Effect of long-term exposure to oxazepam on whole-body cortisol concentration in three-spined stickleback (*Gasterosteus aculeatus*)

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Innehållsförteckning

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Abstract
Pharmaceuticals are common contaminants in aquatic ecosystems. Pharmaceutical residues in aquatic systems have gained increased interest the last decades and are now recognized as a major threat to aquatic ecosystems around the globe. Benzodiazepines (BZDs) are a class of psychiatric pharmaceuticals classified as anxiolytics, i.e. pharmaceuticals used to treat anxiety disorders. A study reports that dilute concentrations of the BZD oxazepam influences fish behavior, which potentially can damage ecosystems. The objective of this study was to investigate the effect of a 7-day exposure to the benzodiazepine oxazepam (1 or 100 μg/L tank water) on whole-body cortisol concentration in three-spined stickleback (Gasterosteus aculeatus). Cortisol concentrations were measured using RIA (Radioimmunoassay). Our results show that the highest oxazepam concentration (100 μg/L) we tested displayed a significantly reduced cortisol concentrations compared to controls when analyzed separately. But when fish were exposed to the lower concentration of oxazepam (1 μg/L), an effect on cortisol levels was not shown. After all, our results show that oxazepam have no effect on cortisol levels and therefore will not influence the behavior of three-spined stickleback regarding cortisol. Our result contradicts several earlier studies and it is imperative to do further studies regarding this manner using other protocols. The complexity of how benzodiazepines affect aquatic life and ecosystems remains largely unknown and environmental predictions are difficult to make. However, it is necessary to study how pharmaceuticals potentially can affect aquatic life because of its alarming effects.
1. Introduction

Pharmaceuticals are common contaminants in aquatic ecosystems. Pharmaceutical residues in aquatic systems have gained increased interest the last decades and are now recognized as a major threat to aquatic ecosystems around the globe (Boxall et al. 2012, Brodin et al. 2013). Psychiatric pharmaceuticals are among the most prescribed active substances in the world (Calisto & Esteves 2009). Benzodiazepines (BZDs) are a class of psychiatric pharmaceuticals classified as anxiolytics, i.e. pharmaceuticals used to treat anxiety disorders. BZDs are not completely metabolized by the human body (Heberer 2002, Brodin et al. 2014) and therefore the main pathway for BZD to enter aquatic systems is via excretion by patients (Calisto & Esteves 2009). Once BZDs are present in the aquatic environment they are usually biochemically active for a long period of time. This is because BZD generally are persistent against degradation, such as photodegradation (Calisto et al. 2011).

BZDs increase the effect of the endogenous neurotransmitter gamma-aminobutyric acid (GABA), which is the major inhibitory neurotransmitter in the central nervous system. BZDs bind to GABA_A receptors resulting in hyperpolarization of the neuron through inflow of chloride ions through the GABA receptors’ central pore (Sieghart 1995, Sieghart et al. 2012). The GABA_A receptor is evolutionary conserved throughout the vertebrates, which means that BZD has a physiological effect on many organisms besides humans (Gunnarsson et al. 2008). A recent study by Brodin et al. (2012) shows that dilute concentrations of the BZD oxazepam influences European perch (Perca fluviatilis) behavior, and that it possibly may affect the ecology and evolution in aquatic systems over time, which is alarming. Concentrations of BZD in aquatic systems are usually below 1 µg/liter which is enough to alter gene expression and behavior of fish (Oggier et al. 2010, Brodin et al. 2013). Studies show that fish have several folds as much BZD in tissue compared to surrounding water, indicating that bioconcentration occurs (Heynen et al. 2016).

