Combination of nanoparticles from polystyrene and the hormone 17alpha -ethinylestradiol (EE2) in water

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Aerial View of the Braided Eyak River, Copper River Delta, Alaska. [Photograph]. *Encyclopædia Britannica ImageQuest*. Retrieved 2 Jun 2017, from http://quest.eb.com/search/107_283125/1/107_283125/cite

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Abstract

Plastic materials and synthetic hormones are found in aquatic environments globally. Plastic in water can degrade to nanoparticles (NP) with special characteristics, in the environment nanoparticles and hormones have a proven effect on wildlife. Synthetic hormones are widely used in contraceptives and medications and one of the most common synthetic hormones used is *17-alpha-ethinylestradiol* (EE2). The aim of this study was to investigate if nanoparticles from polystyrene interact with the synthetic steroid hormone (EE2) in water. A second aim was to study if the NP and EE2 interacted differently in ISO 6341 standardized freshwater compared to copper free tap water, and if the interaction was different with the common zooplankton *Daphnia magna* and algae present in the water. This was tested by contaminating water samples separately with EE2 and NP, but also with EE2 and NP together to compare the results. The analysis method used for measuring the hormone had to be developed during this project. The results from this study implies that interaction between NP and EE2 seem to benefit from the standardized freshwater and from *D. magna* and *algae* present in the water samples. The interaction does not tell anything about the effect of the combination, which have to be studied further. Studies have been done with NP and EE2 separately and its effects in water before, but studies about both pollutants are very limited. This study is important as it investigate if NP and EE2 interact in water, and if they do the effects of the interaction is of further importance and interest.

Content

Introduction

During 2012, 28 million tons of plastic were produced globally (Rochman et al., 2013), of the total production, 6% of the material polystyrene were produced (Andrady, 2011). Polystyrene is a thermoplastic polymer and is used for packaging products, electronics and building constructions (Plastics Europe, n.d.). When plastics occur in the environment a significant part will end up in aquatic environments. (Cole et al., 2011, Gregory and Andrady, 2003). The content of the marine waste are approximately 60-80% of plastic materials (Derraik, 2002) and of that 80% originates from land (Andrady, 2011). Plastics enter the marine environments in different ways, a large volume of the plastics appears from shipgenerated waste and run-offs with rivers and sewage systems (Pruter, 1987). There are mainly two sources that contribute to plastic particles in the marine environment. The first source is from particles that are manufactured to be in a specific size and the second source is from particles which originates from larger structures (Mattson et al., 2015). Manufactured particles enters the water directly via run-off into aquatic environments (Andrady, 2011). Plastics can degrade through different processes and become nanoparticles in water. It can degrade through thermal degradation, hydrolysis, mechanical/physical degradation, thermooxidative degradation, photodegradation and biodegradation. (Andrady, 2011). The degradation of plastic into nanoparticles changes its characteristics, availability and biological impact (Maynard 2006)

Nanoparticles

Nanoparticles (NP) are materials with at least one dimension between 1 and 100 nm (Matsson et al., 2015). The biological fate, mobility and bioavailability of the NP depends on its charge, shape and size. NP have a high surface to volume ratio, high surface reactivity and the small size of the particle makes it possible for different uptake rates and biodistribution. These characteristics makes the particle very dynamic in the environment (Lowry et al., 2012) and NP in aquatic environments will interact with its surroundings (Mattson et al., 2015).

NP can transform while in water or in biological systems and transformation can occur in a few different ways. Aggregation of the same kind of particles, aggregation of different NP and interaction with natural organic materials. When NP interact with organic materials, the particle creates a new surface around the core, which changes the biological properties of the particle. (Mattson et al., 2015)

NP from polystyrene has been shown to have negative effect on fish's brain, fish which were exposed with NP had a more swollen and whiter brain compared to fish not exposed with NP. The fish exposed to NP also showed negative behavioral and metabolic changes (Mattson et al., 2015). The fish *Oryzias latipe* were exposed with NP from polystyrene in different sizes. The result showed that the uptake rate was much larger for the smaller particles and the excretion were more difficult for the smaller nanoparticles. This means that the smaller particles entered the organism easier and it was more difficult for the smaller particles to leave the organism (Manabe et al., 2011). Because of the extremely small size of the particles, NP can pass through biological barriers, tissues and eventually accumulate and damage organs (Kashiwada, 2006).

