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Treatment of pharmaceutical pollution and the release of illicit drugs into the environment.

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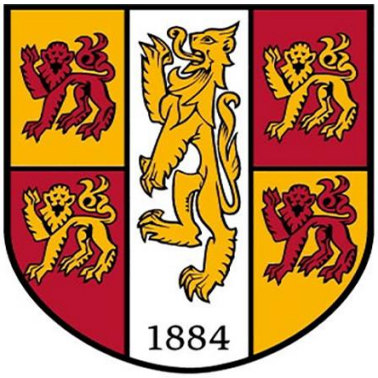

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Treatment of pharmaceutical pollution and the release of illicit drugs into the environment.

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Contents

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Author declaration

I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

Yr wyf drwy hyn yn datgan mai canlyniad fy ymchwil fy hun yw'r thesis hwn, ac eithrio lle nodir yn wahanol. Caiff ffynonellau eraill eu cydnabod gan droednodiadau yn rhoi cyfeiriadau eglur. Nid yw sylwedd y gwaith hwn wedi cael ei dderbyn o'r blaen ar gyfer unrhyw radd, ac nid yw'n cael ei gyflwyno ar yr un pryd mewn ymgeisiaeth am unrhyw radd oni bai ei fod, fel y cytunwyd gan y Brifysgol, am gymwysterau deuol cymeradwy.

Pharmaceuticals in the environment; presence and release.

Pharmaceuticals and personal care products (PPCPs) is a term used for classifying emergent contaminants which contain diverse organic groups and range from over the counter painkillers to cancer treatment drugs (Liu and Wong, 2013). Advances in analysis techniques have led to the ability to detect these chemicals in the aquatic environment such as rivers and lakes (Beek *et al.*, 2016). PPCPs, when taken, are only partially metabolised and subsequently released in the urine where they enter the sewage network, due to the complexity of these compounds they are rarely effectively removed by WWTPs which routinely results in their environmental release (Luo *et al.*, 2014).

PPCPs is a broad classification term that includes multiple different drug groups which are taken in the millions of tonnes worldwide. The two most commonly tested for drug groups are anti-inflammatory/analgesic drugs and antibacterial drugs as they are widely prescribed and pose a significant risk to the environment through bioaccumulation and promotion of antibiotic resistance (Hughes, Kay and Brown, 2013; Luo *et al.*, 2014; Beek *et al.*, 2016). Beek *et al* (2016) found these two groups were the most commonly found compounds in European and North American waters. Global research into PPCP pollution has a significant western bias with the majority of research being conducted in Europe and North America, closely followed by Asia (Hughes, Kay and Brown, 2013; Beek *et al.*, 2016). The small dataset sizes in Africa and other countries do not give as broad of a representation of the PPCP pollution present in the country as the majority of research will have been conducted in pollution hotspot locations where significant levels will be expected. Further research in areas with low sampling rates will expand the map of worldwide pharmaceutical pollution allowing an accurate assessment of worldwide risk.

Environmental presence

Paracetamol is a commonly used painkiller available over the countertop when consumed 80% of the paracetamol dose is released in the urine as conjugates (Kasprzyk-Hordern, Dinsdale and Guwy, 2008). In Wales in 2006, 140,680kg of paracetamol was bought over the counter, when the river Taff (located in South Wales) was tested for pharmaceutical presence a mean concentration of 968ng/L^{-1} of paracetamol was detected (Kasprzyk-Hordern, Dinsdale and Guwy, 2008). The highest concentration recorded in the River Taff was 2382ng/L^{-1}, yet paracetamol has been found at concentrations as high as 230,000ng/L^{-1} in European rivers(Beek *et al.*, 2016). Despite these high concentrations Beek et al (2016) reported the average paracetamol concentration in surface waters in western Europe at 46ng/L^{-1} (n=629) showing a significant difference from the previously mentioned studies. The high environmental concentrations typically are found close to WWTP's

where treated waste containing paracetamol is released into surface waters, for example, the river Taff showed decreasing paracetamol concentrations with increased distance from the WWTP's situated on its banks(Kasprzyk-Hordern, Dinsdale and Guwy, 2008). High environmental concentrations of pharmaceuticals are more common with chemicals that have high usage rates and high unmetabolized release such as paracetamol. Ibuprofen despite being another over the counter painkiller has lower environmental concentrations as only 14% of a dose is released as conjugates (Kasprzyk-Hordern, Dinsdale and Guwy, 2008). The lower urine release rate coupled with lower overall usage in comparison to paracetamol (11,000kg/140,690kg) resulted in ibuprofen concentrations in the river Taff never exceeding 100ng/L^{-1} with an average concentration of 33ng/L^{-1}. Despite the low concentrations in the River Taff, the mean concentration of ibuprofen in western Europe was recorded at 97ng/$L^{-1,}$ almost triple the levels of the River Taff, Ibuprofen is more widely tested for than paracetamol with the average concentration calculated from a sample size of 6264 (Beek *et al.*, 2016). The high mean concentration for ibuprofen in Western Europe suggests that the main factor for high environmental presence of a pharmaceutical compound is high usage rates with little effect by metabolic release percentages. However more potent pharmaceuticals that are only available via prescription such as Tramadol, carbamazepine and diclofenac are widely reported in the environment despite lower usage rates in comparison to paracetamol and ibuprofen.

Tramadol is an opiate commonly prescribed for pain relief and is only available via prescription due to its addictive nature, this results in lower usage rates in comparison to paracetamol and ibuprofen as shown in Wales in 2006 where only 2145kg were prescribed (Kasprzyk-Hordern, Dinsdale and Guwy, 2008). Tramadol is released unchanged in urine at a 15-35% rate, significantly lower than paracetamol (Kasprzyk-Hordern, Dinsdale and Guwy, 2008). Despite the difference in urine release rate, Tramadol was found in the River Taff at a mean concentration of 2774 ng/L^{-1} and with a maximum concentration of 5970ng/L^{-1}. Tramadol was detected in only five studies in Western Europe but was found in significant concentrations with a median of 801ng/L^{-1} and a maximum of 7731ng/L^{-1}, this shows that despite its lower usage rates it is persistent enough to be present in the environment at significant concentrations (Hughes, Kay and Brown, 2013).

Carbamazepine is the most tested for antiepileptic drugs and has been found in 98 studies with a detection frequency of 85% (Hughes, Kay and Brown, 2013). Worldwide median concentrations were low at 174ng/L^{-1} yet the highest recorded concentration was 11,561ng/L^{-1}, significantly higher than the median showing a high potential for significant pollution levels(Hughes, Kay and Brown, 2013). Concentrations in the River Taff reached the same range as ibuprofen with a mean concentration of 157ng/L^{-1} and a maximum concentration of 356ng/L^{-1} (Kasprzyk-Hordern, Dinsdale and Guwy, 2008). The similarity with ibuprofen is continued with the low release of unchanged compounds in urine as

only 3% of a carbamazepine dose is released in urine. Carbamazepine had lower usage rates than ibuprofen in Wales with 2515kg of carbamazepine prescribed as appose to 11,000kg of ibuprofen, the relatively equal environmental rates are caused by the higher excretion rate of carbamazepine in urine counteracting the lower prescription rate.

The anti-inflammatory drug Diclofenac is of particular environmental concern as bioaccumulation of diclofenac in carrion from herd animals lead to the >95% population decline of Oriental White-backed Vultures in the Indian subcontinent in the 90s (Oaks *et al.*, 2004). Overuse of diclofenac in veterinary practices led to bioaccumulation at high enough levels to cause liver failure in vultures, the persistence of the compound in animal tissue is however not echoed by its persistence in water where it has a half-life of 7.3 days (Araujo *et al.*, 2014). Despite its low half-life diclofenac had a detetection rate of 75.5% and a median worldwide concentration of 136ng/L^{-1} with a max found concentration of 18,740ng/L^{-1} (Hughes, Kay and Brown, 2013). Diclofenac has a high metabolism percentage with only 5-10% released unchanged in the urine, this low percentage released unchanged coupled with low usage rates in Wales (2,200kg) led to low environmental concentrations in the Taff with the highest recorded concentration of 85ng/L^{-1} (Kasprzyk-Hordern, Dinsdale and Guwy, 2008).

Removal rates in WWTPs

The presence of these drugs in the environment is correlated with high usage rates coupled with incomplete metabolism yet environmental presence should be prevented by removal by WWTP's. Removal rates vary not only between drugs but also between types of treatment, time of year and between influent loads. Differences between treatment types are best exemplified by Kasprzyk-hordern et al (2009) who compared the removal rates of an activated sludge bed with a trickling filter bed showing the activated sludge bed had significantly higher removal rates for all drugs tested. In some drugs such as paracetamol, the difference in removal was small with trickling beds removing 92% of paracetamol while sludge removed 100%. Despite the 8% difference paracetamol removal was still high at both WWTP, however, due to the quantity of paracetamol that travels through WWTP's 8% still results in the significant release into the environment (615ng/L^{-1}). This explains the high concentrations of paracetamol found in the previous study by Kasprzyk-hordern *et al* (2008) (968ng/L^{-1}). However, even Activated sludge treatment would occasionally fail to removal all paracetamol resulting in an average of 299ng/L^{-1} in the river Ely. Paracetamol was only detected in 60% of samples from the river Ely as opposed to the river Taff where it was found in 100% of samples showing that the activated sludge treatment plant on the river Ely occasionally removed all paracetamol and prevented environmental contamination. Ibuprofen had lower removal rates than

paracetamol with 92% for activated sludge and 82% for trickling bed; again trickling beds had lower removal rates. Ibuprofen influent concentration for both WWTP's was a minimum of 77x lower than paracetamol concentration resulting in much lower environmental concentrations for ibuprofen with average concentrations for both rivers below 30ng/L^{-1}. This highlights how despite removal rates having a direct effect on environmental release the deciding factor on significant environmental levels is the quantities of drugs consumed and released. This can lead to the conclusion that to reduce environmental contamination reduction in pharmaceutical consumption is the most direct fix, however suggesting reduction of pharmaceutical use is both dangerouos and morally wrong and would create more problems through increased strain on the healthcare sector. The most effective and morally sound course of action is to increase removal efficiency at WWTPs through upgrading of pre-existing WWTPs and creation of tertiary removal techniques such as Constructed treatment wetlands (CTWs). CTWs applied as a polishing technique could provide the extra barrier needed to reduce environmental contamination as already exemplified by the use of CTWs for treatment of nitrogen rich pollution such as landfill leachate and sewage (Mæhlum, 1995; Fan, Zhang, *et al.*, 2013).

The need for a final treatment method for removing pharmaceuticals is further reinforced by the low removal rates for pharmaceuticals such as tramadol, carbamazepine and diclofenac. Carbamazepine and diclofenac both had negative removal rates from the activated sludge and trickle filter beds previously mentioned which resulted in an increase in concentration in the effluent for both of these compounds (Kasprzyk-Hordern, Dinsdale and Guwy, 2009). Negative removal is caused by the hydrolysis of pharmaceutical conjugates causing the release of the parent compound and relevant ions, such as with diclofenac sulphate conjugates which deconjugate to diclofenac and sulphate ions, this causes the concentrations of the parent compound to be higher in the effluent than the influent (Vieno and Sillanpää, 2014). Tramadol also had negative removal rates for the trickling bed filter, again caused by deconjugation but also had removal rates of 36% for activated sludge treatment. Positive removal rates for tramadol were most likely due to the adsorption of the parent compound to the activated sludge rather than any degradation process (Kasprzyk-Hordern, Dinsdale and Guwy, 2009). Diclofenac and carbamazepine removal rates were collated by Luo *et al* (2014) and average removal rates were calculated from surrounding literature and both chemicals had average removal rates below 40% (Diclofenac-n=10; Carbamazepine-n=8) with the highest data point being 81% removal for diclofenac. Activated sludge treatment effectiveness is improved with higher hydraulic retention times (HRT) as many pharmaceuticals have slow intermediate kinetics and experience less effective biodegradation with low HRTs (Kasprzyk-Hordern, Dinsdale and Guwy, 2009a; Verlicchi *et al.*, 2013; Luo *et al.*, 2014). WWTPs are continuously fed with waste for treatment and they are

designed and tailored to handle the quantity of waste released from the population they connected to, increase of HRTs is often impossible due to the continuous quantity of waste that needs treating. Installation of a tertiary treatment method such as a series of CTWs would reduce the need for increasing HRTs and allow an additional step of treatment that could be tailored to high risk pollutants unique to each WWTP.

Constructed Treatment Wetlands efficacy for PPCP removal

CTWs are man-made wetland habitats designed to optimise degradation processes of wetland ecosystems to enable efficient removal of pollutants (Cole, 1998). CTWs can be added as a final polishing stage in the water treatment system to ensure removal of harder to degrade compounds such as pharmaceuticals. CTWs have proven to be incredibly effective at pharmaceutical removal with removal rates ranging from 86.2-99% for paracetamol (Ávila *et al.*, 2013; Li, Zhou and Campos, 2017; Vymazal *et al.*, 2017; Li *et al.*, 2019). However, some CTWs occasionally experience removal rates below 50% caused by either a spike in paracetamol levels or dramatic shifts in temperature(Verlicchi *et al.*, 2013; Li *et al.*, 2014). When used as a tertiary treatment method any inconsistent removals have less of an impact as the CTW is acting as a failsafe for the already released wastewater. This is best exemplified by a study by Verlichi *et al* (2013) who connected a CTW to a WWTP which was already removing 96% of the paracetamol yet the extra 45% removal by the CTW resulted in total removal of 98% and an effluent concentration of 16ng/L^{-1}. The removal rates for pharmaceuticals by CTWs echo the removal rates of WWTP's with paracetamol and ibuprofen being readily removed whereas chemicals such as tramadol, diclofenac and carbamazepine having significantly lower removal rates. Ibuprofen is readily degraded in CTWs but not as consistently as paracetamol with removal rates ranging from 20-99% (Matamoros *et al.*, 2007; Ávila *et al.*, 2013; Li *et al.*, 2014; Vymazal *et al.*, 2017). Zhang *et al* (2014) when conducting a review of the surrounding literature found that when CTWs are employed as a tertiary treatment method ibuprofen removal had a significantly higher range of 75-98% in comparison to when used as a secondary treatment where removal ranged from 41-99%. This shows that the lower concentrations typically found in tertiary treatments are more readily and regularly removed as opposed to the higher concentrations experienced in a secondary treatment role (Zhang *et al.*, 2014).

CTW removal of Tramadol shows similar rates to WWTPs with variation between positive and negative removal with some CTWs showing variation from -253% to 60% (Nuel *et al.*, 2018). Average removal rates of around 50% are typical for tramadol yet significant variation occurs with regular dips to -30% removal (Vymazal *et al.*, 2017). Tramadol is not as widely studied as diclofenac, paracetamol or carbamazepine despite it having significant environment release. Carbamazepine

and diclofenac both have less variation of removal rates between CTWs, carbamazepine removal is recorded between the 20-25% range when used as a secondary treatment(Matamoros *et al.*, 2007; Li *et al.*, 2014; Auvinen *et al.*, 2017). When used as a tertiary treatment method carbamazepine removal ranges from 5-97% with one instance of negative removal (-4%) (Verlicchi *et al.*, 2013; Zhang *et al.*, 2014). The lower removal rates referenced in Zhang *et al* (2014) are all from free water surface constructed wetlands which have lower removal rates due to the absence of plants resulting in fewer degradation pathways for pollutants (Matamoros and Salvadó, 2012). The higher removal rates for carbamazepine are found in CTWs with longer HRTs due to the increased time for degradation processes to occur (Zhang *et al.*, 2014). The effect of longer HRTs is supported by diclofenac removal which is typically in the range of 30-40% but once HRTs are extended removal rates reach 70-98% (Ávila *et al.*, 2013; Li *et al.*, 2014; Auvinen *et al.*, 2017; Vymazal *et al.*, 2017). Higher removals are again found when CTWs are used as a tertiary treatment as a pose to secondary with secondary treatments ranging from 0-87% with an average of 40% while tertiary treatments range from 38-98% with an 80% average (Zhang *et al.*, 2014). The higher removals in tertiary treatment are caused by the lower concentrations of pollutants in the influent which doesn't overload the CTW, the effectiveness of CTWs coupled with the low costs and maintenance of the systems makes them a great choice for final polishing of effluent.

Research into removal rates for pharmaceuticals by CTWs has produced promising results, yet criticism has been drawn that research often only measures influent and effluent with no attempt at understanding the removal process occurring inside the CTW (Zhang *et al.*, 2014). The effect of aeration on the removal of different pharmaceuticals is also lacking, aeration provides oxygen to the system which allows aerobic degradation processes to occur and could enhance the removal rate of some pharmaceuticals. Through a comparison between unaerated and aerated CTWs, this study will deduce whether the main degradation pathway of the aforementioned pharmaceuticals by biofilm is aerobic or anaerobic.

Illicit drugs in the environment and wastewater system

As previously described, pharmaceutical drugs are released into the environment due to incomplete metabolism and subsequent poor removal by WWTP. This same process occurs when illicit drugs such as cocaine are consumed. The literature on this topic is very broad with many conflicting values for drug metabolism rates and the environmental impact of these psychoactive compounds.

Zuccato *et al* (2005) found that the river Po in Italy transported roughly 4kg of cocaine a day. While this initial figure is of great significance for an environmental reason where the paper excels is in its

use of sewage epidemiology for detection of drug abuse rates. This paper is the first example of back calculating drug concentrations in sewage influents to estimate the drug abuse rates of a population. Back calculation of cocaine abuse in this paper is calculated by measuring the concentration of cocaine's main metabolite benzoylecgonine in sewage influent and multiplying it by the water flow rate (m^3/sec) to calculate the benzoylecgonine load (g/day). This daily load is then multiplied by a factor of 2.33, which represents the average molar fraction of a cocaine dose that is excreted as benzoylecgonine (45%) as well as the molar mass ratio of the two compounds to each other (0.954). This equals the g of cocaine taken before release in the wastewater system. Due to WWTP's being connected to urban areas with regular population censors an accurate estimate of cocaine doses per 1000 inhabitants can then be calculated for the population connected to the WWTP. Zuccato *et al* (2005) found that the previous estimates for drug use had vastly underrepresented the drug use in Italy with levels of cocaine found in the influent suggested a total of 40,000 doses of cocaine taken per day in the river Po catchment area, this was significantly lower than survey estimates which recorded 15,000 cocaine usage events per month. Technically both survey and epidemiology could be accurate estimates as survey data did not record the amount of cocaine a cocaine user would use so multiple doses of cocaine could be being taken by a single person, therefore, lowering the number of usage events. However, the sheer amount of cocaine that would have to be consumed per usage event per month to equal 40,000 doses per day makes it more likely that surveys were underrepresenting the problem.

This paper effectively proposed sewage epidemiology as a fast-accurate method for monitoring drug abuse in communities. This is particularly evident when compared to drug use rates derived from surveys which underestimated the cocaine use in Italy with a rate of 15,000 cocaine use events per month whereas this paper recorded 40,000 doses per day. The usage rates presented by this study show the unreliability of surveys as a method for recording drug abuse figures, particularly when compared to the results from sewage epidemiology.

