Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

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Oxidative stress effects in the Arctic amphipod *Gammarus oceanicus* after expousure to the two pharmaceuticals carbamazepine and ibuprofen

Master's thesis in Environmental Toxicology and Chemistry
Supervisor: Bjørn Munro Jenssen
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Abstract

Pharmaceuticals are released into the Arctic environment through wastewater treatment and have been detected in the Arctic Ocean, invertebrates and plankton in the Arctic. The focus of the present study is to investigate oxidative stress effects in the amphipod *Gammarus oceanicus* after exposure to the two pharmaceuticals carbamazepine and ibuprofen. *G. oceanicus* individuals were collected by the shoreline at Ny-Ålesund at Svalbard, Norway. The gammarids were then exposed to carbamazepine or ibuprofen for 72 or 96 hours respectively in two separate exposure experiments at a water temperature of 6°C to simulate relevant Arctic water temperature. The exposure concentrations for the carbamazepine exposure experiment were 0, 50 and 1000 ng/L, while 0, 10, 100 and 1000 ng/L were used for the ibuprofen exposure experiment. The biomarkers analysed were catalase (CAT), glutathione (GSH), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and malondialdehyde (MDA). Generally, only a few effects were found in exposed individuals compared to the control group for both the carbamazepine and ibuprofen exposures. In the carbamazepine exposure experiment, a significant increase in GSH levels at 50 ng/L exposure compared to the control was found. Differences between males and females were also investigated. In males, there was a significant increase in SOD activity at 10 ng/L exposure compared to the control group after exposure to ibuprofen. A significant difference was also found in GST activity between 10 and 100 ng/L ibuprofen exposure in males, where the 10 ng/L exposure led to significantly lower GST activity than 100 ng/L exposure. In the carbamazepine exposure experiment, a high positive correlation between SOD, GSH and GST was found. SOD, CAT, GSH and GST were positively correlated in the ibuprofen exposure. In both the carbamazepine and ibuprofen exposure experiment, SOD and GSH were well correlated with each other and with CAT or GST. In the carbamazepine exposure experiment, the MDA levels were significantly higher than in the ibuprofen exposure experiment. GSH levels were significantly higher in the control group of the ibuprofen exposure experiment than in the carbamazepine exposure experiment. The differences in MDA and GSH levels in the carbamazepine and ibuprofen exposure control groups could be due to the different exposure durations and number of individuals in the exposure tanks for the two experiments.

Few studies have investigated oxidative stress using amphipods exposed to carbamazepine or ibuprofen. In studies with amphipods, most studies have investigated effects related to oil, oil dispersants and PAHs. However, multiple studies have been performed with bivalves where various effects on CAT, GSH, GST, GPx and MDA have been detected after exposure to carbamazepine or ibuprofen. The effects of these two pharmaceuticals varied a lot between studies indicating that there are species differences in the effects of these biomarkers. In the present study, higher exposure concentrations did not necessarily lead to stronger effects, which have been found in several other studies. Very few exposure studies have been performed in Arctic-relevant environments. Comparable studies have mostly been performed in temperatures of 15-20°C, which is much higher than the exposure temperatures in the present study. Even though carbamazepine and ibuprofen have been detected at relatively high concentrations in the Arctic marine environment, the result of the present study suggests that there is no immediate risk for *G. oceanicus* due to oxidative stress caused by exposure to carbamazepine or ibuprofen. However, it is unknown how the effects would be at long-term exposure and other species, which should be investigated in future studies.

Sammendrag

Legemidler slippes ut i det Arktiske miljøet gjennom avløpsvann og har blitt detektert i Nordishavet og er påvist i invertebrater og plankton i Arktis. Fokuset for denne studien er å finne ut om eksponering av de to legemidlene karbamazepin og ibuprofen forårsaker oksidativt stress i amfipoden *Gammarus oceanicus*. *G. oceanicus* ble fanget i fjæra i Ny-Ålesund på Svalbard, Norge. De ble så eksponert for karbamazepin eller ibuprofen for 72 eller 96 timer i to separate eksponeringsforsøk med vanntemperatur på 6°C tilsvarende vannet i fjorden utenfor laboratoriet. Konsentrasjonene brukt i eksponerings forsøket med karbamazepin var 0, 50 og 1000 ng/L, mens forsøket med ibuprofen ble utført med eksponerings konsentrasjoner på 0, 10, 100 og 1000 ng/L. Biomarkørene som ble analysert var katalase (CAT), glutathione (GSH), glutathione-S-transferase (GST), glutathione peroksidase (GPx) og maldondialdehyd (MDA).

I karbamazepin eksponerings forsøket ble en signifikant økning i GSH funnet med eksponering for 50 ng/L sammenlignet med kontroll. Men, generelt var det ikke forskjeller mellom ueksponerte og eksponerte individer hverken i karbamazepin eller i ibuprofen forsøket. Det ble også undersøkt forskjeller mellom hannene og hunnene. En signifikant økning i SOD ble funnet når hannene ble eksponert for 10 ng/L ibuprofen sammenlignet med kontroll. En signifikant forskjell ble også funnet i GST-aktivitet mellom 10 ng/L og 100 ng/L eksponeringen for ibuprofen der 10 ng/L eksponering førte til signifikant lavere GST-aktivitet. I karbamazepin forsøket ble det funnet en høy positiv korrelasjon mellom SOD, GSH og GST. SOD, CAT, GSH og GST hadde høy positiv korrelasjon i ibuprofen forsøket. I både karbamazepin og ibuprofen eksponerings forsøket hadde SOD og GSH høy korrelasjon med hverandre og med enten CAT eller GST. I karbamazepin forsøket var MDA nivåene høyere enn i ibuprofen forsøket, men GSH nivåene var høyere i kontrollgruppen i ibuprofen forsøket enn i kontrollgruppen i karbamazepin forsøket. Disse forskjellene mellom de to eksponeringsforsøkene kan skyldes de ulike eksponerings varighetene eller ulikt antall individer per tank i de to forsøkene. Men, de individuelle forskjellene var ganske store, så forskjellene kunne til en viss grad vært forårsaket av individuelle forskjeller.

Få studier har fokusert på oksidativt stress med amfipoder eksponert for karbamazepin eller ibuprofen. I studiene med amfipoder har de fleste fokusert på effekter relatert til olje, oljedispereringsmidler og PAH-er. Flere studier har blitt gjort med bivalver der ulike effekter på CAT, GSH, GST, GPx og MDA har blitt funnet. Effektene varierte mye mellom studiene, noe som indikerer at det kan være store artsforskjeller på effekten av disse biomarkørene. I denne studien ga ikke høyere eksponeringsdoser nødvendigvis større effekter, noe som også har blitt funnet i mange andre studier. Veldig få eksponeringsstudier har blitt gjennomført i et miljø relevant for Arktis. Sammenlignbare studier har stort sett blitt gjennomført i temperaturer på 15-20°C, noe som er mye høyere enn temperaturene brukt i denne studien. I miljøet har karbamazepin og ibuprofen blitt funnet i relativt høye nivå i sjøvann ved Svalbard, der det også har blitt funnet i invertebrater og plankton. Resultatene av denne studien indikerer at det ikke er umiddelbar risiko for at *G. oceanicus* skal streve på grunn av oksidativt stress fra eksponering for karbamazepin eller ibuprofen. Men, det er ikke avklart hvordan effektene kan være etter mer langvarig eksponering noe som bør undersøkes i framtidige studier.

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Abbreviations:

AIC	—	Akaike's Information Criterion
ANOVA	—	Analysis of variance
BHT	—	Butylated hydroxytoluene
BIC	—	Bayesian Information Criterion
CAT	—	Catalase
CDNB	—	1-chloro, 2,4-dinitrobenzene
CECs	—	Contaminants of emerging concern
CV	—	Coefficient of variation
DTNB	—	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	—	Dithiothreitol
dw	—	Dry weight
EDTA	—	Ethylenediaminetetraacetic acid
GPx	—	Glutathione peroxidase
GR	—	Glutathione reductase
GSH	—	Glutathione
GSSG	—	Glutathione disulfide
GST	—	Glutathione-S-transferase
IO PAN	—	Institute of Oceanology Polish Academy of Sciences
LOEC	—	Lowest observed effect concentration
LPO	—	Lipid peroxidation
MDA	—	Malondialdehyde
NA	—	Not analysed
NADPH	—	Dihydronicotinamide adenine dinucleotide phosphate
NSAID	—	Nonsteroidal anti-inflammatory drug
NTNU	—	Norwegian University of Science and Technology
PMFS	—	Phenylmethanesulfonylfluoride
PPCPs	—	Pharmaceuticals and personal care products
RCF	—	Relative centrifugal force
ROS	—	Reactive oxygen species
RV	—	Research vessel
SOD	—	Superoxide dismutase
SSA	—	5-Sulfosalicylic Acid
TBA	—	Thiobarbituric acid
TBARS	—	Thiobarbituric acid reactive substances
UG	—	The University of Gdańsk
ww	—	Wet weight
WWTPs	—	Wastewater treatment plant

1. Introduction

1.1 Pharmaceutical releases to the Arctic environment

Most pharmaceuticals are released into the Arctic environment through wastewater, sewage and domestic and municipal waste (Gunnarsdóttir et al. 2013; Kallenborn et al. 2018). In Arctic settlements the release of pharmaceuticals can be relatively high since most settlements lack modern wastewater treatment plants (WWTPs) due to the cold climate and high cost relative to population size (Gunnarsdóttir et al. 2013; Kallenborn et al. 2018). Sewage containing various pharmaceuticals and other contaminants can therefore be directly released untreated into the marine environment (Kallenborn et al. 2018). Pharmaceuticals can also end up in the Arctic environment by long-range transport by transport with ocean currents (Brumovský et al. 2022). Carbamazepine and ibuprofen are two of the pharmaceuticals detected the most in the marine Arctic environment (Weigel et al. 2004; Sokołowski et al. 2024). Carbamazepine is an antiepileptic and mood-stabilising drug(Amann et al. 2007). Ibuprofen is a nonsteroidal anti-inflammatory drug (NSAID) used as a painkiller and against inflammation and fewer (Rainsford 2009). Although there are regulations for wastewater releases from cruise ships, at least in the area around Svalbard (Ministry of Justice and Public Security), wastewater from cruise ships can be a potential source of pharmaceutical release to the Arctic since the cruise ship traffic can be relatively high in the Arctic during summer. Various pharmaceuticals, including carbamazepine and ibuprofen, have been found in wastewater from cruise ships and settlements, which could be a local contamination source of pharmaceuticals (Weigel et al. 2004; Westhof et al. 2016). Due to their high release and relatively high persistence, some pharmaceuticals can be classified as contaminants of emerging concern (CECs).

1.2 The Arctic environment

The harsh conditions and cold temperatures in the Arctic can reduce the degradability and removal of pharmaceutical residues, and therefore increase their persistency compared to more southern latitudes (Kallenborn, Fick, et al. 2008; Kallenborn et al. 2018). This can cause the ecotoxicological consequences in the Arctic to differ compared to more temperate environments (Kallenborn et al. 2018). Due to their low mobility, pharmaceuticals and personal care products (PPCPs) in the Arctic are often restricted to the vicinity of local anthropogenic sources (Kallenborn et al. 2018), which can enhance their longevity in the area where they are released. During half of the year the photodegradation in the Arctic is very limited due to lack of light half of the year and lower light intensity compared to areas further south (Kallenborn, Fick, et al. 2008). However, in summer the sun never sets, which can increase photodegradation compared to areas further south. Release of pharmaceuticals to wetlands, which freeze in the winter could result in no chemical removal by photodegradation or microbial activity when low temperatures and ice cover block light penetration (Chaves-Barquero et al. 2016). In addition, the low temperatures in the Arctic and the generally low densities of Arctic microbes in Arctic sediments and water make microbial degradation negligible compared to more mid-latitudes (Kallenborn, Fick, et al. 2008). A potential continuous release of antimicrobial substances with wastewater can also change the bacterial community affecting the degradation of pharmaceuticals (Kallenborn et al. 2018). The rate of degradation also depends on the stability and metabolism of pharmaceuticals, which may vary between pharmaceuticals and environmental conditions. Carbamazepine

is one of the more stable and persistent pharmaceuticals and is often not degraded or adsorbed much during wastewater treatment using UV radiation (Clara et al. 2004). In addition, in a study by Tonski et al. (2019) both carbamazepine, ibuprofen, and most of their metabolites seemed to be quite hydrolytically stable with a half-life of over a year at 25 degrees (Tońki et al. 2019). However, information about how stable they are at lower temperatures are lacking.

Ny-Ålesund is a small Arctic settlement located at the south side of Kongsfjorden at the north-west part of Spitsbergen, Svalbard Norway, and is the world's northernmost permanent settlement (Ny-Ålesund - Cruisehåndbok for Svalbard 2015). Ny-Ålesund was earlier a mining town (Ny-Ålesund - Cruisehåndbok for Svalbard 2015). However, the mining activity was decommissioned at the end of 1962 after several big accidents and low coal demand (Ny-Ålesund - Cruisehåndbok for Svalbard 2015). The settlement is now mainly an Arctic and environmental research station (Ny-Ålesund - Cruisehåndbok for Svalbard 2015). The settlement is also a popular destination for tourism, and a lot of tourists and cruise vessels visit in the summer season. However, tourism is also restricted due to local environmental restrictions at Svalbard, its isolated location, travel expenses, harsh weather conditions, and no tourism during winter.

1.3 Pharmaceuticals

Pharmaceuticals are primarily human-made chemicals made to induce a physical and biochemical effect and used at low levels in humans to treat, prevent, and diagnose diseases or other illnesses (Kallenborn et al. 2018). However, they can cause unwanted toxicological effects on non-target biota when released into the environment (Kallenborn et al. 2018). Pharmaceuticals are developed to be metabolised to their active form and resist degradation by biochemical activities long enough to be biologically active at their target location, even at low concentrations (Bu et al. 2016). Pharmaceuticals can be quite persistent, especially where they are continuously released into the environment (Lam et al. 2004).

Very little is known about the fate and effects of pharmaceuticals on the organisms living in the Arctic, nevertheless over 100 different pharmaceuticals have been detected in wastewater, seawater, sediments, and shorelines in Arctic areas (Weigel et al. 2004; Chaves-Barquero et al. 2016; Huber et al. 2016; Choi et al. 2020; Stroski et al. 2020). The pharmaceuticals carbamazepine and ibuprofen, along with naproxen diclofenac, paracetamol, paroxetine, paraxanthine, fluvoxamine, cetirizine and more have been detected in Arctic seawater and sediments (Kallenborn, Eggen, et al. 2008; Vasskog et al. 2008; Chaves-Barquero et al. 2016; Huber et al. 2016; Choi et al. 2020; Stroski et al. 2020). In Kongsfjorden outside Ny-Ålesund at Svalbard, Norway, pharmaceuticals have also been detected in various concentrations in marine invertebrates (Sørensen et al. 2023). Ibuprofen has been found in concentrations up to over 4000 ng/g wet weight (ww) in copepods and 2000 ng/g (ww) in benthic amphipods (Sørensen et al. 2023). In this study, the focus will be on carbamazepine and ibuprofen, which were chosen since they are commonly used pharmaceuticals and are two of the most frequently detected pharmaceuticals found in Arctic areas (Weigel et al. 2004; Kallenborn et al. 2009).

1.3.1 Carbamazepine

Carbamazepine is one of the most studied pharmaceuticals in marine environmental toxicology (Brausch et al. 2012). As mentioned, carbamazepine is an antiepileptic and mood-stabilising drug. It is used to control grand mal, psychomotor epilepsy, and bipolar disorder (Clara et al. 2004). In humans, carbamazepine is usually metabolized by

cytochrome P450 in the liver, leading to the formation of the major metabolite, carbamazepine-10,11-epoxide (Kerr et al. 1994). However, many other metabolites are also formed, either by epoxidation or peroxidation (Lertratanangkoon and Horning 1982).

It is one of the most persistent pharmaceuticals (Lam et.al.). It is resistant to photochemical and biodegradation in the environment with a half-life of up to 100 days in an environment similar to surface water at higher latitudes (above 50°) and can resist up to 4 weeks with exposure to full sunlight (Andreozzi et al. 2003; Calisto et al. 2011). In a microcosm study carbamazepine was found to have a mean half-life of 82 days and be resistant to photodegradation in pond water, where the water was kept at 4°C before the start of the experiment (Lam et al. 2004). Carbamazepine is resistant to removal in WWTP, which can lead to high levels being released with wastewater (Hai et al. 2018). In addition to other effects, carbamazepine has been reported to affect the biochemical and physiological performance of marine invertebrates (Freitas et al. 2015). In Nunavut Canada, carbamazepine has been found in concentrations up to 107-2740 ng/L in bays and lakes (Chaves-Barquero et al. 2016; Stroski et al. 2020). In Tromsø, carbamazepine has been found in a concentration of 270 ng/L in sewage effluent (Weigel et al. 2004). Carbamazepine has been detected in invertebrates in Isfjorden at Svalbard with a detection frequency of 56.3 % and levels up to 20.8 ng/g dry weight (dw) in hermit crab (*Pagurus pubescens*) (Sokołowski et al. 2024). In other studies, carbamazepine has been reported to affect biomarkers of oxidative stress by both upregulation and downregulation at various concentrations in various invertebrates, like mussels, crustaceans, and polychaetes (Maranho et al. 2014; Freitas et al. 2015; Almeida et al. 2017; Almeida et al. 2018).

1.3.2 Ibuprofen

As mentioned, ibuprofen (2-(4-isobutylphenyl) propionic acid) is a pharmaceutical in the drug group NSAIDs (nonsteroidal anti-inflammatory drugs) and is used as a painkiller for mild to moderate pain, often used against fewer, rheumatic diseases and menstrual pain (L17.1.1.7 Ibuprofen | Legemiddelhåndboka). It is one of the most widely used drugs in the world due to its high accessibility (Fent et al. 2006). Ibuprofen inhibits cyclooxygenase (Jan-Roblero and Cruz-Maya 2023). Cyclooxygenase is an enzyme converting the unsaturated fatty acid arachidonic acid to cyclic endoperoxides, which then are transformed into prostaglandins and thromboxanes (Jan-Roblero and Cruz-Maya 2023). Prostaglandins have been found in marine invertebrates where they are involved in processes related to reproduction, defence, and ion transport (Di Costanzo et al. 2019). Some of the detected prostaglandins in invertebrates are cytotoxic, while others have been found to have protective properties (Di Costanzo et al. 2019).

Due to its relatively high therapeutic dose and possible high excretion from the human body (up to 70-80%) high levels of ibuprofen can be detected in sewage (Marchlewicz et al. 2015). Usually, about 15% of ibuprofen administered orally in humans is excreted, either as the parent compound, conjugated, hydroxylated, or carboxylated (Jan-Roblero and Cruz-Maya 2023). In humans, ibuprofen is mostly metabolised by binding to GSH or glucuronic acid, conjugation with sulfates, or hydroxylation (Jan-Roblero and Cruz-Maya 2023). Ibuprofen and its metabolites (Ibu-OH, Ibu-CX) have been detected in sewage and seawater in Tromsø with concentrations in the range 0.7-7.8 ng/L in seawater and 0.07-20.1 µg/L in wastewater (Weigel et al. 2004). In addition, ibuprofen has been found at quite high levels in invertebrates in Kongsfjorden (Sørensen et al. 2023). The highest levels were found in copepods, with levels up to around 4000 ng/g (ww), lower levels were found in benthic and pelagic amphipods and krill, where the levels were about 500-

2000 ng/g (ww) (Sørensen et al. 2023). From other studies, ibuprofen has been found to mostly upregulate biomarkers like glutathione-S-transferase (GST) and downregulate superoxide dismutase (SOD), while catalase (CAT) has been both upregulated and downregulated (Contardo-Jara et al. 2011; Milan et al. 2013; André and Gagné 2017). There have also been studies with ibuprofen where no effects were found on the studied biomarkers of oxidative stress in invertebrates (Gómez-Oliván et al. 2014; Maranho et al. 2014; Martyniuk et al. 2022). Ibuprofen is relatively persistent at room temperature, with a half-life of about 100 days in raw river water or lake water at a temperature of 26-37°C (Araujo et al. 2014). Information about persistence in water at lower temperatures has not been found.

1.4 *Gammarus oceanicus*

G. oceanicus is an amphipod crustacean part of the Gammaridae family. Gammarids are a diverse group with more than 200 known species found in coastal and inland waters across the Holarctic with both marine and freshwater species (Chaumot et al. 2015). *G. oceanicus* is a species living in brackish and marine water (Siegismund 1985). They are commonly found in shallow water under rocks or in algae beds (Siegismund 1985), and their diet consists of mostly jellyfish, macroalgae, and crustaceans (Dischereit et al. 2024). They usually get 11-38 mm long and live for about 2 years (Węsławski et al. 2020). *G. oceanicus* is an abundant and widely distributed species important for the food web (Turja et al. 2014; Dischereit et al. 2024). It is mostly located in the North Atlantic Ocean, both on the east and west sides, and the Arctic Ocean by Canada, Greenland Svalbard, the northern part of Norway and northwest Russia, and in the Baltic Sea (Figure 1) (Kaschner et al. 2019). In Svalbard, *G. oceanicus* coexists with *Gammarus setosus*, which is a more subarctic species compared to the more boreal *G. oceanicus* (Węsławski et al. 2021).