Hormones and its way to the aquatic environment

Humans and livestock excrete hormones which run off via surface water, ground water systems and through sewage treatment plants. The hormone *17alphaethinylestradiol* (EE2) is used in contraceptives, medicine for menopausal and postmenopausal syndrome, physiological replacement therapy, treatment of breast cancer and osteoporosis (Aris et al., 2014).

EE2 is a semisynthetic (PubChem, 2017) derivate from the natural hormone estradiol (E2) (Aris et al., 2014). EE2 is excreted from urine and feces from individuals taking medications containing EE2 (Hannah et Al., 2009). Globally EE2 has been found in surface water in the range of ng/L (Aris et al., 2014) and concentration of EE2 between 0.073-0.831 ug/L has been found in the watercourses of Unites States (Kolpin et al. 2002).

EE2 is as an endocrine disruptor because of its estrogenic activity, this means that the substance can interact with physiological systems and cause changes in development, growth and reproduction (Jobling et al., 2004). The hormone is a very potent (Desbrow et al., 1998) and hydrophobic compound (Park, 2010) and more resistant to degradation than natural hormones and are therefore very persistent in the environment. EE2 has shown effects on wildlife, the freshwater fish fathead minnow, Pimephales promelas produced fewer eggs when exposed to EE2 (Kidd et al., 2007), snails have shown decreased hatchling growth (Segner et al., 2003) and Daphnia magna produced fewer neonates (Dietrich, et al. 2010). Male fish exposed to EE2 can undergo sex-reversal from genetic males to females (Papoilias et al., 2000)

Combinational effects

A study has shown that the combination of EE2 and silver nanoparticles have different effects compared to when they are separated (Völker et al., 2014). The combination of the manufactured nanoparticle nC60 and EE2 made the

bioavailability of EE2 decrease with higher concentrations of nC60. The size and the zeta potential of the nanoparticles were changed in combination with EE2 (Park et al., 2010).

Hypothesis

- There will be interaction between nanoparticles from polystyrene and EE2 in water.
- The interaction between the nanoparticles and EE2 will be different with *Daphnia magna* and *algae* present in the water.
- The interaction in the water will be different in standardized freshwater compared to copper free tap water.

Aim

The aim with this study is to investigate if nanoparticles from polystyrene interact with EE2 in water and also to investigate if the interaction is different with *D. magna* and *algae* in the water. A second aim is also to study if the interaction is different in standardized freshwater compared to copper free tap water.

Method and Materials

Nanoparticles

80 nm negatively charged nanoparticles (NP) from polystyrene were used in this study. A solution of nanoparticles was dialyzed with milli-q water during two days and the water was changed three times periodically. The concentration of the nanoparticle solution were 50 mg/ml, after dialysis the solution were diluted with milli-q water into a stock with the concentration 0.5 mg/ml, which was used in the experiment to acquire the exposure concentration 5 mg/L. This concentration were chosen, because effects of NP is shown between 0.22-103 mg/ml (Besseling et Al. 2014). The solution was contained in a dark glass bottle in the fridge.

17alpha-ethinylestradiol

Concentrated EE2 (0.2 mg/ml) were diluted with de-ionized water into an EE2solution with the concentration 10 ng/ml, which was used in the experiment to get the exposure concentration 0.1 µg/ml. (Luna et Al. 2015). The solution was kept in a dark glass bottle in the fridge before analysis not to degrade.

Water

ISO 6341 Standardized freshwater (FW) was mixed according to a protocol and copper free tap water (TW) from the Ecology building, Lund University, were used in this experiment. Glass beakers were used, the water samples with one substance (NP or EE2) were filled with 495 ml water, and the water samples with both substances (NP + EE2) were filled with 490 ml water. Freshwater were used in three of the water samples and the rest were filled with copper free tap water, this was done to investigate if the substances interacted differently in freshwater due to copper free tap water (Fig.1). After 11 days all the water samples were put in dark glass bottles and kept in freezer until analysis.

Organisms

Zooplankton, *Daphnia magna* from a population that has been kept in the Ecology Building, Lund University for more than 100 generations and algae, *Scendemus sp* were present in some of the water samples to simulate environmental conditions.

