EFFECTS OF TWO ENDOCRINE DISRUPTING COMPOUNDS (17β-ESTRADIOL & ALKYPHENOL) IN NAIROBI RIVER-WATER ON BOAR TESTICULAR DESCENT AND SEMINIFEROUS EPITHELIUM

A thesis submitted in fulfilment of the requirements for Doctor of Philosophy Degree of University of Nairobi (Theriogenology)

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DECLARATION

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To:

Calvin Kipkoech and Hope Chebet

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LIST OF ABBREVIATIONS

BDL	-Below detection level
CBD	-Central business district
DES	-Diethylstilboestrol
EDCs	-Endocrine disrupting compounds
E-EDCs	-Estrogenic endocrine disrupting compounds
FSH	-Follicle Stimulating Hormone
GC-MS	-Gas chromatography-Mass spectrometry
H&E	-Haematoxylin and Eosin
INLS3	-Insulin like 3 gene
KAP	-Knowledge attitudes and practices
LH	-Luteinising Hormone
MIS	-Mullerian inhibiting substance
PCBs	-Polychlorinated biphenyls
SPE	-Solid phase extraction
T3	-Testosterone

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ABSTRACT

Concerns were raised by farmers in Nairobi that suggested the presence of compounds within the water of Nairobi River capable of affecting boar fertility through increased incidence of retained testis in piglets. Initial clinical observation of piglets born of sows reared using sections of Nairobi River draining informal settlements confirmed the farmers' concerns of a seemingly increased incidence of retained testis (cryptorchidism). These observations were suspected to be caused by access to compounds within the river water that are capable of affecting the normal functioning of androgens like testosterone; similar effects had been demonstrated in aquatic animals that had been exposed to pollutants within the water containing compounds affecting functioning of androgens.

It then became prudent to design a study encompassing a series of methodologies to verify the concerns of the farmers, test for any compounds in the river water that could affect testicular descent and spermatogenesis, and also use experimental model to test the effect of the river water on experimentally exposed visa vis the control group of animals. The null hypotheses were that the water of the Nairobi river does not contain significant levels of estrogenic-like endocrine disrupting compounds and that the use of its water in rearing pigs does not affect testicular descent and seminiferous tubule histology.

A semi-structured questionnaire was used to assess the urban farming situation and to determine the knowledge, attitudes and practices of residents along the riparian on the use of river water for farming and its effects on the boar. Eighty (80) pig farms in three study zones located a distance of 50 metres on either side of the riparian of Nairobi river tributaries were purposively selected. Questionnaires were pre-tested and administered to the pig owners as

respondents. River water from the three zones were collected and analysed to determine the levels of two known E-EDCs (17β estradiol and alkylphenol) using Gas chromatography-Mas spectrophotometry (GC-MS). Objective three was achieved by purchasing 15 randomly selected boars raised along the the riparians of the three zones (n=5 for each zone), and another 5 from known non-contaminated area. Each boar was then castrated and the seminiferous tublues examined for any lesions visible under a microscope at X200 magnification. For each testis, three sections of $1mm^3$ cube were used to prepare the embedded tissue. Twenty (20) male mice were purchased and 10 of them kept in the laboratory with *ad-libitum* access to the suspected water for two months as their only source of drinking water. Similarly, 10 other mice accesed clean water for a similar period to serve as the control. Both groups were then castrated to test for any lesions within the seminiferous tubules.

A Significant number (x^2=72, p≤0.05, n=80) of the respondents along the riparian were involved in urban agriculture and engaged in mixed farming that utilized the water of Nairobi river tributaries. The residents also reported that 10% (n=180) of the piglets born to sows accessing such river water had retained testes. The levels of 17β-estradiol and alkylphenol in the water sample ranged from between non clinical levels to clinically significant levels (p≤0.05) of 0.95µg/L and 0.36µg/L for 17β-estradiol and alkylphenol respectively. The seminiferous tubules of boars reared within the riparian revealed a significant (P≤0.05) presence of lesions like epithelial vacuolations, sloughed germ cells and patches of depleted tubules. The seminiferous tubule effects on naive experimental mice exposed to the water showed a significant level of disruption (p≤0.05) of the histology within the seminiferous tubules similar to those seen in the castrated boars.

The results of this study suggest presence of clinically significant levels of 17β-estradiol and alkylphenol capable of affecting testicular descent and the histology of the seminiferous tubules in males. There is need for a policy to address the pollution due to these compunds in order to limit adverse effects of such compounds on animals and/or humans.

CHAPTER ONE

1 GENERAL INTRODUCTION

Environmental contamination due to human activities is an increasing phenomenon in developing countries (Sibanda *et al.*, 2015). This contamination emanates from released natural and synthetic substances that include; pesticides, detergents, hormones, and heavy metals among others. Some of these contaminants are called endocrine disrupting compounds (EDC) because of the potential to disrupt hormone functions (Kavlock, 1996).

Some reproductive and developmental problems observed in human beings and animals have been attributed to EDCs from household, agricultural and industrial sources (Colborn *et al.*, 1993; Brevini *et al*., 2004; Sharpe and Irvine, 2004). To achieve the observed effects, EDCs seem to disrupt the endocrine signalling process at the receptor level at reproductive or embryological processes (Colborn *et al.,* 1993), mainly altering and/ or reversing effects of hormones such as androgens (Svechnikov *et al*., 2014).

Previous studies have indicated levels of EDCs in waste-water discharge (Kolpin *et al.,* 2002; Stevens *et al.,* 2003), which has been linked to testosterone and other related steroid hormone functional alterations (Hecker *et al.,* 2002) and impaired reproductive development in aquatic life (Hemming *et al.,* 2001; Sheahan *et al.,* 2002). These effects in aquatic life have been demonstrated to be due to estrogenic-like actions that led to anti-androgenic disrupting activities of EDCs (Andersen *et al.*, 2002).

Literature on the dangers posed by EDCs on large animals is scarce. Exposure of these animals to these chemicals would likely be through the diet (Norstrom, 2002). Although such levels would be argued to be low, the levels can be significantly high in animal diets constituted from sludge-fertilized pastures and waste-water (Meijer et al 1999; Rhind *et al*., 2010). Pigs raised along the riparian of contaminated water are likely to be exposed to drinking water polluted with such EDCs (Figure 1: a case of Nairobi urban pig rearing).

Figure 1: Sections of Nairobi River showing pigs reared along the riparian and exposed to polluted water

The boars reared under these conditions could serve as models to investigate the effects of environmental pollution by EDCs because it has a peculiar spermatogenesis that is highly dependent on androgens and oestrogens (Mutembei *et al*., 2005).

Nairobi residents have been known to practice urban farming that includes rearing of pigs. This farming is significantly higher along the informal settlements dependent upon the riparian zones of the tributaries of Nairobi River. The farming along these riparian tends to utilize the polluted water within their farms. Clinical observation of the reared pigs revealed a seemly increased incidence of retained testis/es (mono-/ cryptorchidism). Testicular descent is dependent upon testosterone through regulation of INSL-3 gene (Mutembei *et al.,* 2005). Thus, this observation was suspected to be caused by compounds that are capable of reversing the effects of testosterone such as estrogenic-like compounds or oestrogens themselves (Sharpe *et al.,* 2004). The testosterone reversal like testicular effect of such compounds contained in such polluted water has previously been demonstrated in fish, man and wildlife.

The observed clinical signs of retained testis/es in boars reared along the riparian of the tributaries of Nairobi River led to a scientific question of whether estrogenic-like compounds that reverse the effects of testosterone are present in this water to significantly affect testicular function. To test this question a study was designed with a null hypotheses that the tributaries of Nairobi river does not contain significant levels of estrogenic-like compounds and that use of the water in urban farming is not detrimental to reproduction at testicular level.

To elucidate the effects of EDCs on the reproduction of the boar, the current study was set up using the Nairobi River as the likely source of EDCs due to its high contamination level (Ndeda and Manohar, 2014). Nairobi River receives urban waste-water effluent likely to contain varying levels of EDCs as previously demonstrated for other urban rivers with such effluents (Kolpin *et al*., 2002; Lintelmann *et al*., 2003). The boar was chosen because of his genomic resemblance to that of man in order to use it as a model to inform policy on effects of EDCs on human beings. The mouse was used as the experimental animal due to its spermatogenesis resembling that of the boar. The use of a questionnaire to investigate the knowledge and the practices of the farmers along the riparian keeping pigs was informed by previous studies by Rhind, (2002).

The study was designed first to establish if the reproductive problem of cryptorchidism observed in piglets was also widely observed by the farmers and their perceptions on the same through a KAP study. Then an examination of the histology of the testis of reared pigs would follow to check for any other related lesions within seminiferous tubules. There was also need to confirm if the effects seen in the boar could be replicated through an animal model which could then serve to connect the contamination with the observed effects. The levels of two previously reported estrogenic-like EDCs in the river water were determined to validate if the noticed reproductive problems were actually due to such EDCs.

The information generated from the study would be used to inform policy on use of river water for farming, the level of contamination of the river with the two EDCs and the effect it might have on testicular descent and seminiferous epithelium. This information could be used by the relevant authorities to advice on better ways of protecting the water resource to avoid effects of such EDCs.

1.1 Study hypothesis

Two null hypotheses were derived:-

a) Tributaries of Nairobi River do not contain significant levels of estrogenic-like compounds.

b) Use of the water in urban farming does not affect testicular descent and has no detrimental effects at seminiferous tubule level.

1.2 Objectives

1.2.1 General objective

To study the effects of E-EDCs in the water of Nairobi river on the testis of the boar.

1.2.2 Specific objectives

The study specifically sought to:

i) Conduct a survey of the urban farming situation along the Nairobi River riparian to confirm presence of pig rearing and also assess the farmer knowledge, attitudes and practices (KAP) on the rearing in relation to observed reproductive conditions.

ii) Evaluate histology of the seminiferous tubules of boars reared along the riparian to examine for pointer lesions related to E-EDCs exposure in drinking water.

iii) Use mice (a naive animal model in the laboratory) to test if exposure to the suspected water was the cause of the lesions detected in the testis of the boars examined.

iv) Analyse the water for presence of two known E-EDCs (17β estradiol and alkylphenol) that cause similar lesions in seminiferous tubules in order to confirm if they were present in significant levels to cause such lesions in seminiferous tubules.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 The testis

2.1.1 Morphology and location

In adult life, the boar testicles are enclosed in a sac called the scrotum which lie ventral to the anal opening (Olukole and Oke, 2016). Within the scrotum, a capsule; tunica albuginea encloses each testis. From the tunica albuginea, emerges the septula testis which converge centrally within the testis to form the mediastinum testis. The mediastinum testis divides the testis into lobules and contains the rete testis which provides a route for spermatozoa and accompanying liquids leave the testis (Fig 2).

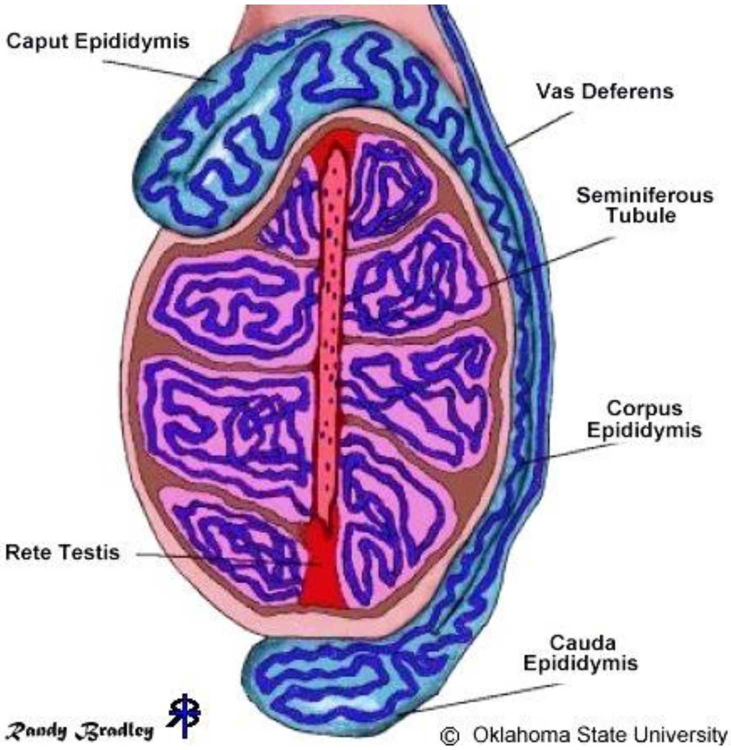

Figure 2: Cross section of a testicle showing the septula testis, testicular lobules and seminiferous tubules (source: www.animalsciences.missouri.edu)

In the boar testis, the seminiferous tubule makes up 60-70 % while the remaining 30-40% is contributed by the interstitial tissue (Wrobel, 1998).