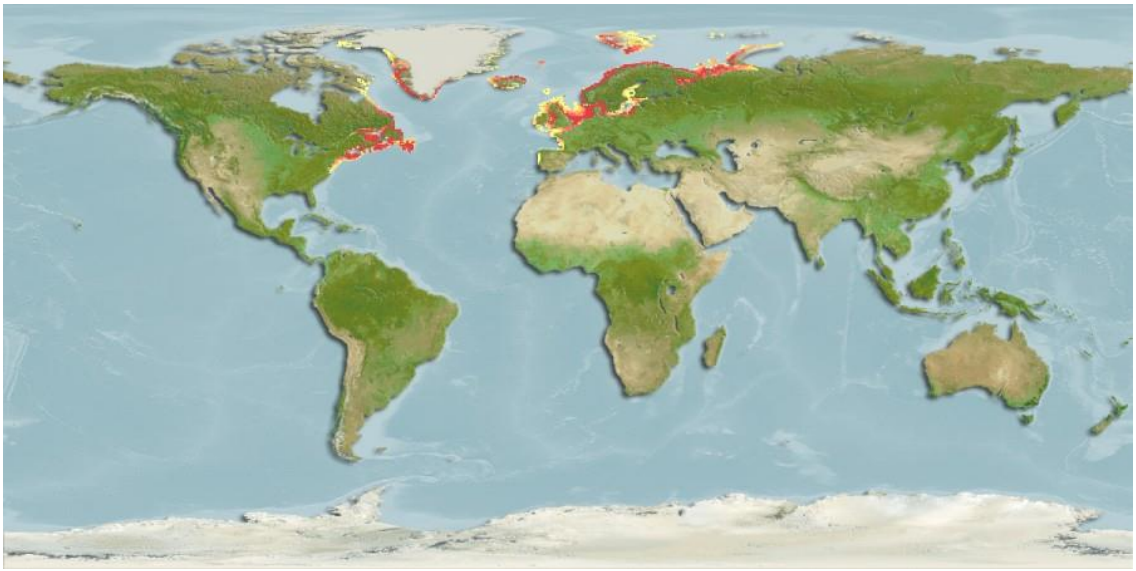

Figure 1: World distribution of *G. oceanicus*. They are likely to be found in areas marked in red and yellow, the probability of occurrence is highest in the red areas. Map retrieved from AquaMaps SeaLifeBase (Kaschner et al. 2019).

Gammarus species are suitable organisms for ecotoxicology studies (Chaumot et al. 2015). However, they were not extensively used until the 1970s and their use increased after the 1980s (Chaumot et al. 2015). Most of the research done is more related to oil

spills and PAHs (Sanz-Lázaro et al. 2008; Turja et al. 2020; Cikcikoglu Yildirim et al. 2023).

1.5 Oxidative stress

Oxidative stress can be defined as a disturbance of the balance between free radicals and antioxidant defences, which can lead to cell injury (Betteridge 2000). Reactive oxygen species (ROS) are generated by normal mitochondrial respiration by all cells and are often considered as byproducts of aerobic metabolism (Lubos et al. 2011; Ghosh and Majee 2023). ROS can be radicals, like superoxide anion (O_2^-) and hydroxyl radicals (•OH), or non-radical molecules like hydrogen peroxide (H_2O_2) or singlet oxygen ($^1O_2$) (Ghosh and Majee 2023). The most common ROS are molecules like O_2^- and H_2O_2 (Lubos et al. 2011). ROS can generate reactive radicals, which can damage cells and tissues by attacking proteins, lipids, and nucleic acids (Endale et al. 2023). A universal response to ROS is the induction of the antioxidant defence system (Turja et al. 2014). Higher ROS levels therefore generally lead to higher levels of antioxidant enzymes, like GST, SOD, CAT, and glutathione peroxidase (GPx) (Turja et al. 2014). However, ROS does not have only negative effects, it is also important for various processes, like growth-factor-mediated signal transduction, mitochondrial function, and regulating thiol redox balance (Lubos et al. 2011). Endogenous ROS are mostly produced in the mitochondria, endoplasmic reticulum, peroxisomes, and the plasma membrane (Snezhkina et al. 2019).

1.6 Biomarkers of oxidative stress

1.6.1 SOD and CAT

SOD is an important antioxidant that converts O_2^- radicals to H_2O_2 and O_2 (Ghosh and Majee 2023). It is produced at various locations in a cell and is present in compartments like mitochondria, chloroplast, peroxisomes, and cytosol (Ghosh and Majee 2023). It is present in about all eukaryotic organisms and protects the cells from damage caused by reactive oxygen species (Ali et al. 2022). CAT is an enzyme catalysing the reaction of H_2O_2 to O_2 and H_2O and is present in most aerobic organisms (von Ossowski et al. 1993). Catalases are mostly present in the peroxisomes and use H_2O_2 as a substrate and break down two hydrogen peroxides into one oxygen molecule and two water molecules in a two-step process (Lubos et al. 2011; Nandi et al. 2019). CAT is important in defence against oxidative stress by reducing the oxidating agent H_2O_2 to hinder it from dissociating to create •OH that can attack protein, lipids, and nucleic acids (Nandi et al. 2019).

1.6.2 Glutathione system

The glutathione system is a very important enzyme system for the protection of cells from ROS (Boelsterli and Boelsterli 2002). Glutathione (GSH) is an endogenous tripeptide with a cysteine residue with a reactive SH-group and can directly interact with radicals non-enzymatically (Boelsterli and Boelsterli 2002). GSH is also a co-substrate for GST and is the main antioxidant thiol derivative (Dringen 2000; Boelsterli and Boelsterli 2002). GST is a superfamily of enzymes catalysing GSH-transfer to xenobiotics via a cysteine thiol (Boelsterli and Boelsterli 2002). GST is a phase II biotransformation enzyme that often inactivates reactive metabolites (Boelsterli and Boelsterli 2002). However. it can also lead to bioactivation and are abundant in most cells, where they are mostly found in the cytosol (Boelsterli and Boelsterli 2002). GST is often induced by reactive metabolites, which are then detoxified by GST (Boelsterli and Boelsterli 2002).

Therefore, GST is an important detoxifying enzyme protecting cells and tissues from harm from reactive metabolites. (Boelsterli and Boelsterli 2002). Reduced GSH is an important antioxidant, which is important to reduce the amount of ROS (Boelsterli and Boelsterli 2002). GSH is synthesized in the cytosol and the GSH levels are regulated through negative feedback by inhibiting ɣ-glutamylcysteine synthetase, which is needed for GSH synthesis (Couto et al. 2016). Disulphide bonds Oxidized proteins can be regenerated back to sulfhydryls by GSH reducing disulphide bonds back catalysed by thiol reductase (Boelsterli and Boelsterli 2002). When GSH is utilized, it gets reduced to glutathione disulfide (GSSG), which then can be regenerated to GSH by glutathione reductase (GR) (Couto et al. 2016). Glutathione peroxidase (GPx) is an intracellular antioxidant enzyme that reduces hydrogen peroxide to water (Lubos et al. 2011).

1.6.3 Lipid peroxidation

ROS can lead to lipid peroxidation (LPO), which is when oxidants, like free radicals, attack lipids containing carbon-carbon double bonds (Endale et al. 2023). LPO is therefore an effect caused by oxidative stress. Lipids can be important signalling molecules (Fernandis and Wenk 2007). Therefore, disruption of these lipids might disrupt signalling pathways. Malondialdehyde (MDA) is a common biomarker for LPO since MDA is one of the final products of peroxidation of polyunsaturated fatty acids in cells (Gaweł et al. 2004). Measurement of thiobarbituric acid reactive substances (TBARS) is a common way to measure LPO since TBARS are formed as a byproduct of LPO (Aguilar Diaz De Leon and Borges 2020). However, measuring TBARS is unspecific for MDA since other peroxidation products than MDA can also be TBARS (Aguilar Diaz De Leon and Borges 2020).

1.7 Aims of the study

This study aims to investigate how carbamazepine and ibuprofen exposure affects biomarkers of oxidative stress in Arctic *G. oceanicus,* by examining responses in CAT, GSH, GST, SOD and MDA, of individuals in an environmental setup relevant to the Arctic summer. The experiments were conducted in Ny-Ålesund, Svalbard, in the Arctic to increase understanding of how carbamazepine and ibuprofen can affect *G. oceanicus* in the Arctic environment.

2. Method description

2.1 Study area and collection of *G. oceanicus*

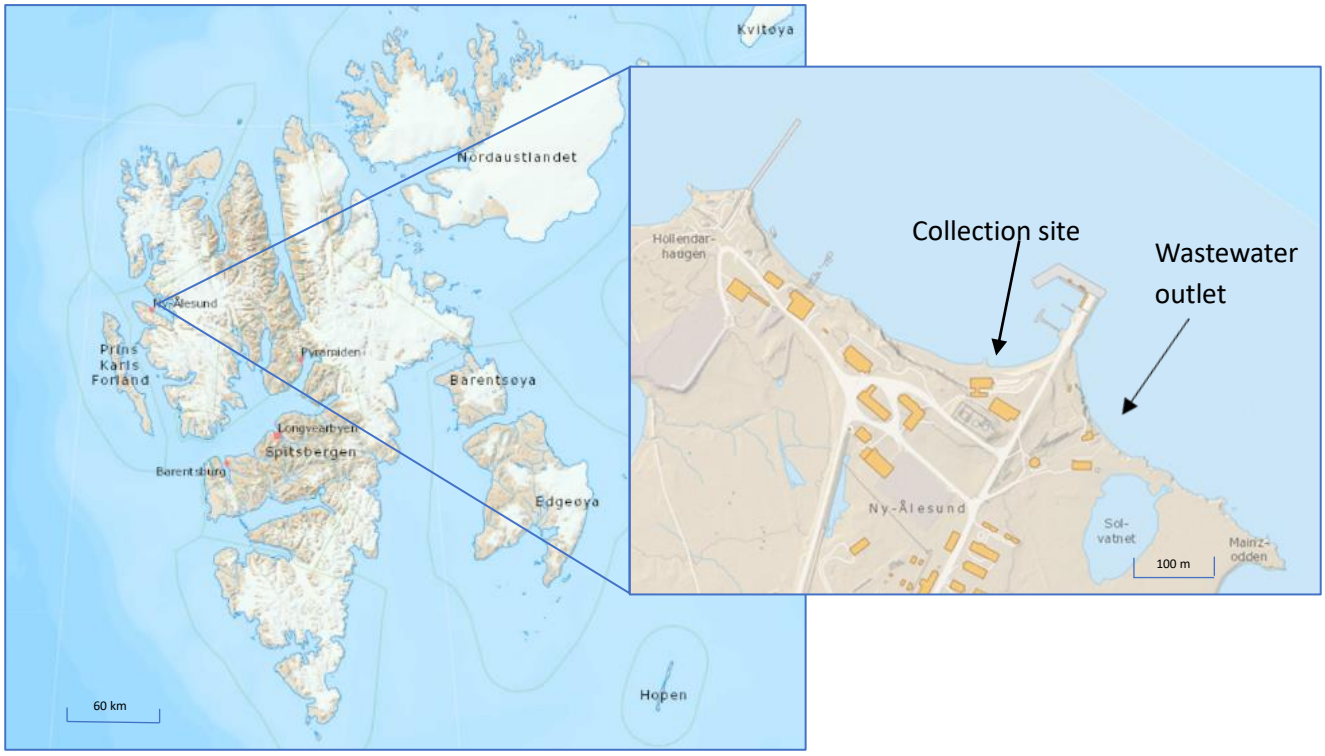

Figure 2: Study area and location for collection of the *G. oceanicus* individuals. Map retrieved from TopoSvalbard (Norsk Polarinstitutt).

Individuals of *G. oceanicus* were collected by hand picking in the intertidal zone outside the Kings Bay Marine Laboratory in Ny-Ålesund located in Kongsfjorden at Svalbard (78°55'39.23"N, 11°55'46.55"E) in August 2023 (Figure 2). Individuals for the carbamazepine experiment were collected at a water temperature of 5.9°C and salinity 33.3 PSU, while the individuals for the ibuprofen experiment were collected at a temperature of 6°C and salinity 34.0 PSU. The individuals were kept in plastic buckets, with an air supply from an air pump (HAILEA ACO-2208, Guangdong China) and filled with water from the fjord, placed in a climate room in the marine lab kept at 6-7 degrees for acclimatisation for 3 days. The water from the fjord was measured for the presence of pharmaceuticals. Concerning feeding, a preliminary study was performed to investigate if *G. oceanicus* preferred cod or pasta. They preferred the pasta, therefore, during the acclimatisation and exposure they were fed dry tagliatelle pasta. Temperature, salinity, and oxygen in the exposure tanks were monitored, and a 24-hour continuous light regime was performed to mimic the natural condition in Svalbard in summer during both the acclimatisation and the exposure period.

2.2 Chemicals and kits

Carbamazepine (C4024, CAS: 298-46-4) and ibuprofen (I4883, CAS: 15687-27-1) powder used for the exposure was purchased from Sigma-Aldrich (St. Louis, MO 63103 USA). The Glutathione assay kit (CS0260) and Glutathione S-Transferase (GST) assay kit (CS0410) were purchased from Sigma (St. Louis, MO 63103 USA). The MDA analysis was performed using components from the Lipid Peroxidation (MDA) Assay Kit (MAK085) from

Sigma (St. Louis, MO 63103 USA). Analysis of SOD activity was done using the 19160 SOD determination kit from Sigma-Aldrich, MilliporeSigma (St. Louis, MO 63103 USA). For analysis of GPx activity the Glutathione Peroxidase Activity Colorimetric Assay Kit (K762-100) from BioVision (Milpitas, CA 95035 USA) was used for the carbamazepine exposure samples, for the ibuprofen exposure samples the Glutathione Peroxidase Cellular Activity Assay Kit (CGP1) from Sigma-Aldrich (St. Louis, MO 63103 USA) was used. Catalase activity and protein amount in the samples were analysed without kits, and the chemicals originated from various producers (see sections 2.4.2 and 2.4.8).

2.3 Exposure experiments

Exposure experiments were performed at the Kings Bay Marine Laboratory in Ny-Ålesund, Svalbard in August 2023 following the acclimatisation period as described above. A stock solution of each pharmaceutical (5 mg/L) were prepared in distilled water in a borosilicate bottle and mixed overnight with a magnetic stirrer before the start of the experiment. For the setup 2L borosilicate beakers were washed and filled to 2000 mL with seawater from the fjord before 12 (carbamazepine exposure) or 15 (ibuprofen exposure) random *G. oceanicus* individuals were added to each beaker before the pharmaceutical was added. Stock solution was added to the beakers to obtain nominal concentrations of 0, 50 and 1000 ng/L in the carbamazepine exposure experiment. There were three replicates of each exposure concentration and 15 individuals per beaker (Figure 1). In the ibuprofen exposure experiment, stock solution was added to the beakers to obtain nominal concentrations of 0, 10, 100 and 1000 ng/L. Here, there were three replicates of each exposure concentration and 12 individuals per beaker (Figure 2). The duration of the carbamazepine exposure experiment was 72 hours, while the ibuprofen exposure lasted 96 hours. During the exposure, the beakers were supplied with air from an air pump (HAILEA ACO-2208, Guangdong China) and temperature and salinity were measured each day. Teflon tubing was used inside the beakers to avoid contact with the regular silicone pump tubing. The temperature was kept at 5.7-6.0 °C and the salinity at 33.1-33.4 ppt during the whole exposure time for both experiments. The gammarids were fed a piece of pasta (0.1 – 0.2 grams) for about 30 minutes each day, except the first and last day. The carbamazepine exposure experiment had two feedings, and the ibuprofen exposure experiment had three feedings during the whole exposure period. The water was not changed during the exposure and water samples (5-10 mL) were taken from the aquaria on the first and the last day of the exposure period. After the exposure period, the individuals were taken out of the beakers, transferred to Eppendorf tubes individually, frozen in liquid nitrogen and stored at -80 °C. The samples were transported with RV Oceania (a sailing research vessel owned by the Institute of Oceanology Polish Academy of Sciences (IO PAN)) to Gdynia in Poland where they were kept in a -80°C freezer at the University of Gdańsk (UG) until analysis in January 2024.

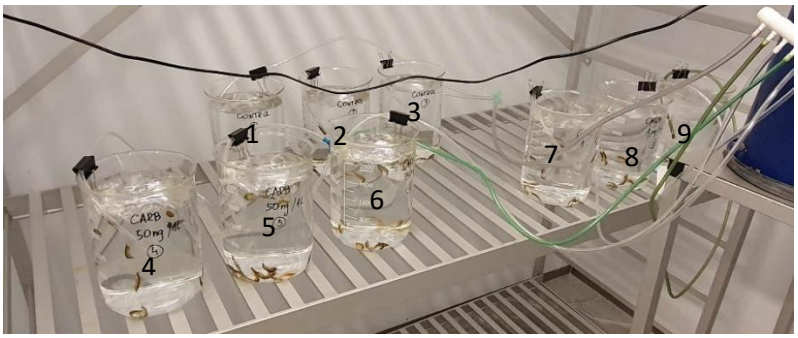

Figure 3: Setup of the carbamazepine exposure experiment with 15 individuals of *G. oceanicus* per beaker. Beakers 1-3 are control, 4-6 50 ng/L and 7-9 are 1000 ng/L exposure.

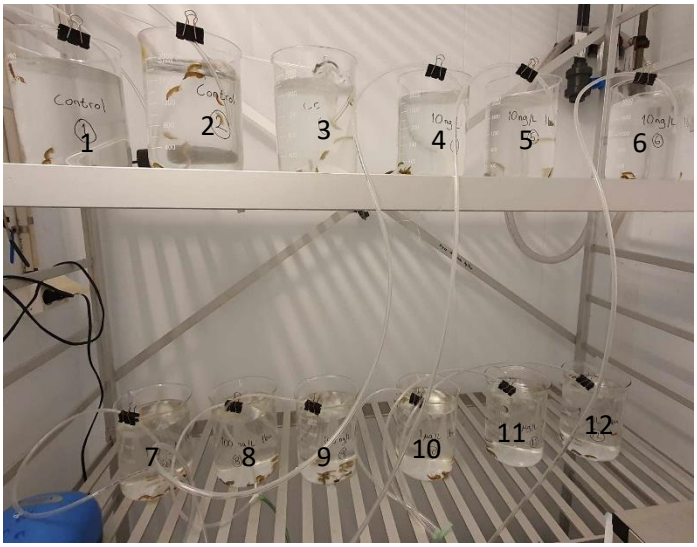

Figure 4: Setup of the ibuprofen exposure experiment with 12 individuals of *G. oceanicus* per beaker. Beakers 1-3 are control, 4-6 10 ng/L, 7-9 100 ng/L and 10-12 are 1000 ng/L exposure.

2.4 Analyses of biomarkers

Analyses of the biomarkers CAT, SOD, GSH, GST, GPx and MDA were performed at The University of Gdańsk (UG) at the Institute of Oceanology in Gdynia, Poland. Four individuals, preferably two males and two females, were chosen from each replicate for analyses of GST, GSH, GPx, CAT, SOD and protein concentrations. This gave a total of 36 individuals for the carbamazepine exposure experiment and 48 individuals for the ibuprofen exposure experiment, with 12 individuals per exposure group. Before the homogenisation of each individual, the wet weight (ww) of the individuals was recorded. Individuals were analysed separately, and 10 or 12 individuals were analysed for all biomarkers per day.

2.4.1 Homogenisation of individuals

Each individual was homogenised separately and the whole individual was homogenised with ice-cold Tris sulfate buffer (pH 7.8) with 50nM Tris (Pol-Aura, Olsztyn Poland, CAS: 77-86-1), 0.1 mM ethylenediaminetetraacetic acid (EDTA, from Pol-Aura, Poland, CAS: 60-00-4), 1 mM Phenylmethanesulfonylfluoride (PMFS, from Acros organics, Germany, CAS: 329-98-6, Lot: A0364241), 2mM Dithiothreitol (DTT from Fisher Chemical, Loughborough UK, CAS: 3483-12-3, Lot:1684889), 0.1% Triton (R) X-100 (Chempur, Piekary Śląskie Poland, CAS: 9002-93-1) and H_2SO_4 (P.O.CH, Poland), used to adjust pH,

in a glass hand tissue grinder (Kontes glass co. DUALL 22 or F EU 5 ml FB 56703). The homogenates were centrifuged at 15 000 relative centrifugal force (RCF) for 30 minutes at 4°C in Centrifuge 5430 R (R 404 A, Hamburg Germany). The supernatant (suspended cytosolic fraction) was collected and kept on ice. Before homogenisation antennae, gnathopods, and the third hindleg were taken off for species and sex identification. The sex determination was uncertain due to the identification being mostly done using size and antennae with the assumption that all individuals were adults.

2.4.2 CAT activity analysis

The CAT activity was determined using the method from Cohen et al. (1970), using five times less volume (Cohen et al. 1970). Prior to analysis, a 0.01 M phosphate buffer and 6 N H_2SO_4 solution were prepared. The 0.01 M phosphate buffer was prepared by diluting 1mL 1 M phosphate buffer in 99 mL water and pH was adjusted to 7 with HCl (Merck) and stored at room temperature in a fume hood. The 6N H_2SO_4 solution was prepared by adding 8.26 mL 95% H_2SO_4 (P.O.CH, Poland) to 50 mL distilled water and stored at room temperature in a fume hood. Just before analysis, a 6mM H_2O_2 solution, and a 0.01 N $KMnO_4$ solution were prepared. The 6mM H_2O_2 solution was prepared by diluting 0.031 mL 30% H_2O_2 (CAS 7722-84-1) in 50 mL of the phosphate buffer prepared earlier. The 0.01 N $KMnO_4$ solution was prepared by diluting 0.0158g $KMnO_4$ (Chempur) in 50 mL distilled water. Reactions were conducted with samples in duplicates in an ice-cold water bath (0-2°C) with a tube rack with 15 mL tubes. The sample homogenates were 2 times diluted with distilled water. For the two blanks, the tubes were filled with 0.1 mL distilled water and 1.0 mL H_2O_2. For the two standards, the tubes were filled with 1.1 mL buffer. For analysis of the samples, 0.1 mL diluted homogenate was mixed in 1.0 mL H_2O_2. All the tubes were vortexed and incubated for 3 minutes before 0.2 mL H_2SO_4 was added to all the tubes to stop the reaction. Just before measurement, 1.4 mL $KMnO_4$ was added to all the tubes before they were vortexed, and the absorbance was measured using cuvettes in a spectrophotometer (Shimadzu UV-Visible spectrophotometer (UV-1601)) at wavelength 480 nm within 1 minute after addition of the $KMnO_4$.

The catalase activity was estimated using equation I to obtain the catalase constant rate (k):

(I) $$k = \log(S_0/S_3) \times 2.3 / 3\ min$$

Where S_0 is the blank subtracted from the standard and S_3 is substrate concentration at 3 minutes.

(II) $$k \times 2 \times \frac{2.7}{0.1} mL$$

Equation II was used to account for dilutions before analysis (two times dilution) and during analysis where the total volume was 2.7 mL where 0.1 mL was the sample homogenate.

