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Uranium Speciation, Bioavailability and Uptake in Atlantic Salmon (Salmo salar L.) Parr and the Influence of pH.

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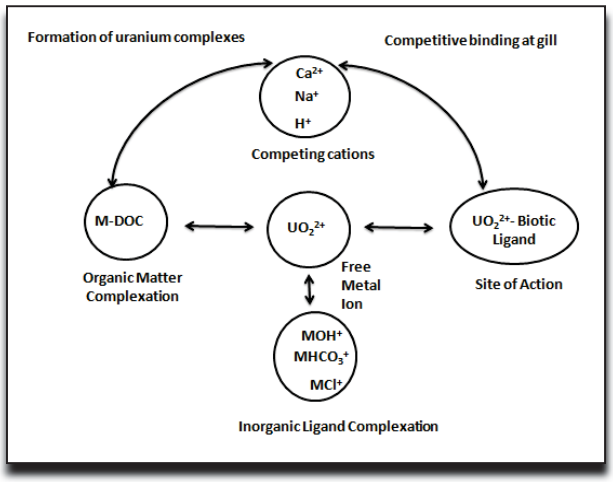
URANIUM SPECIATION, BIOAVAILABILITY AND UPTAKE IN ATLANTIC SALMON (SALMO SALAR L.) PARR AND THE INFLUENCE OF pH.



A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Radioecology

Department of Environmental Sciences (IMV)

Norwegian University of Life Sciences (NMBU)

Aas, Norway

BRICE NKWELLE SONE

Aas, Norway **May, 2015**

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Brice Nkwelle SONE, MSc.

STATEMENT OF DECLARATION

In presenting this thesis in partial fulfillment of the requirements for a Master degree from the Norwegian University of Life Sciences (NMBU), Aas, Norway, I hereby declare that every data is part of the EU STAR project (421151). Most importantly, all waters and the experiments were set up and performed by others within the project. Fish exposures were done in three sessions, the first in the end of November 2012, the second in early January 2013 and the last in early December 2013. I participated in the November and December sessions during which I did onsite water size fractionation for U speciation information, dissection of the exposed fish to obtain fish tissues (gill, liver and kidney), and digestion of the fish samples on ultraclave for ICP-MS measurements of U concentrations.

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LIST OF ACRONYMS

AWQC: Ambient Water Quality Criteria

BLM: Biotic Ligand Model

CCME: Canadian Council of Ministers of the Environment

CRM: Certified Reference Material

DOC: Dissolved Organ Carbon

dw: dry weight

IAEA: International Atomic Energy Agency

ICP-MS: Inductively Coupled Plasma Mass Spectroscopy

kDa: Kilo Dalton

LMM: Low Molecular Mass

OECD: Organization for Economic Cooperation and Development

OSPAR: Oslo/Paris Convention

pH: the negative log of the concentration of the hydrogen cation ($pH= -\log_{10}[H^+]$)

STAR: Strategy for Allied Radioecology

TOC: Total Organic Carbon

U-BLM: Uranium Biotic Ligand Model

US EPA: United States Environmental Protection Agency

ww: wet weight

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ABSTRACT

Many chemicals move readily in the environment and can be transported with surface water runoff to a nearby stream or lake. So, risk assessments need to be mindful of environmental movement of chemicals and the fact that exposure to biota could occur through a variety of pathways. Regularly, exposures can lead to uptake of a chemical by more than one route. The present study was based on controlled acute experiments (96h) with uranium (U) and Atlantic salmon (*Salmo salar* L.) parr systematically exposed to synthetic waters with the same U concentration at different pH water groups comprising of pH 5.4, pH 5.7, pH 6.5 reference water, pH 6.8, pH 7.2 and pH 7.7 respectively, at 1.0 mg/L nominal U concentration. The aim was to have a general idea of U speciation and accumulation across a wide range of pH, as input in the development of an acute U-Biotic Ligand Model (BLM). Speciation information of U based on charge and size fractionation for pH 5.1, pH 5.4, pH 5.7 and pH 6.8 at 1.0 mg U/L and in addition pH 5.5 at varying concentrations (0.46, 0.96, and 1.92 mg U/L) was obtained. Results showed that 93-99 % of the 1.0 mg/L total U was found in the dissolved (< 0.45 μm) fraction whereas the remaining (< 7 %) was found in the particulate form. Of the dissolved fraction, about 90-97 % was present as LMM species (cutoff < 10 kDa). Meanwhile, at pH 5.5 with varying U concentrations, there seemed to be no significant changes in the speciation of U even when changing the concentration at the same pH. It can be assumed that, the U species in the water was considered to be highly mobile and potentially bioavailable. High levels of LMM species suggest that there were insignificant concentrations of particles and colloids. In fish exposed at 1.0 mg U/L, the mean U concentrations ranged from 3.2 mg/kg dw to 58.6 mg/kg dw in gills, 0.13 mg/kg dw to 1.17 mg/kg dw in liver, and 0.014 mg/kg dw to 0.80 mg/kg dw in kidney. The observed trend in mean U concentration in tissues was gill > liver > kidney. The highest accumulation of U in gills, liver and kidney was observed at pH 5.4 and pH 5.7 waters. Statistical test comparing mean U concentration accumulated in tissues and all pH water groups, showed significant ($p < 0.01$, $r^2 = 89.7$ %) differences in mean gill U concentration between pH 5.4 and pH 5.7, and the other pH groups. There was also evidence of significantly different U concentration in liver ($p < 0.01$, $r^2 = 65.9$ %) and kidney ($p < 0.01$, $r^2 = 46.9$ %). The good relationship between concentrations of U in tissues and pH water groups indicated that pH is a significant factor influencing U speciation in freshwater, bioavailability, uptake and accumulation in fish. So, speciation information on U and its fate in the environment depending on pH being an important factor can be very useful in U-BLM modeling and thereby in risk assessment of natural waters.

CHAPTER ONE

1. INTRODUCTION

Uranium (U) occurs ubiquitously in the environment with the most significant source of anthropogenic contribution to aquatic systems arising from U mining (Poston, 1982; Domingo, 1995; Bonin & Blanc, 2001). Aquatic systems downstream of mining areas are susceptible to receiving U discharges through runoff, thus, raising environmental concerns. Such concerns relate to the water quality and biota in terms of increasing U levels, potential risk on aquatic organisms which could range from chronic to acute effects in fish. Though radioactive U has characteristically low specific activity (Colley et al., 2001) its chemical toxicity is considered of much greater concern (Sheppard et al., 2005). This is because it is chemically very reactive as opposed to its radiologic risk (Domingo, 1995; Harper & Kantar, 2008). As such, to understand the chemical toxicity of U in aquatic systems knowledge of its speciation in solution is very vital (Markich, 2002). U toxicity is highly dependent upon its speciation governed by water chemistry parameters, particularly pH, alkalinity, hardness, redox potential (Eh), solubility and complexation with dissolved organic and inorganic ligands (Harper & Kantar, 2008). In freshwater systems, the mobility of U becomes very important with oxic and acid conditions given that dominant U species are dependent on the pH-Eh conditions and the concentrations and availability of complexing ions (Gascoyne, 1992; Ragnarsdottir & Charlet, 2000). Of the four oxidation states of U, the +4 and +6 states are the most dominant with U (VI) prevailing in oxic and acid waters as opposed to U (IV) which predominates in anoxic waters and is poorly soluble and potentially not readily bioavailable (Ragnarsdottir & Charlet, 2000). U generates different species in solution from the uncharged complex UO_2CO_3, the divalent anion complex $UO_2 (CO_3)_2^{2-}$, the four-valent anion complex $UO_2 (CO_3)_3^{4-}$, UO_2OH^+ and inorganic phosphate complexes (Raff & Wilken, 1999). The free uranyl ion (UO_2^{2+}) dominates dissolved U speciation as the toxic chemical species (Markich et al., 2000), with modeling results having shown that low pH favour its formation in aquatic systems (Reithmuller et al., 2000, 2001). Variations in water chemistry parameters may considerably modify U toxicity by altering the speciation, bioavailability, and rate of uptake of U in aquatic organisms such as fish tissues. There is little information relating the speciation of U to its bioavailability in aquatic systems. So more information about U bioavailability is needed to understand U behaviour in terms of its mobility and toxicity in biological systems and to be able to develop models to predict U toxicity based on water quality parameters.

1.1. OBJECTIVES AND HYPOTHESES

In order to improve ecological risk assessment of U, it is necessary to have knowledge about how different water quality parameters influence the bioavailability, uptake and toxicity of U toward aquatic organisms. As a part of the EU STAR development of an acute U biotic ligand model (BLM) for Atlantic salmon (*Salmo salar*), in the EU STAR project, this study is undertaken with objectives to:

- Examine the relationship between U speciation, bioavailability and uptake in f ish as a part of developing a BLM for U;
- Examine the influence of pH on U bioavailability;
- Examine the accumulation of U species on gills and the uptake distribution in fish (liver and kidney);
- Link the uptake in fish to U speciation in water.

Atlantic salmon was selected because it is reported to be economically valuable and sensitive to freshwater pollution (Poléo et al. 1991).

The hypothesis for this study is that:

- Low pH will increase the bioavailability, uptake and toxicity of U in fish.

CHAPTER TWO

2. BACKGROUND

2.1. Ecological Risk Assessment of Metals

When fish are exposed to contaminants in a polluted system they become susceptible, and more often tend to accumulate metals in their tissues. To appreciate how accumulation and distribution to tissues possibly occurs, it is important to have a clear understanding of how metals such as U behave in solution. An assessment of the impact of an element cannot be made based solely on its total concentration because it provides no information concerning its fate in terms of its ability to mobilize and cross biological membranes (bioavailability), or its resultant toxicity (Christie, 2000). Changes in speciation due to differences in water chemistry parameters may dramatically affect the transfer and thus toxicity of a metal. Metal uptake and toxicity have been demonstrated to generally correlate with their free metal ion rather than their total concentration (Campbell, 1995). Metals may be partitioned between the exchangeable fraction (which is considered to be that which is primarily available and immediately for biological uptake), the carbonate fraction, the hydrous metal oxide fraction and the organic/residual fraction. According to Florence (1986), metal speciation analysis involves the fractionation of total metal concentration by physicochemical methods. This is essential in the assessment of the potential biological uptake and the toxicity of metals in a water sample.

2.2. Uranium Speciation in Freshwaters

The aqueous speciation of U is associated with tremendous changes in the presence of organic and inorganic ligands commonly found in natural waters (Fortin et al., 2004). The speciation of U is affected by organic and inorganic ligand concentrations which contribute to U mobility and apparently change its bioavailability. It is assumed that the uranyl ion ($UO_2^{2+)}$ is the most bioavailable species (Moulin et al., 1992). Since the concentration of UO_2^{2+} is dependent upon pH, changes in pH is assumed to significantly alter the bioavailability and toxicity of U to aquatic organisms such as fish (Goulet et al., 2012). Beyond pH 5, hydroxide and carbonate uranyl complexes prevail in neutral and alkaline conditions with hydroxylated forms appearing above pH 6 and carbonated forms at pH higher than 8 (Goulet et al., 2012). In essence, at varying pH different uranyl compounds are formed that is reported to altering the availability of the soluble, mobile and bioavailable UO_2^{2+}. Hence, pH is thought to strongly influence the toxicity of UO_2^{2+} to aquatic organisms such as fish. The presence of

cations and anions may significantly influence U speciation, but also competing with U depending on their concentration and conditions which permit them to prevail. Calcium (Ca) and magnesium (Mg) can contribute to reduce metal uptake and toxicity through competition for surface binding sites (Reithmuller et al., 2001). The presence of Ca at high concentration (40 mg/l) slightly modifies U (VI) toxicity in a relatively narrow pH range 4-5 by competing with H^+ ions for uptake sites (Moulin et al., 1992).

2.3. Biotic Ligand Model (BLM)

The BLM serves as a mechanistic tool integrating the concept of bioavailability into ambient water quality criteria (AWQC) taking into account competition of the free metal ion with other naturally occurring cations together with complexation by abiotic ligands (e.g., dissolved organic carbon, chloride, carbonates, sulphide) for binding with the biotic ligand, the site of toxic action on the organism (Nigoyi & Wood, 2004). The reason for developing a BLM is that it helps to quantitatively relate short-term binding to acute toxicity as well as predict chronic toxicity and thereby generate chronic AWQC (Nigoyi & Wood, 2004). BLM considers the pollutant species as the environmental ligand and the site of accumulation as the biotic ligand, with the gill of fish as the primary uptake route in contact with water (Figure 1). Major cations, such as Ca^{2+} and Mg^{2+} may reduce U uptake through competition with U for uptake sites especially at calcified tissues (e.g. gills), thus, potentially reducing its toxicity (Goulet et al., 2012). In addition, it accounts for the effect of H^+ as a competing ion at the biotic ligand (Di Toro et al., 2005), where H^+ ions compete with UO_2^{2+} ions for binding at the uptake site as proposed by Fortin et al. (2004, 2007). In this respect the much possible effects of UO_2^{2+} at lower pH may be altered as a result of reduced uptake of UO_2^{2+}.

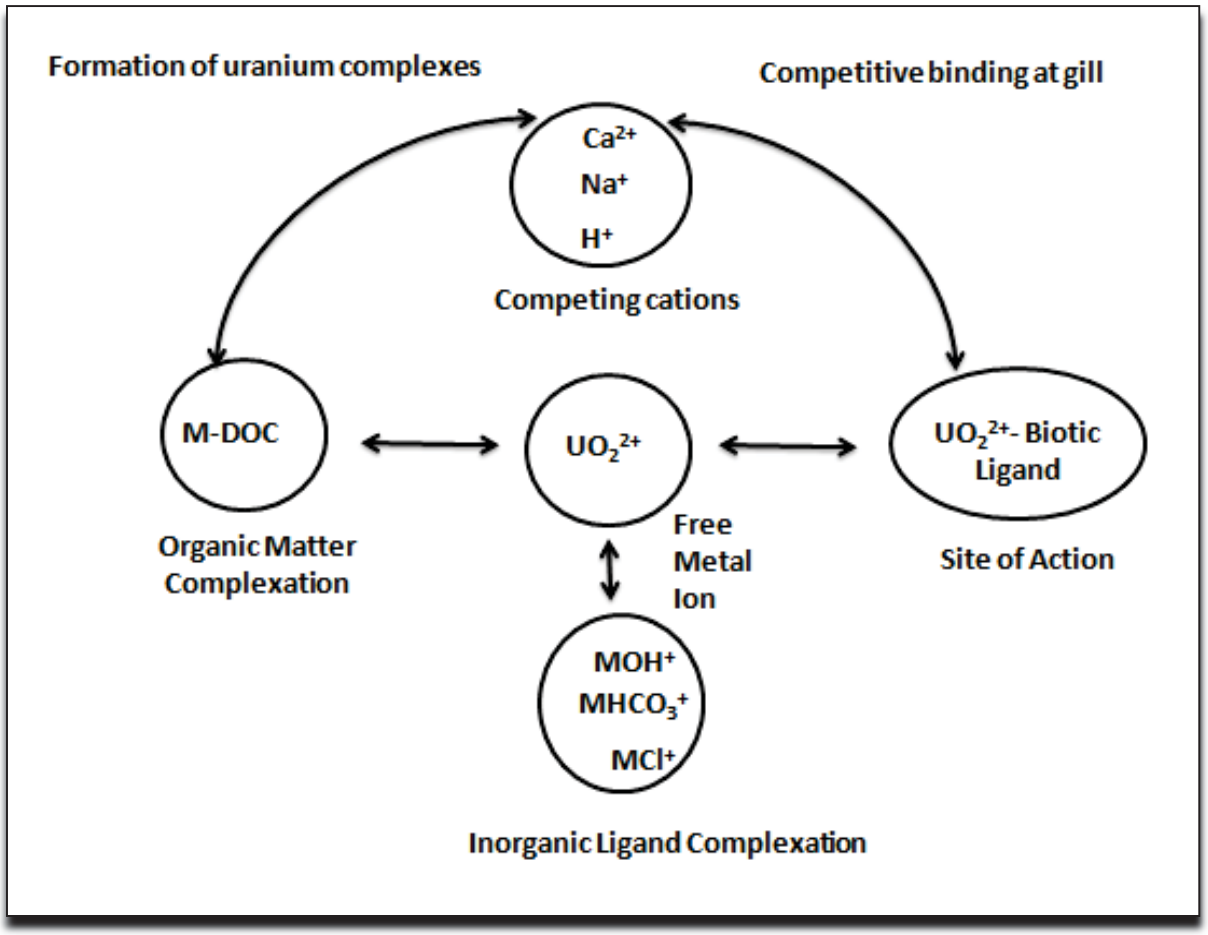

Figure 1. Schematic outline of BLM showing competitive interaction of UO_2^{2+} with other ions in solution and uptake in fish. M represents uranium species (Di Toro et al., 2005).

2.4. Atlantic Salmon (*Salmo Salar*)

The choice of Atlantic salmon is based on its economic importance as a valuable food source and its sensitivity to water pollutants. Atlantic salmon (*Salmo salar*) is an anadromous fish species (OSPAR, 2008), and as such the growth condition requirements tend to vary depending on the developmental stage. The early life stages (i.e. eggs, alevins, fry and parr) of the wondrous anadromous life cycle reside in freshwater (Figure 2). During the smoltification phase, the parr undergoes some physiological changes, making it ready for salt water. The adults only return to freshwater to spawn and the smolt migrates to sea with most of the adult life being spent there. Most importantly the different life stages have different sensitivity to environmental parameters. The smolt stage is the most sensitive stage. However, in this study the parr life cycle stage is focused on.

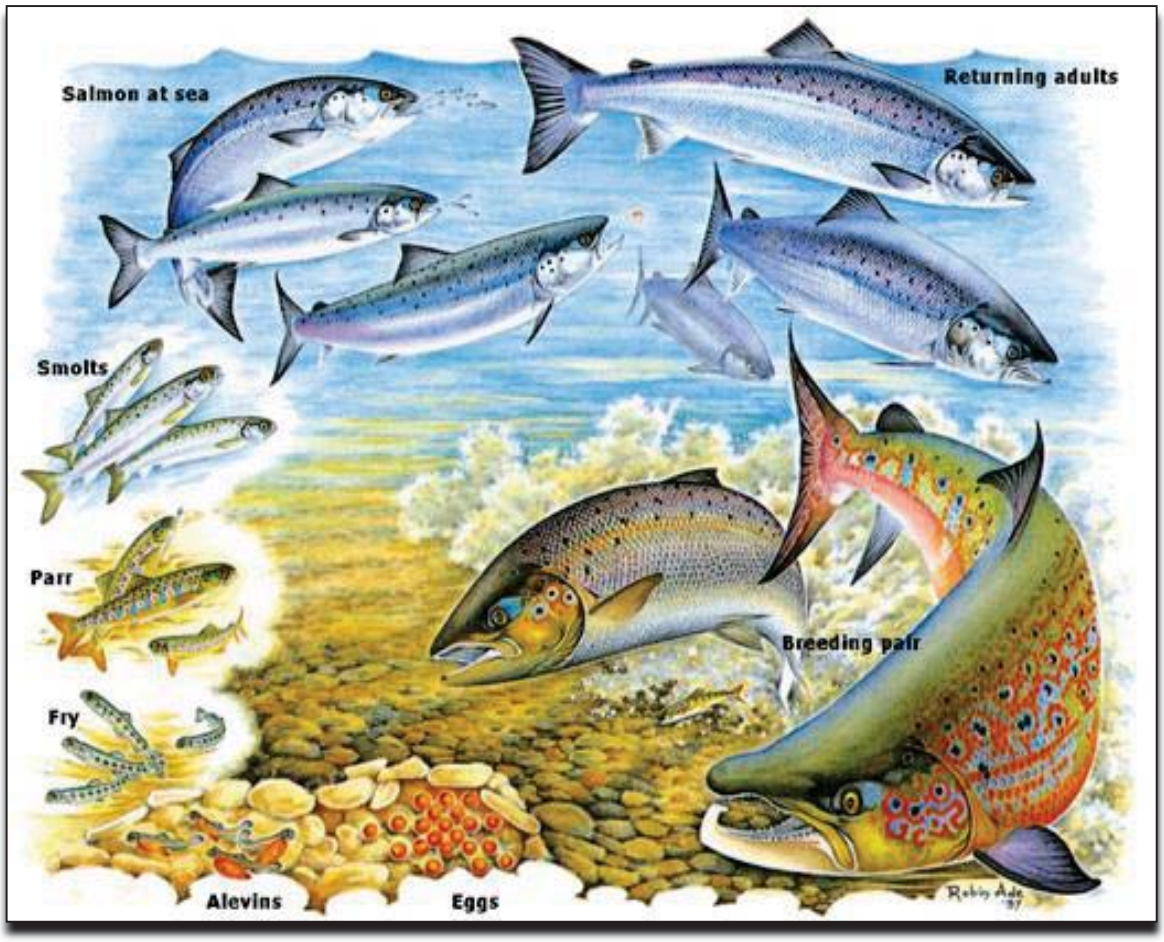

Figure 2. The life cycle of Atlantic salmon (illustration courtesy of the Atlantic Salmon Trust and Robin Ade). Source: http://www.nasco.int/atlanticsalmon.html.

2.5. Uptake, distribution and toxicity of uranium in aquatic organisms

Changes in U concentration may result in significant differences in terms of uptake rate, accumulation and potential effects depending on the nature of exposure (Goulet et al., 2012). In freshwater organisms, chronic and acute exposure concern a wide variety of endpoints (Zeman et al., 2008), which include structural lesions (depletion of plasma chlorides, excessive mucus secretion, corrosion of the gill epithelium, breathing deficiency), functional disturbances (swelling, hyperplasia, hypertrophy and necrosis) and mortality. As a result of acute and chronic scenarios of exposure, some guidelines have been set. The water chemistry of U is very complex, and the specific forms and concentrations of the various U species is strongly determined by water characteristics such as pH, temperature, and hardness (CCME, 2011). While U speciation has been reported to affect its toxicity, at this time there is insufficient information available to quantitatively evaluate the influence of these toxicity modifying factors, and consequently, they were not taken into account during guideline

derivation (CCME, 2011). Canadian water quality guidelines (CWQGs) for U (Tolerable recoverable, Unfiltered) in freshwater is:

Long-Term Exposure: 15 µg/L, and

Short-Term Exposure: 33 µg/L.

In addition, the French Institute of Radiation Protection and Nuclear Safety (IRSN), has proposed the novel approach of 5 µg U/L above background (Beaugelin-Seiller et al., 2009) for soft water. Also, important and worth noting is that, though ubiquitous in natural waters at trace concentrations ranging from 0.02 to 6 µg U/L, higher concentrations may reach 2 mg U/L, reflecting mainly the composition of the surrounding rocks (Bonin & Blanc, 2001).

Exposure time of fish to acute or chronic releases of U is very important in terms of accumulation and distribution to various organs, and key to the scope of the present study. According to Jezierska and Witeska (2006), metal distribution in various organs of fish is also time-related given that at the start of waterborne exposure, metal is absorbed and accumulated at a high rate in the gills, then the level stabilizes when equilibrium of metal uptake and excretion is attained. This seems to allow for accumulation in other internal tissues such as the gastrointestinal tract, liver and kidney, after some time. In aquatic environments, the primary routes of uptake to aquatic organisms include ingestion (dietary exposure) and dermal/gill absorption (Harper & Kantar, 2008). Ahsanullah and Williams (1989), suggest that the primary route of exposure of aquatic organisms is likely from the water as opposed to through food. Available data indicate that the primary sites of U accumulation are gill, bone, gastrointestinal tract, kidney and liver (Waite et al., 1988). Fish gills are important target organs as they are the dominant primary physiological organs directly in contact with the contaminated water. They also give a reflection of the speciation and concentration of the metal in water (Rosseland et al., 1992). Liver (metabolic organ) and kidney (excretory organ) are important because both are sites of significant accumulation after uptake from the water by the gills or through dietary exposure, and provide biologically relevant information because of their vulnerability to U toxicity (Cooley et al., 2000; Cooley & Klaverkamp, 2000). Thus, the tissues would serve as sensitive and reliable biomarkers of U bioavailability and toxicity. Changes in environmental conditions such as the degree of acidification tend to modify the availability and uptake and effect of metals in fish tissues and thereby the following effects. Acidification may have negative effects and pH values near 5 may

represent a lower tolerance limit (Jones, 1964) with alterations of gill surface tissues below 5.2 (Daye & Garside, 1976). Water acidification also affects bioaccumulation of metals by fish, either by altering the distribution of labile and non-labile metal fractions or damaging gill epithelia making them more permeable to labile fractions (Jezierska & Witeska, 2006). In the present study the pH are therefore above 5.3. U having complex speciation chemistry, the ultimate focus in the present study is based on exposure of Atlantic salmon parr to specific acute concentrations of U and varying H^+ concentrations. The idea was to perform two types of experiments using either a range of U concentrations at a specific pH or, a specific concentration and varying pH values to further understand the mechanism of H^+ as a competing ion or the pH influenced U speciation, U absorption on gill, its accumulation and distribution in internal tissues such as the liver and kidney.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. FISH

Juvenile Atlantic salmon parr were obtained from the fish laboratory at the Norwegian University of Life Sciences (NMBU, Ås, Norway).