2.1.2 Testicular descent

During development, the testis is attached to the abdominal wall by a suspensory ligament while the gubernaculum attaches its caudal pole to the inner inguinal ring. Hormonal and paracrine factors interplay to achieve testicular descent from its initial para-renal position to the scrotum. According to Hutson and Hasthorpe (2005), testicular descent occurs through the degeneration of the cranio-suspensory ligament and the thickening of the gubernaculum. In male piglets, the testes are in the scrotum at birth or descend a week after.

The gubernacular differentiation together with cranio-suspensory ligament degeneration are pre-natal activities mediated by androgens and insulin-like factor INSL-3 gene (Fig 3) (Nef *et al*., 2000; Overbeek *et al*., 2001; Mutembei *et al.*, 2005). INSL-3 controls testicular descent during the trans-abdominal stage; its expression is regulated by T3 and expressed by the leydig cells (Adham *et al*., 1993).

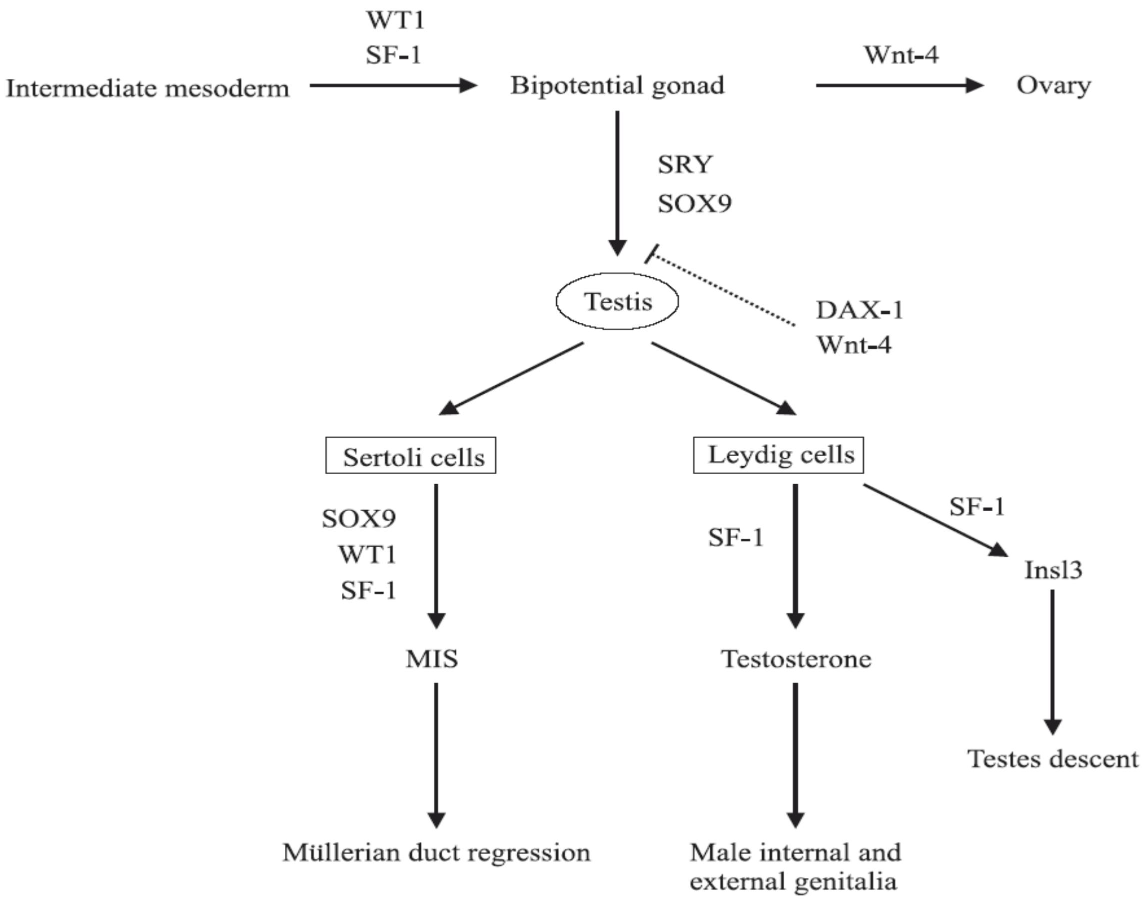

Figure 3: Genes controlling gonadal development. Solid lines = activation, dotted lines= inhibition (from Mutembei *et al*., 2006).

Pre-natal diethylstilbestrol (DES) treatment, exogenous oestrogens and/or anti-androgens have been shown to lead to failure of testicular descent in animals (Skakkebaek *et al*., 2001; Damgaard *et al*., 2002; Main *et al*., 2007; Bay *et al*., 2011). DES impairs INSL3 (Fig 3) expression in mouse testis and interferes with gubernacular development (Emmen *et al*., 2000). Testicular descent may therefore be impaired by any condition associated with decrease of fetal testosterone production or action.

2.2 Histology of the testis

The histological appearance of the seminiferous tubule of the boar has been reviewed (Mutembei *et al*., 2006). A mature testis is characterised by a fully developed seminiferous epithelium (Fig 4) (Russell *et al*., 1990; Mutembei *et al*., 2005). The seminiferous tubule is lined by an epithelium consisting of Sertoli and germ cells (Fig 4) (Russell *et al*., 1990).

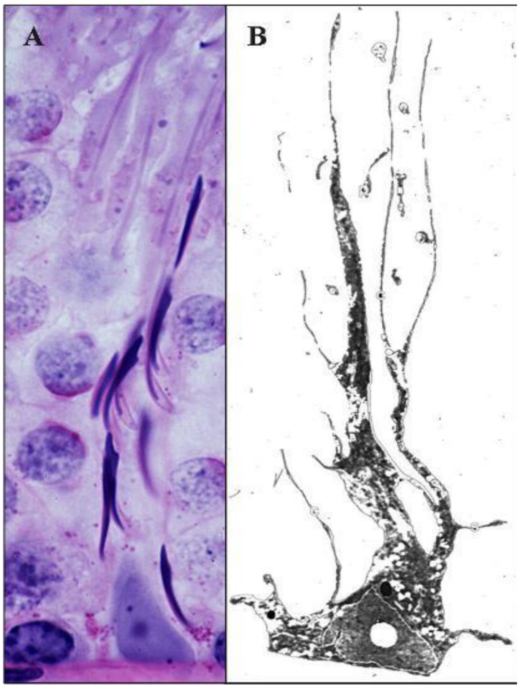

Figure 4: The Sertoli cell (A – on histology and B – on electron micrograph) (Source: Russell 1990).

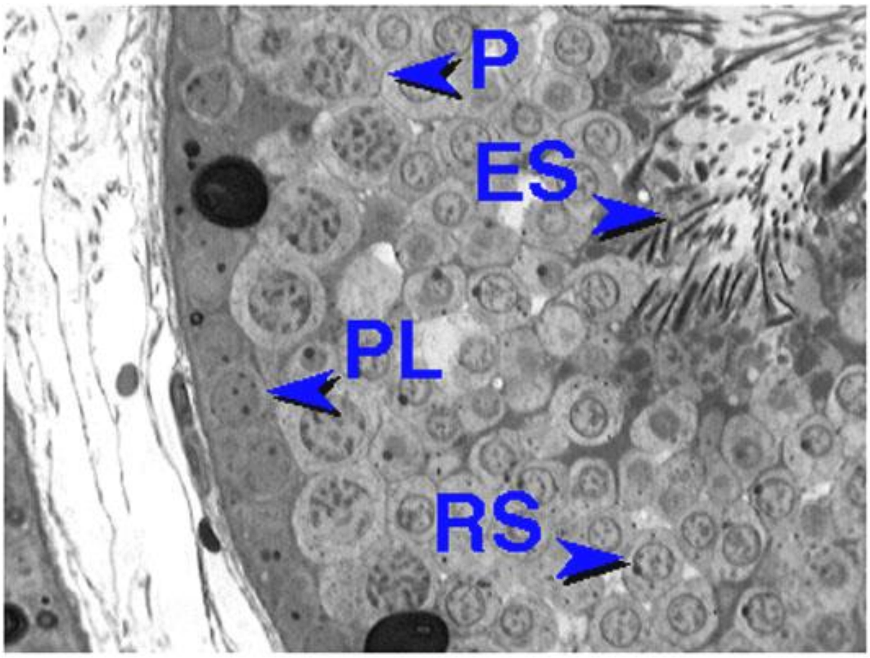

Figure 5: Spermatogenesis in the boar showing the maturation phase spermatids (ES), spermatocytes (P) and spermatids (RS)

Sertoli cells have an extensively branching cytoplasmic structure that form tight occluding junctions with each neighbouring colleague Sertoli cell to separate the tubule into two compartments and form the blood testis barrier (Mutembei *et al*., 2006). These Sertoli cells emanate from the base and extend perpendicularly to the luminal surface of the seminiferous tubule. The Sertoli cell has a large angular and lightly staining nucleus. They are responsible for the seminiferous epithelium structure (De Kretser and Kerr, 1994).

Leydig cells are in clusters of 2-20 cells in the interstitium. Their nucleus is located eccentrically in the cell and is strongly acidophilic (Christensen, 1975). They are abundant in the boar and secrete androgens and sulfo-conjugated oestrone (Rostalski *et al*., 2000).

The normal architecture of the seminiferous tubules exhibits close interaction of germ cells that constitute the stages of spermatogenesis (Fig 5, Mutembei *et al.*, 2005).

Within the seminiferous tubule, the spermatogonia are situated on the basement membrane. Spermatogonia (A0), the true stem cells (Mutembei *et al*., 2005), multiply mitotically to replenish itself and also form new generations of type A1 spermatogonia which proceed on in spermatogenesis (Frankenhuis *et al*., 1982) to give rise to primary spermatocytes.

Primary spermatocytes are large cells found luminal to the spermatogonia. They are visible in large numbers within the seminiferous tubule section. They divide to give rise to secondary spermatocytes.

Secondary spermatocytes rapidly complete the meiotic division and are thus rarely seen on histological preparations.

Spermatids initially appear small with a light staining nucleus (Dadoune, 1994). Chromatin condensation during maturation results in a smaller darker staining nucleus.

2.2.1 Testicular histopathology

Sertoli cells are sensitive to functional perturbation (Creasy, 2001); their injury is manifested by loss of function rather than death, since their cytoskeleton offer structural support to the seminiferous tubule (Wang *et al*., 2008). Exfoliation of germ cells indicates Sertoli cell dysfunction, in such cases the exfoliated cells appear morphologically normal (Creasy, 2001). Prolonged toxin exposure will lead to disorganisation of the seminiferous epithelium with variable germ cell loss and irregularly arranged germ cell layers (Fig 6). Histopathology is considered the most reliable and sensitive means of detecting toxic effects on spermatogenesis (Creasy, 2001).

Germ cell injury manifests as degeneration and loss. Cellular sloughing is evidence of detachment and separation of germ cells; it may involve a portion of the seminiferous epithelium or encompass the circumference of the seminiferous epithelium (Fig 6).

