indoors and outdoors. In each study village, six human dwellings and three cattle sheds were randomly selected and numbered. Collections were done during morning hours (0600 to 0800 h) in the villages at bimonthly interval from August 2005 to March 2006 and at monthly interval from May 2006 to July 2007.

Anopheles mosquitoes were collected for 10 min in each dwelling using a flashlight and an oral aspirator. Equal time was spent for collection from eaves, walls and roofs. The collected mosquitoes were labelled in the field and brought to the laboratory for identification and processing. The mosquitoes were classified according to gonotrophic stages and their age was graded using ovariole dilatation method⁴. The number of female mosquitoes collected per man-hour is expressed as density of the vector species. All suitable specimens of known and potential vector species were subjected to gut and salivary gland dissection for the presence of malaria parasites.

To study preferential resting sites of the vector mosquitoes day time resting collections were done at different sites and at different heights of the walls of the dwellings from December 2006 to November 2007 in three villages viz., Mamulipusi, Puradihi and Dhankuniasahi, where *An. minimus* and *An. fluviatilis* were collected more in numbers. The collected mosquitoes were kept separately according to different sites as well as different heights of the walls.

Resting collections outdoors: Pit shelters (ten numbers in each village) were dug outdoors within one-kilometer radius in each of the three study villages namely; Mamulipusi, Puradihi and Dhankuniasahi. Mud pots with a body width of about 30-60 cm and mouth width of 15-20 cm were fixed horizontally at the sides of shaded embankments or earth mounds. While fixing, it was adjusted in such a way that the mouth of the pot was slightly inclined downwards, so as to prevent the entry and accumulation of rainwater. Searches for the presence of *Anopheles* mosquitoes in these shelters were made at monthly intervals from June 2006 to

July 2007. Time spent and number of *An. minimus* and *An. fluviatilis* collected in each habitat was recorded separately.

Laboratory processing: After dissection, the body parts of the individual specimen of *An. minimus* and *An. fluviatilis* were kept in Eppendorf tube, dried for 4-5 h at 90°C and subjected to polymerase chain reaction (PCR) assay for molecular identification of *An. minimus* and *An. fluviatilis* using the method described by Garros *et al*⁵ and Singh *et al*⁶, respectively.

Month-wise meteorological data for the study period was obtained from the Orissa University of Agriculture and Technology Research Station, Keonjhar.

Statistical analysis: One-way analysis of variance (ANOVA) was used to compare the mean density of *An. minimus* and *An. fluviatilis* between the seven study villages. Pair-wise comparison of mean density of the vector species was done using the post-hoc test based on least significant difference (LSD). The seasonal variation in mean density of *An. minimus* and *An. fluviatilis* as well as variations in distribution of the vector species at different heights of the walls were compared between the villages using two-way analysis of variance. The parity rates of the vectors were compared between the villages using χ^2 test. Correlation analysis was done to understand the relationship between rainfall and density of *An. minimus* and *An. fluviatilis*.

To compare the vector density between different seasons (after pooling the data of the seven villages as they did not differ significantly), one-way analysis of variance was performed. Chi square test was used to find association, if any, for resting of the vectors with different heights of the walls and also to compare the parity of mosquitoes between different seasons. SPSS version 16 and EPI-info softwares (SPSS, Inc. Chicago, IL, USA) were used for the statistical analysis.

Results

Thirteen anopheline species were collected from human dwellings, and 17 were collected from cattle sheds. *An. fluviatilis* was the predominant (41.5%) species among those resting in human dwellings followed by *An. minimus* (26.3%). In cattle sheds, *An. culicifacies* was the predominant species constituting 31.6 per cent followed by *An. subpictus* (21.6%) and *An. vagus* (13.2%). Since the density of *An. minimus* and *An. fluviatilis* in cattle sheds and outdoor shelters was low (Table I), for further analysis the density of the

Table I. Number of *Anopheles* species collected in different habitats in the study area