Acclimation

After transport from the fish lab, fish were allowed a 7-day acclimation period as recommended in the OECD guideline 203 (OECD, 1992) in the same water quality composition (e.g. reference water etc) and at the same temperature (i.e. varying from 8.8 to 9.4 °C) as used for the U exposure. The fish were then fed 1 g food /100 g fish during the acclimation period until three days before the start of the exposure. The feeding period was reduced prior to exposure to ensure that all food was consumed by the parr, thus, allowing control of the dose of U administered to parr. Glass fibre tanks (1000 L) were used for seven (7) days acclimation of Atlantic salmon parr (49 fish) in water with similar ion composition but with variable pH and Cl^- concentrations (Figure 3).

3.2. EXPERIMENTAL TANKS

After acclimation, 7 fish were transferred to each exposure tank lined with transparent plastic bags for 96h acute exposure (Figure 4). To obtain constant oxygen concentrations, one air stone (diameter: 30 mm) continuously aerating the water was placed in each tank connected to an aquarium air pump. A static procedure was applied whereby there was no water replacement of the experimental units following OECD (1992) and Environmental Canada (1990/2007) guidelines. Each of the experimental tanks was randomly assigned seven fish according to the OECD guideline 203 (OECD, 1992). In addition, the experiments were performed devoid of light by covering each experimental unit with a lid. The fish exposures were done in three sessions, first in the end of November 2012, the second in early January 2013 and the last in early December 2013.

Figure 3. Glass fibre tanks with different water qualities used for acclimation of Atlantic salmon parr (Photo: Brice Sone).

Figure 4. Experimental units with different water qualities for randomly assigned fish used during the 96h exposure period (Photo: Brice Sone).

3.3. WATER

Synthetic US EPA very soft water (12 mg/L $NaHCO_3$, 7.5 mg/L $CaSO_4.2H_2O$, 7.5 mg/L $MgSO_4$, 0.5 mg/L KCL, US EPA, 2002) with low ionic strength was selected as the reference water. The choice of reference water was based on sensitive aquatic ecosystems of low ionic strength, but also to represent typical Norwegian river and lake waters. To obtain different water qualities, stock solutions for the reference water were prepared by dissolving the desired amount of $NaHCO_3$ (Assay 100%, VWR International, Haasrode, Belgium), $MgSO_4$ (Reagent Plus ≥ 99.5%; Sigma-Aldrich) and KCl (Pro Analysis min. 99.5%; Merck, Darmstadt Germany) in a plastic can with distilled water. $CaSO_4.2H_2O$ (ACS reagent ≥ 99%, Sigma-Aldrich) was dissolved in a separate plastic container and mixed on a magnetic stirrer according to US EPA (2002). The stock solutions were mixed with deionized water (1:100 and 1:200, respectively) and aerated for 1 to 2 days before it was used for acclimation or distributed to experimental units. The different pH groups were made by adding either HCl (1M) or NaOH (1M) to the reference water. The water were produced at least two days prior to use, mixed and aerated using circulation and aquaria pumps to equilibrate the dissolved CO_2 with the atmosphere. To obtain the different pH levels tested, low pH water were added NaCl partially instead of Na_2CO_3, while high pH water was added NaOH. When the desired pH was reached, all solutions, except the high pH waters were added NaCl (1M) to achieve an equal concentration of Na in all pH groups. During the U exposure period pH was controlled and when needed adjusted every day by adding 0.1 M HCl or NaOH accompanied by NaCl adjustments of the total Na level. The pH, alkalinity, temperature and measured concentrations of major cations and anions in the US EPA very soft water ("reference water") and in the modified water qualities are shown in Table 1.

Table 1. pH, alkalinity, temperature and measured concentrations (mg/L) of major cations and anions in US EPA very soft water ("reference water") and in the modified water qualities.

Group	pH	Alkalinity	Temperature °C	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	CO_3^{2-}	SO_4^{2-}	Cl^{-}
Ref. water	**6.5**	**0.2**	**8.9**	**1.9**	**1.7**	**3.8**	**0.5**	**8.57**	**10.3**	**0.2**
Low pH	5.4	0.01	8.8	1.7	1.5	33.0	0.3	8.57	10.2	55
Low pH	5.7	0.01	8.8	1.7	1.5	33.0	0.3	8.57	10.2	55
Ref. water +Na	6.8	0.2	9.0	1.8	1.6	34.7	0.3	8.57	10.2	46
Medium pH	7.2	0.4	9.1	1.7	1.5	36.8	0.5	8.57	10.2	43
High pH	7.7	1.5	9.1	1.8	1.6	36.8	0.5	8.57	10.2	1.5

3.4. EXPOSURE

As recommended in STAR deliverable 5.1 and by the OECD guideline 203 (1992) one control plus 5 different concentrations of U were included in the experiment to generate a dose response curve. The Canadian Council of Ministers of the Environment (CCME) has performed a comprehensive review of the available literature concerning acute and chronic effects of U on freshwater fish (CCME, 2011). There are no acute (LC_{50}) U toxicity data for Atlantic salmon, but information is available for two fish species belonging to the same family (Salmonidae). Rainbow trout (*Oncorhynchus mykiss*) was found to have a 96-h LC_{50} range of 3800–4200 µg U/L found by Vizon Scitech Inc. (2004) at differing hardness values ranging from 15 to 240 mg/L as $CaCO_3$. The lowest water hardness is approximately the same as for the US EPA very soft water (10.6 mg/L). However, to narrow the scope and stay within the focus of this study as part of the project the effect of pH on bioavailability of 1 mg U/L on various pH levels only were included: pH 5.4, pH 5.7, pH 6.5 "Reference water-Control group", pH 6.8, pH 7.2 and pH 7.7. To simulate freshwater based U contamination, a U stock solution was prepared by dissolving depleted U, 5.3 g uranyl nitrate hexahydrate ($UO_2(NO_3)_2.6\ H_2O$, Sigma Aldrich) per L of ultrapure water (Milli-Q/Millipore, 18MΩcm) in a plastic bottle, giving a concentration of 2.51 g U/L. The stock solution was made one day before use. The volume of the stock solution added to the different pH groups (Table 1) giving final concentrations of U and NO_3 is specified in Table 2.

Table 2. Nominal concentration of U and nitrate in the different pH exposure groups.

Nominal U concentration		Nominal NO_3^- concentration	
mg /L	µM	mg/L	µM
1	**4.2**	**0.5**	**8.4**

The U was added to the water 3 days (72 h) prior to addition of the fish, and during this period the solutions were bubbled with air using aquaria pumps to mix and equilibrate the solution before the start of exposure. The exposure lasted for 96 h and no food was given during the experiment according to OECD guideline 203 (1992).

3.5. SAMPLING

Water quality parameters such as pH, dissolved oxygen, conductivity and temperature were continuously logged every 30 min in two selected control groups. For the different U treatments the water quality parameters were measured before and after experimental period,

at 0, 24, 48, 72 and 96 h of exposure. Water samples were taken for analysis of U concentrations immediately before the fish was added (0 h) and after 96 h or at the time 100% mortality was observed. In sampling fish, viability was used as biological endpoint. The test was accepted if the mortality of the control fish was less than 10% during the 96h exposure following recommended guidelines (Environment Canada 1990/2007; OECD, 1992). Viability was registered after 24, 48, 72, and 96 h of exposure according to the OECD (1992) and Environment Canada (1990/2007) guidelines. Viable fish was sampled after 96h for measurement of accumulation of U on the gills, in liver and kidney.

3.5.1. Size and Charge Fractionation of Water

To obtain information on the speciation of U in water, at site size and charge fractionation of subset sampled water from the different experimental units representative of different water qualities was performed. The water samples were taken for analysis of total U concentrations and other elements immediately before the fish was added (0 h) and after 96 h to provide quality control. Prefiltration through various size membranes was performed with subset sampled water fractionated to exclude specific molecular weight cutoff components. The purpose of this was to understand the mechanism of U speciation in relation to the size of various components in the water. To obtain information if U was dissolved or present as particles, filtration (PALL® 0.45 μm high capacity in-line membrane filter) was applied. To distinguish between colloidal and low molecular mass (LMM species) form of U, ultrafiltration (Amicon PPall hollow fiber, nominal cut off 10 kDa and 3 kDa) was applied at controlled pressure according to Pall guidelines (Teien et al., 2005). To obtain information of negatively charged U species, a charge fractionation system with ion exchange chromatography (anion: AG_{1x8} resin) was used in combination with ultrafiltration. For changes in speciation of U in water in relation to H^+ ion concentration in water, the following groups was fractionated: pH 5.1, pH 5.4, pH 5.7 and pH 6.8 at 1.0 mg U/L.

3.5.2. Blood and Tissue Sampling of Fish

After exposure, fish were killed by a blow on the head, placed on the right side with head toward the left and blood sampled from the ventral aspect of the tail (Rosseland et al., 2001). Blood was then analyzed by I-Stat for levels of plasma glucose. Table 9 shows the mean blood glucose levels of fish exposed to different water qualities of varying pH. Fish tissues (gill, liver and kidney) of interest were sampled following the procedures in the EMERGE protocol (Rosseland et al., 2001) using scalpels and slicers. The secondary gill arch

(biomarker for metal contamination) on the right side was extracted by means of a scalp and scissor before determination of the total U concentrations from all fish at all U concentrations and water qualities. Whole kidney and half liver was sampled from all fish and stored at -20 ºC and -80 ºC, respectively until further processing for determination of U concentrations.

3.6. ANALYSIS OF WATER AND FISH SAMPLES

3.6.1. General Water Parameters

Based on the subset water samples collected, other water quality parameters; dissolved organic carbon (DOC), total organic carbon (TOC), major anions (Cl^-, SO_4^{2-}, NO^{3-}) and major cations (Na^+, K^+, Mg^{2+}, Ca^{2+}) were determined from all groups at the start and at the end of the exposure. Major cations (Mg^{2+}, Ca^{2+}, Na^+, K^+) were determined in 5% HNO_3 acidified water samples using ICP-MS (8800 ICP-MS Triple Quad, Agilent Technologies). Non acidified filtered samples (0.45µm syringe filter Millexwater®-AA, MF Millipore membrane, Millipore Ireland) were analyzed for major anions using Iachat IC5000 ion chromatography. Sub samples for TOC and DOC were not acidified. The water samples for determination of DOC were filtered through a 0.45 µm syringe filter (Millexwater®-AA, MF Millipore membrane, Millipore Ireland). TOC and DOC were determined using a total organic analyzer (Shimadzu TOC cpn, Kyoto, Japan) at IMV.

3.6.2. Uranium in Water

Unfiltered water samples and water fractions were acidified with 5% (v/v) ultrapure nitric acid (HNO_3) prior to the determination of U concentrations using inductively coupled plasma mass spectrometry (8800 ICP-MS Triple Quad, Agilent Technologies). The accuracy of the U measurements was checked by analyzing samples of a reference (1643H in-house) water standard. Different physicochemical forms (i.e., particles, colloids and LMM species) can influence the mobility and bioavailability of metals (Salbu, 2007), so it is important to define them. Of the different physicochemical forms of metals, particles are the entities with diameters > 0.45 µm, while colloids or pseudocolloids are defined as localized heterogeneities ranging in size from 1 nm to 0.45 µm, and the LMM species < 1 nm are believed to be mobile and bioavailable (Salbu, 2007). Thus, the following U species (dissolved and colloidal species) fractionated on the basis of physicochemical characteristics were included in the analyses:

U_{tot}: uranium in unfiltered water;

$U_{particulate}$: derived by difference, U in unfiltered water subtracting U in 0.45 µm filtered water;

i. e. $U_{particulate} = U_{tot} - U_{0.45µm.}$

$U_{colloids}$: derived by difference, U in 0.45 µm filtered water subtracting U in ultrafiltered water;

i. e. $U_{colloids} = U_{0.45µm} - U_{LMM.}$

U_{LMM}: U in ultrafiltered water (cutoff 10 kDa and 3kDa).

U_{anions}: negatively charged U, filtered (cutoff 10 kDa) and retained in the anion exchanger (AG_{1x8}).

3.6.3. Uranium in Fish Tissues

After storage (-20 °C) gill samples were freeze dried and weighed. Dried samples were added ultrapure HNO_3 (65%), ultrapure water (Milli-Q type 3 water, 18 MΩ cm) and internal standard (^{115}In) prior to digestion at increasing temperatures (up to 250 °C) and pressure (up to 160 bar) for 2 h using an ultraclave (Mile-stone, Leutkirch, Germany). After digestion, samples were diluted with MQ Type 3 water (10% acidic solution) before the U concentration was determined using ICP-MS. To obtain information of accuracy, samples of Standard Reference Material (1570a for Trace Elements in Spinach Leaves) were digested and analyzed at the same time as the samples in separate batches on ultraclave. Certified and measured values for U are shown (Table 3). For data handling, detection limits for U concentrations in sampled fish tissues were obtained as follows:

Calculations performed;

Mean (±SD) of blank samples for control.

Limit of Detection (LOD) = 3 x Standard Deviation of blanks = 3(SD).

Limit of Quantification (LOQ) = 10 x Standard Deviation of blanks = 10(SD).

Average weight of gill samples = 0.014 g
LOQ for gill digest (µg/g) = **[(**LOQ in µg/l**) / (**100 x Average weight of sample in grams**)]**
= **[(**0.15 µg/l**) / (**100 x 0.014 g**)]**
= 0.11 µg/g gill
So, quantification limit was approximately 0.11 µg/g gill taking into account that the average weight of gill was 0.011 g.

Table 3. Certified and measured values for U in fish tissues and percentage (%) differences between certified and measured values.

Reference Material	Certified value µg/g	Measured value µg/g	% difference between certified and measured values
1570a	0.155±0.023*	0.162	9.0
1643H (water)	1 (0.93 - 1.003)	0.98 - 1.03	0.0

(*) - Reference value, N/B: 1643H value is in µg/l.

3.7. STATISTICAL ANALYSIS

Different statistical tools (Minitab 16 and Excel 2007) were used for data handling. Data for separate tissues- gill, liver and kidney from different exposed pH groups were tested for normality (Appendix 3) with the assumption of homogeneity in variance (constant variance) at α=0.01 using MINITAB 16 Statistical package. For normal data, Bartlett's Test p-value was used and for non-normal data Levene's Test p-value, respectively to test the assumption of homogeneity in variance. Where the p-value was less than α=0.01, the null hypothesis of constant variance was rejected. Then, one-way ANOVA was used to determine the differences in sample mean variations. The ANOVA test does not tell where we have the difference. So, to investigate for differences between treatments, Turkey's *Post hoc* Test for multiple comparisons of mean concentrations of U accumulated in fish tissues from different pH groups was used. Turkey's *Post hoc* Test gave information on where the significant difference in sample means exist. Taking into consideration the experimental setup, it was important to consider the possibility of pseudo-replication. There is pseudo-replication in the data, but, for practical experimental reasons it was not taken into account given that it will need at least 4 representative exposure tanks for each treatment group in order to get true representative means for concentrations of U accumulated in tissues. So, on this basis, subsequent discussions pertaining to analysis will not be over stated.

CHAPTER FOUR

4. RESULTS AND DISCUSSION

4.1. DETECTION LIMITS

The limit of detection (LOD) and limit of quantification (LOQ) for U in water were 0.04 µg/l and 0.15 µg/l, respectively. Based on the digestion of samples in several batches with three blanks as control and average weight of tissues, the detection LOD and LOQ were obtained as presented in table 4. The accuracy of the measurements was good and within 91 % of standard reference materials (e.g., 1570a-Trace Elements in Spinach Leaves) for traceability to control sample values and the accuracy of the ICP-MS measurements.

Table 4. Tissue Digest ICP-MS Detection Limits.

Tissue Digest	Detection Limit	Units (µg/l)	U µg/g tissue
Water	LOD	0.04	
	LOQ	0.15	
Gill	LOD		0.03
	LOQ		0.11
Liver	LOD		0.004
	LOQ		0.015
Kidney	LOD		0.005
	LOQ		0.02

4.2. WATER CHEMISTRY

4.2.1. General Water Quality

Table 1, showed the mean values for general water quality parameters (alkalinity, temperature and ion composition) recorded during the experimental period from the different pH exposure groups. The results suggest that they were no major differences in measured water quality parameters. So, it can be assumed that the exposure conditions were similar at different pH levels with only H^+ and Cl^- having varied during the experimental period. This suggests that any measured U concentrations in fish tissues was influenced by varying pH as all other parameters were held constant. Measured alkalinity giving indirect information about carbonate concentration in water due to increased solubility of CO_2 as carbonate with

increasing pH was important in figuring out how buffered the pH groups were against sudden changes in pH, so as to protect the exposed fish.

4.2.2. Uranium Speciation in Water

Results showed that 93-99 % of the 1.0 mg/L total U was found in the dissolved (< 0.45 µm) fraction whereas the remaining (< 7 %) was found in the particulate form. Of the dissolved fraction, about 90-97 % was present as LMM species (cutoff < 10 kDa). Meanwhile, at pH 5.5 with varying U concentrations, more than 95 % of the total U was found in the dissolved (< 0.45 µm) fraction with 87-98 % of the U present as LMM species (cutoff < 10 kDa). There seemed to be no significant changes in the speciation of U even when changing the concentration at the same pH. It can be assumed that, the U species in the water was considered to be highly mobile and potentially bioavailable. High levels of LMM species suggest that there were insignificant concentrations of particles and colloids.

Table 5. Information on Uranium (U) speciation in water (mean, n=3) based on size and charge fractionation.

pH	Nominal U concentration (mg/L)	U total (mg/L)	<0.45µm (mg/L)	< 10kDa (mg/L)	< 10kDa (Ag_{1x8}) (mg/L)
5.1	1.0	n.a	0.97	0.96	n.a
5.4	1.0	0.97	0.91	0.87	n.a
5.5	0.5	0.46	n.a	0.44	n.a
5.5	1.0	0.96	0.91	0.87	n.a
5.5	2.0	1.92	1.92	1.83	n.a
5.7	1.0	0.98	0.92	0.82	0.03
6.8	1.0	n.a	0.93	0.90	n.a

% large colloids = [0.45µm - 10kDa]/ U-total and, n. a. - not analyzed.
% small colloids = [10kDa - 3kDa] / U-total.

4.3. MEASURED URANIUM CONCENTRATION IN FISH TISSUES

Below, tables and figures showed nominal and measured U concentrations in water. Comparisons between pH water groups were made on the basis of U concentrations measured in tissues from fish exposure at 1.0 mg U/L in water. Subsequently, comparisons were made between all pH water groups at nominal 1.0 mg U/L in order to have a general idea of possible accumulation in tissues at much lower pH and lower U concentrations in water.

4.3.1. Uranium Concentration (mg/kg dw) in Gill

Based on measured U in tissues of fish exposed at 1.0 mg U/L in water, maximum concentration of U determined in gills were found at pH 5.4 with a mean concentration of 58.6 mg/kg dw, while the minimum concentration was found at pH 7.7 with a mean U concentration of 3.2 mg/kg dw (Table 6). Results also showed that gills accumulated the highest levels of waterborne U from pH groups 5.4 and 5.7 (Figure 5) with at least a factor of 3 and 2 times compared to accumulated levels from pH 6.5, respectively. In addition, gills accumulated U from reference water group pH 6.5 with at least a factor of 1.5 and 5 times higher levels compared to accumulated levels from pH water groups 7.2 and 7.7, respectively. Figure 5, shows U concentration in gills according to pH. It indicates a risk of more U uptake and accumulation on the gill at lower pH, with increasing labile and LMM U species

Table 6. Mean (± SD) values of U accumulated in gill (mg/kg dw), with minimum-maximum values in parenthesis, and sizes of Atlantic salmon (Salmo salar) parr after 96h exposure to 1.0 mg U/L nominal concentration in different pH water groups. (n = fish sample size)

Nominal U	Measured U	Measured pH	n	Mean ± SD	(min-max)	Length (cm)	Weight (g)
1.0	0.8	5.4	7	58.6±11.5	(40-69)	11.7±1.3	13.1±4.2
1.0	0.8	5.7	6	42.3±9.2	(27-53)	12.2±1.6	14.9±4.5
1.0	1.0	6.5	7	20.3±3.9	(14-26)	11.7±0.8	13.1±2.9
1.0	1.0	6.8	7	16.4±2.8	(13-21)	12.5±0.6	15.7±2.5
1.0	1.0	7.2	7	10.9±4.6	(6.3-20)	12.1±0.9	14.9±3.7
1.0	1.0	7.7	7	3.2±0.8	(1.9-4.3)	12.7±1.0	16.3±4.4

Nominal 1.0 mg U/L based comparisons showed that there was a strong negative significant (r^2 = 85.7%) relationship between gill U concentration and pH (Figure 6). The difference in pH in terms of gill U accumulation was tested statistically using Turkey's ANOVA *Post hoc* tests for pair wise comparison at nominal 1.0 mg U/L between pH water groups (See: Appendix 6.4). The test showed significant (p-value = 0.00 < 0.01, r^2 = 89.7%) differences in mean U concentration in gill. pH groups 5.4 and 5.7 were significantly different from each other and from pH 6.5 - 7.7, while between pH 6.5 - 7.2 there were no significant differences. The following trend was observed: pH 5.4 > 5.7 > 6.5=6.8 > 7.2=7.7 (See: Appendix 6.4). This suggests that a decrease in pH results in increased U accumulation in the gills, even taken into account that the concentration in water at the lowest pH is lower than the nominal

values. This demonstrates that U is more bioavailable at low pH than at high and that pH is an important variable for explaining the concentrations of U in the fish gills.

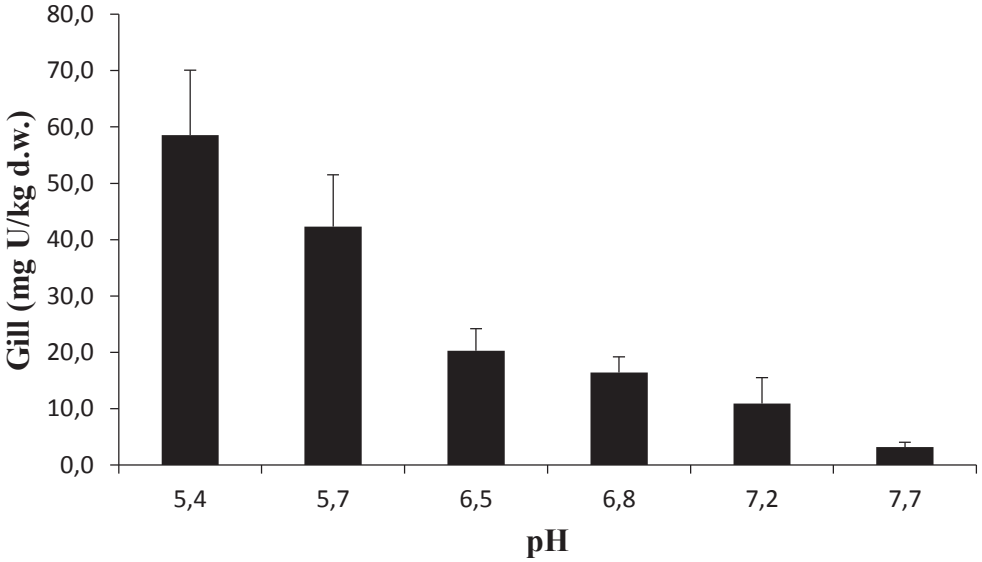

Figure 5. Gill U concentration (mg U/kg dw) in Atlantic Salmon parr as a function of pH.

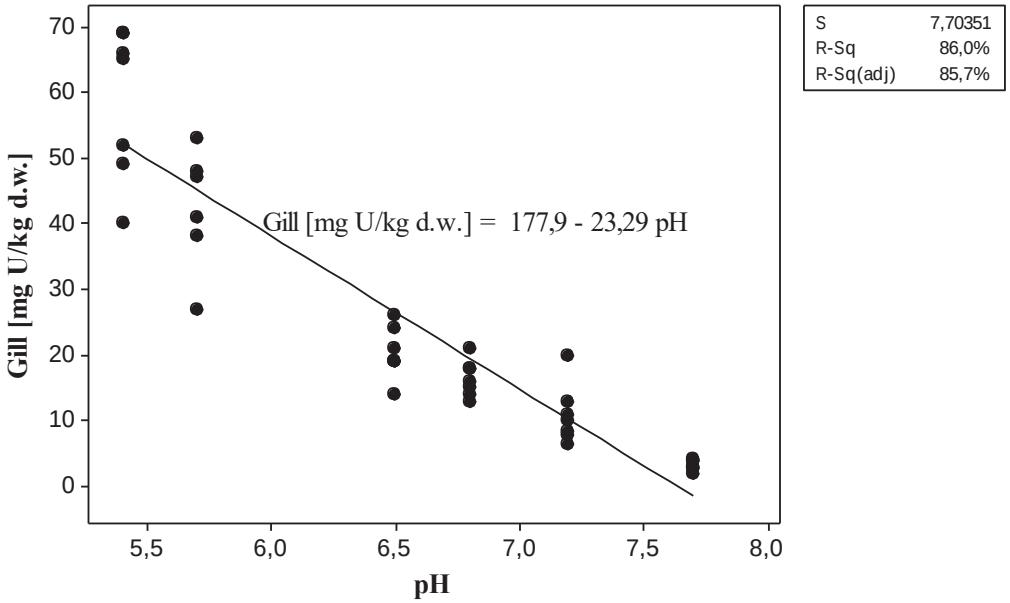

Figure 6. The U concentrations (mg U/kg dw) in gill as a function of pH following 96 h exposure of Atlantic salmon parr at 1 mg U/L. n= 7 fish from the same experimental unit (pseudoreplicates).