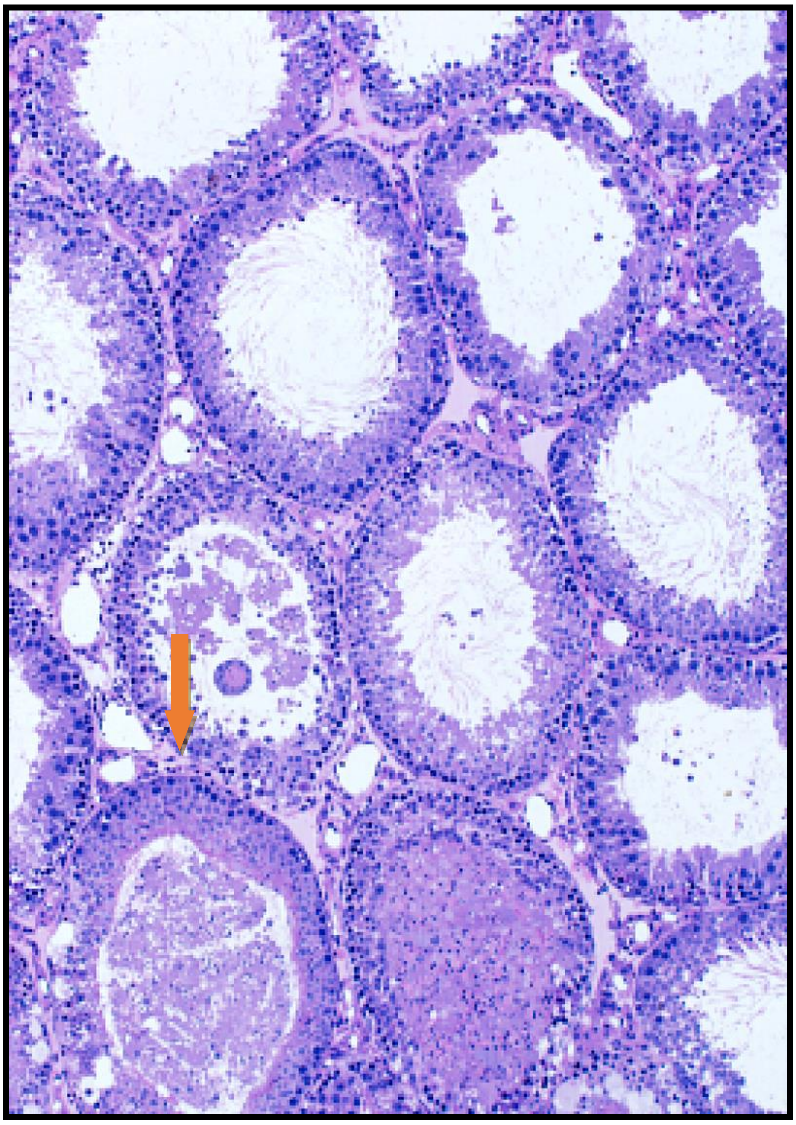

Figure 6: Germ cell toxicity showing sloughed germ cells (Arrow) within the lumen of the seminiferous tubule of rats. (Creasy, 2001)

The germ cell loss causes vacuolation of the seminiferous epithelium; these vacuoles consist of large non-membrane bound spaces at various depths within the tubule. Sertoli cell cytoplasmic vacuolation are seen as discrete round structures located towards the basement membrane (Fig 7).

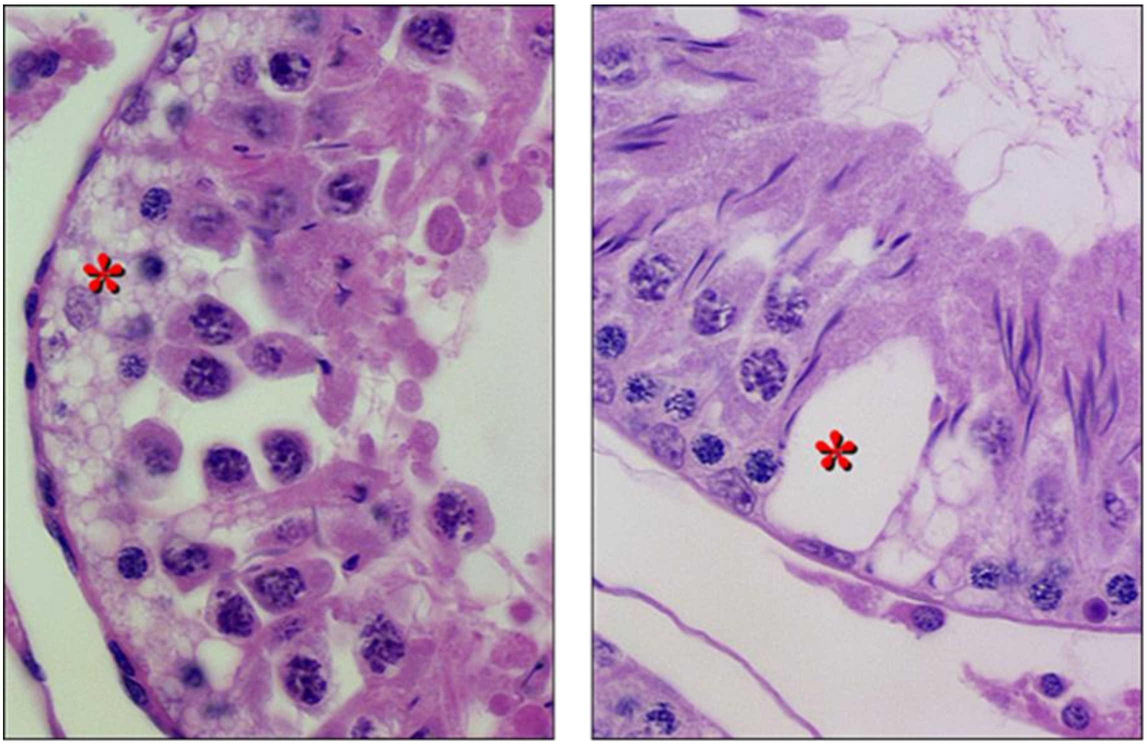

Figure 7: Germ cell toxicity within Sertoli cells showing vacuolation (Asteric) (Creasy, 2001)

Leydig cell function is impaired by agents that interfere with the steroidogenesis or its regulatory hormones. Most testicular toxicants cause varying extent of germ cell degeneration and depletion. Severe and prolonged injury often results in a depletion of all germinal cellular epithelium except the Sertoli cells (Creasy, 2001).

2.3 Spermatogenesis

Spermatogenesis is a complex process involving mitosis, meiosis, and morphological changes to convert primodial germ cells into motile spermatozoa (Mutembei *et al*., 2005). Sertoli cells anchor germ cells into place, provide structural support and also nutrition. Successful spermatogenesis is accomplished through coordination and interactions of various testicular cells (O'Donnell *et al*., 2001) and mediated through the hypothalamo-pituitary-gonadal (HPG) axis together with paracrine and autocrine factors (Huleihel and Lunenfeld, 2004). Endogenous oestrogens are involved in epididymal sperm concentration, an essential process for maintenance of normal fertility the boar, stallion and mice secrete remarkably high amounts of testicular oestrogens with an essential physiological role for male fertility (Hess *et al*., 1997).

2.3.1 Endocrine regulation of spermatogenesis

Spermatogenesis is a unique strictly timed process involving proliferation and differentiation of immature spermatogonia into mature elongated spermatids (Franca *et al*., 1998). It occurs in the testis in a topographically well-coordinated manner (Russel *et al*., 1990).

Gonadotrophins are the major endocrine regulators of spermatogenesis. Follicle stimulating hormone (FSH) targets Sertoli cells and regulates spermatogenesis by promoting spermatogonial proliferation (Sharpe, 1994; Mc'Lachlan *et al*., 2002). Luteinizing hormone (LH) targets leydig cells to stimulate testosterone (T3) secretion, thus maintaining T3 in circulation and in the testis. Optimal spermatogenesis is initiated and maintained by FSH and T3 (Sharpe 1994; Mc'Lachlan *et al*., 2002). Clear-cut control of germ cell development by androgens is seen during; spermatid adhesion and development, spermiation, progression through meiosis and spermatogonal differentiation. Therefore, a delicate balance of Hypothalamo-Pituitary- Testis axis is required for full fertility (O'Donnell *et al*., 2001).

Exogenous androgen administration decreases the levels of circulating LH which leads to a drop in intra-testicular T3, consequently blocking the progression from round to elongated spermatids (Sharpe, 1994). Additionally, low levels of T3 results in a compromise in the apical endoplasmic specialization which leads to premature sloughing of round spermatids (Scott *et al*., 2009).

2.4 Environmental contamination and the male gonad

2.4.1 Endocrine disrupting compounds

Endocrine disrupting compounds (EDCs) interfere with endogenous hormone homeostasis to alter normal body functions (Kavlock, 1996). These compounds include natural and synthetic hormones, alkylphenols, phthalates, bisphenols A, some pharmaceuticals, personal care products and some pesticides (Gray and Metcalfe, 1997).

These chemicals are called "*endocrine disrupting compounds*" because they antagonise natural hormone function at receptor level (Sharpe and Irvine, 2004). By so doing they can either block access to receptors by hormones (Andersen *et al*., 2002) and/or alter the endocrine systems functions (Rhind, 2002). The hormone modulating effect of EDCs can either be estrogenic-like and/ or anti-androgenic (Svechnikov *et al*., 2014). EDCs with estrogenic-like and anti-androgenic activities are the most common in disruption of cellular and hormonal aspects of male reproduction (Sharpe and Irvine, 2004).

Exposure of animals to these chemicals occurs via ingestion of polluted feed and water (Norstrom, 2002). EDCs like pesticides have been demonstrated to cause detrimental results on male reproduction (Rhind *et al*., 2010) some of which can be identified on testicular histopathology (Scott *et al.,* 2009). The major seminiferous epithelium changes attributed to EDCs exposure include; Sertoli cell vacuolation, germ cells sloughing, loss of Sertoli cell tight junctions and loss of germ cell associations (Evans *et al*., 2004; Scott *et al.,* 2009). Malformations of testicular descent and ducts have been noted (Jobling *et al*., 2002) and reduced size of gonads and gonadal intersex has also been observed (Meijer *et al.,* 1999; Rhind, 2002; Jobling, 2002; Paul *et al*., 2005).

Notable testicular dysfunctions associated with the exposure of EDCs are cryptorchidism and hypospadias (Gabel *et al*., 2011; De Falco *et al*., 2015). Other reports by Colborn et al. (1993) indicate general seminiferous tubule functional alterations.

These findings are a clear indication that EDCs within the environment are capable of disrupting normal reproduction due to their detrimental effects during testicular descent and disruptive effects within the seminiferous tubules.

2.4.2 Occurrence of endocrine disrupting compounds

Endocrine disrupting compounds have been demonstrated in surface waters (Kolpin *et al.,* 2002; Ndeda and Manohar, 2014), ground water, river sediments (Petrovic *et al*., 2003) and waste-water (Snyder, 2008). Human population growth and urbanization are implicated to be the main contributors of presence of EDCs in water bodies (Kolpin *et al*., 2002). These trends have been shown to be true for developing countries, including Kenya, Egypt and South Africa (Wandiga, 2001; Sibanda *et al*., 2015). In the city of Nairobi, the sources of surface water contamination include effluents from hospitals, industries, motor vehicle garages and households.

Industrial effluents commonly contain surfactant and phenolic compounds from paints that heavily contain EDCs (Gray and Metcalfe, 1997). On the other hand, domestic waste-water has been shown to contain personal care products that contain estrogenic-like EDC steroids (Arnon *et al*., 2008; Kipyegon *et al*., 2016).

2.4.3 Endocrine disrupting compounds in Urban River waters

Urban rivers are threatened by heavy contamination from human population growth and inadequate sewerage facilities (Sibanda *et al*., 2015). This is more so for urban rivers in close proximity to informal settlements, industries, agricultural activities and waste-water plants (Chezhien *et al*., 2010; Ahmad *et al*., 2013).

EDCs have been reported globally within urban rivers (Kolpin *et al*., 2002; Lintelmann *et al*., 2003). Some of these EDCs isolated from polluted urban water are phenols and phenolic like compounds (Sharpe and Irvine, 2004; Jafari *et al*., 2009). These compounds are demonstrated to be estrogenic disruptors (Sharpe and Irvine, 2004). The main phenol and phenolic like compounds isolated in such waters are nonyl-phenol, bisphenol A and triclosan (Furuichi *et al*., 2004; Kuch and Ballschmitter 2001).

Some of the hormonal compounds isolated in water are estrogenic-like compounds with anti-androgenic effects (Huang and Sedlak, 2001; Kolpin *et al*., 2002). Oestrogen-like chemicals, have been reported in the USA (Zhang *et al*., 2014), Europe (Kuch and Ballschmitter, 2001) and Japan (Furuichi *et al*., 2004).

The levels of estrogenic EDCs that cause detrimental testicular effects can be as low as 0.1ng/l (Aerni *et al*., 2004). Polluted urban water are reported to contain higher concentration of such estrogenic-like compounds (Jafari *et al*., 2009; Zhang *et al*., 2014), making it a matter global concern.

2.4.4 Nairobi River water and its association with EDCs

The Nairobi River (Fig 8) has three main tributaries namely Mathare, Nairobi and Motoine, which downstream becomes Ngong River. They all join to become Nairobi River which joins Athi River downstream. The three tributaries each pass through areas of settlement with industrial and agricultural.