BZDs are recognized as an anxiolytic and affect the body in several ways. Their objective is to suppress the activity of neuroendocrine systems associated with anxiety and stress responses. One of the systems it affects in fish is the neuroendocrine axis controlling cortisol concentrations when exposed to a stressful situation, the hypothalamic-pituitary-interrenal (HPI) axis. Abreu et al. (2014) showed that fish acutely exposed to the benzodiazepine diazepam had lower cortisol response when submitted to an acute stress test. Cortisol is a corticosteroid that plays important roles during stress, and its plasma concentrations increase drastically during stress. Cortisol is a glucocorticoid and its physiological function is to increase blood glucose concentration and to increase mean blood pressure, which is vital when coping with stress and to be able to react to potential danger. Also, stress is an energy-demanding state where metabolic rate and oxygen uptake increases, where increased glucose concentrations is beneficial to sustain the stressed state. Although cortisol has an important role in stress it also has other vital housekeeping roles which should not be ignored when discussing cortisol, therefore disturbances in cortisol regulation can also negatively affect osmoregulation, growth and reproduction (Mommsen et al. 1999). Importantly the effect of BZD on cortisol concentrations has been shown to be strongly dependent on the concentration of BZD the fish has been exposed to, where low and medium BZD concentrations produced an effect on cortisol concentrations whereas the high BZD concentration was not different from the control (Abreu et al.). This phenomenon is commonly referred as U-shaped dose–response hypothesis (Calabrese & Baldwin 2001).

The release of cortisol is strictly regulated by the hypothalamus-pituitary-interrenal axis (HPI axis). The HPI axis describes the physiological response to a stressor, and is activated when fish are exposed to a stressor. In short, exposure to a stressor increases the release of corticotropin-releasing hormone (CRH), which in turn stimulates the anterior pituitary to release adrenocorticotropic hormone (ACTH). ACTH then stimulates the release of cortisol in the interrenal cells located in the head kidney. Typically, plasma cortisol levels rise rapidly within minutes after exposure to the
stressor. The negative feedback regulation of cortisol acts on all levels of the HPI axis, such as the inhibitory effect of high cortisol concentrations on CRH and ACTH production. This ensures that cortisol concentrations return to baseline after exposure to a modest stressor (Mommsen et al. 1999, Grone & Maruska 2015, Külerich et al. 2018). Exposure to BDZs can inhibit the activity of HPI-axis. Neurons in hypothalamus are hyperpolarized and therefore it is believed that less CRH is released and therefore less cortisol is produced (De Souza 1990, De Boer et al. 1990, Pivac & Perićić 1993, Imaki et al. 1995, Arvat et al. 1999).

The objective of this study was to investigate the effect of a 7-day exposure to the benzodiazepine oxazepam (1 or 100 ug/L tank water) on whole-body cortisol concentration in three-spined stickleback (Gasterosteus aculeatus). This is done to get a better understanding on how oxazepam affects fish physiologically and how aquatic ecosystems potentially can take damage from BZD effects on fish. And what consequences the release of oxazepam has in aquatic systems.

2. Method

Juvenile three-spined stickleback (Gasterosteus aculeatus) were caught at Vikhög harbor, Sweden (55°43'N 12°57'E) on 26 February 2017. The animals were initially kept at the Department of Zoology, Stockholm University, and were transferred to the Biomedical Center at Uppsala University on 20 July 2017 where they were kept in two 500L holding tanks with flow-through of tap water at ~16°C and under constant aeration. Animals were fed daily with frozen artemia (Gibbon, Sweden). Two weeks before the start of the experiment, fish were individually tagged with visible implant elastomer tags (Northwest Marine Technology, USA).

Exposure experiment

We used a static system for oxazepam treatment, consisting of 12 aerated 22L glass aquaria with 4 oxazepam concentrations (0, 1, 10 or 100 μg L⁻¹). On the first day of the experiment, behavioral testing was performed using a 15-minute long novel tank diving test, after which fish were transferred to exposure tanks until each treatment tank contained 20 fish. During the 8 days of treatment, animals were fed with frozen artemia daily, after which tanks were cleaned of feces.

Experimental design

On the 7th day of treatment, animals were tested for behavior in the novel tank diving test (15 min), and subsequent scototaxis test (15 min). On day 8 of the experiment, animals were individually caught from the treatment tank and exposed to confinement stress for 30 minutes in plastic beaker containing 100 mL water. Immediately thereafter, animals of were euthanized with an overdose of MS-222 and a cut through the spinal cord. For the animals from the tanks containing 0 and 100 μg L⁻¹, the brain was removed from the skull and dissected into 5 parts (telencephalon, olfactory bulbs, cerebellum, brain stem and the remaining midbrain), and the remainder of the head was cut off. These brain parts as well as the body were frozen on dry ice and stored at -80°C. Brain dissection was not performed for the animals exposed to 1 and 10 μg L⁻¹, instead the head was removed and the body was frozen on dry ice and stored at -80°C.