4.3.2. Uranium Concentration (mg/kg dw) in Liver

Maximum concentration of U determined in liver were found at pH below 6 with a mean concentration of 1.12 mg/kg dw at pH 5.4, while the minimum concentrations were found at pH 7.2 and 7.7 (the highest pH value tested) with a mean concentration of 0.13 mg/kg dw (Table 7). Results also showed that at pH 5.4-5.7, liver accumulated U to factors of 9 times higher compared to U concentrations at pH 7.2 and pH 7.7, respectively.

Table 7. Mean (± SD) values of U accumulated in liver (mg/kg dw), with minimum-maximum values in parenthesis, and sizes of Atlantic salmon (Salmo salar) parr after 96h exposure to 1.0 mg U/L nominal concentration in different pH water groups. n = sample size.

Nominal U	Measured U	Measured pH	n	Mean ± SD	(min-max)
1.0	0.8	5.4	7	1.12±0.44	(0.65-1.67)
1.0	0.8	5.7	7	1.17±0.45	(0.47-1.89)
1.0	1.0	6.5	7	0.55±0.30	(0.21-0.96)
1.0	1.0	7.2	7	0.13±0.16	(0.02-0.50)
1.0	1.0	7.7	7	0.13±0.07	(0.07±0.27)

Figure 7 shows the U concentration (mg U/kg dw) in liver as a function of pH in 1.0 mg U/L in water. Comparing all pH water groups at nominal 1.0 mg U/L, significant differences was obtained (p-value = 0.00 < 0.01, r^2=65.9%; See: Appendix 7.4) in mean U concentration in liver. The following trend was observed: pH {5.4 = 5.7} > {6.5=7.2=7.7}.

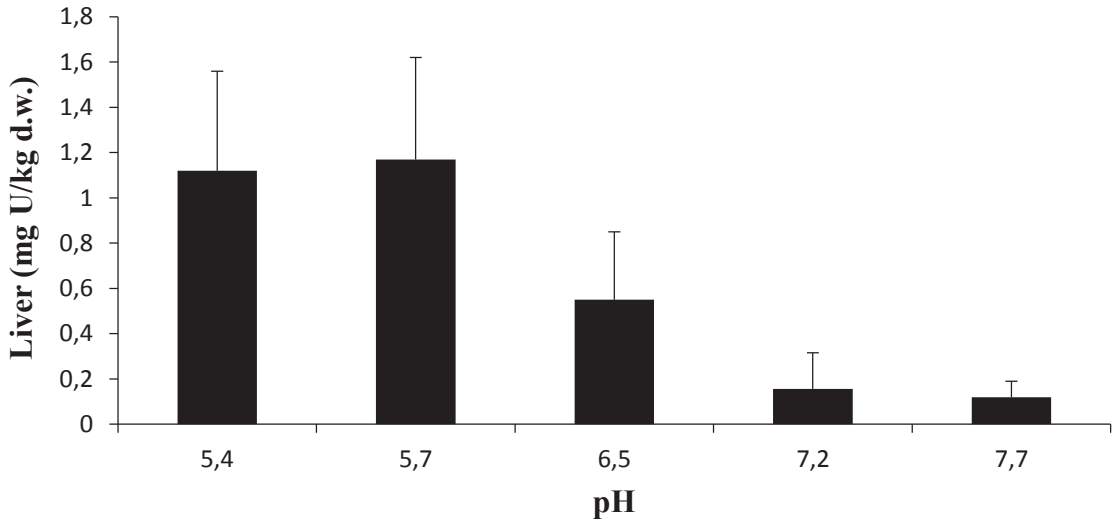

Figure 7. Liver U concentration (mg U/kg dw) in Atlantic Salmon parr as a function of pH.

The linear regression line (Figure 8), showed a negative significant relationship (r^2=53.1 %) between U concentration in liver and pH. So, U accumulation in liver seems to follow the

same trend as in gill with low pH leading to significantly higher uptake, distribution and accumulation, although the trend was not as strong as for the gills. In addition, it seems more gill U is linked to increased distribution through blood and accumulation at secondary target organs such as the liver in this case.

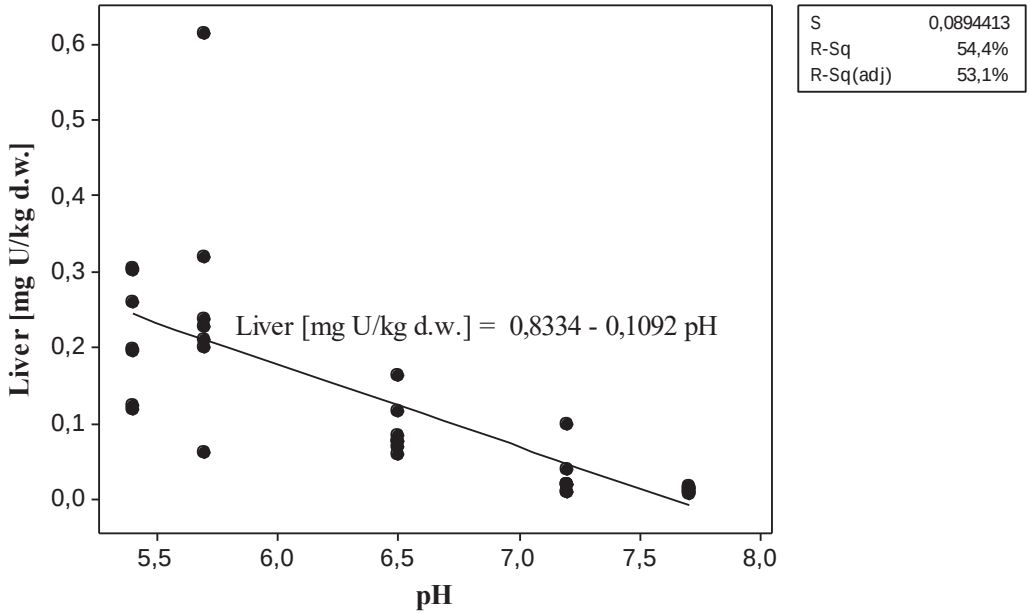

Figure 8. The U concentrations (mg U/kg dw) in liver as a function of pH following 96 h exposure of Atlantic salmon parr at 1 mg U/L. n= 7 fish from the same experimental unit (pseudoreplicates).

4.3.3. Uranium Concentration (mg/kg dw) in Kidney

Table 8. Mean (± SD) values of U accumulated in kidney (mg/kg dw), with minimum-maximum values in parenthesis, and sizes of Atlantic salmon (Salmo salar) parr after 96h exposure to 1.0 mg U/L nominal concentration in different pH water groups.

Nominal U	Measured U	Measured pH	Sample size (n)	Mean ± SD	(min-max)
1.0	0.4	5.4	7	0.66±0.44	(0.22-1.40)
1.0	0.4	5.7	7	0.80±0.46	(0.09-1.31)
1.0	1.0	6.5	7	0.24±0.11	(0.11-0.44)
1.0	1.0	7.7	7	0.014±0.01	(0.01-0.02)

The highest mean U concentration of 0.7-0.8 mg/kg dw determined in kidney was found at pH 5.4-5.7 (Table 8), with highest levels of waterborne U being 70-80 times the levels determined in the group exposed at pH 7.7, respectively. The difference in pH in terms of

kidney U accumulation was tested statistically using Turkey's ANOVA *Post hoc* tests for pair wise comparison at nominal 1.0 mg U/L between pH waters (See: Appendix 8.4). The test showed significant (p-value = 0.00 < 0.01, r^2 = 46.9%) differences in mean U concentration in kidney. pH water groups 5.4-5.7 were significantly different from pH water 7.7. The regression line in figure 10 shows a significant negative relationship between U concentration in kidney and pH although the goodness of fit was rather low (r^2=43.8%). There is a large variation in U concentration in the kidney of fish exposed in water at pH 5.4 and 5.7.

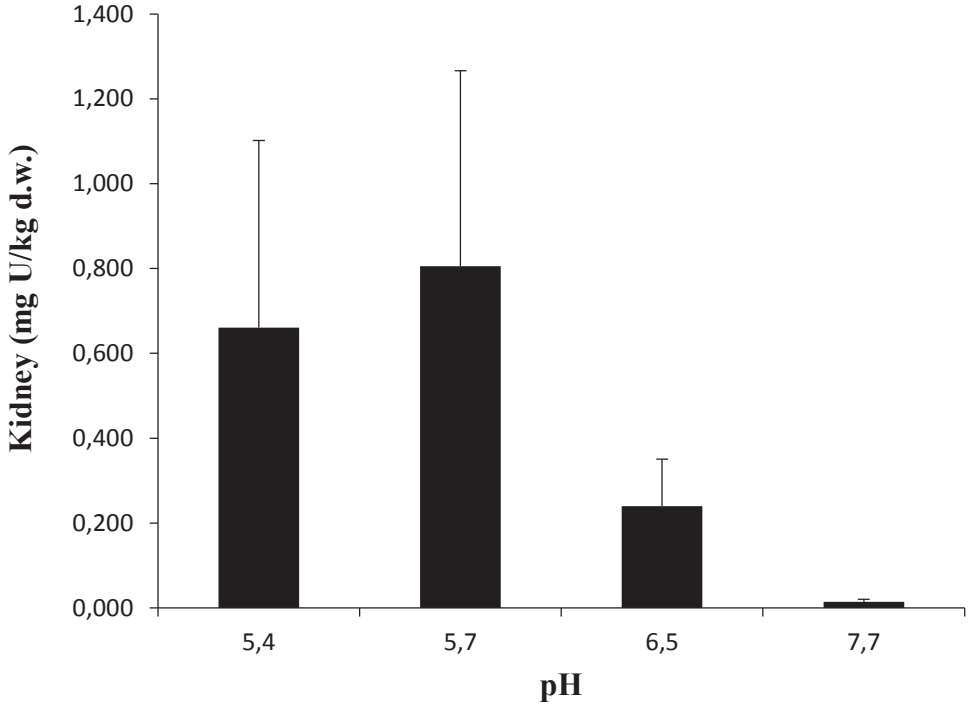

Figure 9. Kidney U concentration (mg U/kg dw) in Atlantic Salmon parr as a function of pH.

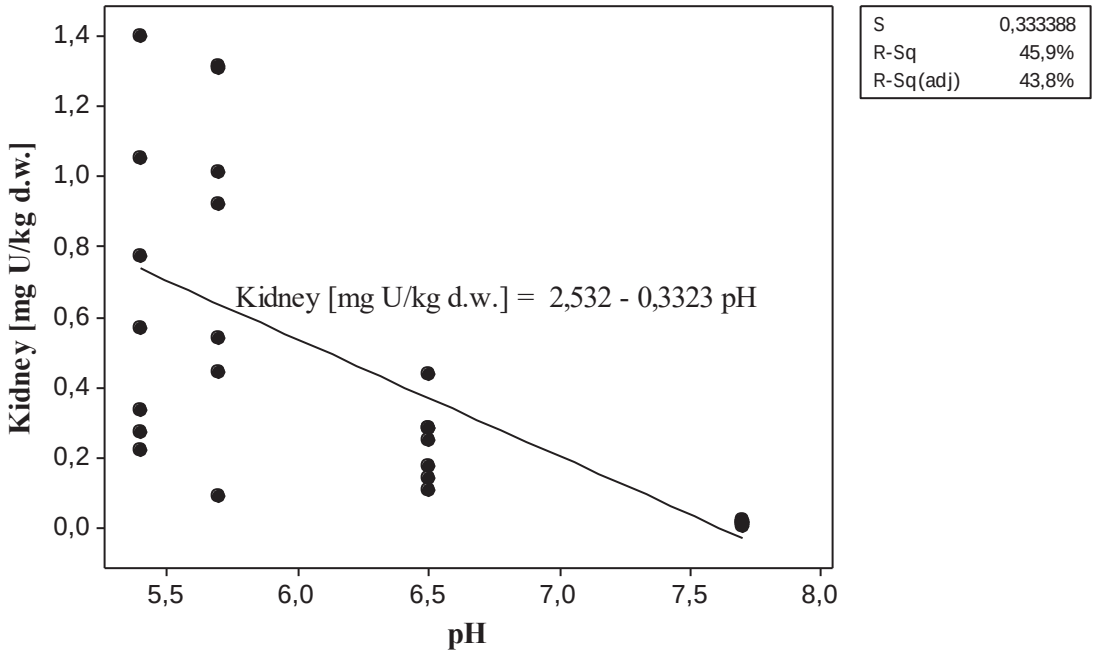

Figure 10. The U concentrations (mg U/kg dw) in kidney as a function of pH following 96 h exposure of Atlantic salmon parr at 1 mg U/L. n=7 fish from the same experimental unit (pseudoreplicates).

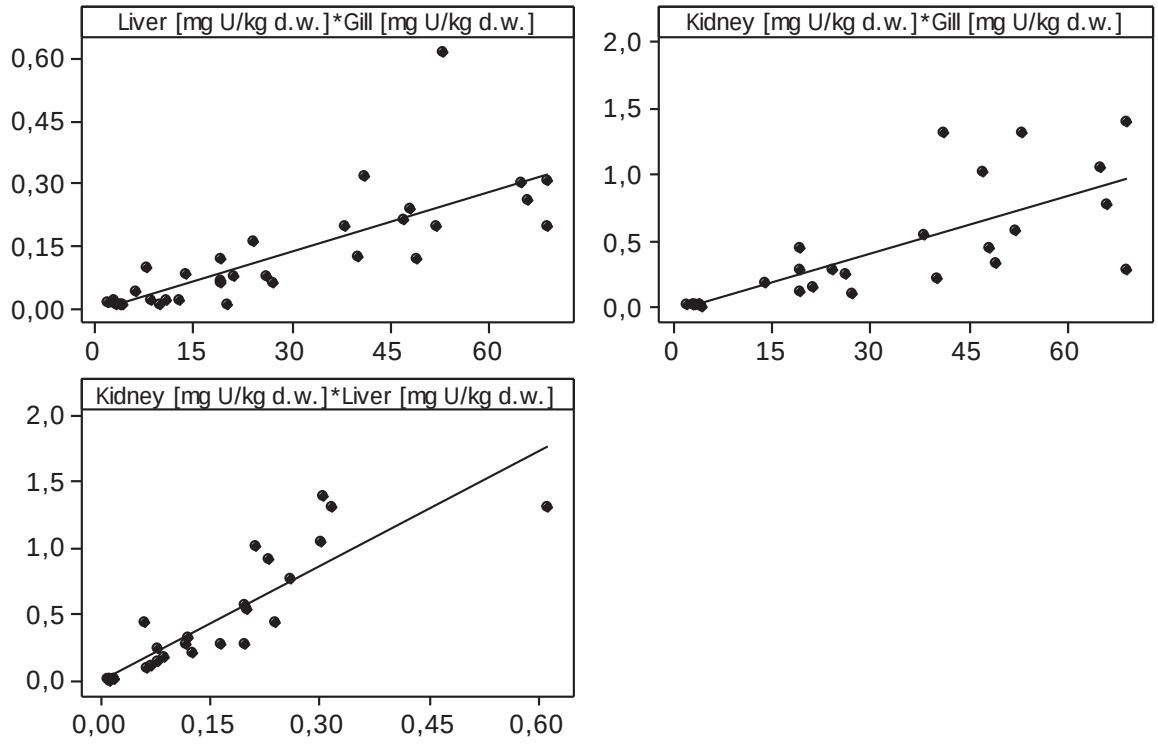

Figure 11. Correlation between U concentrations (mg/kg dw) in tissues.

Based on the above results, the following trends for tissue U accumulation were observed for U concentrations of 1.0 mg U/L in water.

Trend in accumulation for all tissues can be calculated based on correlation between gills and liver, and gills and kidney, respectively (Figure 11): gill > liver > kidney.

The general trend of pH water group contribution and influence on tissue U accumulation in terms of significant differences, were:

Gill: pH {5.4 > 5.7} > pH {6.5 = 6.8 = 7.2}

pH {5.4 > 5.7} > pH 7.7

pH {pH 6.5-7.2} > pH 7.7

Liver: pH {5.4 = 5.7} > pH {7.2 = 7.7}

Kidney: pH {5.4 = 5.7} > pH 7.7

The high U concentrations in gills observed in all the pH water groups with a mean concentration factor of at least 3 and 4 times the highest mean U concentrations in liver and kidney, respectively, may be attributed to direct contact of the gills with waterborne U. The presence of U in the gills in significantly large amounts reflects the sensitivity of the gills to changes in the water pH, thus affecting the water quality to which the fish was directly

exposed. It is understood that the waterborne U is mainly taken up by the gills since the fish are not fed during exposure and fish do not drink water in freshwater systems. The reasons for differences in gill accumulation of U with respect to different pH groups could be a result of the U speciation which affects its availability to the exposed fish. Of importance, is that the presence of H^+ ions can as well inhibit binding and uptake of a metal (Croteau et al., 1998) through competitive interaction for active binding sites. However, the toxicity of U depends on solubility and complexation with organic and inorganic ligands even though qualitatively, its complexation leads to a decrease in its bioavailability (Fortin et al., 2004). Based on thermodynamic data, UO_2^{2+} ion forms different complexes with H^+ ion at pH values less than 6 (Langmuir, 1978) and carbonato-complexes predominating at much higher pH levels. These uranyl hydroxide species and free UO_2^{2+} ions may prove to be more toxic because they may be more biologically active than the carbonate complexes which predominate at higher pH levels (Poston et al., 1983). In accordance with the afore mentioned, results obtained showing much higher U concentrations in tissues at low pH (5.4 and 5.7) and lower U concentration (< 1.0 mg U/L), seem to be in good agreement with the fact that acidification may further enhance U speciation, bioavailability and exacerbate U toxicity in fish. It is also possible that the more active U species prevail at lower pH, as observed within this study. Hence, U toxicity could be highly dependent on pH as opposed to hardness (i.e., to competition with Ca^{2+} and Mg^{2+}) and U concentration. A decrease in pH due to H^+ addition promotes the solubility and mobilization of U, thus increasing the pool of dissolved and bioavailable U and enhancing gill uptake and accumulation. The solubility of UO_2^{2+} is very low at high pH as U is transferred to U-carbonates. Thus, the reduced gill concentration could be a result of reduced concentration of UO_2^{2+} in water. The resulting differences in gill uptake and bioaccumulation of U may result in effects on many diverse endpoints given that the gills participate in many important physiological functions such as respiration, osmoregulation and excretion.

Analogies based on significant differences obtained from statistical tests among pH groups for determined U concentrations in tissues, suggest the following:

- U appears to be most labile and bioavailable at lower pH values in waters (increased H^+ ion concentrations). As a result, low pH may induce more free UO_2^{2+} ions and other biologically active species in solution.

- U is most gill reactive at low pH, such as pH 5.4 and pH 5.7 with observed significant differences when compared to higher pH waters, hence there is a need to identify effects of smaller changes in the H^+ ion concentrations in low conductivity waters.

- Unit differences in the H^+ ion concentrations in water, could significantly alter the U speciation and bioavailability, posing significant risk in terms of physiological effects that could be harmful to fish health in due course even at naturally low U concentrations.

In addition, the presence of U concentrations in liver and kidney indicate that the gill not only is a site of uptake and bioaccumulation of U, but, also a potential pathway for distribution and accumulation of waterborne U to secondary (internal) target organs. Worth noting is that as the U concentration increases in the gill, there are high chances of measuring significant amounts in the liver and kidney as well (Tables 6-8). Hence, it appears that low pH water groups have a greater potential to produce and release readily labile and bioavailable U species with a high gill reactive tendency. These bioavailable U species appear to posses the ability of not only binding to gills but being able to pass across the gill surface membrane into the blood and interact with internal tissues such as the liver. This line of thought is in accordance with Song et al. (2012), who substantiate that waterborne U may be taken up and accumulate in the liver as one of the main target organs in Atlantic salmon. In addition, according to Handy (1992), bioavailable and absorbed metal species are redistributed from active uptake sites through the blood and accumulate at other target organs distant from the point of entry resulting in systemic effects. Metals differ in their ability to accumulate at specific uptake sites, as such, though specific metals may target specific tissues such as bone, spleen, kidney, muscle and intestines. Thus, the pattern of distribution in tissues may reflect the route of metal uptake in fish. For example, continuous interaction of waterborne U with the gills can be a valuable indicator of acute lethal exposure as referenced for Cu (van Hoof & van San, 1981) due to accumulation, but dietary uptake might be different. Tissue localization studies have shown that fish liver tissue generally accumulates highest concentrations of trace metals (Bendell-Young et al., 1986; Ewers & Schlipkoter, 1991). The liver is the major producer of the metal binding protein metallothionein (Kalay & Canli, 2000) and also acts as the organ for storage and detoxification of contaminants. On the contrary, this study seems to indicate otherwise, given that U levels in the liver are very low compared to levels accumulated in the kidney. As U source is in the water, it is expected that the gill concentration should be higher than liver, as also demonstrated in Song et al. (2012). The

liver accumulates slightly higher levels than the kidney (Table 7 and 8). Also, the behaviour of a toxicant on surfaces or within an organism it encounters can be modified. For example Zn, Cd, Co, Pb, and Sr mimic calcium on the calcified tissues of fish by competing for active binding sites on calcium rich bone and filaments on the gills arch (Bury et al., 2003). Within the fish tissue itself, U tends to accumulate in mineralized tissue, such as bone and scales, and to a lesser extent in the kidney (Cooley and Klaverkamp, 2000). Albeit, at certain pH, high U levels persist in the liver, this may not necessarily result in toxic effects. This is possible as a result of homeostatic control through which levels in liver can be regulated by metabolic processes. It is also possible that levels in liver may be poorly regulated leading to increased resident times in tissues resulting in adverse acute and chronic effects. Though U concentrations in kidney are markedly low compared to the gills and liver, the kidney just like the liver is susceptible to significant U accumulation at low pH. This indicates that the kidney is a target organ for U accumulation and toxicity and also a potential pathway for U detoxification or excretion.

4.4. Stress Response

Blood physiology after 96hrs exposure of fish (n=42) was used as indicator of fish response to stress to the different pH water groups. The results in table 9, shows that pH water groups 5.4-5.7 had the highest mean blood glucose levels of 8.7 mmol/l and 5.7 mmol/l, respectively. The lowest mean blood glucose level was 2.2 mmol/l measured at pH water 7.2.

Table 9. Mean (±SD) blood glucose levels (mmol/l) of fish after exposure to different pH water groups containing 1.0 mg U/L nominal concentration. n = sample size of fish.

Nominal U	Measured U	Measured pH	Sample Size (n)	Mean (±SD)
1.0	0.8	5.4	7	8.7±2.9
1.0	0.8	5.7	7	5.7±2.1
1.0	1.0	6.5	7	3.5±0.8
1.0	1.0	6.8	7	3.4±0.5
1.0	1.0	7.2	7	2.2±0.4
1.0	1.0	7.7	7	3.1±0.7

The regression line in figure 12, showed a negative relationship between glucose levels and pH with a correlation of good fit of 53 %. At low pH water groups 5.4 and 5.7, fish appeared

to show signs of elevated levels of glucose indicative of possible high levels of stress. As criteria for 'no effect' on stress levels, 3-6 mmol/l is used for glucose (Kroglund et al., 2001). Fish exposed to water pH groups 5.4 and 5.7 had glucose levels above the no effect criteria of 6 mmol/l glucose. This suggests that, the lower the water pH group, the more U accumulates on gill and the more likely the effect of stress on fish. There was a significant correlation (r^2 = 65.5 %, Figure 13) between gill U accumulation and glucose levels. Worth noting, is that pH water groups 5.4 and 5.7 had measured U concentrations in water below 1.0 mg/L U nominal concentration. So, higher gill U concentrations could be expected with more significant correlation than shown (Figures 13).