Experimental design

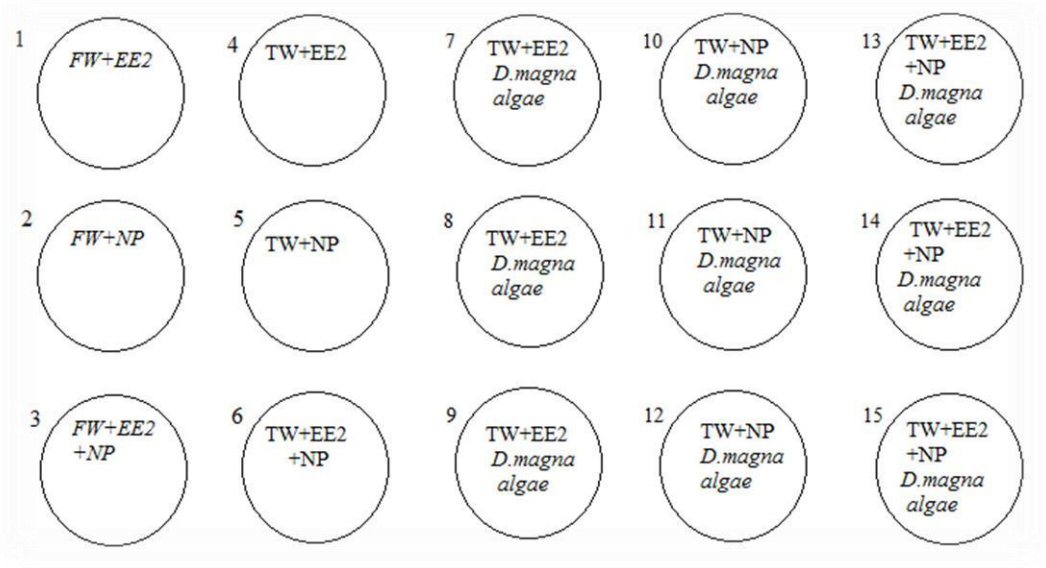

Figure 1.
Experimental Set-up. Water sample 1 filled with FW and EE2, 2 filled with FW and NP and 3 filled with FW, EE2 and NP. Water sample 4 is filled with TW and EE2, 5 is filled with TW and NP and 6 is filled with TW, EE2 and NP. Water sample 7-9 are replicates filled with TW, EE2, *D. magna* and *algae.* Water sample 10-12 are replicates filled with TW, NP, *D. magna* and *algae.* Water sample 1315 are replicates filled with TW, EE2, NP, *D. magna* and *algae.*

Analysis

Some of the water samples contained two substances, this made the analysis of the samples a bit difficult, and therefore the analysis method had to be developed further during the project. The absorbance was measured in a spectrometer in all water samples, before and after centrifugation. This was done to compare the spectra in the water samples and thereby deciding how much of the nanoparticles that had interacted in some way. The size of the particles and size distribution in the water samples were measured before and after centrifugation in a DLS-machine (Dynamic Light Scattering). The machine detected a radius 1 and a radius 2 of the particles in the water samples. If radius 2 was larger compared to radius 1 aggregation had occurred. Centrifugation were done to let the nanoparticles fall to the bottom of the test tubes, this made it possible to remove the water samples without the nanoparticles and thereby analyze the concentration of EE2.

Before the analysis of the hormone, liquid-liqiud extraction method was used to extract EE2 from the aqueous media to organic phase. Ethyl acetate was used in the extraction because of its similar solubility with EE2. To ensure the method, 30 ml of milli-q water was spiked with EE2 (1µg/ml) and 30 ml of ethyl acetate was added. The solution were mixed in a vortex and the upper layer (organic phase) was collected. The liquid was removed by evaporation under nitrogen flow until dryness was achieved. The extract of EE2 was then re-dissolved in 1 ml of acetonitrile and injected in high performance liquid chromatography (HPLC). Same procedure were followed with the water samples, except the extracts of EE2 were re-dissolved in 50 µl acetonitrile to achieve a higher enrichment factor. An EE2 stock solution was prepared by weighing 5.0 mg pure powder of EE2 and dissolved in 5 ml of acetonitrile to make the concentration 1 mg/ml. The stock solution was diluted with water:acetonitrile (70:30) to make different concentrations (10 ng/ml, 50 ng/ml, 100 ng/ml, 100 ng/ml, 250 ng/ml, 500 ng/ml and 1000 ng/ml), this made it possible to plot a calibration curve of the concentration and peak area of EE2 and to compare the water samples with. 10µl from the calibration solutions and extracts was injected to (HPLC) connected to, C_{18} column, photodiode array and fluorescence detectors. The mobile phase consisted from water and acetonitrile. The gradient started with 40% acetonitrile, increased to 100 % in 5 min, then kept at 100% for 2 min and then back to 40% in 0.5 min and finally re-equilibration of the column in 4 minutes. The flow rate was kept at 0.400 ml/min and column temperature set at 45 °C. Eluted peaks were detected in DAD at 210, 280 and 410 nm. The fluorescence was set 280 nm as excitation wavelength and 310 nm as emission wavelength. EE2 was eluted after 4.5 minutes.