MHS species	Indoor		Outdoor
	HD (140)	CS (70)	(42)
<i>An. aconitus</i>	0	1	0
<i>An. annularis</i>	8	25	0
<i>An. barbirostris</i>	0	2	0
<i>An. culicifacies</i>	231	242	0
<i>An. fluviatilis</i>	748	6	5
<i>An. jamesi</i>	1	16	0
<i>An. jeyporiensis</i>	48	96	0
<i>An. karwari</i>	0	1	0
<i>An. maculatus</i>	6	25	0
<i>An. minimus</i>	473	0	1
<i>An. nigerrimus</i>	0	15	0
<i>An. pallidus</i>	2	5	0
<i>An. splendidus</i>	11	49	0
<i>An. subpictus</i>	152	166	4
<i>An. tessallatus</i>	0	1	0
<i>An. theobaldi</i>	1	5	0
<i>An. vagus</i>	94	101	2
<i>An. varuna</i>	26	11	4
Total	1801	767	16

HD, human dwelling; CS, cattle shed; MHS, man hour spent

vector species based on human dwelling collections was only considered.

One-way ANOVA showed that the mean density of *An. minimus* between the seven villages differed significantly ($P < 0.001$). The mean density was significantly higher in two villages *viz.*, Dhankuniasahi (6.80 ± 7.2) and Mamulipusi (5.85 ± 4.9) than the other five villages; between Dhankuniasahi and Mamulipusi the mean density did not differ significantly. In the remaining five villages, the density ranged from 1.35 ± 2.4 to 3.75 ± 2.9 and there was no significant difference between them.

The mean density of *An. fluviatilis* also varied significantly between the seven villages ($P < 0.001$). The village, Mamulipusi recorded the maximum density of 10.35 ± 8.1 followed by the village, Puradihi (8.20 ± 7.4). There was no significant difference in density between these two villages. The density in the remaining five villages ranged from 2.10 ± 2.7 to 5.60 ± 4.5 and they did not differ significantly.

Two-way analysis of variance, considering villages and seasons as the main factors, showed a similar seasonal trend in vector density in all the seven villages

without any significant difference. Therefore, the data of the seven villages were pooled for analyzing seasonal prevalence of the two vector species.

The per man-hour density (PMD) of *An. minimus* and *An. fluviatilis* recorded during different months are shown in Fig. 1. The PMD of *An. minimus* varied from 0.4 (July 2007) to 11.7 (October 2006 and November 2006). With the onset of southwest monsoon in July, the density started increasing and peaked during October and November in both the years of observation. Though, there was a negative correlation ($r=-0.201$; $P=0.396$) between the quantum of rainfall and indoor resting density of *An. minimus*, it was not statistically significant. The density of *An. minimus* was higher during rainy season than winter and summer seasons (Fig. 2), but the difference was not significant.

The PMD of *An. fluviatilis* was the lowest in June (summer month), increased with the onset of monsoon in July, and peaked during September and October (rainy months). The indoor resting density of this species also did not show any significant relationship with rainfall ($r=-0.033$; $P=0.891$). Like *An. minimus*, there was no significant difference in *An. fluviatilis* density between the three seasons (Fig. 2).

Human dwelling versus cattle shed: As many as 140 man hours, spent over 20 collections in human

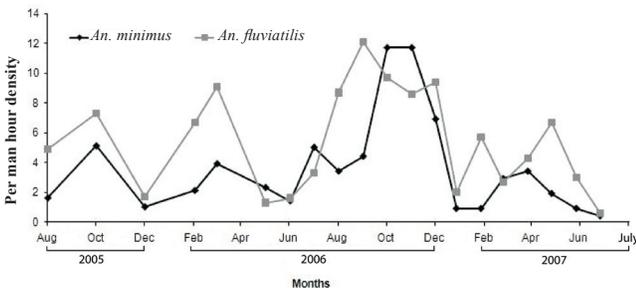


Fig. 1. Indoor resting density of *An. minimus* and *An. fluviatilis* in human dwellings in different months.