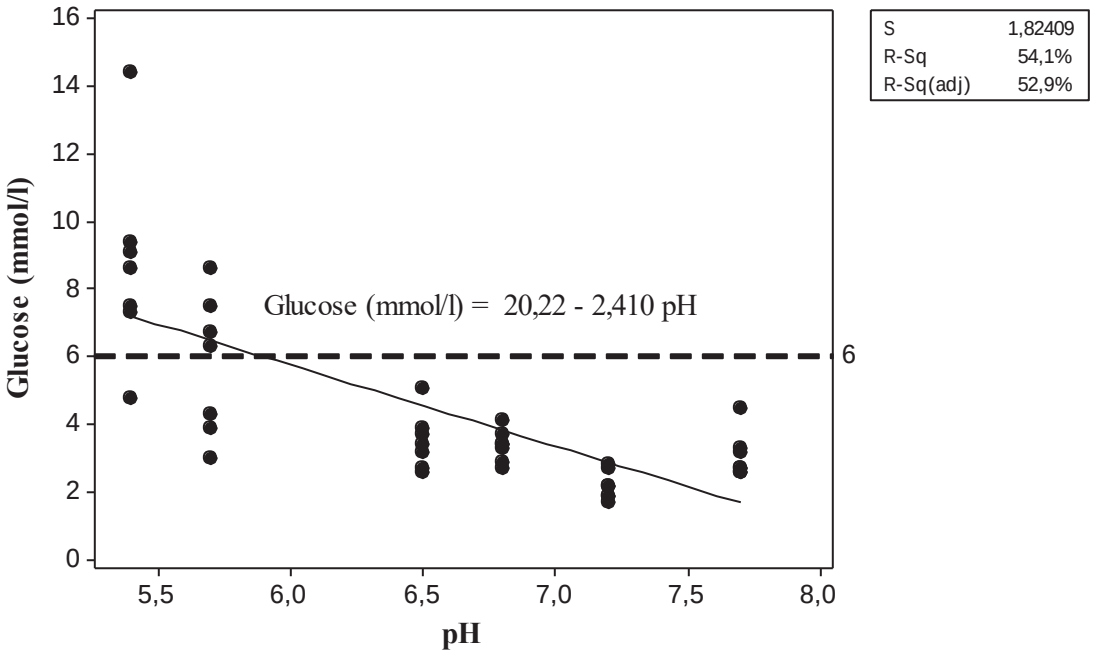

Figure 12. Regression fit of blood plasma glucose (mmol/l) versus pH. The "no effect" value is indicated by dotted line.

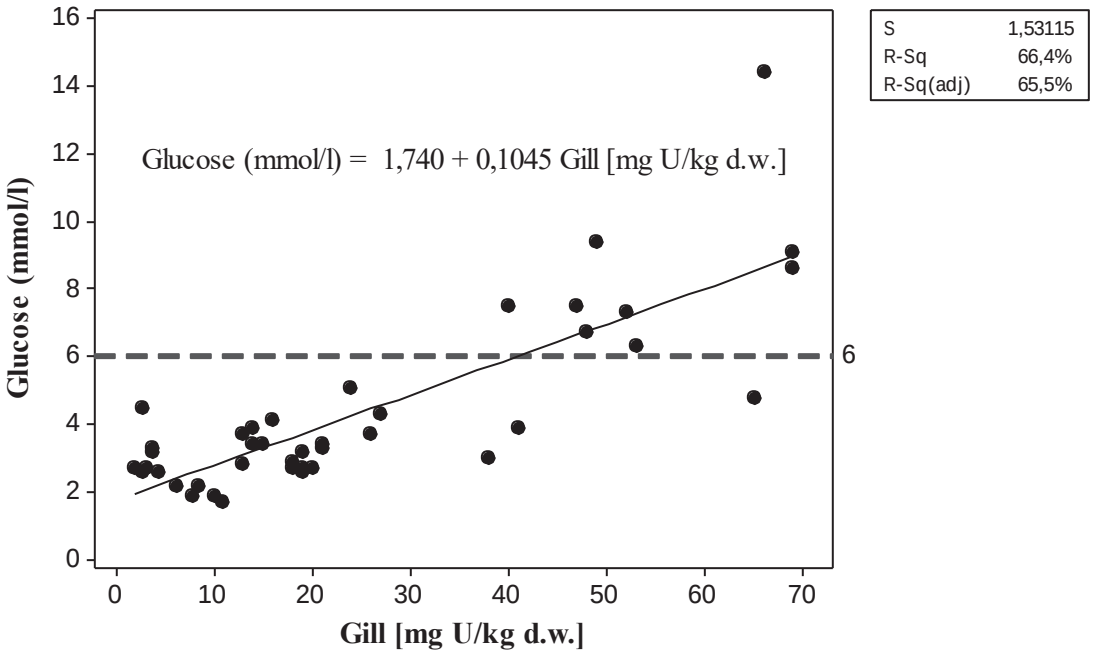

Figure 13. Relationship between gill U (mg U/kg dw) accumulation and responses to stress (glucose) level. The "no effect" value is indicated by dotted line.

4.5. U-BLM

Linking speciation information of U in water and the accumulation of U in sampled tissues (Figure 14) could serve as a quantitative tool in the risk assessment of U toxicity in fish by developing a biotic ligand model (BLM) for U. Results showed (Tables 6-8) that pH is an important factor influencing U uptake in sampled fish tissues (gill, liver and kidney). The gill is the biotic ligand and primary target organ for subsequent uptake and distribution of the environmental ligand (U) to internal secondary organs (e.g., liver and kidney). In addition, toxic effects are related to U concentration in tissues and not levels in water since fish do not drink water in freshwater systems. In developing the U- BLM model, sampling of kidney and liver is not necessary, but might give some useful information concerning the accumulation potential in these critical organs during chronic exposure in different water qualities, as illustrated in table 7 and 8.

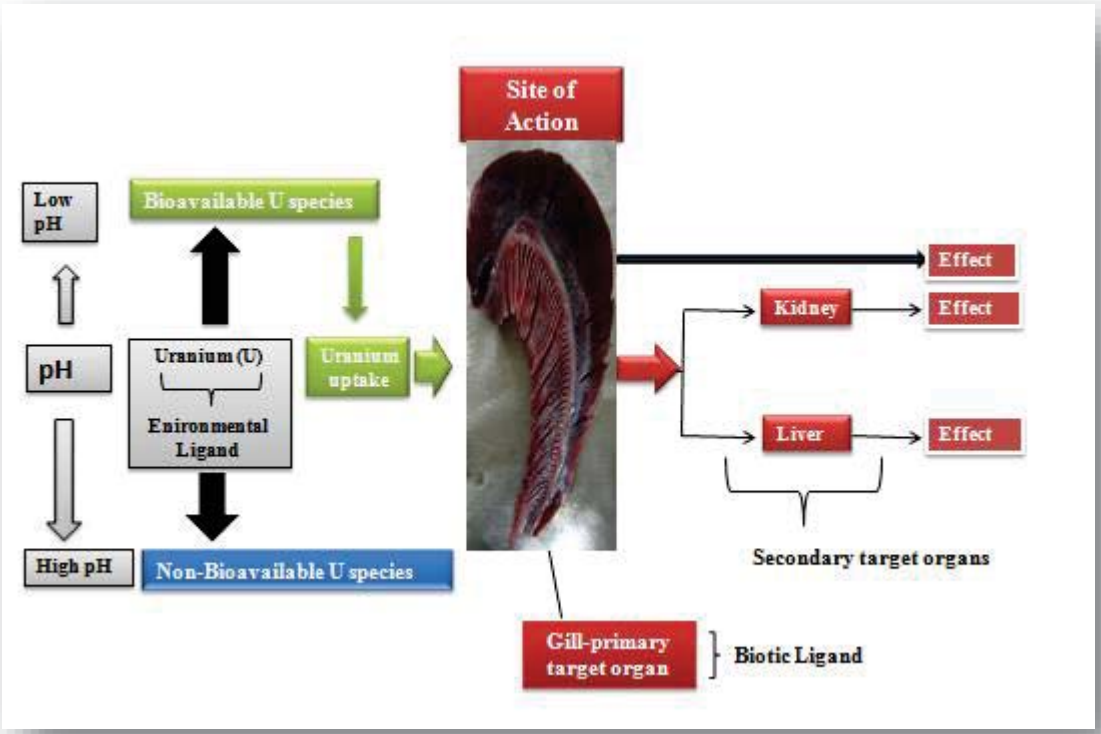

Figure 14. Schematic presentation of U-BLM for Atlantic salmon parr.

CHAPTER FIVE

5. CONCLUSION

The present study showed that physicochemical analyses of U in waters with focus on different pH levels in exposure water provide important information about the possible variations in U speciation. Size and charge fractionation of different water qualities help provide information in terms of possible labile and bioavailable LMM forms of U. pH can significantly influence U speciation as 90-97 % of the dissolved fractions are present as LMM (cutoff < 10kDa) species. Between pH 5.4 to pH 7.7, significant modifications in the speciation and bioavailability of waterborne U appear to have occurred which influenced the uptake in fish, primarily in the gills. In very soft water and at environmentally relevant U concentrations of up to nominal 1.0 mg U/L in water, there was a significantly negative correlation between gill U uptake and pH. With significant discrimination in U accumulation across the different water qualities, the gills appeared to be most susceptible to U uptake at pH < 6. In addition, the gills equally reflect different sensitivities to its external environment with respect to changes in water pH, accumulating U at levels higher than compared to lever and kidney. Also, the gills serve as excellent external biomarkers of U toxicity in view of stress response to measured glucose levels. Measured U concentrations in liver and kidney tissues sampled, showed that the gill is an important pathway for uptake and distribution of U through blood to secondary target organs distant from the point of entry. pH is an important factor for U bioavailability and bioavailability is vital for U toxicity. Increased acidity will lead to higher tissue U concentrations, thus increasing levels of glucose associated with increased stress response (Kroglund et al., 2001). Subsequent toxic effects are related to U concentrations accumulated in fish tissues and not to U concentrations in water, since fish do not drink water in freshwater systems. Linking speciation information of U and its accumulation in tissues could help in developing a U-BLM and serve as a quantitative tool in the risk assessment of U toxicity in fish.

Finally, the overall results of this study seem to confirm the general hypothesis that pH may significantly influence U speciation in water based on amounts accumulated in tissues and thereby the bioavailability, uptake and potential toxicity to fish. Also, that accumulation of U in fish gills, liver and kidney may be as a result of exposure to bioavailable free UO_2^{2+} and other neutral U species. So, characterizing the exposure medium and uptake routes is fundamental in developing the U-BLM for risk assessment of U toxicity in fish.

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Appendix 1: WORKING DATA I

		Measured	Measured	Gill weight	U-gill	U-liver	U-kidney	Length	Weight	Glucose	%dead
Date	Water quality	U	pH	g	mg/kg dw	mg/kg dw	mg/kg dw	cm	g	mmol/l	
30.11.2012	Reference water	1010,5	**6,5**	0,008	26,0	0,30	0,251	11,6	12,5	3,7	**0,00**
30.11.2012	Reference water	1010,5	**6,5**	0,028	19,0	0,37	0,438	12,7	16,5	2,6	
30.11.2012	Reference water	1010,5	**6,5**	0,006	14,0	0,40	0,177	10,8	10,44	3,9	
30.11.2012	Reference water	1010,5	**6,5**	0,009	19,0	0,82	0,283	11,2	11,42	2,7	
30.11.2012	Reference water	1010,5	**6,5**	0,013	24,0	0,97	0,283	13,2	17,9	5,1	
30.11.2012	Reference water	1010,5	**6,5**	0,007	21,0	0,81	0,142	11,2	10,79	3,4	
30.11.2012	Reference water	1010,5	**6,5**	0,008	19,0	0,22	0,108	11,3	12,28	3,2	
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	0,0105	15,0			12,4	15,41	3,4	**0,00**
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	0,0086	18,0			11,8	12,57	2,9	
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	0,0125	18,0			13,3	18,8	2,7	
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	0,009	13,0			11,7	12,5	3,7	
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	0,0131	21,0			12,8	18,03	3,3	
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	0,0091	14,0			12,9	16,8	3,4	
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	0,0117	16,0			13	16,39	4,1	
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	0,014	8,4	0,02		13,2	19,32	2,2	**0,00**
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	0,012	7,8	0,092		13	17,76	1,9	
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	0,009	20,0	0,502		10,9	10,2	2,7	
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	0,014	11,0	0,064		13	18,76	1,7	
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	0,008	10,0	0,068		10,9	11,16	1,9	
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	0,012	13,0	0,103		11,9	13,9	2,8	
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	0,011	6,3	0,185		11,7	12,97	2,2	

Cont'd

Date	Water quality	Measured U	Measured pH	Gill weight g	U-gill mg/kg dw	U-liver mg/kg dw	U-kidney mg/kg dw	Length cm	Weight g	Glucose mmol/l	%dead
14.12.2012	High pH: 8.5	1021,1	**7,7**	0,012	3,8	0,011	0,010	12,5	16,09	3,2	**0,00**
14.12.2012	High pH: 8.5	1021,1	**7,7**	0,015	2,8	0,018	0,022	13,6	20,94	2,6	
14.12.2012	High pH: 8.5	1021,1	**7,7**	0,015	1,9	0,016	0,019	13,4	20,7	2,7	
14.12.2012	High pH: 8.5	1021,1	**7,7**	0,012	3,1	0,008	0,012	13,1	17,65	2,7	
14.12.2012	High pH: 8.5	1021,1	**7,7**	0,013	3,8	0,010	0,017	13	17,62	3,3	
14.12.2012	High pH: 8.5	1021,1	**7,7**	0,011	2,8	0,016	0,016	12,8	11,5	4,5	
14.12.2012	High pH: 8.5	1021,1	**7,7**	0,007	4,3	0,012	0,005	10,5	9,47	2,6	
14.12.2012	Lav pH: 5.5	884,2	**5,4**	0,0137	65,0	0,302	1,052	13,1	17,52	4,8	**0,00**
14.12.2012	Lav pH: 5.5	884,2	**5,4**	0,0056	40,0	0,124	0,222	9,5	6,6	7,5	
14.12.2012	Lav pH: 5.5	884,2	**5,4**	0,0083	69,0	0,195	0,274	11,2	11,47	9,1	
14.12.2012	Lav pH: 5.5	884,2	**5,4**	0,0082	49,0	0,120	0,334	11,2	10,1	9,4	
14.12.2012	Lav pH: 5.5	884,2	**5,4**	0,0091	69,0	0,305	1,399	11,7	12,2	8,6	
14.12.2012	Lav pH: 5.5	884,2	**5,4**	0,0139	66,0	0,260	0,776	13,2	18,32	14,4	
14.12.2012	Lav pH: 5.5	884,2	**5,4**	0,0115	52,0	0,197	0,568	12,4	15,24	7,3	
14.12.2012	Medium pH: 6.0	863,2	**5,7**	0,0042	27,0	0,062	0,093	8,7	5,2	4,3	**0,00**
14.12.2012	Medium pH: 6.0	863,2	**5,7**	0,0087	48,0	0,237	0,442	12,1	14,32	6,7	
14.12.2012	Medium pH: 6.0	863,2	**5,7**	0,0113	47,0	0,211	1,012	12,7	16,6	7,5	
14.12.2012	Medium pH: 6.0	863,2	**5,7**	0,0132	53,0	0,614	1,312	13,3	18,4	6,3	
14.12.2012	Medium pH: 6.0	863,2	**5,7**	0,0112	38,0	0,200	0,544	12,7	15,9	3	
14.12.2012	Medium pH: 6.0	863,2	**5,7**	0,0132	**130,0**	0,229	0,924	12,7	16,03	8,6	
14.12.2012	Medium pH: 6.0	863,2	**5,7**	0,0107	41,0	0,318	1,310	13,1	18,1	3,9	

For obtaining concentration of U in fish tissues based on dry weight (mg/kg dw),

Concentration (mg/kg dw) = measured ICP-MS value in digested tissue / (100 * dry weight of tissue)

N/B: Measured values were divided by 100 because samples were digested in 10 ml instead of 1000 ml.

Wet weight tissue values were transformed by a factor of 5, i.e., 1 mg/kg ww = 5 mg/kg dw.

Appendix 2A. WORKING DATA II

		Measured	Measured	0h	96h	stop	stop	stop	stop	stop	stop	stop	stop
Date	**Water quality**	**U**	**pH**	Alkalinity	Alkalinity	**TOC**	**Chloride**	**Nitrate**	**Sulfate**	**Na**	**Mg**	**K**	**Ca**
30.11.2012	Reference water	1010,5	**6,5**	0,164	0,171					3,8	1,7	0,5	1,9
30.11.2012	Reference water	1010,5	**6,5**	0,164	0,171					3,8	1,7	0,5	1,9
30.11.2012	Reference water	1010,5	**6,5**	0,164	0,171					3,8	1,7	0,5	1,9
30.11.2012	Reference water	1010,5	**6,5**	0,164	0,171					3,8	1,7	0,5	1,9
30.11.2012	Reference water	1010,5	**6,5**	0,164	0,171					3,8	1,7	0,5	1,9
30.11.2012	Reference water	1010,5	**6,5**	0,164	0,171					3,8	1,7	0,5	1,9
30.11.2012	Reference water	1010,5	**6,5**	0,164	0,171					3,8	1,7	0,5	1,9
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	0,161	0,164	1,88	46,00	0,15	10,35	34,7	1,6	0,3	1,8
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	0,161	0,164	1,88	46,00	0,15	10,35	34,7	1,6	0,3	1,8
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	0,161	0,164	1,88	46,00	0,15	10,35	34,7	1,6	0,3	1,8
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	0,161	0,164	1,88	46,00	0,15	10,35	34,7	1,6	0,3	1,8
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	0,161	0,164	1,88	46,00	0,15	10,35	34,7	1,6	0,3	1,8
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	0,161	0,164	1,88	46,00	0,15	10,35	34,7	1,6	0,3	1,8
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	0,161	0,164	1,88	46,00	0,15	10,35	34,7	1,6	0,3	1,8
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	0,391	0,395					36,8	1,5	0,5	1,7
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	0,391	0,395					36,8	1,5	0,5	1,7
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	0,391	0,395					36,8	1,5	0,5	1,7
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	0,391	0,395					36,8	1,5	0,5	1,7
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	0,391	0,395					36,8	1,5	0,5	1,7
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	0,391	0,395					36,8	1,5	0,5	1,7
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	0,391	0,395					36,8	1,5	0,5	1,7

Cont'd

		Measured	Measured	0h	96h	stop	stop	stop	stop	stop	stop	stop	stop
Date	**Water quality**	**U**	**pH**	Alkalinity	Alkalinity	**TOC**	**Chloride**	**Nitrate**	**Sulfate**	**Na**	**Mg**	**K**	**Ca**
14.12.2012	High pH: 8.5	1021,1	**7,7**	1,480	1,544					36,8	1,6	0,5	1,8
14.12.2012	High pH: 8.5	1021,1	**7,7**	1,480	1,544					36,8	1,6	0,5	1,8
14.12.2012	High pH: 8.5	1021,1	**7,7**	1,480	1,544					36,8	1,6	0,5	1,8
14.12.2012	High pH: 8.5	1021,1	**7,7**	1,480	1,544					36,8	1,6	0,5	1,8
14.12.2012	High pH: 8.5	1021,1	**7,7**	1,480	1,544					36,8	1,6	0,5	1,8
14.12.2012	High pH: 8.5	1021,1	**7,7**	1,480	1,544					36,8	1,6	0,5	1,8
14.12.2012	High pH: 8.5	1021,1	**7,7**	1,480	1,544					36,8	1,6	0,5	1,8
14.12.2012	Lav pH: 5.5	884,2	**5,4**	0,010	0,020	3,48	57,00	0,15	10,52	37,9	1,6	0,6	1,8
14.12.2012	Lav pH: 5.5	884,2	**5,4**	0,010	0,020	3,48	57,00	0,15	10,52	37,9	1,6	0,6	1,8
14.12.2012	Lav pH: 5.5	884,2	**5,4**	0,010	0,020	3,48	57,00	0,15	10,52	37,9	1,6	0,6	1,8
14.12.2012	Lav pH: 5.5	884,2	**5,4**	0,010	0,020	3,48	57,00	0,15	10,52	37,9	1,6	0,6	1,8
14.12.2012	Lav pH: 5.5	884,2	**5,4**	0,010	0,020	3,48	57,00	0,15	10,52	37,9	1,6	0,6	1,8
14.12.2012	Lav pH: 5.5	884,2	**5,4**	0,010	0,020	3,48	57,00	0,15	10,52	37,9	1,6	0,6	1,8
14.12.2012	Lav pH: 5.5	884,2	**5,4**	0,010	0,020	3,48	57,00	0,15	10,52	37,9	1,6	0,6	1,8
14.12.2012	Medium pH: 6.0	863,2	**5,7**	0,010	0,044					36,8	1,6	0,5	1,9
14.12.2012	Medium pH: 6.0	863,2	**5,7**	0,010	0,044					36,8	1,6	0,5	1,9
14.12.2012	Medium pH: 6.0	863,2	**5,7**	0,010	0,044					36,8	1,6	0,5	1,9
14.12.2012	Medium pH: 6.0	863,2	**5,7**	0,010	0,044					36,8	1,6	0,5	1,9
14.12.2012	Medium pH: 6.0	863,2	**5,7**	0,010	0,044					36,8	1,6	0,5	1,9
14.12.2012	Medium pH: 6.0	863,2	**5,7**	0,010	0,044					36,8	1,6	0,5	1,9
14.12.2012	Medium pH: 6.0	863,2	**5,7**	0,010	0,044					36,8	1,6	0,5	1,9

Appendix 2B: WORKING DATA III

		Measured	Measured	0h	24h	48h	72h	96h
Date	Water quality	U	pH	Temperature	Temperature	Temperature	Temperature	Temperature
30.11.2012	Reference water	1010,5	**6,5**		8,9		9,0	9,0
30.11.2012	Reference water	1010,5	**6,5**		8,9		9,0	9,0
30.11.2012	Reference water	1010,5	**6,5**		8,9		9,0	9,0
30.11.2012	Reference water	1010,5	**6,5**		8,9		9,0	9,0
30.11.2012	Reference water	1010,5	**6,5**		8,9		9,0	9,0
30.11.2012	Reference water	1010,5	**6,5**		8,9		9,0	9,0
30.11.2012	Reference water	1010,5	**6,5**		8,9		9,0	9,0
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	9	8,9	9,0	9,0	9,0
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	9	8,9	9,0	9,0	9,0
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	9	8,9	9,0	9,0	9,0
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	9	8,9	9,0	9,0	9,0
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	9	8,9	9,0	9,0	9,0
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	9	8,9	9,0	9,0	9,0
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	9	8,9	9,0	9,0	9,0
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	9,1	9,1		9,1	8,9
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	9,1	9,1		9,1	8,9
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	9,1	9,1		9,1	8,9
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	9,1	9,1		9,1	8,9
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	9,1	9,1		9,1	8,9
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	9,1	9,1		9,1	8,9
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	9,1	9,1		9,1	8,9

Cont'd

		Measured	Measured	0h	24h	48h	72h	96h
Date	**Water quality**	**U**	**pH**	**Temperature**	**Temperature**	**Temperature**	**Temperature**	**Temperature**
14.12.2012	High pH: 8.5	1021,1	**7,7**	9,1	9,2		9,2	9,0
14.12.2012	High pH: 8.5	1021,1	**7,7**	9,1	9,2		9,2	9,0
14.12.2012	High pH: 8.5	1021,1	**7,7**	9,1	9,2		9,2	9,0
14.12.2012	High pH: 8.5	1021,1	**7,7**	9,1	9,2		9,2	9,0
14.12.2012	High pH: 8.5	1021,1	**7,7**	9,1	9,2		9,2	9,0
14.12.2012	High pH: 8.5	1021,1	**7,7**	9,1	9,2		9,2	9,0
14.12.2012	High pH: 8.5	1021,1	**7,7**	9,1	9,2		9,2	9,0
14.12.2012	Lav pH: 5.5	884,2	**5,4**	8,6	8,8		8,9	8,7
14.12.2012	Lav pH: 5.5	884,2	**5,4**	8,6	8,8		8,9	8,7
14.12.2012	Lav pH: 5.5	884,2	**5,4**	8,6	8,8		8,9	8,7
14.12.2012	Lav pH: 5.5	884,2	**5,4**	8,6	8,8		8,9	8,7
14.12.2012	Lav pH: 5.5	884,2	**5,4**	8,6	8,8		8,9	8,7
14.12.2012	Lav pH: 5.5	884,2	**5,4**	8,6	8,8		8,9	8,7
14.12.2012	Lav pH: 5.5	884,2	**5,4**	8,6	8,8		8,9	8,7
14.12.2012	Medium pH: 6.0	863,2	**5,7**	8,8	9,0		9,0	8,7
14.12.2012	Medium pH: 6.0	863,2	**5,7**	8,8	9,0		9,0	8,7
14.12.2012	Medium pH: 6.0	863,2	**5,7**	8,8	9,0		9,0	8,7
14.12.2012	Medium pH: 6.0	863,2	**5,7**	8,8	9,0		9,0	8,7
14.12.2012	Medium pH: 6.0	863,2	**5,7**	8,8	9,0		9,0	8,7
14.12.2012	Medium pH: 6.0	863,2	**5,7**	8,8	9,0		9,0	8,7
14.12.2012	Medium pH: 6.0	863,2	**5,7**	8,8	9,0		9,0	8,7