Results

Absorbance measuring

The results from measuring the absorbance shows that the water sample with FW+EE2+NP have a shorter wavelength than the water sample with FW+NP after centrifugation (Table 1). There is no difference in wavelength between the water sample with TW+NP and the sample TW+EE2+NP, either before or after centrifugation (Table 1). The wavelength for the water samples with TW+EE2+NP, D. magna and algae are shorter compared to the wavelength for the water sample with TW+NP, D. magna and algae, after centrifugation (Table 1). The difference in wavelength is most obvious between the water samples with FW+EE2+NP and FW+NP.

Table 1.
Detected wavelength [nm] before and after centrifugation for the water samples. The wavelength for the samples with replicates is a mean value of the three replicates. Water samples exposed to EE2 or NP or the combination of EE2+NP.

Water Sample	Before centrifugation (Wavelength) [nm]	After centrifugation (Wavelength) [nm]
FW + EE2 (1)	399. 9936	399. 7109
FW + NP (2)	385. 8315	399. 7109
FW + EE2 + NP (3)	399. 9936	385. 2639
TW + EE2 (4)	398. 297	399.7109
TW + NP (5)	399.7109	399.9936
TW + EE2 + NP (6)	399.7109	399.9936
TW + EE2 (*D. magna, algae)* Mean value for the three replicates	399. 9936	399. 899
TW + NP (*D. magna, algae)* Mean value for the three replicates	395.084	399.805
TW + EE2 + NP (*D. magna, algae)* Mean value for the three replicates	399.852	399.7109

DLS – measuring of the particle size

The results from the DLS-measuring of the particle size in the water samples shows if aggregation has occurred between particles in the water samples. Aggregation could occur between all particles in the water sample, it is therefore not possible to decide if NP and EE2 have interacted. Aggregation had occurred in all of the water samples (Appendix A), however due to the sensitivity of the DLS machine some values of the radius are missing for several of the water samples. The aggregation does not seem to follow a specific pattern but is larger for the water samples with NP in it. Nanoparticles from polystyrene seem to aggregate in greater extent than other particles (Appendix A).

HPLC – measuring of EE2

Injection of spiked water and EE2 in HPLC created a perfect peak after approximately 4.5 minutes, (Fig.2) which implies that the method was reliable. To be able to calculate the concentrations of EE2 in the water samples, a calibration curve was created with the linear equation: $y = 7{,}7865x - 0{,}1493$, where y is peak area of the compound and x the concentration of the compound, (Fig.3). Injection of the water samples in HPLC did not create a peak area at 4.5 minutes in any of the samples (Appendix B), which made it impossible to compare with the calibration curve. In all of the water samples more than one peak area were detected, which implies that there were more than one compound in the samples (Appendix B). This also made it impossible to compare with the calibration curve.

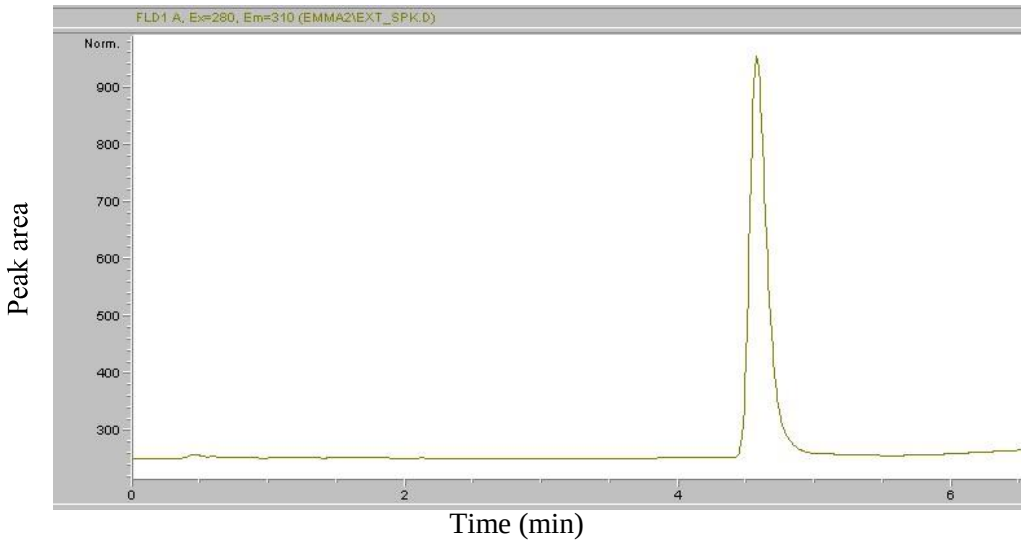

Figure 2.
Peak area of spiked EE2 diluted in acetonitrile. Elution of EE2 occurs after 4.5 minutes.

