Life cycle responses of the midge of *Chironomus* species (Diptera: Chironomidae) to sugarcane and paper pulp effluents exposure

Nyakeya K.*[1, 3], Nyamora J.M.[2, 3], Raburu P.O.[4], Masese F.O.[4], Kerich E.[5] and Magondu E.W.[2]

Kenya Marine and Fisheries Research Institute, P.O. Box 31, Kampi ya Samaki, Baringo Station, Kenya[1]
Email: *kobinginyakeya@gmail.com

Faculty of Agriculture and Natural Resource Management, Kisii University[3]

Department of Fisheries and Aquatic Sciences, University of Eldoret, P.O. Box 1125 – 30100, Eldoret, Kenya[4].
Emails: praburu2002@yahoo.com; f.masese@gmail.com

Directorate of Research and Extension, University of Eldoret, P.O. Box 1125 – 30100, Eldoret, Kenya[5]
Email; emmycheptoo@gmail.com

Abstract

The degradation of freshwater systems is on the increase due to anthropogenic activities. In the recent past, there has been a rapid shift of assessment and monitoring from the conventional methods to biomonitoring of these ecosystems globally. Although, an organism's response to degradation varies, little is known as far as their sensitivities are concerned. The aim of this study, therefore, was to assess the life cycle responses of the midge of Chironomus species (Diptera: Chironomidae) to sugarcane and paper pulp effluents exposure. This is in order to ascertain their use in the monitoring of freshwater ecosystems. Fourth instar of Chironomid midges were sampled at two locations on the Rivers Nzoia and Mbogo. River Mbogo is a tributary joining River Nyando within the Lake Victoria Basin. All midges were taken to the laboratory on the same day of collection and acclimatized in a controlled climate room for 24 hours. They were then exposed to the effluent dilutions (i.e. 100%, 50%, 25%, 12.5% and lastly 6.25%) collected from Webuye Paper Mill Factory and Chemelil Sugar Factory treatment ponds/lagoons. A completely randomized experimental design was employed in which ten midges were placed in each of the treatments replicated four times and the experiment ran for 28 days. It was found that emergence of the Chironomus species decreased with an increase in the effluent concentration. An increase in effluent concentration, led to a delay in emergence of chironomids over time. However, there was no significant difference ($p<0.05$) between the emergence of Chironomus species exposed to the two effluents. Emergence is one of the most sensitive endpoints in toxicological studies. This is because effects on emergence involve three moultings and the very complex pupation process. Therefore, not all surviving larvae would have managed to undergo all the four stages of their life cycle due to adverse effects of the effluents. The study concluded that pulp paper and sugar cane effluents delayed the development of life cycle stages of Chironomus species and it was recommended that life cycle stages of the midges, Chironomus species can be a good indicator of environmental degradation.

Key words: Pollution, toxicology; *Chironomus species*; and effluent

INTRODUCTION

Water is the basic resource upon which society relies for its quality of life; including its health and recreation (Kobingi *et al*., 2009). However, because of the unsustainable use of freshwater, coupled with its scarcity in most countries including Kenya, the quantity and quality available for human use continue to be a major challenge. Thus posing the world's greatest threats over the coming decades on biodiversity loss, climate change and water shortages. In Kenya the situation has been worsened by degradation of the country's water towers through land use and land cover change (Mati *et al*., 2008). Also environmental impacts are already manifesting themselves in form of frequent and destructive flooding, receding lake levels and supra-reduced base flow conditions in streams and rivers, with occasionally or completely drying up of rivers (Masese *et al*., 2009 a).