2.4.3 SOD activity analysis

SOD activity was analysed using the SOD Determination kit and performed as in the protocol with a 96-well plate. The SOD assay was performed utilising the WST-1 tetrazolium salt (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium). The WST working solution was prepared by diluting 1 mL of the WST solution in 19 mL Buffer Solution. The Enzyme working solution was prepared by dilution of 15 µL Enzyme solution in 2.5 mL Dilution Buffer. For the SOD analysis, three blanks

were used. Blank 1 was to test if the enzyme solution was working, blank 2 was to correct for colour differences, and blank 3 was to account for possible effects of the dilution buffer. First, 20 µL distilled water was added to the blank 1 and blank 3 wells in the 96-well plate. 20 µL undiluted sample homogenate was added to wells for the sample and blank 2. For samples 1-20, 35, and 53-84 an individual blank 2 was performed per sample. For samples 21-34 and 37-52, the blank 2 was a mix of the sample with the most and least amount of colour, with 10 µL of each. 200 µL of WST working solution was added to all the wells. Then, 20 µL enzyme working solution was added to the sample and blank 1 wells and 20 µL dilution buffer was added to blank 2 and blank 3 wells. The plate was incubated at 37°C for 20 minutes before the absorbance endpoint (450 nm) was measured in a microplate spectrophotometer (Multiskan SkyHigh thermoscientific) with samples and blank in duplicates.

The SOD-inhibition rate was calculated using the equation III:

$$\text{(III)} \qquad \text{SOD inhibition}(\%) = \frac{(A_{\text{blank }1} - A_{\text{blank }3}) - (A_{\text{sample}} - A_{\text{blank }2})}{(A_{\text{blank }1} - A_{\text{blank }3})} \times 100\%$$

To estimate the SOD activity the standard curve in Figure 4 in the 19160-1KT-F SOD determination kit bulletin was used with the equation:

$$\text{(IV)} \qquad y = 0.4516\,x + 0.1505$$

Where y is the inhibition rate and x is the amount in U/mL.

For samples 1-20, 35 and 75-84, the supernatants were frozen at -80°C and analysed for SOD in May 2024. The SOD activity in the carbamazepine exposure samples analysed later (samples 1-20 and 35) were in the same range as those analysed the same day as the homogenisation. However, the ibuprofen exposure samples analysed later (samples 75-84), were generally lower in SOD activity than the other samples from the ibuprofen exposure experiment.

2.4.4 GSH analysis

Total glutathione (GSH + GSSG) was measured using undiluted samples. The 5x Assay Buffer for the GST assay kit was diluted to 1x Assay Buffer with distilled water. The enzyme solution was prepared before each round of measurements by mixing 3.8 µL Glutathione reductase in 246 µL 1x Assay Buffer. A working mix was made by mixing 5.4 mL 1x Assay Buffer, 154 µL Enzyme solution and 154 µL 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB). A dihydronicotinamide adenine dinucleotide phosphate (NADPH) solution was repared by diluting 7.2 µL NADPH Stock in 1.8 mL 1x Assay Buffer. The Glutathione standard solution was made just before use by mixing 1 µL GSH stock in 199 µL 5% 5-Sulfosalicylic Acid (SSA). All the solutions were prepared a short time before use. The standard curve was made by diluting the Glutathione standard solution in 5% SSA with 2x dilution for each step, where the highest concentration was undiluted. 10 µL 5% SSA (blank), standard curve or undiluted sample homogenate was added to wells in duplicates. 150 µL working mix was added to each well simultaneously using a multi-channel pipette and mixed. After 5 minutes 50 µL NADPH solution was added to each well. The absorbance (412 nm) was measured in a microplate spectrophotometer (Multiskan SkyHigh thermoscientific) immediately after the addition of NADPH solution with reaction kinetics settings for 10 minutes with 1-minute intervals and shaking.

The GSH activity was calculated using the equation:

(V) $$\text{GSH (nmol/mL)} = \frac{\Delta\text{Abs(sample)}}{\Delta\text{Abs(1nmol from std.curve)} \times 0.01\ \text{mL}}$$

Where ΔAbs is the change in the measured absorbance value per minute. Different GSH standard curve equations were used for each sample batch (Table A14 Appendix A4).

2.4.5 GST activity analysis

GST was analysed using the Glutathione S-Transferase (GST) Assay Kit. A 200 mM L-Glutathione solution was made for each round of measurements by dissolving 12.3 mg L-glutathione (Sigma-Aldrich) in 200 µL water. A substrate solution was made just before measurements consisting of 4.9 mL Dulbecco's buffer, 50 µL of the L-Glutathione solution and 50 µL of 100 mM 1-chloro, 2,4-dinitrobenzene (CDNB). The sample homogenate was diluted two times with the sample buffer. Blank, consisting of only substrate solution, positive control, consisting of 4 µL GST positive control in 196 µL substrate solution, and samples, consisting of 10 µL diluted samples in 190 µL substrate solution, were added to a 96-well plate in duplicates and mixed with substrate solution to a total volume of 200 µL. The absorbance (340 nm) was measured in a microplate spectrophotometer (Multiskan SkyHigh thermoscientific) with reaction kinetics for 10 minutes with 1-minute intervals.

The GST activity was calculated using the formula:

(VI) $$\text{GST (nmol/mL/min)} = \frac{\Delta\text{Abs} \times 0.2\ \text{mL} \times 2}{5.3\ \mu\text{mol}^{-1}\ \text{mL}^{-1} \times 0.01\ \text{mL}} \times 1000$$

ΔAbs is the change of absorbance (340 nm) in the samples per minute, and "2" is the dilution factor. 5.3 μmol^{-1} mL^{-1} is the extinction coefficient for CDNB conjugate at 340 nm.

2.4.6 GPx activity analysis

GPx was for the samples from the carbamazepine exposure experiment analysed using the Glutathione Peroxidase Activity Colorimetric Assay kit from BioVision and performed as described in the protocol. A NADPH standard (1 mM) was made by mixing 9 µL 40 mM NADPH in 351 µL water. The standard curve was prepared by mixing 0, 20, 40 or 100 µL NADPH standard with Assay Buffer to a final volume of 100 µL in duplicates directly in the wells on the 96-well plate. The reaction mix was prepared by mixing 891 µL Assay Buffer, 81 µL 40 mM NADPH, 54 µL GR solution and 54 µL GSH solution. The control, blank and samples were processed in duplicates. In the wells for the positive control was a mix of 40 µL reaction mix, 45 µL Assay Buffer and 5 µL GPx positive control. The blank was a mix of 40 µL reaction mix and 50 µL Assay Buffer. For the samples, 10 µL sample supernatant was mixed with 40 µL Assay buffer and 40 µL reaction mix. The filled plate was incubated at room temperature for 15 minutes before the first measurement. The absorbance (340 nm) of the standard curve was measured using a 96-well plate in a microplate spectrophotometer (Multiskan SkyHigh thermoscientific). Then, Cumene Hydroperoxide was added to the positive control, blank and samples and the absorbance was measured immediately (Time 1) and 5 minutes later (Time 2).

The NADPH amount in the samples at Time 1 and Time 2 were calculated from the NADPH standard curves for each batch of samples (Table A16 Appendix A4):

(VII) $$y = ax + b$$

Where Y is the absorbance (340 nm) and x is the NADPH amount in nmol. The GPx activity was calculated using the formula:

$$\text{(VIII)} \qquad \text{GPx activity (nmol/mL/min)} = \frac{\text{NADPH Time 1} - \text{NADPH Time 2}}{5 \text{ min} \times 0.01 \text{ mL}}$$

where NADPH Time 1 is the measured NADPH amount (nmol) at the first measurement and NADPH Time 2 is the measured NADPH amount (nmol) five minutes after the first measurement.

For the samples from the ibuprofen exposure experiment, GPx was measured using the Glutathione Peroxidase Cellular Activity Assay Kit from Sigma-Aldrich. The Assay Buffer was kept at a temperature of 25°C, the temperature was kept as stable as possible using a water bath placed in a heating block. The NADPH Assay reagent was prepared by reconstitution of 1 vial in 1.25 mL distilled water right before and stored on ice. The oxidising reagent working solution was diluted to a 30 mM solution by diluting 4.3 µL oxidising reagent in 995.7 µL distilled water. For the blank, 940 µL assay buffer was mixed with 50 µL NADPH Assay Reagent and 10 µL oxidising reagent working solution in a quartz cuvette. For the first samples, 930 µL Assay Buffer was mixed with 10 µL sample homogenate before 50 µL NADPH Assay Reagent and 10 µL oxidising reagent solution in the quartz cuvette. However, when the use of 10 µL sample homogenate did not seem to give enough signal in the instrument it was chosen to use 50 µL sample mixed with 890 µL Assay Buffer instead, which then were added 50 µL NADPH Assay Reagent and 10 µL oxidising reagent working solution. After the addition of the NADPH Assay Reagent and oxidising reagent solution, the mixture was mixed for about ten seconds in the cuvette before measurement right after. Between each measurement, the quartz cuvette was washed with distilled water and dried. The absorbance (340 nm) was measured one sample at a time with two replicates of each sample using quartz cuvettes inserted in the cuvette port in a microplate spectrophotometer (Multiskan SkyHigh thermoscientific) set at 25°C with reactions kinetics for 90 seconds with 10-second intervals. Only one replicate was performed for the blank.

The GPx activity was calculated using the equation:

$$\text{(IX)} \qquad \text{GPx activity (nmol/mL/min)} = \frac{(\Delta\text{Abs blank} - \Delta\text{Abs sample}) \times \text{dilution}}{6.22 \text{ µmol}^{-1} \times \text{mL}^{-1} \times \text{sample volume (mL)}} \times 1000$$

ΔAbs is the change in measured absorbance (340 nm) per minute.

2.4.7 MDA analysis

LPO was analysed using components from the lipid peroxidation (MDA) assay kit. However, performed a bit differently than described in the protocol. Butylated hydroxytoluene (BHT) was not used and the with a four-point MDA standard curve. Thiobarbituric acid (TBA) solution was made by mixing 88.6 mg TBA (Sigma Aldrich), 2.76 mL acetic acid (P.O.CH, Poland) and 9.24 mL distilled water. The MDA2 solution was prepared by diluting 20 µL MDA1 (stored at -20°C before use) with 980 µL distilled water. A standard curve in duplicates was made by mixing MDA2 standard in distilled water with concentrations of 0, 4, 8 and 16 nmol/well MDA. For the samples, 100 µL concentrated supernatant was added to test vials in duplicates, before 500 µL 42 mM H_2SO_4 (P.O.CH, Poland) and 125 µL phosphotungstic acid solution (Sigma-Aldrich) was added. Samples were vortexed and centrifuged at 13000 g for 10 minutes at 22°C before the supernatant was discarded and 200 µL distilled water was added to the pellet. 600 µL TBA solution was added to samples and standard curve before incubation in a heating block set at 95°C for about 2,5 hours. Before measuring the absorbance, the samples were cooled down to room temperature and centrifuged at 2000-3000 RCF for 2 minutes. The absorbance (532 nm) was measured using a 96-well plate in a microplate

spectrophotometer (Multiskan SkyHigh thermoscientific) with the samples and standards in duplicates.

The MDA amount in the samples was calculated from the MDA standard curve (Table A15 Appendix A4).

(X) $$y = ax + b$$

Where y is the measured absorbance and x is the MDA amount (nmol), and used to get the concentration in nmol/mL with equation XI:

(XI) $$\text{MDA (nmol/mL)} = \frac{(\text{Abs} - b)/a}{0.1\ \text{mL}}$$

Abs is the measured absorbance in the sample at 532 nm and b and a are from the MDA standard curve (Table A15 Appendix A4).

2.4.8 Protein content measurement

The cytosolic protein content was determined using the method by Lowry et al., 1951 (Lowry et al. 1951). For the protein measurements a 5% sodium dodecyl sulphate (SDS) reagent (P.O.CH, Poland), 0.8 N NaOH (P.O.CH, Poland), blue mixture (0.2% potassium tartrate (P.O.CH, Poland), 0.1% copper sulphate (P.O.CH, Poland), 10% sodium carbonate(P.O.CH, Poland)) and Folin-Ciocalteau reagent (Chempur, Poland) diluted two times with distilled water was used. A 5-point standard curve of bovine serum albumin (BSA), with two replicates per point, was performed. For the standard curve, 0, 10, 20, 30 or 50 µL 2.5 mg/mL BSA solution (Sigma-Aldrich) was mixed with distilled water to a total volume of 500 µL. The samples were diluted 10 times with distilled water before 50 µL of the diluted sample was mixed with 450 µL distilled water in test vials in duplicates. SDS reagent, NaOH and blue mixture were mixed in a volumetric ratio of 2:1:1 and 500 µL were added to each sample homogenate or standard curve sample and mixed. Samples and standard curve were incubated for 10 minutes at room temperature before the addition of 250 µL diluted Folin-Ciocalteau reagent, the samples and standard curve were then mixed and incubated for 40 minutes in darkness. Absorbance was measured at 750 nm in microcuvettes in a spectrophotometer (Shimadzu UV-Visible spectrophotometer (UV-1601)).

The protein amount was estimated using a BSA standard curve (equation X) for each sample batch (Table A13 Appendix A4). The protein levels were calculated using equation (XII):

(XII) $$\text{protein amount (mg/mL)} = \frac{\text{Abs} - b}{a} \times 200$$

Abs is the measured absorbance at 750 nm in the spectrophotometer and a and b are from the BSA-standard curve. The multiplication with 200 was to account for dilution (10 times before analysis and 20 times during analysis) and to get the concentration in mg/mL.

The analysed CAT, GSH, GST, GPx and MDA were calculated per mg protein for each individual.

2.5 Quality control

During the exposure experiments pH and temperature were measured in each of the exposure tanks to make sure they were stable. Before the start of the analysis, the methods were tested using extra samples from *G. oceanicus* or bivalves to find the best

sample volume and dilutions to use. Duplicates of blank and standards were used for the analysis of the biomarkers, except GPx from the ibuprofen experiment where only one replicate of the blank was performed for each round of analysis. The order of analyses of the different biomarkers was always GSH and catalase measurement first, before the MDA, protein, GST, SOD and GPx measurements.

Standard curves were performed for analysis of MDA, GSH, protein and GPx (carbamazepine exposure only) with R^2 of at least 0.96 for protein, 0.999 for MDA, 0.997 for GSH and 0.993 for GPx. For the SOD measurements, a standard curve from an earlier experiment with the same kit using bovine erythrocytes was used.

2.6 Data processing and statistics

All the data were exported to Microsoft Excel for calculations before exporting to RStudio for data processing and statistics. Standard deviations were calculated using the STDEV.S() formula in Excel and the coefficient of variation (CV) was calculated by dividing the standard deviation by the mean.

The data were plotted using ggplot() from the ggplot2 package and ggarrange() from the ggpubr package and annotate_figure() was used to plot the graphs together. The dplyr package was used for calculations of mean, standard error, and standard deviation. The showSignificance() function from the superb package in RStudio was used to plot significant differences between exposure concentrations. P-value ≤ 0.05 was used as the limit for significance. The normality of the data and residuals of the models were checked using the Shapiro-Wilk normality test (Shapiro.test() function). Levene test (leveneTest() function), Barlett's test (barlett.test() function), and Flinger's test (flinger.test() function) were used to test for homoscedasticity. The data and residuals of the models tested were plotted using the hist() function to see if the data were normally distributed. In addition, fitted vs residuals were plotted using the plot() function, and the residuals were plotted in a qqplot using the qqnorm() and qqline() functions. Cook's distance was plotted using the plot() function to investigate normality, homoscedasticity, and outliers of the models to be able to select the best model. Normal distribution was only found for CAT, GST, and SOD. Log transformation was tested for MDA, GSH, GST, and GPx. From the carbamazepine exposure measurements normality was obtained for GSH, GST, and GPx with log-transformation. For the ibuprofen exposure samples normality was obtained for MDA, GSH, and GST with log transformation.

Different models with various combinations of variables were made using the glm() function and different models with and without interactions between exposure concentration, exposure tank, and sex were tested (Table A17, Appendix A5). The model used for each of the biomarkers for each exposure experiment was chosen based on normality, homoscedasticity, lack of outliers, Akaike information criterion (AIC), using the aictab() function in the AICcmodavg package, and Bayesian information criterion (BIC), using the BIC() function in the "flexmix" package (Table A17, Table A18, Appendix A5). Differences between groups were tested using analysis of variance (ANOVA) (aov() function) using the chosen model, followed by Tukey's multiple comparisons tests (TukeyHSD() function). Non-parametric tests were chosen for MDA measurement from the carbamazepine exposure and GPx measurements from the ibuprofen exposure since the data were still not normally distributed after log transformation. For these data, differences between exposure concentration groups were tested using the Kruskal-Wallis statistics test (Kruskal.test() function) followed by Dunn's test (dunn.test() function, "holm"). To test significance between exposure concentrations investigating sex

differences non-parametric tests were chosen, due to the sample size being too low to assume normal distribution and there were unequal numbers of male and female individuals between some of the exposure concentrations in the ibuprofen exposure experiment and in the SOD for the carbamazepine exposure experiment (Appendix A3, Table A11, Table A12). Therefore, the Kruskal-Wallis test (Kruskal.test() function) followed by Dunn's test (dunn.test() function, "holm") was performed to investigate differences between the exposure groups.

Linear regression was performed to investigate correlations between the biomarkers investigated and correlation tests were performed using the cor.test() function. The correlation graphs were made by using the ggscatter() function in the ggpubr package and plotted together using the ggarrange() and annotate_figure() functions. For correlations between CAT, GST, SOD, and weight Pearson correlations were used, while correlations involving MDA, GSH, or GPx were done using Kendall Tau's correlations. In the carbamazepine exposure experiment samples, the correlation between weight and protein were done with Person correlation, Kendall Tau's correlation was performed in the ibuprofen exposure samples. Differences between the control groups of the carbamazepine and ibuprofen exposure experiments were tested with T-test using the built-in the t.test() function.

3. Results

G. oceanicus was exposed to carbamazepine or ibuprofen in seawater from Kongsfjorden by Ny-Ålesund, Svalbard, and the biomarkers CAT, SOD, GSH, GST, GPx, and MDA was measured after the exposure period of 72 hours for the carbamazepine exposure and 96 hours for the ibuprofen exposure.

3.1 Effect on biomarkers of oxidative stress

The CAT activity was not significantly different between the control and exposure groups for carbamazepine or ibuprofen exposure (Figure 5 A, B). The CAT activity was similar in the control samples in the carbamazepine exposure experiment and the control samples in the ibuprofen exposure experiment. The SOD amount was very similar in the control group of the carbamazepine exposure samples and the control group of the ibuprofen exposure samples (Figure 5 C, D). The SOD activity was not significantly different between the control and exposure groups for either the carbamazepine exposure or ibuprofen exposure experiment.

The GSH levels were generally higher in the samples from the ibuprofen exposure experiment, which had a mean of 1.49 ± 0.89 nmol/mg protein, than in the samples from the carbamazepine exposure samples, which had a mean of 1.07 ± 0.57 nmol/mg protein (Figure 5 E, F). A significant difference in the GSH levels was found between the control groups in the carbamazepine exposure experiment and the ibuprofen exposure experiment ($p=0.04$). For the samples from the carbamazepine exposure experiment, there was a significant increase in GSH levels from the control at 50 ng/L ($p=0.02$, Figure 5 E).

The GST activity was similar between the control groups in the carbamazepine exposure experiment and the ibuprofen exposure experiment (Figure 5 G, H). However, there was a higher sample variation in GST activity between the samples in the carbamazepine, with a CV of 29%, compared to the ibuprofen exposure experiment, with a CV of 20%. In the carbamazepine exposure experiment, the GST activity had a similar trend as GSH where 50 ng/L exposure had higher GSH levels than control and 1000 ng/L exposure concentration (Figure 5 G), however for GST the difference was not significant. A significant difference in GST activity was found between one of the exposure tanks in the control group and one of the exposure tanks in the 1000 ng/L exposure group. However, the variation between the exposure tanks within the same exposure concentration was too high to lead to significant differences between the exposure concentrations (mean CV of 29%).

The GPx activity was very different in the carbamazepine and ibuprofen exposure experiments (Figure 5 I, J). The GPx activity was significantly higher in the carbamazepine exposure experiment compared to the ibuprofen exposure experiment ($p=0.00$). The GPx activity in the control group in the carbamazepine exposure experiment was significantly higher than the control group in the ibuprofen exposure experiment ($p=0.0005$). In addition, the variation of measured levels in the ibuprofen exposure experiment was much higher, CV of 252% compared to 30% in the carbamazepine exposure experiment.