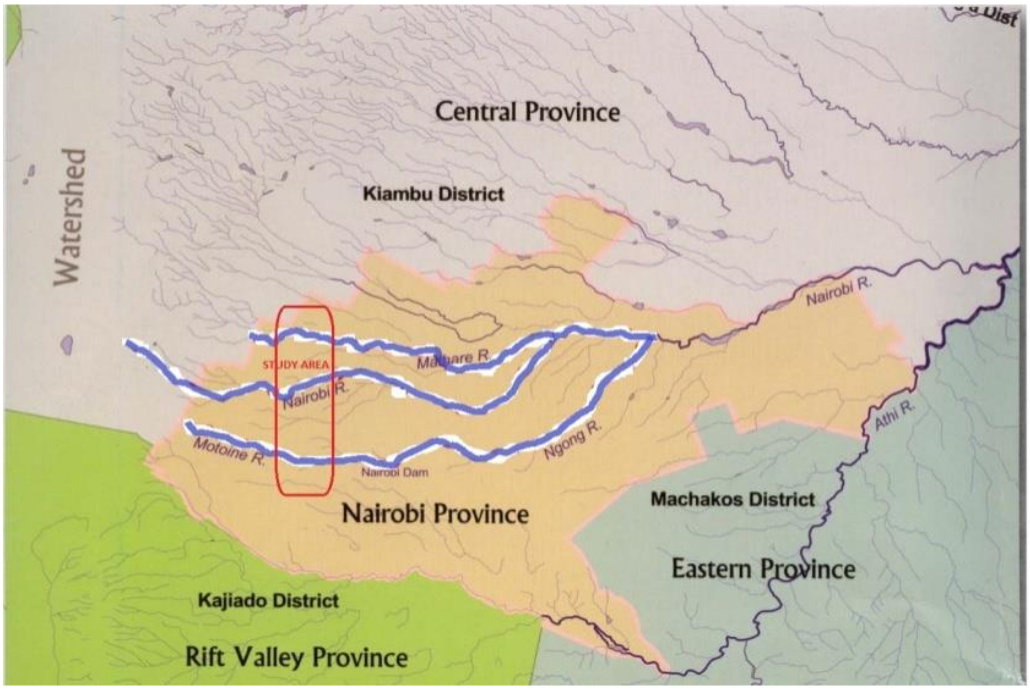

Figure 8: The Nairobi River Basin (sourced from Nairobi maps, survey of Kenya)

According to Karisa (2010), the Nairobi river basin has over time has been under intense pressure from human activities which include: habitation, agriculture and industrial activities.

These activities have contributed to the degradation of this riparian environment by various wastes like raw sewage among others. The riparian areas of the three tributaries of Nairobi River (Nairobi Mathare, Ngong,) are characterised by several informal settlements which have inadequate or lack of proper sewerage management systems. This phenomenon predisposes the nearby rivers and streams to pollution by raw sewage discharge, partially treated or untreated industrial effluents. For these reasons, the Nairobi River tributaries are arguably the most polluted river in Kenya (Wandiga, 2001; Musyoki *et al*., 2013; Ndeda and Manohar, 2014).

The Nairobi River water is likely to contain a combination of EDCs detrimental to reproduction in animals and man dependent upon its riparian. This is the basis for its use during the current study.

CHAPTER THREE

3 KNOWLEDGE, ATTITUDE AND PRACTICES OF THE RESIDENTS LIVING ALONG THE NAIROBI RIVER RIPARIAN ON THE USE OF RIVER WATER FOR FARMING AND ITS EFFECTS ON TESTICULAR DESCENT

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3.1 Abstract

Residents practising pig farming along the Nairobi River Riparian in Kenya were contacted to evaluate their knowledge attitudes and practices on use of the polluted river for farming and its effects on animal reproduction. Eighty (80) pig farms were randomly selected and questionnaires administered to the pig owners as respondents. A Significant number (x^2=72, p≤0.05, n=80) of the respondents were involved in urban farming and reared pigs as a supplementary source of income. Over a third of them (38.8%) had attained secondary level of education. A majority (x^2=59.7, p≤0.05, n=80) utilized the polluted river water for pig farming. The main reproductive effect noted on the boars was retained testes with no significant difference on the side of retention. The reported reproductive defect points towards environmental estrogenic endocrine disrupting chemicals with anti-androgenic effects.

3.2 Introduction

Farming is an important undertaking in many urban informal settlements of the world (Kagira and Kanyari, 2010; Lee-Smith, 2010). At least 35% of households in African cities are engaged in some form of agriculture (Nabulo *et al*., 2006). This practice contributes to food security, offers employment and income for the poor (Lynch *et al*., 2001). In Kenya, the reasons for urban farming by the poor were listed by Kagira and Kanyari (2010) as a response to limited alternative livelihood options and food security. Due to the increasing scarcity of fresh water resource, waste-water re-use of for farming (WHO 2006) is the practise albeit the health and environmental risk associated with its use.

The characteristic high population in informal settlement areas with absent or inadequate sewerage infrastructure result in substantial volumes of untreated domestic and industrial waste-water discharge into surface water; which are the main source of water for the urban poor farmers. Urban rivers are for this reason heavily polluted with toxic contaminants likely to affect users of such water. Urban livestock farming is more often unplanned and takes place in densely populated neighbourhoods. The animals kept include ruminants, pigs, chicken, ducks, dogs and cats (Kagira and Kanyari, 2010). Among these, pig farming is characterised by low inputs and free ranging system. In these areas pigs scavenge in garbage disposal points, waste-water drainage channels and in polluted rivers (FAO, 2012).

Globally, urban draining rivers have been reported to be polluted by many chemicals including endocrine disrupting compounds (Huang and Sedlak, 2001; Kolpin *et al*., 2002; Snyder, 2008) and aquatic organisms in such rivers have shown perturbation in reproductive function (Hecker *et al*., 2002; Hemming *et al*., 2001; Jobling *et al*., 2002; Sheahan *et al*., 2002). Similarly, lambs born of ewes exposed to sewage sludge showed a disruption in their reproductive function (Paul *et al*., 2005).

Low level exposure to these chemicals is mainly through the animal diet (Norstrom, 2002); however, exposure is very high in certain production systems where animals are provided with polluted water as the only source of their water consumption. This study aimed at investigating the knowledge and perception of urban informal settlement pig farmers on the use of waste-water or effluent polluted water on the reproductive health of boars.

3.3 Materials and methods

3.3.1 Study area

The study took place in the informal settlements of Nairobi County, Kenya. Residents in these areas have poor access to clean water supply, sanitation and solid waste management facilities. The sites selected for this study were Kibera (representing upper zone of the riparian), Mathare (representing the middle zone of the riparian) and Dandora (representing the lower zone of the riparian). The locations were also selected based on: its proximity to a city river, the physical appearance of the water and pig keeping activities with animals scavenging along the polluted river water.

Kibera is roughly five kilometres away from the Nairobi central business district (CBD) towards Ngong. It lacks drainage facilities, wastes are scattered all over and sights of open sewers are common (Hodson & Marvin, 2009). Mathare is also about five kilometres east of the Nairobi CBD. Dandora is about 10 KM from the CBD and hosts the largest dump site and a waste-water treatment plant. Limited agricultural activities and keeping of livestock occurs in the three locations along the riparian.

3.3.2 Study design

A semi structured questionnaire (Appendix 1) was used to obtain information from randomly selected pig owners living within 50 meters close to the polluted rivers in each selected location. The interview aimed at establishing the use of polluted water for pigs and the observed type of diseases and reproductive problems that the pigs suffer due to access to polluted water.

3.3.3 Sampling

Pig farmers selected for interviews in every location were within 50 metres from a visibly polluted river. An adult member of the family was picked as a respondent to take the interview; this was regardless of the education status, gender and occupation.

3.4 Data collection

To gain information on the disposal of household generated effluents, water contamination and their effects on livestock and humans health, a questionnaire was administered to each respondent face to face as the investigator filled out the questionnaire. Field observations were also made together with photographic evidence.

Pig farmers adjacent to the river bank within selected informal settlements of Nairobi city were the targeted population, a total of 80 farmers were interviewed. The questionnaire (Appendix I) provided information on the farmer knowledge and attitude towards river water use and their perception on health issues arising from the use of such waters.

3.5 Data management and analysis

Data from the questionnaire responses were assigned numerical numbers and entered into an Excel sheet. The data was then encoded using a statistical package (IBM® SPSS® statistics version 20) and analysed using chi-square ($p \leq 0.05$, $n=80$).

3.6 Results

Most of the respondents (69%) had primary - secondary level of education and many were involved in urban farming (Table 1). A significant number of them reported incidences of retained testis in piglets reared using waste-water. This incidence was highest in Kibera and Dandora, which apparently also had more of the farmers using waste-water for farming. Majority of the respondents also seemed aware that use of polluted water was dangerous on the pigs reared. This awareness was higher in respondents who had noticed retained testis of the pigs, who mostly came from Kibera and Dandora.

Table 1: Respondent's knowledge, attitudes and practices on use of waste-water for pig rearing in three riparian zones of Nairobi River.

	Kibera n=21	**Mathare** n= 38	**Dandora** n=21
Respondents with Primary-secondary level of education	53.1 ±1.20	90.3±0.22	59.7±2.41
Use of waste-water in farming	61.8±1.69	34.2±2.01	88.2±0.33
Observed testicular retention in reared pigs	57.2±0.19	33.5±2.02	89.1±0.23
Knowledge awareness on potential danger of use of waste-water	98.5±1.51	23.6±1.85	85.6±1.72

Table 2: Chi-square analysis on the pooled data based on responses from the three zones (n=80).

Waste-water use, location and retained testis	Analysis
Association of consumption of polluted water by pigs and failure of the testis to descend	$X^2 = 22.93$, P= 0.01
Association level of education to use of waste-water	$X^2 = 2.08$, P= 0.01
Association of testicular retention and riparian zone reared (Kibera, Mathare and Dadora)	$X^2 = 35.95$, P= 0.01

There was an association between failure of the testis to descend (cryptorchidism) and the consumption of polluted water in the affected pigs ($X^2 = 22.93$, P= 0.01, n=80). Similarly, the retention of the testis was associated with the riparian zone the pigs were reared in (extent of water pollution, $X^2 = 35.95$, P= 0.01, n=80). The preference in the choice of water source used for pig rearing was not associated with the level of education ($X^2 = 2.08$, P= 0.01, n=80).

3.7 Discussion

The results revealed that most pig keepers in the areas surveyed were literate and were aware of the potential danger of using polluted water for pig rearing and continued to use the same for rearing the pigs anyway (Table 1). The residents also reported that at least 10% of the piglets born to sows accessing such river water had retained testes. This incidence was highest in Kibera and Dandora, which apparently also had more of the farmers using waste-water for farming. In contrast, a prevalence of ≤1% and 2-4% in normal pigs has been reported (Grant, 1952; Barthold and Gonzalez, 2003) respectively. Amann and Veeramachaneni, (2007) also reported an increased prevalence of 2-12% in piglets.

Majority of the respondents seemed aware that use of polluted water was dangerous on the pigs reared. This awareness was higher in respondents who had noticed retained testis of the pigs, who mostly came from Kibera and Dandora. The use of waste-water was however fuelled by its ease of availability with no cost attached to its use. This observation is in agreement with findings by Kagira and Kanyari (2010) that livestock keeping was a secondary occupation for urban farmers. This is expected because according to Ellis and Sumberg, (1998), pig farming is an easy source of income and an alternative livelihood option.

The use of polluted water for farming has been reported previously (Kagira and Kanyari, 2010). Thus, it was not surprising to find out that farmers from Kibera, Mathare and Dandora utilized such water for pig rearing. Such water has been reported to contain effluents from households and small industries, due to lack of or inadequate sewerage facilities (Norah *et al.*, 2015). Additionally, the farmers have been reported to use such water because it is not associated with costs, easy to access and is convenient to them (Kagira and Kanyari, 2010; Ndunda and Mungatana, 2013). Access to polluted water is associated with various adverse reproductive issues (Jobling *et al*., 2002; Bellinghan *et al*., 2012). The respondents in this study are aware of such dangers.

There was an association between failure of the testis to descend (cryptorchidism) and the consumption of polluted water by the affected pigs (X^2 = 22.93, P= 0.01, n=80). This observation was expected following suspicion that the water contained EDCs. Similarly, the retention was associated with the riparian zone where the pigs were reared (extent of water pollution, X^2 = 35.95, P= 0.01, n=80) the association is thought to emanate from the differences in river catchment are characteristics and the access to waste-water by the pigs. The preference in the choice of water source used for pig rearing was not associated with the level of education (X^2 = 2.08, P= 0.01, n=80).