		Measured	Measured	0h	24h	48h	72h	96h
Date	**Water quality**	**U**	**pH**	pH	pH	pH	pH	pH
30.11.2012	Reference water	1010,5	**6,5**	**6,55**		**6,53**	**6,56**	**6,51**
30.11.2012	Reference water	1010,5	**6,5**	**6,55**		**6,53**	**6,56**	**6,51**
30.11.2012	Reference water	1010,5	**6,5**	**6,55**		**6,53**	**6,56**	**6,51**
30.11.2012	Reference water	1010,5	**6,5**	**6,55**		**6,53**	**6,56**	**6,51**
30.11.2012	Reference water	1010,5	**6,5**	**6,55**		**6,53**	**6,56**	**6,51**
30.11.2012	Reference water	1010,5	**6,5**	**6,55**		**6,53**	**6,56**	**6,51**
30.11.2012	Reference water	1010,5	**6,5**	**6,55**		**6,53**	**6,56**	**6,51**
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	**6,82**			**6,86**	**6,88**
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	**6,82**			**6,86**	**6,88**
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	**6,82**			**6,86**	**6,88**
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	**6,82**			**6,86**	**6,88**
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	**6,82**			**6,86**	**6,88**
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	**6,82**			**6,86**	**6,88**
18.01.2013	High Na: 33 mg/L	1010,5	**6,8**	**6,82**			**6,86**	**6,88**
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	**7,21**	**7,13**	**7,32**	**7,27**	**7,21**
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	**7,21**	**7,13**	**7,32**	**7,27**	**7,21**
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	**7,21**	**7,13**	**7,32**	**7,27**	**7,21**
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	**7,21**	**7,13**	**7,32**	**7,27**	**7,21**
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	**7,21**	**7,13**	**7,32**	**7,27**	**7,21**
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	**7,21**	**7,13**	**7,32**	**7,27**	**7,21**
14.12.2012	Medium pH: 7.0	1000,0	**7,2**	**7,21**	**7,13**	**7,32**	**7,27**	**7,21**

Cont'd

		Measured	Measured	0h	24h	48h	72h	96h
Date	**Water quality**	**U**	**pH**	pH	pH	pH	pH	pH
14.12.2012	High pH: 8.5	1021,1	**7,7**	**7,74**	**7,70**	**7,94**	**7,81**	**7,89**
14.12.2012	High pH: 8.5	1021,1	**7,7**	**7,74**	**7,70**	**7,94**	**7,81**	**7,89**
14.12.2012	High pH: 8.5	1021,1	**7,7**	**7,74**	**7,70**	**7,94**	**7,81**	**7,89**
14.12.2012	High pH: 8.5	1021,1	**7,7**	**7,74**	**7,70**	**7,94**	**7,81**	**7,89**
14.12.2012	High pH: 8.5	1021,1	**7,7**	**7,74**	**7,70**	**7,94**	**7,81**	**7,89**
14.12.2012	High pH: 8.5	1021,1	**7,7**	**7,74**	**7,70**	**7,94**	**7,81**	**7,89**
14.12.2012	High pH: 8.5	1021,1	**7,7**	**7,74**	**7,70**	**7,94**	**7,81**	**7,89**
14.12.2012	Lav pH: 5.5	884,2	**5,4**	**5,42**	**5,81**		**5,98**	**5,85**
14.12.2012	Lav pH: 5.5	884,2	**5,4**	**5,42**	**5,81**		**5,98**	**5,85**
14.12.2012	Lav pH: 5.5	884,2	**5,4**	**5,42**	**5,81**		**5,98**	**5,85**
14.12.2012	Lav pH: 5.5	884,2	**5,4**	**5,42**	**5,81**		**5,98**	**5,85**
14.12.2012	Lav pH: 5.5	884,2	**5,4**	**5,42**	**5,81**		**5,98**	**5,85**
14.12.2012	Lav pH: 5.5	884,2	**5,4**	**5,42**	**5,81**		**5,98**	**5,85**
14.12.2012	Lav pH: 5.5	884,2	**5,4**	**5,42**	**5,81**		**5,98**	**5,85**
14.12.2012	Medium pH: 6.0	863,2	**5,7**	**5,77**	**6,17**		**6,17**	**6,27**
14.12.2012	Medium pH: 6.0	863,2	**5,7**	**5,77**	**6,17**		**6,17**	**6,27**
14.12.2012	Medium pH: 6.0	863,2	**5,7**	**5,77**	**6,17**		**6,17**	**6,27**
14.12.2012	Medium pH: 6.0	863,2	**5,7**	**5,77**	**6,17**		**6,17**	**6,27**
14.12.2012	Medium pH: 6.0	863,2	**5,7**	**5,77**	**6,17**		**6,17**	**6,27**
14.12.2012	Medium pH: 6.0	863,2	**5,7**	**5,77**	**6,17**		**6,17**	**6,27**
14.12.2012	Medium pH: 6.0	863,2	**5,7**	**5,77**	**6,17**		**6,17**	**6,27**

Appendix 3. Normality Test for U concentration in Fish Tissues

One of the tools used to analyze normality is Anderson-Darling Test for which a data output with p-value > α = 0.01, would imply the data is normally distributed with a probability being greater than 99%. In fact p-value > α, basically says the data is normal.

Hypothesis at α=0.01

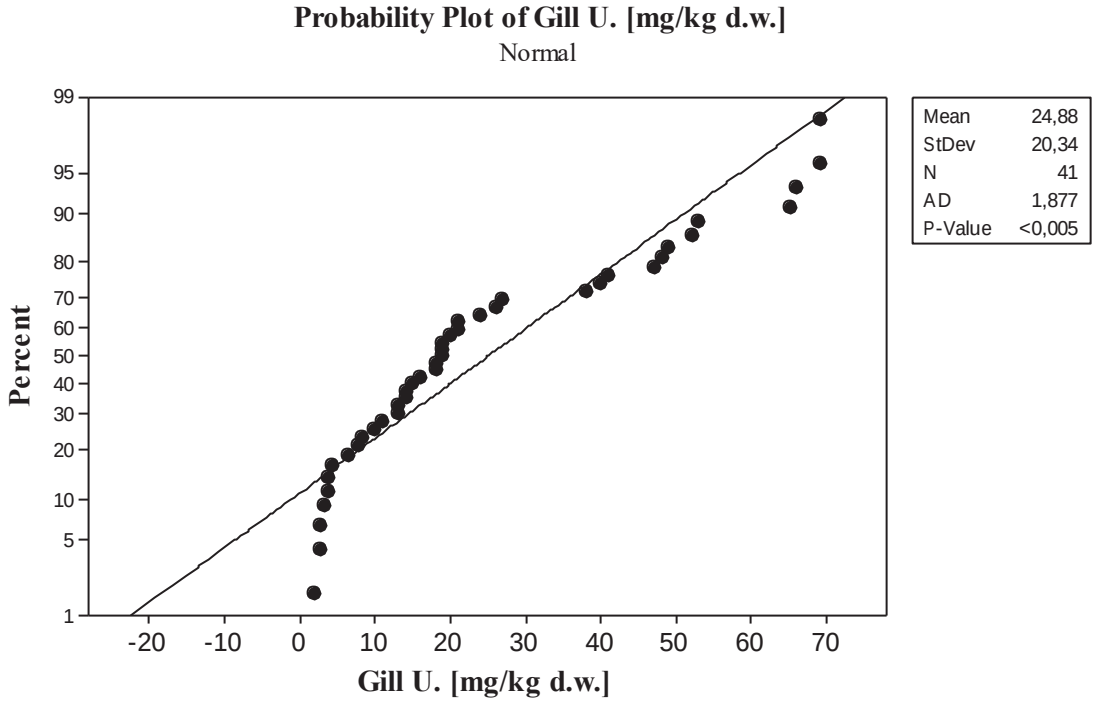
H_0: data is normal

H_1: data is not normal

Appendix 3.1. Normality Test Probability Plot for Gill Tissue

Minitab Operation

1. Select, **Stat >Basic Statistics >Normality >Variable (Gill U. [mg/kg dw]**

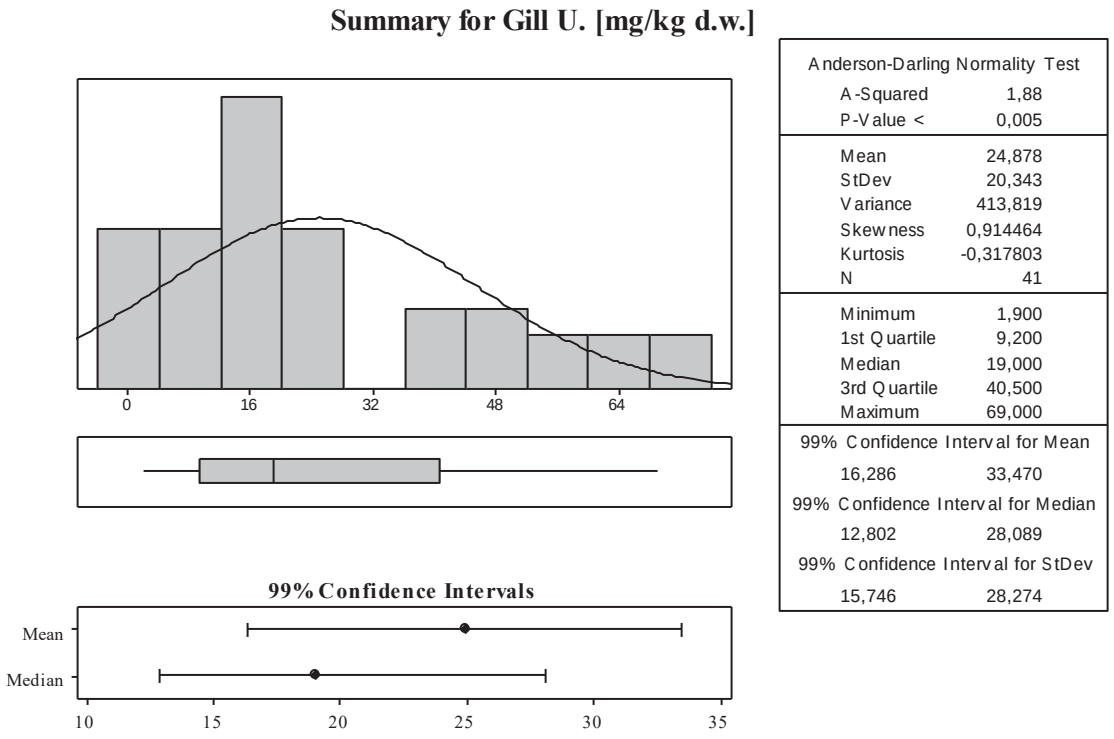


From the above plot, **p-value < 0.005 << 0.01=α** suggests the data is not normal.

Appendix 3.1.1. Summary Plot for Gill U concentration

Minitab Operation

1. Select, **Stat > Basic Statistics> Graphical Summary> Variable (Gill U. [mg/kg dw]**



Anderson-Darling Normality Test	
A-Squared	1,88
P-Value <	0,005
Mean	24,878
StDev	20,343
Variance	413,819
Skewness	0,914464
Kurtosis	-0,317803
N	41
Minimum	1,900
1st Quartile	9,200
Median	19,000
3rd Quartile	40,500
Maximum	69,000
99% Confidence Interval for Mean	
16,286	33,470
99% Confidence Interval for Median	
12,802	28,089
99% Confidence Interval for StDev	
15,746	28,274

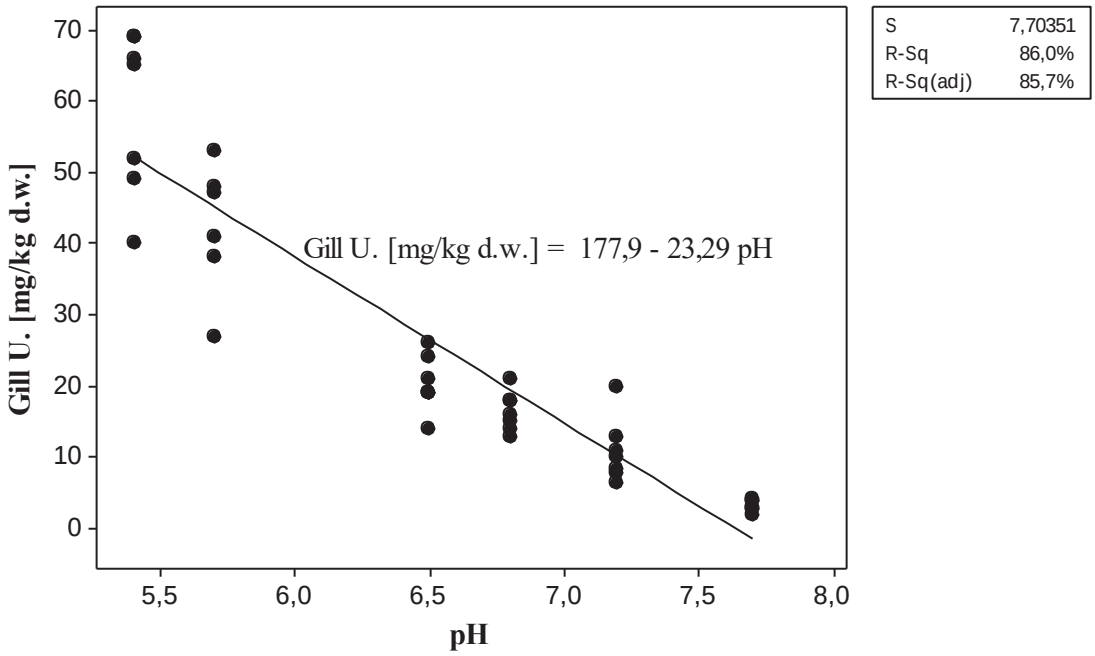
The above output shows that the data is right-skewed.

3.1.2. Fitted Line Plot for gill U concentration

Minitab Operation

1. Select, **Stat > Regression> Fitted Line Plot > Variable**

2. Select, **Response (Y): Gill U. [mg/kg dw] and Predictor (X): pH**



Regression Analysis: Gill U.[mg/kg dw] versus pH

The regression equation is

Gill U. [mg/kg d.w.] = 178 - 23,3 pH

Predictor	Coef	SE Coef	T	P
Constant	177,924	9,954	17,88	0,000
pH	-23,292	1,504	-15,49	**0,000**

S = 7,70351 R-Sq = 86,0% R-Sq(adj) = **85,7%**

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	14238	14238	239,93	**0,000**
Residual Error	39	2314	59		
Total	40	1655			

The p-value = 0.00 < 0.01, means that there is a significant relationship between pH and U accumulated in gill tissue. It also implies that pH is a significant factor in influencing U accumulation on the gill. But, the p-value doesn't explain all of the variation in the data, as it explains only 85.7% [R^2 (adjusted)]. The R^2 indicates how much variation in the data is being explained. So, they may be other variables which may explain the rest of the variation in the data or it could be that the rest of the variation is really just random and there is no pattern to it, or the relationship between pH and U accumulation in gill is not a completely linear relationship but rather something more complicated. Based on this, it is important to take a look at the residual plots.

The main assumption for regression is that of constant variation (homogeneity of variance).

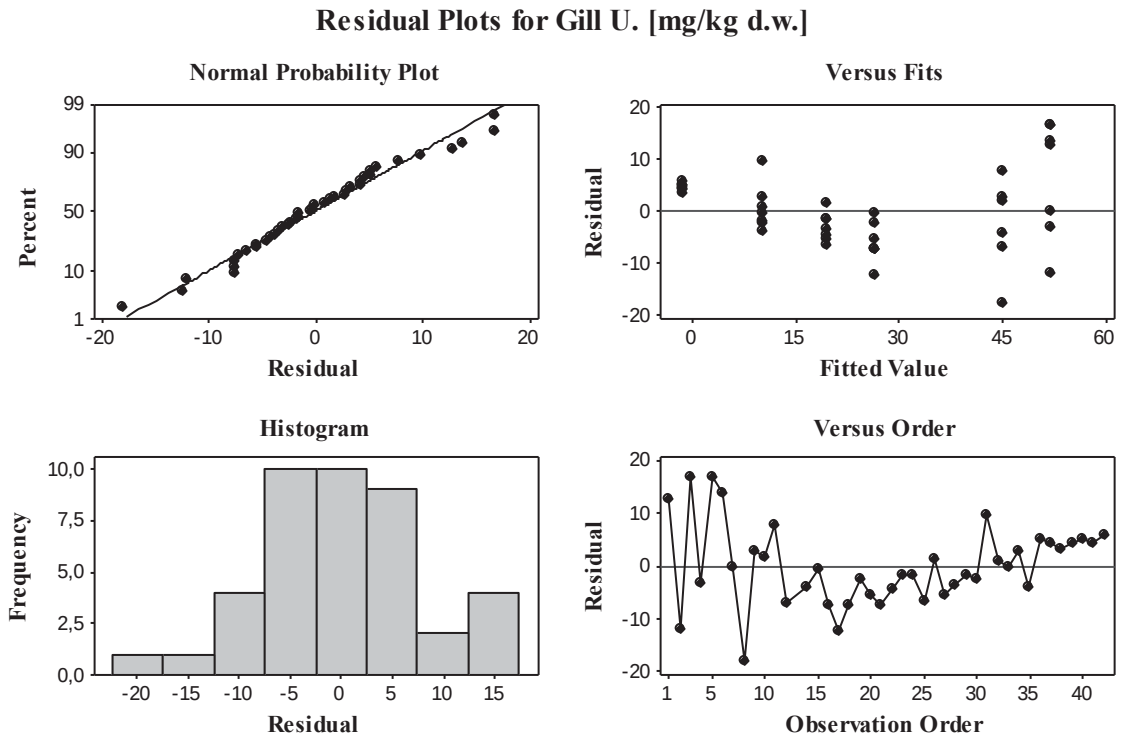
So, is there any pattern to the data the model is not explaining?

To answer this, we use residual plots. The residuals are the difference between the observed U accumulation in gill and what is predicted. It is expected that the residuals are normally distributed about zero. A p-value > α=0.01 is a strong evidence that the residuals are normally distributed. **If there is a pattern, is it significant?**

Appendix 3.1.3. Residual Value Plots of Gill U concentration

1. Select, **Stat> Regression> Regression> Graph> Residual Plots> Four in One > Ok**

2. Select**, Response : (Gill U. [mg/kg dw]) , Predictor : pH.**



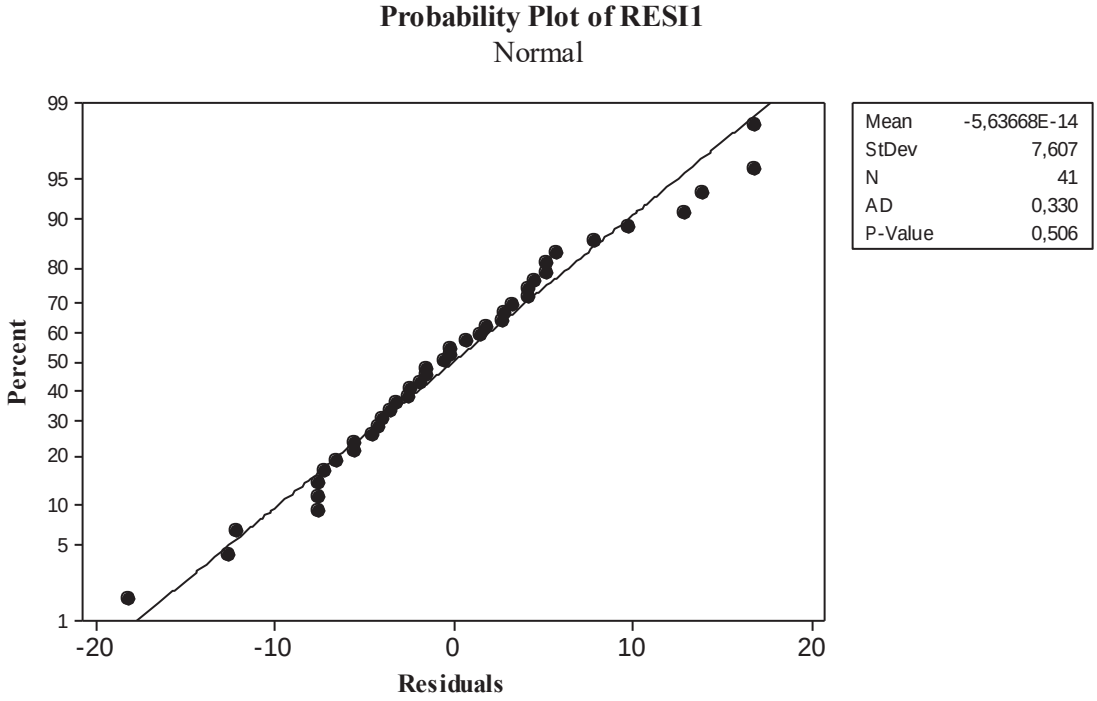
Answer: Based on the residual plot there is no clear cut pattern to the errors that the regression model is not explaining. There is also no significant increase in variance over time.

In addition to this, we plot the residuals for normality.

Appendix 3.1.4. Probability Plot of Residuals

Minitab Operation

1. Select, **Stat> Basic Statistics> Normality Test > Variable (Residuals: RESI 1)**



Since the p-value = **0.51 > 0.01**, it can be concluded the residuals are normally distributed.

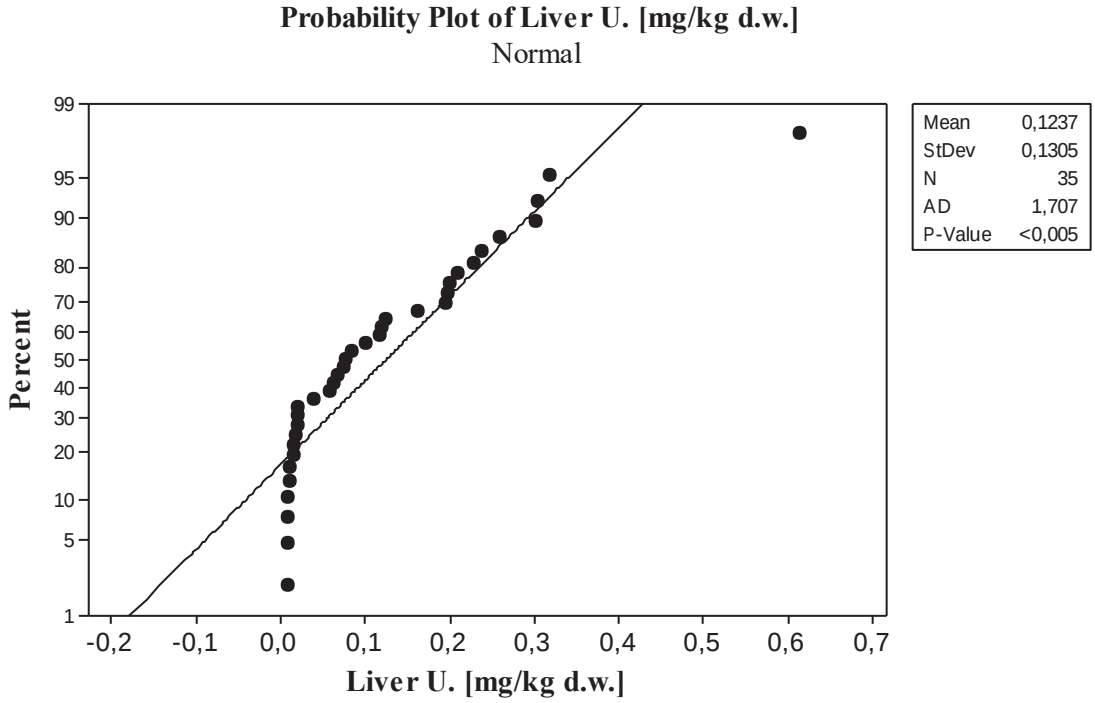
Hence the data for gill is normal.

LIVER TISSUE

Appendix 3.2. Normality Test Probability Plot for Liver Tissue

Minitab Operation

1. Select, **Stat >Basic Statistics >Normality >Variable (Liver U. [mg/kg dw]**

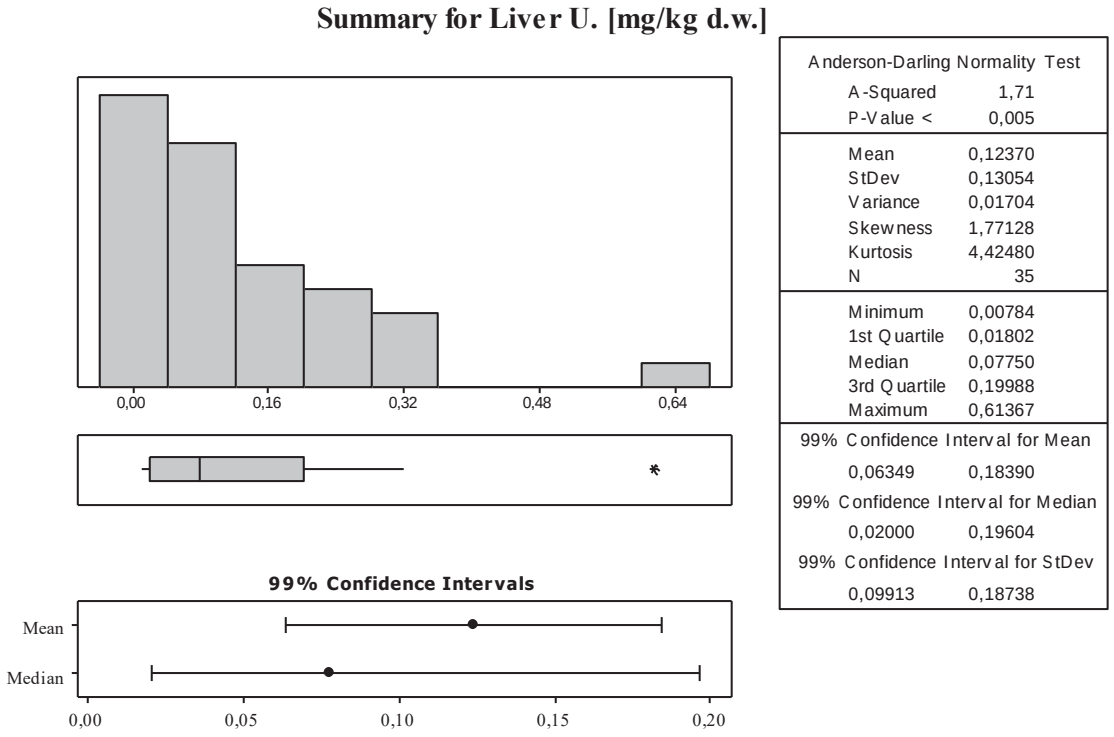


From the above plot, **p-value < 0.005 < α = 0.01** suggests the data does not seem to be normal.