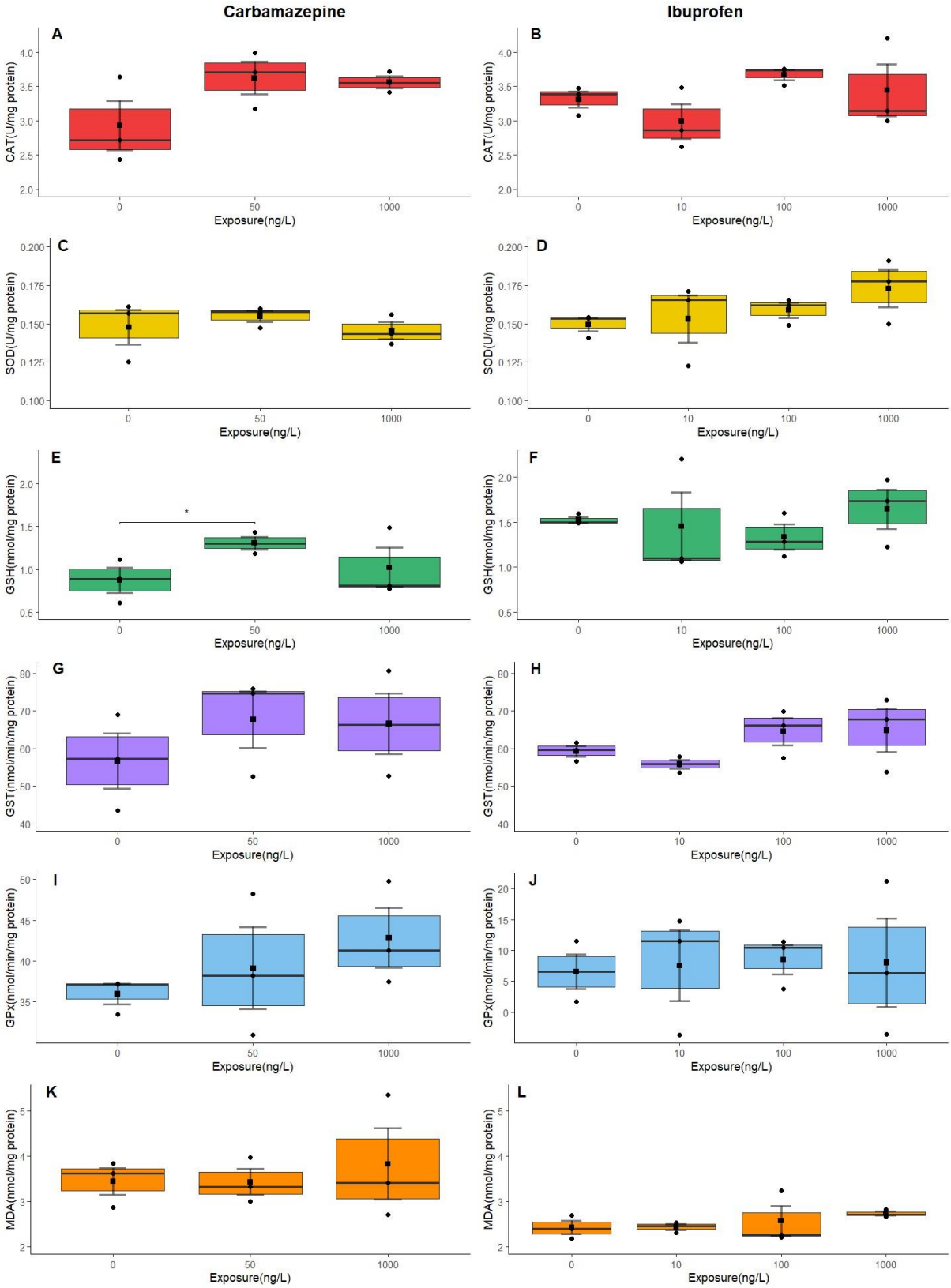

Figure 5: CAT (A, B), SOD (C, D), GSH (E, F), GST (G, H), GPx (I, J) and MDA (K, L) in *G. oceanicus* exposed to carbamazepine (A, C, E, G, I, K) or ibuprofen (B. D. F. H, J, L). The points are the mean for each of the exposure tanks per exposure concentration (n=4 per tank, except SOD see Table A11, Table A12), the squares are the mean per exposure concentration, and the black line marks the median per exposure concentration. The error bars are standard errors per exposure concentration. Significant differences between exposure concentrations are marked with a line with an asterisk. Note the different y-axis scales for GPx.

The MDA levels in the control groups were significantly lower in the samples from the ibuprofen exposure experiment, which had a mean of 2.54 ± 0.68 nmol/mg protein, compared to the carbamazepine exposure experiment samples, which had a mean of 3.56 ± 1.49 nmol/mg protein ($p=0.02$, Figure 5 K, L). However, the measured MDA levels were similar between the exposure concentration within the same exposure experiment in both the carbamazepine and ibuprofen exposure experiment (Figure 5 K, L). In the carbamazepine exposure, the mean MDA level was higher in the highest exposure concentration (Figure 5 K). However, the variation in the highest exposure concentration was also quite high with a CV of 51%, compared to 37% and 36% in the 0 and 50 ng/L exposure concentrations respectively (Table A6).

Overall, the levels of the biomarkers were relatively similar between the carbamazepine exposure experiment and the ibuprofen exposure experiment for CAT, SOD, and GST. More difference was found between the carbamazepine and ibuprofen exposure experiments for GSH, GPx, and MDA. Apart from elevated GSH concentrations after 50 ng/L carbamazepine exposure, no significant effects were found on the biomarker levels when *G. oceanicus* was exposed to carbamazepine or ibuprofen.

3.2 Differences between males and females

For the samples from the carbamazepine exposure, the CAT activity did not increase with exposure concentration when considering males and females together (Figure 5 A). By investigating males and females separately still no significant differences were found between the exposure concentration groups (Figure 6 A, B). However, the males had an increasing tendency in CAT activity with exposure concentration (Figure 6 A), while the females generally had a higher CAT activity at 50 ng/L than at 1000 ng/L (Figure 6 B). Investigating sex differences in CAT activity for samples from the ibuprofen exposure the females had a significantly lower CAT activity than the males ($p=0.001$, Figure 6 C, D). However, no significant differences or trends were found between the different exposure groups within each sex.

No significant differences were found in SOD activity between the exposure concentrations for males or females in the carbamazepine exposure experiment (Figure 7 A, B). However, the males generally had a lower SOD activity when exposed to carbamazepine (Figure 7 A), while the females had generally a higher SOD activity when exposed to ibuprofen (Figure 7 B). Investigating sex differences of SOD levels for the ibuprofen exposure experiment the males had a significant increase in SOD at 10 ng/L compared to control ($p=0.03$, Figure 7 C). However, at higher exposure concentrations the SOD levels in the males were similar to the control (Figure 7 C). In the females, no significant differences were found between the ibuprofen exposure concentration (Figure 7 D).

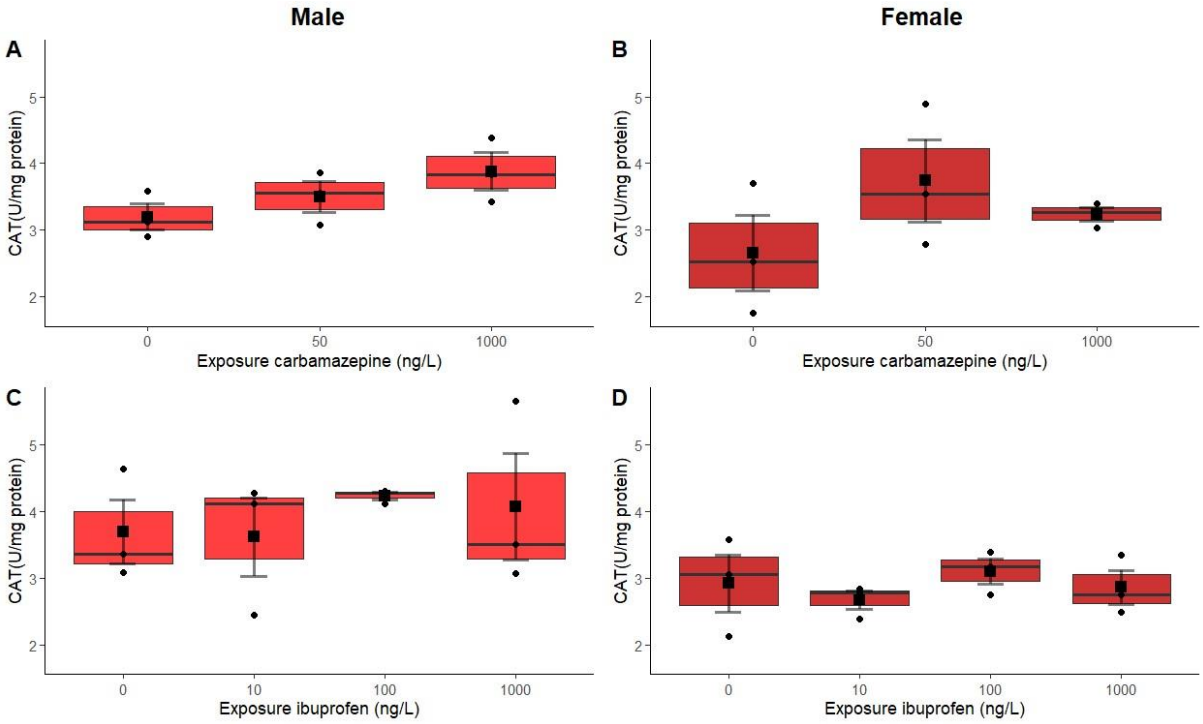

Figure 6: CAT amount in *G. oceanicus* males (A, C) and females (B, D) after exposure to carbamazepine (A, B) or ibuprofen (C, D). The points are the mean for each of the exposure tanks per exposure concentration (n= 2 per tank, exceptions see Table A11, Table A12), the squares are the mean per exposure concentration, and the black line marks the median per exposure concentration. The error bars are standard errors per exposure concentration.

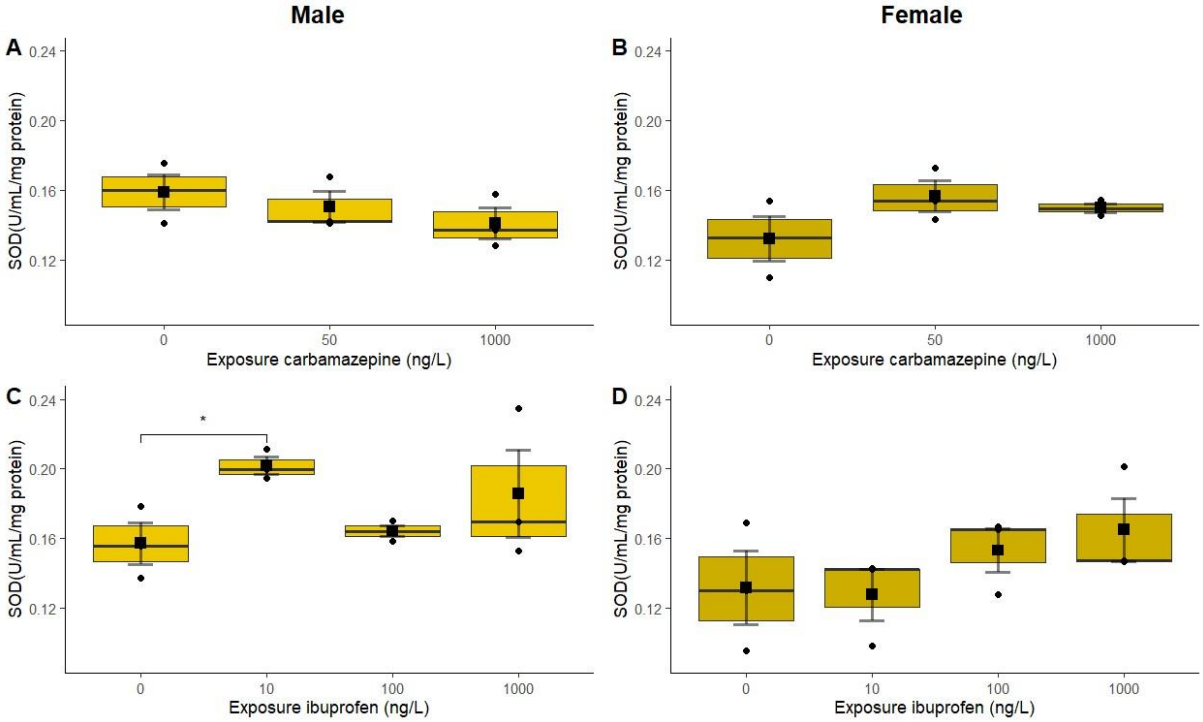

Figure 7: SOD amount per mg protein in G. oceanicus males (A, C) and females (B, D) exposed to carbamazepine (A, B) or ibuprofen (C, D). The points are the mean for each of the exposure tanks per exposure concentration (n= 2 per tank, exceptions see Table A11, Table A12), the squares are the mean per exposure concentration, and the black line marks the median per exposure concentration. The error bars are standard errors per exposure concentration. Significant differences between exposure concentrations are marked with a line with an asterisk.

When investigating males and females separately the GSH levels were quite similar between males and females in the carbamazepine exposure experiment (Figure 8 A, B). The females had a close to significant increase in GSH at 50 ng/L compared to the control (p=0.05), while 1000 ng/L was very similar to the control (Figure 8 B). The males had no significant differences between the exposure concentrations (Figure 8 A).

For the ibuprofen exposure, no significant differences in GSH levels were found between the exposure concentrations for males and females (Figure 8 C, D). The GSH levels at the various exposure concentrations were similar for both males and females. The females generally had lower GSH levels than the males, however not significantly.

In the carbamazepine exposure experiment, the GST activity was quite similar for males and females, and no significant differences were found between the exposure concentrations for either (Figure 9 A, B). The trends in males and females in GST activity were very similar to the trends in GSH in the carbamazepine exposure experiment. In the ibuprofen exposure experiment, a significant difference in GST was found between the 10 ng/L and 100 ng/L exposure concentrations in the males, where individuals exposed to 10 ng/L had significantly lower GST activity than individuals exposed to 100 ng/L ibuprofen (p=0.04, Figure 9 C). No exposure groups were significantly different from the control group (Figure 9 C, D). The males had significantly higher GST activity than the females overall (p=0.005).

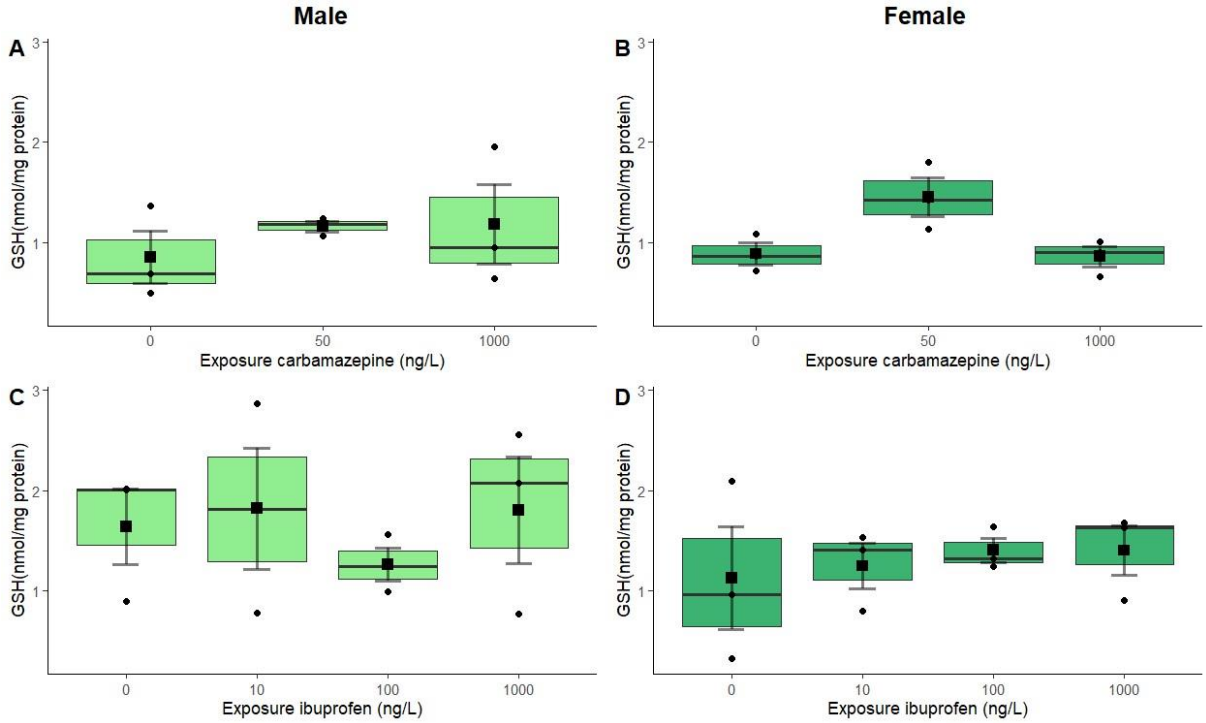

Figure 8: GSH amount in *G. oceanicus* males (A, C) and females (B, D) exposed to carbamazepine (A, B) or ibuprofen (C, D). The points are the mean for each of the exposure tanks per exposure concentration (n= 2 per tank, exceptions see Table A11, Table A12), the squares are the mean per exposure concentration, and the black line marks the median per exposure concentration. The error bars are standard errors per exposure concentration.

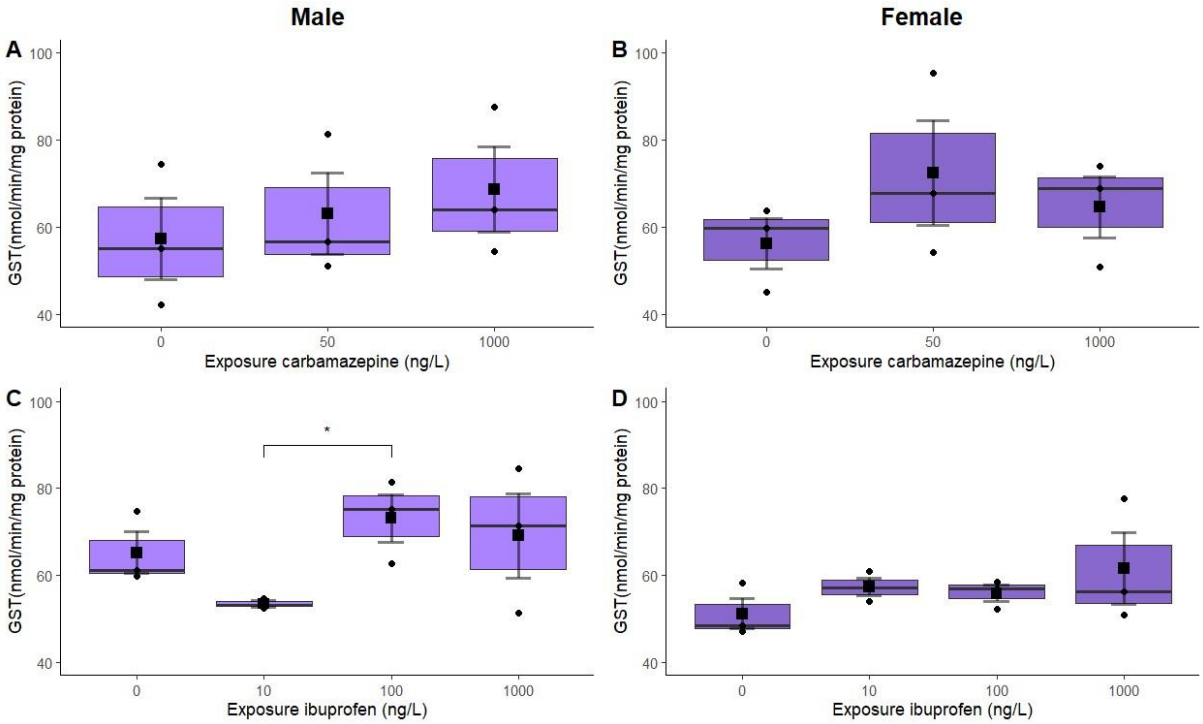

Figure 9: GST activity in *G. oceanicus* males (A, C) and females (B, D) exposed to carbamazepine (A, B) or ibuprofen (C, D). The points are the mean for each of the exposure tanks per exposure concentration (n= 2 per tank, exceptions see Table A11, Table A12), the squares are the mean per exposure concentration, and the black line marks the median per exposure concentration. The error bars are standard errors per exposure concentration. Significant differences between exposure concentrations are marked with a line with an asterisk.

In the carbamazepine exposure experiment, no significant differences were found in GPx activity between the exposure concentrations for males and females (Figure 10 A, B). However, in the females, the variance in GPx activity was much higher in the exposure groups, with a CV of 50% and 38% in the 50 and 1000 ng/L exposure group respectively, compared to the control group, with a CV of 8%. In the ibuprofen exposure experiment, no significant differences were found in GPx activity between the exposure concentrations for the males and females (Figure 10 C, D). However, the males had an increasing tendency with exposure concentration, while the females had a decreasing tendency in GPx activity with exposure concentrations.

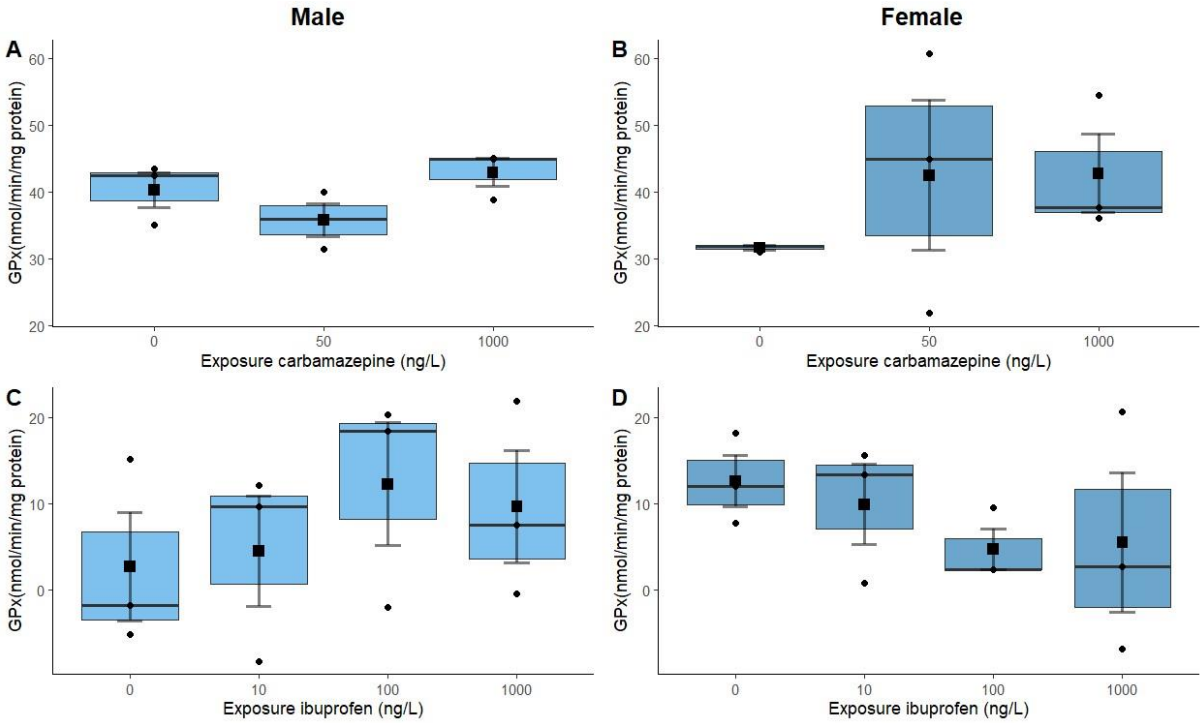

Figure 10: GPx activity in G. oceanicus males (A, C) and females (B, D) exposed to carbamazepine (A, B) or ibuprofen (C, D). The points are the mean for each of the exposure tanks per exposure concentration (n= 2 per tank, exceptions see Table A11, Table A12), the squares are the mean per exposure concentration, and the black line marks the median per exposure concentration. The error bars are standard errors per exposure concentration. Note the different y-axis scales for A, B and C, D.