Cryptorchidism is a predisposing factor for testicular cancer in human beings (Svechnikov *et al*., 2014). The exact cause of testicular retention is not fully explained. However, exogenous oestrogens and anti-androgens have been linked to this condition (Damgaard *et al*., 2002). EDCs with estrogenic (Main *et al*., 2007) or anti-androgenic effects can disturb events that drive testicular descent (Svechnikov *et al*., 2014). Consequently, polluted water is known to contain a complex mixture of EDCs (Rhind *et al*., 2010), which are associated with the testicular retention (De Falco *et al*, 2015) by either reducing the production or release of T3 which is essential for this activity.

Paul *et al*., (2005) demonstrated a disruption of testicular growth in lambs whose dams were exposure to EDCs in polluted water. Similarly Gabel *et al*., (2011) pointed out a higher risk of having cryptorchid sons by women exposed to EDC pollution. Other epidemiological studies showed links between environmental EDCs and cryptorchidism (De Falco *et al*, 2015).

It is concluded that despite the awareness that waste-water is unsafe, the residents of the informal settlements continue to avail it to their pigs which end up developing reproductive defect consistent with environmental oestrogens within the water. The residents need to be made aware of the dangers such water may pose not only to boars but potentially human beings.

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CHAPTER FOUR

4 LEVELS OF 17β ESTRADIOL AND ALKYLPHENOL ESTROGENIC ENDOCRINE DISRUPTING COMPOUNDS IN NAIROBI RIVER

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4.1 Abstract

Water samples from Nairobi river were obtained to test for the levels of two compounds; 17β-estradiol and alkylphenol. Samples were collected along the informal settlements of Kibera, Dandora and Mathare using glass amber bottles and transported to the laboratory at 4°C. Water was then analysed to determine the levels of two EDCs (17β-estradiol and alkylphenol) using Gas chromatography-Mass Spectrophotometry. The levels of alkylphenol and 17β-estradiol in the sampled water were between below detection level 0.08µg/L to 0.917µg/L and BDL to 0.3005µg/L for 17β –estradiol and alkylphenol, respectively. The mean values were 0.0953µg/L and 0.360µg/L for 17β steroid and alkylphenol, respectively. The detected levels of 17β-estradiol and alkylphenol point towards a suggestion that the effects observed in the boars and mice are caused by estrogenic disrupting compounds in the water. It is concluded that there is need to have a policy in place to control effects of such EDCs like 17β estradiol and alkylphenol on humans and/or animals.

4.2 Introduction

Boars raised along the riparian within the informal settlements of Nairobi are predisposed to cryptorchidism (Kipyegon *et al*., 2016a). Testicular retention has been associated with exposure to polluted river water with levels of EDCs of 0.1ng/l (Aerni et al., 2004). Various authors have reported that urban rivers are heavily polluted through release of raw domestic and industrial effluents into waterways (Johnson and Sumpter, 2001; Clara *et al*., 2004). The Nairobi river has been demonstrated to contain heavy loads of microbial organisms (Musyoki *et al*., 2013), heavy metals (Ndeda and Manohar, 2014) and pesticides (Wandiga, 2001). These pollutants have been suspected to be containing endocrine disrupting compounds (EDC) with a potential to disrupt the endocrine function (Falconer *et al*., 2006) and also cause reproductive problems (Mendes, 2002).

The observed male reproductive abnormalities in the boar accessing the polluted Nairobi River were suspected to be caused by estrogenic compounds in such water (Kipyegon *et al*., 2016b). The present study therefore sought to determine the levels of two known estrogenic endocrine disrupting compounds (Alkylphenol and 17β estradiol) in the Nairobi urban river flowing through informal settlements. Natural and synthetic oestrogens in large quantities have previously been reported in water (Jafari *et al*., 2009; Ying *et al*., 2012; Knez, 2013). This paper tries to shed light on the extent of the contamination of the Nairobi River with alky phenol and 17β estradiol endocrine disrupting compounds to find out the main cause of the observed effects of its water on testicular function. This data would be used to inform policy on governance of the contamination to avert reproductive effects in animals and humans.

4.3 Materials and methods

The study took place in the settlements dependent upon the tributaries of Nairobi River for mixed agricultural activities; vegetable farming and rearing of domestic animals among them pigs. The water of the tributaries is heavily polluted with household, industrial and farm wastes. The sampling sites selection was based on presence of mixed farming activities that included rearing of pigs that accessed the polluted river (Figure 1).

Two and half (2.5) litres river water from each of the three sampling sites, were collected into amber coloured bottles prepared beforehand by rinsing with hexane. The water samples were maintained at 4°C and transported to the laboratory for processing through solid phase extraction (SPE).

In the laboratory, suspended matter was removed through a filtration process using first a 1µm filter (Whatmann, USA) and then a 0.45µm (MN GF5) membrane filter. After filtration, the samples were subjected to SPE following the procedure described previously (Zhang *et al*., 2006). The cartridges were vacum dried, wrapped in aluminium foil previously rinsed with hexane and stored at -18°C.

For analysis, the frozen C-18 cartridges were placed in a fume hood for 2 hours to thaw. The analytes were eluted into autovials using 5 ml of methanol at flow rate of 1mL/min. GC-MS analysis was carried out using Agilent6890N gas chromatograph interface with a 5973C mass selective detector equipped with Agilet 7683B auto sampler and a DB-5 fused silica capillary column of 30m x 0.25µm i.d. x 0.25µm film thickness coated with cross-linked 5 % phenyl dimethyl polysiloxane. The temperature of the machine oven was initially maintained at 70°C for 1minute and then slowly raised to 175°C at a rate of 15°C/minute, it was then raised to

215°C at a rate of 2°C/minute then to 265°C at a rate of 10°C/minute. It was finally raised to 290°C at a rate of 20°C/min and maintained for 8 minutes. Helium (99.9% purity) was used as a carrier gas at a flow rate of 1.0 ml/minute. The analyte was injected at a volume of 1μL, in split less mode and the injection temperature kept at 250°C. The two EDCs under investigation were identified based on retention time and abundance of confirmation ions in their standards. Confirmation of identity of the analytes was done using NIST/EPA/NIH MASS SPECTRAL LIBRARY (NIST 05) and NIST MASS SPECTRAL SEARCH PROGRAM Version 2.0d.

The data was stored in Microsoft Excel programme and the significance level was tested using Student T-test at $P \leq 0.05$.

4.4 Results

The mean values of the levels of the two EDCs are presented in Table 3. The two EDCs (alkylphenol and 17β-estradiol) were found varying concentrations in all the samples analyzed except Mathare sampling point where estradiol was below detectable levels. Alkylphenol and 17β-estradiol levels ranged from 0.08 to 0.9174µg/L and BDL to 0.3005µg/L respectively (Table 3).

Table 3: Summary of mean levels of 17β-estradiol and alkylphenol

Location	17β-estradiol (μg/L)	Alkylphenol (μg/L)
Kibera	0.3005±0.02*	0.4428±0.2*
Dandora	0.0807±0.05*	0.08±0.01*
Mathare	BDL	0.9174±0.32*

*Means significantly higher than toxic value $P \leq 0.05$

The results (Table 3) indicate the two EDCs were present in significantly detrimental levels in Kibera and Dandora water ($p \leq 0.05$). However, it was only alkylphenol EDC which was in significant levels in Mathare ($p \leq 0.05$).

4.5 Discussion

Detrimental levels of the two EDCs were reported in Kibera and Dandora water and only, alkylphenol EDC was detected in significant levels in Mathare. Interestingly, the incidence of testicular retention reported earlier was highest in Kibera and Dandora which had more farmers using waste-water (Kipyegon *et al*., 2016b) and both recorded detrimental levels of the two compounds in this study. Similarly, the low incidence of reported testicular retention in Mathare area is hereby attributed to the below detectable levels of 17β-estradiol in this area.

The levels of these EDCs within the analyzed water as reported by this study, although comparable to reports by Sole et al. (2000), exceed those reported in Iran's surface water. Jafari et al. (2009) reported 0.003μg/L and 0.009 μg/L for estrogens and alkylphenol respectively in similar polluted water situations. The difference might be due to levels of contamination in different study cases as pointed out by others (Johnson and Sumpter, 2001; Zhang *et al.*, 2014).

In all cases the levels detected for the two EDCs are significantly higher than the detrimental levels demonstrated in previous studies (Aerni *et al*., 2004). This confirms presence of the two EDCs that could be associated with the reported testicular retention in chapter 3 Kipyegon *et al*., 2016b). Thus it is in order to speculate that alkylphenol and 17β-estradiol EDCs within the polluted water of Nairobi River have the potential to disrupt reproduction by contributing towards testicular retention. Estrogenic contamination of river water has been suspected to be a concern for male infertility (Sim *et al*., 2010; Zhang *et al*., 2014) because of their ability to cause endocrine disruption similar to reversal of androgen effects (Baronti *et* *al*., 2000). There are reports of detected 17β estradiol in polluted river water (Peng *et al*., 2008; Jafari *et al*., 2009; Knez, 2013), and in waste-water (Ma *et al*., 2007; Ying *et al*., 2012). High levels of 17β-estradiol were detected from the sampled water in this study making it a major suspect EDC.

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CHAPTER FIVE

5 EFFECTS OF POLLUTED NAIROBI RIVER WATER ON THE SEMINIFEROUS TUBULES OF BOARS

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5.1 Abstract

A study was carried out to determine the effects of exposure to polluted water on the boar seminiferous tubule histology. Adult boars accessing polluted river water were acquired and castrated for testicular histopathological observations. Several lesions were observed; seminiferous epithelial vacuolations, sloughing of germ cells and patches of depleted seminiferous tubules. The findings indicate that exposure of boars to polluted water with environmental chemicals led to lesions within seminiferous tubules that would render them infertile.

5.2 Introduction

Rapid Industrialization and urbanization especially in developing countries has resulted in an increased contamination of surface water (Chezhien *et al.,* 2010). This situation is worsened by the coming up of urban informal settlements along river banks. Informal settlements mainly have no proper sewerage systems and usually discharge raw sewage into nearby rivers. Urban rivers are thus polluted by waste-water effluents indiscriminately discharged from industries, households and farmlands. Waste-water effluents contain a wide array of contaminants which include; heavy metals (Ahmad *et al.,* 2013; Ndeda and Manohar, 2014) pathogens (Musyoki *et al*., 2013) and endocrine disrupting compounds (Ying *et al.,* 2012). Such chemicals have been detected in urban draining rivers globally (Huang and Sedlak, 2001; Kolpin *et al.,* 2002; Snyder, 2008) and demonstrated to negatively affect reproduction in males (Jobling *et al*., 2002) through estrogenic-like effect and/or anti-androgenic mediated processes (Knez, 2013) which regulate male reproductive functions like histology of the seminiferous tubules (Svechnikov *et al.,* 2014). Known endocrine disrupting compounds include: estradiol, alkylphenol, plasticizers, surfactants, pesticides and pharmaceuticals (Ankley *et al.,* 2007; Ahmad *et al*., 2013). Waste-water effluents, contains a mixture of chemicals, pharmaceuticals, and hormones (Stevens *et al.,* 2003).

River water polluted with endocrine disrupting compounds are known to perturb reproduction in aquatic organisms through steroid hormones alteration (Hecker *et al.,* 2002) and impairment of gonadal development (Hemming *et al.,* 2001; Jobling *et al*., 2002; Sheahan *et al.,* 2002; Shalaby and Migeed, 2012). Paul *et al*, (2005) reported similar observation in ruminants exposed to sewage sludge.

Effluents from waste-water treatment plants together with discharges from informal settlements along the river banks have contributed to the massive contamination of rivers (Sibanda *et al.,* 2015). In Kenya, studies on urban draining rivers in Nairobi city have revealed heavy contamination by microbial organisms (Musyoki *et al.,* 2013), heavy metals (Ndeda and Manohar, 2014) and pesticides (Wandiga, 2001). These contaminants emanate from informal settlements, surface run-off from the city, urban farmlands and light industries like garages along the banks of the Nairobi River.

With urban agriculture in the increase in developing countries, urban rivers and waste-water effluents are increasingly becoming reliable water sources for urban agriculture due to water scarcity. Thirty percent (30%) of Nairobi residents keep animals and cultivate crops in the city's unused land and in their backyards (FAO, 2012). Majority use untreated or partially treated sewage to irrigate crops and fodder (Karanja *et al.,* 2010). In informal settlements, people commonly scavenge in rivers in search of valuables to sell while others plant food crops like bananas, kales and arrow roots along river banks and raise livestock like poultry, goats and pigs which supplement their nutritional requirement and also their income. Food crops grown using polluted river water have been shown to contain microbial organisms and heavy metals which pose a health risk to consumers of such products.

