1.1A Introduction

The problem of environmental pollution on account of essential industrial growth is mainly arising due to the problem of disposal of industrial waste, whether solid, liquid or gas (Kamlesh et al., 2007). Water pollution occurs as a result of the presence of any objectionable or waste material capable of damaging the water quality. With the global shortage of fossil energy, especially oil and natural gas, and heavy biomass energy consumption, a major focus has developed worldwide on biofuel production (Barbara, 2007). Ethanol is a major fuel additive and promising energy alternative in the future (Sasidhar et al., 2007). Ethanol has already been introduced on a large scale as a fuel additive in Brazil, USA, and some European countries, and it is expected to be one of the dominating biofuels in the transport sector within the coming twenty years. Ethanol is being blended with diesel as well as with petrol. It is also used as a neat alcohol in dedicated engines, taking advantage of the higher octane number and higher heat of vaporization. Furthermore, it is regarded as an excellent fuel for future advanced flex fuel hybrid vehicles (Hahn et al., 2006). Gasoline and diesel which are currently being blended with ethanol contaminates the environment during spills. The presence of gasoline, diesel and ethanol together brings about deleterious impacts on living organisms present in soil and water. The presence of ethanol delays the degradation of gasoline.
present in the aquifer. Spills and leakage of pure ethanol from storage tanks can create toxicity up to 150% more than a spill from non-ethanol fuel. Ethanol dissolves oxide scale from the walls of pipes and tanks, subjecting the system to internal corrosion, which leads to leaks. In the United States, ethanol constitutes 99% of all biofuels (Farrell et al., 2006). Fermenting and distilling corn ethanol requires large amounts of water. Thus, a total of about 12 l of wastewater must be removed per liter of ethanol produced. More than 1,700 gallons of water is required to produce one gallon of ethanol (Pimentel and Patzek, 2008). The production and usage of ethanol contributes to pollution of air, soil, water and global warming (Pimentel et al., 2008). Ethanol rapidly biodegrades in water which lowers the amount of dissolved oxygen in an aquatic system resulting in fish kills. Further ethanol is toxic to fish and other aquatic and terrestrial organisms at high concentrations. Fuel ethanol being heavier than gasoline is completely miscible in water, which will separate ethanol from gasoline at high concentrations of water. Ethanol has also got the potential to transport the components of gasoline through ground water and surface water and to spread the floating product to a larger area due to the solvency of ethanol with gasoline components and water. Ethanol when released into water will volatilize and rapidly biodegrade. The potential decrease in dissolved oxygen as a result of ethanol degradation can upset microbial functions as well as aquatic systems. Biodegradation of ethanol can decrease the dissolved oxygen in surface waters resulting in fish kills. The presence of ethanol in water lowers the dissolved oxygen levels and results in increased chemical oxygen demand (COD) and biological oxygen demand (BOD). Emergency Planning and Community Right Know Act (EPCKA) has considered waste ethanol as a hazardous chemical (USEPA, 2008). Sugar industry and jaggery industry are the major problematic sources of pollution to both aquatic and terrestrial ecosystems. The liquid wastes discharged by these industries contain large amounts of alcohols and organic compounds which seem to be lethal to aquatic flora and fauna (Kudesia, 1980). During processing, a large amount of freshwater is used by the mills which accordingly produce a huge amount of the effluent. These untreated or partially treated effluents are discharged in surface water bodies or on land. Oxidation of biodegradable organic component of the effluent results in the depletion of dissolved
Quantitative determination of ethanol and other primary alcohols is often a challenge in the food and microbiological industry, clinical studies, and forensic science. The usage of online ethanol measurements plays an important role in food industry and in clinical analysis (Salgado et al., 1998). The available techniques of ethanol detection include gas chromatography, electrochemical and enzymatic assays. A novel method for the determination of ethanol in jaggery effluent has been developed using GC/MS. Optimization of the mass spectrometer parameters and mobile-phase composition was performed to maximize the sensitivity and reproducibility of the method. This sensitive and rapid method does not require any sample preconcentration, although bioethanol production has been greatly improved by new technologies. The short chains being volatile, remain less time in the aquatic environment, but possess high toxic potential for aquatic life. GC/MS method was mainly used to determine the presence of ethanol in an effluent, since this method is considered to be rapid, accurate, sensitive and was validated according to EMEA guide line. Chromatographic analysis of GC-MS was performed by a calibration technique, employing standard ethanol. The headspace analysis method is very frequently used for the determination of trace organic solvent impurities in effluent samples, and in solid painted materials, etc. Headspace analysis (HSA) is a method that gives information on the nature or composition of liquid and solid samples based on the results of the analysis of the contacting gas phase (headspace) using various static and dynamic versions of gas extraction (Ioffe, 1981). Static headspace gas chromatography (GC) is a technique used for the analysis of volatile organic compounds (Ioffe and Vitenberg 1984). An important application of headspace analysis mainly includes the quantitative determination of trace organic substances present in drinking, natural and industrial waters, sewage (Cruwys et al., 2002). It has also been employed for determining the presence of ethanol in blood (Deng et al., 2003; Zuloaga et al., 2000). Hence the present study was carried out to detect the presence of ethanol in jaggery effluent sample using Perkin Elmer AutoSystem XL Gas Chromatograph equipped with head space sampler. Nevertheless, preconcentration and extraction steps were necessary before the analysis by gas chromatography, due to the complexity and low concentration of the sample.
1.1 B Materials and Methods