The findings of Zuccato *et al* (2005) caused an increase in illicit drug testing in surface and sewage waters across the world with the majority of research concentrated in Europe and North America (Yadav *et al.*, 2017). Cocaine levels in surface waters are typically in the low ng/L^{-1} level and range from 0-25ng/L^{-1} with occasional examples reaching as high as 59.8ng/L^{-1} (van Nuijs *et al.*, 2009; Yadav *et al.*, 2017). Levels in surface waters are low due to cocaine's high biodegradability as 80% of a cocaine concentration will degrade after 24 hours in 20°C, pH 6 river water (Gheorghe *et al.*, 2008). Cocaine's main metabolite benzoylecgonine, however, is more stable and experiences no notable degradation after 24 hours in the same conditions, this stability coupled with the higher release in urine (45%) results in benzoylecgonine reaching high concentrations in surface waters (Zuccato *et*

al., 2005; Gheorghe *et al.*, 2008). The river Zenne in Belgium, where the 59.8ng/L^{-1} cocaine concentration was recorded had a benzoylecgonine concentration of 222.3ng/L^{-1}, almost 4 times the concentration(van Nuijs *et al.*, 2009). Higher concentrations of Benzoylecgonine are found in wastewater influents and effluents with concentrations in the thousands of ng/L^{-1}; influent concentration range from 1000-7500ng/L^{-1} with effluent ranging from 1-1500ng/L^{-1} (Yadav *et al.*, 2017). Removal rates for illicit drugs, as with pharmaceuticals show significant variation and are influenced by the same factors(Yadav *et al.*, 2017). Illicit drug usage as previously mentioned can be tracked through sewage analysis allowing trends in usage to be detected such as higher usage during weekend periods and during large public social events such as the Super Bowl or new year's eve (Gerrity, Trenholm and Snyder, 2011; Irvine *et al.*, 2011). When drug use spikes in such a short time it has been shown to overload the WWTP's and reduce their efficacy resulting in lower removal rates, this is best exemplified by sewage analysis from a music festival on the Spanish coast (Bijlsma *et al.*, 2014). During the music festival MDMA concentrations in the influent reached 27µg/L^{-1} when it was barely present before the festival, benzoylecgonine concentrations increased from around 3,000ng/L^{-1} to 10,000ng/L^{-1} (Bijlsma *et al.*, 2014). The spike in concentrations caused removal rates of benzoylecgonine to drop from 95% to 52% while MDMA dropped from 60% to 20% (Bijlsma *et al.*, 2014). This drop-in removal caused effluent with the equivalent weekly load of 405g of MDMA, 16.21g of cocaine and 121.67g of benzoylecgonine to be released into the environment (Bijlsma *et al.*, 2014). The levels released the week of the music festival were 597 times higher than the previous weeks MDMA load while cocaine and benzoylecgonine were 36 and 217 times higher. The significant release by the WWTP during the festival shows that spikes of drug use can cause failures in treatment methods. The higher concentrations found in the influent show that music festivals are hotspots for drug use and through the use of sewage epidemiology more accurate recordings of drug use can be taken.

THESIS AIMS

- Test the ability of simple unplanted gravel-based CTWs to treat pharmaceutical waste and the effect of aeration on removal rates.
- Develop colourimetric methods for detection of key pharmaceuticals
- Investigate direct environmental release of illicit drugs into freshwater systems due to drug use at music festivals.
- Evaluate the validity of sewage epidemiology calculations when using environmental concentrations of illicit drugs.

Effect of aeration on the degradation of paracetamol in constructed treatment wetlands using a colourimetric based detection method

ABSTRACT

Over the counter pharmaceuticals such as paracetamol are consumed in a significant volume each year and released in urine due to their partial metabolism. This results in vast quantities of paracetamol entering WWTP's and then the freshwater environment when not fully removed. CTWs have been used with great results as a final polishing stage for paracetamol removal where the main pathway for removal is biodegradation. Although the biodegradation of paracetamol is suspected to be anaerobic every test wetland has had a significant form of oxidation of the wetland matrix by either plants or an aeration system. This study compared the removal rates of paracetamol in aerated and non-aerated unplanted vertical subsurface flow wetlands. Paracetamol concentrations were measured using a colourimetric method based on the oxidation of paracetamol by Fe(III) which forms a Prussian blue solution. The colourimetric method has a LOD range of 0.2 – 2 ±0.036 mg/L^{-1}. The colourimetric method provided a fast, cheap, reliable way to measure paracetamol concentrations. 95% of all paracetamol was removed in 24 hours in all CTWs and sitting water systems designed to mimic natural degradation conditions. Water-based systems had 95% removal in 23 hours further confirming paracetamol typically lasts for 0.8 days in the environment. Unaerated gravel-based wetlands removed 95% of paracetamol in 13 hours on the first day of the experiment but then removed 95% in four hours for consecutive days. Aerated wetlands initially took 23 hours for 95% removal but then took 4 hours for 95% removal in consecutive days. The increased removal rate for consecutive days suggests adaption of the biofilm to the biodegradation of paracetamol resulting in more efficient CTWs. The initial higher removal rate for unaerated CTWs provides further evidence for the biodegradation of paracetamol being anaerobic. The high removal rates in such a short time show the effectiveness of CTWs as a treatment method for paracetamol pollution.

INTRODUCTION

PPCPs have been detected in 37% of tested rivers worldwide at concentrations ranging from ng/L^{-1} to mg/L^{-1} (Jones, Voulvoulis and Lester, 2001; Hughes, Kay and Brown, 2013; Santos *et al.*, 2013). PPCPs are chemicals that affect human biological processes and are used for diagnosis, treatment, alteration or prevention of diseases and includes both prescribed and over the counter medications

such as paracetamol (Jones, Voulvoulis and Lester, 2001). Due to the significant effect these chemicals have on people, the environmental impact of these chemicals could be significant at high enough concentrations. The EU has developed risk assessment guidelines to calculate the concentration for chemicals to no longer have an impact on an ecosystem, this value is called a PNEC value (Predicted No Effect Concentration) (Stuer-Lauridsen *et al.*, 2000; Bound and Voulvoulis, 2006). PNEC values for paracetamol are reported at values ranging from 65.4µg/L^{-1} to 210ng/L^{-1} in papers but a value of 134µg/L^{-1} is reported by the ECHA (European Chemicals Agency) (Bound and Voulvoulis, 2006; Stuer-Lauridsen *et al.*, 2000; ECHA, 2020).

The official ECHA PNEC value is higher than the majority of environmental cases for paracetamol, the collated median concentration from 35 studies was 148.2 ng/L^{-1}, significantly lower than the PNEC value (Hughes, Kay and Brown, 2013). Some of the highest environmental levels don't exceed the PNEC value with concentrations reaching 2382ng/L^{-1} in the River Taff in South Wales and reaching 15,700ng/L^{-1} in the river Dahan in Taiwan (Kasprzyk-Hordern, Dinsdale and Guwy, 2008; Lin and Tsai, 2009). Environmental levels lower than the PNEC value suggests that paracetamol pollution is negligible in the rivers tested and has no effect on the ecosystems it is present in. However multiple studies have found negative effects on various organisms after exposure to paracetamol concentrations below PNEC values. Zebrafish experienced increased embryonic incubation and reduction in body length and mass of embryos after exposure of 1µg/L^{-1} of paracetamol (David and Pancharatna, 2009). Concentrations of 0.25µg/L^{-1} caused the closure of valves in clams (*R. philippinarum*), a behaviour typically used to reduce exposure to contaminants such as paracetamol which would otherwise cause oxidative stress and membrane damage (Nunes *et al.*, 2017). Rainbow Trout, when exposed to paracetamol at concentrations from 12.5-50µg/L^{-1} for 28 days, showed increased levels of oxidative stress further confirming that below PNEC values still have detrimental effects on organisms of various trophic levels (Ramos *et al.*, 2014). Detrimental effects are also experienced in biofilm which experienced decreased cyanobacteria composition and activity by peptidase enzyme, resulting in a significantly lower primary productivity for the stream (Proia, Osorio, Soley, Köck-Schulmeyer, *et al.*, 2013). All these examples show PNEC values for paracetamol should be calculated at lower concentrations as numerous effects on various ecosystem levels are caused at lower concentrations.

PPCP usage is in the millions of tonnes with paracetamol having a usage rate of 140 tonnes in 2006 in Wales alone (Kasprzyk-Hordern, Dinsdale and Guwy, 2008). PPCPs when taken are rarely fully metabolised with paracetamol metabolism rates ranging from 30-90% the release of unmetabolized paracetamol in urine is a guarantee (Prescott, 1980; Bertolini *et al.*, 2006). Paracetamol has a half-life of 1.4-2.5 hours in plasma but lasts significantly longer in the environment with a half-life of 0.7-

1.1 days (Prescott, 1980; Lam *et al.*, 2004; Ranieri, Verlicchi and Young, 2011). PPCPs are classed as pseudo persistent pollutants as their continuous release into the environment results in the replacement of any concentrations lost via degradation resulting in a constant presence of PPCPs. WWTPs vary with their reliability for removing paracetamol with the majority reporting >99% removal however due to the high usage rates of paracetamol any removal efficiencies lower than 99% can result in large amounts of paracetamol being released into the environment. (Ternes, 1998; Luo *et al.*, 2014; Burns *et al.*, 2018). This is best exemplified by the river Taff which despite having a higher volume than the river Ely had 200% more paracetamol per litre due to the removal efficiencies for the WWTP situated on the Taff having an efficiency of 92% rather than the >99% found on the river Ely (Kasprzyk-Hordern, Dinsdale and Guwy, 2009). Removal efficiencies of WWTP's can also decrease when levels of PPCPs increase, during a music festival in Spain levels of benzoylecgonine increased from <2500ng/L^{-1} to <11,000ng/L^{-1} and caused a drop in removal efficiency of at least 25% with many removal efficiencies dropping to below 50% (Bijlsma *et al.*, 2014). The varying removal efficiencies of WWTP's due to differing treatment types and shifting pollutant levels shows a need for a third "polishing" system for treatment of paracetamol and other PPCPs to ensure minimal release into the environment.

CTWs are man-made systems that tailor and enhance the natural processes of a wetland ecosystem to remove and store any pollutants in wastewater. They are often used as the final stage of water cleaning as a polishing stage to remove any residual concentrations of pollutant. They have exceptional results at removing paracetamol, with removal rates of >99% in 5.16-10.2 hours (Ranieri, Verlicchi and Young, 2011). Removal efficiencies vary not only from the type of CTW but are also affected by the length of the HRT with CTWs with longer HRTs having higher removal rates even when treating higher pollutant concentrations (Vymazal *et al.*, 2017). Removal efficiencies ranged from 86.2 to 99.6% in four Horizontal sub-surface flow wetlands which were exposed to paracetamol concentrations ranging from 7486 to 45,571ng/L^{-1} with HRTs of 6.3 to 11.6 days (Vymazal *et al.*, 2017). This significant range in concentrations and removal rates is evidence of the variability and inconsistency in CTW treatment types. Most of the breakdown of the paracetamol in CTWs is argued to be from biodegradation assisted by the biofilm of the roots of the plants rather than a mechanism from the plants themselves.

CTWs are often fitted with aeration systems which bubble air through to system causing oxidation of the wetland matrix allowing aerobic degradation process to occur (Maltais-Landry, Maranger and Brisson, 2009). Aeration systems typically run intermittently throughout the day to allow both aerobic and anaerobic processes to occur (Fan, Liang, *et al.*, 2013; Fan, Zhang, *et al.*, 2013). The addition of aeration systems to CTWs greatly increases the removal of nitrogen-rich pollutants such

as landfill leachate and sewage (Mæhlum, 1995; Nivala *et al.*, 2007; Fan, Zhang, *et al.*, 2013). The main drawback of aeration systems is the increased running cost and complexity from installing them as well as the possibilities for blockages forming in the aeration pipes (Nivala *et al.*, 2007). Aerated CTWs have been used to treat PPCPs to great effect with the removal of paracetamol at or above 90% along with other compounds such as caffeine and triclosan (Matamoros *et al.*, 2007; Auvinen *et al.*, 2017; Li, Zhou and Campos, 2017; Li *et al.*, 2019). All these studies had planted CTWs fitted with aeration beds to further increase the availability of oxygen in the wetland matrix. Plants enhance various removal processes in CTWs such as sorption and microbial degradation while also being responsible for phytoremediation and plant uptake (Zhang *et al.*, 2014). Ranieri *et al* (2011) recorded a removal rate of 51.3-97.7% in an unplanted control horizontal subsurface flow wetland while planted beds ranged from 46.7->99%. This variation between planted and unplanted showed that the effect of plants for the biodegradation of paracetamol is minimal. Although the planted beds statistically had higher removal rates of paracetamol it was deduced to not be due to plants but due to the increased surface area provided by the plants which allowed a larger surface area of biofilm to develop (Ranieri, Verlicchi and Young, 2011). The HSFCW's had a HRT of 35.8-36.7 h when the flow was at its highest ($1m^3/d$) which was also found to have the highest removal rate. Research into the removal of other pharmaceuticals by Matamoros *et al* (2007) suggested that vertical flow wetlands can provide faster removal rates for PPCPs as they achieved 95% removal of caffeine and other common PPCPs after just four hours.

The widespread effect of plants on the processes of CTWs means the specific effect of aeration on the processing of PPCPs cannot be assessed by CTWs with plants. With the main removal of paracetamol being biodegradation, the presence of plants overcomplicates test systems where artificial aeration on a gravel bed could provide a more efficient treatment system.

Further research into the ability of CTWs to process PPCPs is limited by the cost and time limitations of high performance liquid chromatography (HPLC) methods. The precision of HPLC methods is unrivalled but is often time-consuming and with removal occurring in a matter of hours a fast methodology for analysis would be beneficial for research as well as field testing. Through the use of colourimetry, this study will test the effect of aeration on the removal rate of paracetamol in simple biofilm gravel sub-surface flow wetlands.

METHOD

Construction of test wetlands

The constructed wetlands used were vertical subsurface flow wetlands consisting of an unplanted gravel substrate. 10 CTWs were made in plastic pint glasses each with 750g +/- 0.03g of washed gravel and 5 had air stones fitted in the bottom for aeration (appendix 1, 2). Aerated wetlands were intermittently aerated with an airflow of 20ml/min for four hours starting on the fourth hour of treatment (appendix 1). Before the experiment was started all CTWs were filled with river water from Afon Cegin and changed weekly for three weeks for biofilm establishment. 5 additional empty pint glasses were used to compare the natural decomposition of paracetamol in river water. All pint glasses were fitted with a sampling tube down the side to allow sampling from the bottom of the CTWs. Wetlands were fitted with covers to reduce evaporation. All CTWs were stored in a temperature control room at 25°C away from direct sunlight to eliminate photolytic degradation.

Experiment length

Two experiments were conducted, the first one involved sampling 3 times over 4 days to test whether biofilm only CTWs could still remove paracetamol. Sampling occurred at the 24, 48, and 96-hour mark after the addition of pollutant.

The second experiment occurred over 24 hours and was repeated four times. Sampling occurred on the 4^{th}, 8^{th}, 13^{th} and 23^{rd} hour each day.

Paracetamol concentration, conductivity and pH were measured for each water sample.

Paracetamol detection method

The level of paracetamol in water samples was estimated using a spectrophotometric method based on the oxidation of the drug with Iron(III). Paracetamol reduces Iron(III) to Iron(II) on a 1 to 1 ratio, Iron(II) ions react with potassium hexacyanoferrate(III) in an acidic medium to make a Prussian blue solution(RSC, no date; Nagendra, 2011). The intensity of the Prussian blue measured at 700nm can be used to estimate the paracetamol concentration.

176µl of water sample was reacted in a clear microplate with 70µl 0.02 mol dm^{-3} Iron(III) Chloride and 35µl 0.002 mol dm^{-3} potassium hexacyanoferrate(III), after 10 minutes 15µl of 5 molar HCl was added and the microplate was left to stand for 20 minutes. An endpoint reading of the absorbance at 700nm gives an optical density of the Prussian blue which can then be converted to paracetamol concentration using the line equation from a standard curve (appendix 3, fig.1). Through the use of a

microplate up to 20 samples and a standard curve consisting of 8 points could be tested in 40 minutes (appendix 4).

The Iron(III) Chloride solution consists of 0.544g of Iron(III) Chloride-$6H_20$, 300µl of 32% HCL and 1g of Potassium Chloride dissolved in a $100cm^3$ volumetric flask with deionised water. The addition of acid and potassium chloride increases the stability of the solution in samples and reduces precipitation and adhesion of iron to the surface of the volumetric flask (appendix 5). The Potassium hexacyanoferrate(III) solution is 0.066g of potassium hexacyanoferrate dissolved in $100cm^3$ of deionised water.

Agitation of the sample was found to increase the reaction rate. However, due to the staggering of samples in the microplate, the agitation would not be applied evenly between samples.

The oxidation reaction is temperature-sensitive, so a single standard curve was run in conjunction with samples to allow comparison between sample days. The reaction was also never done in direct sunlight or near heat sources. To minimise variation between sample parameters all water sourced for the experiment was collected from the same area on the Afon Cegin and was filtered with a 0.45µm filter paper before being analysed for paracetamol.

The paracetamol method had a range of 0.2-2mg/L^{-1} and a standard error of 0.036mg/L^{-1}, the standard curve is displayed in figure 1. The quantification is limited to 2mg/L^{-1} as the Iron(II) ions precipitate at higher concentrations and produce unreadable samples as shown in appendix 5.

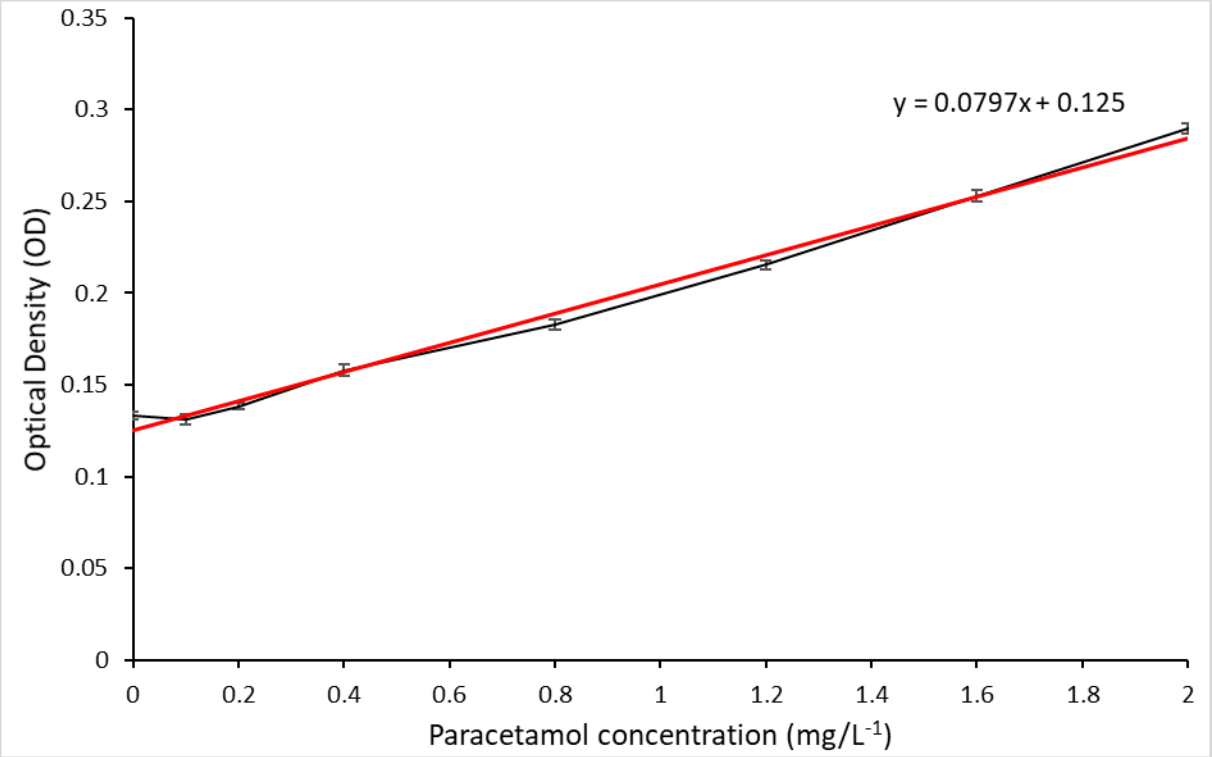


Figure 1: Standard curve for paracetamol colourimetric reaction. Line equation is presented in the top right. Shows the relationship of optical density with paracetamol concentration (mg/L^{-1}).