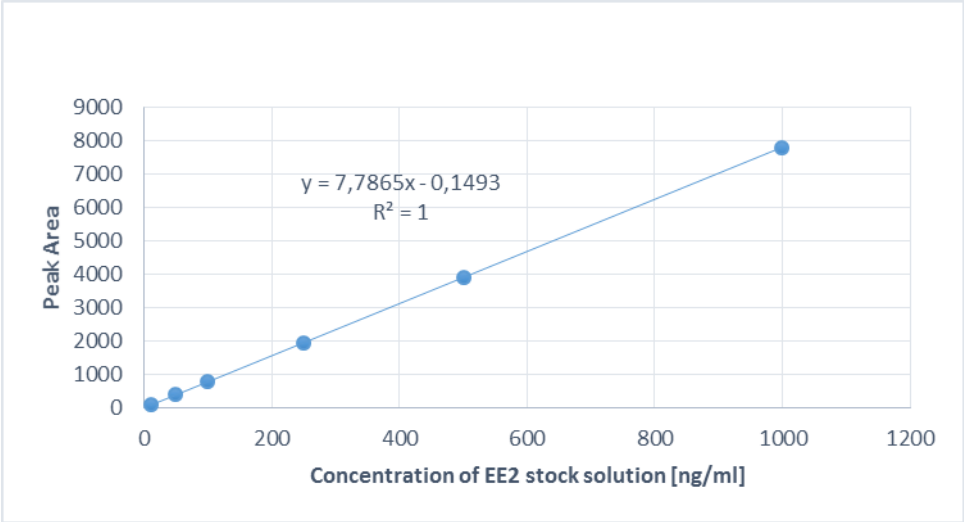

Figure 3. Calibration curve of EE2 stock solution. Peak area on y-axis and concentration on x-axis.

Discussion

The results from this study implies that the nanoparticles have interacted with EE2 in the freshwater and in the water with *D. magna* and algae present. The combination of NP and EE2 in freshwater had a shorter wavelength than the sample containing only NP. Polystyrene absorb more as free particles, compared to when bonded with other particles. This implies that the nanoparticles have interacted with EE2 in the freshwater. However there were no difference between the water sample with copper free tap water and NP and the sample with the combination in the tap water. The same wavelength implies that the NP from polystyrene has the same amount of free particles in both samples. Interaction seems not to have occurred between the NP and EE2 in copper free tap water while, when *D. magna* and *algae* are present in the mixture, interaction between NP and EE2 seem to have occurred. The difference in wavelength is most obvious in the freshwater according to the absorbance measuring, this implies that interaction between NP and EE2 benefits from the freshwater. This is an interesting result considering that the freshwater simulate real environmental conditions.

Aggregation occurred in all water samples, before and after centrifugation. It is not possible to decide which particles that had aggregated in the samples. By comparing the radius of the particles in the water samples it was possible to see that the aggregation was larger for the samples with NP in it. Unfortunately it does not tell if the NP and EE2 have interacted. In real environmental conditions aggregation between NP and other pollutants may occur all the time. The result from the analysis of the size distribution implies that NP are more likely to aggregate with other compounds than EE2. It is therefore interesting to further study the combination of NP with other compounds and the effects it may have.

Injection of the water samples in the HPLC did not create a peak at 4.5 minutes, this implies that there were not pure EE2 in the water samples. An explanation to this could be that the hormone had degraded to another compound and therefore were not detected at the same time as EE2. Unfortunately this made it impossible to compare with the calibration curve and the concentration of EE2 in the water samples could not be calculated.

More than one peak were detected in all water samples, this indicates that there are more than one compound in the samples. Because of centrifugation before analysis of EE2, the machine should only inject one compound (EE2). The

explanation for this could be the solvent that were containing DMSO (*dimethyl sulfoxide*) which the machine might have detected. It could also be rests from nanoparticles, *D. magna* or algae in the samples.