1.1.1B Description of the Sample used for GC/MS studies: Jaggery effluent sample was collected in dark brown coloured bottles for the purpose of the investigation. It was obtained from local industries at the point of discharge. The samples were brought to the laboratory under cold conditions. The collected samples were stored at -20°C until assayed (Carolyn, 1999).

1.1.2B Estimation of ethanol in jaggery effluent using an analytical technique: Gas chromatography–mass spectrometry (GC–MS).

Principle

GC–MS is based on a combination of two analytical techniques: capillary gas chromatography and mass spectrometry. This is further based on two analytical parameters: the retention time and the mass spectrum. The analyte molecules which arrive at a vacuum chamber get ionized by 70-eV electrons. The resulting ions are then focused with an electromagnetic lens system and get directed to an analyzer (quadrupole mass filter), where they get separated under the action of an electromagnetic field and get detected based on the mass-to-charge ratios (m/z) and get recorded as a mass spectrum. GC/MS instruments were further equipped with mass spectral libraries, which contain upto 500000 mass spectra. The components of a test sample were identified either between a library mass spectrum and the experimental mass spectrum. Mass spectrometric detection is performed in the full-scan mode (SCAN) in which, mass spectra were measured and identified using a library of mass spectra.

1.1.3B Standard Conditions of GC/MS Analysis: Instrumentation

The experiment was carried out using a Perkin Elmer AutoSystem XL Gas Chromatograph equipped with Perkin Elmer TurboMass Gold Mass Spectrometer, Perkin Elmer TurboMatrix 40 Trap Headspace Sampler and SUPELCO 24103-U SPB™-608 column (30m X 0.25 mm X 0.25μm film). A 5μL of effluent was injected under split mode.
1.1.4B Standard Conditions of GC/MS Analysis: Chromatographic Separation

The temperature of the column thermostat was programmed from an initial value of 60°C for 20 minutes then increased to 150°C at a rate of 25°C/minute and kept at 150°C for 1 minute. The flow of the gas was set at 1ml/minute. The carrier gas employed was helium.