Statistical analysis

On each sampling day/hour, the paracetamol concentration from each treatment type was analysed by one-way ANOVA. Effects were analysed by the Tukey's b post hoc test unless equal variance was not assumed then a Games Howell test was used. Statistical significance was set at $p = 0.05$. The analysis was performed using SPSS Version 25.

For the 24-hour experiment analysis between days was conducted with the same statistical techniques.

RESULTS

Four-day experiment

The concentration of paracetamol in all treatments decreased to below the limit of detection after only 24 hours as shown in figure 2. No noticeable or significant difference was found between biofilm, aerated and water treatment methods (24hr $F(2, 42) = 0.00$, $P = 1.00$; 48hr $F(2, 42) = 0.00$, $P = 1.00$; 96hr $F(2, 42) = 0.00$, $P = 1.00$; 168hr $F(2, 42) = 0.00$, $P = 1.00$). Concentrations stayed below the limit of detection for the rest of the week showing that any increase in concentration from evaporation was not significant enough to counteract removal by the biofilm.

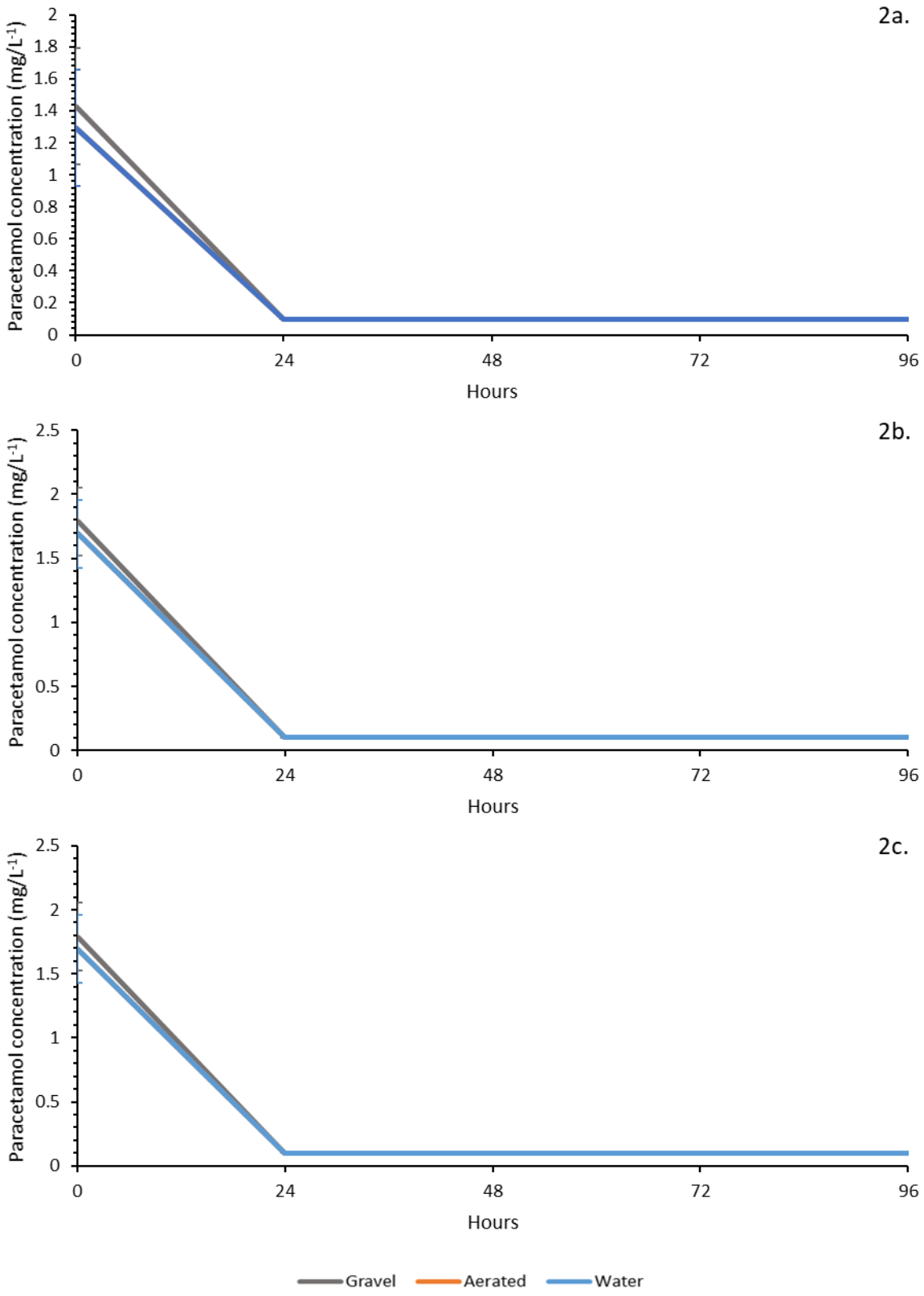


Figure 2: Paracetamol concentration in the biofilm CTWs over 4 days. Each graph is a different week of experiment with 2a being the first week. Error bars are not present for datapoints from the 24hr point onwards as all samples were below the limit of quantification.

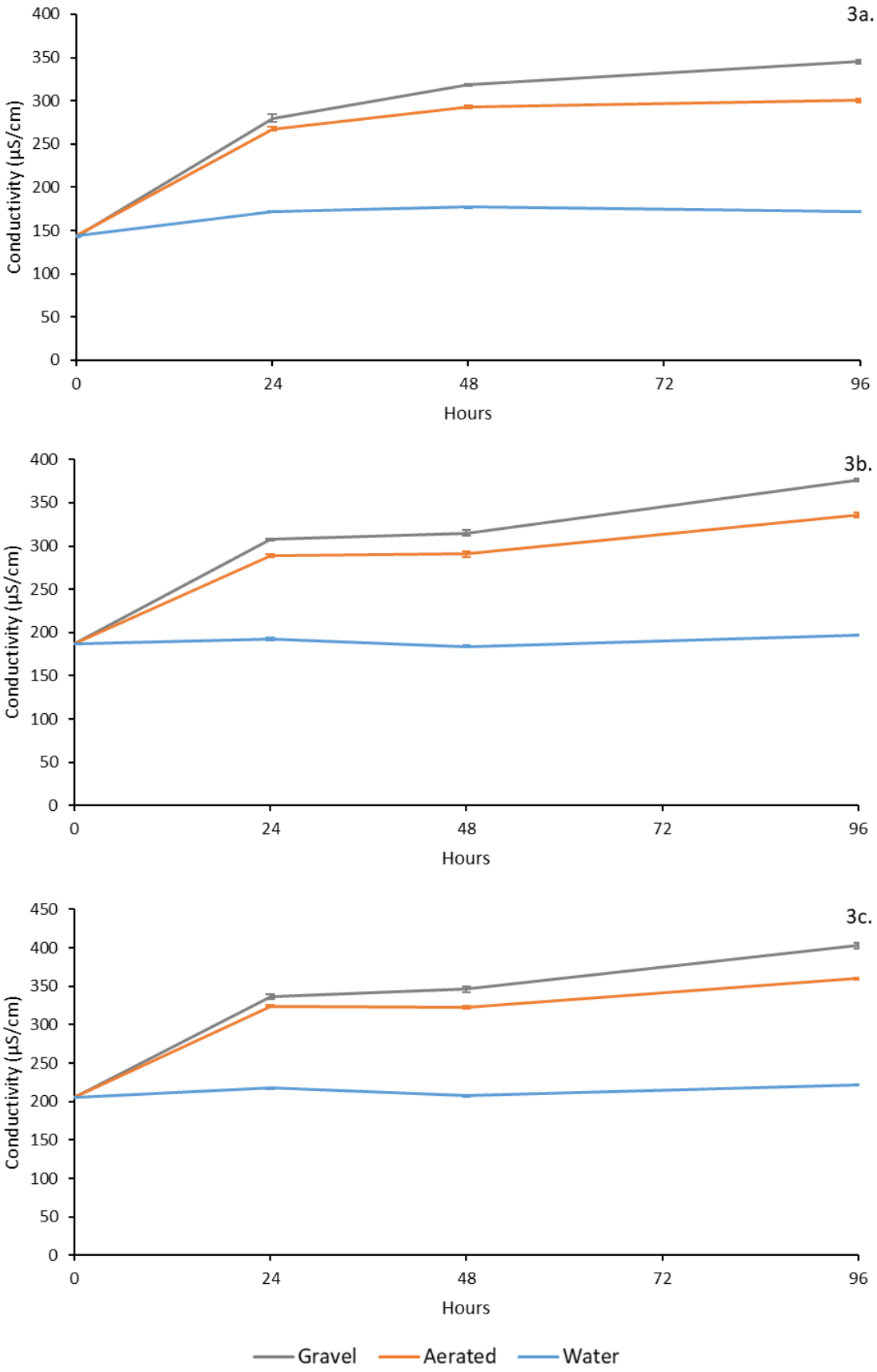


Figure 3: Conductivity levels in the test wetlands over 96 hours in microsiemens per cm. Each letter represents a different weekly run.

Conductivity trends across the four-day experiment as shown in figure 3, show a large increase in the conductivity of water in gravel and aerated CTWs in the first 24 hours as this was the period with the highest removal of paracetamol (figure 2). Gravel has a higher conductivity than aerated CTWs at all data points suggesting unaerated wetlands have higher microbial activity. Despite this difference, paracetamol was removed at in the same amount of time in both aerated and unaerated. Figure 2 shows water systems had paracetamol levels as low as the CTWs after 24 hours, despite this high removal rate, conductivity levels remained relatively constant indicating low microbial activity in the water systems.

24-hour experiment

The 24-hour experiment shows a similar endpoint when compared to the 4-day experiment as all treatments reached the limit of detection after 23 hours as shown in figure 4. Day 1 of the experiment (4a) shows gravel has the fastest removal rate of paracetamol with a 0.28±0.09mg/L^{-1} concentration after four hours and reaching the limit of detection by the 13th hour. In figure 4a the concentration found in the gravel and aerated CTWs reaches the limit of quantification at hour 2 where the concentrations diverge and increase before slowly declining again; the initial decrease measured at hour 2 suggests either inaccurate measurement or an increase in concentration at hour 4 due to evaporation. The paracetamol concentration in 4b shows no increases suggesting no effect from evaporation or increased removal negating any effect from evaporation. Removal rates for gravel based CTWs increase over the following days with day 2, 3 and 4 all reaching the limit of detection after four hours. Aerated CTWs had slower removal rates for day 1 with the limit of quantification being reached at the same time as the water degradation system on the 23rd hour. Aerated wetlands for the rest of the days had faster removal rates with a limit of quantification being reached after four hours. The water systems, which were designed to replicate the degradation of paracetamol in rivers and lakes had lower removal of paracetamol than the CTWs. Removal of paracetamol became more efficient as the days went on with the limit of detection being reached by the 13th hour for days 2, 3 and 4; this increase was likely to be caused by early stages of biofilm establishment on the insides of the test container.

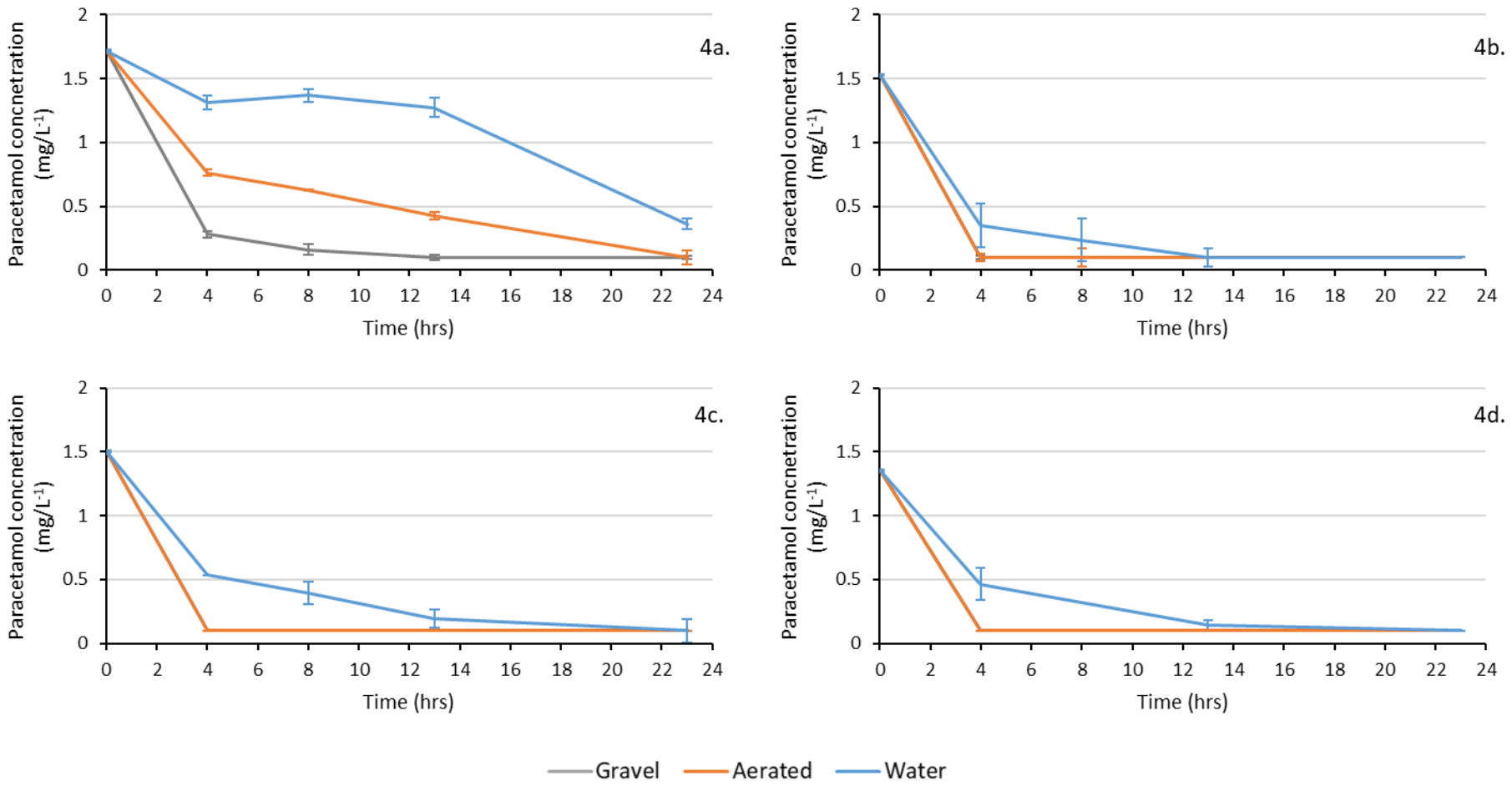


Figure 4: Graphs of paracetamol removal over 24 hours from gravel and biofilm-based CTWs. Each letter represents an experimental day with 4a being day one

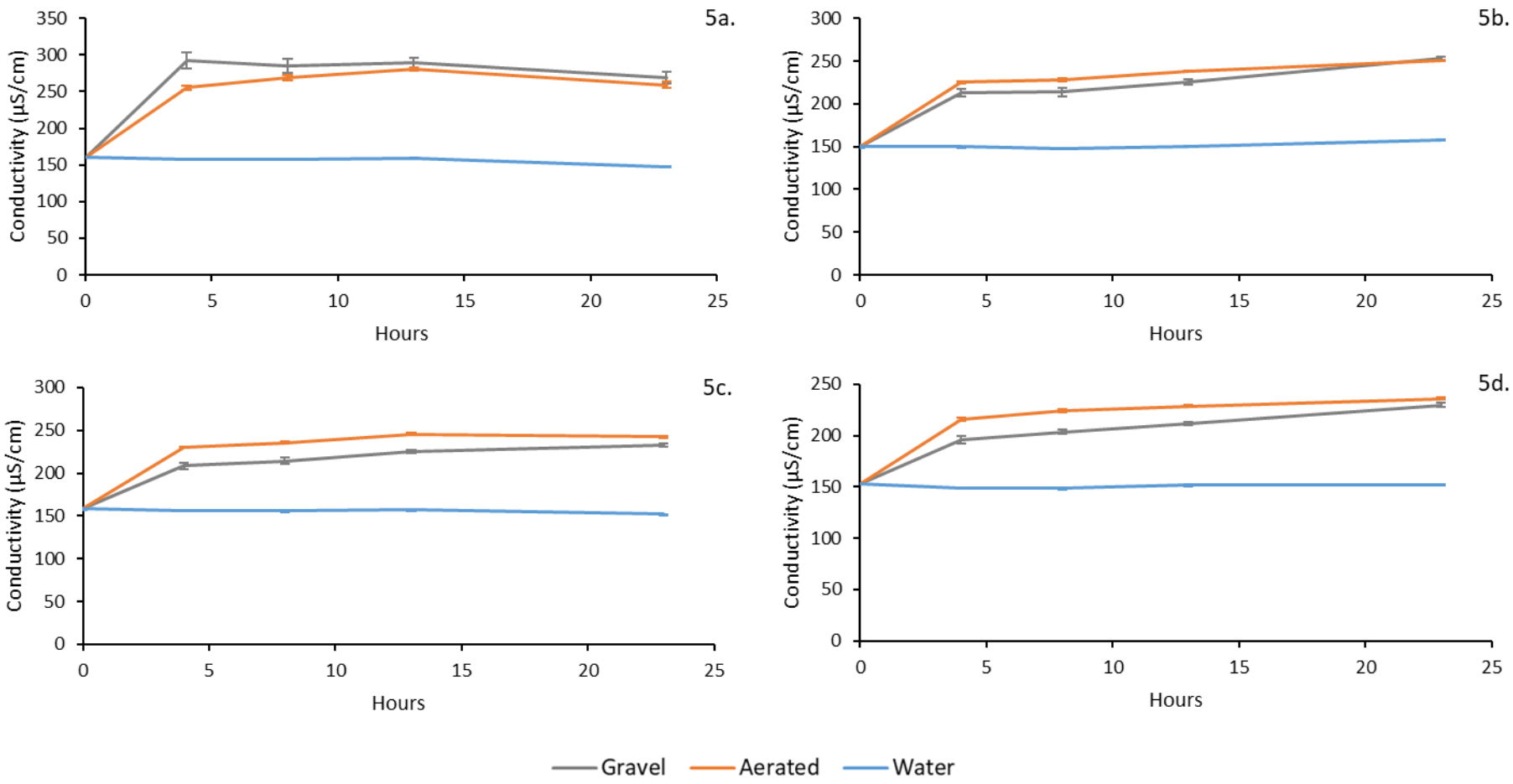


Figure 5: Conductivity levels in the test wetlands over 24 hours in microsiemens per cm. Each letter represents a different daily run.

Between days

Due to increased removal rates as days went on statistical analysis was used to test for a significant difference between each sample day. ANOVA tests, as presented in table 1 found a significant difference (p= < 0.05) between days for all treatments except Gravel at 13hrs, Aerated at 23hrs and Gravel at 23hours. The statistically significant difference between days is evidence for an increase in the efficiency of the CTWs as treatment went on suggesting adaption of the biofilm to paracetamol removal. A Games-Howell test found the significant difference found by the ANOVA tests was between day 1 of the experiment with every other day (P=<0.05). This shows that after exposure to paracetamol concentration for 24 hours the biofilm adapts and has increased removal efficiency for subsequent days. The Statistical analysis between treatment types must then be conducted separately for day 1. Days 2, 3 and 4 showed no significant difference between each day suggesting that the biofilm has reached peak efficiency after exposure of 24 hours.

No significant difference was found between days for gravel at 13 and 23 hours as paracetamol concentrations had reached the limit of detection by 13 hours on all days, the same applies for the concentration in aerated wetlands at 23 hours.

Table 1: Results from a one-way ANOVA comparing the sample days for each sample hour of each treatment type.