In the catchments of Lake Victoria Drainage Basin (LVDB) - Kenya, the most serious environmental concern is the degradation of the streams, rivers and the lake itself. Consequently, loss of water quality and biological diversity threaten the well-being of millions of lake side riparian communities. This is because studies have shown that portable water supply by relevant authorities is less than 60% hence, most people use the water directly without prior treatment (Ntiba *et al*., 2001; Njiru *et al*., 2008; Kobingi *et al*., 2009; Masese *et al*., 2009b). The major cause of degradation is increased intensity of agriculture and deforestation coupled with the rapid growth of urban centers and industrial activities that have been linked to increasing magnitude and frequency of run-off events (Mutie, 2006; Mati *et al*., 2008), pesticide contamination (Osano *et al*., 2003), reduced base flow (GEF, 2004), erosion and sedimentation of streams and rivers (Mutie, 2006). Others include habitat loss as a result of deforestation and horticulture, river bank degradation by animals, sand mining (Masese *et al*., 2009a; Masese *et al*., 2009b; Raburu *et al*., 2009a; Raburu *et al*., 2009b; Raburu and Masese, 2010) and drainage of wetland areas in the upper reaches (Bavor and Waters, 2008; Njuguna, 1996). Overall, the hydrological characteristics of flow and water retention have been altered resulting in massive and destructive flooding in the lowlands during spates (UNEP, 2003; Kadomura, 2005). As a result, these activities have led to sedimentation and eutrophication that have not only affected domestic and industrial water supply (Ntiba *et al*., 2001), but also led to massive fish kills and proliferation of the water hyacinth (*Eichhornia crassipes*) (Gichuki, 2000; Lung'aiya *et al*., 2000; Mugidde *et al*., 2005; Wawire and Ochiel, 2005; Njiru *et al*., 2008).

In view of the foregoing, it is clear that the degradation of Lake Victoria and its entire catchments areas is a challenge not only to the riparian communities who derive their livelihood from it, but also to scientists (Kibichii *et al*., 2007). In the recent past , scientists have shifted their approaches from the traditional way of monitoring aquatic systems to the most current, convenient and efficient approach that employs a more integrated and holistic ecosystem approach – biomonitoring (Masese *et al*., 2009a; Ojunga *et al*., 2009; Raburu and Masese, 2010; Raburu *et al*., 2009b). For example, in the upper catchments of L. Victoria different authors have shown that macroinvertebrates can respond in different ways to pollutants depending on the nature of the stress in the aquatic ecosystem (Masese *et al*., 2009a ; Raburu *et al*., 2009b; Aura *et al*., 2010) whereas in the lower catchments *Chironomus* species has been depicted as tolerant to pollution as opposed to EPT which are intolerant (Kobingi *et al*., 2009). Most of these studies have been useful in laying a firm foundation for developing indices to guide monitoring practices not only in the basin, but in Kenya as a whole. Although, their response to degradation varies, little is known as far as their sensitivities are concerned. Such sensitivities can only be obtained by toxicity bioassay tests using different contaminants like effluents, inorganic salts and pesticides.

Over the years, toxicity bioassays have been described and put to routine use that are based on a variety of organisms, from well-established fish and *Daphnia* toxicity tests to cell based systems (Blaise, 1991). The observed toxicity in any given assay system is influenced by numerous environmental factors and is strongly dependant on the test organism (Bancon-Montigny *et al*., 2001). Members of the test panel should be selected from among species of ecological relevance to the study area, and combine a broad chemicals detection spectrum and a sufficiently high sensitivity. Hence, the current trend internationally is to include native species in the suite of toxicity test species, in order to generate ecologically relevant and environmentally realistic data for use in water quality guidelines. Similar studies have not been done in Kenya and information is lacking on the sensitivity and precision of various indigenous taxa to various contaminants in rivers and streams. Hence this study aimed at assessing the life cycle responses of the midge of *Chironomus* species (Diptera: Chironomidae) to sugarcane and paper pulp effluents in the catchments of Lake Victoria Basin (LVB).

MATERIALS AND METHODS

Chironomids

Chironomids used in this study belong to the order Diptera (flies, midges and mosquitoes) in the family of Chironomidae. This is the largest insect order (Cranston, 1995). Its lifecycle includes eggs, larvae, pupae and adult with the adult phase differing dramatically from the larvae. The egg, larval and pupal stages are normally aquatic, with mating and oviposition occurring in the adult phase (Figure 1). The larval stage is, for most chironomids, spent in tubes in the sediment from which they feed on the deposited detritus (Rasmussen, 1984) whereas the adult stage is terrestrial (Groenendijk, 1999). *Chironomus* species are commonly used for toxicity tests because they are easy to culture (McCahon and Pascoe, 1988).