Procedure

10 ml of pure ethanol was used for carrying out GC/MS analysis. This serves as the standard. From this 5μL of pure standard ethanol was directly injected for carrying out GC/MS analysis. This was run to confirm the retention time and spectrum. An ethanol peak as well as corresponding standard area was obtained. It was then compared with the data in the Perkin Elmer library. Similarly 5μL of the jaggery effluent sample was directly injected for GC/MS analysis, which resulted in obtaining a peak as well as area. The mass spectral data obtained were compared to the data in the Perkin Elmer library. Perkin Elmer AutoSystem XL Gas Chromatograph equipped with TurboMass Gold Mass Spectrometer and TurboMatrix 40 Trap Headspace Sampler provided excellent accuracy and precision in carrying out the analysis of effluent sample. The measurements were repeated three times.

1.1C Results

GC/MS method was used to determine the presence of ethanol in jaggery effluent. The mass spectrum of the unknown chromatographic peak is matched with the mass spectrum of a pure compound (ethanol) from the Perkin Elmer library, and the mass spectrum is quantitatively interpreted. It was found that about 0.34μl of ethanol was present in 5μL of the effluent. The chromatographic peak and corresponding mass spectrum of pure ethanol are shown in Figures 1A to 1G. Similarly Figure 2A to Figure 2G exhibits chromatographic peak and corresponding mass spectrum of jaggery effluent.
Chapter 1

Figure 1A Standard chromatogram with solvent peak and ethanol peak

Figure 1B Standard chromatogram with ethanol peak after masking solvent peak

Figure 1C Standard chromatogram with ethanol peak after masking solvent peak
Chapter I

Figure 1D Standard chromatogram with ethanol peak after masking solvent peak

Figure 1E Standard chromatogram with ethanol peak

Figure 1F Standard chromatogram with ethanol peak and area (for 5μl/10ml)

Figure 1G Mass spectrum of pure ethanol obtained from the PE library.
Figure 2A Sample chromatogram with solvent peak and ethanol peak

Figure 2B Sample chromatogram with ethanol peak after masking solvent peak

Figure 2C Sample chromatogram with ethanol peak after masking solvent peak

Figure 2D Sample chromatogram with ethanol peak
1.1 Discussion

A headspace gas chromatography method has been developed for determining C1-C3 alcohols. GC/MS method was found to be rapid, accurate, sensible and was validated according to EMEA guide line. Moshonas and Shaw (1994) has found ethanol as a major compound in fresh unpasteurised orange juice using GC/MS technique. Similar findings were also stated by Nisperos-Carriedo and Shaw (1990). GC-mass spectrometry (MS) methods have been applied to measure the concentrations of ethanol present in biological specimens (Liebich et al., 1977; Liebich et al., 1982; Tang, 1987). The present study revealed the occurrence of ethanol in an effluent quantitatively. It was found that the concentration of ethanol present in jaggery effluent was almost equal to the sub lethal dosage value (1/20 of LC50) which indicates the potential toxic impacts of ethanol.
1.2 DETERMINATION OF BLOOD ETHANOL IN OREOCHROMIS MOSSAMBUS (PETERS): STUDIES USING GAS CHROMATOGRAPHY

1.2A Introduction

Population explosion together with urbanization and industrialization has created innumerable ecological problems including environmental pollution. With the rapid rise in the price of crude oil and projected decreases in oil supplies, alternative fuels receive considerable attention (Hill et al., 2006). The production of ethanol from agricultural products such as starch, sugar or cellulose has been in practice for the past 80 years. Effluents of these industries cause severe pollution problems which impart the need of better waste management techniques. The effect of ethanol on zebra fish has been studied by a number of investigators, which establish the importance of fish being used as a model organism to study ethanol teratogenicity (Michael et al., 2008). Gas chromatographic (GC) assays provide the greatest amount of flexibility and specificity in analyzing volatile compounds. Analysis time and resolution are the two critical factors taken into account when developing a GC assay for blood ethanol. This is based on the separation of substances on a chromatographic column, the inner surface of which is coated with a layer of a stationary phase (a viscous liquid). The process of chromatographic separation is based on various affinities of substances (sample components) to the stationary phase. The test sample components migrate along the column with the help of a carrier gas (helium); the emergence time of a substance from the column (i.e., the chromatographic retention time) is an analytical parameter. The substances
that emerge from the chromatographic column (as narrow zones) arrive at the
detection system and form an analytical signal (chromatographic peak). In 1992,
Tagliaro et al. reviewed methodologies of blood alcohol determination in which GC
is referred to as the most precise and reliable method for alcohol determination in
blood and other biological fluids. It has also become the gold standard in forensic
toxicological studies also. In order to prevent from polluting the injection port,
precolumn and column, proper dilution of the blood before analysis was carried out
(Taligaro et al., 1992). The objective of the present investigation was to develop a
sensitive, reliable, easy-to-use, and rapid procedure for the determination of ethanol
in whole blood, by using the direct injection GC technique. Direct injection and
headspace GC are the two most often used GC techniques for measuring ethanol in
biological specimens (Albert Tangerman, 1997).