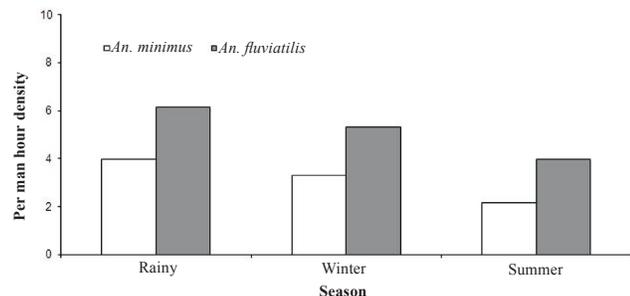


Fig. 2. Per man-hour density of *An. minimus* and *An. fluviatilis* in human dwellings during the three seasons.

dwellings during the study period yielded a total of 473 (26.3%) and 748 (41.5%) females of *An. minimus* and *An. fluviatilis*, respectively. In cattle sheds, 70 man-hours spent over the same number of collections yielded only 0 and 6 (0.8%) females of *An. minimus* and *An. fluviatilis*, respectively. The average per man-hour density of *An. minimus* recorded in human dwellings and cattle sheds was 3.6 and 0, respectively and the corresponding values for *An. fluviatilis* were 5.7 and 0.1.

Preferential resting sites in human dwellings: The number of the vector species collected from different resting sites in human dwellings was analyzed for their preferential resting (Table II). All the 204 *An. minimus* collected were found resting on walls; none was found resting either on roof or on hanging objects. Of the 296 *An. fluviatilis* collected indoors, 99.3 per cent was found resting on walls, 0.7 per cent on hanging objects and none was found resting on roof.

The distribution of both the vector species at different heights of the walls was not significantly different between the three villages studied (interaction of village and height: *An. minimus*: $F=1.71$; $df=7,112$; $P=0.115$ and *An. fluviatilis*: $F=0.70$; $df=8,163$; $P=0.69$, by two-way ANOVA). Hence, data of the three villages were combined and distribution pattern, overall, at different heights of the walls was studied (Fig. 3). The maximum number of *An. minimus* (45.6%, $n=204$) was collected at a height of 3 to 4 ft of the wall; below 3 ft (21.6%) and above 4 ft (32.8%) the number collected was low. Similarly, of the total wall collections of *An. fluviatilis* ($n=296$), 47.3 per cent was from a height of 3 to 4 ft and below 3 ft (23.0%) and above 4 ft (29.7%) the number collected was low. However, the height-wise association for both the vector species was not statistically significant.

Gonotrophic condition: The unfed *An. minimus* females constituted only 2.4 per cent of the total indoor resting population. The percentage of fully fed, semi-gravid

Table II. Collection of *An. minimus* and *An. fluviatilis* from different resting sites in human dwellings during the three seasons

Season	Species	Wall	Eave	Roof	Hanging object
Rainy	<i>An. minimus</i>	74	0	0	0
	<i>An. fluviatilis</i>	142	0	0	0
Winter	<i>An. minimus</i>	90	0	0	0
	<i>An. fluviatilis</i>	118	0	0	0
Summer	<i>An. minimus</i>	40	0	0	0
	<i>An. fluviatilis</i>	36	0	0	2

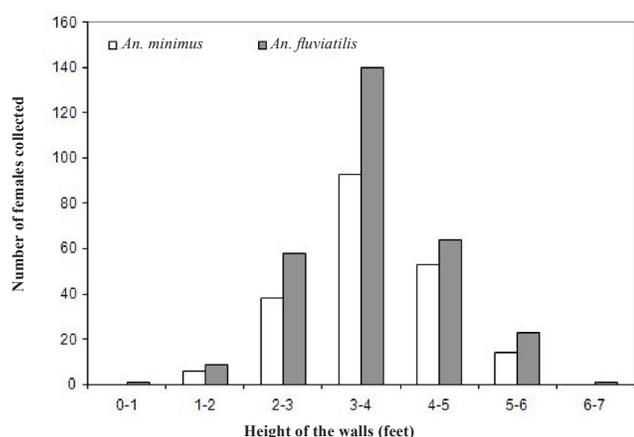


Fig. 3. Number of vector species collected in different heights of the wall in human dwellings.

and gravid was 83.1, 12.3 and 2.2, respectively. In the case of *An. fluviatilis*, the proportion of unfed, fully fed, semi gravid and gravid was 2.5, 87.0, 9.4 and 1.1 per cent, respectively.