Cortisol extraction

Fish samples were stored at -20°C and taken out to thaw. 50 mL falcon tubes were used to each individual fish, and were weighed before insertion of fish sample. Samples were cut with scissors to small pieces (>1mm) before homogenisation in PBS buffer using a tissue homogenisator VWR CD112 with S12N-7S rotor (VWR International, Sweden). Cortisol was extracted from the tissue homogenate by adding ethyl acetate, after which samples were centrifuged at 1000g for 5 minutes. The supernatant was transferred into a borosilicate glass tube (VWR, Sweden) and the ethyl acetate was removed by evaporation using a nitrogen gas sparge with heat block at 40°C (Bibby Scientific, USA). Ultimately samples were reconstituted in 2400 μl ethyl acetate.
Chemical analysis using radioimmunoassay (RIA)

For detailed method, see attached protocol in appendix. The technique that was used to analyze cortisol concentration of each fish sample was radioimmunoassay (RIA). RIA is a common method to measure concentration of substances, cortisol in this case. By using a standard curve using a dilution series of cortisol we can determine the cortisol concentration in analyzed sample. A scintillation machine (Tri-Carb 2910TR®, PerkinElmer, USA) was used to perform RIA and data was registered by a software (Quantasmart™, PerkinElmer, USA). Count time of scintillation machine was 5 min/sample.

Statistical analysis

To determine if cortisol response is influenced by different concentrations of oxazepam, we analyzed the data using R (R Development Core Team, 2018), using packages ‘lme4’ (Bates et al., 2015;) and ‘lmerTest’ (Kuznetsova A, Brockhoff PB, Christensen RHB, 2017) to fit a linear mixed-effects model (LMM) to the data. We included oxazepam concentration, time of day caught, weight of the fish before dissection and all interactions as fixed factors, and sample and tank as random factors. Because of the high complexity of this LMM, the model is simplified using backward step-wise selection (step function in lmerTest; ref), which resulted in a minimum adequate model (MAM) with oxazepam concentration and weight as fixed factor, and sample as random factor.

3. Results

As shown in table 1 and figure 1, the minimum adequate LMM revealed a close to significant effect of oxazepam treatment on whole-body concentrations of cortisol in stickleback. However, there is a significance effect of the weight of fish after dissection.

Visual inspection of the differences between replicate tanks revealed that the groups exposed to 1 μg/L differed strongly between replicates (figure 2). If this concentration is removed from the model and only control and fish exposed to 100 μg/L is considered, oxazepam treatment had a significant lowering effect on whole-body cortisol concentration after confinement stress (table 2, p=0.032). On average, oxazepam treated fish have whole body cortisol concentrations 0.114 ng/mg fish which is lower than in control treated animals.

Table 1
The effect of treatment (0, 1 μg/L or 100 μg/L) and bodyweight on cortisol levels (ng/mg fish).

<table>
<thead>
<tr>
<th></th>
<th>Sum Sq</th>
<th>Mean Sq</th>
<th>NumDF</th>
<th>DenDF</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxazepam concentration</td>
<td>0.040</td>
<td>0.020</td>
<td>2</td>
<td>94.090</td>
<td>3.046</td>
<td>0.0523</td>
</tr>
<tr>
<td>Weight after dissection</td>
<td>0.053</td>
<td>0.053</td>
<td>1</td>
<td>94.082</td>
<td>8.022</td>
<td>0.0057</td>
</tr>
</tbody>
</table>
Figure 1. Cortisol concentrations in fish exposed to 0 (control), 1 and 100 μg/L of oxazepam.

Fish exposed to 1 μg/L had an unusual variation in their cortisol response as it can be seen between the 3 replicated in figure 2.