An earlier study has shown that the combination of silver nanoparticles and EE2 had an effect on fresh water wildlife (Völker et al., 2014) and EE2 in a mixture with other compounds have effects on freshwater wildlife (Luna et al., 2015). It is therefore interesting to further study the effects of NP from polystyrene and EE2 on freshwater wildlife, but to also study the effects from the combination of other compounds. In future studies it would be interesting to do the experiment with freshwater from a lake or sea water, to have even greater environmental relevance.

Manufactured NP have been suggested that in the presence of EE2 makes the nanoparticles aggregate in greater extent (Park et al., 2010). The result from this study indicates that NP aggregate in water and the result from another study shows that NP that are not combined with other pollutants have effects on fish's brain (Mattson et al., 2015). This suggests that aggregated nanoparticles have an effect on fish.

The result from this study does not tell anything about the effects of the combination, but like mentioned above almost similar compounds as used in this study have had effect on wildlife. It is known that nanoparticles can accumulate in organs (Kashiwada, 2006), when nanoparticles bond to EE2 it is probably possible for both compound to accumulate. If smaller organisms like zooplankton accumulate the compounds, it can accumulate it larger organisms and bioaccumulate in the food chain. If the compounds bioaccumulate in larger organisms like fish, it may bioaccumulate in humans eating the fish. This scenario might be something that already occur, since there are no measurements or treatments for nanoparticles or hormones today.

Today there are no methods for cleaning out or even detect nanoparticles in the environment, it is therefore difficult to measure the amount of nanoparticles in the environment. Sewage treatment plants do not clean out hormones and medications. To regulate the amount or even detect these compounds in the environment, new techniques is needed. Techniques that cleans the environment from nanoparticles could lower the amount of nanoparticles in the environment and thereby prevent interaction with other compounds. Developed methods for sewage treatment plants could prevent hormones from entering the aquatic environments.

Laws and regulations about plastic waste and hormones in aquatic environment is one way to prevent the compounds from entering the environment from the beginning. Information and knowledge to the general public about the effects of the compounds in the nature could make people more caring and more willing to make changes for the environment.

The result from this study might contribute to a further understanding of the environmental impact of NP and EE2 combined. It might also contribute to a further interest in studying the impact of pollutants in the environment.

Thank you

I would like to thank my mentor Lina Nikoleris for guidance during this project. I want to thank Charlotta Turner for helping with the analysis and for developing the analysis method. I want to thank Said Alhamimi for helping me in the laboratory with the analysis and for developing the analysis method. I want to thank Tommy Cedervall for helping me with analysis of the nanoparticles.

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Appendix A

The table shows detected radius for the particles in the water samples. Detection were done before and after centrifugation in all samples. Unfortunately many of the water samples could not be detected due to the sensitivity of the machine. The machine detected a radius 1 and a radius 2. If radius 2 were larger compared to radius 1, aggregation had occurred.

Before Centrifugation		
Sample	Radius 1	Radius 2
TW,EE2,D,A	75,8	1659,3
TW,EE2,D,A	50,1	858,3
TW,NP,D,A	95,4	1346,9
FW,EE2,NP	13,5	207,9
TW,NP	25,2	116,6
After centrifugation		
FW,EE2,NP	70,4	544,4
TW,EE2,NP,D,A	92,2	638,4
TW,NP	13	3736,7
TW,EE2,NP	83,7	345,3
TW,EE2,NP,D,A	10	802,5
TW,NP,D,A	13,5	3761,1
TW,NP,D,A	88,7	1053,9

Appendix B

The figures shows detected compound in the water samples. Peak area on Y-axis and time (min) on X-axis. The optimal detection creates a peak for EE2 after 4.5 minutes. The machine did not detect one compound after 4.5 minutes in any of the water samples.