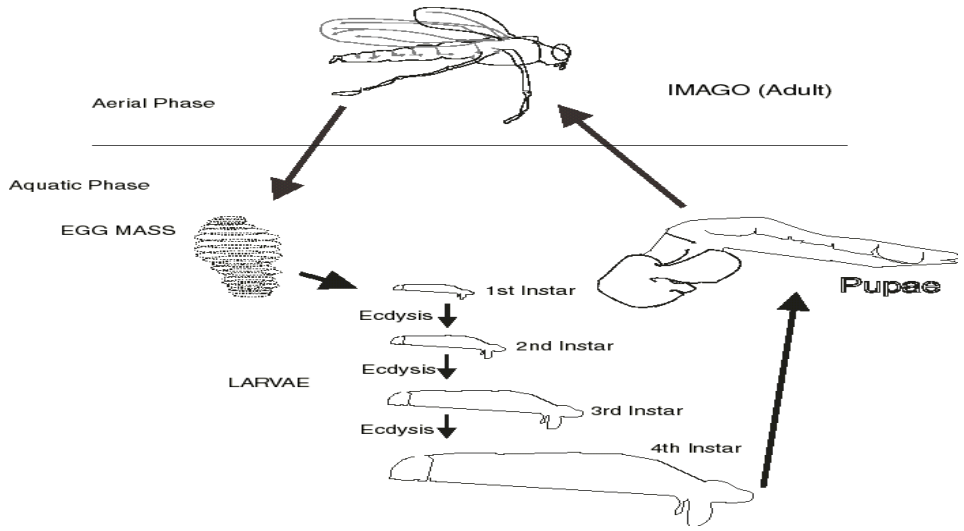


Figure 1: Life cycle of a typical Chironomid

Study Area

Indigenous *Chironomus* species for toxicity tests in the laboratory were sampled on two locations in the Rivers Nzoia and Mbogo, a tributary joining River Nyando within the LVB, i.e. polluted site (C for River Nzoia and D for River Mbogo) and pristine site (R for River Nzoia and S for River Mbogo) (reference site). Station R of River Nzoia was characterized by thick riparian vegetation,

slightly turbid water and a sandy substratum whereas on the other hand, station C of the same river was characterized by paper kraft effluents emanating from Webuye Factory. The water was highly turbid, the riparian vegetation was totally cleared and the substratum was muddy. Effluent contamination in R. Nzoia comes from a paper factory, Webuye Paper mill which creates a distinct point source of pollution in the river, whereas R. Mbogo receives effluent contaminants mainly from the Chemelil Sugar factory. The pristine sites (reference) were points upstream in the forest from the two factories whereas the polluted sites were just downstream from the point of the factories.

Field sampling

Indigineous *Chironomus* species for toxicity tests in the laboratory were sampled from the two locations of each river under study as described above. It is only the indigenous Chironomid midges (Plate 1). that were used in the effluent toxicity test because a majority of the organisms including macroinvertebrates are most sensitive to effluents and/or toxicants when at their larval stages (USEPA, 2002; Fonseca and Rocha, 2004). Given that enough larvae for successful toxicity tests couldn't be realized at one particular sampling occasion, two to three occasions were made.

The larval sampling took place in sediment banks, on loosely hanging vegetation from the banks, sweeping along the exposed stones on the shallow waters of each respective river using a hand net and checking on any litter bug that was found on the sampling site. The upper mud layer of the sediment bank was scraped over three metres using nylon nets with a mesh size of 300 µm. The sediment was then sieved by nylon nets with mesh size of 400 µm in order to collect larvae belonging to the genus *Chironomus* and then placed in clear plastic vials.

Collection of effluents and preparation

Liquid effluent samples from the above mentioned factories (Webuye and Chemelil) were sampled at a point between the final treatment and the discharge outfall. Samples were then transported to the laboratory and either used immediately or kept at 0-4^{o}C. These were later used to inhibit microbial degradation, chemical transformations, and loss of highly volatile toxic substances. However, samples once collected were used within the 36-h period.