Figure 2. Cortisol concentrations in fish exposed to 0 (control), 1 and 100 μg/L of oxazepam. Each bar represents one exposure tank.
It is important to take into account that there is a mixture of organic chemical compounds in aquatic systems which means that fish are exposed to several chemical compounds that can cause a cocktail effect (Loos et al. 2013). Therefore, the fish response on an individual pharmaceutical does not

### Table 2

| The effect of treatment (0 or 100 µg/L) and bodyweight on cortisol levels (ng/mg fish). |
|-----------------|----------|----------|----------|----------|----------|----------|
| Oxazepam concentration | Sum Sq  | Mean Sq  | NumDF | DenDF | F       | P       |
| Weight after dissection | 0.029   | 0.029    | 1      | 58.952 | 4.456   | 0.039   |

**4. Discussion**

The results of this study show no significant difference in cortisol concentrations between control and oxazepam treated fish. However, the high oxazepam concentration (100 µg /L) displayed significantly reduced cortisol concentrations compared to controls when analyzed separately. This means that the 7-day treatment with oxazepam (100 µg/L) partially impaired the HPI axis.

Abreu et al. (2014) reported decreased cortisol concentrations in zebrafish (*Danio rerio*) treated with the benzodiazepine diazepam (16 µg/L) for 15 minutes. Moreover, they found that only certain concentrations of diazepam affected fish cortisol concentrations when exposed to acute stress. This states that the concentrations used has a major importance on fish physiological response. Therefore, certain concentrations may cause an effect while others might not. Abreu and coworkers found an effect on cortisol concentrations at diazepam concentration of 16 µg/L while 0.88 µg/L and 100 µg/L showed no effect on zebrafish. Their result supports the U-shaped dose–response hypothesis. Diazepam is about ten times more potent than oxazepam which means that theoretically a concentration of 160 µg/L of oxazepam would have potentially affected fish cortisol levels in our case.

Concentrations of BZD in aquatic systems are usually below 1 µg/liter, but fish usually experience higher concentrations of BZD because of bioconcentration. Interestingly, the effect of bioconcentration seem to be species-specific. Brodin et al. (in prep.) found that bioconcentration factors (BCFs) of oxazepam in perch (*Perea fluviatilis*) and crucian carp (*Carassius carassius*) was 9.7 and 1.4 even though they had been exposed to the same water concentration. Another study found that three-spined stickleback have BCFs reaching 4 (Sundelin 2015). These differences in BCFs indicate that pharmaceuticals can have a wide range of effects depending on species.

The reason our results did not reach the level of statistical significance might be explained by the fact that in fish exposed to 1 µg/L oxazepam showed large variance in whole body cortisol. Fish that were exposed to 1 µg/L were treated slightly differently during sampling compared to the animals exposed to 0 and 100 µg/L, since for the latter groups, also the brain was removed. This might have resulted in that some fish samples had parts of the head kidney left, which may have increased variation in cortisol concentration between samples. Interestingly, if 1 µg/L samples are removed from our data a significant effect of oxazepam on cortisol concentrations was detected. Hence, this might explain why we do not find an effect of oxazepam on cortisol concentrations in our complete dataset. Additionally, the GABA<sub>A</sub> receptor is evolutionary conserved throughout the vertebrates, and studies on rodents have shown that benzodiazepines inhibit the activity of HPI-axis which means that BZD is believed to have a physiological effect on our three-spined stickleback. However, even though three-spined stickleback have all physiological requisites that oxazepam influences, they did not get affected.

It is important to take into account that there is a mixture of organic chemical compounds in aquatic systems which means that fish are exposed to several chemical compounds that can cause a cocktail effect (Loos et al. 2013). Therefore, the fish response on an individual pharmaceutical does not
Our results show that the highest oxazepam concentration (100 μg/L) we tested displayed a significantly reduced cortisol concentrations compared to controls when analyzed separately. But when fish were exposed to the lower concentration of oxazepam (1 μg/L), an effect on cortisol levels was not shown. This might have been caused by the way we did our sampling. After all, our results show that oxazepam have no effect on cortisol levels and therefore will not influence the behavior of three-spined stickleback regarding cortisol.

Our result contradicts several earlier studies that supports the fact that BZD influences the HPI axis. However, it is imperative to do further studies regarding this manner using other protocols. The complexity of how benzodiazepines affect aquatic life and ecosystems remains largely unknown and environmental predictions are difficult to make. For further studies, it is crucial to study how the mix of pharmaceuticals in aquatic systems might cause a cocktail effect. Another idea for future studies is to examine how higher concentrations of BZD affect fish and examine if the U-shaped dose-response curve is occurring. This will contribute to a better understanding on the importance of concentration in affecting fish.