Parity: Of the total *An. minimus* females dissected (n=473), 62.8 per cent was parous (number of mosquitoes having one or more dilatations) (Table III). Since, the proportion of parous females of *An. minimus* and *An. fluviatilis* did not vary significantly between the seven study villages, the seven villages data were combined for further analysis. The proportion parous showed significant association with seasons ($\chi^2=7.3$; df=2; $P=0.026$); the highest during winter (0.70), followed by summer (0.64) and rainy (0.56) season. A total of 748 *An. fluviatilis* were dissected, and of these 57.0 per cent was parous. Although, the proportion parous was higher during winter months (0.60) than summer (0.56) and rainy (0.55) months, the association with season was not significant.

Infection: Besides the two vector species, *An. minimus* (n=461) and *An. fluviatilis* (n=722), *An. culicifacies* (n=185) was also dissected for gut and gland infection. Ten specimens of *An. minimus* were found gland positive; the sporozoite rate was 2.2 per cent. *An.*

fluviatilis showed a sporozoite rate of 1.1 per cent. None of the *An. culicifacies* dissected was found positive either for gland or for gut infection.

Sibling species composition: The diagnostic PCR assay of 247 adult specimens of *An. minimus* identified all as species A. In the case of *An. fluviatilis*, out of the 245 samples PCR assayed, 87.0 per cent were of species S and 13.0 per cent species T.

Discussion

An. minimus s.l. is one of the primary malaria vectors in the hilly-forested regions of Southeast Asia¹. Despite a large number of studies over its range of distribution, it is difficult to have an overall view of its ecological and bionomical characteristics⁷. *An. minimus*, which was earlier believed to have disappeared from this area consequent to the introduction of DDT spraying under the National Malaria Eradication Programme (NMEP), was later found to have reappeared^{1,2}. Our findings are in conformity with those of the earlier reports that in northeastern region of India, *An. minimus*, which was thought to have disappeared after the introduction of DDT residual spraying, is still present and transmits malaria parasites in some areas, with poor or no spray coverage⁸⁻¹⁰. Further, in the study area, *An. minimus* was the incriminated vector of malaria^{2,3}.

The present study was undertaken in a hilly and forested area, which has been hyper-endemic for *falciparum* malaria^{2,3}. The study villages are situated at an altitude of 573.4 - 601.2 m mean sea level and are of almost similar ecotype. However, variations in density of the two vector species were observed between the study villages. The variations were due to the difference in extent of forest cover and other vegetation and dissimilarity in contour and water flow rates in the streams surrounding the villages. The density of both the vector species did not correlate with rainfall in the study area and their abundance throughout the year might be due to the presence of perennial streams, the preferential breeding habitat of these two-vector species¹¹. The increase in density during rainy and post rainy months could be due to enhanced breeding in paddy fields as reported by Rao¹¹. In Assam, peak abundance of *An. minimus* was noticed during rainy season¹².

An. minimus generally rests in houses and cattle sheds during day time¹¹. During the present study, *An. minimus* was collected only from human dwellings and not from cattle sheds, indicating its preference to rest in human dwellings. In Assam also, *An. minimus* was

Table III. Parous rate of the vector species during the three seasons

Season	Species	Number dissected	Parous rate (%)
Rainy	<i>An. minimus</i>	202	56.4
	<i>An. fluviatilis</i>	313	55.0
Winter	<i>An. minimus</i>	164	70.1
	<i>An. fluviatilis</i>	239	60.3
Summer	<i>An. minimus</i>	107	63.6
	<i>An. fluviatilis</i>	196	55.6

reported to rest predominately in human dwellings^{12,13}. *An. fluviatilis*, though collected from cattle sheds; a significantly higher number was obtained from human dwellings. Contrary to this finding, a study conducted in Jeypore hills of Orissa state¹⁴ showed that the ratio of *An. fluviatilis* resting in human dwellings to cattle sheds was 1.0: 1.4. The reason for the absence of *An. minimus* and the very low number of *An. fluviatilis* in cattle sheds in the present study was because of the open type of cattle sheds without walls which are the preferential resting sites of these species.