1.2B MATERIALS AND METHODS

1.2B.1 Maintenance of Fish

Fresh water fish, Oreochromis mossambicus (Peters, 1852) commonly
known as Tilapia was selected as the animal model for the study considering its
hardy nature, ease of rearing, maintenance, availability, resistance, and economic
viability. They were collected from Kerala Agricultural University, Fisheries station,
Puduvyppu. They were acclimatized to the laboratory conditions for 15 days in large
cement tanks filled with dechlorinated water (500L). The tanks were previously
washed with potassium permanganate, to free the walls from fungal infections. The
tank had a continuous and gentle flow of tap water. The physico chemical
parameters of water were estimated daily according to the procedure of APHA
(1998) and were maintained constant throughout the experiment. The mean values
for the parameters were as follows: as dissolved oxygen of 8.16 ppm, total hardness
13 ± 2 mg/l, total alkalinity 4 ± 2 mg/l, temperature 26 ± 2°C, pH 7.0 ± 0.33 and
salinity 0 ppt. They were fed on commercial diet ad libitum. For experimental set up
the laboratory acclimatized fish were sorted into batches of six each and were kept
in 60 L tubs. Water in the tubs were changed daily. During the experimental period
the animals were fed on the same commercial diet so as to avoid the effects of
starvation on normal physiological processes.
Determination of LC$_{50}$ of ethanol in Oreochromis mossambicus (Peters).

Lethal toxicity studies give information about the relative lethality of a toxicant. LC$_{50}$ test was designed to determine the highest concentration of ethanol that was sufficient to kill 50% of Oreochromis mossambicus and was carried out using semi static method. The median lethal concentration LC$_{50}$ (concentration of ethanol at which 50% mortality of test population occurred) for an exposure period of 96 hr was determined by trial and error. Each experiment was repeated three times at the selected ethanol concentration, every time noting the number of fish killed at each concentration up to 96 hours. A control without the toxicant was also maintained for both lethal and sub lethal studies (Bijoy et al., 2003). The average mortality in each concentration was taken to determine the LC$_{50}$ by graphic method in which the probit mortality was plotted against log concentration of ethanol fractions by the procedure of Finney (1971). In the present investigation the effect of various concentrations of ethanol on O. mossambicus as a function of different exposure periods indicated that the mortality of fishes were dependent on dose and duration. Several tubs of 60 litre capacity were taken. Each tub contained 6 fishes. They were exposed to different concentrations of ethanol ranging from 1.27 g/l to 127 g/l. It was observed that at 13.01 g/l dose, 50% of the fishes were dead within 96 hrs. As per probit analysis, LC$_{50}$ was recorded at 13.107 g/l. In this the 95% confidence limit ranged between 12.786 and 13.382 g/l.

1.2B.3 Behavioural changes observed

When fishes were exposed to different lethal concentrations of ethanol, they exhibited erratic movements, increase in opercular movement followed by a decrease, frequent surfacing and gulping, loss of equilibrium, grouping, increase in respiratory rhythm, excess secretion of mucus followed by a gradual onset of inactivity.