5. Acknowledgements
A big thank you to my supervisors, Laura Vossen and Svante Winberg for making this study possible and guiding me through this process.

6. References


Sundelin A. 2015. Ecotoxicological effects on a food-web exposed to pharmaceuticals: Uptake and effects of oxazepam, fexofenadine and a mixture of both in algae, zooplankton and sticklebacks.


7. Appendix

Protocol; 23/3/2010
Three-spined stickleback whole-body cortisol quantification
Radioimmunoassay (RIA)

Adapted from plasma protocol Tom Pottinger

Cortisol extraction
1. Collect body samples from -20 freezer and thaw on ice.
2. Weigh the falcon tube before insertion of sample and tare the weighing scale.
3. Cut the body into small (1 mm) pieces and transfer these to a 50 ml falcon tube.
4. Weigh each sample to the nearest milligram and write down in labbook.
5. Add 2 mL PBS buffer to each sample.
6. Homogenize the first sample using the tissue homogenizer VWR VDI12 with S12N-7S rotor.
7. Wash the rotor in between each sample with PBS, 96% ethanol, again 96% ethanol and finally MilliQ water. Be sure to remove any tissue pieces from the rotor with tweezers.
8. Repeat step 4 and 5 for all samples.
9. Add 10 mL ethyl acetate to each sample inside the fume hood. Vortex each sample thoroughly for 1 minute.
10. Centrifuge at 1000g for 5 minutes using the falcon tube centrifuge in the Birnir lab.
11. For each sample, transfer 6 mL of the supernatant (containing the cortisol) to a borosilicate glass tube (VWR, 100 x 12,00 x 0.8-1.0 mm, catalogue no. 212-0015).
12. Evaporate the ethylacetate in the BIBBY Scientific evaporator with the heat block at 40° C.

Prepare standard curve samples
14. Prepare the standard curve samples according to the table below. Use small glass bottles with screw lids.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Content</th>
<th>Cortisol Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10 mg cortisol + 10 mL 96% ethanol</td>
<td>1 ng mL⁻¹</td>
</tr>
<tr>
<td>B</td>
<td>50 ul A + 9500 ul ethylacetate</td>
<td>5 ng mL⁻¹</td>
</tr>
<tr>
<td>C</td>
<td>100 ul B + 5000 ul ethylacetate</td>
<td>50 ng mL⁻¹</td>
</tr>
<tr>
<td>D</td>
<td>1.6 mL C + 8.4 mL ethylacetate</td>
<td>8 ng mL⁻¹</td>
</tr>
<tr>
<td>E</td>
<td>5 mL D + 5 mL ethylacetate</td>
<td>4 ng mL⁻¹</td>
</tr>
<tr>
<td>F</td>
<td>5 mL E + 5 mL ethylacetate</td>
<td>2 ng mL⁻¹</td>
</tr>
<tr>
<td>G</td>
<td>5 mL F + 5 mL ethylacetate</td>
<td>1 ng mL⁻¹</td>
</tr>
<tr>
<td>H</td>
<td>5 mL G + 5 mL ethylacetate</td>
<td>500 pg mL⁻¹</td>
</tr>
<tr>
<td>I</td>
<td>5 mL H + 5 mL ethylacetate</td>
<td>250 pg mL⁻¹</td>
</tr>
<tr>
<td>J</td>
<td>5 mL I + 5 mL ethylacetate</td>
<td>125 pg mL⁻¹</td>
</tr>
</tbody>
</table>

These samples are going to contain known amounts of cortisol with bound radio-active labeled antibodies. The standard curve is needed to interpolate the cortisol concentration of your body samples.
At this stage we will need to add 3 negative controls:

T: 100 µl ethylacetate, ¹H-cortisel, no antibodies (gives highest count);
B: 100 µl ethylacetate, ¹H-cortisel, no antibodies, DCC (lowest count);
N: 100 µl ethylacetate, ¹H-cortisel, antibodies, DCC (max bound ¹H-cortisel).