Appendix 3.2.1. Summary Plot for Liver U concentration

Minitab Operation:

1. Select, **Stat > Basic Statistics> Graphical Summary> Variable (Liver U. [mg/kg dw]**



Anderson-Darling Normality Test	
A-Squared	1,71
P-Value <	0,005
Mean	0,12370
StDev	0,13054
Variance	0,01704
Skewness	1,77128
Kurtosis	4,42480
N	35
Minimum	0,00784
1st Quartile	0,01802
Median	0,07750
3rd Quartile	0,19988
Maximum	0,61367
99% Confidence Interval for Mean	
0,06349	0,18390
99% Confidence Interval for Median	
0,02000	0,19604
99% Confidence Interval for StDev	
0,09913	0,18738

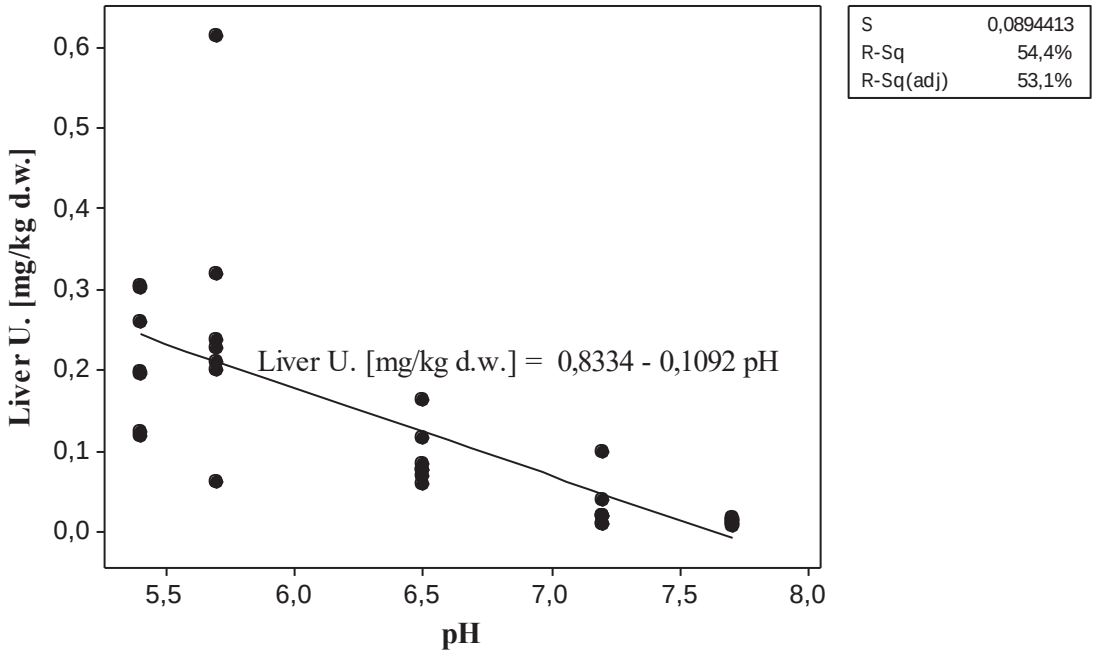
From the above plot, **p-value < 0.005 < α = 0.01** implies the data appears not to be normal and the data is also right-skewed.

Appendix 3.2.2. Fitted Line Plot for Liver U concentration

Minitab Operation

1. Select, **Stat > Regression> Fitted Line Plot > Variable**

2. Select, **Response (Y): Liver U. [mg/kg dw] and Predictor (X): pH**



Regression Analysis: Liver U.[mg/kg dw] versus pH

The regression equation is

Liver U. [mg/kg d.w.] = 0,833 - 0,109 pH

Predictor	Coef	SE Coef	T	P
Constant	0,8334	0,1140	7,31	0,000
pH	-0,10918	0,01739	-6,28	**0,000**

S = 0,0894413 R-Sq = 54,4% R-Sq(adj) = **53,1%**

Analysis of Variance

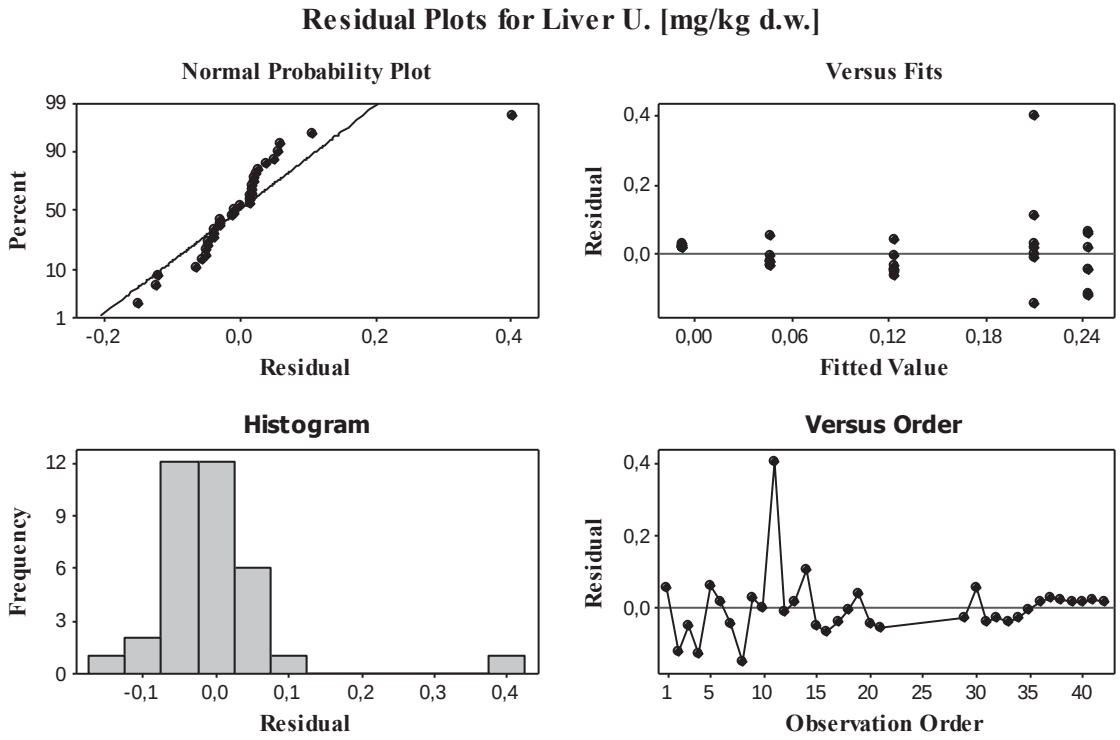
Source	DF	SS	MS	F	P
Regression	1	0,31540	0,31540	39,43	**0,000**
Residual Error	33	0,26399	0,00800		
Total	34	0,57939			

Appendix 3.2.3. Residual Value Plots of Liver U concentration

Minitab Operation

1. Select, **Stat> Regression> Regression> Graph> Residual Plots> Four in One > Ok**

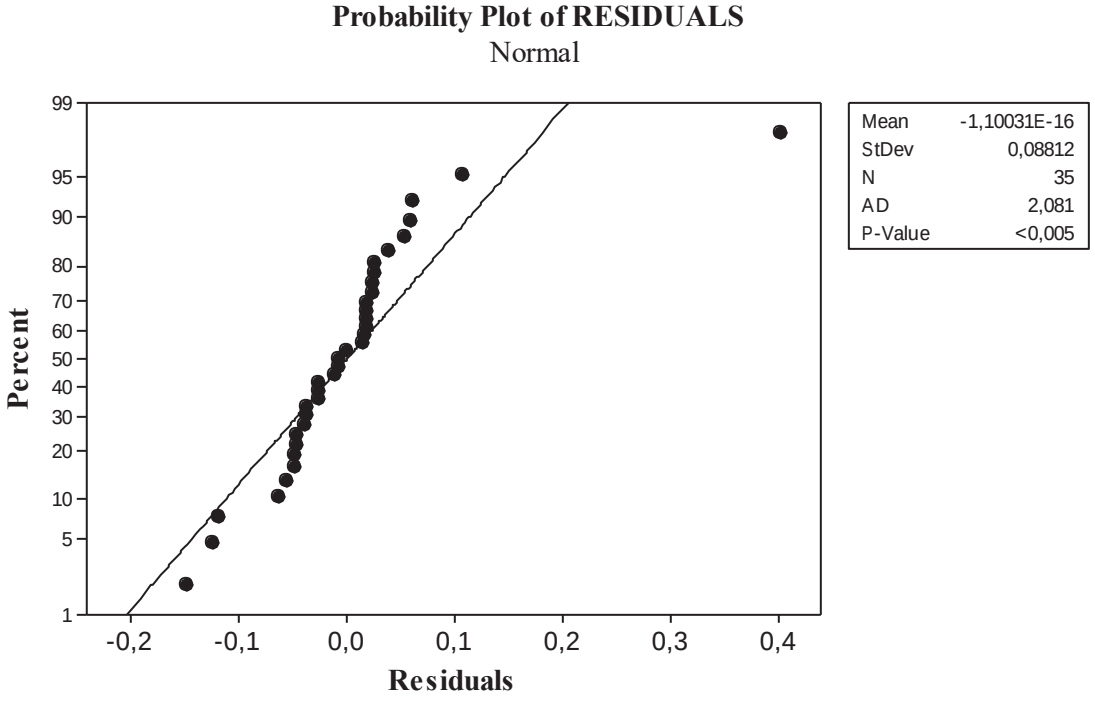
2**. Select, Response : (Liver U. [mg/kg dw]) , Predictor : pH**



Appendix 3.2.4. Probability Plot of Residuals

Minitab Operation

1. Select, **Stat> Basic Statistics> Normality Test > Variable (Residuals: RESI 1)**



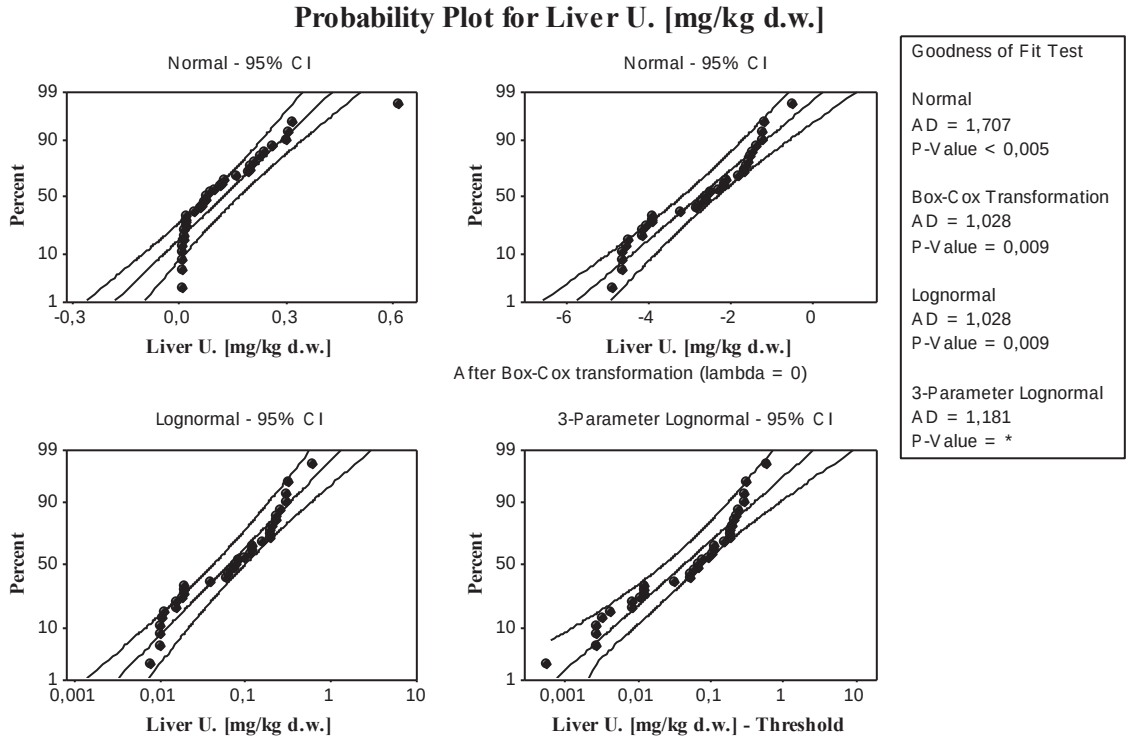
From the p-value < 0.005 << 0.01=α, it can be concluded the residuals are not normally distributed. Hence the data for liver is not normal. So, the data under consideration needs to be transformed to obtain a set a data that will be relatively well modeled to meet a normal distribution.

Appendix 3.2.5. Data Transformation for Liver

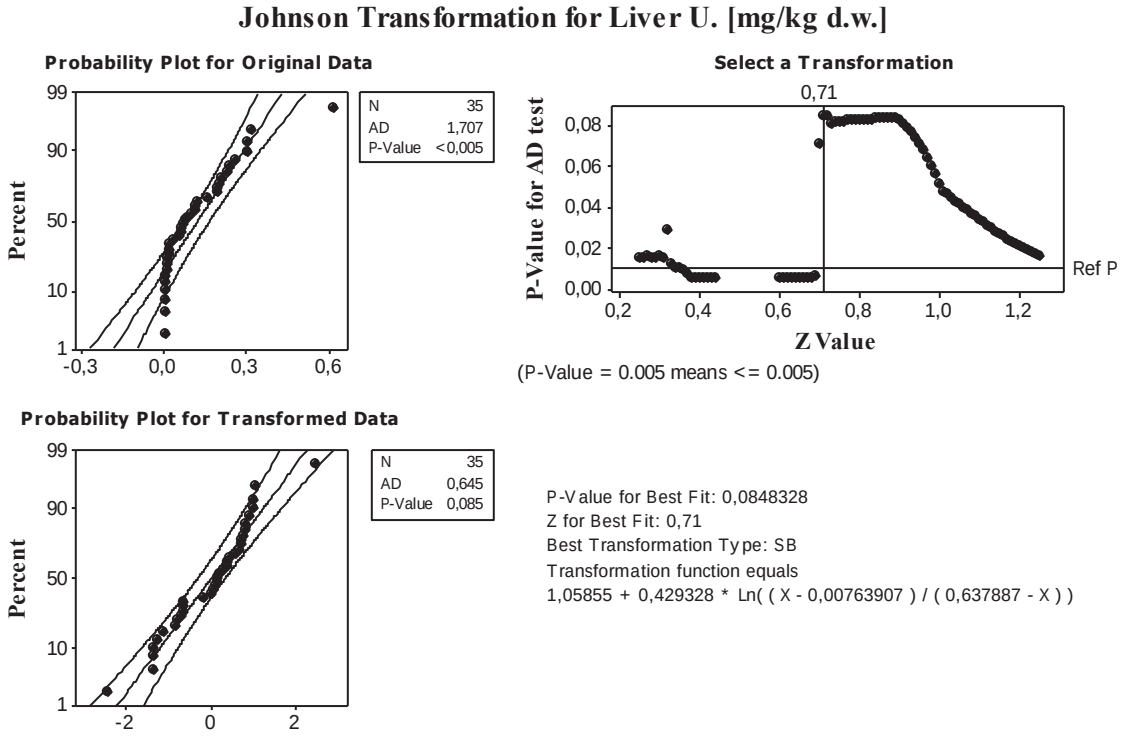
Minitab Operation

1. Select, **Stat**> **QualityTool**> **Individual Distribution Identification**> **Single Column** (Liver U mg/kg dw)

Box-Cox Transformation Probability plot for Liver U Concentration



Johnson Transformation Probability plot for Liver U concentration



The probability plot and associated Anderson-Darling Test after Johnson transformation (AD=0.645 and p-value=0.085) indicate that the transformed data fit a normal distribution reasonably well.

Descriptive Statistics

N	N*	Mean	StDev	Median	Minimum	Maximum	Skewness	Kurtosis
35	7	0,123698	0,130541	0,0774970	0,00784	0,613669	1,77128	4,42480

Box-Cox transformation: Lambda = 0

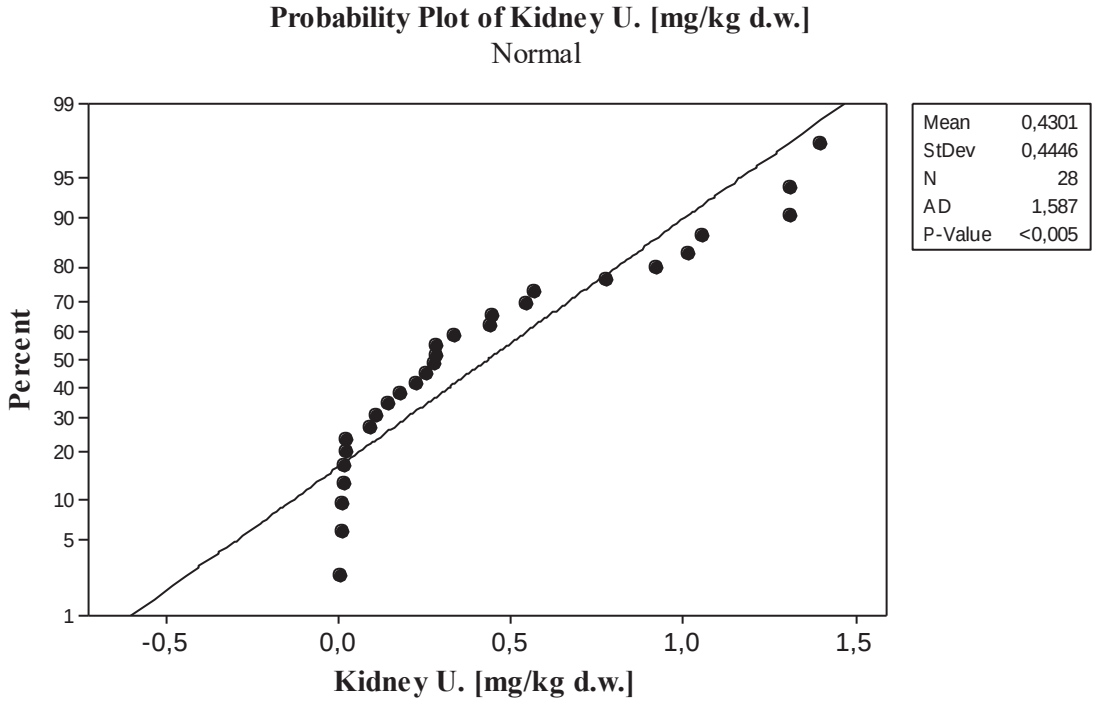
Johnson transformation function:
1,05855 + 0,429328 * Ln((X + 0,00763907)/(0,637887 - X))

KIDNEY

Appendix 3.3. Normality Test Probability Plot for Kidney Tissue

Minitab Operation

1. Select, **Stat >Basic Statistics >Normality >Variable (Kidney U. [mg/kg dw])**

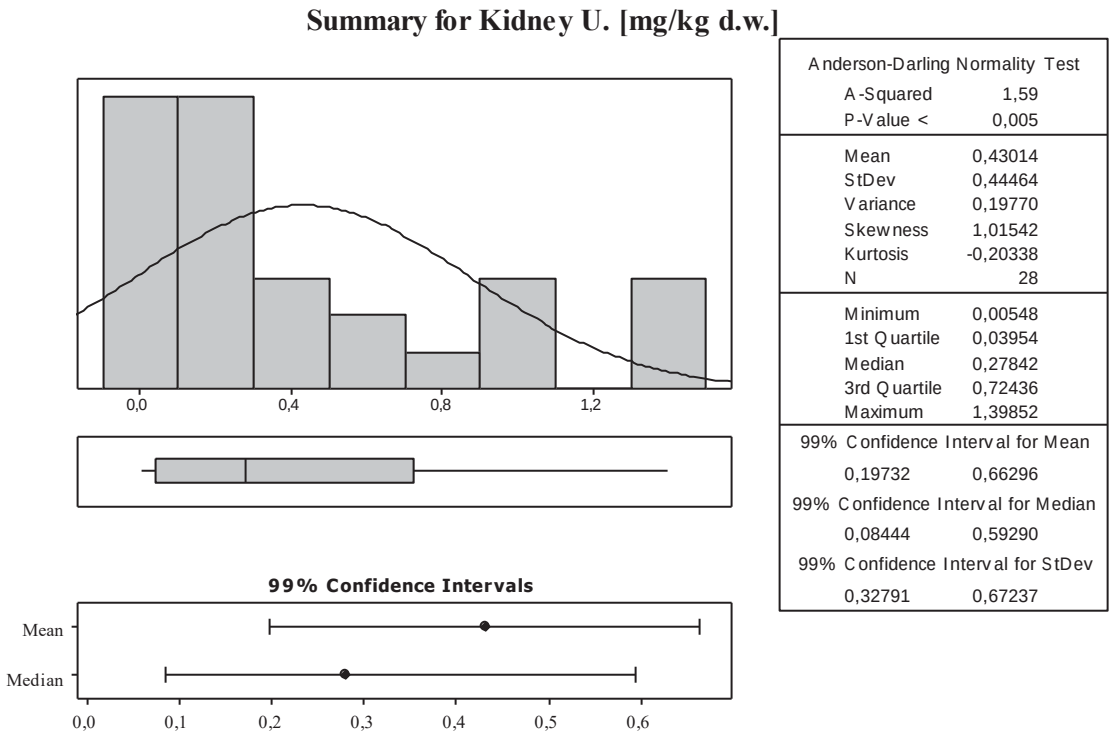


From the above plot, p-value <0.005 << 0.01 = α, suggest the data is not normal.

Appendix 3.3.1. Summary Plot for Kidney U concentration

Minitab Operation

1. Select, **Stat > Basic Statistics> Graphical Summary> Variable (Kidney U. [mg/kg dw])**



Anderson-Darling Normality Test	
A-Squared	1,59
P-Value <	0,005
Mean	0,43014
StDev	0,44464
Variance	0,19770
Skewness	1,01542
Kurtosis	-0,20338
N	28
Minimum	0,00548
1st Quartile	0,03954
Median	0,27842
3rd Quartile	0,72436
Maximum	1,39852
99% Confidence Interval for Mean	
0,19732	0,66296
99% Confidence Interval for Median	
0,08444	0,59290
99% Confidence Interval for StDev	
0,32791	0,67237

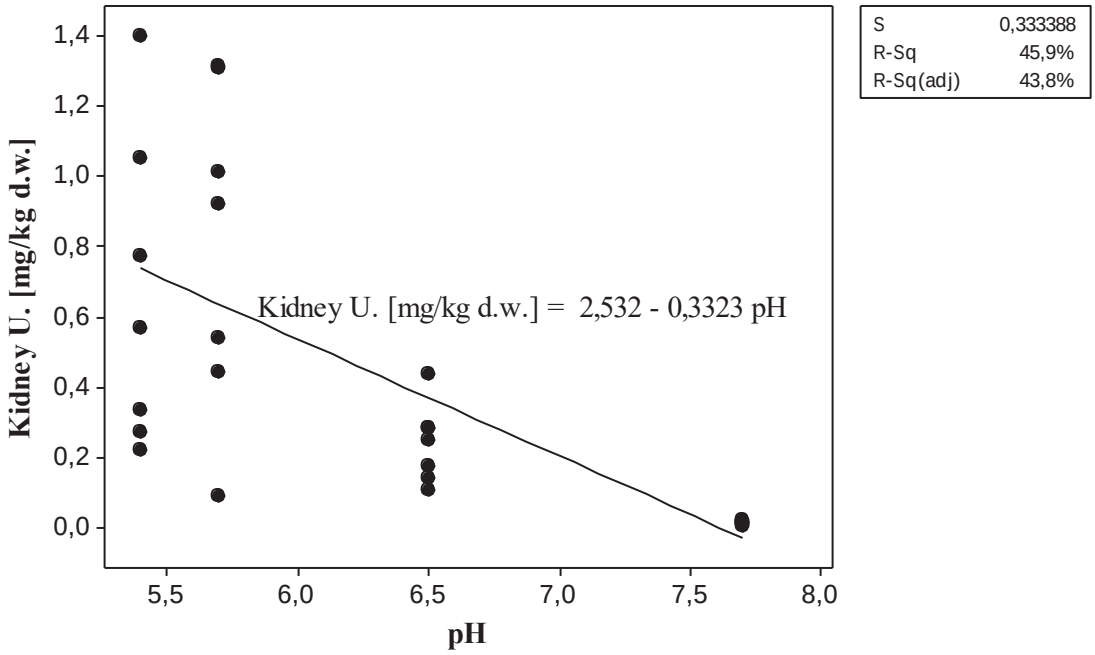
The above output shows that though the data does not seem normal as per the p-value =α, it is also skewed to the right.