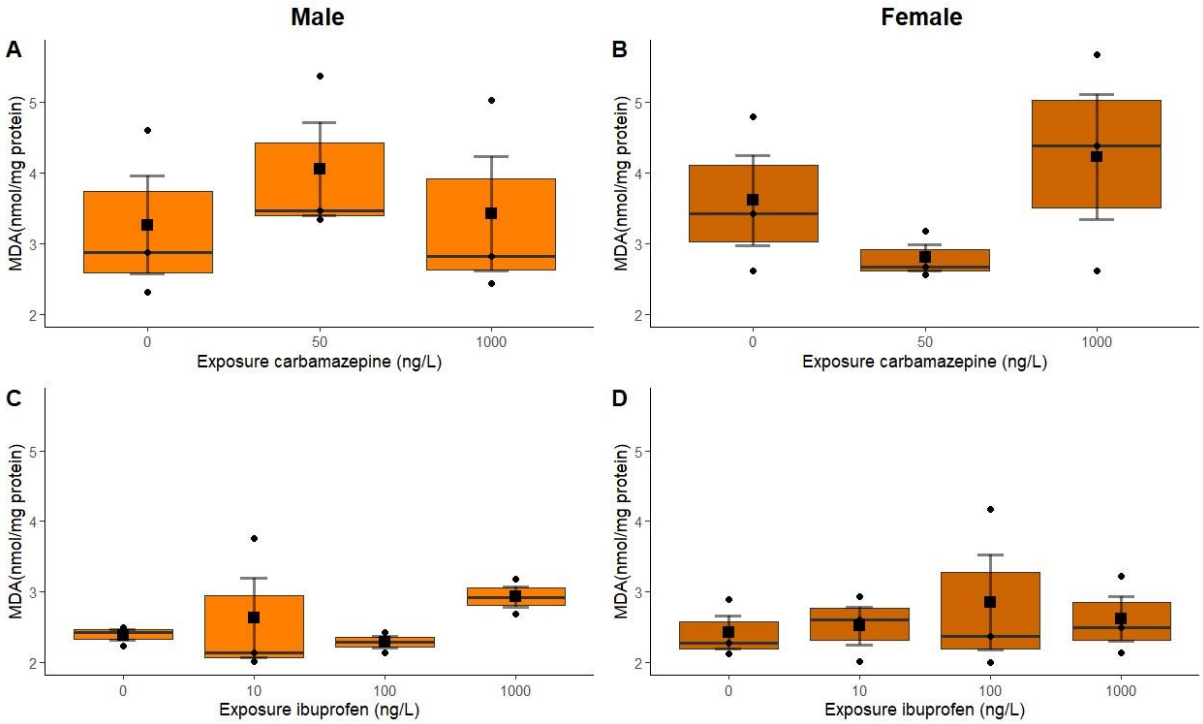

Figure 11: Malondialdehyde (MDA) amount in G. oceanicus males (A, C) and females (B, D) exposed to carbamazepine (A, B) or ibuprofen (C, D). The points are the mean for each of the exposure tanks per exposure concentration (n= 2 per tank, exceptions see Table A11, Table A12), the squares are the mean per exposure concentration, and the black line marks the median per exposure concentration. The error bars are standard errors per exposure concentration.

No significant differences in MDA levels were found between the exposure concentrations in males and females in the carbamazepine exposure experiment (Figure 11 A, B). However, the males had an increasing tendency in MDA levels in individuals exposed to 50 ng/L (Figure 11 A), while the females had a decreasing tendency of MDA in individuals exposed to 50 ng/L (Figure 11 B). No significant differences between the exposure concentrations were found in males or females in the ibuprofen exposure experiment (Figure 11 C, D). The MDA levels in the control group were similar in males and females for both of the exposure experiments (Figure 11).

3.3 Correlations between biomarkers

Correlation analyses were performed between the biomarkers, including weight, in the two exposure experiments to investigate how well they correlate with each other. The correlation values for the carbamazepine exposure experiment are shown in Table 1, while the correlations for the ibuprofen exposure experiment are shown in Table 2. The graphs for the correlations can be found in Appendix A6.

Table 1: Correlation of biomarkers from the carbamazepine exposure with p-values below the correlation coefficient (R). Significant correlations are in bold.

	Weight	CAT	SOD	GSH	GST	GPx	MDA
Weight	1	0.27 p=0.079	0.11 p=0.55	-0.19 p=0.11	-0.20 p=0.23	0.11 p=0.34	-0.003 p=0.99
CAT		1	**0.48** p=0.004	**0.25** p=0.03	0.06 p=0.75	**0.27** p=0.02	-0.02 p=0.90
SOD			1	**0.27** p=0.03	**0.59** p=0.0002	**0.42** p=0.0003	0.07 p=0.56
GSH				1	**0.38** p=0.0009	0.09 p=0.47	0.00 p=1.00
GST					1	**0.26** p=0.03	0.02 p=0.90
GPx						1	0.08 p=0.49
MDA							1

Table 2: Correlation of biomarkers from the ibuprofen exposure with p-values below the correlation coefficient (R). Significant correlations are in bold.

	Weight	CAT	SOD	GSH	GST	GPx	MDA
Weight	1	**0.45** p=0.001	0.21 p=0.15	0.14 p=0.16	**0.42** p=0.003	0.06 p=0.53	0.007 p=0.95
CAT		1	**0.74** p=0.00	**0.38** p=0.0001	**0.64** p=0.00	-0.10 p=0.34	0.07 p=0.51
SOD			1	**0.49** p=0.00	**0.44** p=0.002	-0.07 p=0.50	0.07 p=0.52
GSH				1	**0.3599** p=0.0002	-0.17 p=0.09	0.19 p=0.06
GST					1	0.06 p=0.58	0.12 p=0.22
GPx						1	-0.03 p=0.76
MDA							1

Correlation analysis was also performed between weight and protein levels. In the both the carbamazepine and ibuprofen exposure experiment a significant negative correlation was found between weight and protein levels, with a correlation coefficient of -0.46 (p=0.005) for the carbamazepine exposure experiment samples and -0.42 (p=2.5e-05) for the ibuprofen exposure experiment samples (Figure A1 Appendix A6).

Investigating correlations between the biomarkers, including weight, of the *G. oceanicus* individuals from the carbamazepine exposure experiment (Table 1), significant positive correlations were found between SOD and CAT, GSH and CAT, and GPx and CAT. Significant correlations were also found between GSH and SOD, GST and SOD and GPx and SOD, and between GSH and GST and GPx and GST. None of the biomarkers were significantly correlated with the weight of the individuals.

Significant positive correlations were found between SOD and CAT, GSH and CAT, and GST and CAT in the ibuprofen exposure experiment (Table 2). Significant positive correlations were also found between GSH and SOD, GST and SOD, and GST and GSH. So together, GST, GSH, CAT, and SOD were well correlated with each other. All the glutathione-related biomarkers were quite well correlated except for GPx, which did not correlate with any of the other biomarkers. No correlations were found between MDA and the other biomarkers either. Significant positive correlations with weight were found for CAT and GST.

3.4 Mortality

During the exposure period in the exposure experiments, only one individual died. The individual who died was from the 1000 ng/L exposure concentration in the carbamazepine exposure experiment. The cause was probably cannibalism since only a few remnants of the individual were left in the exposure tank at the end of the exposure period.

4. Discussion

Only a few biomarkers of oxidative stress were affected after exposure of *G. oceanicus* to carbamazepine or ibuprofen. However, a significant increase of GSH was found at 50 ng/L after exposure to carbamazepine compared to control. In the ibuprofen exposure, a significant increase of SOD was observed in the males at 10 ng/L exposure compared to control. There was also a significant difference in GST activity in the males between 10 ng/L and 100 ng/L exposure in the ibuprofen exposure experiment. In general, there was not a consistent pattern of which biomarkers were affected by the pharmaceutical exposure.

4.1 CAT activity

No significant effects were found on CAT activity after exposure to carbamazepine in *G. oceanicus* (Figure 5 A). However, in general, there was a tendency of increasing CAT activity. The males had an increasing tendency with exposure concentration and the females had a tendency of higher CAT activity at 50 ng/L (Figure 6 A, B). The effect of carbamazepine on CAT activity in other comparable studies with aquatic invertebrates show both increase, decrease and no change. A non-significant increasing tendency was also found in the marine bivalve mollusc *Rudistapes philippinarum* exposed to 1000 ng/L carbamazepine (Almeida et al. 2018). However, in that study, only one exposure concentration was used, and the exposure duration was 28 days, which is much longer than the exposure duration in the present study (Almeida et al. 2018). While in another study with *R. philippinarum* a decrease was found in CAT activity at 30 ng/L, and an increase at 3000 ng/L after 96 hours exposure to carbamazepine (Almeida et al. 2014). In the marine bivalve mollusc *Rudistapes decussatus,* it was found a decrease in CAT activity in all exposure concentrations, 30, 300, 3000, and 9000 ng/L, after a 96-hour exposure (Almeida et al. 2014). In the marine polychaete *Diopatra neapolitana,* no significant effects on CAT were found compared to control as well when exposed to 300, 3000, 6000, and 9000 ng/L for 28 days (Freitas et al. 2015). In the marine and freshwater polychaete, *Hediste diversicolor* a decrease in CAT activity was found when exposed to 300, 3000, 6000, and 9000 ng/L carbamazepine for 28 days (Pires et al. 2016). In the marine bivalve mollusc *Scrobicularia plana* increase in CAT was found at concentrations of 300 ng/L and 3000 ng/L after 28 days of exposure to carbamazepine (Freitas et al. 2015). In summary, most studies have been done with bivalve molluscs and polychaetes, which can have different effects compared to amphipods. Differences in exposure time and concentrations are also factors that influence the responses of CAT.

From the ibuprofen exposure, there were no trends in CAT activity with exposure concentration, no trend was found when investigating males and females separately either (Figure 5 B, Figure 6 C, D). Compared to other studies investigating the effects of ibuprofen on CAT a decrease in CAT activity was found in the freshwater zebra mussel gills (*Dreissena polymorpha*) when exposed to 206 ng/L, 2060 ng/L, and 206 µg/L ibuprofen for one day, a significant increase was found after exposure at 206 µg/L for four days and a significant decrease was found after exposure to 2060 ng/L for seven days (Contardo-Jara et al. 2011). No effect on CAT was found in the digestive glands of the marine bivalve mollusc *R. philippinarum* after 14 days of exposure to 15000 ng/L ibuprofen (Trombini et al. 2019). However, an increase was found in gills after a depuration period of seven days in clean water after the exposure (Trombini et al. 2019). In a study by Yildirim et al. (2022) on the freshwater amphipod *Gammarus pulex*

exposed to 2075, 1037.5, and 518.75 ng/L ibuprofen was found to have a significant increase in CAT activity in all exposure groups when exposed for 24 hours. After 96 hours of exposure there was a significant increase in CAT when exposed to 2075 ng/L and 1037.5 ng/L (Yildirim et al. 2022). The CAT activity was most upregulated at the highest exposure concentration, 2075 ng/L, which was much higher than the exposure concentrations used in the present study (Yildirim et al. 2022). If the exposure concentrations were higher in the present study, significant increases in CAT may have been found. However, higher concentrations would also be less environmentally relevant. In the study by Yildirim et al. (2022) the water temperature used was around 18°C and 12h light and dark periods compared to 6°C and 24-hour light regime in the present study. Temperature and light could possibly influence behaviour, metabolism and enzyme activity. It could be an impact from the difference in freshwater and saltwater as well compared to the present study when components in the water could interact with the uptake of the pharmaceuticals. In the study by Yildrim et al. (2022), sediment was used in the aquaria, which was not used in the present study, and could interact with the uptake of pharmaceuticals. In addition, only males were used in the study by Yildirim et al. (2022), which also could influence the enzyme activity as discussed later (4.7). In addition, the homogenate was frozen before analysis, which also could influence the measured enzyme activities (Yildirim et al. 2022).

In a study by Gómez-Oliván et al. (2014) where the freshwater amphipod *Hyalella azteca* was exposed to 0.17 mg/L ibuprofen for 72 hours an increase in CAT was found compared to control. However, this was a much higher concentration than what was used in the present study (Gómez-Oliván et al. 2014). In a study on zebra mussels (*D. polymorpha*) by Parolini et al. (2011) increase in CAT was found after ibuprofen exposure at 206 ng/L for 48 and 96 hours, after exposure to 1856 ng/L for 48 hours and 7200 ng/L for 48 hours (Parolini et al. 2011). In the study by Parolini et al. (2011), no significant effects were found at other exposure durations at each exposure concentration. The effects found could therefore depend on how long they were exposed. However, when there is no clear tendency with time in literature it seems to be quite random at what exposure duration you would find the highest effect.

In summary, no effects on CAT activity were observed in the present study after carbamazepine or ibuprofen exposure. In literature inconsistent, but mostly increasing effects on CAT have been observed, however many studies have used higher exposure concentrations and different experimental conditions than in the present study.

4.2 SOD activity

In the present study, no significant effects were found on SOD activity when exposed to carbamazepine (Figure 5 C). Compared to a study by Almeida et al. (2014) SOD activity was found to increase in the marine bivalve mollusc *R. deccusatus* after carbamazepine exposure for 96 hours at 300, 3000 and 9000 ng/L exposure, with the highest increase at 3000 ng/L exposure (Almeida et al. 2014). In the bivalve mollusc *R. philippinarum* SOD was increased much less when exposed to carbamazepine, and only significantly at 9000 ng/L exposure (Almeida et al. 2014). In another study with *R. philippinarum,* carbamazepine had no significant effect on SOD (Almeida et al. 2018). However, there was an increasing tendency when exposed to 1000 ng/L carbamazepine (Almeida et al. 2018).

No significant effects on SOD were found in the combined group of males and females of *G. oceanicus* exposed to ibuprofen in the present study (Figure 5 D). However, there was

a significant increase in SOD in the males at 10 ng/L exposure concentration compared to control (Figure 7 C). In a study by Yildirim et al. (2022), the freshwater amphipod *G. pulex* exposed to 2075 ng/L, 1037.5 ng/L and 518.75 ng/L, which are concentrations comparable to the levels used in this study. A significant decrease in SOD activity was observed after 24-hours exposure at 1037.5 ng/L and 518.75 ng/L (Yildirim et al. 2022). However, when exposed for 96 hours no significant effects on SOD activity were found (Yildirim et al. 2022). As mentioned, the freezing of the homogenate before the analysis could influence the measured enzyme activity. In the present study, the SOD enzyme activity measured in sample homogenates which had been frozen were similar to the non-frozen samples in the carbamazepine exposure samples. However, in the ibuprofen exposure samples generally lower SOD activity was found in the samples which had been frozen. A reason for this could be that the samples from the carbamazepine exposure experiment were frozen sooner after homogenisation, implying that the SOD activity could be influenced by the time of measurement after homogenisation.

In a study by Gómez-Oliván et al. (2014) where the freshwater amphipod *H. azteca* was exposed to ibuprofen, a decrease in SOD-activity was found when exposed to 0.17 mg/L ibuprofen (Gómez-Oliván et al. 2014). In a study of the freshwater zebra mussels (*D. polymorpha*) by Parolini et al. (2011), an increase in SOD was found after ibuprofen exposure at 206 ng/L for 96 hours and 7200 ng/L for 48 or 72 hours (Parolini et al. 2011). No significant differences were found at other times and not at 1856 ng/L exposure (Parolini et al. 2011).

In summary, an increase in SOD was found in males at 10 ng/L exposure to ibuprofen. However, no significant effects were found after carbamazepine exposure. In comparable studies with aquatic invertebrates, carbamazepine exposure primarily caused increase or no effect, as in the present study, on SOD activity. The effects on SOD activity after ibuprofen exposure in the present study are in line with the literature, where both increase, decrease and no effect on SOD activity have been observed.

4.3 GSH concentration

The amount of total GSH can be upregulated or depleted when exposed to agents inducing oxidative stress (Boelsterli and Boelsterli 2002). Normally, GSH will be upregulated in response to oxidative stress (Gómez-Oliván et al. 2014). However, if GSH is constantly conjugated to reactive electrophiles by agents inducing oxidative stress GSH will decrease (Boelsterli and Boelsterli 2002; Gómez-Oliván et al. 2014). In the carbamazepine exposure experiment in the present study, there was a significant increase in GSH levels when exposed to 50 ng/L carbamazepine (Figure 5 E). However, when investigating the males and females separately, no significant effects were found between the exposure conditions (Figure 8 A, B). In a study with the marine bivalve mollusc *R. philippinarum,* total GSH was found to increase when exposed to carbamazepine at all exposure concentrations, 30, 300, 3000, and 9000 ng/L, with the highest increase at 3000 ng/L exposure (Almeida et al. 2014). In the marine bivalve mollusc *Rudistapes decussatus*, a low significant increase in GSH was found after exposure to 300 and 3000 ng/L carbamazepine exposure (Almeida et al. 2014).

In the ibuprofen exposure experiment, there was no significant effects found on the GSH levels between the exposure conditions (Figure 5 F). In a study with the freshwater bivalve mollusc *Unio tumidus*, no significant effect on GSH was found either when exposed to 800 ng/L ibuprofen for 14 days (Martyniuk et al. 2022). In another study with

U. tumidus, a significant increase in GSH was found in the digestive gland after exposure to 250 ng/L ibuprofen for 14 days (Falfushynska et al. 2014).

In summary, an increase in total GSH was observed after carbamazepine exposure, which is in line with relevant literature data. No effects were observed on ibuprofen, while in literature both increase and decrease have been reported after ibuprofen exposure.

4.4 GST activity

In the carbamazepine exposure experiment, no significant effects on GST activity were found when exposed to carbamazepine (Figure 5 G, Figure 9 A, B). However, the GST activity generally had an increasing tendency in *G. oceanicus* when exposed to carbamazepine, where the males had an increasing tendency with exposure concentration and the females a tendency for higher GST activity only at 50 ng/L. In another study investigating GST activity after carbamazepine exposure in the marine amphipod *Ampelisca brevicornis*, a non-significant increasing tendency when exposed was found as well (Maranho et al. 2015). In the marine bivalve mollusc *R. philippinarum* exposed to carbamazepine for 96 hours, GST was found to decrease at 30, 3000, and 9000 ng/L (Almeida et al. 2014). In the marine bivalve mollusc *R. decussatus* an increase in GST was found at 30 and 3000 ng/L exposure concentration (Almeida et al. 2014). In another study, the marine and freshwater polychaeta *H. diversicolor* was exposed to carbamazepine for 14 days in sediment (Maranho et al. 2014). In that study, a significant increase in GST was found at 0.05 ng/g, 50 ng/g, and 500 ng/g exposure. However, not at 0.5 and 5 ng/g exposure (Maranho et al. 2014). In the bivalve mollusc, *R. philippinarum* exposed for 28 days to 1000 ng/L carbamazepine, there was found a decrease in GST when exposed to carbamazepine (Almeida et al. 2014).

In the ibuprofen exposure experiment, no significant effects were found in GST activity in the combined group of males and females when exposed to ibuprofen compared to control (Figure 5 H). However, males had a significantly lower GST activity at 10 ng/L exposure compared to 100 ng/L exposure, which was similar to control (Figure 9 C). In another study, GST activity increased in the marine amphipod *A. brevicornis* when exposed to 0.05, 0.5, and 50 ng/g ibuprofen in sediment for 10 days (Maranho et al. 2015). However, the increase was not found when exposed to 5 or 500 ng/g (Maranho et al. 2015). It can be challenging to compare sediment concentrations with water concentrations since pharmaceuticals can act differently depending on whether they are in the water or the sediment. GST activity increased at 1000 ng/L and 100 µL exposure to ibuprofen in a study with the freshwater zebra mussel (*D. polymorpha*) after 96 hours exposure (André and Gagné 2017). In the freshwater and brackish crustacean *Daphnia magma* exposed to ibuprofen for 48 hours, GST was found to increase at 500 ng/L (L. Wang et al. 2016). In a study with the marine and freshwater polychaetae *H. diversicolor* no significant effects were found on GST activity when exposed to ibuprofen for 14 days in sediment (Maranho et al. 2014). In a study on the freshwater zebra mussels (*D. polymorpha*) by Parolini et al. (2011) an increase in GST was found after ibuprofen exposure at 206 ng/L for 48 or 96 hours, 1856 ng/L for 24 hours and 7200 ng/L for 24-, 48-, 72- and 96-hours exposure (Parolini et al. 2011). In another study with *D. polymorpha,* a significant increase in GST was found at 1000 ng/L and 100 µg/L ibuprofen exposure (André and Gagné 2017). However, no significant effects were found when the GST levels were corrected for TBARS or after air stress (André and Gagné 2017). In a study with the freshwater bivalve mollusc *U. tumidus* from a clean site no significant effects, yet a decreasing tendency, on GST were found when exposed to 800 ng/L ibuprofen for 14 days (Martyniuk et al. 2022). However, a significant increase in

GST was found in the population from the contaminated site (Martyniuk et al. 2022). In another study with *U. tumidus* digestive glands, a significant increase was found in GST activity after exposure to 250 ng/L ibuprofen for 14 days (Falfushynska et al. 2014).

In summary, no significant effects on GST activity were observed after exposure to carbamazepine or ibuprofen compared to control. However, in males a significant difference was found between the 10 ng/L and 100 ng/L exposure groups after ibuprofen exposure. Literature shows inconsistent patterns of GST responses after carbamazepine exposure, including studies showing no effects like the present study. After ibuprofen exposure, most literature has reported an increase or no effect on GST activity which is in line with the present study.

4.5 GPx activity

In the carbamazepine exposure experiment, no significant effects were found on the GPx activity in *G. oceanicus* when exposed to carbamazepine (Figure 5 I). However, there was generally an increasing tendency with exposure concentration. Compared to another study investigating GPx activity in the marine amphipod *A. brevicornis* exposed to carbamazepine for 10 days through sediment, no effect or strong tendencies were found either (Maranho et al. 2015). In a study with the marine and freshwater polychaete *H. diversicolor*, an increase in GPx activity was found only at 0.05 ng/g exposure after 14 days sediment exposure, however not at higher concentrations (Maranho et al. 2014).