In areas where pigs are kept, these animals are seen roaming around freely, searching for food in drainage canals and rooting in water ways. In so doing they come in contact with a cocktail of chemicals in the water through ingestion and also skin contact. Whereas it has been shown that effluent polluted water has adverse effects on the reproduction of aquatic organism (Sheahan *et al*., 2002), literature on the possible effects of such chemicals on domestic animals especially pigs is scanty.

This study aimed at investigating the possible effects on reproductive system of exposure to environmental contaminants in waste-water on the boar (*Suis scrofa domestica*) by examining their testicular histopathology. This follows the observation by Svechnikov et al. (2014) that human beings dwelling in areas close to dump sites had an increased risk of reproductive abnormalities in their male children.

5.3 Materials and methods

5.3.1 Study site

The study was carried out in the informal settlements in Nairobi County, Kenya. The sites selected were the three areas served by the tributaries of the Nairobi River namely; Kibera, Mathare, and Dandora. The locations were also selected based on: its proximity to a city river, the physical appearance of the water and pig keeping activities with animals scavenging along the polluted river water. In these areas pigs are housed in temporary structures only at night and left to roam freely during the day.

5.3.2 Animal selection and sample collection

A total of 15 free roaming Large White adult boars, reared and fed on the polluted Nairobi River since birth were selected from the three study sites (n=5 for each site) castrated and examined for any notable abnormality of the seminiferous tubules. Another group of five boars were selected from known non-polluted area to serve as the control animals.

The boars were castrated under general anaesthesia achieved using a combination of Xylazine 4.4mg/kg, Telasol 4.4mg/kg and Ketamine 2.2mg/kg. The testes were cleaned, epididymis trimmed off and testis cut longitudinally in half for histopathology.

5.3.3 Sample processing, examination and analysis

From each half of the testis, thin sections were made from the area between the mediastinum testis and the tunica albuginea. These sections were fixed in freshly prepared 10% phosphate buffered formalin for 24 hours. The sections were further processed for microscopic evaluation. Ten (10) sites were examined per slide. The affected seminiferous tubules were counted and the mean values subjected to a Student T-test ($p \leq 0.05$).

5.4 Results

Table 4 shows the number of seminiferous tubules with lesions in boars castrated from the three riparian zones. Figure 9 shows the testicular histological appearance of a boar from the control group while figures 10 to 12 show the histological appearance of the seminiferous tubules of the testicular sections examined from the same boars. The histopathological lesions observed were similar within and across boars castrated in Kibera and Dandora ($p \leq 0.05$). Boars from Mathare had less severe lesions of the seminiferous tubule (Fig 12).

Significant ($p \leq 0.05$) lesions observed were vacoulation and germ cell sloughing (disruption in the layered architecture of the seminiferous tubule and sloughing of germ cells in the apical compartment into the seminiferous tubule lumen (Table 4; Fig 10-12).

The seminiferous tubules in control animals showed active spermatogenesis without any significant lesions ($p \leq 0.05$). Thus the normal seminiferous epithelia containing Sertoli cells, spermatogonia closely associated with the basal lamina, spermatocytes, spermatids and their differentiation into spermatozoa (Fig 9). In both experimental and control boars, no significant lesions were observed in Leydig cells, which remained normal and intact (Fig 9-12).

Table 4: Number of seminiferous tubules showing lesions in the experimental and control group of boars (Mean ±SEM)

Type of lesion	Location			
	Kibera (n=5)	**Mathare (n=5)**	**Dandora (n=5)**	**Control (n=5)**
Vacuolation	72.3±9.4*	2.1±3.5	57.9±9.2*	1.7±3.0
Germ cell sloughing	68.6±5.4*	2.3±9.4	80.8±11.1*	2.0±2.1

** Significantly higher than control*

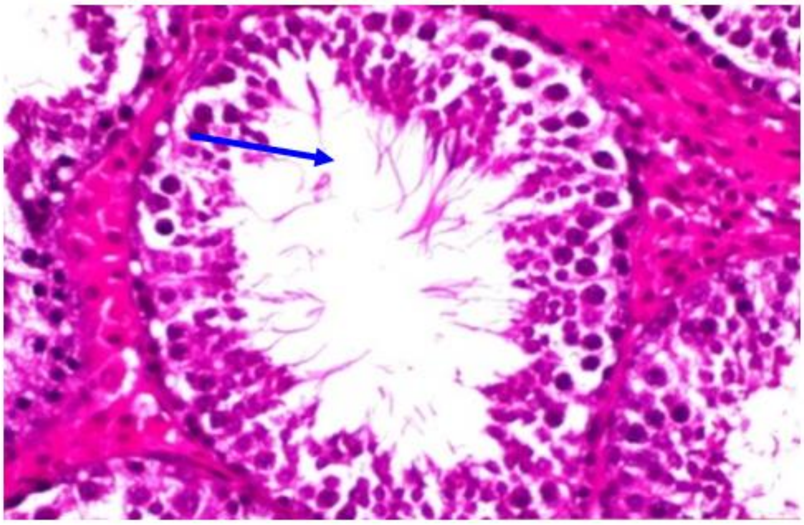

Figure 9: Seminiferous tubule from the control group showing normal spermatogenesis (Magnification×400 H&E stain).

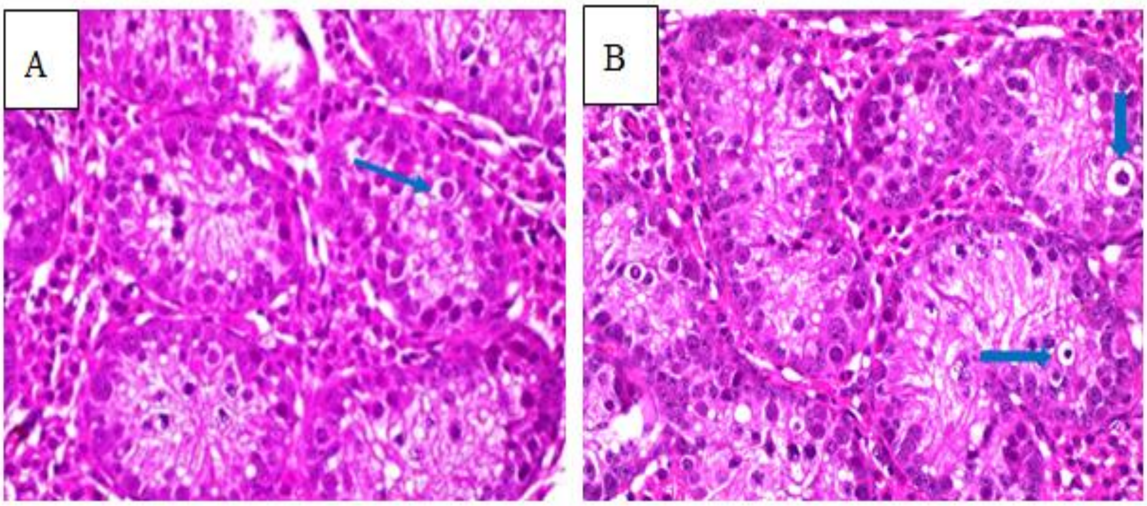

Figure 10 (A&B): Photomicrograph of the testis of a boar accessing polluted river water in Kibera. The seminiferous tubules are lined by a few germ cells, cell vacuolation is also noted (arrow) as a sign of toxicity (Magnification×400 H&E stain).

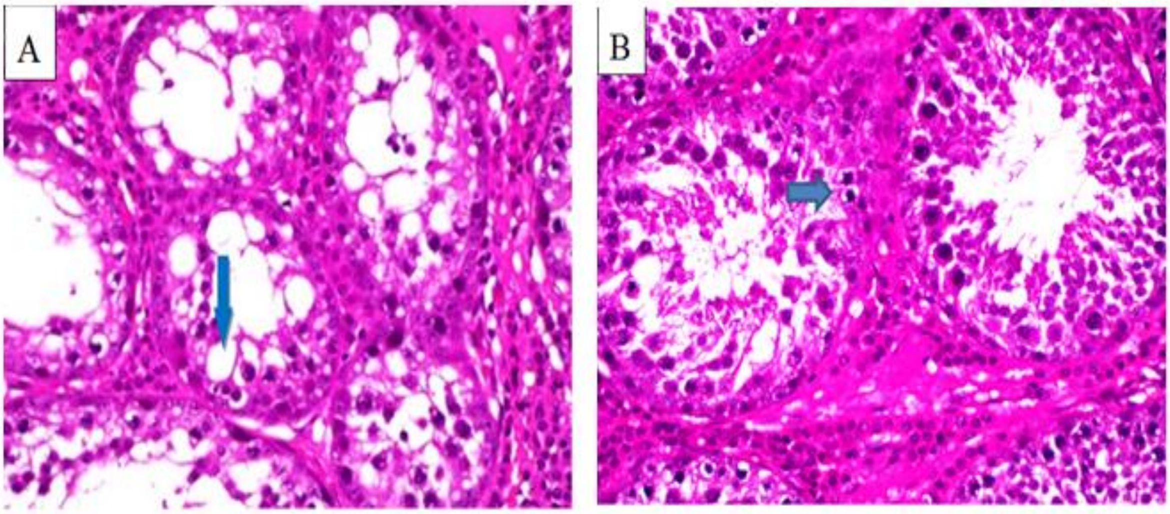

Figure 11 (A&B): Photomicrograph of the testis of a boar accessing polluted river water in Dandora. There is a disruption of the seminiferous epithelium architecture due to desquamation, cell vacuolation is more evident here (arrow) (Magnification×400 H&E stain).

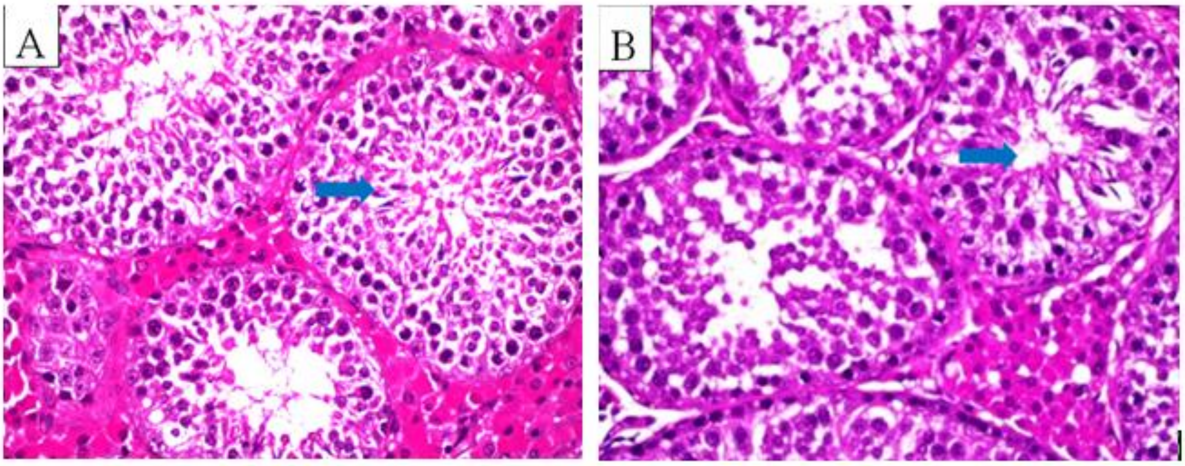

Figure 12 (A&B): Photomicrograph of the testis of a boar accessing polluted river water in Mathare. There is little germ cell displacement. Some tubules exhibit normal spermatogenesis (Arrow) (Magnification×400 H&E stain).

5.5 Discussion

This study reports two major seminiferous tubule lesions (Vacoulation and germ cell sloughing) that are associated with consumption of polluted water by boars. The severity of the changes observed varied from one study area to another; in Mathare, where most of the farmers reared their boars using clean water, the seminiferous tubule histology of the boars closely resembled that of the control group ($p \leq 0.05$, Fig 9 and 12). In Kibera and Dandora where the survey indicated the use of waste-water for pig rearing, the seminiferous epithelium disruption was severe (Fig 10 to 11), this finding concurs with that of Shalaby and Migeed (2012) and clearly indicates that the pathological lesions observed are associated with drinking polluted water by the boars. Similar findings have been reported in rats exposed to polluted water with known estrogen-like or anti-androgenic EDCs (Adamkovicova *et al.,* 2014), which then suggest the water the boars consumed might contain such EDCs.