1.2B.4 Bioassay method

For each sub lethal experimental set up the laboratory acclimatized fishes were sorted into batches of six each and the bioassays were conducted in tubs (capacity 60 litres) static waters. For each experimentation fishes weighing 10 ± 2g
were used. To know the effect of higher concentrations as well as lower concentrations of ethanol, apart from 1.3 g/l, two more concentrations were also selected for the present study. Group 1 served as a control and was maintained in the water without addition of ethanol. Group 2 (0.65 g/l), Group 3 (1.3 g/l), Group 4 (2.6 g/l) were also selected for the present studies. While in the sub lethal toxicity study, water was changed daily and the test solutions were renewed every 24 hours to maintain the dissolved oxygen concentration at optimum level (USEPA, 1975). The fishes were fed on commercial diet ad libitum.

1.2B.5 Experimental design for study of the effects of ethanol on immediate and prolonged exposure

For conducting experimental studies, *O. mossambicus* of 10 ± 2 g were taken in three separate tubs which contained desired concentration of ethanol (0.65 g/l, 1.3 g/l and 2.6 g/l respectively) along with tap water. Six replicates were kept for each experiment. The experimental animals were exposed for 21 days with a periodical sampling at 7 days also. During the experimental period of 21 days the animals were fed on the same diet so as to avoid the effects of starvation on normal physiological processes and antioxidant stress. Any other factor likely to influence toxicity was nullified by maintaining a suitable control.

1.2B.6 Preparation of blood sample for Gas chromatographic studies

On completion of fixed exposure periods, blood was drawn from the common cardinal vein using 1ml sterile plastic insulin syringe (Smith et al., 1952) (26 mm gauge size). It was then mixed with an anticoagulant (Sodium fluoride, 2 mg/ml). The total volume was made up to 2 ml. It was then stored at 4°C prior to analysis (Mc Carver and Durisin, 1997).

1.2B.7 Estimation of ethanol in blood using an analytical technique: Gas Chromatography (GC)

**Principle**

GC is a powerful and widely used technique employed for the separation, identification and quantification of components present in a mixture. In this technique, sample is converted to the vapor state and a flowing stream of carrier gas
(helium) sweeps the sample through a thermally-controlled column where the separation of components occurs. The separated components are then made to pass through a hydrogen flame detector where a complex ionization process occurs. As a consequence carbon atoms are given up in proportion to the amount of organic material present. These carbon atoms are counted as they pass through the detector and appropriate signals are transmitted to the recorder where they are transcribed in the form of a peak. Each compound that elutes from the column has a characteristic retention time, defined as the time interval from injection to peak detector response for that compound. The retention time identifies the compound; the magnitude of the detector response measures the quantity.

1.2B.8 Standard Conditions employed for GC Analysis: Instrumentation and Chromatographic separation conditions

All samples were analyzed on a GC-Varian CP 3800 gas chromatograph (GC-Varian CP 3800), equipped with a Flame ionization detector. The Flame ionization detector temperature was maintained at 150°C. The initial column temperature was raised automatically from 60 -100°C at a rate of 5°C/minute. Then the final column temperature was maintained by rising the temperature from 100 to 120°C at a rate of 20°C/minute. The carrier gas employed was helium and its flow rate was 2ml/minute and its pressure was 1 kg/cm². The make up gas was nitrogen. The fuels used were of H₂/Air. The injection technique employed was of splitless mode. The sample injection volume was 1µL.