Treatment type	Sampling hour	Degrees of freedom		F value	P-value
		between groups	within groups		
Aerated	4	3	16	84.997	< 0.001
	8	2	12	333.063	< 0.001
	13	3	16	38.224	< 0.001
	23	3	16	0.000	1.000
Gravel	4	3	16	16.729	< 0.001
	8	2	12	9.867	0.003
	13	3	16	0.506	0.684
	23	3	16	0.000	1.000
Water	4	3	16	14.216	< 0.001
	8	2	12	83.043	< 0.001
	13	3	16	95.768	< 0.001
	23	3	16	4.332	0.020

Between treatments

Comparison between treatments for paracetamol concentrations at the 4-hour sampling point on day 1 found a significant difference between treatment methods F(2, 12)= 184.96, P= <0.001; with a Tukey test showing a significant difference between all treatment types (P=<0.01). An ANOVA for

days 2, 3 and 4 found a significant difference between treatments F(2, 42)=21.70, P=<0.01; A Tukey test found a significant difference between all treatments except gravel and aeration (P=>0.05). The lack of a significant difference between gravel and aerated at the 4-hour mark shows how the efficiency of both treatments has reached the same level.

At the 8 hour mark on day 1 an ANOVA showing a significant difference between all treatments F(2, 12)= 178.22, P= <0.001 and a Tukey test showed the significant difference (P= <0.01) was again between all treatments. Days 2, 3 and 4 also showed a significant difference between treatments F(2, 27)= 12.28, P= <0.001; with a Tukey test showing water were significantly different from gravel and aerated treatments. No significant difference was found between gravel and aerated treatments again.

The day 1 samples, taken at 12 hours showed a statistically significant difference between treatments F(2, 12)= 213.44, P= <0.001) and a subsequent games-Howell test showing a significant difference (P=<0.05) between all treatments. In contrast, the ANOVA for days 2, 3 and 4 showed no significant difference between treatment types F(2, 42)=1.86, P=0.169. This not only shows that all treatments had reached the limit of detectable removal but also shows that the subsequent days had a faster removal rate.

The 23-hour samples from day 1 showed a significant difference between treatments under an ANOVA test F(2, 12)= 4.33, P=0.038 yet a subsequent Tukey test found no difference between treatment types (P=>0.05). This contradiction by statistical tests is likely due to the majority of data points for treatments at 23hours being the limit of quantification (0.1mg/L^{-1}) which resulted in a weak significant global effect and a P value close to significance for the ANOVA. Days 2, 3 and 4 showed the same results but with the ANOVA showing no significant difference between treatment methods F(2, 42)=0.00, P=1.00. This is due to all CTWs reaching the limit of detection so all data points are equal and have no variation.

Conductivity values echoed the results from the 4-day experiment with gravel and aerated CTWs having a spike in conductivity at the fist sample point, the four-hour sample point was also the point with highest paracetamol removal (figure 5). Water systems again remained relatively constant with little variation showing minimal microbial activity. Gravel CTWs however only had higher conductivity than aerated CTWs on the first day. The higher conductivity values for aerated CTWs on days 2, 3 and 4 shows further adaption by the biofilm resulting in higher rates of biodegradation. The difference between aerated and gravel CTWs closes as time goes on with day 4 showing almost equal conductivity values, this conforms with conductivity data from the 4-day experiment (figure 3) which had similar conductivity at the 24-hour mark. The 24-hour experiment in conjunction with the

4-day experiment suggests that aeration will initially have higher microbial activity for 24 hours before being overtaken by gravel-based CTWs.

DISCUSSION

Treatments

Results from the 4-day experiment show removal in 24 hours for all treatments with no significant differences between any of the treatments (fig. 2). Low concentrations after 24 hours in the water systems provides further evidence that the biodegradation of paracetamol in river water occurs in 0.7-1.1 days (Lam *et al.*, 2004; Ranieri, Verlicchi and Young, 2011). For the first day of the 24-hour experiment (fig. 4a), water-based systems removed 95% of paracetamol in 18-20 hours (0.8 days) showing the fast degradation of paracetamol in rivers in the natural ecosystem. In consecutive days (fig. 4b, 4c, 4d) the half-life decreased to 0.17 days showing an increase in degradation of paracetamol caused by the growth of a biofilm on the inside of the container.

The gravel-based CTWs removed 95% of the paracetamol in 4 hours (fig.4b, 4c, 4d), this is significantly faster than rates recorded by Ranieri *et al* (2011) who recorded 97.7% removal after 35.8-36.7 hours. The removal graphs by Ranieri show decreasing removal rate as the paracetamol concentrations decrease suggesting that the lower the concentration of paracetamol, the lower the biodegradation rate. This conforms with collision-based reaction rate theory where higher concentrations cause increased rates of reaction. The starting pollutant concentration for this study was on average 2000 times higher than the concentrations used in Ranieri *et al* (2011) (750ng/L^{-1}). The significant difference between starting pollutant concentrations coupled with the effect of collision theory reaction rates implies that for lower concentrations the gravel systems in this study would have lower removal rates for the same time.

The aerated systems had significantly slower paracetamol removal than the non-aerated gravel systems on the first day (fig. 4a) with the aerated systems taking 23 hours to reach the limit of detection. This provides evidence for the biodegradation of paracetamol by biofilm being an anaerobic process. Surrounding literature on the treatment of paracetamol by CTWs all have some form of oxidation of the wetland matrix by plant communities and sometimes in tandem with aeration systems. These systems have removal rates ranging from 82.6-99% similar to removal rates of the aerated and gravel based systems from this study (Ranieri, Verlicchi and Young, 2011; Li, Zhou and Campos, 2017; Vymazal *et al.*, 2017; Li *et al.*, 2019). However, comparisons between unplanted and planted systems showed a significant but only slightly higher removal rate in planted systems which was attributed to the increased surface area provided by the root systems of the plants rather

than any oxidation by the plants (Ranieri, Verlicchi and Young, 2011). The low removal rate in the aeration systems on day 1 shows that the presence of oxygen reduced the removal rate of paracetamol in the wetland. This suggests that the biodegradation of paracetamol by biofilm is inhibited by the presence of oxygen.

Research into paracetamol degradation pathways has revealed two main pathways; the pyrocatechol pathway and hydroquinone pathway (Wu, Zhang and Chen, 2012). The hydroquinone pathway is the major degradation pathway most commonly used by microorganisms and is an aerobic process whose main intermediate compound is hydroquinone. This pathway is reliant on the presence of hydrolytic enzymes which catalyse the hydroxylation of paracetamol to yield hydroquinone and acetamide (Zhang *et al.*, 2013). Hydroquinone and acetamide are then broken down into simple chain-based compounds via monooxygenase catalysing the breaking of rings before complete biodegradation occurs, resulting in the release of CO_2. The pyrocatechol pathway is also reliant on the presence of oxygen as the first stage is the oxidation of paracetamol which produces a variety of intermediate compounds such as *p* aminophenol. Intermediate compounds then undergo further oxidation resulting in pyrocatechol before complete degradation to CO_2 and H_2O via chain based intermediate compounds (Wei *et al.*, 2011). Both pathways are aerobic at some stage, this contradicts the ability of unaerated gravel-based systems to be more efficient at removal than aerated CTWs. Paracetamol removal in unaerated systems could be from either an anaerobic pathway, absorption and storage of paracetamol by the biofilm or a combination of both.

The batch fed systems used in this study have a higher redox potential than continuous systems as the process of draining and filling of the wetlands increases the availability of oxygen in the system (Ávila *et al.*, 2013; Auvinen *et al.*, 2017). The addition of slight oxidation from refilling of the systems could potentially be significant enough for the aerobic stages of either of the two biodegradation pathways. The presence of oxygen could potentially be enough for the first stage of the hydroquinone pathway which would convert all paracetamol to hydroquinone which would not be detected by the colourimetry method. Avila *et al* (2013) compared batch and continuous fed systems and found similar removal rates for paracetamol between them suggesting paracetamol biodegradation was not affected by the redox potential caused by batch refilling. Both Avila *et al* (2013) and this study only tested for parent compound PPCPs and did not test for any biodegradation intermediates, this could lead to 99% of the paracetamol being converted into its intermediate compounds due to incomplete degradation and subsequently evading detection.

Research into the removal of PPCPs by alternative treatment methods such as CTWs is typically scoped very narrowly with the main research aim focusing on measuring removal rates rather than

delving deeper into the actual process of removal. Of the seven papers that focus on removal of paracetamol by CTWs only one measured for metabolites or intermediate compounds of paracetamol (Ranieri, Verlicchi and Young, 2011; Ávila *et al.*, 2013; Verlicchi *et al.*, 2013; Li *et al.*, 2014, 2019; Li, Zhou and Campos, 2017; Vymazal *et al.*, 2017; Nuel *et al.*, 2018). Nuel et al (2018) tested for Paracetamol-β-D-glucoronide alongside paracetamol, Paracetamol-β-D-glucoronide is a metabolite of paracetamol usage by humans rather than an intermediate compound from a degradation pathway. Despite the lack of testing for degradation intermediates, all seven papers concluded that paracetamol was mainly removed by anaerobic biodegradation despite the presence of oxygen from plants, aeration or refilling redox. It is most likely that various pathways for paracetamol biodegradation are possible with both aerobic and anaerobic options. Anaerobic options are probably the most viable or any aerobic process have a low enough oxygen threshold to appear anaerobic. This study also did not test for any degradation of intermediate compounds and concluded the same findings as the surrounding CTW literature.

Removal of paracetamol in the wetland systems could potentially be via sorption of paracetamol to the biofilm rather than degradation of the paracetamol. River biofilms have been shown to sorb PPCPs in the ng/g level (Corcoll *et al.*, 2015; Huerta *et al.*, 2016; Aubertheau *et al.*, 2017). The sorption of the PPCPs is not limited by the sorption capabilities of the target compounds as Carbamazepine, a compound with low sorption ability has was recorded at accumulation levels of 583ng/g (Aubertheau *et al.*, 2017). Paracetamol sorption in biofilms has not been measured but the sorption of diclofenac another anti-inflammatory drug has been recorded at levels ranging from 190–20ng/g however ibuprofen did not show sorption capabilities to biofilm (Corcoll *et al.*, 2015; Huerta *et al.*, 2016; Aubertheau *et al.*, 2017). The highest recorded concentration of accumulated PPCP was for propanolol which reached 965ng/g, sorption of PPCPs was found to have no correlation with different types of ionisation with positively charged, negatively charged and uncharged PPCPs all being stored in biofilms (Aubertheau *et al.*, 2017). Storage of paracetamol in biofilm would result in the removal of paracetamol from the influent where it would then act as either a carbon and nutrient source or pose a toxic risk to the algal biomass (Lawrence *et al.*, 2012). The conductivity levels recorded (fig.5) show a spike in the first four hours showing an increase in degradation in the systems, this coincides with the decreasing paracetamol concentration (fig.4) which suggests that paracetamol is being degraded rather than stored in the biofilm of the wetlands.

Days

The significant difference between days found in this study is evidence towards the ability of freshwater biofilms to adapt and acclimate to concentrations of PPCPs found in rivers. As shown in

figure 4 paracetamol removal was more efficient as days went on with removal being significantly lower for the first day of analysis in comparison to the others. This increase over days suggests the biofilm is adapting and optimizing its processes towards the removal of paracetamol. Translocation of biofilms from low to high concentrations of analgesic pharmaceuticals such as paracetamol and ibuprofen caused decreased photosynthetic activity and a decrease of the green algae/cyanobacteria ratio (Proia, Osorio, Soley and Barceló, 2013). The decreased green algae/cyanobacteria ratio suggests a negative effect of paracetamol on green algae and/or a positive effect on cyanobacteria (Proia, Osorio, Soley and Barceló, 2013). Exposure to a cocktail of pharmaceuticals that did not include paracetamol caused the opposite effect in the biofilm community with increased green algae and decreased cyanobacteria populations (Corcoll *et al.*, 2015). Reduction in biofilm community diversity has also been reported when comparing biofilms exposed to hospital effluents as a posed to regular urban effluent with hospital effluents causing a reduction in diversity and development of biofilm communities (Chonova *et al.*, 2016). These studies show that paracetamol exposure produces a natural selection-based shift in the biofilm community reducing the diversity resulting in the adaption of the biofilm towards paracetamol. This would cause the remaining biofilm to be composed of only pharmaceutical tolerant microorganisms that are adept at degrading PPCPs.

This shift in biofilm community composition due to exposure to pollutants is a negative consequence of the environmental release of PPCPs, it can however be harnessed to tailor treatment at WWTPs. Through tandem use of activated sludge treatment with a moving bed biofilm reactor (MBBR), PPCPs can be partially degraded before exposure to biofilm resulting in higher removal rates (Escolà Casas *et al.*, 2015). Activated sludge treatment is one of the most effective treatments for PPCP based pollutants; comparisons with trickle bed filters show activated sludge being significantly more effective in terms of both removal efficiency and the breadth of compounds effectively removed (Kasprzyk-Hordern, Dinsdale and Guwy, 2009). Activated sludge treatment removes 100% of paracetamol from influent yet when WWTP's are exposed to higher concentrations of pollutants than expected, removal efficiencies typically decrease (Kasprzyk-Hordern, Dinsdale and Guwy, 2009a; Bijlsma *et al.*, 2014). Hospitals and other medical buildings are sources of effluent with high PPCP contamination and often require a further water treatment step to reduce effluent concentrations before release into the environment (Jones, Voulvoulis and Lester, 2001). The tandem activated sludge and MBBR is one example of using biofilms as the extra step to effectively treat PPCP pollutants, this study shows how simple biofilm coated gravel beds could be used as a final polishing technique to treat PPCP pollution. Gravel beds could be built and incubated with high levels of PPCP pollutant to force the biofilm community to adapt to the removal of PPCP pollution.

Gravel beds could then be set up to act as a final polishing stage to divert influent to during times of high PPCP contamination.

Method

The results obtained by this study support the use and reliability of the colourimetric method as the concentrations of paracetamol were recorded accurately enough to conclude statistically significant degradation was occurring as shown in figure 2 and 4. Despite its limitations, it has shown it can produce conclusive results on paracetamol concentrations. However, it is important to note that paracetamol is never the sole pollutant in the environment and other PPCP chemicals could disrupt the reaction for the method. HPLC analysis has numerous advantages over the colourimetry method but has a significantly higher cost and skill set requirement, unlike colourimetry which can be conducted in almost all labs. The colourimetry method also has significantly faster analysis times which allowed the 24-hour experiment to be such a success.

CONCLUSION

Aeration of the gravel bed and biofilm reduced the biodegradation rate of paracetamol suggesting the pathway is anaerobic. Freshwater biofilms removed 95% of paracetamol in four hours and adapt to paracetamol presence resulting in higher removal rates. Aerated wetlands gradually increased in efficiency as time went on suggesting adaption of aerated biofilms. The colourimetric method of paracetamol detection was successful at detecting paracetamol and provided accurate enough results for a significant conclusion on removal rates by CTWs to be concluded. The method however cannot detect concentrations as low as HPLC but is significantly faster so is preferable for shorter experiments.

Release of illicit drugs into the environment by Glastonbury festival.

ABSTRACT

Analysis of wastewater for illicit drugs has been used extensively to show short- and long-term trends in drug abuse. Music festivals are hotspots for drug use and cause drug concentrations in wastewater to spike. Many UK festivals occur in rural areas and require the installation of temporary toilets which when coupled with the high intoxication of festival-goers' results in high public urination rates. Glastonbury Festival is recognised worldwide and draws in a crowd of 201,000 people to Worthy Farm over its four-day period. Worthy Farm is situated in Somerset near the village of Pilton and is intercepted by the River Whitelake. The proximity of Whitelake coupled with the high drug use and public urination on the festival site provides the potential for release of illicit drugs into the environment. Water samples from the River Whitelake were taken on the weekends before, during and after the festival and analysed for illicit drugs, the neighbouring River Redlake was also analysed for comparison. The mass load (mass of a substance that flows through a point in a river per unit of time) of cocaine flowing through the River Whitelake downstream of the festival was greater than that of the upstream sites for the weekend of the festival (1.25-4.23 mg/hr vs 50.38 mg/hr; $p<0.01$). This also applied for the other tested drugs, Benzoylecgonine (22.7-81.4 mg/hr vs 854.6 mg/hr; $p<0.01$) and MDMA (1.1-61.0 mg/hr vs 114.7mg/hr; $p<0.01$). Mass loads for cocaine, benzoylecgonine and MDMA for the weekend before the festival never exceeded 7.6, 54.9 and 4.2mg/hr, significantly lower than the weekends afterwards. MDMA reached its highest level during the weekend after the festival with a mass load and concentration of 282.7mg/hr and 322.77ng/L^{-1}, this provides evidence for continuous release after the festival due to a possible leaching effect of MDMA from the festival site. Cocaine and benzoylecgonine reached their highest recorded concentrations the weekend of the festival with concentrations of 32.3±2.9 ng/L^{-1} and 548.4±27.8 ng/L^{-1}. The concentrations of tested drugs found in the River Whitelake for the weekend of the festival and onwards were all above levels found to cause damage to ecosystems in literature. The River Redlake experienced no significant changes ($p > 0.05$) in any illicit drug levels further confirming that drug release was dependent on the festival site. This study shows that music festivals situated in the countryside provide a large risk to aquatic ecosystems as they are sources of illicit drug pollution in freshwater ecosystems.

INTRODUCTION

Advancements in analysis techniques in recent years such as the development of HPLC and ultra-performance liquid chromatography (UPLC) has revealed the presence of illicit drugs and their metabolites in the surface waters across the world. UPLC is faster and more accurate than HPLC as the higher pressure and larger surface area of the UPLC column stationary phase results in sharper peaks and higher sensitivity (Churchwell *et al.*, 2005). The higher-pressure tolerance of the UPLC columns allows for higher mobile phase flow rates, and therefore significantly shorter methods (Van De Steene and Lambert, 2008).

The presence of these emergent contaminants was first reported in Italy in 2005 with an estimated 4kg of cocaine being transported by the River Po per day (Zuccato *et al.*, 2005). This research led to widespread sampling of rivers for illicit drugs and revealed their prevalence in surface waters across the world with concentrations ranging from 0.2-183 ng/ L^{-1} found in the River Thames(UK), Po(Italy), Ebro(Spain), Beiyunhe(China) and Liberia(Costa Rica) (Zuccato *et al.*, 2008; Postigo, de Alda and Barcelo, 2010; Causanilles *et al.*, 2017; Hu *et al.*, 2019). Substances found in surface waters range from class A to class C with cocaine, methamphetamine, amphetamines, and opiates being a common occurrence at the ng/L^{-1} level in rivers, higher concentrations of the metabolites for these compounds are often found alongside.

Illicit drugs typically have a short half-life due to their high psychoactive nature, however constant usage in populations causes a continuous release into the environment from WWTP's (Postigo, de Alda and Barcelo, 2010; van Nuijs *et al.*, 2012). The continuous release creates a pseudo-persistence in surface water as decaying concentrations are replaced by fresh release from WWTP's, this process has led to the classification of illicit drugs as pseudo-persistent contaminants (Postigo, de Alda and Barcelo, 2010; Rosi-Marshall *et al.*, 2015).

Illicit drugs, once taken are released in urine and faeces both as their unaltered and metabolic forms (Castiglioni *et al.*, 2008). Illicit drugs then travel through the sewage system and enter WWTP's where they are then released into the environment as removal rates can be <30% depending on the method of treatment (Baker and Kasprzyk-Hordern, 2013). Drug levels in the influent of WWTP's can be measured using HPLC and then back-calculated using pharmacokinetic assumptions to estimate the drug use of populations connected to that sewage network(Zuccato *et al.*, 2005; Castiglioni *et al.*, 2008). This non-intrusive method of monitoring drug abuse has been used in countries across the world, such as Italy, The UK, Canada, Croatia and India (Zuccato *et al.*, 2008; Metcalfe *et al.*, 2010; Terzic, Senta and Ahel, 2010; Subedi *et al.*, 2015).