Appendix 3.3.2. Fitted Line Plot for Kidney U concentration

Minitab Operation

1. Select, **Stat > Regression> Fitted Line Plot > Variable**

2. Select, **Response (Y): Kidney U. [mg/kg dw] and Predictor (X): pH**



Regression Analysis: Kidney U.[mg/kg dw] versus Water quality (pH)

The regression equation is

Kidney U. [mg/kg d.w.] = 2,53 - 0,332 pH

Predictor	Coef	SE Coef	T	P
Constant	2,5318	0,4522	5,60	0,000
pH	-0,33229	0,07080	-4,69	**0,000**

S = 0,333388 R-Sq = 45,9% R-Sq(adj) = **43,8%**

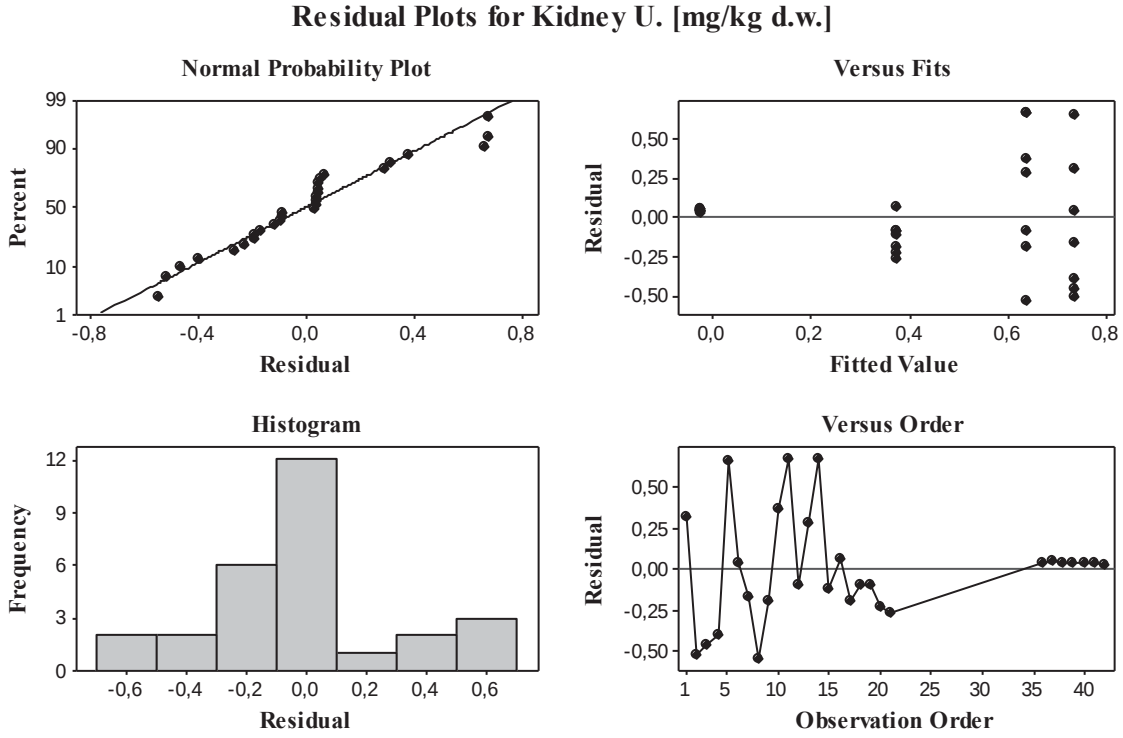
Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	2,4481	2,4481	22,03	**0,000**
Residual Error	26	2,8898	0,1111		
Total	27	5,3380			

Appendix 3.3.3. Residual Value Plots of Kidney U concentration

1. Select, **Stat> Regression> Regression> Graph> Residual Plots> Four in One > Ok**

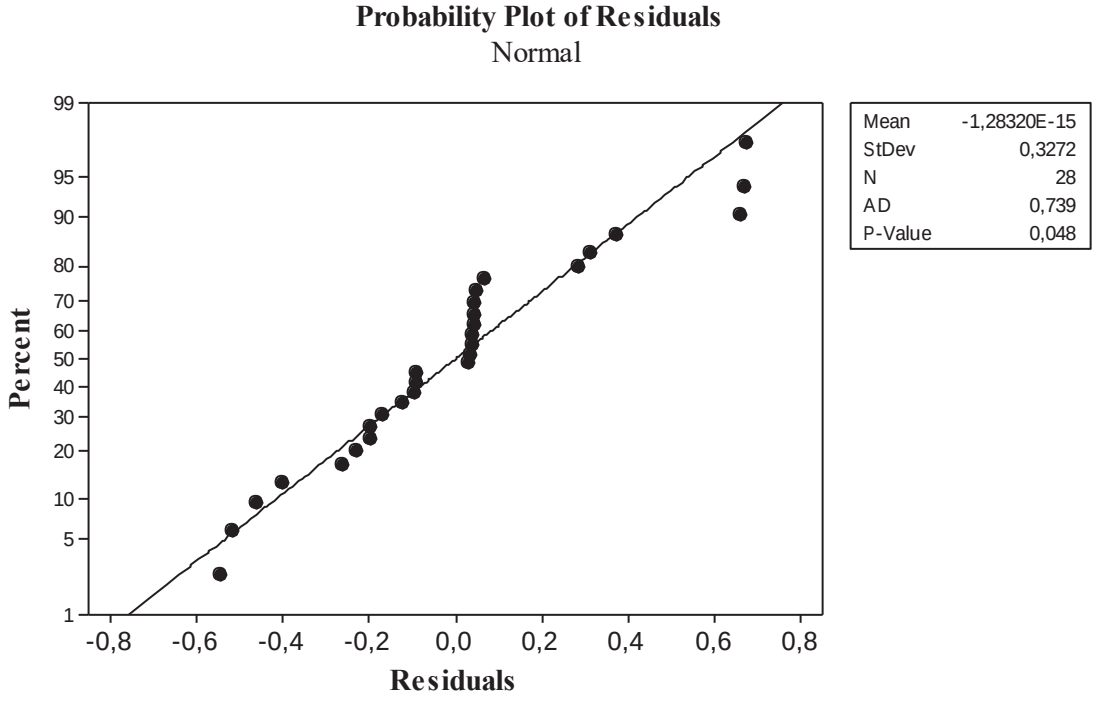
2**. Select, Response : (Kidney U. [mg/kg dw]) , Predictor : pH**



Appendix 3.3.4. Probability Plot of Residuals

Minitab Operation

1. Select, **Stat> Basic Statistics> Normality Test > Variable (Residuals: RESI 1)**



From the **p-value = 0.05 > 0.01=α** it can be concluded the residuals seem to be normally distributed. Hence, suggesting the data for kidney is normal.

Appendix 4.0. General Descriptive Statistics of U concentration in Tissues and fish sizes

Descriptive Statistics: Gill U. [mg/; Liver U. [mg; Kidney U. [m; ...

Variable	pH	Total Count	N	N*	Mean	StDev	Minimum	Maximum
Gill U. [mg/kg dw]	6,5	7	7	0	20,29	3,90	14,00	26,00
	6,8	7	7	0	16,43	2,76	13,00	21,00
	7,2	7	7	0	10,93	4,57	6,30	20,00
	7,7	7	7	0	3,214	0,811	1,900	4,300
Liver U. [mg/Kg dw]	5,4	7	7	0	1,118	0,439	0,649	1,677
	5,7	7	7	0	1,166	0,449	0,470	1,892
	6,5	7	7	0	0,554	0,299	0,215	0,965
	7,2	7	7	0	0,1322	0,1653	0,0200	0,5024
	7,7	7	7	0	0,1286	0,0725	0,0705	0,2677
Kidney U. [mg/kg dw]	6,5	7	7	0	0,2401	0,1110	0,1080	0,4375
	6,8	7	0	7	*	*	*	*
	7,2	7	0	7	*	*	*	*
	7,7	7	7	0	0,01449	0,00561	0,00548	0,02176
Length (cm)	6,5	7	7	0	11,714	0,888	10,800	13,200
	6,8	7	7	0	12,557	0,613	11,700	13,300
	7,2	7	7	0	12,086	0,992	10,900	13,200
	7,7	7	7	0	12,700	1,036	10,500	13,600
Weight (g)	6,5	7	7	0	13,12	2,91	10,44	17,90
	6,8	7	7	0	15,786	2,476	12,500	18,800
	7,2	7	7	0	14,87	3,73	10,20	19,32
	7,7	7	7	0	16,28	4,36	9,47	20,94

Asterix (*) denote missing data

Appendix 5.0. One-Way ANOVA (Analysis of Variance)

Appendix 5.1. Problem Description

In this experiment, five treatment groups were set up in water quality of different pH but with the same nominal concentration of 1.0 mg U/L. So, fish were exposed to nominal 1.0 mg U/L at pH 6.5 (Reference water), pH 5.4, pH 5.7, pH 6.8, pH 7.2 and pH 7.7. In each of the experimental tanks, 7 fish were exposed and extracted after 96hr exposure. Gill, Liver and Kidney tissues were extracted from the fish and accumulated uranium levels determined. In lieu of the objectives and hypothesis stated in the main introduction, the main questions to answer within this experiment to form the basis for any analysis are:

i. What is the effect of water quality (pH) on Uranium levels in the sampled fish tissues-gill, liver and kidney?
ii. Which water quality contributes the most uranium in each sampled tissue?
iii. Are there significant statistical differences in tissue U accumulation between water quality groups?
iv. Is there a correlation between water quality and Uranium levels measured in extracted tissues?

For one-way ANOVA we have one dependent or response variable and one independent variable [predicting factor] which has at least two levels. From the above problem description, we have four different levels or groups of pH as the predictor factor and one response variable which is U concentration in sampled tissues (gill, liver and kidney). So, the problem description suits a one-way ANOVA statistical analysis.

Appendix 6.0. Gill Tissue U Concentration (mg/kg dw)

Gill U.[mg/kg d.w.] determined					
pH 5.4	**pH 5.7**	**pH 6.5**	**pH 6.8**	**pH 7.2**	**pH 7.7**
65	27	26	15	8.4	3.8
40	48	19	18	7.8	2.8
69	47	14	18	20	1.9
49	53	19	13	11	3.1
69	38	24	21	10	3.8
66		21	14	13	2.8
52	41	19	16	6.3	4.3

The above data was entered in Minitab 16 Statistical software such that the response variable was in one column and the factor in a separate column. The data was simply stacked as in Appendix 2 (e.g. C1: Gill U; C2: pH).

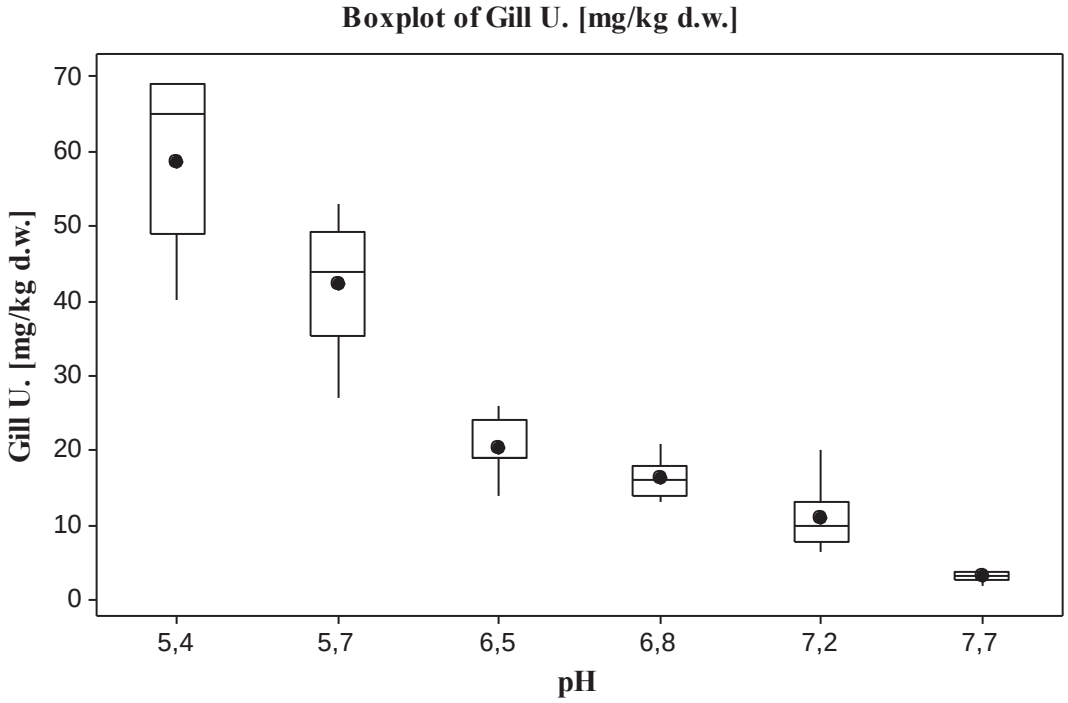
Where, C1=Colum 1, C2= Column 2. **N/B:** Same was done for the liver and kidney data.

ANOVA was performed by assessing the necessary assumptions of normality and equal variance. To do this there is need to create box plots and normal plots.

Appendix 6.1. Boxplot of Gill U concentration (mg/kg dw)

1. Select the tab: **Graph** > **Boxplot** > "**with groups**" option.

2. Select the appropriate variables. The *graph variable* is the dependent variable [Gill U. (mg/kg dw)] and the *categorical variable* for grouping is the independent variable, which is pH.

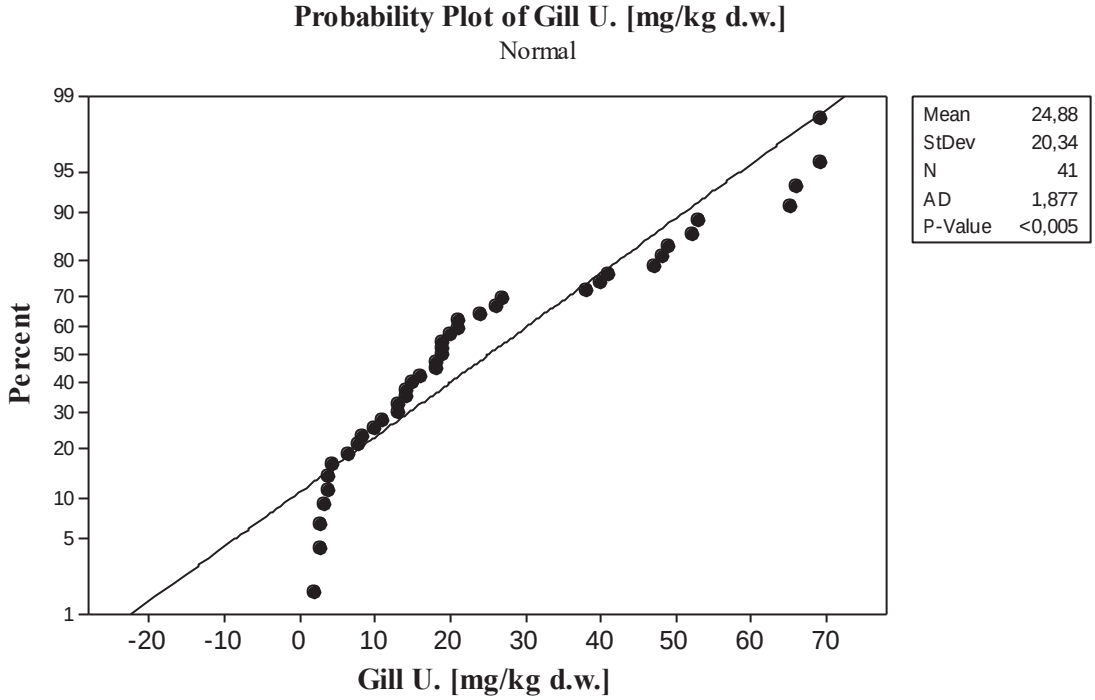


Appendix 6.2. Normality Probability Plot of Gill U concentration (mg/kg dw)

1. Select **Graph** > **Probability plot**

2. The graph variable is [Gill U. (mg/kg dw)], which is the dependent measure.

3. Select **OK**

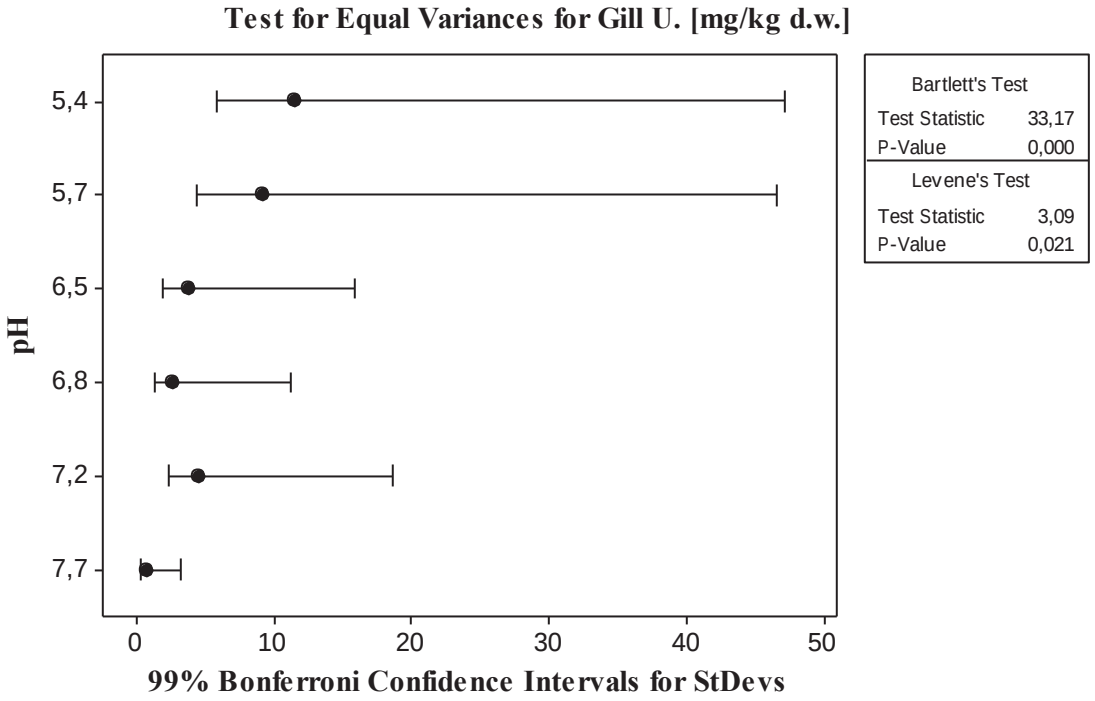


Appendix 6.3. Plot of Equal Variance Test for Gill U concentration (mg/kg dw)

1. Select **Stat** > **ANOVA** > **Test of Equal Variances**

2. **Again**, select variables- response variable: **[Gill U. (mg/kg dw)],** factor: **pH**

3. Select **Ok**



H_0: samples have equal variances

H_1: at least one of the sample variances is significantly different, at **α**=0.01

Since the data for [Gill U. (mg/kg dw)] is normally distributed, based on residual plots, we look at the Bartlett's Test. The Bartlett's Test p-value = 0.00 < 0.01, so we reject the null hypothesis (**H_0**), and we cannot conclude homogeneity in variances. Therefore, there is a significant difference in at least one of the variances of the samples.

Appendix 6.4. Multiple Comparison of Gill U concentration in different pH groups

Post hoc Tests

Since the null hypothesis is rejected and at least one of the means is different from the others, we wish to find out how different they are.

1. **Stat** > **ANOVA** > **One Way**

2. Select **Comparisons**

3. Select the first option, **Turkey's family error rate**

For pair wise comparison, if intervals do not include zero (0), then, it means they are significantly different ($p < 0.01$). Thus, mean concentration of U in gills is significantly different in the different pH groups. One way ANOVA is used to see whether accumulation of U in gills is dependent on changes in pH.

One-way ANOVA: Gill-U (mg/kg d.w.) versus pH

```
Source  DF       SS      MS      F      P
pH       5  15069,6  3013,9  71,12  0,000
Error   35   1483,2    42,4
Total   40  16552,8

S = 6,510   R-Sq = 91,04%   R-Sq(adj) = 89,76%
```

```
                          Individual 99% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  --+---------+---------+---------+-------
5,4    7  58,571  11,502                                (--*---)
5,7    6  42,333   9,201                          (--*---)
6,5    7  20,286   3,904             (--*--)
6,8    7  16,429   2,760           (--*---)
7,2    7  10,929   4,565       (--*---)
7,7    7   3,214   0,811  (---*--)
                          --+---------+---------+---------+-------
                            0        20        40        60

Pooled StDev = 6,510
```

```
Grouping Information Using Tukey Method

pH   N    Mean  Grouping
5,4  7  58,571  A
5,7  6  42,333    B
6,5  7  20,286      C
6,8  7  16,429      C
7,2  7  10,929      C D
7,7  7   3,214        D
```

Means that do not share a letter are significantly different.

Tukey 99% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of pH

```
Individual confidence level = 99,92%


pH = 5,4 subtracted from:

pH     Lower   Center    Upper  ---------+---------+---------+---------+
5,7  -29,478  -16,238   -2,998             (--*---)
6,5  -51,006  -38,286  -25,565      (---*---)
6,8  -54,863  -42,143  -29,422     (---*---)
7,2  -60,363  -47,643  -34,922    (--*---)
7,7  -68,078  -55,357  -42,637  (--*---)
                                 ---------+---------+---------+---------+
                                        -35         0        35        70


pH = 5,7 subtracted from:

pH     Lower   Center    Upper  ---------+---------+---------+---------+
6,5  -35,288  -22,048   -8,808          (---*--)
6,8  -39,145  -25,905  -12,665         (---*--)
7,2  -44,645  -31,405  -18,165        (---*---)
7,7  -52,359  -39,119  -25,879      (---*---)
                                 ---------+---------+---------+---------+
                                        -35         0        35        70

pH = 6,5 subtracted from:

pH     Lower   Center   Upper  ---------+---------+---------+---------+
6,8  -16,578   -3,857   8,863               (---*---)
7,2  -22,078   -9,357   3,363              (--*---)
7,7  -29,792  -17,071  -4,351           (---*---)
                               ---------+---------+---------+---------+
                                      -35         0        35        70


pH = 6,8 subtracted from:

pH     Lower   Center   Upper  ---------+---------+---------+---------+
7,2  -18,221   -5,500   7,221               (--*---)
7,7  -25,935  -13,214  -0,494             (--*---)
                               ---------+---------+---------+---------+
                                      -35         0        35        70


pH = 7,2 subtracted from:

pH     Lower  Center  Upper  ---------+---------+---------+---------+
7,7  -20,435  -7,714  5,006             (---*--)
                             ---------+---------+---------+---------+
                                    -35         0        35        70
```

Based on the above output display, some intervals for pair wise comparison between all pH groups contain zero. There are observed significant differences in mean gill U accumulation between pH < 6 and pH {6.5 - 7.7}.