Laboratory acclimatization of the test organisms

The sampled larvae were transported to the laboratory where they were acclimatized on the basis of the maintenance methodology developed by different authors (Fonseca and Rocha, 2004; Santos *et al*., 2007) and which have been adopted by many environmental protection bodies: tray length (45 cm x 6 cm x 35 cm) as shown by the sketch on Fig. 2 covered with a mesh cage with a control sand layer at the bottom (1 cm, sieved through 0.5 mm mesh size and sterilized at 550ºC for 2 h), and 4 litres of dechlorinated water (USEPA, 1999). Dechlorinated water was also used for toxicity testing of the *Chironomus* species. Such water has been used for toxicity tests of *Chironomus xanthus* (Fonseca and Rocha, 2004) and *Chironomus tentans*, (USEPA, 1999).

Chironomid larvae were fed on chick mash. A 12:12, light: dark photoperiod regime was maintained. The water temperature of the aquaria was maintained at 25^{o}C to avoid temperature variations. All toxicity experiments were done under static non-renewal acute toxicity test.

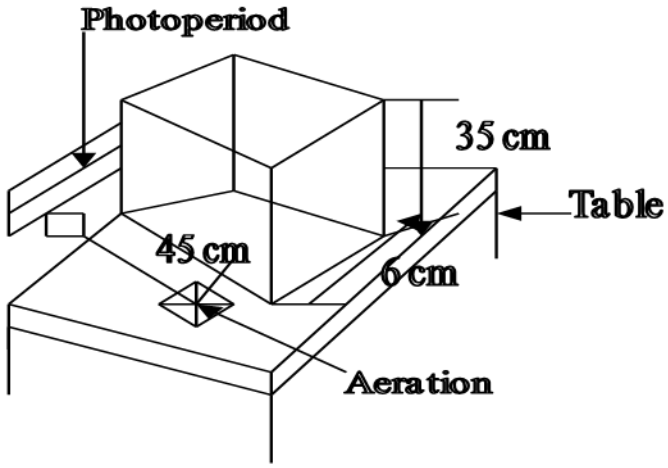


Figure 2: General view of the aquarium that was used for maintaining *Chironomus* species in the laboratory

Bioassays/Bio-assessment

Experimental design

Five industrial effluent sample concentrations plus control for each test with four replicates was used for the test of the *Chironomus* midges (USEPA, 2002). Ten live (midges) test organisms were exposed to each industrial effluent sample concentration (i.e. six concentrations x ten midges per concentration for each test). Serial dilutions of the effluent for each test was done with a factor of 0.5 i.e. 100%, 50%, 25%, 12.5% and lastly 6.25% (USEPA, 2002). Ten midges in all the experiments were added to 500 ml beakers, containing the appropriate serial effluent dilutions, with approximately 1 cm layer of sterilized sand and 1.0 ml of food suspension. A temperature of 25^{o}C and a 12:12, light: dark regime was maintained. The experiment ran for 28 days when the adults started emerging (Fonseca and Rocha, 2004). Consequently, additional feeding of suspended chick mash was added to provide an extra source of food for the organisms under study. This was done three times a week.

Identification of the Chironomids

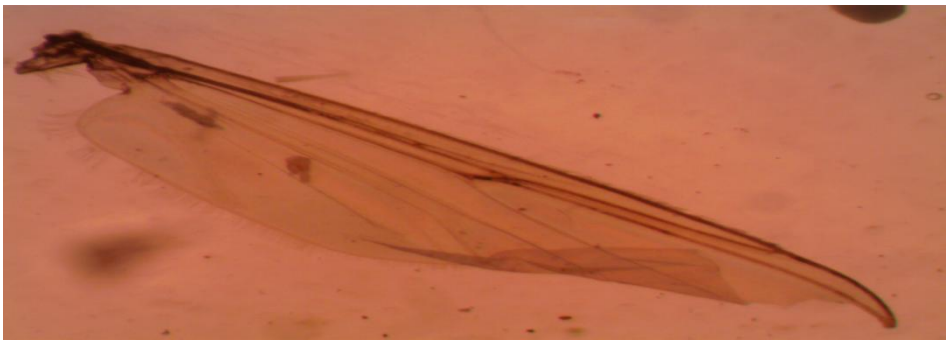
The Chironomid midges were identified to the lowest level possible using identification keys (Epler, 1995) in order to know the different species available for the study. Larval specimens sampled from the four stations of the two rivers and stored in 70% ethanol were observed under a dissecting microscope in order to ascertain the physical features before the head capsule was removed for further investigations (Plate 1 below).