Vacuolation within seminiferous epithelium and sloughing of germ cells with partial depletion of the seminiferous epithelium have been previously demonstrated to be indicative of environmental endocrine disrupting toxicity pointing towards effects of oestrogens-like or anti-androgens on the testicular tissue (Knez, 2013). These EDCs are the toxicants expected with increasing human population and industrialization that lead to an increase in the factory, farm land and domestic waste discharges of a wide variety of chemicals into the environment (Bustos-Obregón and Hartley, 2008; Knez, 2013).

These EDCs in water affect animals through ingestion of polluted water or feed (Norstrom, 2002). Although it can be argued that the exposure rates of such contaminants are low and the reproduction of the majority of ruminants is unaltered by such low levels of exposure (Rhind *et al*., 2010), certain production systems expose animals to a higher concentration of environmental contaminants already shown to have reproductive side effects (Norstrom, 2002). It is therefore postulated from these results that these effects could be due to prolonged exposure of EDCs in polluted water.

Histopathological evaluation of the testis as a measure of toxicity on fertility has been used by previous authors in various animals (Leino *et al.,* 2005; Svechnikov *et al.,* 2014). In this study, the seminiferous epithelium histopathological changes varied widely; while some tubules showed developing spermatozoa (Figures 14 and 15), other tubules showed a disrupted epithelium, without the signature basal to luminal germ cells maturation pattern (Figures 10 and 12). Such disruption has been reported in fish living in a polluted lake and shown to cause a decrease in spermatogenesis (Shalaby and Migeed, 2012). Experimentally, comparable findings were recorded in rats exposed to EDCs (Adamkovicova *et al.,* 2014.

Exposure to exogenous chemicals including EDCs is reported to damage the Sertoli-Sertoli cell interaction and Sertoli- Germ cell interaction leading to germ cell sloughing (Bello *et al.,* 2014; Adamkovicova *et al.,* 2014). Evidence of such loss in cellular interaction is reported in this study. The loss of germ cells through sloughing into the lumen further led to some tubules with almost complete absence of spermatozoa; a consequence of testicular toxicity. These changes would render the affected boars to produce reduced number of spermatozoa thus reducing their fertility.

The findings of this study indicate that exposure to urban waste-water can result in testicular disruption seen as vacuolation and sloughing of germ cells. Similar findings have been demonstrated in rams exposed to polluted water (Paul *et al.*, 2005), and postulated to be caused by EDCs.

In conclusion, exposure of boars to polluted water leads to lesions within seminiferous tubules that would render them infertile. The noted findings strongly pointed towards the effects of 17β-estradiol and alky phenol EDCs (Bello *et al*., 2014).

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CHAPTER SIX

6 EFFECTS OF POLLUTED RIVER WATER ON TESTICULAR HISTOLOGY OF MICE

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6.1 Abstract

Mature naive male mice were used to test if consumption of polluted water led to lesions in seminiferous tubules. Water was collected from polluted river water in Kibera, Mathare and Dadora and utilized as drinking water to the 10 caged mice on daily basis *ad-libitum*. Ten (10) other mice were used as a control group by providing them with clean water on daily basis *ad-libitum* for similar period of 60 days (One-and-half cycles of spermatogenesis). The mice were euthanized and testicles obtained for histology. The seminiferous tubules of experimental mice showed significant lesions ($p \leq 0.05$) of vacoulation and sloughing of germ cells, consistent to the lesions observed in boars reared using the same polluted water. Like in the case of boars, results indicate that exposure of mice to polluted water with environmental chemicals led to lesions within seminiferous tubules. The noted findings strongly pointed towards the effects of estrogenic EDCs (Bello *et al*., 2014).

6.2 Introduction

Polluted water bodies are known to contain many chemicals which include (Huang and Sedlak, 2001; Kolpin *et al*., 2002). Endocrine disrupting compounds negatively affects the reproductive system of animals (Paul *et al*., 2005; Rhind *et al*., 2010; Shalaby and Migeed, 2012; Bello *et al*., 2014). Surface water contamination emanate from discharge from sewage treatment works, industries, farmlands and informal settlements close to the river (Stevens *et al*., 2003; Sibanda *et al*., 2015). In Kenya urban draining streams and rivers are contaminated by microbial organisms (Musyoki *et al*., 2013), heavy metals (Ndeda and Manohar, 2014) and pesticides (Karanja *et al*., 2010; Wandiga, 2001).

This study was aimed at validating the observed reproductive disruption of effluent polluted water on the boar by using the mice as an animal model.

6.3 Materials and methods

6.3.1 Animals and treatment

The current study involved twenty (20) mature male mice purchased and kept in cages with polypropylene sides and floors, and stainless-steel grid tops (22 × 40 × 15 cm) within the animal house in the Department of Public Health Pharmacology and Toxicology (PHPT) University of Nairobi. Standard mice pellets were available *ad-libitum* and the room conditions were maintained at an average of 60% relative humidity with approximately 12 hours light and dark cycle. An acclimatisation period of two weeks was allowed before commencement of the experiment. The twenty mice were then randomly separated into two groups of ten mice each (control and experimental), their use and care followed the guidelines set by the Faculty of Veterinary Medicine, University of Nairobi Animal use and ethical Committee.

Polluted river water was collected in the morning and supplied to the caged experimental group *ad-libitum*. The control group received clean tap water. After 60 days the mice were sacrificed in an anaesthesia chamber and the testes were dissected out and processed for histology.

6.3.2 Sample preparation

For light microscopy, the testicular tissue samples were cleaned off blood and excess tissue trimmed; the tissue was then sectioned into smaller blocks and immediately fixed in freshly prepared 10% phosphate buffered formalin for 24 hours. The sections were further processed for microscopic evaluation. Ten (10) sites were examined per slide. The affected seminiferous tubules were counted and the mean values subjected to a Student T-test ($p \leq 0.05$).

6.3.3 Light microscopy

The Haematoxylin Eosin stained sections were observed and digital images taken by a digital camera coupled to Zeiss light microscope for histopathological analyses of seminiferous tubules. In every slide, 10 seminiferous tubules were examined.

6.4 Results

Figure 13 shows the histological appearance of a normal mouse testis. The results of the seminiferous epithelium changes are shown (Fig 14-16 and Table 5). Two main lesions; vacuolation and sloughing of germ cells were observed. The finding from the control and the experimental groups showed a significant difference ($p \leq 0.05$, Table 5). In control group (Fig 13) no significant histopathology was recorded. The various lesions observed in the experimental group (Fig 14-16) are demonstrated.

Table 5: Mean (±SEM) number of seminiferous tubules showing lesions in the experimental and control group of mice in an average of 10 views.

Type of lesion	Experimental Group (n=10)	Control Group (n=10)
Vacuolation	40.71±12.3*	8.02±8.09
Germ cell Sloughing	45.67±10.1*	3.21±5.4

** Significantly higher than control (p≤0.05)*

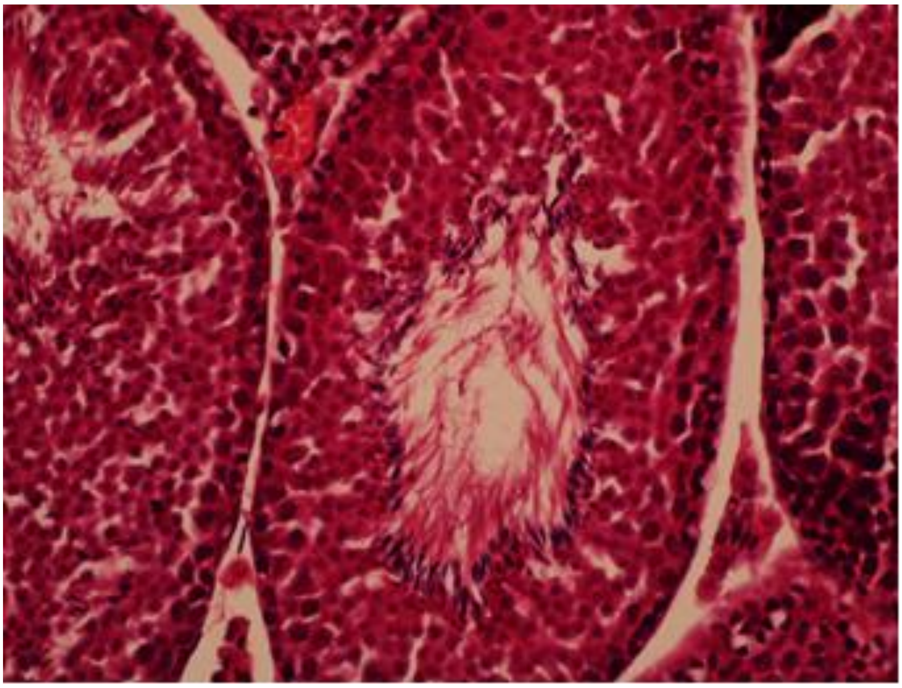

Figure 13: Histological section of the mouse testis showing normal architecture of seminiferous tubular epithelium with developing germ cells (Magnification×400 H&E stain).

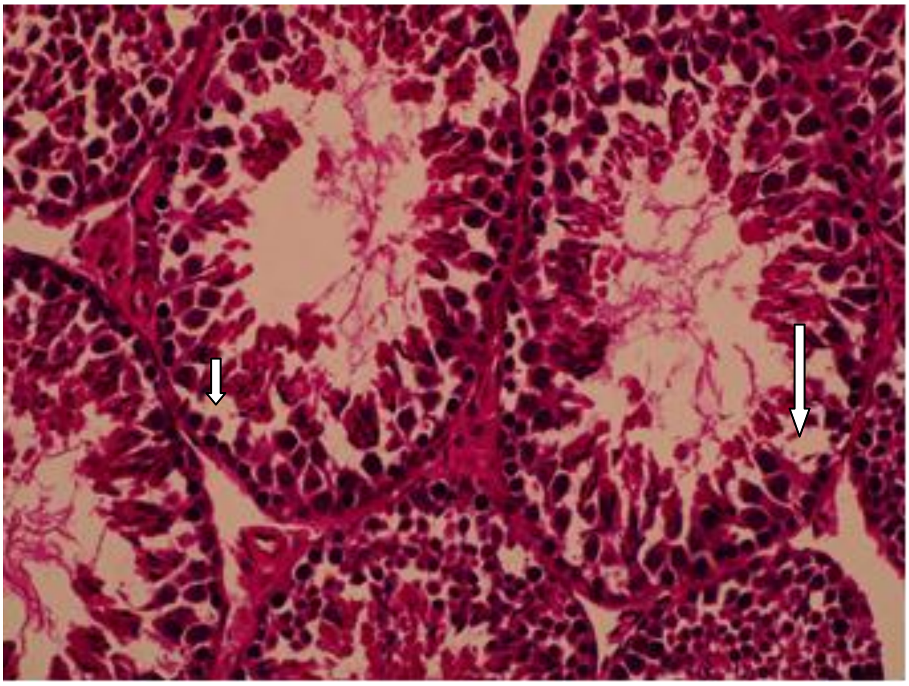

Figure 14: Histological section of the testis from polluted river water treated mice vacuolation (Arrow) and decline in the number of germ cells (Magnification×400 H&E stain)

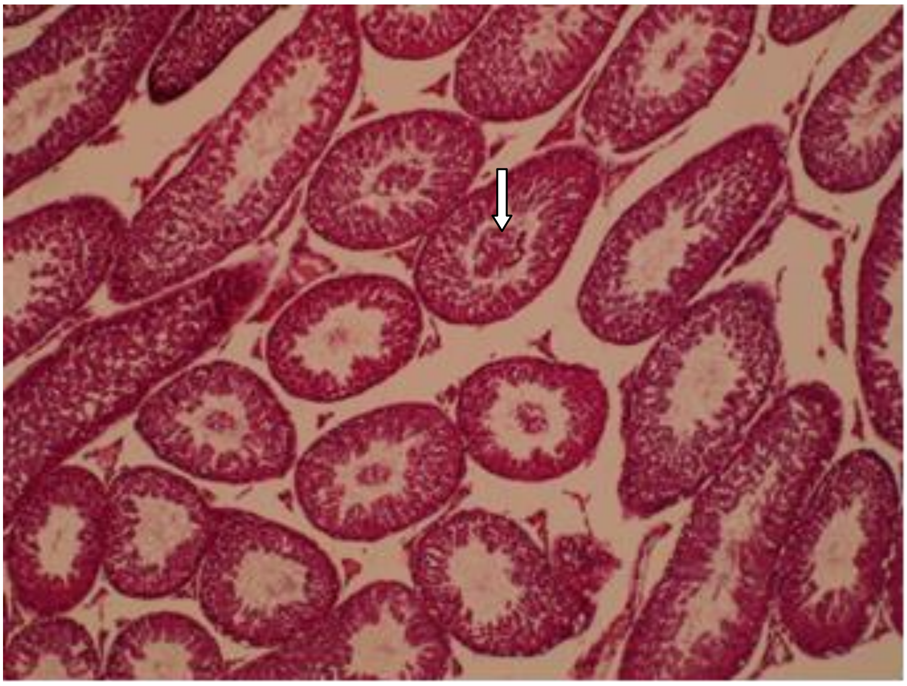

Figure 15: Histological section of the testis from polluted river water treated mice showing sloughed germ cells (Arrow) in the seminiferous epithelium lumen (Magnification ×100 H &E stain).