In the ibuprofen exposure experiment, no significant effects were found on GPx activity (Figure 5 J, Figure 10 C, D). There were no trends in GPx activity in any direction when investigating males and females together (Figure 5 J). However, when separating males and females an increasing tendency with exposure concentration up to 100 ng/L exposure was found for the males, while the females generally had a decreasing tendency in GPx activity with exposure concentration (Figure 10 C, D). In a study with the marine amphipod *A. brevicornis* exposed to ibuprofen in sediments for 10 days it was found an increase in GPx activity at 0.05 ng/g exposure concentration and the increase in activity had a decreasing tendency with exposure concentration, where the two highest concentrations, 50 and 500 ng/g, were very similar to control, which is a trend quite similar to the trend found in the females in the present study (Maranho et al. 2015). In another study with the freshwater amphipod *H. azteca* exposed to 0.17 mg/L ibuprofen for 72 hours, an increase in GPx activity was found when exposed compared to control (Gómez-Oliván et al. 2014). In a study where the marine and freshwater polychaete *H. diversicolor* was exposed to ibuprofen through sediment, no significant effects were found on GPx activity (Maranho et al. 2014). In a study on the freshwater zebra mussels (*D. polymorpha*) by Parolini et al. (2011), increase in GPx was found after ibuprofen exposure at 206 ng/L for 24, 48, 72 and 96 hours, 1856 ng/L for 72 hours and 7200 ng/L after 48- and 72-hours exposure (Parolini et al. 2011).

No effects were observed after carbamazepine or ibuprofen exposure in the present study. This is in line with literature where also no significant effects were found at similar carbamazepine exposure concentrations. Other ibuprofen exposure studies have reported an increase or no effects, as in the present study, on GPx activity.

4.6 LPO concentration

In the carbamazepine exposure experiment, no significant effects were found on MDA levels (Figure 5 K, Figure 11 A, B). However, there was a slight increasing tendency with exposure concentration (Figure 5 K). The males had an increasing tendency of MDA

levels at 50 ng/L, while the females had a decreasing tendency in MDA levels at 50 ng/L exposure (Figure 11 A, B). In the present study LPO was analysed by measuring the MDA levels. However, in other comparable studies LPO have been analysed by measuring either MDA or TBARS, which are unspecific for MDA. In a study with the marine amphipod *A. brevicornis* TBARS were significantly decreased after 50 ng/L sediment exposure for 10 days (Maranho et al. 2015). When the bivalve mollusc *R. philippinarum* was exposed to carbamazepine, MDA was found to increase at 9000 ng/L exposure. (Almeida et al. 2014). While when the other bivalve mollusc *R. deccusatus* was exposed to carbamazepine MDA was found to decrease at 9000 ng/L exposure (Almeida et al. 2014). In a study with the marine and freshwater polychaete *H. diversicolor* exposed to carbamazepine, a significant increase in TBARS was only found at 500 ng/g exposure after 14 days of exposure to carbamazepine through sediment (Maranho et al. 2014). However, at 5 ng/g exposure, a decreasing tendency was found (Maranho et al. 2014). In the bivalve mollusc *R. philippinarum* exposed for 28 days to 1000 ng/L carbamazepine, an increase in MDA was found (Almeida et al. 2018).

In the ibuprofen exposure experiment, no significant effects were found on MDA levels (Figure 5 L, Figure 11 C, D). However, the highest exposure concentration (1000 ng/L) had a tendency of higher MDA levels than the control and 10 ng/L exposure in the males (Figure 11 C). In a study with the marine amphipod *A. brevicornis*, a significant increase in TBARS was found at 0.05 ng/g exposure to ibuprofen through sediment for 10 days, a non-significant increase in TBARS was found at 0.5 ng/g exposure (Maranho et al. 2015). In a study where the freshwater amphipod *H. azteca* was exposed to 0.17 mg/L ibuprofen, also no significant effects were found on MDA levels (Gómez-Oliván et al. 2014). In the study by Gómez-Oliván et al. (2014) much higher concentrations were used for the exposure than in the present study, suggesting that the use of higher exposure concentrations might not necessarily lead to stronger effects on MDA. In the marine and freshwater polychaete *H. diversicolor* a significant increase in TBARS was only found at 5 ng/g exposure after exposure for 14 days through sediment, where no effects were found at higher or lower exposure concentrations (Maranho et al. 2014). In a study by André and Gagné (2017) in the zebra mussel *D. polymorpha*, a significant increase was found in TBARS after 1, 10 or 100 µg/L exposure to ibuprofen for 96 hours at 15°C (André and Gagné 2017).

In the present study, no effects were observed on MDA concentration after carbamazepine or ibuprofen exposure. In comparable studies varying effects on MDA or TBARS were observed after carbamazepine exposure, while ibuprofen exposure mostly showed an increase or no effect. However, several studies had no effects at several exposure concentrations, as in the present study.

4.7 Sex differences

In the present study, there were found some differences in trends of the biomarkers between males and females. In another study, females were generally found to have higher antioxidant enzyme levels, while males had higher MDA levels generally in the freshwater amphipod *Gammarus roeseli* (Sroda and Cossu-Leguille 2011). In the study by Sroda and Cossu-Leguille (2011), the sex determination was done more extensively than in the present study. In the present study the sex was not very well determined since it was done quickly, and sometimes determined mostly from the size. This could have influenced the results of the sex differences in this study, and the differences found between the sexes could therefore be more from weight or size than from sex. There were also some differences in the number of males and females analysed in the

ibuprofen exposure since it was hard to find two females and two males from all the exposure tanks (Table A11, Table A12). This could have influenced the results and increased the influence of individual variation since the sample size was quite low for males and females separately. In the present study, there were in addition some uncertainties if all the individuals were the species *G. oceanicus* when there were some suspicions that some of the individuals could be *G. setosus*. In the study by Sroda and Cossu-Leguille (2011), the MDA and lipids were found to increase in *G. roeseli* in the summer season when the temperature was higher, they also generally had lower lipids levels in summer than in winter. The GPx levels were generally higher in the females than the males in the summer, while the levels in the females were more similar to the levels in males the rest of the year (Sroda and Cossu-Leguille 2011). The GPx levels in the males were quite stable throughout the year (Sroda and Cossu-Leguille 2011). In the same study, CAT was found to be high in males in June, while for females CAT was highest in November (Sroda and Cossu-Leguille 2011). This could explain some of the sex differences in the biomarkers found in the present study since it shows that there can be fluctuations in the levels of the analysed biomarkers throughout the year, which can vary between males and females.

4.8 Correlations

In both the carbamazepine and ibuprofen exposure experiments the CAT, SOD and GSH activity were strongly correlated (Table 1, Table 2). And in both exposure experiments, GST was well correlated with GSH and SOD. GST was however only significantly correlated with CAT in the ibuprofen exposure experiment. It makes sense that CAT and SOD are well correlated since they are subsequent reactions when CAT catabolises hydrogen peroxide, which SOD produces when it reacts with ROS. The same accounts for the correlation between SOD and GST, when GST is involved in the catabolism of hydrogen peroxide. The correlation between GST and GSH also makes sense since GSH is a co-substrate for the GST reaction. The positive correlation with weight found for CAT and GST in the ibuprofen exposure experiment could indicate that bigger individuals generally had higher CAT and GST activity, which could explain some of the differences between the males and females when the males generally were bigger than the females. The correlation coefficients between weight and protein levels in the carbamazepine and ibuprofen exposure experiment were very similar (Figure A1 Appendix A5). Both of them were negative meaning that heavier individuals generally had lower protein levels. A reason for this could be water, since the individuals were measured in ww, making the individuals heavier and water in the organisms could dilute the protein levels measured in the homogenate.

4.9 Differences between carbamazepine and ibuprofen exposure

Despite carbamazepine and ibuprofen did not have many significant effects on the biomarkers analysed in *G. oceanicus*, carbamazepine generally seemed to affect *G. oceanicus* more than ibuprofen (Figure 5). However, in the carbamazepine experiment, 15 individuals were put in the same tank, compared to 12 in the ibuprofen exposure. So, in the carbamazepine exposure experiment, there could be additional stress due to more individuals being in the tank. However, the effect should have been evened out within the same exposure experiment, since the effect of the number of individuals should have been similar between all the tanks in the same exposure experiment. However, it could have led to differences between the carbamazepine and ibuprofen exposure experiments. The molecular structures of carbamazepine and ibuprofen are different, where

carbamazepine has a tricyclic structure with a nitrogen atom connected to an amide group and ibuprofen consists of a benzene ring with two substituents where one of them has a carboxyl group (PubChem a; PubChem b). Since the two pharmaceuticals are quite different in structure it is not expected that they would give the same response in organisms, however the control in the two exposure experiments should still be similar.

The differences between the control of the carbamazepine exposure experiment and ibuprofen exposure experiment for GSH and MDA (Figure 5) could be due to the differences in the experimental setup with more individuals in each tank in the carbamazepine exposure experiment. More individuals could have led to additional stress in the carbamazepine exposure experiment, which could explain the higher MDA levels in that experiment compared to the ibuprofen exposure experiment. The difference could also be due to the different exposure times between the exposure experiments when the individuals in the ibuprofen exposure had an exposure period of 24 hours more than the carbamazepine exposure. However, the difference could also be due to individual variation since only 12 individuals were analysed per exposure concentration and it was found quite high variation between the exposure tanks in the same exposure group for several of the biomarkers. The difference in the GPx activity between the individuals in the carbamazepine exposure experiment and ibuprofen exposure experiment was likely due to different methods being used when analysing the GPx activity from the two experiments. One of the methods might have been more affected by the components used in the homogenizing buffer, like DTT, EDTA and Trinton-X, which could affect enzyme levels, than the other method in addition to GPx being measured in different ways. It would have been better to do a separate homogenisation for each biomarker response, however then it would not be possible to analyse all the biomarkers per individual. When measuring GPx in the carbamazepine exposure experiment a standard curve was used, making the measurements more precise compared to the ibuprofen exposure experiment measurements of GPx.

4.10 Methods and mortality

The biomarker responses found could differ a lot depending on which methods that were used, making it hard to compare studies, especially when there also can be high intra and inter species variation in the measured responses. For the present study, it would have been better to use more individuals for the biomarker response analyses to increase the statistical power and decrease the influence of individual variation to obtain more trustworthy results. In the present study only four individuals were analysed from each exposure tank, which is a very small sample size. The method was not optimal for the GPx measurements for ibuprofen exposure when the measured values varied a lot, and some of the samples had lower values than the blank. In addition, different volumes of sample supernatant were used for some of the measurements since the lower sample volumes used in the beginning did not give a satisfying response. This was corrected for in the calculations, however it could still influence the measured values. The GPx measurements for ibuprofen seemed to be affected by the time of measurement when the measured levels decreased after the order they were measured, which could have affected the results significantly depending on what samples were measured first. DTT and EDTA used in the homogenising buffer could depress GPx activity measured, Triton-X in the buffer could also have an effect (Sigma-Aldrich). Therefore, it is uncertain how trustworthy the GPx results for the ibuprofen samples are. Protein levels could also be affected by the Tris and EDTA in the homogenisation buffer used since ethanol and Tris can interfere and increase the protein content by 20% (Lucarini and Kilikian 1999).

However, the Lowry method can quite well determine the increase of purity from fractionation (Lucarini and Kilikian 1999). EDTA and Tris present in the homogenizing buffer could have interfered with the protein levels. However, the buffer used for solubilization should usually not interfere much so the results should still be reliable (Lucarini and Kilikian 1999). In the present study MDA were not measured in the cytosolic fraction, which could make the MDA results less reliable since it was calculated per mg protein, which were measured in the cytosolic fraction. The measurements for CAT, GST and GPx (ibuprofen exposure experiment) were not super accurate when standard curves are not used, and for the SOD a standard curve from an earlier analysis was used (Sigma-Aldrich). Nevertheless, the major part of the study is a comparison between treatments, so it would not have that much impact since the same procedure where performed each time.

In this study the nominal concentrations were used, as the water sample analysis was not performed in time. Therefore, it is not known what the real concentrations were in the water during the exposure and how they changed during the exposure time. So, it could be that the pharmaceutical concentration decreased significantly during the experiment due to evaporation or metabolism by the individuals, which could have affected the results and led to different results depending on the time of measurement during the exposure and could have led to different results between the exposure tanks. The actual concentrations of the pharmaceuticals could be quite different from what was added in the beginning, meaning that the effects found in this study could be effects from lower concentrations than the concentrations added. In a study by Contardo-Jara et al., the measured water concentrations of the pharmaceuticals were 20.6% of the nominal concentration for ibuprofen and 23.6% of the nominal concentration for carbamazepine at the end of the exposure (Contardo-Jara et al. 2011). This suggests that the levels could have decreased a lot during the exposure period. However, carbamazepine is found to be quite persistent (Andreozzi et al. 2003; Lam et al. 2004; Calisto et al. 2011), so it is reasonable to assume that it was still carbamazepine left in the exposure tanks at the end of the exposure period. Ibuprofen is not as resistant, however still found to be quite persistent in freshwater (Araujo et al. 2014).

The concentration of the pharmaceuticals or metabolites were not measured in the organisms, so it was not known how much of the pharmaceuticals were taken up. It was also not known if carbamazepine or ibuprofen were present in the individuals from control as well, which could indicate previous exposure before the exposure experiment. If they had been exposed to the pharmaceuticals in the environment earlier, they could have developed adaption to the exposure, which could explain some of the nonsignificant results found in the present study. Especially since the animals were collected quite close to the sewage outlet and the harbour where many cruise vessels and boats had been. The studied pharmaceuticals have been detected in plankton and invertebrates in Kongsfjorden in earlier studies (Sørensen et al. 2023; Sokołowski et al. 2024). In addition, ibuprofen and other PPCPs and contaminants have been detected in sewage and sediments in Kongsfjorden (Choi et al. 2020; Rauseo et al. 2024). Therefore, it is quite likely that they had already been exposed to the pharmaceuticals and other compounds that may induce oxidative stress before the exposure experiments started. However, it is hard to know for sure when the pharmaceuticals were not measured in the animals before the experiment started.

In the study by Yildirim et al. (2022) on *G. pulex* it was observed an LC50 of 8300 ng/L for ibuprofen (Yildirim et al. 2022). This indicates that the concentrations used in this

study were well under lethal doses. However, in this study, one individual died during the exposure period. Since it was only one individual it is reasonable to assume the exposure concentrations used were sublethal for the timespan of the exposure. Cannibalism was found in another exposure experiment with *G. oceanicus* where they were exposed to cyanobacteria bloom extract and benzo(a)pyrene where 9.2% of the individuals probably were victims of cannibalism, not affected by the treatment (Turja et al. 2014). Cannibalism within the Gammarus family has also been found in various other studies (Macneil et al. 1997). So, it is likely that the cause of death was cannibalism. However, it might be that it was also weak and sick. Generally, no significant effects were found, which indicates that the concentrations used were mostly under NOEC. However, for chemicals like pharmaceuticals, the effects could be more significant at lower exposure concentrations making it hard to determine what exposures that are causing a significant effect without testing them.

4.11 Environmental relevancy and temperature

In Longyearbyen, Svalbard, carbamazepine has been found in concentrations up to 400 ng/L in effluent and 1 ng/L in seawater in (Kallenborn et al. 2009). Carbamazepine has additionally been detected in invertebrates in Isfjorden Svalbard (Sokołowski et al. 2024). Carbamazepine has been detected in concentrations up to 47.7 ng/L in seawater, Hudson Bay in Manitoba, Canada (Kallenborn et al. 2018). In a study by Brumovský et al. (2022) investigating various CECs in a Europe-Arctic transect offshore carbamazepine had a 100% detection frequency in the Arctic Ocean and found in concentrations 0.02-0.16 ng/L, while ibuprofen had a 5% detection frequency and found in concentrations <0.15-0.26 ng/L in the Arctic Ocean (Brumovský et al. 2022). Ibuprofen has usually been detected in higher concentrations in the environment close to human settlements compared to carbamazepine, while carbamazepine is more long-range transported than ibuprofen, which might be due to higher consumption and release of ibuprofen than carbamazepine to the environment and carbamazepine seemingly being more persistent in the environment (Andreozzi et al. 2003; Lam et al. 2004; Calisto et al. 2011; Hai et al. 2018). Compared to the concentrations found in the Arctic environment there does not seem to be an immediate risk for *G. oceanicus*, when it comes to oxidative stress, since few significant effects were found. However, the environment for the exposure experiments was quite different from the environment in nature. There could be additional stress from the handling and being kept in a compartment of two Litres with 11 or 12 other individuals that could have concealed the effects of the exposure if the individuals in the control and the exposure concentrations were stressed from the handling, and the additional exposure did not cause additional stress.

Most comparable exposure experiments had been conducted using temperatures of about 15-20°C, which is not relevant for the Arctic environment where the climate is much colder. So, the effects could differ with the temperature for the exposure, which could affect the behaviour of the chemicals, uptake in the animals and the metabolism. It could also be that higher environmental temperature could increase the effects of oxidative stress. However, in a study by Rastrick and Whiteley (2017), the cost of protein synthesis was similar for subarctic and temperate climates for *G. oceanicus* (Rastrick and Whiteley 2017), which could imply that the enzyme activity and metabolism could be quite similar independent of the environmental temperature as long as the organism is adapted to the environment they are kept in. However, more studies are needed to verify this.

Many comparable studies also used longer exposure periods and higher exposure concentrations than those used in the present study. So, it could be that more significant

effects have been found if higher concentrations were used. However, the use of higher exposure concentrations would also decrease the environmental relevancy when the concentrations in nature usually are not above the exposure concentrations used in the present study (Weigel et al. 2004; Brumovský et al. 2022; Rauseo et al. 2024). There were also found very different effects between species (4.1-4.6), even species from the same family (Almeida et al. 2014). This suggests that the species differences of exposure to these two pharmaceuticals could be quite high, at least on the biomarkers in the present study,

The intertidal zone in the Arctic is subject to high fluctuations in water levels, ice cover, temperature, salinity and gases (Węsławski et al. 2018). Gammarus are species known to be quite tolerant towards temperature and salinity fluctuations and are found at salinities ranging from 0 to 35 and temperatures from -1.5 to 25 °C (Węsławski et al. 2018). An adaption to these fluctuations is to hide under rocks during low tides for protection (Węsławski et al. 2018). This suggests that *G. oceanicus* is a quite tolerant species, which could explain the lack of significant effects found in the present study.

4.12 Limitations and influencing factors

Probably the biggest limitation of the present study is that ibuprofen or carbamazepine were not measured in the individuals such that the uptake of the pharmaceuticals in the individuals is unknown, which could highly influence the effects found on the investigated biomarkers. Also, the concentration of the pharmaceuticals in the water used for the exposure has not been analysed yet so what the concentrations of the pharmaceuticals were at the beginning and end of the exposure are also still unknown. Another limitation is the low sample size analysed, which lowered the statistical power and could have led to a higher influence of individual variance. The number of individuals for the analysis are dependent on the constrains of cost and time, which makes it challenging to obtain an optimal number of samples for the statistics. In the environment, the mixture of contaminants and other substances is much more complex. Carbamazepine and ibuprofen could interact with other contaminants, which could lead to different outcomes. Especially carbamazepine is known to interact with other drugs (Pippenger 1987). Mixture effects were not the focus of this study however it should be investigated in future studies. Another limitation of the present study is not investigating the effects of metabolites of carbamazepine and ibuprofen, which may be released in higher quantities with wastewater than the parent compound.

During the carbamazepine exposure, some iron oxide was formed from the clips used for the aeration in some of the exposure tanks due to some contact with the water, which could have contaminated the water and led to an effect on the individuals or the chemicals. Since seawater from the fjord was used it is likely that some contaminants were present in the water used for the control as well (Choi et al. 2020). The feed could also have affected the results since the intake of pasta could differ between individuals. It is unknown how pasta could affect Gammarus since that is not what they normally eat. Starvation has been found to decrease enzyme levels and metabolism. Sex differences could affect enzyme levels and metabolism, which could have affected the results if some individuals did not eat the pasta. Smaller individuals are likely to consume more pharmaceuticals per gram body weight than bigger individuals since they can take up about the same amount independent of size and have a smaller body to distribute it increasing the concentration. Size could therefore be a bias for the uptake of pharmaceuticals. In the exposure tank where one individual had died, the other

individuals could have been affected by the fact that they consumed most of the dead individual.

4.13 Wastewater treatment

Better wastewater treatment could decrease the release of pharmaceuticals, like carbamazepine and ibuprofen. However, most conventional wastewater treatment facilities existing today are not very efficient in removing PPCPs, especially carbamazepine, and different treatment processes are efficient for different PPCPs (Wang and Wang 2016). Furthermore, some wastewater treatment processes can generate harmful byproducts. For instance, chlorination and ozonation could lead to more reactive and harmful products, like radicals, or more persistent products, like chlorinated derivatives (Quero-Pastor et al. 2014; Wang and Wang 2016; Pohl et al. 2020). In a study on zebrafish (*Danio rerio*), some of the byproducts of wastewater treatment were more toxic than the parent compound (Pohl et al. 2020). However, ozonation has also been found to be quite efficient in removing many PPCPs and has been found to have a removal efficiency of 99% for ibuprofen and 100% for carbamazepine at 25°C in ultrapure water or drinking water (Wang and Wang 2016). However, in another study, a removal rate of 82% was found for carbamazepine (Pohl et al. 2020). The removal rate can vary dependent on how the measurements were done and on what other compounds and organic matter that were present in the water as well (Y. Wang et al. 2016; Pohl et al. 2020). Ozonated carbamazepine has been found to have embryotoxic effects in zebrafish larvae (Pohl et al. 2020). Ozonation has also been found to have cytotoxic effects in cells, greater than chlorination (Han et al. 2018). Chlorination or chlor-amination of carbamazepine has also been found to lead to DNA damage in cells (Han et al. 2018). Ozonation of ibuprofen can lead to more toxic byproducts found to be toxic in algae and daphnia (Quero-Pastor et al. 2014; Merkus et al. 2022). In addition, these removal processes could act differently in the cold Arctic environment, which could make the removal by ozonation or chlorination less efficient or lead to more or less toxic byproducts compared to warmer environments. The wastewater might also need to be warmed up for efficient removal, which could lead to more use of energy and higher costs for the operation and maintenance of the WWTP.