X5 magnification

Plate 1: Fourth instar Chironomid larvae used in this study (Photo by Kobingi Nyakeya, 7th May, 2014).

The head capsule of each individual specimen was carefully removed while observed under a dissecting microscope (GallenKamp) using a needle and a sharp thin metal plate. The clean head capsule was then put in a 10% potassium hydroxide (KOH) for 6-24 hours in order to remove the soft obscuring tissues. After 6-24 hour period the head capsule was removed cautiously from the 10% KOH and mounted on the slide using Euparal mountant with the ventral side facing upward. In order to expose the mouth parts properly and the antennae, the head capsule was depressed gently. The mentum teeth were examined and counted as well as the antennae segments as this could be used in differentiating different species.

The wings of the reared Chironomid adults were also carefully removed while being observed under the dissecting microscope and mounted on the slides using Euparal mountant and observed under the compound microscope (see plate 2) with different ranges of magnification (i.e. x5, x10 and x20). This is because different *Chironomus* species have different characteristics on the wings



X40 magnification

Plate 2: A wing of an adult *Chironomus* species (Photo by Kobingi Nyakeya, 7th May, 2014)

Emergence

Inspection for the emerged midges began from day seven and it was done daily on subsequent days until termination of the experiment at day 28. Also, from the 7th day onwards, cages were placed on the jars to catch emerging adults. However, because there were chances of mortality, the experiment was inspected regularly to remove any dead larvae in the concentrations. At the end of the experiment (28 days), the sediment was sieved through a 350µm sieve and surviving larvae were counted. Emergence at each treatment concentration was calculated using the equation below:

$$E = n_e/n_a$$

Where, E = emergence,
n_e = the number emerged, and
n_a = the total introduced.

Data Analysis

Data was expressed as the proportion emerged and was first transformed by the Probit transformation to stabilize the variance and satisfy the normality requirement before analysis. Emergence of the *Chironomus* species midges was presented as the accumulated number that emerged against time (days).

Two way ANOVA for the tests of the *Chironomus* species midges was run with the station and concentration as the main factors with the station x concentration interaction effect and where there was no interaction then 1-way ANOVA was run with the main individual factors only (i.e. effluent, station and concentration). *Post hoc* Tukey test was then performed to identify means of concentrations of the different test parameters for the *Chironomus* midges differing from one another. One-Way ANOVA was used to determine significant difference between the control and the effluent concentrations. To determine at which actual toxicant concentrations emergence was significantly delayed compared to the control for the effluents and compared to the two effluents, two-way ANOVA was used. All analysis was done in Sigma Plot and the significant differences were inferred at α=0.05 level.

RESULTS

***Chironomus* species identified in the study**

Four species were identified and classified in this study. They included *Chironomus decorus*, *Chironomus riparius*, *Chironomus stigmaterus* and *Goeldichironomus c.f. natans*.

Emergence of *Chironomus* species

From the results, it was observed that generally paper and pulp mill effluents delayed the emergence of the Chironomid midges with time such that the higher the concentration the more the delay in time (number of days). However, from the graphs below it is evident that emergence was more delayed in station R as compared to station C, but in both stations emergence succeeded as from the 7th day in the control compared to all other concentrations (Figures 3 and 4).

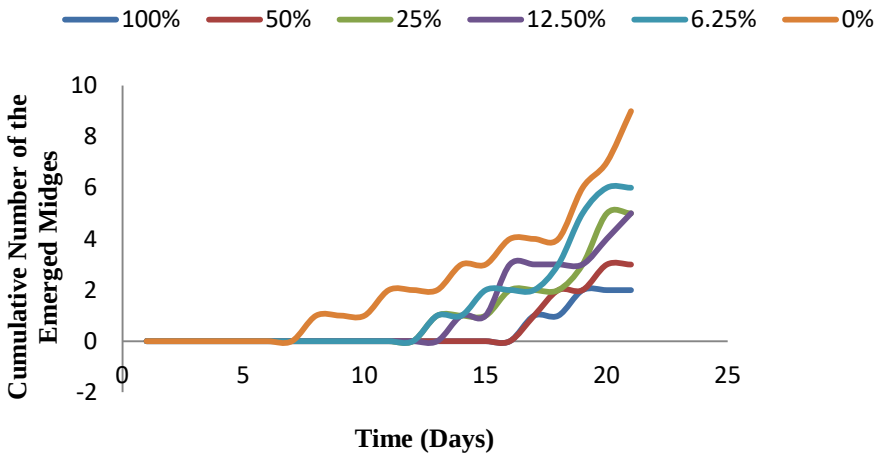


Figure 3: Emerged Chironomid midges against time for station R (reference point) of R. Nzoia during the study period