15. Transfer 100 µl of each sample to each of 3 eppendorf tubes, in the order as outlined below.
16. Transfer 100 µl of the standard curve samples (D-J) to 3 eppendorf tubes.
17. Transfer 100 µl ethyl acetate into three eppendorf tubes for the T, B and N controls.
18. Add 50 µl ¹H-cortisel to each eppendorf.
19. Use the SpeedVac to evaporate the ethylacetate from the eppendorf tubes for 30 minutes (max 4 samples per run).
   For example, for 42 samples you need 42 * 200 µl per sample = 8.4 ml
   antibody buffer. Add 8400/2000 = 4.2 µl antibody to this.
21. Add 200 µl antibody containing buffer to each tube, EXCEPT FOR T1, T2, T3
   and B1, B2, B3. These samples get 200 µl antibody buffer without antibody.
22. Incubate all samples in the fridge overnight.

<table>
<thead>
<tr>
<th>Order</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T1</td>
</tr>
<tr>
<td>2</td>
<td>T2</td>
</tr>
<tr>
<td>3</td>
<td>T3</td>
</tr>
<tr>
<td>4</td>
<td>B1</td>
</tr>
<tr>
<td>5</td>
<td>B2</td>
</tr>
<tr>
<td>6</td>
<td>B3</td>
</tr>
<tr>
<td>7</td>
<td>N1</td>
</tr>
<tr>
<td>8</td>
<td>N2</td>
</tr>
<tr>
<td>9</td>
<td>N3</td>
</tr>
<tr>
<td>10</td>
<td>J1</td>
</tr>
<tr>
<td>11</td>
<td>J2</td>
</tr>
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<td>J3</td>
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<td>13</td>
<td>J4</td>
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<td>14</td>
<td>J5</td>
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<td>15</td>
<td>J6</td>
</tr>
<tr>
<td>16</td>
<td>H1</td>
</tr>
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<td>17</td>
<td>H2</td>
</tr>
<tr>
<td>18</td>
<td>H3</td>
</tr>
<tr>
<td>19</td>
<td>G1</td>
</tr>
<tr>
<td>20</td>
<td>G2</td>
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<td>21</td>
<td>G3</td>
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<td>22</td>
<td>F1</td>
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<td>25</td>
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<tr>
<td>29</td>
<td>D2</td>
</tr>
<tr>
<td>30</td>
<td>D3</td>
</tr>
</tbody>
</table>

- 30 - ? Samples in triplicates
The next day:
23. Turn on the centrifuge to let it cool down to 4°C.
24. Mark up scintillation tube lids for all your eppendorf tubes.
25. Fill the scintillation tubes with 5 mL scintillation liquid (AquaSafe).
26. Add 100 μL stirred, chilled DCC to each eppendorf, EXCEPT FOR T1, T2, T3 (add 100 μL PBS instead). Incubate on ice exactly 5 minutes (use timer). Max 24 samples at a time.
27. Centrifuge eppendorf tubes at 2000g at 4°C for 5 minutes.
28. Add 200 μL of the supernatant to each corresponding scintillation tube and shake by hand.
29. Run the scintillation tubes in the scintillator. Be sure to add flag 21 to the first rack with the flag pointing outwards.

Analysis:
30. Report1.csv is your datafile. Check that the date is the date that the scintillator finished. Rename as: RIA_20180322 (for example).
31. Save the csv file from the scintillator onto a memory stick. Location: Desktop > Users > Flag 21.
32. Save the .csv as an .xlsx file.
33. Reorganize the csv file by adding columns (alt+click) and using the Text to Columns command (Data > Text to Columns > Delimited > tick semicolon > continue > finish). Delete the original column names. New column names: Vial; Count Time; cpm; SIS; Messages.
34. Add a column "Sample". The first 30 samples are the controls + standard curve samples, from vial 31 it’s your samples in the order of the scintillation racks.
35. Add a column "Cortisol_pg_mL". Copy the concentrations for the standard curve samples from an old RIA csv. For your samples, write "NA".
36. Add a column "Weight".
37. Add a column "cpm_nound".
38. Save as text file (Save as > Tab Delimited Text (.txt)).
39. Email text file to Laura for calculation of cortisol concentration (in ng / mg fish).