Monitoring of WWTP's shows both short and long-term trends in drug use of populations. "party drugs" such as cocaine and MDMA typically show usage spikes over weekends whereas large social events such as The Super Bowl have been shown to cause increases in all drug use (Metcalfe *et al.*, 2010; Irvine *et al.*, 2011; van Nuijs *et al.*, 2011; Lai, Bruno, *et al.*, 2013). During The 2010 Super Bowl, 45% more benzoylecgonine (BE, main metabolite of cocaine) was detected in the influent when compared with the baseline values (494 g/day)(Gerrity, Trenholm and Snyder, 2011). The same increase in cocaine and other illicit drug use was reported for independence day as well as the 2017 solar eclipse observation day (Foppe, Hammond-Weinberger and Subedi, 2018).

Music festivals are large social events where drug use rates are higher than average with 26% of UK festival attendees admitting to taking drugs while at a festival (UK festival awards, 2019). The average UK marijuana rate is 12.3% for the 16-34 age category which makes up 47% of all festival attendees(EMCDDA, 2019; UK festival awards, 2019). The festival usage rate is more than double the UK marijuana rate which is significantly higher than usage rates for drugs such as cocaine (4.7%) and MDMA (3.3%)(EMCDDA, 2019). WWTP's experience large increases in MDMA, ketamine and methamphetamine concentrations during music festivals (Lai, Thai, *et al.*, 2013; Bijlsma *et al.*, 2014a; Jiang *et al.*, 2015). Increased concentrations of cocaine and its metabolites have also been reported but at lower values in comparison to MDMA (Bijlsma *et al.*, 2014), The increases in concentrations for a variety of drugs in the influent of WWTP's is caused by higher drug use at festivals, the large increase in drug concentrations causes removal rates in WWTP's to decrease by up to 70% resulting in the inefficient removal and release of illicit drugs into connected rivers (Lai, Thai, *et al.*, 2013; Bijlsma *et al.*, 2014a; Jiang *et al.*, 2015).

Due to the psychoactive nature and harmful effects of illicit drugs on humans the consequences for ecosystems could be severe. Cocaine concentrations of 20ng/L^{-1} have been shown to disrupt the endocrine systems of European eels (*Anguilla Anguilla*) after exposure of 30 days. Brain dopamine in eel's post-exposure to cocaine was almost four times higher than when constantly exposed to cocaine showing that aquatic vertebrae can experience withdrawal symptoms. The endocrine system is responsible for growth, reproduction and migration and it has been suggested that environmental cocaine contamination has been responsible for the decline of the European eel (*Anguilla anguilla)* (Sébert *et al.*, 2008; Gay *et al.*, 2013). The impact of cocaine on aquatic vertebrates affects all lifecycle stages with DNA fragmentation and cytotoxicity in Zebrafish embryos being recorded after exposure to concentrations of 1.2µg/L^{-1} (Parolini *et al.*, 2017). BE caused similar levels of damage at a lower concentration of 112ng/L^{-1} showing a higher cytotoxic potential than its parent compound (Parolini *et al.*, 2017).

The negative effects of illicit drugs are also evident in lower trophic levels with cocaine causing oxidative stress and destabilizing lysosomal membranes in zebra mussels exposed to a 40ng/L^{-1} (Binelli *et al.*, 2012). MDMA also caused cytotoxic damage to the haemocyte cells of zebra mussels at concentrations of 500ng/L^{-1} for 14 days (Parolini, Magni and Binelli, 2014). Exposure to 50ng/L^{-1} concentrations of MDMA did not cause a significant effect yet the cytotoxic potential of the molecule suggests long term exposure to the lower concentration would have a negative effect on zebra mussels (Parolini, Magni and Binelli, 2014). Environmental pollution of illicit drugs is rarely limited to a single substance and is often a cocktail of illicit compounds, the genotoxic and oxidative effects of illicit drugs are increased higher when zebra mussels are exposed to multiple substances at a time(Parolini *et al.*, 2015, 2016).

BE at low concentrations (1ng/L^{-1}) has been found to cause a strong inhibitory effect on mitochondrial activity of fern spores after 48 hours(García-Cambero *et al.*, 2015). High concentrations of BE (>10g/L^{-1}) causes a measurable decrease in root growth over 24 hours for Field Lupin(Macht and Livingston, 1922). To inhibit root growth to the same degree a concentration of 3g/L^{-1} of cocaine was needed, both concentrations tested were well above environmental levels yet lower concentrations would undoubtedly effect at a cellular level (Macht and Livingston, 1922).

Previous literature focused on analysing illicit drugs in wastewater from urban environments as WWTP's provide direct access to sewage from large populations. Many festivals in the UK are "greenfield festivals" situated in the countryside and are not connected to a WWTP due to the lack of sewage infrastructure and subsequent substitution with porta-loos and other temporary toilets. Public urination is a widespread issue at festivals as toilets often have poor conditions and long queues. Mardal *et al* (2017) analysed urine samples from pools of urine in soil at a music festival and found cocaine and MDMA in 66% of soil samples analysed (n=12). Public urination is such a large problem that Glastonbury festival-goers now have to sign a green pledge to not urinate on the site (Digital, 2020c, 2020b). This was implemented after the festival breached environmental regulations when a sewage system leaked and killed 43 fish in the nearby river(Association, 2016). With public urination and drug use at festivals both being higher than the average, the possibility for significant environmental release of illicit drugs is likely.

Glastonbury festival is a five-day music and arts festival in Pilton Somerset that has been running since 1970 and is the most famous UK festival having won the Best Festival Award at the New Musical Express (NME) awards 10 times. The 2019 festival had a total of 203,000 visitors and was the first festival after a one year break to rest the land, known as a fallow year (Digital, 2020a). The

festival site is split through the middle by a tributary of the Whitelake River which then continues through the surrounding farmland towards the Shapwick Heath and Westhay Moor NNRs.

This study hypothesises that the high level of drug use and public urination common with music festivals, coupled with proximity to a river will cause the release of illicit drugs into the ecosystem with potentially harmful effects downstream.

METHODS

Sample collection

This study aimed to detect the presence or absence of illicit drugs in the surface water surrounding the Glastonbury festival site and to see if any change occurred in the concentrations of any drugs during the festival period. Glastonbury festival site is intersected by the River Whitelake. As shown in figure 6 the River Whitelake consists of two tributaries that converge on the festival site, both were sampled upstream from the festival (WLUP1(appendix 6) and WLUP2(appendix 7)) and one site downstream was sampled (WLDWN(appendix 8)). The neighbouring river (Redlake) was sampled upstream and downstream (RLUP(appendix 9) and RLDWN(appendix 10)) to allow a comparison between a river directly in contact with the festival and one in a neighbouring valley.

Collection of four 1 litre grab samples were collected at each site in 1L amber glass bottles and then acidified to pH 2.0 and kept at 5°C(Gheorghe *et al.*, 2008). Each bottle was consecutively washed with tap water, 5% decon 90 solution, 5% HCl, distilled water, MeOH and Ultra-pure water before the sample was collected. Each site was sampled on the weekend before, during and after the festival (22/06/19, 29/06/19, 06/07/19). Samples were transported to the laboratory at Bangor University within 24hrs and then filtered through a GF/C glass fibre filter paper and a 0.45 µm Nalgene filter paper before being stored at 5°C in the dark to minimise photolytic and microbial degradation.

The literature on this topic typically focusses on the sampling of WWTP's where the use of 24hr composite samples is preferable (Ort *et al.*, 2010). However, some papers have opted to use grab sampling due to sample degradation concerns(Baker and Kasprzyk-Hordern, 2013). Samples are also typically 2.5L for surface water sampling but due to the anticipated higher concentrations than normal 1L bottles were used(Gheorghe *et al.*, 2008; Kasprzyk-Hordern, Dinsdale and Guwy, 2009a; van Nuijs *et al.*, 2009). Samples would have preferably been stored at -20°C but due to the volume of samples collected each weekend there was not enough freezer space, samples were analysed as quickly as possible to minimise degradation (Gheorghe *et al.*, 2008).

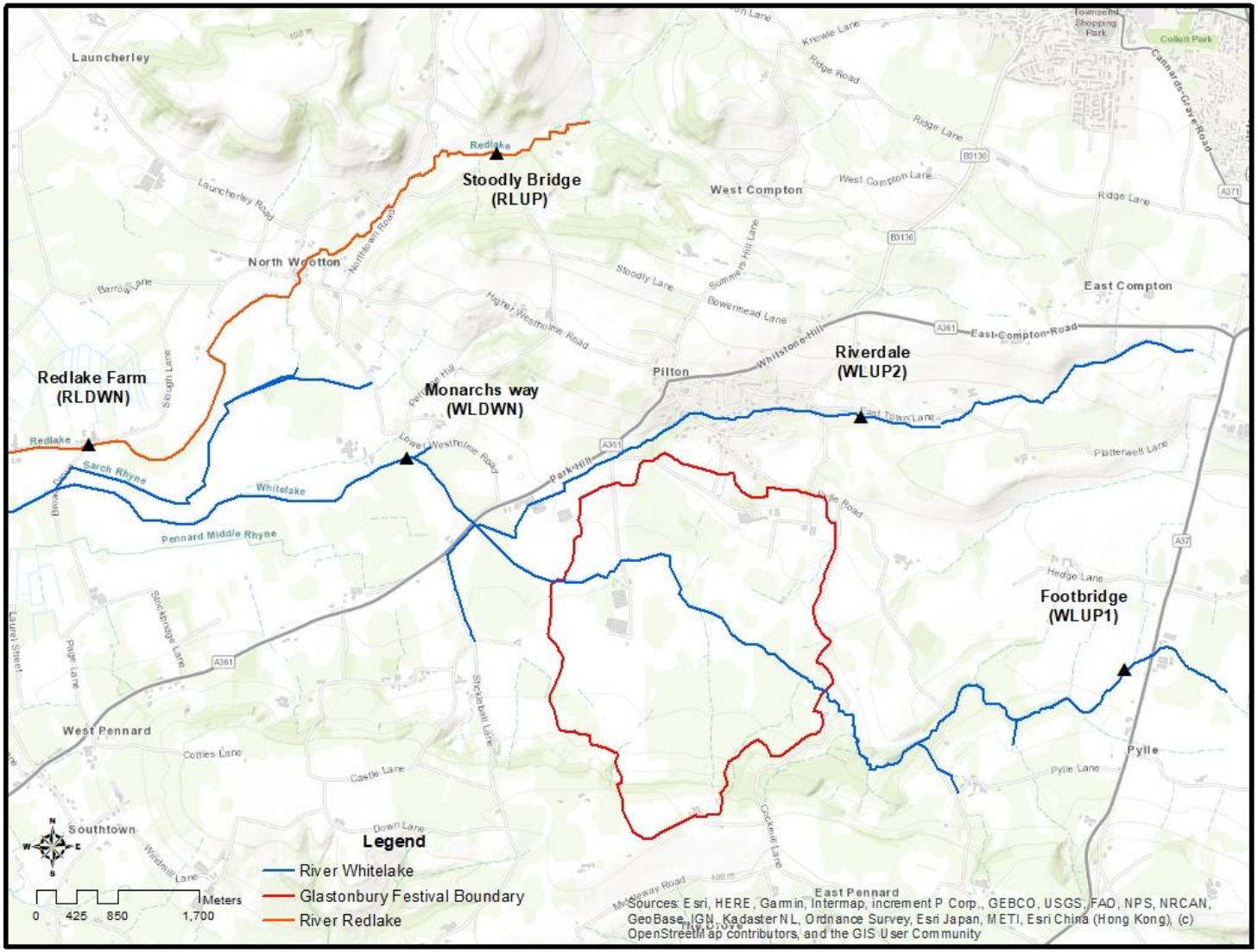


Figure 6: Map of the Pilton area at 1:50.000 with festival boundary and river courses. Position of sample sites along the rivers Whitelake and Redlake marked by triangles and labelled with geographic and shorthand names.

River temperature, conductivity, flow velocity and cross-sectional area were measured at each site. All field techniques and river calculations(river discharge) were conducted following the WHO guidelines(Bartram *et al.*, 1996).

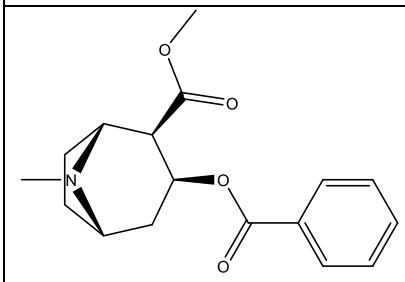
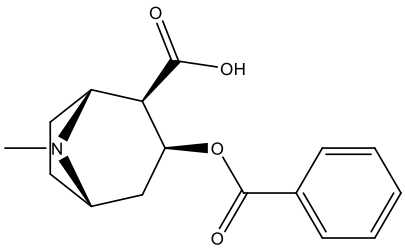
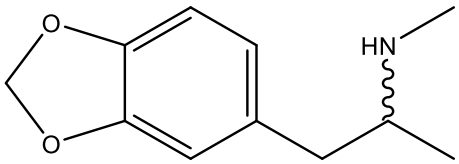
Sample preparation method

Samples were concentrated and filtered via a solid phase extraction (SPE) method using Oasis MCX PRIME (150mg, 6cc) cartridges. Each cartridge was primed with 2ml of MeOH and 2ml of pH 2.0 water, each priming solution was flowed through via gravity. Water samples were then vacuum pulled through at 2ml per minute (appendix 11a,b). Cartridges were cleaned with 2ml of pH 2.0 water and then washed with 1ml of MeOH via vacuum filtering (appendix 12). Each cartridge was eluted 3X with 2ml of ACN:5% NH3 MeOH at a 60:40 ratio via gravity (appendix 13). Each elution had a 300µl aliquot extracted and filtered using a 0.2µm PTFE syringe filter. The Aliquot was stored in Acquity I-Class sample manager at 10°C in preparation for injection.

Target Analytes

Illicit drugs were selected on their popularity with festival users with the focus on Cocaine and MDMA. Target analytes were Cocaine, Benzoylecgonine and MDMA. Percentage recovery from the SPE method was tested for each compound with the results presented in table 2.

Table 2: Compound list of analytes with chemical structure, formula, exact mass, percentage recovery from SPE method (n=6), Limit of Detection and Limit of Quantification. Compound structures were drawn on Chemdraw.

Compound	**Percentage recovery (SPE) (%)**	**Limit of Detection (µg/L^{-1})**	**Limit of Quantification (µg/L^{-1})**
 Cocaine Chemical Formula: $C_{17}H_{21}NO_4$ Exact Mass: 303.1471	118 +/- 3	0.03	0.1
 Benzoylecgonine Chemical Formula: $C_{16}H_{19}NO_4$ Exact Mass: 289.1314	96 +/- 3	0.03	0.1
 MDMA Chemical Formula: $C_{11}H_{15}NO_2$ Exact Mass: 193.1103	111 +/- 2	0.05	0.15

Analytical Method

The analytical analysis was conducted using the methodology outlined in the Waters application notes for analysis of drugs of abuse (Danaceau, Freeto and Calton, no date). UPLC-TQMS experiments were carried out on an Acquity I Class UPLC coupled to a Xevo TQ-XS triple quadrupole mass spectrometer. An Acquity UPLC BEH C_8 column (75 mm × 2.1 mm, 1.7 µm) from Waters Corporation (Wilmslow, UK) was used in the UPLC separation. The column was kept at a constant temperature of 40°C with the aid of an oven and preheater connected before the column. The

Mobile Phase consisted of MilliQ water with 0.1% formic acid (A) and Acetonitrile with 0.1% formic acid (B). The gradient program was as follows: 98% A at 0 min, 50% A at 3.33 min, 10% A at 3.50 min, 98% A at 3.60 min, 98%A at 4 min (Figure 7). The flow rate was set at 0.6 mL min^{-1}. The Injection volume was 1 µL and the autosampler was cooled to 15°C. Analytes were ionized with an ESI source in positive ion mode under the following conditions: Desolvation gas 1000 L/hr, Collision gas 0.15ml/min, cone gas 150 L/hr; Ion spray nebulizer gas 7.0 bar; Capillary voltage 2.57 kV; Detector voltage 0.56 kV; Cone voltage 53 V; Entrance Potential -1.7 V; Exit Potential -2.1 V. Additional parameters were fixed as for the tuning file. Analysis occurred in a polarity specific to each compound. MRM conditions are detailed in table 3. All samples were ran on the UPLC-TQMS by Dr Daniel Chaplin.

Fisher Scientific Optima grade acetonitrile, water and formic acid were purchased from Fisher Scientific UK. Standard compounds were purchased from Sigma Aldrich UK and used without further purification.

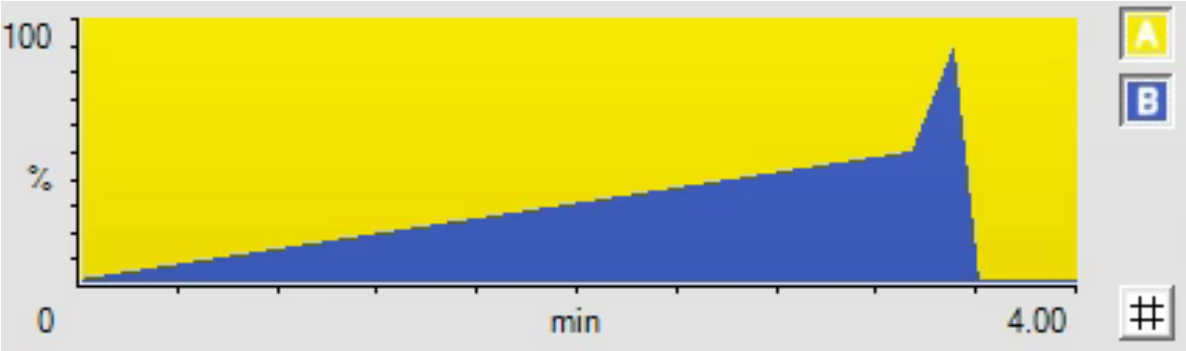


Figure 7: UPLC gradient applied in the method. A – MilliQ Water with 0.1% Formic Acid, B – Acetonitrile with 0.1% Formic acid.

Table 3: Details of MRM method.

Drug	Parent ion (mass)	Fragment ion (mass)	Cone voltage (V)	Collision energy (eV)	Retention time (decimal minutes)
Cocaine	304.2	82.1, 304.2	25	20	1.80
Benzoylecgonine	290.1	168.1	53	20	1.56
MDMA	194.1	163.1	25	12	1.27

RESULTS

All samples at every site had values above the limit of detection showing that the illicit drugs tested for are present in even small streams and tributaries. As shown in figure 8 levels of BE in the RLUP site on the weekends before and after the festival were below the limit of quantification (LOQ; 0.1ng L^{-1}). All other sites were above the LOQ.

Concentrations

The concentrations of illicit drugs (fig.8) in the Whitelake River (WL) and Redlake River (RL) vary greatly, with WL having higher concentrations than RL for all drugs at every site. RL sites only exceeded a concentration of 1ng L^{-1} five times, whereas every concentration for WL except one was above 1 ng L^{-1}. BE had the highest concentrations for most sites with a peak of almost 1µg L^{-1} for WLUP1 after the festival. Concentrations of MDMA were highest on the weekend after the festival for all sites except WLUP2 which experienced its highest concentration for the weekend of the festival. WL sites for the weekend before the festival all had higher concentrations than the RL sites.

Mass loads

Figures 9-12 show the mass load of the river in mg/hour to provide a more accurate comparison between sites as the flow rate of the river varies throughout the watercourse. Each graph shows the mass of each drug that would travel through the river in an hour. This is calculated by multiplying the concentration(ng/L^{-1}) by the flow rate of the river(L^{-1}/hr). Sewage epidemiology studies typically measure by gram per day but due to the grab sampling method, any 24-hour estimates would be under the assumption that the site measured would not experience any significant changes(Ort *et al.*, 2010).