Although emergence delayed with increased concentration, midges emerged earlier in 50% and 100% concentration (i.e. the 10th day) in station C as compared to station R where they emerged as from day16. Another point worthy noting is that emergence was somehow accelerated at 6.25% paper and pulp mill concentration of station C as compared to any other concentration in both stations including the control.

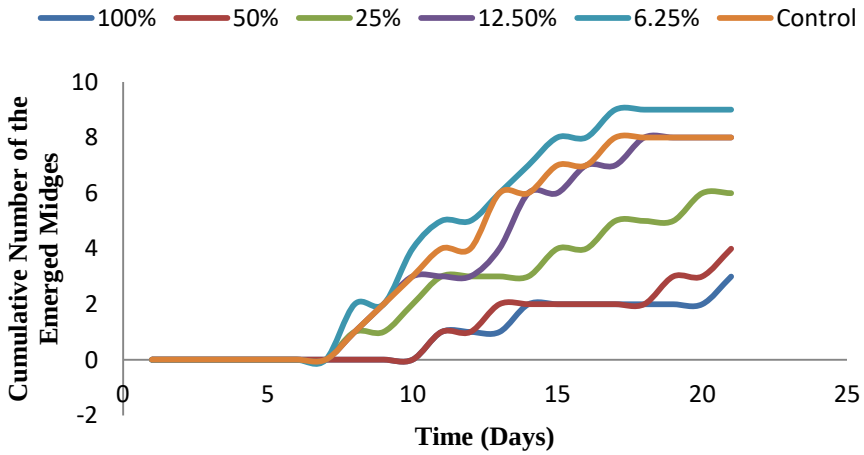


Figure 4: Emerged Chironomid midges against time for station C (downstream) of R. Nzoia during the study period

A similar trend in emergence was observed in the two stations of R. Mbogo (i.e. an increase in sugar cane effluent concentration resulted in delayed emergence of the Chironomid midges). With an exception of the control in station S of R. Mbogo where emergence began at day 7, emergence was delayed until day 10 for 6.25%, 50% and 100%. It was further delayed until the 13th and 14th day for the 12.5% and 25% respectively (Figure 5).

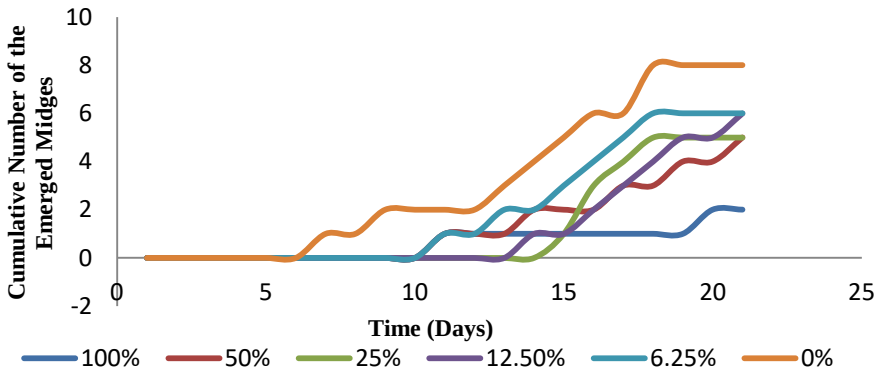
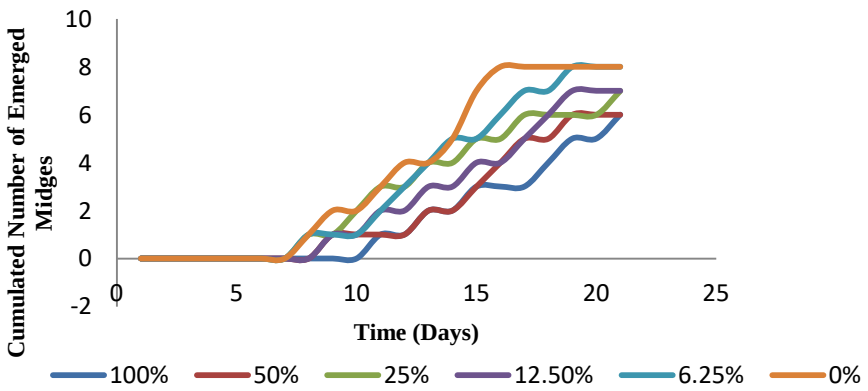