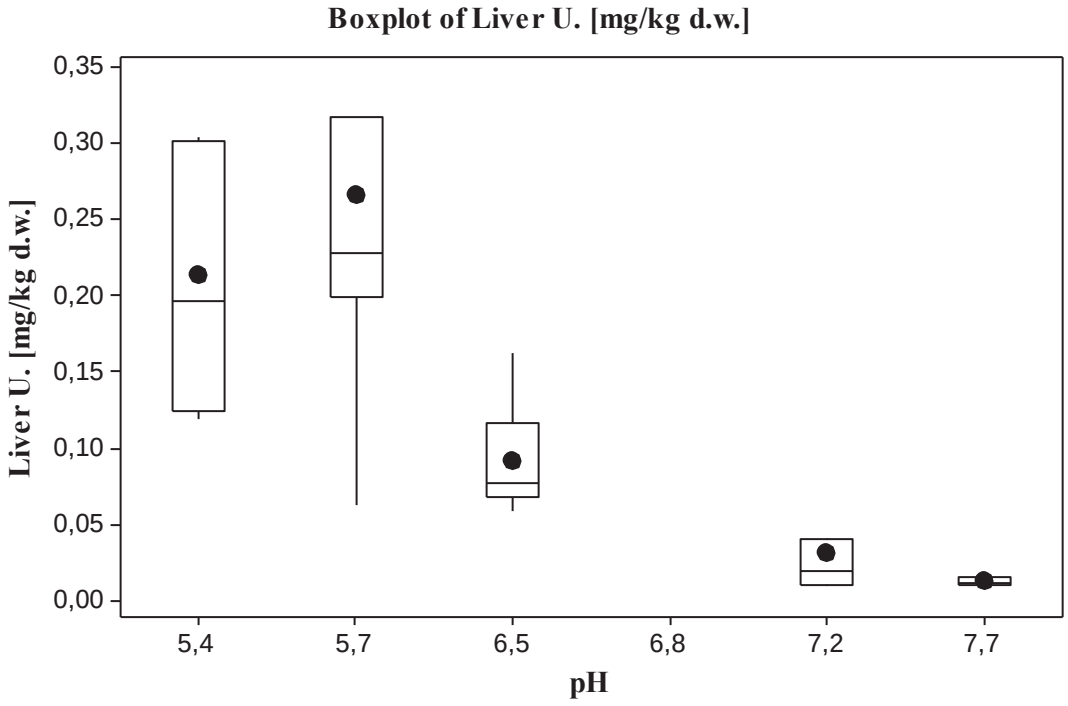
Appendix 7.0. Liver Tissue U Concentration (mg/kg dw)

Liver U.[mg/kg dw] determined					
pH 5.4	**pH 5.7**	**pH 6.5**	**pH 6.8**	**pH 7.2**	**pH 7.7**
0,302	0,062	0.076	*	0.02	0.011
0,124	0,237	0.060	*	0.09	0.018
0,195	0,211	0.085	*	0.50	0.016
0,120	0,614	0.117	*	0.06	0.008
0,305	0,200	0.163	*	0.08	0.010
0,260		0.077	*	0.07	0.016
0,197	0,318	0.068	*	0.10	0.012

Appendix 7.1. Boxplot of Liver U concentration

1. Select the tab: **Graph** > **Boxplot** > "**with groups**" option.

2. Select the appropriate variables. The *graph variable* is the dependent variable [Liver U. (mg/kg dw)] and the *categorical variable* for grouping is the independent variable, which is pH.

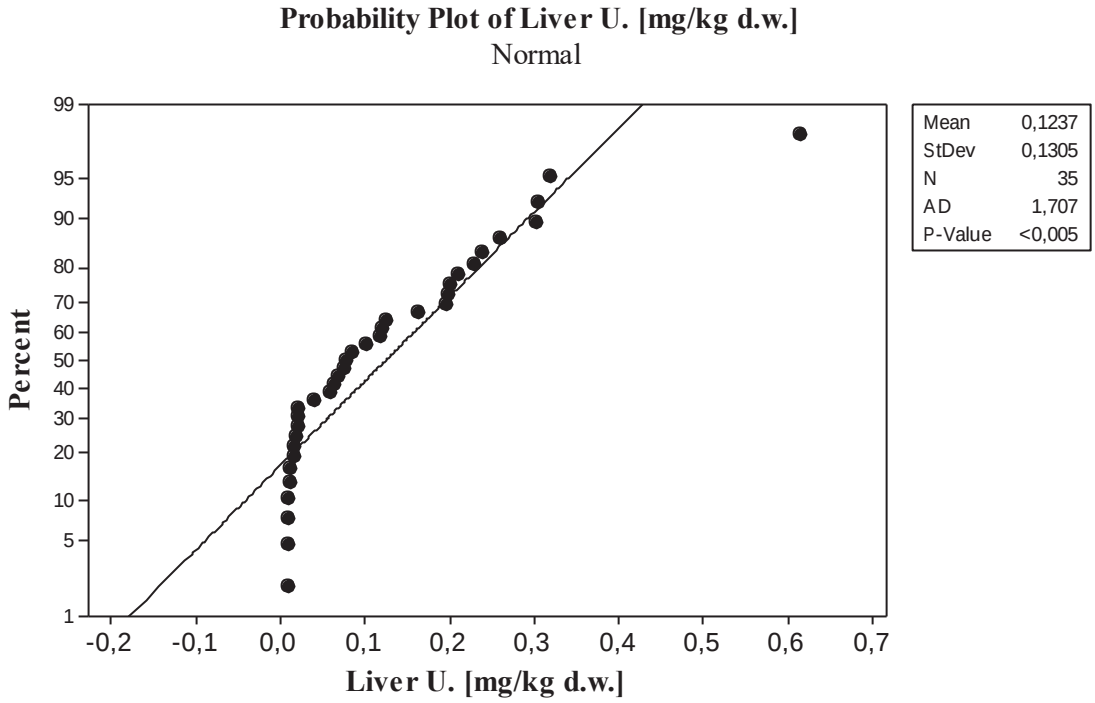


Appendix 7.2. Normality Probability Plot of Liver U concentration (mg/kg dw)

1. Select **Graph** > **Probability plot**

2. The graph variable is [Liver U. (mg/kg dw)], which is the dependent measure.

3. Select **OK**



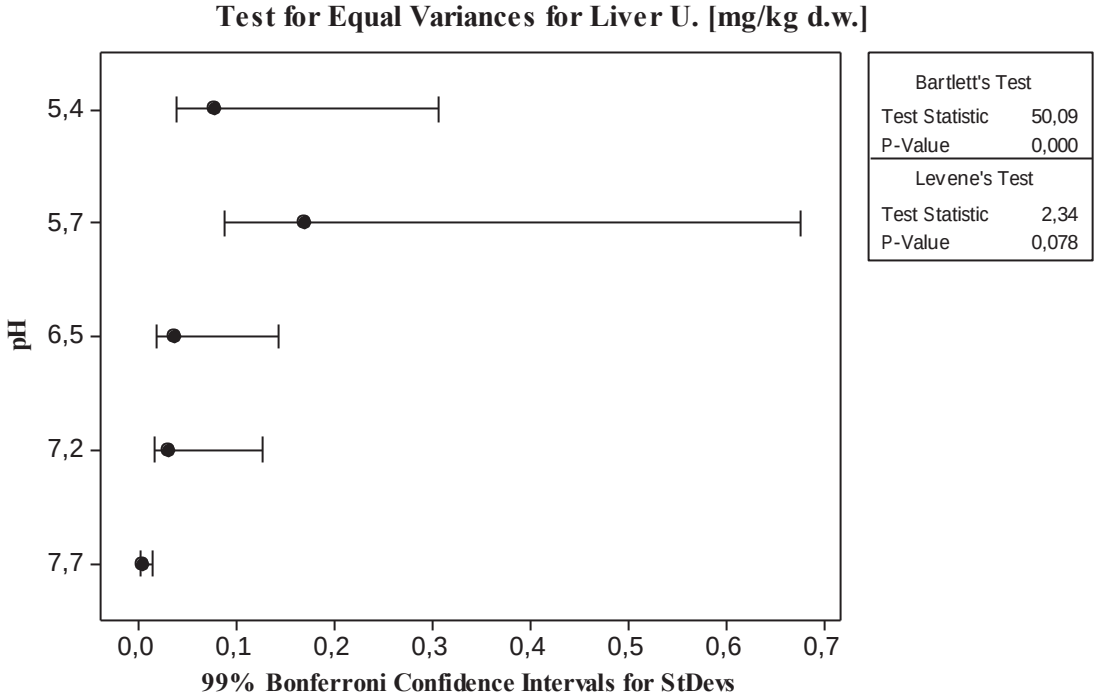
The p-value < 0.005 <0.01 = α, suggests the data is not normally distributed.

Appendix 7.3. Plot of Equal Variance Test for Liver U concentration (mg/kg dw)

1. Select **Stat** > **ANOVA** > **Test of Equal Variances**

2. Again, select variables- response variable: **[Liver U. (mg/kg dw)]**, factor: **pH**

3. Select **Ok**



H_0: Liver samples have equal variances

H_1: at least one of the Liver sample variances is significantly different, at **α**=0.01

Since the data for [Liver U. (mg/kg dw)] is normally distributed, we look at the Levene's Test. The Levene's Test p-value = 0.08 > 0.01, so we reject the null hypothesis (**H_0**), and we cannot conclude that there is no significant difference in the variance of the samples.

Appendix 7.4. Multiple Comparison of Liver U concentration in different pH groups

Post hoc Tests

We wish to find out if at least one of the means is different from the others,

1. **Stat > ANOVA > One Way**

2. Select Comparisons

3. Select the first option, **Turkey's family error rate**

One-way ANOVA: Liver U.[mg/kg dw] versus pH

Source	DF	SS	MS	F	P
pH	4	7,211	1,803	17,46	**0,000**
Error	30	3,098	0,103		
Total	34	10,309			

S = 0,3214 R-Sq = 69,95% R-Sq(adj) = **65,94%**

Individual 99% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	----+---------+---------+---------+-----
5,4	7	1,1183	0,4388	(-----*------)
5,7	7	1,1660	0,4491	(-----*------)
6,5	7	0,5538	0,2992	(------*------)
7,2	7	0,1322	0,1653	(------*-----)
7,7	7	0,1286	0,0725	(------*-----)
				----+---------+---------+---------+-----
				0,00 0,50 1,00 1,50

Pooled StDev = 0,3214

Grouping Information Using Tukey Method

pH	N	Mean	Grouping
5,7	7	1,1660	A
5,4	7	1,1183	A
6,5	7	0,5538	A B
7,2	7	0,1322	B
7,7	7	0,1286	B

Means that do not share a letter are significantly different.

Tukey 99% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of pH

```
Individual confidence level = 99,88%


pH = 5,4 subtracted from:

pH     Lower   Center    Upper  -------+---------+---------+---------+--
5,7  -0,5657   0,0476   0,6610             (-----*------)
6,5  -1,1779  -0,5646   0,0488       (-----*-----)
7,2  -1,5994  -0,9861  -0,3727   (-----*-----)
7,7  -1,6031  -0,9897  -0,3764   (-----*-----)
                                -------+---------+---------+---------+--
                                    -1,0       0,0       1,0       2,0


pH = 5,7 subtracted from:

pH     Lower   Center    Upper  -------+---------+---------+---------+--
6,5  -1,2256  -0,6122   0,0012       (-----*-----)
7,2  -1,6471  -1,0337  -0,4203   (-----*-----)
7,7  -1,6508  -1,0374  -0,4240  (------*-----)
                                -------+---------+---------+---------+--
                                    -1,0       0,0       1,0       2,0


pH = 6,5 subtracted from:

pH     Lower   Center   Upper  -------+---------+---------+---------+--
7,2  -1,0349  -0,4215  0,1919        (-----*-----)
7,7  -1,0385  -0,4252  0,1882        (-----*-----)
                                -------+---------+---------+---------+--
                                    -1,0       0,0       1,0       2,0


pH = 7,2 subtracted from:

pH     Lower   Center   Upper  -------+---------+---------+---------+--
7,7  -0,6170  -0,0037  0,6097             (-----*-----)
                                -------+---------+---------+---------+--
                                    -1,0       0,0       1,0       2,0
```

Based on the above output display, since not all intervals for pair wise comparison between all pH groups contain zero, there is therefore some significant difference in liver U accumulation between pH < 6 and pH > 7.

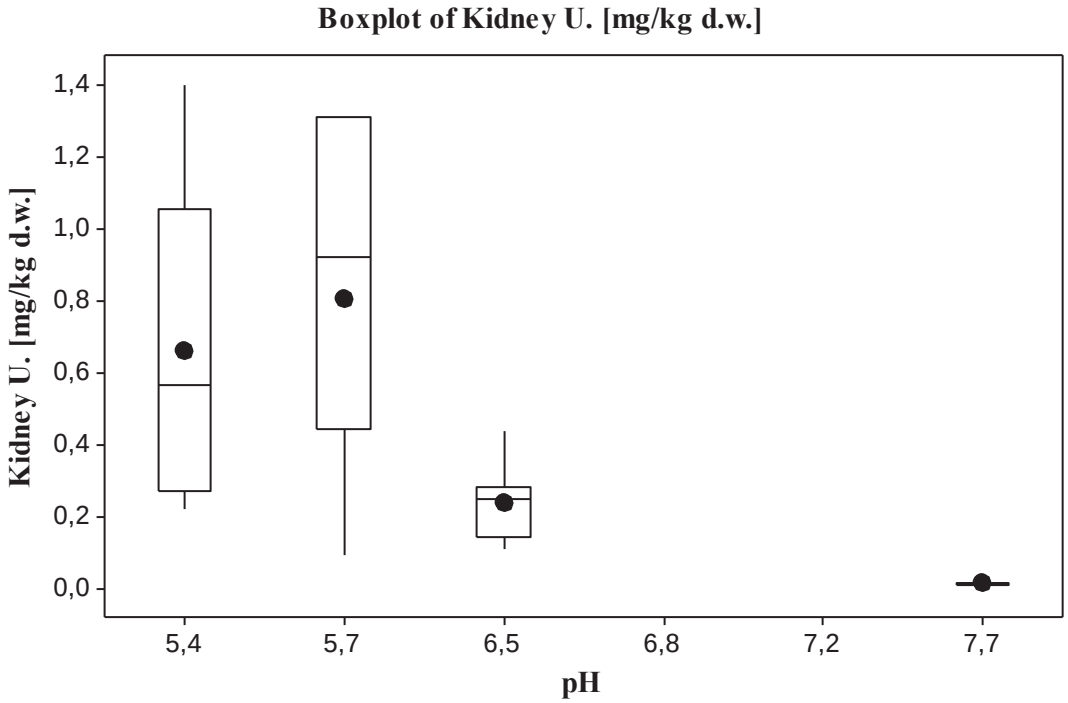
Appendix 8.0. Kidney Tissue U Concentration (mg/kg dw)

Kidney U.[mg/kg dw] determined			
pH 5.4	**pH 5.7**	**pH 6.5**	**pH 7.7**
1.052	0.093	0.251	0.010
0.222	0.442	0.438	0.022
0.274	1.012	0.177	0.019
0.334	1.312	0.283	0.012
1.399	0.544	0.283	0.017
0.776	0.924	0.142	0.016
0.568	1.310	0.108	0.005

Appendix 8.1. Boxplot of Kidney U concentration (mg/kg dw)

1. Select the tab: **Graph** > **Boxplot** > "**with groups**" option.

2. Select the appropriate variables. The *graph variable* is the dependent variable [Kidney U. (mg/kg dw)] and the *categorical variable* for grouping is the independent variable, which is pH.

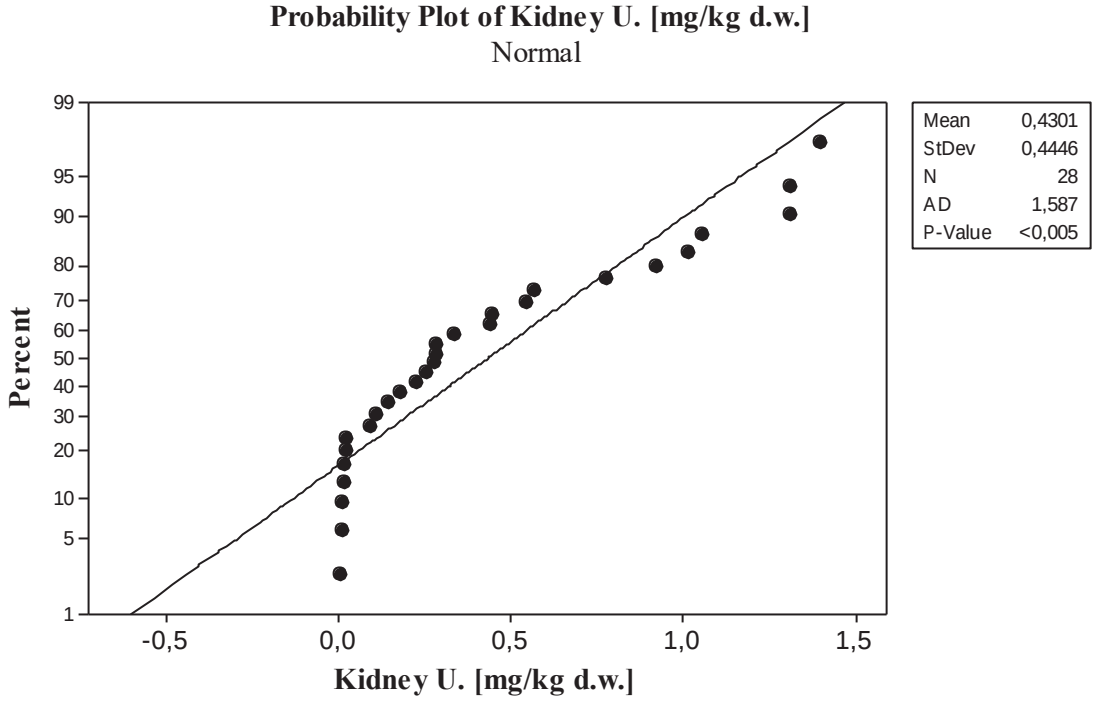


Appendix 8.2. Normality Probability Plot of Kidney U concentration (mg/kg dw)

1. Select **Graph** > **Probability plot**

2. The graph variable is [Kidney U. (mg/kg dw)], which is the dependent measure.

3. Select **OK**



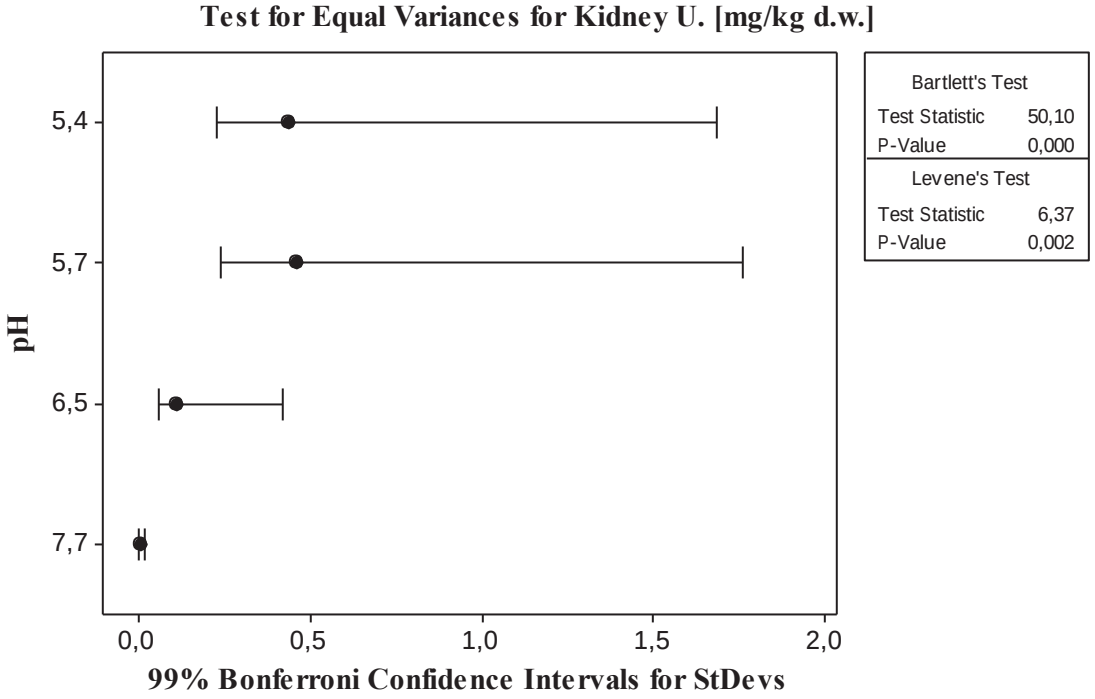
The p-value < 0.005 <0.01= α, suggests the data is not normal. However, from probability plots of residuals for normality, the data is normal. So when we perform the homogeneity of variance test, we will use the Bartlett's test.

Appendix 8.3. Plot of Equal Variance Test for Kidney U concentration (mg/kg dw)

1. Select **Stat** > **ANOVA** > **Test of Equal Variances**

2. Again, select variables- response variable: [Kidney U. (mg/kg dw)], factor: pH

3. Select Ok



H_0: samples have equal variances

H_1: at least one of the sample variances is significantly different, at **α**=0.01

The Bartlett's Test p-value = 0.00 < 0.01, so we reject the null hypothesis (**H_0**), and say there is a significant difference in at least one of the sample variances.

Appendix 8.4. Multiple Comparison of Kidney U concentration in different pH groups

Post hoc Tests

Since the null hypothesis is rejected and at least one of the means is different from the others, we wish to find out how different they are.

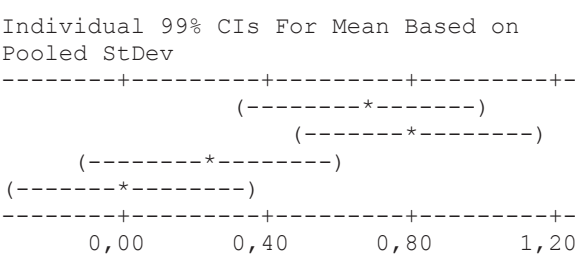
1. **Stat > ANOVA > One Way**

2. Select Comparisons

3. Select the first option, **Turkey's family error rate**

One-way ANOVA: Kidney U. [mg/kg d.w.] versus pH

```
Source  DF     SS     MS     F      P
pH       3  2,819  0,940  8,96  0,000
Error   24  2,519  0,105
Total   27  5,338

S = 0,3239   R-Sq = 52,82%   R-Sq(adj) = 46,92%
```




```
Pooled StDev = 0,3239
```

```
Grouping Information Using Tukey Method

pH   N    Mean  Grouping
5,7  7  0,8053  A
5,4  7  0,6607  A
6,5  7  0,2401  A B
7,7  7  0,0145    B
```

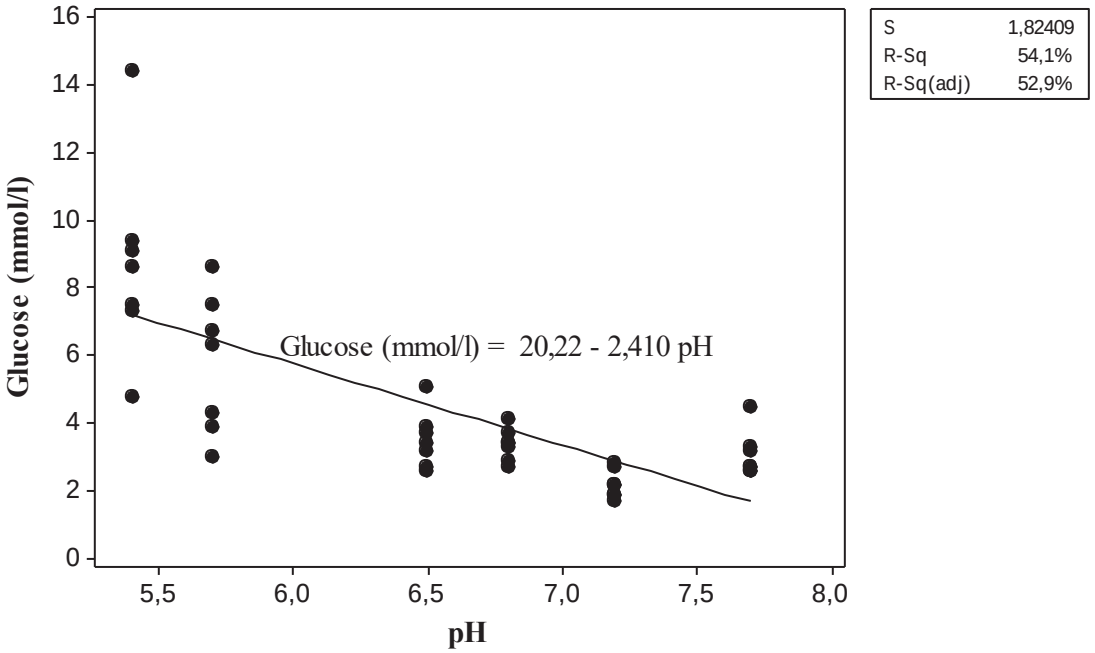
Means that do not share a letter are significantly different.

Tukey 99% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of pH

```
Individual confidence level = 99,80%


pH = 5,4 subtracted from:

pH     Lower   Center    Upper     +---------+---------+---------+---------
5,7  -0,4567   0,1445   0,7457                (--------*--------)
6,5  -1,0218  -0,4206   0,1806          (--------*--------)
7,7  -1,2474  -0,6462  -0,0450       (--------*-------)
                                   +---------+---------+---------+---------
                                -1,40     -0,70      0,00      0,70


pH = 5,7 subtracted from:

pH     Lower   Center    Upper     +---------+---------+---------+---------
6,5  -1,1663  -0,5652   0,0360        (--------*--------)
7,7  -1,3920  -0,7908  -0,1896     (--------*-------)
                                   +---------+---------+---------+---------
                                -1,40     -0,70      0,00      0,70


pH = 6,5 subtracted from:

pH     Lower   Center   Upper     +---------+---------+---------+---------
7,7  -0,8268  -0,2256  0,3756             (--------*-------)
                                   +---------+---------+---------+---------
                                -1,40     -0,70      0,00      0,70
```

Based on the above output display, since not all intervals for pair wise comparison between all pH groups contain zero, there is therefore some significant difference in kidney U accumulation between pH < 6 and pH > 7.

Appendix 9.0. Stress Response



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