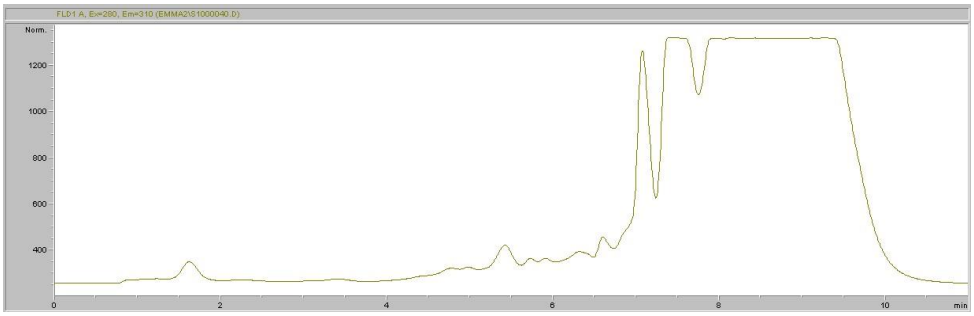

Figure 1.
FW, EE2

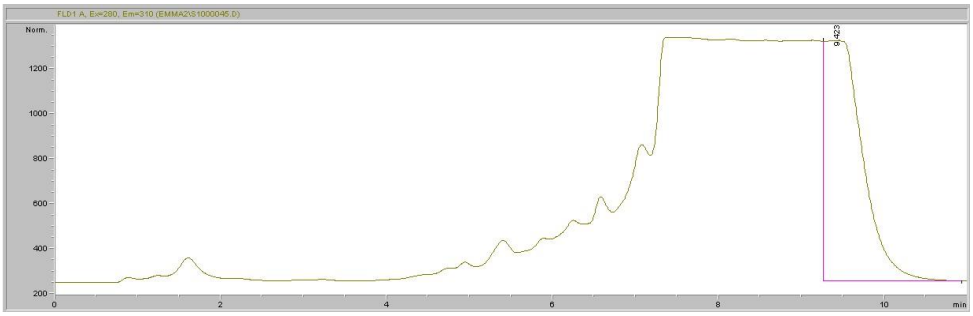

Figure 2.
FW, EE2, NP

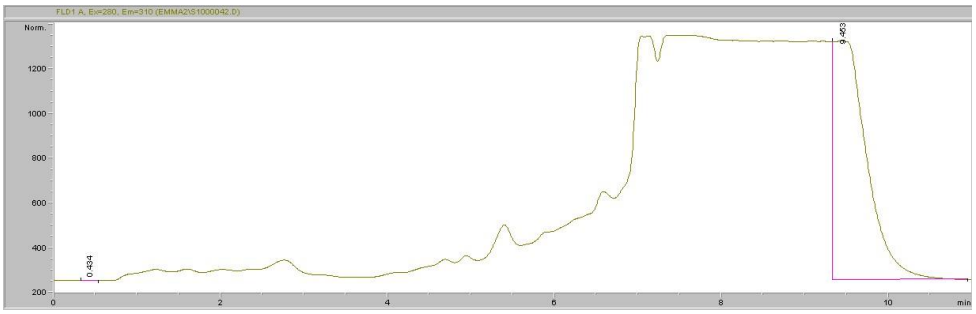

Figure 3.
TW, EE2

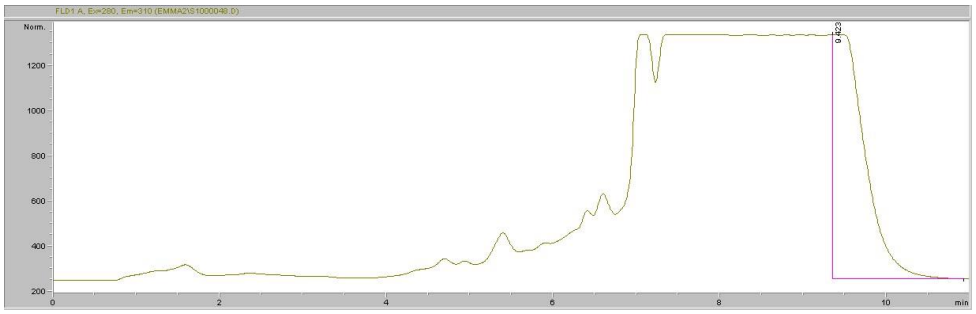

Figure 4.
TW, EE2, NP

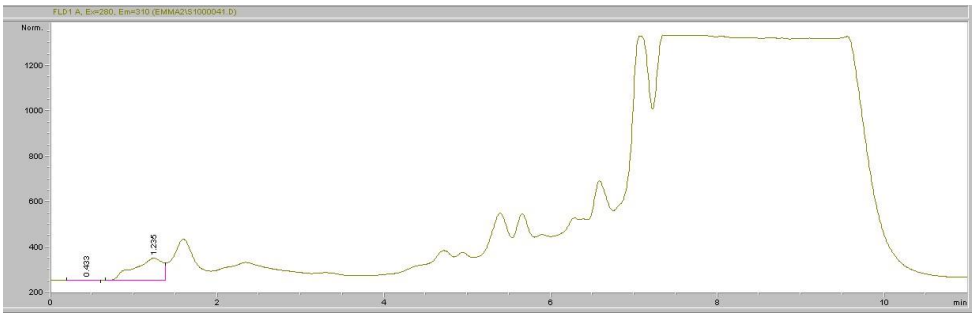

Figure 5.
TW, EE2, D. magna and algae