Figure 5: Emerged Chironomid midges against time for station S (upstream – reference point) of R. Mbogo during the study period

In station D, however, emergence had picked up by the tenth day in all the concentrations including the control and in general account, emergence was more pronounced in sugar cane effluent concentrations for station D (Figure 6) of R. Mbogo.

Figure 6: Emerged Chironomid midges against time for station D (downstream) of R. Mbogo during the study period



The highest emergence achieved was in the control of both effluents and in the 6.25% concentration of the paper pulp effluent whereby in all those instances at least six midges emerged for the entire 21 day study period (see Figures 3-6).

Emergence increased with midges sampled downstream in both rivers. However, the highest rate (17.5%±5%) of emergence occurred on midges sampled from station D of R. Mbogo whereas both stations R and S in the upstream of the two rivers registered same rate of emergence (14.2%±2.5%) as depicted in Figure 7.

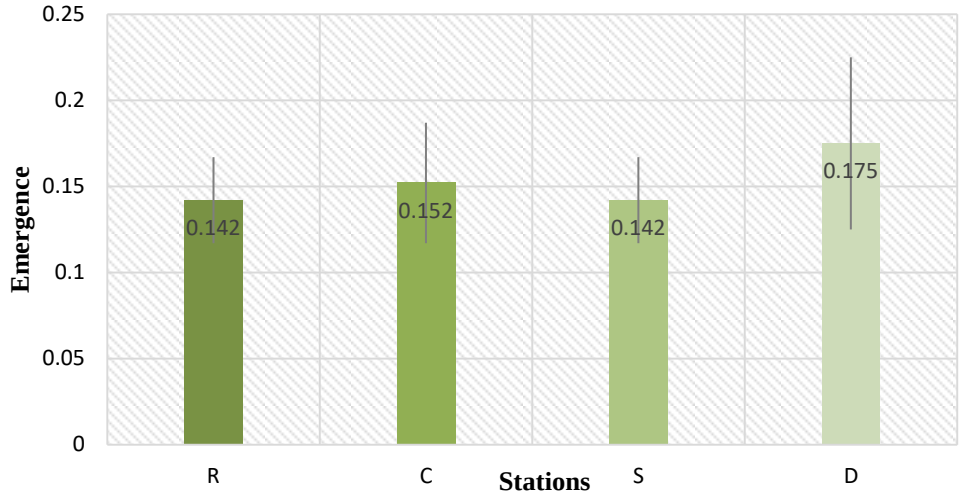


Figure 7: Mean (±SEM) emergence of *Chironomus* species sampled from the four stations of the two rivers (Nzoia, R – reference point; C - downstream and Mbogo, S – reference point; D - downstream) during the study period

All pair wise multiple comparison of the emergence of the exposed midges between stations within all the dilutions/concentrations (i.e. 0%-100% effluent) did not indicate any effect. The same occurrence was observed with the different levels of concentrations. There was no significant difference in the emergence of the midges exposed to different levels of effluent concentrations within the four sampled stations.

However, Two-Way ANOVA showed that there was an interaction in the emergence of the *Chironomus* species and the concentration (F=4.359, p=0.002) but such an effect was not observed in the effluent, station, effluent x concentration and station x concentration (Table 1).