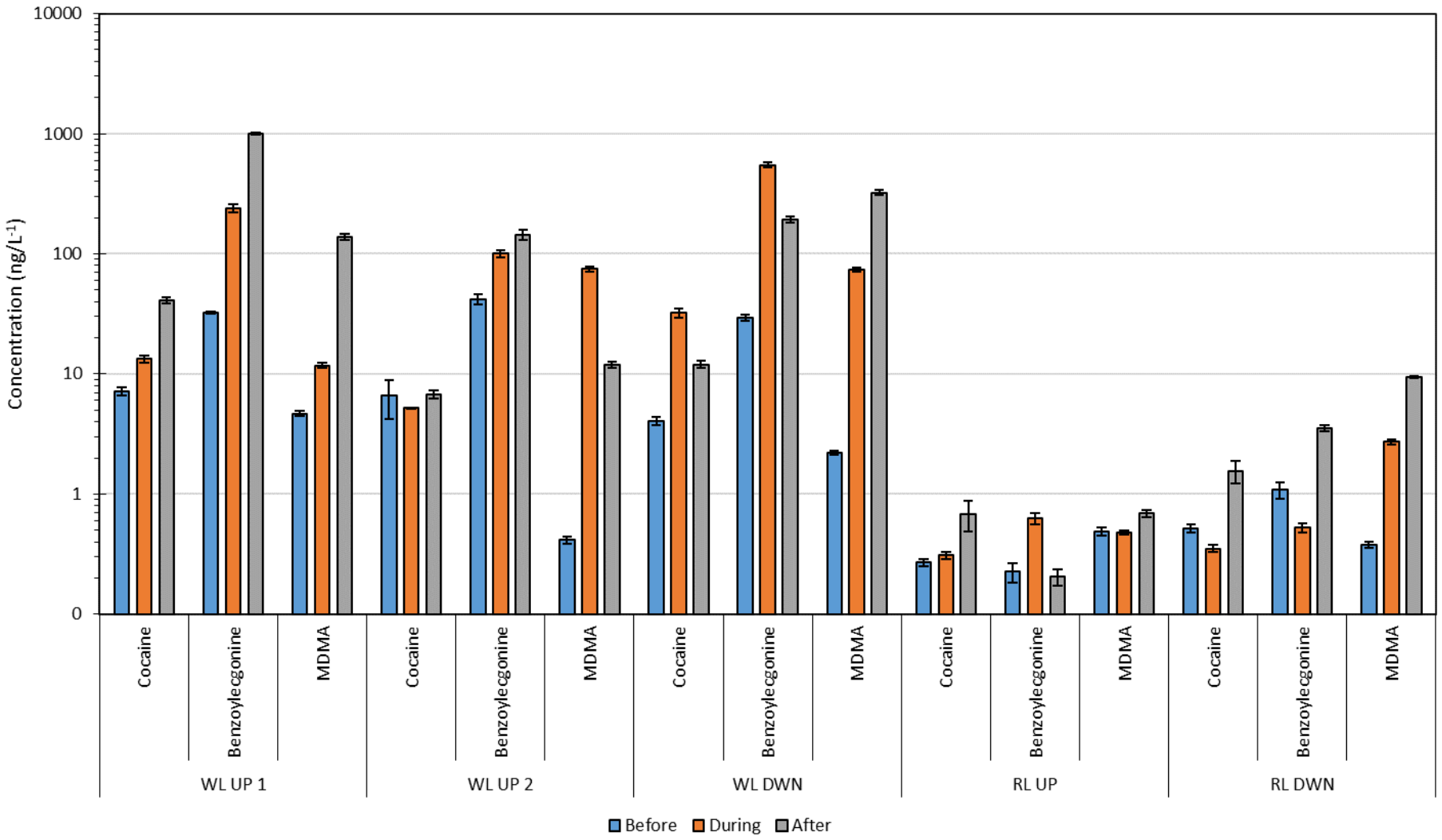


Figure 8: Concentrations of the illicit drugs at each site for the weekend before, during and after Glastonbury Festival in ng/L^{-1}. Y-axis is on a logarithmic scale to the scale of ten.

Cocaine levels, as shown in figure 9 were highest at the Whitelake downstream (WLDWN) site for all weekends with the highest recorded value of 50mg/hr for the weekend of the festival. WL had significant levels of cocaine at both upstream sites for all weekends sampled with a gradual increase in levels as the festival period came and passed. The WLDWN site had significantly higher levels for all weekends in comparison to the upstream sites. The weekend during the festival had over six times the amount of cocaine when compared to the weekend before the festival. An ANOVA test showed a statistically significant difference in cocaine levels between the sites $F(14, 57)= 99.44$, $P = <.001$. A post hoc Tukey test showed a significant difference between the WLDWN site during the festival and every other sample site and day $P < .05$. The WLDWN site showed no significant difference for the weekends before and after the festival suggesting a return to the rivers base level $P > .05$. The WLDWN lowest cocaine load was still twice as high as the highest for Redlake downstream (RLDWN) when comparing sample days differences of up to 128X were recorded for the weekend of the festival.

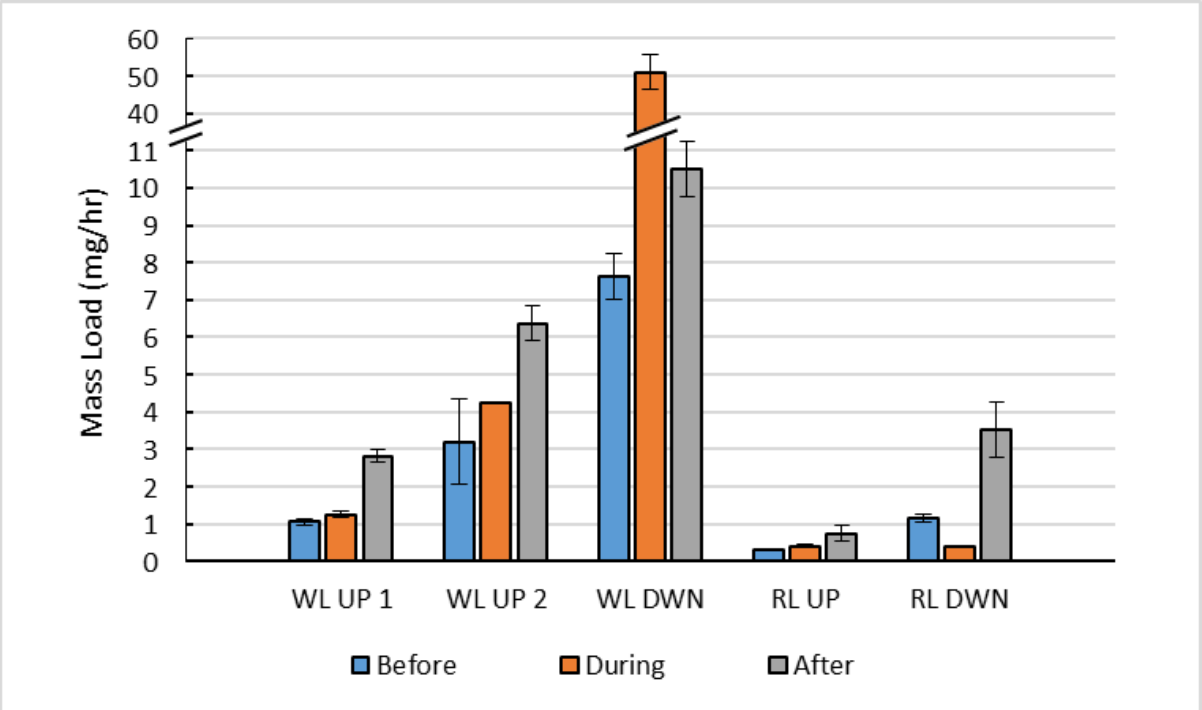


Figure 9: Mg per hour of cocaine flowing through each sampling point in the week before, during and after the Glastonbury music festival.

BE was found at much higher levels than cocaine with every site except RLUP having a minimum of 10X the mass. Figure 10 shows the mass load of BE for all sites in mg/hr. Figure 10 follows a similar trend to the levels found for cocaine (fig.9) with WLDWN during the festival having the highest values of around 850mg/hr. A one way ANOVA test showed a significant difference in the levels of BE between sites $F(14, 58)= 334.16$, $P = <.001$. A post hoc Tukey test showed the WLDWN site had

significantly higher levels of BE during and after the festival when compared with the other sites P < .05. The WLUP sites during and after the festival had a significant increase in drug levels with both these sample days having a higher mass load than WLDWN before the festival. All values for WL after the festival were at least three times higher than for before at their respective sites with WLUP1 being fourteen times higher.

All RL sites had significantly lower mass loads than WL (P> .05). Very little change occurred for the RL sites with the only large increase being for the RLDWN site after the festival however, the difference was not statistically significant P > .05.

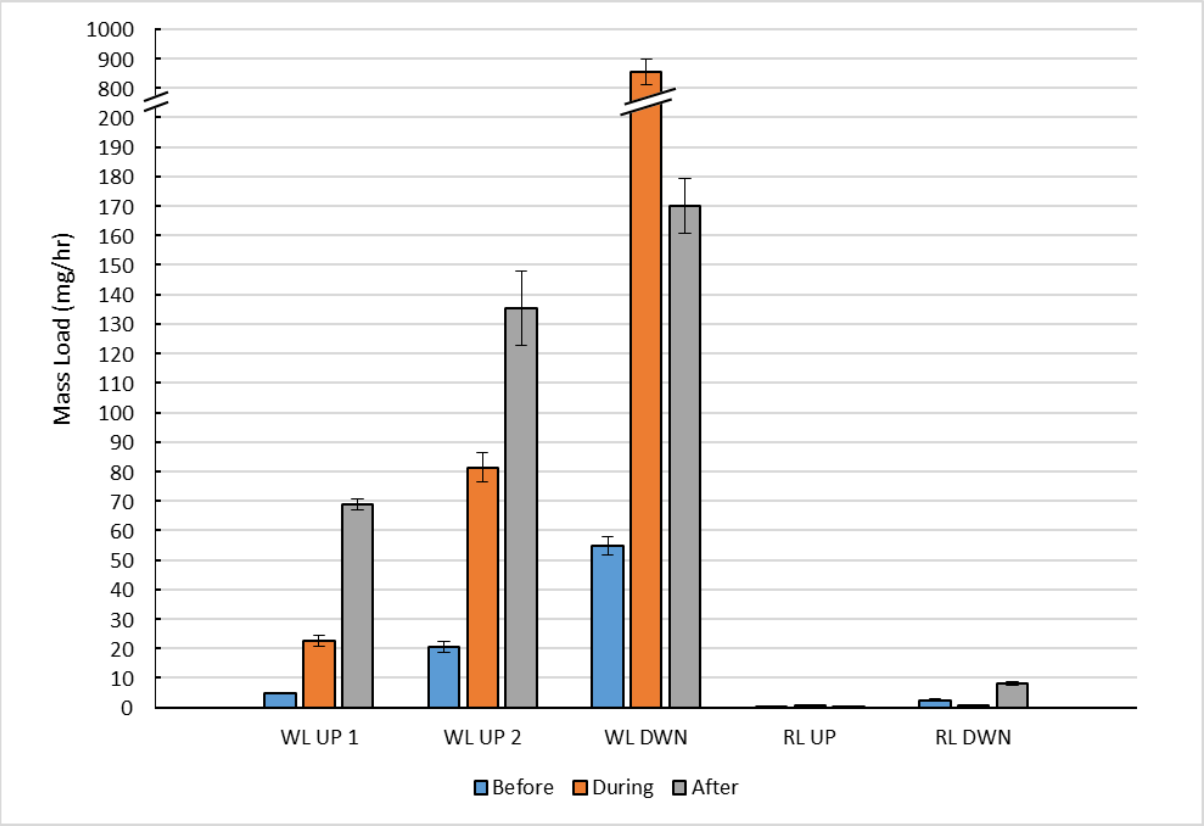


Figure 10: Mg per hour of Benzoylecogonine flowing through each sampling point in the week before, during and after Glastonbury music festival.

MDMA was found at levels higher than cocaine for all WL sites during and after the festival, with mass loads being up to twenty-six times higher. Figure 11 shows the hourly load of MDMA of the river for all sites. MDMA trends differed from cocaine with the levels after the festival being the highest. AN ANOVA showed a significant difference in levels of MDMA between sites F(14, 59)= 422.06, P = <.001. A post hoc Tukey test showed a significant difference between WLDWN during and after the festival with every site. The WLUP2 site was unique with being the only site with its highest level during the festival rather than after. WLUP2 during the festival showed a statistically significant difference between itself and every other site P < .05. The RLDWN had a higher level for

after the festival than every other site except WLDWN however, a Tukey test showed RLDWN was not significantly different from the other sites P > .05. RLUP showed no significant variation between weekends P > .05.

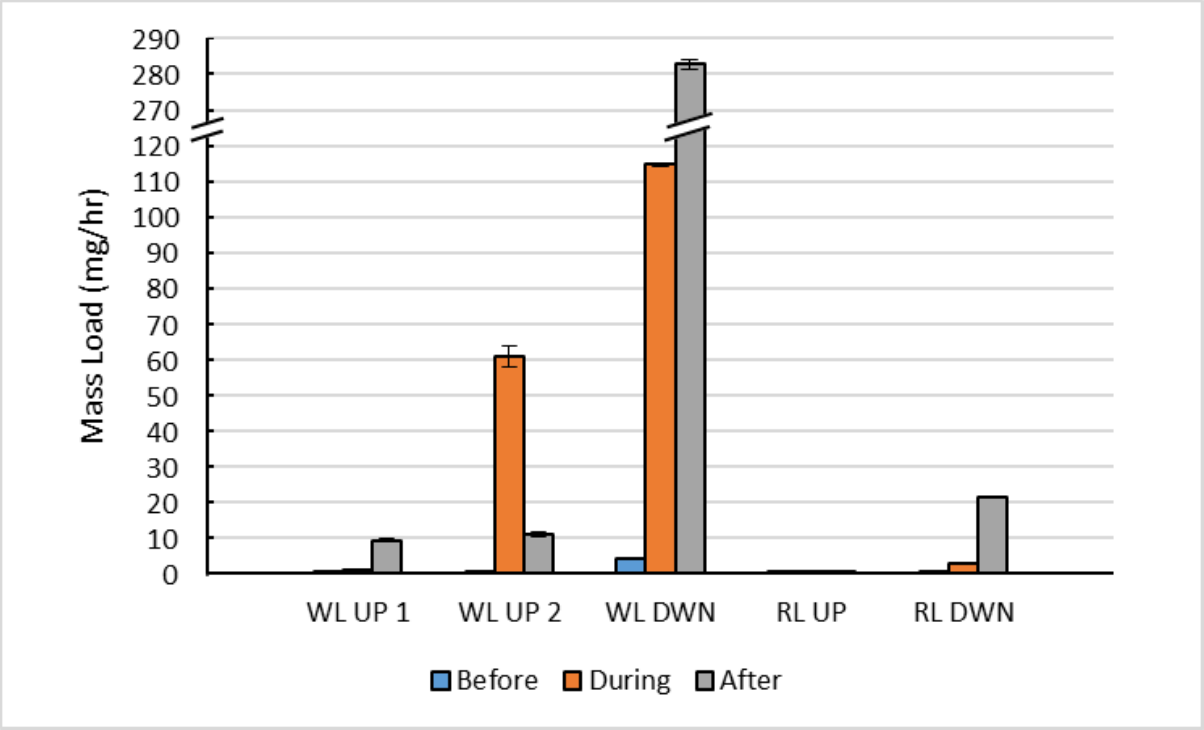


Figure 11: Mg per hour of MDMA flowing through each sampling site in the week before, during and after Glastonbury music festival.

Figure 12 shows the mass load flowing through the WL downstream site per day. Grab sample data, as used in this study provides a snapshot of the river level, so any data is calculated under the assumption that the recorded values were at an average level for the river at the time. A 24-hour composite sample would provide a more accurate average for the day but was not possible due to time and logistical constraints.

Figure 12 shows the highest drug load was the weekend of the festival with an estimated 20g of BE being transported through the river, Cocaine had its highest level during the festival with 1g being transported in the river. The weekend of the festival had the highest values for cocaine and its metabolite but the highest recorded value for MDMA was WLDWN site after the festival. An ANOVA test found a significant difference between each weekend for all drugs found at the site (Cocaine: $F(2,11)= 78.97$, $P =.00$, BE: $F(2, 11)= 282.36$, $P=<.001$, MDMA: $F(2,11)= 302.19$, $P=.00$). The cocaine levels for the weekend before and after the festival were the only samples not statistically significant

from each other P > .05. The levels for both MDMA and BE were found to have a statistically significant difference between every weekend sampled P < .05.

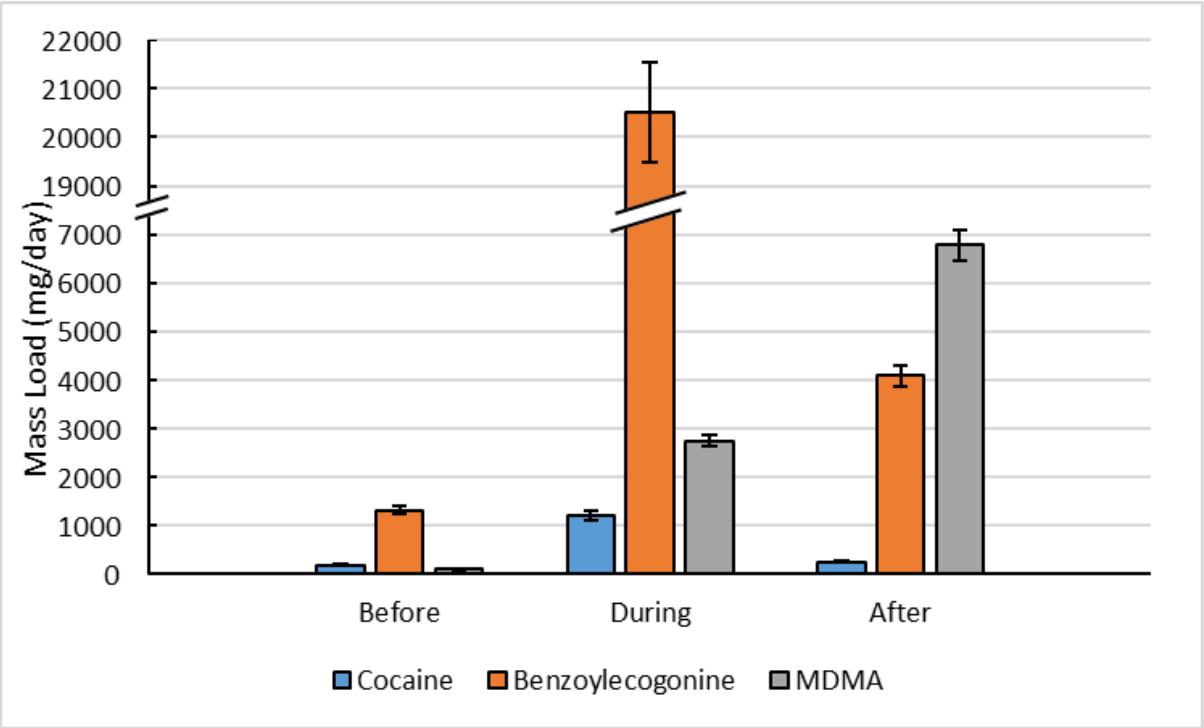


Figure 12: Mg per day of tested analytes flowing through the Whitelake downstream site (Monarchs way) before, during and after the Glastonbury music festival.