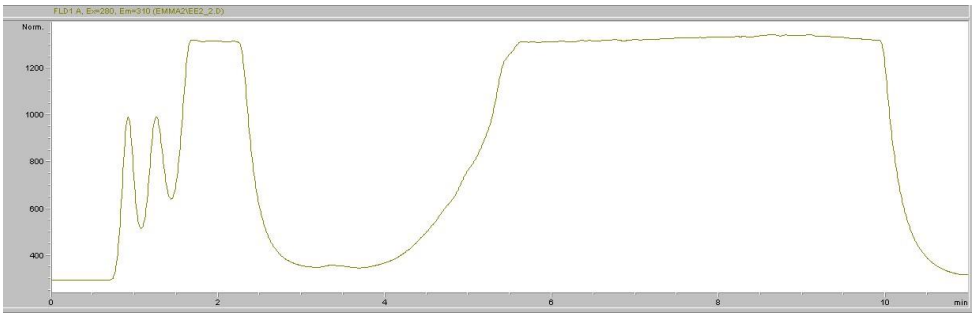

Figure 6.

TW, EE2, D. magna and algae

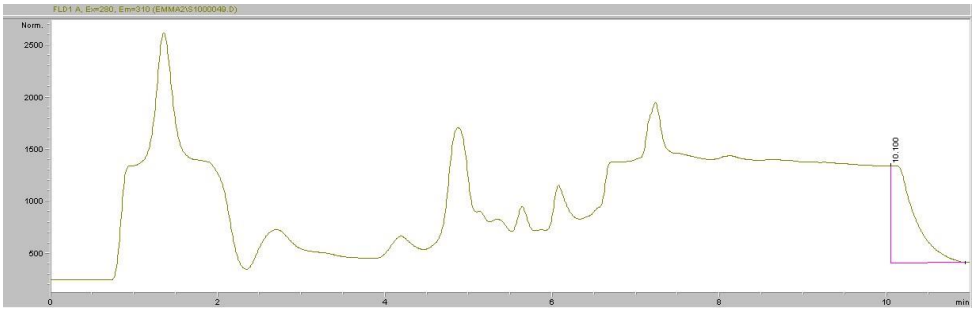

Figure 7.
TW, EE2, D. magna and algae

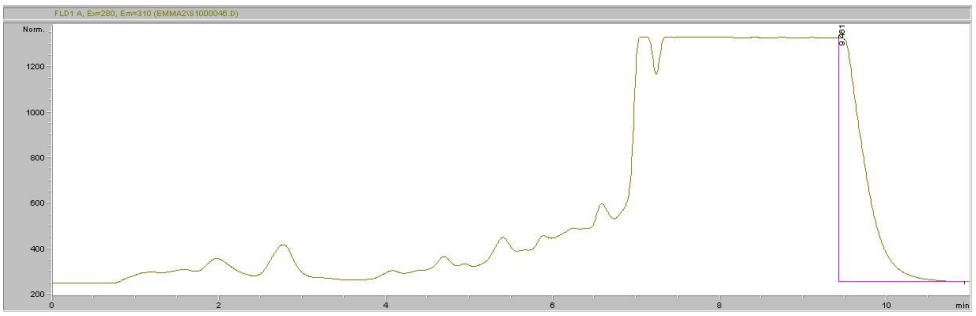

Figure 8.
TW, EE2, NP, D. magna and algae

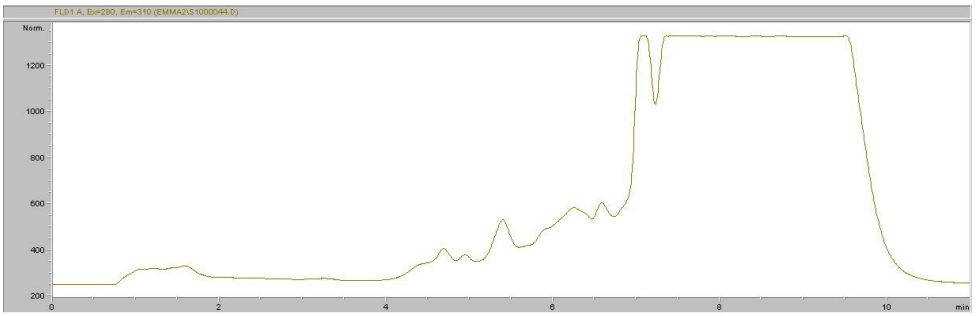

Figure 9.

TW, EE2, NP, D. magna and algae