Procedure

Quantitative calculations were performed by the external calibration standard method. Five standard solutions of pure ethanol in the range of 1.57 mg/l to 7.85 mg/l corresponding to 0.2% to 1% (v/v) was taken. It was then made up to 10ml with distilled water in a standard flask. 1µL of standard ethanol was run first to confirm the retention time. When being subjected into gas chromatograph ethanol is eluted as a single peak within 5 minutes of injection. Amount of the ethanol present in the sample was calculated using standard area. The area under the peak gives an accurate representation of the concentration of ethanol present. For estimating
ethanol in blood, 1μL of the blood was directly injected into the GC injection port without any pretreatment. All the experiments were carried out on the same day. Sources of error were greatly reduced by limiting the number of steps in the protocol for the sample procedure. The area of the single ethanol peak obtained was then calculated by plotting peak area along the Y-axis and concentration along X-axis. The graph was a straight line indicating a direct proportionality. For concentration measurements, the dilution factor was taken into consideration. The results obtained were then expressed as mg/l.

1.2C Results

Effect of 0.65 g/l, 1.3 g/l and 2.6 g/l concentrations of ethanol in the blood of *O. mossambicus* exposed for 21 days with a periodical sampling at 7 days are represented by chromatograms (Figure 1 to 3) and Tables 1.2.1 to 1.2.3 and Figure 1.2.3. Results obtained from the raw data were statistically analyzed by Two way ANOVA (Analysis of Variance) followed by Dunnett’s method.

**Figure 1** Gas chromatograms showing standard ethanol peak

![Figure 1A Gas chromatogram of standard ethanol (3.14mg/l) (0.4%)](image1.png)

![Figure 1B Gas chromatogram of standard ethanol (4.71mg/l) (0.6%)](image2.png)

**Figure 2** Standard Gas chromatograms showing ethanol peak in the blood of *O. mossambicus* upon exposure to different sub lethal concentrations of ethanol for 7days.
Figure 3 Standard Gas chromatograms showing ethanol peak in the blood of *O. mossambicus* upon exposure to different sub lethal concentrations of ethanol for 21 days.
Figure 3c) 1.3g/l  Figure 3d) 2.6g/l

Table 1.2.1 Effect of 7 and 21 days of exposure to different concentrations of ethanol in the blood of *O. mossambicus*.

<table>
<thead>
<tr>
<th>Days of Exposure</th>
<th>Control</th>
<th>Concentrations of ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.65g/l</td>
</tr>
<tr>
<td>7 days</td>
<td>0.762±</td>
<td>25.75±</td>
</tr>
<tr>
<td></td>
<td>0.0117</td>
<td>0.1871</td>
</tr>
<tr>
<td>21 days</td>
<td>1.57±</td>
<td>7.83±</td>
</tr>
<tr>
<td></td>
<td>0.0103</td>
<td>0.0228</td>
</tr>
</tbody>
</table>

Average of six values in each group ± SD of six observations
Blood ethanol concentration was expressed as mg/l

Figure 1.2.2 Levels of ethanol in the blood of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.
A significant increase ($P<0.001$) in blood ethanol concentration was noted in *O. mossambicus* exposed to the three sub lethal concentrations of ethanol as compared to control group (Figure 1.2.3). Investigations using ANOVA substantiates the above statement and the results are shown below (Table 1.2.3a).

**Table 1.2.2a ANOVA Table for Blood ethanol**

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Days of Exposure</td>
<td>1550.754</td>
<td>1</td>
<td>1550.754</td>
<td>125435.96</td>
<td>0.000</td>
</tr>
<tr>
<td>Between Concentrations</td>
<td>4283.715</td>
<td>3</td>
<td>1427.905</td>
<td>115499.04</td>
<td>0.000</td>
</tr>
<tr>
<td>Days of Exposure × Concentration</td>
<td>679.126</td>
<td>3</td>
<td>226.375</td>
<td>18310.839</td>
<td>0.000</td>
</tr>
<tr>
<td>Error</td>
<td>0.495</td>
<td>40</td>
<td>0.012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6514.090</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$df$ - degrees of freedom

$F$ - variance ratio

$\times$ - Interaction effect

Sig. - Significance level

0.000 indicates that the values are significant at $P < 0.001$

Studies conducted on blood ethanol levels exhibited a significant difference between days ($P<0.001$). Between concentrations there was a significant difference ($P<0.001$). Considering the interaction effect of both days as well as concentrations, a significant difference ($P<0.001$) was observed.
A subsequent pair wise comparison between various concentrations with respect to control was carried out using Dunnett’s method. In the case of blood ethanol values, significant difference (P<0.001) was observed in all the three concentrations with respect to control which explains the high specificity.