Back calculation of drug use

Using pharmacokinetic assumptions, the concentration of illicit drugs found in influent waters can be back-calculated into the total amount of the selected drug consumed. This idea was first proposed by Daughton in 2001 and was then subsequently implemented by Zuccato *et al* in 2005 for analysis of cocaine in Italian surface waters(Daughton, 2001; Zuccato *et al.*, 2005). The calculations used by Zuccato in 2005 is the most widely used method. The method calculates a correction factor from the excretion rate of the main metabolites of each drugs parent compound. The concentration of the selected metabolite (ng/L^{-1}) is then multiplied by the flow rate (m^3/sec) and again by the correction factor. Multiple assumptions must be made with sewage epidemiology to allow comparable results: negligible degradation of target compounds, no loss of sewage water from pipes and no direct release of drugs into the system (Postigo et al., 2010; Zuccato et al., 2008).

This paper assumes that many drug users will avoid using the public toilets at the festival and choose instead to urinate directly onto the site. Public urination is a widespread issue in festivals and has led to the implementation of “the Green Pledge” at Glastonbury, the pledge is a terms and condition clause everyone signs when entering the festival where they pledge not to urinate on the land and

to leave no trace (Mardal *et al.*, 2017; Digital, 2020b, 2020c). Urine pools from festivals have been sampled and found to contain significant concentrations of illicit drugs yet little research has been done on the environmental fate of the drugs after release into the soil (Mardal *et al.*, 2017). The flow of urine from the site is an open system with the potential for high loss of urine and target compounds in the substrate of the soil before infiltration into the river. This means any back calculations will provide a much lower estimate of drug use than the actual value.

The area around Pilton is comprised of the Penarth Group shales, a group of impermeable mudstones with lower Lias clays forming the soil on top (Jones *et al.*, 2000; Bristow, 2015). The low permeability of the mudstones prevents percolation of water into the groundwater system resulting in the water collecting in the surface soil and flowing into nearby rivers. The surface soil has a high affinity for water due to its high clay content which slows down any surface runoff and infiltration.

Table 4 presents the back-calculated values for drug use at Glastonbury festival. Values calculated are under the assumption that all drugs measured in the river come from the festival site. Levels before the festival are potentially from festival staff and volunteers setting up the site. Levels after are from either slow leaching from the festival site or drug use from festival staff after. An estimated minimum total of 698.75g of cocaine was taken at the festival which equates to 35 doses per 1000 inhabitants. MDMA had an estimated total of 167.3g taken at the festival which equals around 8 doses per 1000 inhabitants. Due to sampling occurring on the third day of the festival with one day remaining, the total drug use at the festival cannot be calculated without sampling occurring every day at the downstream site. To accurately calculate the total drug use at the festival a hydrograph of WL would be required to calculate stream lag which would be equivalent to the time it takes for drugs to travel from the site into the river. This would still only provide a fraction of the total drug use as most drug users would use the public services provided.

Table 4: Back calculated values of cocaine and MDMA doses that were taken at Glastonbury Festival site 2019. Calculated using the Zuccato *et al* (2008) method for back-calculation of illicit drugs.

	WLDWN – Monarchs way					
	Compound and correction factor	Weekend of sampling (in relation to the festival)	Per entire festival (203,000)		Doses per 1000 inhabitants	Mg per 1000 inhabitants
			Doses	Total mass (g)		
Cocaine	Benzoylecgonine 2.33	Before	355.34	35.53	1.78	178
		During	5531.17	553.12	27.67	2767
		After	1101.02	110.10	5.51	551
		Total	6987.53	698.75	34.96	3496
MDMA	MDMA 1.5	Before	17.30	1.73	0.09	9
		During	477.76	47.78	2.39	239
		After	1177.93	117.79	5.89	589
		Total	1672.99	167.3	8.37	837

Table 5: The ratio of Cocaine to Benzoylecgonine for upstream and downstream sites of the Whitelake River.

Site	**Weekend of sampling in relation to the festival**		
	Before	**During**	**After**
WLUP1	0.22	0.16	0.14
WLUP2	0.06	0.05	0.06
WLDWN	0.04	0.05	0.06

Summary

Drug levels in WL were significantly higher than the levels found in RL both in terms of concentration and river load, this suggests high levels of drug usage in the WL catchment area. WL had significant concentrations in both its upstream and downstream sites which when extrapolated into river load showed the amount of drugs was higher at the downstream site. The largest increase between upstream and downstream was for the weekend of the festival, implying a larger quantity of illicit drugs were released into the watercourse during that weekend in comparison to the other weekends. As shown in figure 6 the area between the sites is mainly dominated by the Glastonbury festival site where drug use would be higher than in a rural village such as Pilton. The weekends after

the festival were still significantly higher than the weekend before for BE and MDMA suggesting either continued drug use on the festival site or a slow leaching effect from pooled drugs on-site(Yu, Liu and Wu, 2013; Rosi-Marshall *et al.*, 2015).

DISCUSSION

The concentrations found in the River Whitelake (WL) (fig. 8) reached higher levels than expected for a river without a WWTP or urban development nearby. Concentrations for the River Ebro, a non-urban river in Spain are reported in the 25ng/L^{-1} range for total drug concentration, whereas WL before the festival and at its lowest had a total drug concentration of 35ng/L^{-1} (Postigo, de Alda and Barcelo, 2010). A more accurate comparison can be drawn between WL and the River Redlake (RL) as they are both similar in size and situated in the same geographical area around the same settlements. When comparing the lowest total drug loads for each river, RL is repeatedly lower with a total drug load of 2ng/L^{-1} in comparison to WL 35ng/L^{-1}. The higher baseline values for WL are indicative of higher drug use in the catchment area of the river in comparison to RL and the River Ebro. This is of important significance as the 2019 festival was the first festival to occur after the 2018 “fallow year” where the festival does not occur to allow the land to rest. The higher levels of drugs in WL before the festival suggest drug use on-site before the festival begins, this could be possible due to drug use by the large amounts of workers and volunteers present for setting up the site. Using back-calculated values from table 4, workers and volunteers would have taken a minimum of 35g of cocaine and 2g of MDMA amongst them that was then urinated onto the site. Another possibility for the higher baseline levels could be long term storage and a slow leaching effect of illicit drugs from the site which will be explored later.

The disparity between RL and WL increases for the weekend during and after the festival, with figure 9,10,11 and 12 all showing increased illicit drug load for WL during and after the festival. The mass loads of cocaine and BE for the weekend of the festival were 6.5X and 15X higher than for the weekend before (fig.9,10). MDMA mass loads were 27X and 68X higher for the weekend during and after the festival when compared to the weekend before (fig.11). The massive increases in drug level in the river are indicative of increased drug use and release from the catchment area of WL which as shown in figure 6, is dominated by the festival site.

Music festivals causing large increases in MDMA concentrations in wastewater is widely reported with the Benicasm WWTP in Spain reporting a peak of 27µg/L^{-1} of MDMA during the festival period while MDMA was barely present in the wastewater before the festival (Bijlsma *et al.*, 2014). The Spring Scream music festival in Taiwan reported an increase to 950ng/L^{-1} from 89.1ng/L^{-1} (Jiang *et al.*, 2015). Large amounts of MDMA release from festival sites are common due to its high popularity

for music events such as raves and festivals (Van Havere *et al.*, 2011). This statement aligns with the levels found in the river Whitelake where MDMA levels increased during and after the festival and reached concentrations as high as 323ng/L^{-1} and mass loads as high as 283mg/hr (fig.8,11). This equates to a minimum of 165.57g being consumed on the Glastonbury site, which equates to 828mg per 1000 inhabitants (mg/1000) or a 0.8% usage rate amongst inhabitants which is vastly under the UK average MDMA usage rate of 3.3% (EMCDDA, 2019). An average usage rate of 240mg/1000 was recorded for an Australian music festival, this is significantly lower than the recorded Glastonbury rate suggesting undetected usage at Glastonbury (Lai, Thai, *et al.*, 2013). It is worth noting that the values recorded for Glastonbury cannot be identified to a single day so could potentially be a representation of MDMA use for the entire festival due to mixing in the soil during the week before release into the river. Considering that drugs found in the river are from users who have urinated on the site rather than the toilets provided MDMA usage can be assumed to be much higher than any amounts found in the literature for festivals.

The large spike in levels of cocaine and its metabolites previously mentioned resulted in peak concentrations and mass loads of 548ng/L^{-1} and 855mg/hr for the weekend of the festival (fig.8,9,10). The spikes in cocaine levels recorded in Whitelake from Glastonbury were significantly higher than levels at the Spring Scream Festival in Taiwan and the unnamed Australian festival. Both these festivals recorded minor amounts of cocaine with Spring Scream festival recording maximum concentrations at the WWTP of 17.7ng/L^{-1} and <0.05ng/L^{-1} in nearby surface waters (Jiang *et al.*, 2015). The Taiwanese festival did not measure for benzoylecgonine or any other of the principal metabolites of cocaine meaning the usage rate estimates would be vastly below the actual value due to the high degradation rate of cocaine in wastewater. Australia also recorded minor amounts of cocaine and its metabolites with an average of 3.5 and 45mg/1000 for cocaine and benzoylecgonine respectively, the low amounts suggest MDMA and other methamphetamine-based drugs to be more popular than cocaine (Lai, Thai, *et al.*, 2013). Both these studies suggested that cocaine usage at festivals would not be high which is a contradiction to our data. High cocaine release was recorded in the Benicasim WWTP after the Spanish music festival where cocaine and benzoylecgonine followed a similar trend to MDMA with a large increase during the festival period however cocaine concentrations did not exceed MDMA concentrations unlike in our study (Bijlsma *et al.*, 2014). The higher cocaine use at the Spanish music festival and Glastonbury but not at either of the other two highlights the differences in drug cultures. Western Europe is the second-largest market in the world for cocaine whereas cocaine in South Asia became more popular in 2015/16 indicated by a tenfold increase in seized imports those years (Pirona, Matias and Giraudon, 2018). The mean daily amount of cocaine found in the wastewater of 10 European cities in 2017 equated to 410mg/1000 while the

spike in Whitelake equated to 2767mg/1000 which is significantly higher and further evidence of the heightened drug use at festivals (Pirona, Matias and Giraudon, 2018).

BE was the most abundant tested chemical at all sites and had the highest concentration and daily load found (1µg/L^{-1} and 850mg/hr; fig.8, 10). BE levels were all significantly higher than what was measured for cocaine, this disparity is a regular occurrence in sewage epidemiology analysis (Zuccato *et al.*, 2005, 2008; Castiglioni *et al.*, 2008). When cocaine is taken only a small percentage (8%) of Cocaine is excreted in the urine unchanged, with the majority being excreted as BE (generally 35% of total dose) this can be presented as a metabolic ratio COC/BE= 0.23 (Ambre *et al.*, 1988; Cone *et al.*, 1998; van Nuijs *et al.*, 2009). Cocaine has low stability in freshwater at environmental conditions (pH 6-7, >4°C) with degradation of 22-36% after three days where it typically undergoes hydrolysis into BE (Gheorghe et al., 2008). The high degradation rate of cocaine in surface waters is often signified with a lower COC/BE ratio as shown by Van Nuijs *et al* (2009) where ratios increased in winter due to reduced hydrolysis of cocaine in the low-temperature water. COC/BE ratios for WL (Table 5) range from 0.22 to 0.14 for before the festival but then decreases to around 0.05 for during and after the festival. The decrease in ratio and thus higher degradation was probably caused by the higher water temperatures for the weekends during and after where surface waters were 2-5°C higher (appendix 15).

The short half-life of illicit drugs would mean the large release from the festival would quickly degrade and no longer be present in the river for the weekends after. Figures 8,9,10,11 and 12 all show higher concentrations and river loads of illicit drugs for the weekend after the festival when compared to the weekend before showing the river has not returned to its base level. The highest MDMA concentrations and mass loads for each site were all recorded for the weekend after the festival (fig.8 and 11). The continued release of drugs into the river suggests either continued drug use and subsequent public urination on the site or a slow leaching effect due to substrate sorption of the illicit compounds.

PPCPs due to their polar nature, interact with charged surfaces such as clay minerals found in soils leading to sorption of PPCPs to soils (Stein *et al.*, 2008; Yu, Liu and Wu, 2013). Illicit drugs such as cocaine and MDMA are also polar and would interact in a similar way with soil (Rosi-Marshall *et al.*, 2015). The sorption/desorption of illicit drugs is reported to be pH-dependent as most illicit drugs gain charge at pH ≥ 8 (Stein *et al.*, 2008; Rosi-Marshall *et al.*, 2015). The polarity of cocaine and MDMA make them highly soluble so will freely dissolve in surface water which is the main method of transport in the environment (Stein *et al.*, 2008; Rosi-Marshall *et al.*, 2015). Charged surfaces such as clay minerals could reduce polar compound mobility in soil and slow their release into ground and

surface waters(Stein *et al.*, 2008; Yu, Liu and Wu, 2013). Dry soils once urinated on could absorb the urine and any illicit drugs in it, which then remain sorbed to the soil until rainfall causes the drugs to desorb from the soil and dissolve into the water. The Somerset area had no rainfall during the festival and an average temperature of 19.8°C, the area had a total of 11.4mm of rainfall over the ten days before the festival with an average temperature of 14.8°C (appendix 14, 15). The low rainfall and high temperature resulted in decreasing water levels in the rivers sampled and drier soils. The low water content in the soil would cause a higher rate of adsorption of drugs to soil particles consequently causing the slow release of drugs from the site after the festival (fig.8 and 12).

MDMA is one of the more stable illicit drugs and experiences small degradation of <10% after 3 days in wastewater (van Nuijs *et al.*, 2012; McCall *et al.*, 2016). Cocaine, on the other hand, has low stability in freshwater at environmental conditions (pH 6-7, >4°C) with degradation of 22-36% after three days where it typically undergoes hydrolysis into BE (Gheorghe et al., 2008). Research into the stability of illicit drugs in soils has found that once sorbed to soil MDMA has a half-life of up to 59 days (Pal *et al.*, 2011). Cocaine soil stability has not been researched as it shows poor sorption to solid particles and is typically dissolved in water (Gheorghe *et al.*, 2008). This increased stability of MDMA when sorbed to soil coupled with subsequent desorption during rainfall could result in the Glastonbury site continually leaching drugs into the nearby surface water and explain the higher concentrations found in WL after the festival (fig. 8). The levels found before the festival were probably not from long term leaching from the site as the stability of illicit drugs even when prolonged in soil would not be enough to reduce degradation for a whole year.

The festival ran from the 26-30th of June with sampling only taking place on the 29th the four days missed have the potential to release the same quantity of drugs into the river. The festival released an estimated 24.5mg of illicit drugs into the River Whitelake on the 29th June (fig.12), if every day of the festival had similar rates of drug use and public urination, an estimated total of 98mg could be released throughout the festival. A full week after the festival the site released 11mg of illicit drugs in the river (fig.12), assuming an immediate drop to that level after the festival rather than a slow decrease, a total of 77mg could be released during the week after.

Since 2016, Glastonbury festival has used compost toilets as a green alternative to the porta-potties and drop toilets normally used (*What the Glastonbury 2019 toilets are really like*, 2019). These compost toilets are used to generate compost for the on-site peace garden, when a festival-goer uses the toilet they put a scoop of sawdust in afterwards to soak up any liquid(Digital, 2020c, 2020d). Drug use at the festival could cause illicit drugs to be present in the compost which, once

spread onto the garden could leach illicit drugs into the environment. The presence of illicit drugs would vary depending on the length of the composting process as degradation of illicit drugs would occur but at a slower rate than in surface water.

As previously mentioned, illicit drugs have negative effects on the environment at ng/L^{-1} concentrations. Cocaine concentrations in WL as shown in fig.6 ranged from 4-41 ng/L^{-1} with a skewness for higher concentration for the weekends during and after the festival. Cocaine levels decreased after the festival and would continue to decline as drug use declined on site.

Exposure to 20 ng/L^{-1} concentrations of cocaine influenced the endocrine system of the European eel and caused various hormone imbalances in the brain such as heightened dopamine release(Gay *et al.*, 2013). Eels post-exposure to cocaine were found to have even higher levels of dopamine in both the brain and plasma when compared to exposed eels. This dopamine imbalance is a symptom and effect of endocrine disruption by cocaine and causes delayed sexual maturity in eels and is hypothesised to be a cause of their population decline(Sébert *et al.*, 2008; Gay *et al.*, 2013). Cocaine exposure also caused oxidative stress and destabilized lysosomal membranes in zebra mussels at concentrations as low as 40 ng/L^{-1} (Binelli *et al.*, 2012). Concentrations of cocaine found in WL are within the range to cause the various impacts previously described, although eels and zebra mussels are not present in WL the negative impacts would undoubtedly be similar for the natural fauna.

BE concentrations were in the 30-998 ng/L^{-1} range and were a minimum of 4X higher than cocaine concentrations (fig.8). Both cocaine and BE caused similar levels of DNA fragmentation and cytotoxicity in zebrafish embryos, but at different concentrations (115 ng/L^{-1} for BE and 1.2 µg/L^{-1} for cocaine)(Parolini *et al.*, 2017). Comparison of biomarker response indexes showed that BE is more cyto-genotoxic than cocaine(Parolini *et al.*, 2017). The concentrations released from the festival often exceed the proven limit for damage top fish embryos showing that the festival has the potential to disrupt fish lifecycles. BE also caused a 20% and 40% decrease in mitochondrial activity in fern spores exposed to a 1 ng/L^{-1} or 10 ng/L^{-1} concentration for 48hrs(García-Cambero *et al.*, 2015). A decrease in mitochondrial activity would reduce a spores germination capabilities meaning that BE is a germination inhibitor just like its parent compound cocaine (Evenari, 1949; García-Cambero *et al.*, 2015). Both Cocaine and BE cause decreased root growth of 11% and 23% in field lupine exposed to concentrations of 0.01 mol/L^{-1} and 0.04 mol/L^{-1}. These concentrations are higher than environment levels, but cell level effects would undoubtedly be caused by lower concentrations. Both these studies show the potential for interference with plant germination and growth from concentrations of BE and cocaine. WL concentrations of BE before the festival ranged from 30-41 ng/L^{-1} meaning long term germination inhibition in the WL area is a possibility.

MDMA concentrations ranged from 12-322 ng/L^{-1} in WL for the weekends during and after the festival, the weekends before were significantly lower and arranged from 0.4-4.5 ng/L^{-1} (fig. 8). MDMA causes cytotoxic damage to haemocyte cells of zebra mussels at a concentration of 500 ng/L^{-1}, exposure to 50 ng/L^{-1} concentrations of MDMA does not cause a significant effect yet the cytotoxic potential of the molecule suggests long term exposure to the lower concentration would have a negative effect on zebra mussels (Parolini, Magni and Binelli, 2014). Illicit drug pollution is at a level where any organisms will not be exposed to one illicit drug at a time they will be exposed to a "cocktail" of illicit drugs in surface water. Studies have found that zebra mussels experienced higher genotoxic effects as well as higher oxidative stress and damage when exposed to a cocktail of drugs as a pose too high levels of a single compound(Parolini *et al.*, 2015, 2016). The drug cocktail used in the study consisted of Cocaine (50 ng/L^{-1}), BE (300 ng/L^{-1}), Amphetamines (300 ng/L^{-1}), MDMA (50 ng/L^{-1}) and morphine (100 ng/L^{-1}), the concentrations in the cocktail for the compounds tested in this study were of a similar level to the WL sites during and after the festival with MDMA often being double the concentration found in the cocktail (fig.8).

Biofilms close to WWTP's have been shown to accumulate PPCPs to almost 1 µg/g levels, and cause a decrease in microbial diversity through reduction of cyanobacteria populations (Aubertheau *et al.*, 2017). Biofilm accumulation occurred for compounds with varying charges and no particular affinity was shown. A similar effect can then be hypothesized to occur for illicit drugs as the impacts of PPCPs and illicit drugs often overlap (Rosi-Marshall *et al.*, 2015). This could cause accumulation of illicit drugs in biofilm populations in the River Whitelake causing disruption to biofilms microbial diversity and photosynthetic ability.