The abdominal condition of day time resting females provide an additional evidence of their resting behavior¹⁵. Among the indoor resting females of *An. minimus*, the proportion of semi gravids was markedly lesser than fully feds and the proportion of gravids was significantly lower, indicating that a large proportion left indoors before completion of their gonotrophic cycle. This leads to believe that the population of *An. minimus* in the study area tends to be more exophilic for resting. However, during the entire study period, only one *An. minimus* was collected from outdoors. It has been reported that the numbers collected outdoors and indoors cannot be compared directly because of the fact that adult anophelines could be collected from houses or cattle sheds with less effort, as the adult tend to become concentrated in indoor resting shelters whereas the effort required is disproportionately more in outdoor shelters as the adults are scattered over wide areas^{11,16}. In the present study, since the study villages are surrounded by jungles in all directions the potential resting shelters outdoors are vast. Probably, this could be the reason for not collecting a good number of *An. minimus* outdoors. However, it is necessary to focus intensively on outdoor collections especially by digging more ideal pit shelters and by fixing light-traps so as to confirm the exophilic behaviour of this vector species.

Recently, endophily of *An. minimus* has been reported in Assam by several workers^{17,18}. In Sonitpur¹² and Tamulpur PHC¹³ most of the *An. minimus* collected from houses were either fully fed, semi gravid or gravid in almost equal proportions indicating its endophilic behaviour. However, it was suggested that *An. minimus* population in Tamulpur PHC; though endophilic, exhibited some degree of exophily and these findings needed further confirmation. There are also earlier reports of some degree of outdoor resting of *An. minimus* in Assam¹⁹ and in Jeypore hills²⁰. Outside India, exophily of *An. minimus* was reported in Thailand²¹.

Similar to *An. minimus*, though direct collections of *An. fluviatilis* yielded low number from outdoors, the proportion of semi gravid indoors was lesser to fully fed specimens, which apparently indicates an exophily of this species in this area. *An. fluviatilis* has also been known to prefer outdoor resting in many parts of India^{11,15}.

Among the indoor collections of the two vector mosquitoes, a higher density was obtained from human dwellings than cattle sheds. An important finding of this study was that both the vector species rest mainly on walls of the houses in all seasons. But Das *et al*¹⁵ reported that in human dwellings *An. fluviatilis* preferred to rest on walls only during winter and summer months and on ceilings during rainy season. Dev¹² observed in Assam that *An. minimus* and *An. fluviatilis* were resting on walls, hanging clothes and other articles and under beds. In the present study, the reason for not finding these vector species on roofs, eaves and hanging objects inside human dwellings could be attributed to the structure of the huts in the study area. There was a gap of 2 to 3 feet between the two side walls and roof which makes the house more lighted immediately after day breaks. Because of this, mosquitoes avoided resting on light exposed portions such as roof, eaves and hanging objects in houses and preferred to rest on walls at a height of 3-4 feet which is relatively darker.

All *An. minimus* collected from the study area were of species A. This sibling species has been incriminated as a malaria vector in Assam²² and also in the study area. In the case of *An. fluviatilis*, though both species S and T were present, the former was the predominant one and the incriminated vector elsewhere²² and also in the study area. It could be possible that the prevalence of *An. minimus* species A and *An. fluviatilis* species S has been accountable for the hyperendemic situation of the area for falciparum malaria.

In the study area, *An. minimus*¹ and *An. fluviatilis* are susceptible to DDT and synthetic pyrethroids. Indoor residual spraying with DDT/ synthetic pyrethroids has been the main vector control measure in the district since 1958. In such a situation, it is expected that malaria transmission is interrupted but it is not so as malaria continues to persist with high morbidity and mortality, indicating an inadequate implementation of indoor residual spraying over the years. Both the vectors, though their gonotrophic conditions indicated higher degree of exophily, were found resting

predominantly on walls in human dwellings after every feeding and there are ample chances for the females to come in contact with the insecticides. Therefore, a quality indoor residual spraying of human dwellings focusing indoor walls would reduce the vector density and thereby interrupting the transmission of malaria in the area.

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Reprint requests: Dr S.S. Sahu, Scientist 'D' & Officer-in-Charge, VCRC Field Station, Koraput, Hati Line, Near Collectorate, Koraput, At/P.o/ Dt. Koraput, Orissa 764 020, India
e-mail: sssahu1961@gmail.com