Table 1: Summary of Two-Way ANOVA for the emergence of the chironomid midges (*=p<0.05)

Source of Variation	DF	SS	MS	F	P
Effluent	1	0.00167	0.00167	0.22	0.637
Station	3	0.0183	0.00611	0.898	0.447
Concentration	5	0.148	0.0297	4.359	<0.002*
Effluent x Concentration	5	0.00833	0.00167	0.22	1
Station x Concentration	15	0.0217	0.00144	0.212	0.999
Residual	72	0.49	0.00681		
Total	95	0.678	0.00714		

*Denotes significant difference

Relationship among the concentrations for emergence of the exposed *Chironomus* species

Post hoc Tukey test for emergence showed a significant difference between the Chironomid midges in the control and all other effluent concentrations (F=55.9, p=0.003). However, the relationship was weak.

Table 2: *Post hoc* Tukey test among means of different concentrations for the emergence ($p<0.05$)

Concentration (%)	Emergence
0	0.2±0.07a
6.25	0.19±0.04a
12.5	0.17±0.02ab
25	0.17±0.02ab
50	0.11±0.06ab
100	0.09±0.05b

A, b, c, d, e shows an effect

DISCUSSION

Several studies have been carried out using emergence of Chironomids as an endpoint (Leslie *et al*., 2004). It is argued that emergence is one of the most sensitive endpoints in toxicological studies compared to growth for example (Marinkovic *et al*., 2011). One of the reasons advanced to this fact is that effects on emergence involve three moultings and the very complex pupation process.

Not all surviving larvae managed to undergo all the four stages of a life cycle of an insect, indicating that larval development was substantially delayed with time. This was in total contrast with Paumen *et al*. (2009) who had reported that all surviving larvae managed to emerge arguing that larval development was not substantially delayed. The author went further to state that emergence equaled survival in metal exposed *C. riparius* larvae. In fact, this is in total contrast with the findings of the current study where serious acute or sublethal effects were observed. It is also reported that accelerated emergence, for example 2,4,5-trichlorophenol and several endocrine-disrupting chemicals have been said to have boosted emergence in chironomids (Groenendijk, 1999) most probably due to disturbance of the moulting and pupation processes.

In the current study it was observed that female emergence was significantly delayed with an increase in effluent concentration in time as compared to males. Differences in sensitivity of males and females have been reported in studies on different types of chemicals, including metals, pesticides, PAHs, surfactants and endocrine-disrupting chemicals (Santos *et al*., 2007;). There is however, no single satisfying explanation that has been given as to why there are always sex-emergence differences in chironomids especially when exposed to different anthropogenic impacts. This observation was not in agreement with Groenendijk (1999) who reported that male emergence was significantly delayed when exposed to certain levels of metal concentrations. Santos *et al*. (2007), equally reported in their study that among the six tested compounds, male emergence was significantly delayed, while no significant difference from the control was found for female emergence.

In general, the results from this study revealed that the two effluents delayed emergence and based on other published studies, toxicants have exhibited similar effects on *C. riparius* larval development and/or emergence (Groenendijk, 1999). The concentration range at which emergence was delayed in both effluents was much narrower an indication that both the two effluents would have had constituent toxic substances that would have affected vital biological processes in the chironomids under investigation.

Relationship among the concentrations for the emergence of the exposed *Chironomus* species

The *Post hoc* test that indicated a significant difference between the control of the measured parameters and the different treatments is an indication that paper pulp and sugarcane effluents had adverse effects on the *Chironomus* species. It may also be explained by the fact that *Chironomus* species are sensitive to both effluents hence can be relied upon in testing for pollution in any aquatic ecosystem.

Emergence of *Chironomus* species was affected by different levels of concentrations whereby those exposed to higher concentrations did not emerge as compared to those exposed to low concentrations hence a significant difference was noted between 6.25% effluent and all other dilutions. However, emergence was not significantly delayed between the midges exposed to 12.5%, 25% and 50% effluent concentrations most probably due to the presence of different species that responded differently to the toxicants in the effluents. The same trend was observed in the deformity of the exposed midges, a clear indication on how *Chironomus* species can behave to different doses of toxicants (Groenendijk, 1998).

CONCLUSION

From the results of this study, emergence of *Chironomus* species decreased with an increase in the effluent concentration, however, it increased downstream. An increase in effluent concentration, led to a delay in emergence of chironomids over time. There was no significant difference between the emergence of *Chironomus* species exposed to the two effluents, but taking into account on the life cycle stages of the midges, C. species can a good indicator of environmental degradation.

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