CONCLUSION

The Glastonbury festival despite its green attitude still poses a risk to the environment through the release of illicit drugs from public urination into the River Whitelake. Greenfield site festivals where high levels of drugs and alcohol are consumed in a condensed area with few toilets pose a significant risk for the release of illicit drugs into both the freshwater and soil of the site. Although the stability of drugs is low, they are often released in high enough amounts to cause substantial damage to the ecosystem. Increased stability in soil complexes coupled with a leaching effect could result in a continued release from festival sites in following weeks after drug use has stopped. Sewage epidemiology back calculations for estimation of drug use do not provide an accurate estimate of total drug use at festivals when analysing nearby surface water levels but could through tandem analysis of wastewater from on-site facilities with environment levels. To minimise the release of

drugs from festivals research into possible treatment via environmentally friendly methods such as CTWs should be conducted.

Thesis conclusions

- Paracetamol is effectively removed by biofilm in anaerobic conditions in simple gravel-based CTWs. Aeration of the CTW does not cause any increase in removal rate of paracetamol. Unaerated systems reported 95% removal in 13 hours on the first day before increasing efficiency on consecutive days with 95% removal in 4 hours. Aerated systems experienced 95% removal in 23 hours on the first day before adapting and increasing efficacy to 95% in four hours. This shows adaption of biofilm to pharmaceutical concentrations as well as the ability of simple CTW systems to effectively treat waste containing high paracetamol concentrations. The presence of aeration provides no increase in paracetamol removal providing further evidence for paracetamol degradation being an anaerobic process.

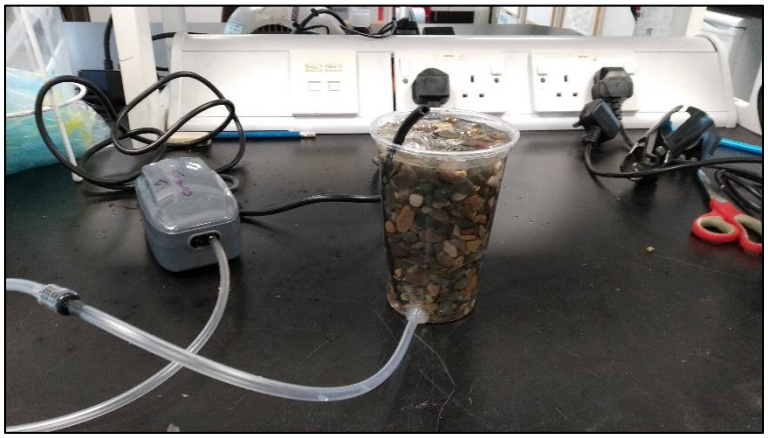
- The use of colorimetric methods for pharmaceutical testing provides a fast, cost-effective method for testing in laboratory conditions. Further research on other high-risk pharmaceuticals such as tramadol, carbamazepine and diclofenac should be conducted to further understand the optimal removal processes for these compounds.

- The direct environmental release of cocaine, benzoylecgonine and MDMA into the freshwater ecosystem surrounding the Glastonbury music festival was proven to occur. The river Whitelake on the Saturday of the festival transported 24,471mg/day of illicit drugs that day with 11,119mg/day of illicit drugs transported by the river the following week after the festival had ended. This is evidence for the continued release of illicit drugs from the festival site after the festival has ended. Highest environmental concentrations in the river Whitelake downstream from the festival were 32.33ng/L^{-1}, 548.41ng/L^{-1} and 322.77ng/L^{-1} for cocaine, benzoylecgonine and MDMA respectively. Although only in the ng/L^{-1} range, illicit drugs have significant effects on aquatic systems at low concentrations. Testing for other illicit drugs and metabolites such as ketamine and 11-nor-9-carboxy-Δ9 - tetrahydrocannabinol (a metabolite of THC) would undoubtedly produce results and was planned for this experiment but due to COVID restrictions was dropped.

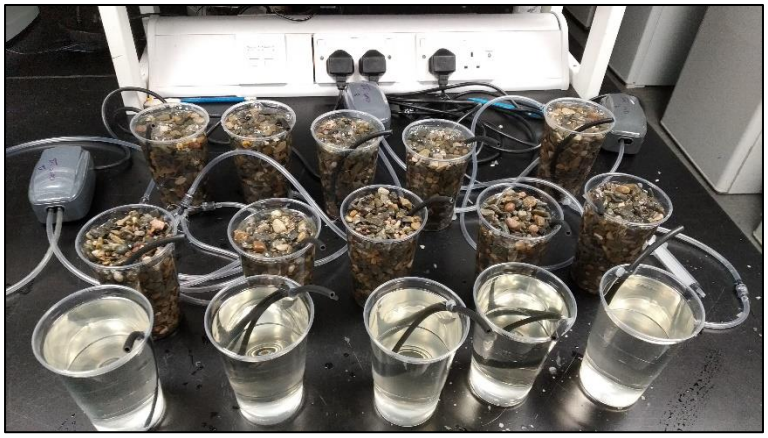
- The use of sewage epidemiology to estimate drug use in populations is effective when used on wastewater from sewage networks but when applied to concentrations found in surface waters underrepresentation is a guarantee. Estimates calculated a usage rate of 35 doses of cocaine and 8 doses of MDMA per 1000 inhabitants for Glastonbury festival. This dosage rate is below the UK average usage rate for the respective drugs and suggests a decreased drug use rate at Glastonbury festival. The low rates calculated can, however, be explained by the high degradation of the test compounds as well as the majority of people using the toilets provided therefore not causing release into local waters which would leave the majority of festival-goers unaccounted for. To calculate a more accurate estimate of drug use at the festival, testing of waste from the public toilets should be conducted as well as increased sampling on the days of the festival rather than once a week.

- Further research into the stability of popular illicit drugs in soil and leaching processes that occur would assist in identifying the level of illicit drugs released due to public urination at music festivals. Glastonbury festival has a river running directly through the site unlike other festivals such as Reading and Leeds which could still pose environmental concern depending on the properties of illicit drugs in soil. Research into the ability of riparian based CTWs to remove ng levels of illicit drugs from river water would provide an environmentally friendly solution to the release from Glastonbury festival whilst also providing a potential tertiary treatment system for WWTPs that could remove both illicit and pharmaceutical chemicals from wastewater. The effectiveness of CTWs at removing complex organic compound pollution is well documented as well as illustrated by the paracetamol research conducted in this thesis.

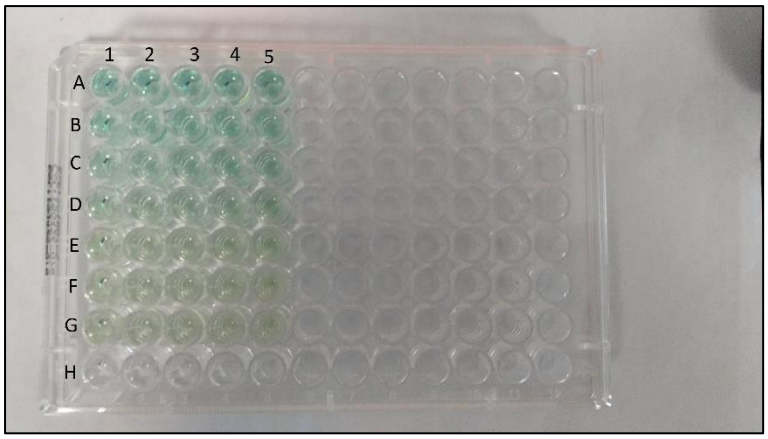
APPENDIX



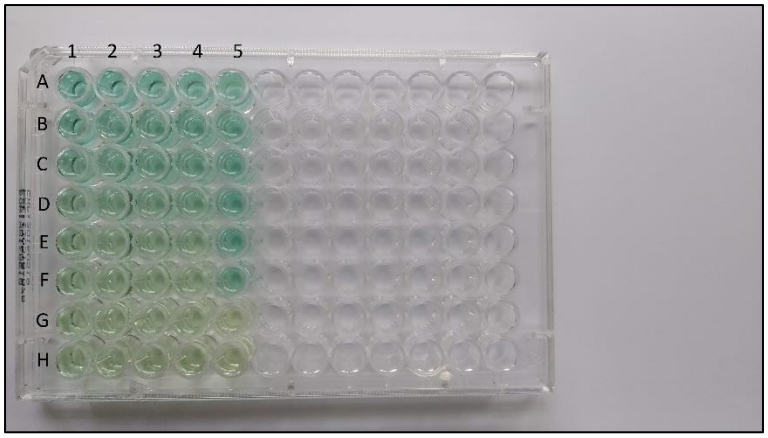
Appendix 1. Photo of a aerated gravel wetland with aeration pump attached. Black tube is the tube used for taking samples.



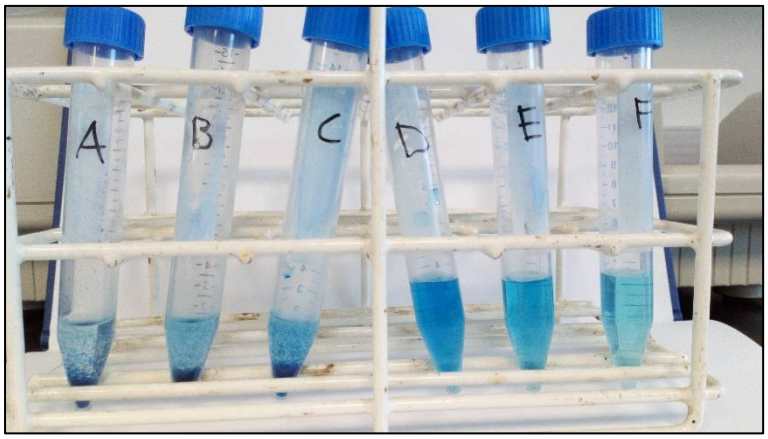
Appendix 2. Photo of all test wetlands. Closest row are the test wetlands for mimicking surface waters. Back two rows are unaerated and aerated wetlands.



Appendix 3. Clear microplate showing 5 standard curve runs. Row A shows 2mg/L^{-1}, each row decreases in concentration by 0.4mg/L^{-1} except row F and G which have concentrations of 0.2mg/L^{-1} and 0.1mg/L^{-1}.



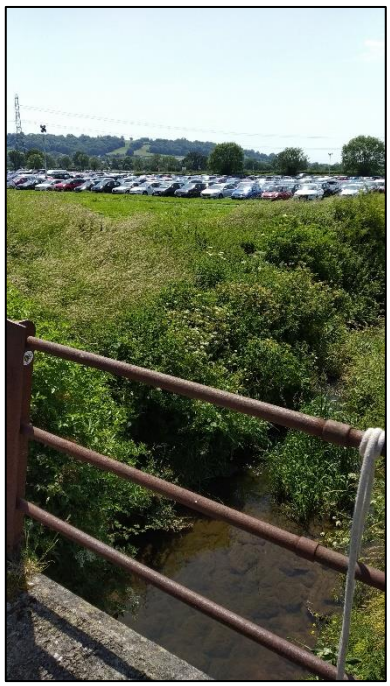
Appendix 4. Clear microplate showing a standard curve run in column 1 and samples in the remaining columns.



Appendix 5a, 5b. 15ml centrifuge tubes showing the Prussian blue solution formed by the redox of Fe. Tubes A, B and C all show the precipitation of Fe(II) caused by high concentrations and low acidity in the sample.

Appendix 6: Picture of site WLUP1. Whitelake upstream 1, Footbridge, Coordinates: 51.149908, -2.547587

Appendix 7: Picture of site WLUP2. Whitelake upstream 2, Riverdale. Coordinates: 51.1648-2.57215



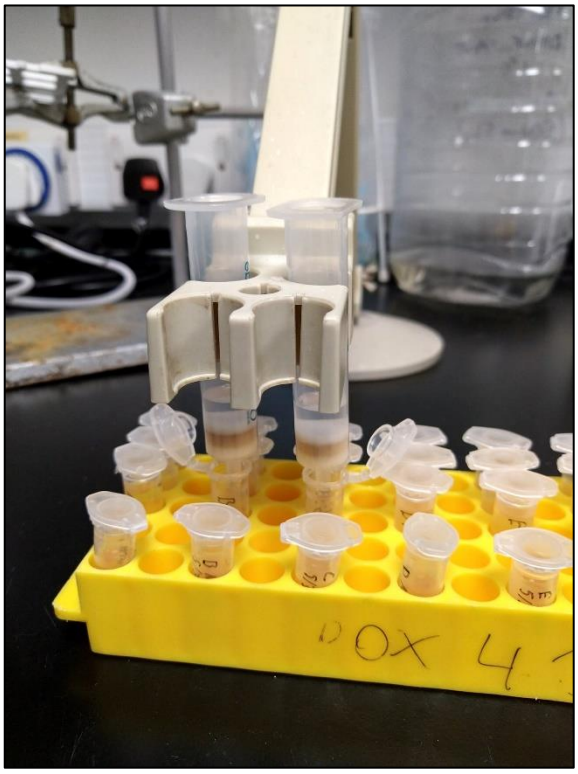
Appendix 8: Picture of site WLDWN. Whitelake downstream, Monarchs Way, Coordinates: 51.16226959-2.61537337

Appendix 9: Picture of site RLUP. Redlake upstream, Stoodly Bridge. Coordinates: 51.19858-2.62730

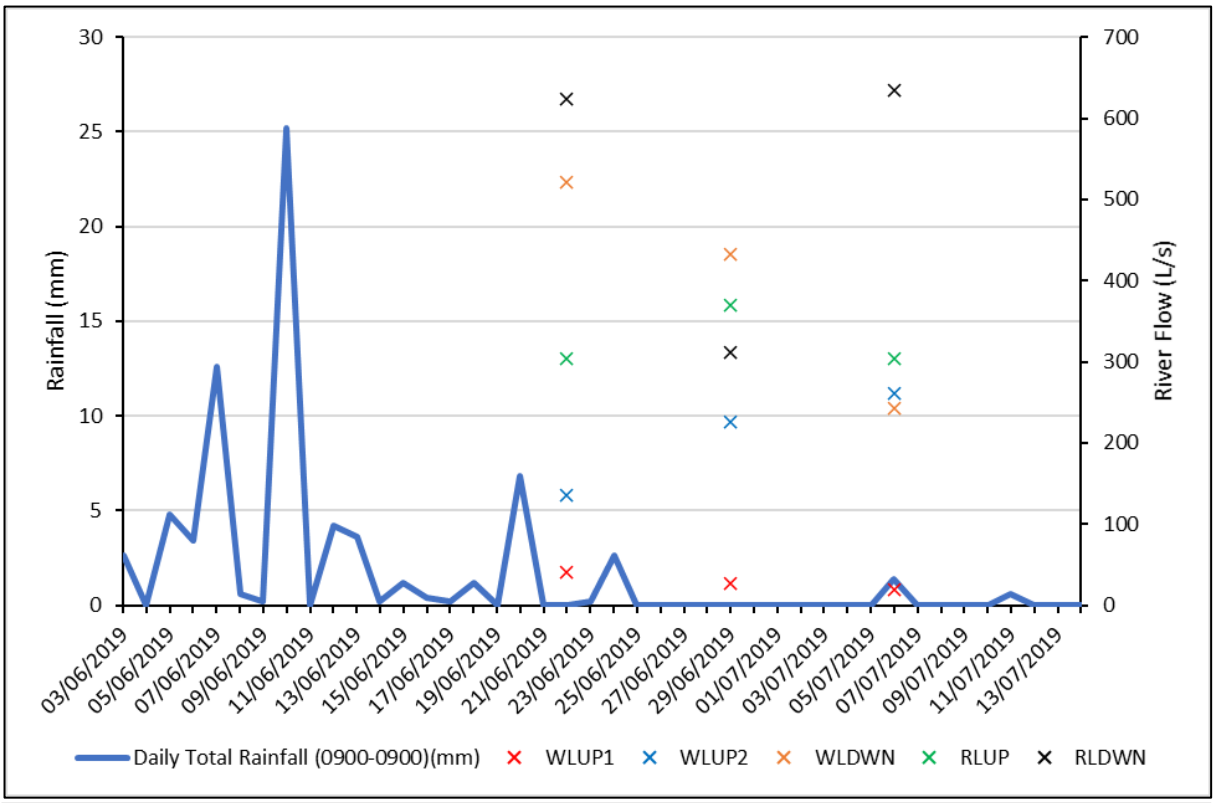
Appendix 10: Picture of site RLDWN. Redlake Downstream, Redlake Farm. Coordinates: 51.16305160-2.64494538

Appendix 11a, 11b: Pictures of vacuum manifold setup used for SPE extraction of water samples.

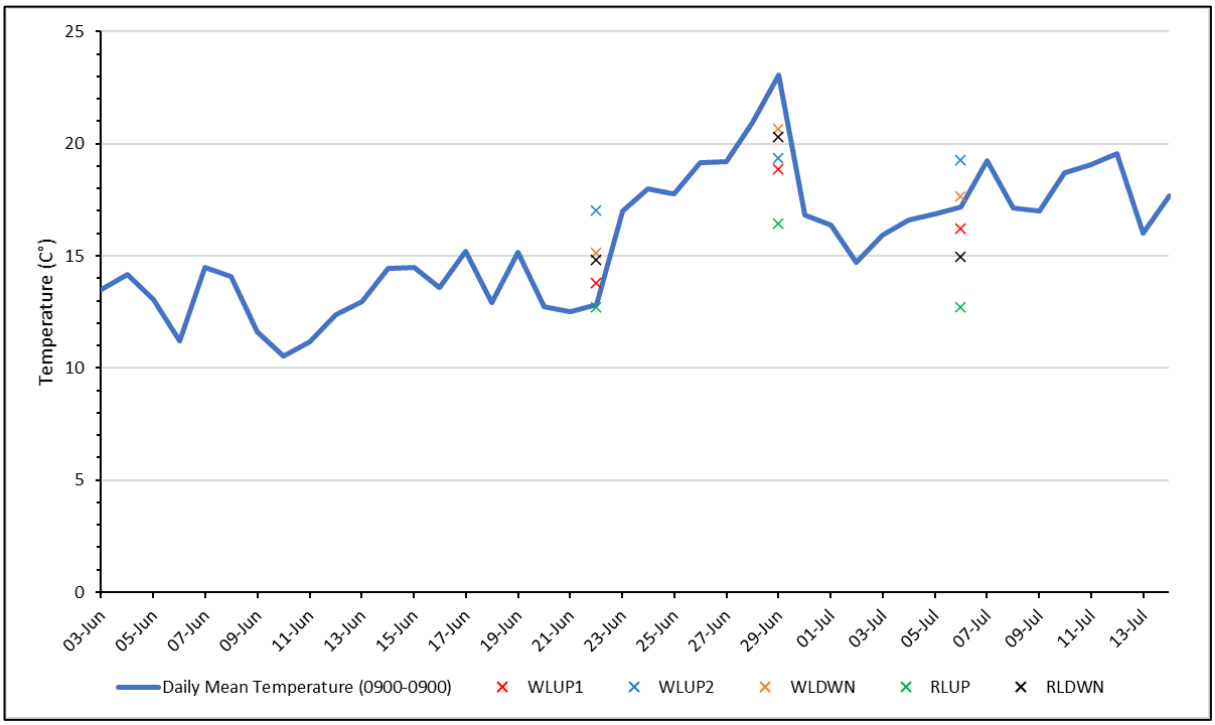
Appendix 12: Picture of 1ml of methanol being pulled through SPE tube to clean tube of contaminants.



Appendix 13: Picture of SPE extraction via gravity flow of 2ml ACN:5% NH3 MeOH at a 60:40 ratio.



Appendix 14: Graph of daily total rainfall (mm) recorded at the Yeovilton MET office weather station, the closest station to Pilton. River flow in litres per second from each sample site is also presented alongside on the right axis.



Appendix 15: Graph of daily mean temperature in Celsius from 0900 to 0900 recorded at the Yeovilton MET office weather station, the closest station to Pilton. Water temperature from each sample site is also presented alongside. Error bars are present for water temperatures of the sites but are too small to be seen as they range from $0.229\text{-}7.25\times10^{-16}$.

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