1.2 D Discussion

The behavioural changes exhibited by *O. mossambicus* upon exposure to various lethal concentrations of ethanol points to the stimulation of peripheral nervous system which results in increased metabolic activities. The higher metabolic rate indicates that more oxygen is being utilized. Increase in opercular movement is in response to the toxicant ethanol. Observations made by Yadav et al. (2007) corroborates the above statement. He also found similar increase in opercular movement in *Channa striatus* exposed to fertilizer industrial waste water. The decrease in opercular movement is referred to as a unique adaptive feature to avoid intake of toxicant. Frequent surfacing and gulping shows the effort of the animal to cope with the deficiency of oxygen, and fill the two lateral highly vascular air sacs with fresh air for accessory respiration. On initial exposure to ethanol, the fish *O. mossambicus* exhibited characteristic avoidance behaviour by rapid swimming, stretching half of their body out of water surface and trying to jump out. Fish secreted copious amount of mucus, a defence mechanism to neutralize the effect of ethanol which gradually covered the entire body, gills etc. In the terminal phase of intoxication, the fish lost their balance and equilibrium and died. Rakesh et al. (2009) also observed similar altered behavioural changes in fresh water air breathing catfish, *Heteropneustes fossilis* (Bloch) when they were subjected to different concentrations of dimethoate for 96 hrs which also supported the present study.
Albert Tangerman (1997) explained the importance of maintaining a lower temperature of about 60°C for ethanol based studies. According to Macchia et al. (1995), ethanol was stable for seven days in urine, serum, plasma and saliva when stored at 4°C. In the present study an increase in blood ethanol concentration was found when *O. mossambicus* was subjected to 7 days of exposure to different sub lethal concentrations of ethanol. This was mainly brought about by the stress in fish when subjected to ethanol toxicity. A state of stress brought about by ethanol made *O. mossambicus* enter into a state of anoxia, which in turn increased the rate of blood alcohol concentration in *O. mossambicus*. This was mainly attributed to the enhanced activity of hepatic ADH or to increased gluconeogenesis. Observations made by Badawy (1998), Mezey (1998), Johnston and Bernard (1983), Richard et al. (2000) and Shoubridge and Hochachka (1980) supported the present data. When *O. mossambicus* was subjected to 21 days of exposure to different sub lethal concentrations of ethanol a significant increase (P<0.001) in blood ethanol concentration was observed. This is due to *O. mossambicus* entering into a state of hypoxia, resulting in the increased production of ethanol. This data suggest the ethanol production as an ubiquitous "anaerobic" end product, which accumulates whenever metabolic demand exceeds the mitochondrial oxidative potential (Milica et al., 2008). Reports by Jorma and Ismo (1986) stated that the hypoxic state occurring in the fish on long exposure periods results in increased ethanol formation. This too supported the present finding. The findings made by Richard et al. (2000) also supports the present data. Apart from the above mentioned facts, very low amount of ethanol detected in control group of *O. mossambicus* in the present study was mainly due to the presence of microorganisms in the gut region of *O. mossambicus*. The observations made by Rod et al. (1997) support this finding.

An aqueous external calibrator of ethanol being used met all the requirements in which a linearity was obtained. The use of ethanol as an external calibrator was supported by Albert Tangerman (1997). Quantification of blood ethanol on the basis of peak area exhibited an excellent result. In conclusion, the direct injection method as presented here is a highly sensitive, rapid and reliable gas chromatographic technique used for measuring ethanol in blood. Once running, the method is easy to perform and does not require highly and specifically trained personnel, making this gas chromatographic method also suited to the field of clinical chemistry.