Few carbamazepine and ibuprofen exposure studies have been performed with aquatic invertebrates for species in the Arctic, which also are exposed to these pharmaceuticals in the environment (Sokołowski et al. 2024). Even though not everything was optimally performed in the present study, and the sample size was quite low, the results can still be used since the results were comparable to results found in previous studies. The study can be repeated to confirm the results observed in the present study and increase the understanding of oxidative stress effects on *G. oceanicus*.

Conclusion

Few significant effects were observed in *G. oceanicus* after exposure to carbamazepine or ibuprofen. This could imply that *G. oceanicus* is a quite tolerant and robust species. There was however a significant increase in GSH concentrations at 50 ng/L exposure to carbamazepine compared to control. It was also investigated if the males and females were affected differently. There was a significant increase in SOD activity in the males after exposure to 10 ng/L ibuprofen compared to control. Different trends in the levels of the biomarkers, especially in GPx activity after ibuprofen exposure and in MDA levels after carbamazepine exposure, were found between males and females. The individual variation in the present study was quite high, which could be due to the low sample size, different feeding activities and other stressors and confounding effects that could have been affecting the results of the present study.

To obtain more certain results more individuals should be analysed to improve the statistical power and limit the individual variance. The environment they were kept in the present study is not fully what they are used to, which could cause more stress. However, during the acclimatisation stones which they could hide under were put in the tank. Nevertheless, it would have been more environmentally relevant to conduct a study in an environment more similar to their natural environment. Since few effects were observed in the present study, future studies could aim to focus on other parameters or species that could be affected by carbamazepine or ibuprofen release in the Arctic. In addition, different life-stages and species could be more sensitive to carbamazepine and ibuprofen exposure than *G. oceanicus*.

There is a lack of exposure studies in invertebrates focusing on the Arctic and using exposure setup relevant to the Arctic environment. This is causing a gap of knowledge on how pharmaceuticals and other contaminants act in colder environments and how they affect organisms living in the Arctic. This study focuses on improving the knowledge of the effects of pharmaceuticals on Arctic invertebrates. However, many more studies are needed to increase the understanding of the risk the release of untreated wastewater and pharmaceuticals can have on the Arctic environment and the organisms living there.

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Appendices

A1: Levels of biomarkers

Table A1: Mean levels ± standard deviation of CAT, SOD, GSH, GST, GPx and MDA levels per mg protein and the protein concentrations per exposure tank from the carbamazepine-exposed *G. oceanicus*.

Exposure (ng/L)	Tank	CAT (U/mg)	SOD (U/mg)	GSH (nmol/mg)	GST (nmol/ min/mg)	GPx (nmol/min /mg)	MDA (nmol/ mg)	Protein (mg/mL)
0	1	2.44 ± 0.99	0.13 ± 0.03	0.61 ± 0.15	43.56 ± 7.44	33.47 ± 4.73	2.86 ± 0.83	8.86± 3.15
0	2	3.64 ± 0.56	0.16 ± 0.02	0.89 ± 0.47	57.37 ± 13.83	37.18 ± 7.52	3.61 ± 1.19	7.37± 1.19
0	3	2.71 ± 0.33	0.16 ± 0.06	1.11 ± 0.90	69.03 ± 20.89	37.25 ± 11.21	3.84 ± 1.75	7.76± 2.49
50	4	3.99 ± 1.23	0.15 ± 0.01	1.43 ± 0.93	52.56 ± 3.68	48.25 ± 20.74	3.00 ± 0.54	5.80± 2.33
50	5	3.71 ± 0.67	0.16 ± 0.02	1.30 ± 0.31	74.60 ± 16.24	30.95 ± 12.04	3.32 ± 0.36	7.42± 1.20
50	6	3.17 ± 0.97	0.16 ± 0.04	1.18 ± 0.38	75.92 ± 26.09	38.19 ± 8.50	3.96 ± 2.16	7.97± 1.83
1000	7	3.55 ± 0.73	0.14 ± 0.02	0.77 ± 0.30	66.36 ± 13.77	37.44 ± 3.09	2.71 ± 0.19	7.10± 0.72
1000	8	3.41 ± 1.06	0.16 ± 0.05	1.48 ± 0.84	80.75 ± 10.99	49.78 ± 14.35	3.41 ± 1.47	7.39± 2.08
1000	9	3.71 ± 1.08	0.14 ± 0.04	0.81 ± 0.20	52.61 ± 16.93	41.31 ± 15.16	5.35 ± 2.59	7.80± 2.50

Table A2: Mean levels of CAT activity, SOD inhibition, GSH levels, GST activity, GPx activity and MDA levels each exposure concentration from carbamazepine-exposed *G. oceanicus*.

Exposure (ng/L)	CAT (U/mg)	SOD (U/mg)	GSH (nmol/mg)	GST (nmol/ min/mg)	GPx (nmol/ min/mg)	MDA (nmol/ mg)	Protein (mg/mL)
0	2.93± 0.82	0.15±0.04	0.87±0.58	56.65± 17.45	35.97± 7.69	3.44± 1.27	8.00±2.28
50	3.62± 0.95	0.15±0.02	1.31±0.56	67.70± 19.66	39.13± 15.22	3.43± 1.25	7.06±1.93
1000	3.56± 0.89	0.14±0.04	1.02±0.59	66.57± 17.52	42.84± 12.27	3.82± 1.95	7.43±1.76

Table A3: Mean levels ± standard deviation of CAT, SOD, GSH, GST, GPx and MDA levels per mg protein and the protein concentration per exposure tank from ibuprofen-exposed *G. oceanicus*.

Exposure (ng/L)	Tank	CAT (U/mg)	SOD (U/mg)	GSH (nmol /mg)	GST (nmol/ min/mg)	GPx (nmol/min /mg)	MDA (nmol/ mg)	Protein (mg/mL)
0	1	3.38 ± 1.51	0.15 ± 0.03	1.49 ± 0.66	61.60 ± 15.36	6.51 ± 17.68	2.69 ± 0.67	8.69± 1.95
0	2	3.47 ± 0.57	0.15 ± 0.02	1.50 ± 0.98	59.66 ± 8.75	11.45 ± 5.22	2.18 ± 0.39	8.01± 0.68
0	3	3.08 ± 1.01	0.14 ± 0.04	1.59 ± 1.63	56.59 ± 10.05	1.67 ± 35.50	2.39 ± 0.26	8.59± 2.19
10	4	2.86 ± 1.13	0.12 ± 0.05	1.06 ± 0.57	55.91 ± 10.91	14.72 ± 5.47	2.45 ± 0.96	8.11± 0.89
10	5	3.48 ± 1.36	0.17 ± 0.04	2.20 ± 1.66	57.84 ± 10.35	-3.74 ± 25.65	2.31 ± 0.53	8.04± 2.05
10	6	2.62 ± 1.07	0.17 ± 0.05	1.10 ± 0.54	53.63 ± 12.89	11.52 ± 11.04	2.53 ± 0.73	9.62± 2.90
100	7	3.75 ± 0.47	0.15 ± 0.04	1.29 ± 0.80	66.15 ± 14.43	3.77 ± 28.62	2.26 ± 0.55	8.22± 1.91
100	8	3.74 ± 0.71	0.17 ± 0.05	1.60 ± 0.42	69.90 ± 13.26	11.35 ± 14.11	2.21 ± 0.38	7.81± 2.05
100	9	3.51 ± 1.16	0.16 ± 0.04	1.12 ± 0.46	57.44 ± 10.12	10.39 ± 13.06	3.23 ± 1.38	8.10± 2.00
1000	10	3.15 ± 0.73	0.18 ± 0.02	1.97 ± 0.89	72.96 ± 3.34	6.31 ± 21.44	2.82 ± 0.28	7.10± 0.33
1000	11	3.00 ± 1.06	0.15 ± 0.03	1.23 ± 0.54	53.77 ± 6.51	-3.57 ± 20.35	2.71 ± 0.66	8.89± 2.00
1000	12	4.20 ± 2.10	0.19 ± 0.06	1.73 ± 0.99	67.74 ± 20.77	21.22 ± 5.60	2.66 ± 0.93	7.43± 3.03

Table A4: Mean levels of CAT activity, SOD inhibition, GSH levels, GST activity, GPx activity and MDA levels in each exposure concentration from ibuprofen-exposed *G. oceanicus*.

Exposure (ng/L)	CAT (U/mg)	SOD (U/mg)	GSH (nmol /mg)	GST (nmol/ min/mg)	GPx (nmol/ min/mg)	MDA (nmol/ mg)	Protein (mg/mL)
0	3.31± 1.01	0.15± 0.03	1.53± 1.05	59.28± 10.84	6.54± 21.30	2.42± 0.48	8.43± 1.60
10	2.99± 1.15	0.15± 0.05	1.45± 1.11	55.80± 10.50	7.50± 17.07	2.43± 0.69	8.59± 2.06
100	3.67± 0.76	0.16± 0.04	1.34± 0.57	64.50± 12.74	8.51± 18.35	2.57± 0.94	8.04± 1.81
1000	3.45± 1.40	0.17± 0.04	1.64± 0.82	64.82± 14.28	7.99± 18.98	2.73± 0.62	7.81± 2.07

A2: Coefficient of variation

Table A5: Coefficient of variation between individuals in each exposure tank in the carbamazepine exposure experiment.

Exposure	Tank	CAT	SOD	GSH	GST	GPx	MDA
0	1	0.40	0.20	0.25	0.17	0.14	0.29
0	2	0.15	0.11	0.53	0.24	0.20	0.33
0	3	0.12	0.36	0.81	0.30	0.30	0.46
50	4	0.31	0.05	0.65	0.07	0.43	0.18
50	5	0.18	0.11	0.24	0.22	0.39	0.11
50	6	0.30	0.25	0.32	0.34	0.22	0.54
1000	7	0.21	0.14	0.39	0.21	0.08	0.07
1000	8	0.31	0.34	0.57	0.14	0.29	0.43
1000	9	0.29	0.30	0.24	0.32	0.37	0.48

Table A6: Coefficient of variation between individuals in each exposure condition in the carbamazepine exposure experiment.

Exposure	CAT	SOD	GSH	GST	GPx	MDA
0	0.28	0.24	0.66	0.31	0.21	0.37
50	0.26	0.15	0.43	0.29	0.39	0.36
1000	0.25	0.26	0.57	0.26	0.29	0.51

Table A7: Coefficient of variation between individuals in each exposure tank in the ibuprofen exposure experiment.

Exposure	Tank	CAT	SOD	GSH	GST	GPx	MDA
0	1	0.45	0.19	0.44	0.25	2.72	0.25
0	2	0.17	0.16	0.66	0.15	0.46	0.18
0	3	0.33	0.28	1.03	0.18	21.23	0.11
10	4	0.39	0.42	0.54	0.20	0.37	0.39
10	5	0.39	0.25	0.75	0.18	-6.86	0.23
10	6	0.41	0.28	0.49	0.24	0.96	0.29
100	7	0.12	0.28	0.62	0.22	7.59	0.24
100	8	0.19	0.29	0.27	0.19	1.24	0.17
100	9	0.33	0.22	0.41	0.18	1.26	0.43
1000	10	0.23	0.10	0.45	0.05	3.40	0.10
1000	11	0.35	0.17	0.44	0.12	-5.70	0.25
1000	12	0.50	0.32	0.57	0.31	0.26	0.35

Table A8: Coefficient of variation between individuals in each exposure condition in the ibuprofen exposure experiment.

Exposure	CAT	SOD	GSH	GST	GPx	MDA
0	0.31	0.20	0.69	0.18	3.26	0.20
10	0.38	0.32	0.76	0.19	2.28	0.29
100	0.21	0.25	0.43	0.20	2.16	0.37
1000	0.41	0.23	0.50	0.22	2.38	0.23

A3: Sample overview and raw data of each individual

Table A9 and Table A10 show the exposure condition, weight, sex and biomarker activity for each of the individuals used for the carbamazepine (Table A9) and ibuprofen exposure experiments (Table A10) respectively. For the sex, M is male and F is female. Samples not analysed for a biomarker are marked with *NA*.

Table A9: Overview samples carbamazepine exposure *G. oceanicus* with the levels of the measured biomarkers for each individual.

Sample	Exposure (ng/L)	Weight (mg ww)	Sex	CAT (U/mL)	SOD (U/mL)	GSH (nmol /mL)	GST (nmol/mL /min)	GPx (nmol/mL /min)	MDA (nmol /mL)
1	0	379	M	2.47	0.12	0.49	34.28	31.17	2.76
2	0	549	M	3.76	0.16	0.51	50.11	38.87	1.85
3	0	263	F	3.02	0.14	0.59	44.72	30.72	2.51
4	50	408	M	2.76	0.14	0.60	50.01	40.36	3.04
5	0	344	M	3.64	0.15	0.63	48.06	40.73	4.23
6	50	232	F	5.60	0.16	2.70	50.27	78.47	2.32
7	50	284	F	4.19	0.15	0.90	57.91	42.85	3.02
8	1000	453	M	3.53	0.13	0.36	53.80	40.00	2.87
9	1000	311	M	4.33	0.12	0.95	35.46	35.48	2.73
10	1000	214	F	3.95	0.20	0.54	59.08	52.90	7.80
11	50	236	F	3.00	0.20	1.33	111.66	47.71	2.72
12	50	320	M	2.53	0.11	0.82	55.10	28.34	7.10
13	1000	390	M	3.85	0.17	1.17	78.60	38.63	2.93
14	1000	207	F	2.53	0.16	1.05	81.89	33.06	2.43
15	50	216	F	2.58	0.15	0.93	78.95	42.15	2.40
16	50	171	F	2.80	*NA*	1.49	80.31	17.04	3.54
17	50	366	M	3.64	0.18	0.85	93.52	45.46	3.36
18	50	307	M	4.09	0.16	1.51	69.28	34.56	3.56
19	0	286	M	3.21	0.23	2.29	95.82	53.96	3.93
20	0	168	F	2.47	0.13	1.35	75.43	33.44	6.09
21	0	154	F	2.11	0.11	0.82	49.00	28.19	2.98
22	1000	242	F	4.63	0.22	0.98	76.66	69.43	5.43
23	1000	266	F	4.01	0.13	0.76	55.77	39.14	2.79
24	0	382	M	3.53	0.17	0.75	61.79	46.08	4.98
25	1000	365	M	4.44	0.15	0.95	73.23	54.32	7.33
26	1000	211	F	2.13	0.10	0.80	42.68	22.53	3.55
27	1000	303	M	4.15	0.13	0.93	73.98	37.56	2.76
28	0	226	F	1.41	0.11	0.62	40.83	35.66	3.87
29	1000	279	M	3.00	0.15	2.74	96.56	51.50	1.94
30	1000	163	F	2.17	0.09	1.04	71.19	39.57	3.34
31	50	270	M	3.39	0.14	1.54	52.06	31.32	3.63
32	50	300	F	4.30	0.14	1.36	55.30	26.72	2.79
33	0	221	F	4.37	0.17	1.59	74.92	31.18	2.71
34	50	392	M	4.59	0.17	1.65	57.98	34.55	3.64
35	0	367	M	2.60	0.12	0.45	52.99	30.91	1.83
36	0	238	F	2.58	*NA*	0.37	51.90	30.70	3.50

Table A10: Overview samples of ibuprofen exposure *G. oceanicus* with the levels of the measured biomarkers for each individual.

Sample Id	Exposure (ng/L)	Weight (mg ww)	Sex	CAT (U/ mL)	SOD (U/ mL)	GSH (nmol /mL)	GST (nmol/mL /min)	GPx (nmol/mL /min)	MDA (nmol /mL)
37	0	145	F	2.16	0.12	0.98	48.69	11.29	3.50
38	1000	371	M	4.32	0.16	1.02	56.61	-18.00	3.56
39	100	132	F	2.11	0.13	1.36	51.26	-6.52	5.12
40	10	260	F	4.11	0.17	1.91	70.50	-14.45	3.08
41	10	152	F	2.52	0.12	1.90	51.02	0.15	3.62
42	1000	296	F	3.35	0.20	1.34	77.62	2.70	3.22
43	1000	424	M	4.34	0.20	2.11	80.18	28.64	2.35
44	0	266	M	4.38	0.18	3.21	70.78	-49.87	2.09
45	1000	171	F	1.79	0.12	2.95	51.49	-23.80	2.90
46	100	281	M	4.27	0.21	1.62	64.05	-35.87	1.94
47	10	343	M	4.71	0.21	2.54	61.84	-34.98	1.86
48	1000	297	M	3.58	0.18	0.98	70.25	-22.52	2.76
49	100	223	F	2.86	0.11	1.88	58.72	-8.78	2.35
50	0	426	M	5.16	0.18	1.74	76.96	-17.44	2.96
51	0	291	F	3.82	0.16	2.76	68.66	4.63	2.55
52	100	308	M	4.94	0.18	1.08	72.57	25.34	2.69
53	10	273	M	3.54	0.19	1.50	47.70	18.46	2.18
54	1000	211	F	3.19	0.17	2.25	61.03	10.28	2.08
55	100	207	F	3.47	0.22	1.63	58.16	13.61	1.65
56	0	223	F	3.34	0.18	1.04	47.73	10.88	1.69
57	10	281	M	4.28	0.19	1.27	52.48	12.15	3.75
58	100	162	F	3.40	0.20	1.55	53.24	11.34	3.23
59	10	203	M	3.71	0.21	1.21	68.64	10.47	2.20
60	100	206	F	3.57	0.15	1.74	52.68	5.98	2.99
61	1000	267	M	6.96	0.27	1.37	89.00	15.04	4.01
62	0	329	M	4.12	0.18	1.25	72.68	7.20	2.03
63	0	314	M	2.92	0.16	0.96	54.81	31.46	2.51
64	1000	315	M	2.68	0.14	0.68	45.95	17.23	2.29
65	10	265	F	3.06	0.17	1.05	57.16	26.63	2.24
66	0	144	F	2.09	0.14	1.49	48.08	24.98	2.29
67	1000	184	F	1.91	0.13	1.50	43.16	20.32	1.90
68	1000	197	F	3.59	0.17	1.03	58.62	20.89	2.38
69	100	278	M	4.23	0.19	1.16	82.41	24.00	2.49
70	1000	293	M	2.07	0.16	3.58	70.84	20.39	2.59
71	0	277	M	4.01	0.16	1.38	60.16	17.11	2.41
72	1000	351	M	3.59	0.17	1.40	73.13	24.65	2.70
73	10	492	M	1.93	0.12	1.62	53.66	13.12	2.69
74	10	200	F	1.57	0.11	0.74	51.33	16.01	2.14
75	10	255	F	3.01	0.12	0.80	45.71	10.34	1.43
76	100	382	M	3.61	0.13	0.57	52.69	11.41	1.88
77	10	186	M	1.19	*NA*	0.61	37.70	8.83	2.06
78	100	276	F	3.20	0.11	0.30	61.33	12.99	1.75
79	0	276	M	2.72	0.12	0.59	62.11	13.17	2.06
80	0	242	F	3.06	0.10	0.37	47.11	11.97	2.27
81	10	254	F	2.62	0.10	0.81	54.12	13.72	2.32
82	100	316	M	4.38	0.14	2.23	80.31	16.57	2.35
83	10	172	F	1.54	0.07	0.79	71.33	22.65	2.29
84	100	324	M	3.98	0.13	0.73	86.53	31.98	2.34

Table A11 and Table A12 show the number of males (M) and females (F) analysed for biomarker responses in each of the exposure tanks per exposure condition.

Table A11: Number of individuals of each sex analysed for all the biomarkers in the ibuprofen exposure experiment for each of the exposure concentrations.

Exposure	Tank	Sex	n	Sex	n	N total
0	1	M	2	F	2	4
0	2	M	2	F	2	4
0	3	M	3	F	1	4
10	4	M	1	F	3	4
10	5	M	2	F	2	4
10	6	M	2*	F	2	4*
100	7	M	2	F	2	4
100	8	M	2	F	2	4
100	9	M	2	F	2	4
1000	10	M	3	F	1	4
1000	11	M	2	F	2	4
1000	12	M	2	F	2	4

*Only one male was analysed for SOD in tank 6, three in total

Table A12: Number of individuals of each sex analysed for SOD* in the carbamazepine exposure experiment for each of the exposure concentrations.

Exposure	Tank	Sex	n	Sex	n	N total
0	1	M	2	F	2	4
0	2	M	2	F	2	4
0	3	M	2	F	1	3
50	4	M	2	F	2	4
50	5	M	2	F	1	3
50	6	M	2	F	2	4
1000	7	M	2	F	2	4
1000	8	M	2	F	2	4
1000	9	M	2	F	2	4

*For all the other biomarkers in the carbamazepine exposure experiment two males (M), two females (F) and four individuals in total were analysed.

A4: Standard curves measurements levels of biomarkers

For the measurements of protein, GSH and MDA standard curves were used. New standard curves were made for each batch of analysis. The equations and R^2 are shown for each round of analysis of protein, GSH and MDA levels in Table A13, Table A14 and Table A15 respectively. The protein analysis was performed with a BSA standard curve. In the carbamazepine exposure experiment, standard curves were also used for the measurement of GPx activity, Table A16, where y is the measured absorbance and x is the NADPH amount (nmol).

Table A13: BSA standard curves protein measurements from carbamazepine or ibuprofen exposure of *G. oceanicus.* In the equation x is BSA (mg/mL) and y is the absorbance (750 nm).

Sample	Exposure	Equation	R^2 value
1-10	Carbamazepine	$y = 8.6686x + 0.1862$	0.9851
11-20	Carbamazepine	$y = 9.1899x + 0.1934$	0.9820
21-30	Carbamazepine	$y = 8.6105x + 0.2229$	0.9915
31-34	Carbamazepine	$y = 8.8693x + 0.1895$	0.9865
35-36	Carbamazepine	$y = 7.1406x + 0.2206$	0.9556
37-42	Ibuprofen	$y = 8.8693x + 0.1895$	0.9865
43-52	Ibuprofen	$y = 8.7166x + 0.2104$	0.9726
53-62	Ibuprofen	$y = 7.8118x + 0.1905$	0.9856
63-74	Ibuprofen	$y = 8.3831x + 0.1957$	0.9808
75-84	Ibuprofen	$y = 7.1406x + 0.2206$	0.9556

Table A14: GSH standard curves GSH measurements in *G. oceanicus* exposed to carbamazepine or ibuprofen. In the equation x is the GSH amount (nmol/well) and y is the absorbance (412 nm).