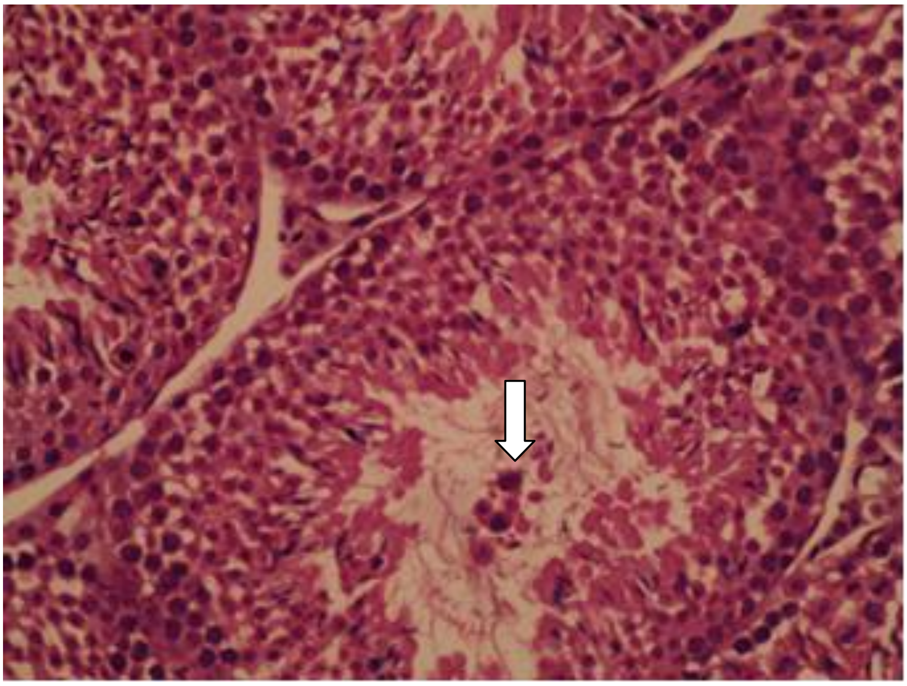

Figure 16: Histological section of the testis from polluted river water treated mice showing sloughed germ cells (Arrow) in the seminiferous epithelium lumen (Magnification ×400 H &E stain).

6.5 Discussion

Two main lesions; vacuolation and sloughing of germ cells have been demonstrated in mice exposed to the polluted water for a period of 60 days. In control group non-significant lesions were observed in the seminiferous tubules, these lesions are thought to be due to the normal apoptotic processes in the testis and those occurring during tissue handling and processing.

Animal models have been used to evaluate testicular toxicity in previous experiments (Leino *et al*., 2005; Svechnikov *et al*., 2014). Similar to the case of boars, results indicate that prolonged exposure of pigs to polluted water with environmental chemicals led to lesions within seminiferous tubules. Similar findings were reported by Bello et al. (2014).

Our observations concur with those by Adamkovicova *et al*, (2014) after administration of estrogenic-like EDCs to male rats. In addition a decrease in the epithelial height has been associations to toxicity of anti-androgens (Damodar *et al*., 2012; Adamkovicova *et al*., 2014).

Androgens like testosterone are important in normal functions of the Sertoli cell, which maintain normal architecture of germ cells (Damodar *et al*., 2012). Thus, estrogenic-like and/ or anti-androgens in water as pollutants would disrupt function of such androgens leading to vacoulation and sloughing of germ cells (Sikka and Wang, 2008). These results are a clear indication that the polluted water contains EDCs capable of affecting seminiferous tubules. Similar experiments have demonstrated the same lesions (Knez, 2013; Kumar and Nagar, 2015).

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CHAPTER SEVEN

7 COMMON DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 Discussion and Conclusions

Results of the present study revealed that most pig keepers in the areas surveyed were literate, aware of the potential danger of using polluted water for pig rearing and continued to use the same for rearing the pigs. In addition majority had primary - secondary level of education and many were involved in urban farming. A significant number reported retained testis in pigs reared using waste-water. This incidence was highest in Kibera and Dandora, which apparently also had more of the farmers using waste-water for farming. Majority seemed aware that use of polluted water was dangerous on the pigs reared. This awareness was higher in respondents who had noticed retained testis of the pigs, who mostly came from Kibera and Dandora.

Retention of the testis has been demonstrated in reared pigs and has been reported previously following chronic exposure of animals to EDCs in polluted water (Paul *et al*., 2005; Bellinghan *et al*., 2012; Svechnikov *et al*., 2014). The condition (failure of testicular descent) has been also attributed to exposure to EDCs of estrogenic or anti-androgenic nature within the water (Skakkebaek *et al.*, 2001; Damgaard *et al*., 2002; Main *et al*., 2007; Bay *et al*., 2011).

Two major seminiferous tubule lesions (Vacoulation and germ cell sloughing) are reported in association with consumption of polluted water by boars. In Mathare where most of the farmers reared their boars using tap water, the seminiferous tubule histology of the boars did not differ with that of the control group ($p \leq 0.05$), clearly indicating that the pathological lesions were associated with drinking polluted water by the boars.

The results of animal model in the laboratory confirmed that the two seminiferous; vacoulation and sloughing of germ cells are highly associated with polluted water. There was also significant difference between experimental and control groups ($p \leq 0.05$). In control group no lesions were observed in the seminiferous tubules while lesions were significant in the experimental group.

The analysis revealed that two EDCs (alkylphenol and 17β-estradiol) were present in significantly detrimental levels in the polluted water of Nairobi river. The two EDCs were also demonstrated to be associated with observed lesions within seminiferous tubules (Kuch and Ballschmiter, 2001). In Mathare area where 17β-estradiol was not detected, the seminiferous epithelium histology was similar to that of the control group (Fig 12); in contrast to Kibera and Dandora where both chemicals were detected and the seminiferous epithelium damage was more significant (Fig 10 & 11). This analogy indicates that 17β-estradiol is a more potent EDC compared to alkylphenol.

It is therefore, based on these observed results that the polluted water of Nairobi river water contains significant levels of 17β-estradiol and alkylphenol EDCs capable of being causing male infertilities relating to retained testis and disruptive seminiferous epithelium. These observations are consistent with effects of estrogenic-like or ant-androgenic endocrine disrupting compounds previously reported (Hecker *et al*., 2002; Jobling *et al*., 2002; Sheahan *et al*., 2002; Paul *et al*., 2005; Rhind *et al*., 2010).

In consistence with other authors (Wandiga, 2001; Falconer *et al*., 2006; Ndeda and Manohar, 2014; Sibanda *et al*., 2015; Sole *et al.,* 2000; Jafari *et al*., 2009; Zhang *et al.,* 2014), we conclude that rivers draining through informal settlements of urban and peri-urban areas pose a threat to male reproduction due to pollutions with EDCs.

7.2 Recommendations

- There is need for policy to raise public awareness on the risks associated with the use of polluted Nairobi river water for pig farming.

- Further studies may also be required to determine if similar effects would be observed in other males, especially in men consuming kales farmed using the polluted water.

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8 APPENDICES

8.1 APPENDIX I: RESEARCH QUESTIONNAIRE

UNIVERSITY OF NAIROBI

DEPARTMENT OF CLINICAL STUDIES

RESEARCH QUESTIONNAIRE ON USE OF WASTE-WATER FOR FARMING AND ITS IMPLICATION ON THE FUNCTIONING OF THE BOAR TESTIS

Investigator: **Ambrose Kipyegon Ng'eno** B.V.M, MSc (UON).

Supervisors:

1. **Dr Henry Mutembei, PhD.** Signed ………………………Date ……………
2. **Dr Victor Tsuma, PhD.** Signed ………………………Date ……………
3. **Dr Jemimah Oduma, PhD.** Signed ………………………Date ……………..

PART ONE: CONSENT

A. RESEARCHER'S DECLARATION

We the researchers declare that:-

1. The information from our respondents will not at any time be obtained by false pretence, coercion or intimidation.

2. The information received from the respondents will not be altered or tampered with in any way.

3. The data collected using this questionnaire will be solely used for research purposes.

B. RESPONDENT'S INFORMED CONSENT

I ____willingly give my

consent to be questioned by the researcher for the purpose of his research work

Signed____________________________ Thumb print_____________________

PART TWO: RESPONDENT'S DATA

I- General Information:

<table>
<tr><td colspan="2">1) Name</td><td>2) Male</td><td>3) Female</td></tr>
<tr><td>4) Occupation</td><td colspan="3">5) Age</td></tr>
<tr><td>6) Level of education</td><td colspan="3">7) Marital status</td></tr>
<tr><td>8) Location of farm</td><td colspan="3">9) Geo coordinates</td></tr>
</table>

II- Social setting and economic factors

10) For what period have you practised livestock farming here? **(1-5 yrs, 5-10 yrs, 10-20 yrs, >20 yrs, Not known)**

11) What animal species do you keep?

12) If pigs, what breeds do you keep?

13) What is your reason for keeping them? **(Easily marketable, Fast growth rate, Large litter size,, Consumes less food, Other)**

14) Herd structure:
Mature sows------ Gilts------Mature boars------Piglets-----Finishers------Growers------

15) What is the method of service? **(AI, Natural)**

16) If natural service, do you own a boar? **(Yes, No)**

17) What is the age of the boar you use? **(1yr, 2yr, 3yr, 4yr, >5yrs, unknown)**

18) How long have you been using this boar? **(1yr, 2yr, 3yr, 4yr, >5yrs, unknown)**

19) How do you detect heat? **(Observe signs, Pressure test, Use a boar, Not done)**

20) How many times do you serve? **(1, 2, 3, unknown)**

21) Are there repeats after service? **(Yes, No)**

22) What is the average number of piglets/sow/farrowing? **(<3, 4, 5, >6)**

23) What is the average piglets weaned per sow per year? **(<3, 4, 5, >6)**

24) At what age do your gilts first show signs of heat? **(6mo, 8mo, 12mo, >12months)**

25) What defects of the external genitalia have you observed in males and females?**(none hypospadias, retained testis, others named)**

26) Do you slaughter them for family consumption? **(Yes, No)**
27) If no, where is your market? **(neighbours, Local market, retailers, Other)**

Section III- Waste-water use patterns

28) What is the source of food for your animals?**(Commercial feed, Kitchen remains, Hotel remains, Food sorted from dumpsites, Fend for themselves, Other)**

29) Which are the water sources for your animals?
(Waste-water, River water, Rainfall, Tap water)

30) What source of water do you use in the dry seasons? **(waste-water, river water, tap water)**

31) If waste-water, what are the reasons for its preference?
(The only one available, Free, Easily accessible, Other)

32) How do you provide the water to the animals? **(Fetch for them, Taken to the river, Other)**

33) Do you encounter problems due to use of waste-water?
(Scarcity, Disease, None, Other)

34) Do you think waste-water have health effect on your animals? **(Yes, No)**

35) If yes name them :

i) ____

ii) ____

iii) ____

iv) ____

v) ____

8.2 APPENDIX II: PUBLICATIONS

A. **Kipyegon AN, HM Mutembei, VT Tsuma, JA Oduma and P Kimeli.** (2016). Knowledge and Practices of the residents living along the Nairobi River Riparian on the use of the contaminated river for farming and its effects on animal reproduction. J Agric and Vet Sci. 9(8):59-61.

B. **Kipyegon AN, HM Mutembei, VT Tsuma and JA Oduma.** (2017). Effects of exposure to effluent contaminated river water on boar reproduction. Inter J Vet Sci, 6(1):49-52.

C. **Kipyegon AN, HM Mutembei, VT Tsuma, JA Oduma.** (2016). Effects of Effluent Contaminated River Water on Testicular Histology of Mice. Sch J Agric Vet Sci; 3(6):411-415.

D. **Kipyegon AN, HM Mutembei, VT Tsuma, and JA Oduma.** (2016). Levels Of 17β Steroid and Alkylphenol Estrogenic Endocrine Disrupting Compounds in Nairobi River. Journal of Physical Science and Environmental Studies. 2 (3):46-49.