Sample	Exposure	Equation	R^2 value
1-10	Carbamazepine	$y = 0.0628x + 0.0003$	0.9999
11-20	Carbamazepine	$y = 0.0476x + 0.0005$	0.9966
21-30	Carbamazepine	$y = 0.0599x - 7E{-}05$	1.0000
31-34	Carbamazepine	$y = 0.0611x + 9E{-}05$	0.9999
35-36	Carbamazepine	$y = 0.0769x + 0.0008$	0.9981
37-42	Ibuprofen	$y = 0.0611x + 9E{-}05$	0.9999
43-52	Ibuprofen	$y = 0.0485x - 0.0001$	0.9999
53-62	Ibuprofen	$y = 0.0658x + 0.0008$	0.9988
63-74	Ibuprofen	$y = 0.0410x + 8E{-}06$	0.9995
75-84	Ibuprofen	$y = 0.0769x + 0.0008$	0.9981

Table A15: MDA standard curves MDA measurements *G. oceanicus* exposed to carbamazepine or ibuprofen. In the equation x is the MDA amount (nmol/well) and y is the absorbance (532 nm).

Sample	Exposure	Equation	R^2 value
1-10	Carbamazepine	$y = 0.0423x + 0.0014$	0.9999
11-20	Carbamazepine	$y = 0.0445x - 0.0004$	0.9998
21-30	Carbamazepine	$y = 0.0414x + 0.0087$	0.9992
31-34	Carbamazepine	$y = 0.0420x + 0.0016$	0.9999
35-36	Carbamazepine	$y = 0.0423x + 0.0064$	0.9994
37-42	Ibuprofen	$y = 0.0420x + 0.0016$	0.9999
43-52	Ibuprofen	$y = 0.0440x + 0.0055$	0.9997
53-62	Ibuprofen	$y = 0.0412x + 0.0009$	0.9999
63-74	Ibuprofen	$y = 0.0433x + 0.0023$	0.9999
75-84	Ibuprofen	$y = 0.0423x + 0.0064$	0.9994

Table A16: Standard curves GPx measurements *G. oceanicus* exposed to carbamazepine. In the equation x is the NADPH amount (nmol/well) and y is the absorbance (340 nm).

Sample	Exposure	Equation	R^2 value
1-10	Carbamazepine	$y = 0.0119x + 0.0924$	0.9964
11-20	Carbamazepine	$y = 0.0117x + 0.0887$	0.9929
21-30	Carbamazepine	$y = 0.0117x + 0.0930$	0.9938
31-36	Carbamazepine	$y = 0.0119x + 0.0859$	0.9951

For the measurement of SOD levels, a standard curve between inhibition (y) and U/mL (x) from an earlier study using bovine erythrocytes from the datasheet for the SOD kit (19160 SOD determination kit, Sigma) was used after testing that the reactions were linear kinetically for 20 minutes. Here, the same equation was used for all the samples.

$$y = 0.4516x + 0.1505$$

Where y is inhibition and x are U/mL. R^2 of 0.9983

A5: Model testing

Table A17 and Table A18 show the results of the model testing using Shapiro Wilk's test with the residuals of the model, AIC, BIC, and plotting fitted vs residuals, qqplot and Cook's distance. More "x" in the "Normal in plots?" column indicates a better fit with normal distribution, homoscedasticity and lack of outliers.

Table A17: Overview models tested for normality, homoscedasticity and model selection.

Exposure experiment	nr	Model variables	Shapiro (residuals)	AIC	BIC	Normal in plots?
CBZ	0	**CAT~Exposure**	0.55	**99.79**	**104.83**	xx**xx**
CBZ	1	**CAT~Exposure*Tank**	0.8776	112.10	119.14	xxx
CBZ	2	CAT~Exposure*Tank*Sex	**0.8924**	151.47	134.05	xx**x**
CBZ	3	**CAT~Exposure + Tank**	0.7799	104.08	110.69	xxx
CBZ	4	CAT~Exposure + Tank + Sex	0.6397	105.90	112.98	xx
CBZ	0	SOD~Exposure	0.0626	**-129.25**	**-124.52**	x**xx**
CBZ	1	SOD~Exposure*Tank	0.2355	-113.27	-107.57	
CBZ	2	**SOD~Exposure*Tank*Sex**	0.3326	-55.73	-81.02	xx
CBZ	3	SOD~Exposure + Tank	**0.4355**	-126.58	-120.53	x**x**
CBZ	4	**SOD~Exposure + Tank + Sex**	0.2398	-123.42	-117.04	xx
CBZ	0	logGSH~Exposure	0.2113	**56.17**	**61.22**	xx**xx**
CBZ	1	**logGSH~Exposure*Tank**	0.8411	69.38	76.42	xxx
CBZ	2	logGSH~Exposure*Tank*Sex	**0.9604**	117.88	100.47	xx**x**
CBZ	3	logGSH~Exposure + Tank	0.1681	58.60	65.20	xx
CBZ	4	logGSH~Exposure + Tank + Sex	0.1289	61.56	68.65	xx
CBZ	0	logGST~Exposure	0.8017	17.04	**22.08**	x**x**
CBZ	1	**logGST~Exposure*Tank**	0.2688	16.66	23.79	xx
CBZ	2	logGST~Exposure*Tank*Sex	0.3899	63.68	46.27	x
CBZ	3	logGST~Exposure + Tank	0.9002	**15.97**	22.57	x**x**
CBZ	4	**logGST~Exposure + Tank + Sex**	**0.9412**	18.93	26.02	xx**x**
CBZ	0	logGPx~Exposure	0.1687	**20.38**	25.42	x**x**
CBZ	1	**logGPx~Exposure*Tank**	0.6938	31.82	38.86	xxx
CBZ	2	logGPx~Exposure*Tank*Sex	**0.9983**	67.27	49.85	x**x**
CBZ	3	logGPx~Exposure + Tank	0.2036	25.91	**32.52**	xx**x**
CBZ	4	logGPx~Exposure + Tank + Sex	0.2315	28.51	35.60	x
IBU	0	CAT~Exposure	0.0970	153.02	**160.95**	xx**x**
IBU	1	CAT~Exposure*Tank	**0.7576**	173.09	186.71	xx**x**
IBU	2	CAT~Exposure*Tank*Sex	0.3188	211.41	199.10	xx
IBU	3	**CAT~Exposure + Tank**	0.1271	158.26	168.56	xxx**x**
IBU	4	CAT~Exposure + Tank + Sex	0.0947	**150.04**	161.32	xx**x**
IBU	0	SOD~Exposure	0.1911	-162.81	**-155.02**	x**x**
IBU	1	**SOD~Exposure*Tank**	**0.5563**	-144.85	-131.83	xxx**x**
IBU	2	SOD~Exposure*Tank*Sex	0.341	-104.34	-119.99	
IBU	3	SOD~Exposure + Tank	0.4811	-158.45	-148.37	x
IBU	4	SOD~Exposure + Tank + Sex	0.1212	**-164.26**	-153.25	x**x**
IBU	0	**logGSH~Exposure**	**0.8113**	**96.85**	**104.78**	xx**xxx**
IBU	1	**logGSH~Exposure*Tank**	0.7897	116.41	130.03	xxx

IBU	2	logGSH~Exposure*Tank*Sex	0.7987	159.60	147.29	xx
IBU	3	**logGSH~Exposure + Tank**	0.668	101.17	111.47	xxx
IBU	4	logGSH~Exposure + Tank + Sex	0.6893	102.87	114.15	x
IBU	0	logGST~Exposure	0.0928	-11.80	**-3.87**	x**x**
IBU	1	logGST~Exposure*Tank	0.2542	4.27	17.89	xx
IBU	2	logGST~Exposure*Tank*Sex	**0.6927**	41.36	29.05	x**x**
IBU	3	logGST~Exposure + Tank	0.1701	-8.41	1.88	xx
IBU	4	**logGST~Exposure + Tank + Sex**	0.6162	**-13.51**	-2.23	xxx**x**
IBU	0	logMDA~Exposure	0.1591	**11.42**	**19.35**	**xx**
IBU	1	logMDA~Exposure*Tank	0.4017	29.55	43.17	xx
IBU	2	logMDA~Exposure*Tank*Sex	0.0319	74.06	61.75	
IBU	3	**logMDA~Exposure + Tank**	0.4858	14.79	25.09	xx
IBU	4	logMDA~Exposure + Tank + Sex	**0.5106**	17.65	28.93	xx**x**

Table A18: Levene test, barlett test and flinger test values for the different models tested. Significant values are red.

Exposure	Model	Levene test	Barlett test	Flinger test
CBZ	CAT~Exposure	0.847	0.8826	0.7624
CBZ	CAT~Tank	0.4228	0.3519	0.3636
CBZ	CAT~Sex	0.05181	0.03832	0.04394
CBZ	CAT~Exposure*Tank	0.7798		
CBZ	CAT~Exposure*Tank*Sex	<2.2e-16		
CBZ	SOD~Exposure	0.352	0.3269	0.2734
CBZ	SOD~Tank	0.1116	0.05866	0.1278
CBZ	SOD~Sex	0.289	0.2923	0.3222
CBZ	SOD~Exposure*Tank	0.4525		
CBZ	SOD~Exposure*Tank*Sex	<2.2e-16		
CBZ	logGSH~Exposure	0.7098	0.6193	0.6349
CBZ	logGSH~Tank	0.9042	0.7532	0.9367
CBZ	logGSH~Sex	0.3824	0.4204	0.3411
CBZ	logGSH~Exposure*Tank	0.1363		
CBZ	logGSH~Exposure*Tank*Sex	<2.2e-16		
CBZ	logGST~Exposure	0.9877	0.9507	0.9855
CBZ	logGST~Tank	0.3963	0.4395	0.2883
CBZ	logGST~Sex	0.8099	0.657	0.8181
CBZ	logGST~Exposure*Tank	0.07333		
CBZ	logGST~Exposure*Tank*Sex	<2.2e-16		
CBZ	logGPx~Exposure	0.3469	0.1261	0.2539
CBZ	logGPx~Tank	0.5198	0.5659	0.4773
CBZ	logGPx~Sex	0.0584	0.01077	0.08892
CBZ	logGPx~Exposure*Tank	0.4915		
CBZ	logGPx~Exposure*Tank*Sex	<2.2e-16		
IBU	CAT~Exposure	0.5265	0.2641	0.4422
IBU	CAT~Tank	0.4701	0.2113	0.5842
IBU	CAT~Sex	0.2762	0.038	0.4303
IBU	CAT~Exposure*Tank	0.5576		
IBU	CAT~Exposure*Tank*Sex	0.02936		
IBU	SOD~Exposure	0.3755	0.4749	0.3419
IBU	SOD~Tank	0.5909	0.5569	0.8493
IBU	SOD~Sex	0.4815	0.6682	0.5119
IBU	SOD~Exposure*Tank	0.8099		
IBU	SOD~Exposure*Tank*Sex	3.06e-05		
IBU	logGSH~Exposure	0.71	0.4643	0.7328
IBU	logGSH~Tank	0.867	0.6528	0.7272
IBU	logGSH~Sex	0.6659	0.5102	0.6981
IBU	logGSH~Exposure*Tank	0.784		
IBU	logGSH~Exposure*Tank*Sex	0.06352		
IBU	logGST~Exposure	0.7789	0.8814	0.6874
IBU	logGST~Tank	0.6337	0.6043	0.5035
IBU	logGST~Sex	0.1511	0.1201	0.1882
IBU	logGST~Exposure*Tank	0.2656		
IBU	logGST~Exposure*Tank*Sex	0.00161		
IBU	logMDA~Exposure	0.7784	0.3968	0.9275
IBU	logMDA~Tank	0.6181	0.4749	0.6474
IBU	logMDA~Sex	0.1697	0.07989	0.2433
IBU	logMDA~Exposure*Tank	0.8081		
IBU	logMDA~Exposure*Tank*Sex	0.03529		

A5: Correlation graphs

Figure A1 show the correlation between the weight and protein concentrations in the individuals from the carbamazepine and ibuprofen exposure experiments. Figure A2 and Figure A3 show the correlation graphs for the correlations between the levels of the biomarkers per mg protein analysed, including weight, in the carbamazepine and ibuprofen exposure experiment respectively. The points symbolise each individual analysed, the line symbolises the correlation line, "R" is the correlation coefficient and "p" is the p-value.

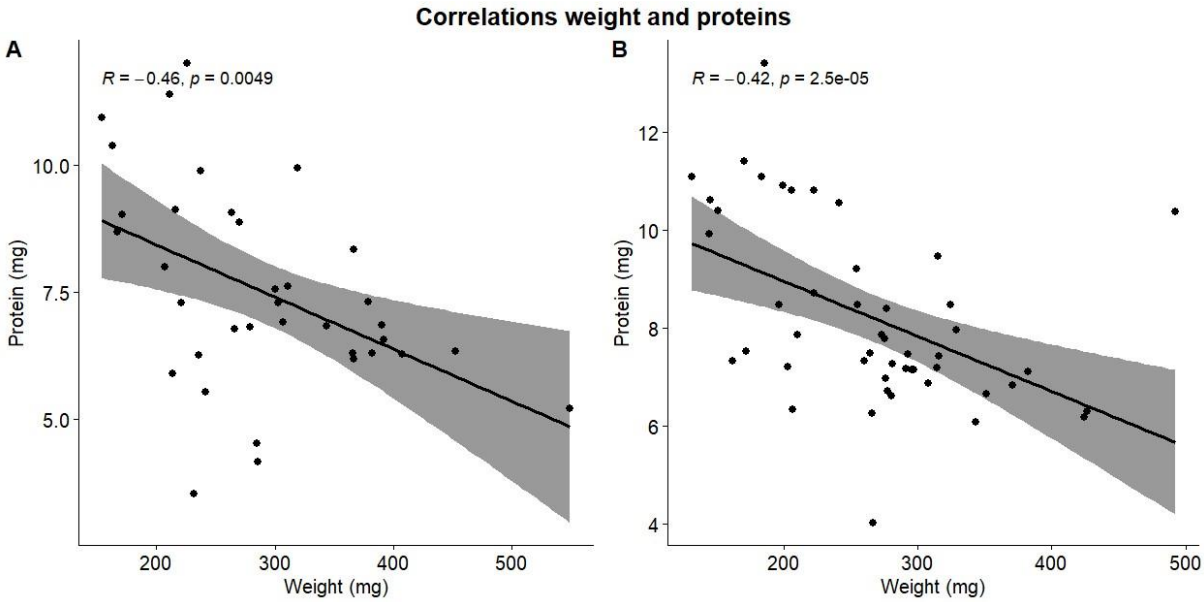


Figure A1: Correlation weight and protein levels in the carbamazepine (A) and ibuprofen (B) exposure experiment individuals.

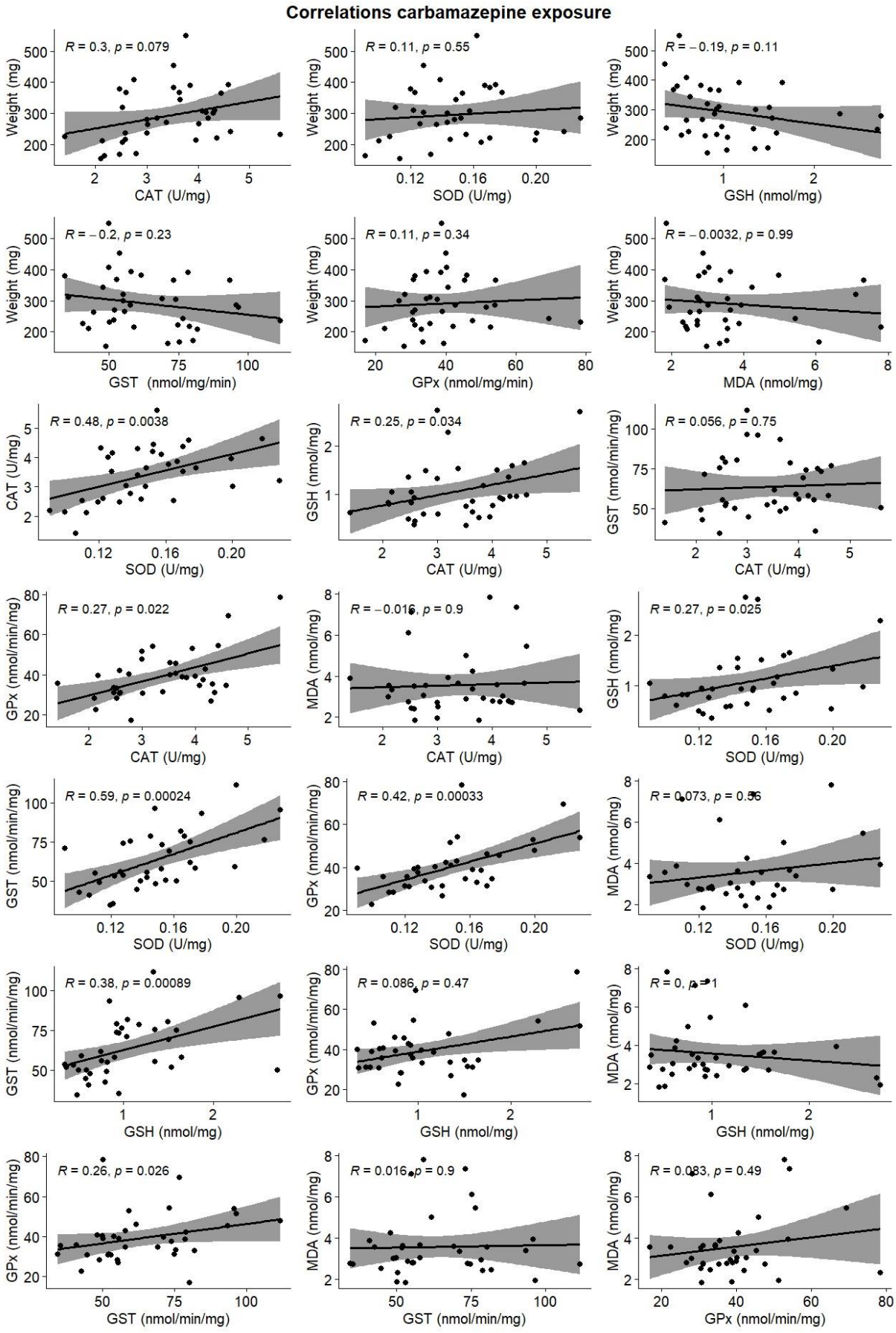


Figure A2: Correlations biomarkers (per mg protein) and weight in the carbamazepine exposure experiment individuals.

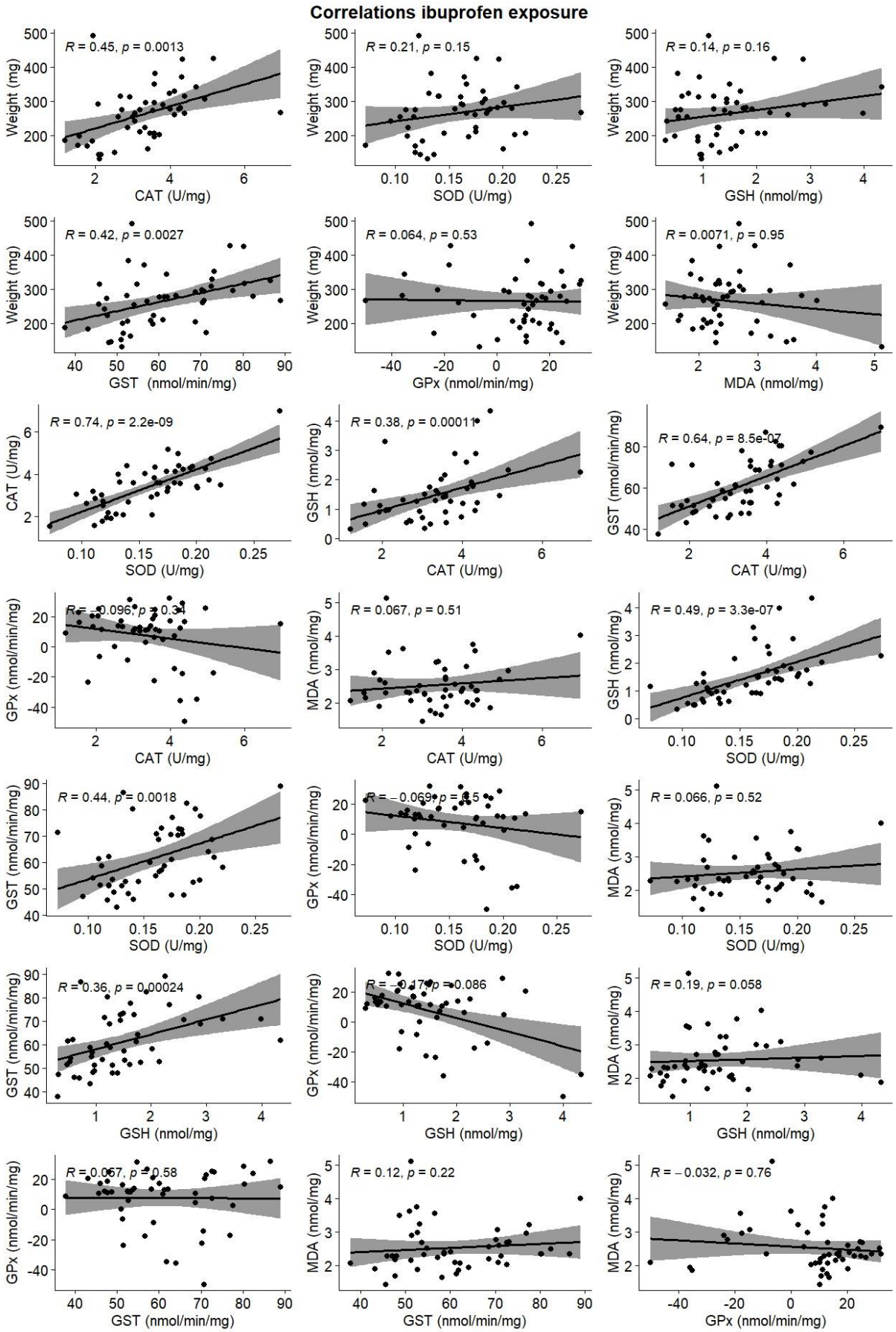
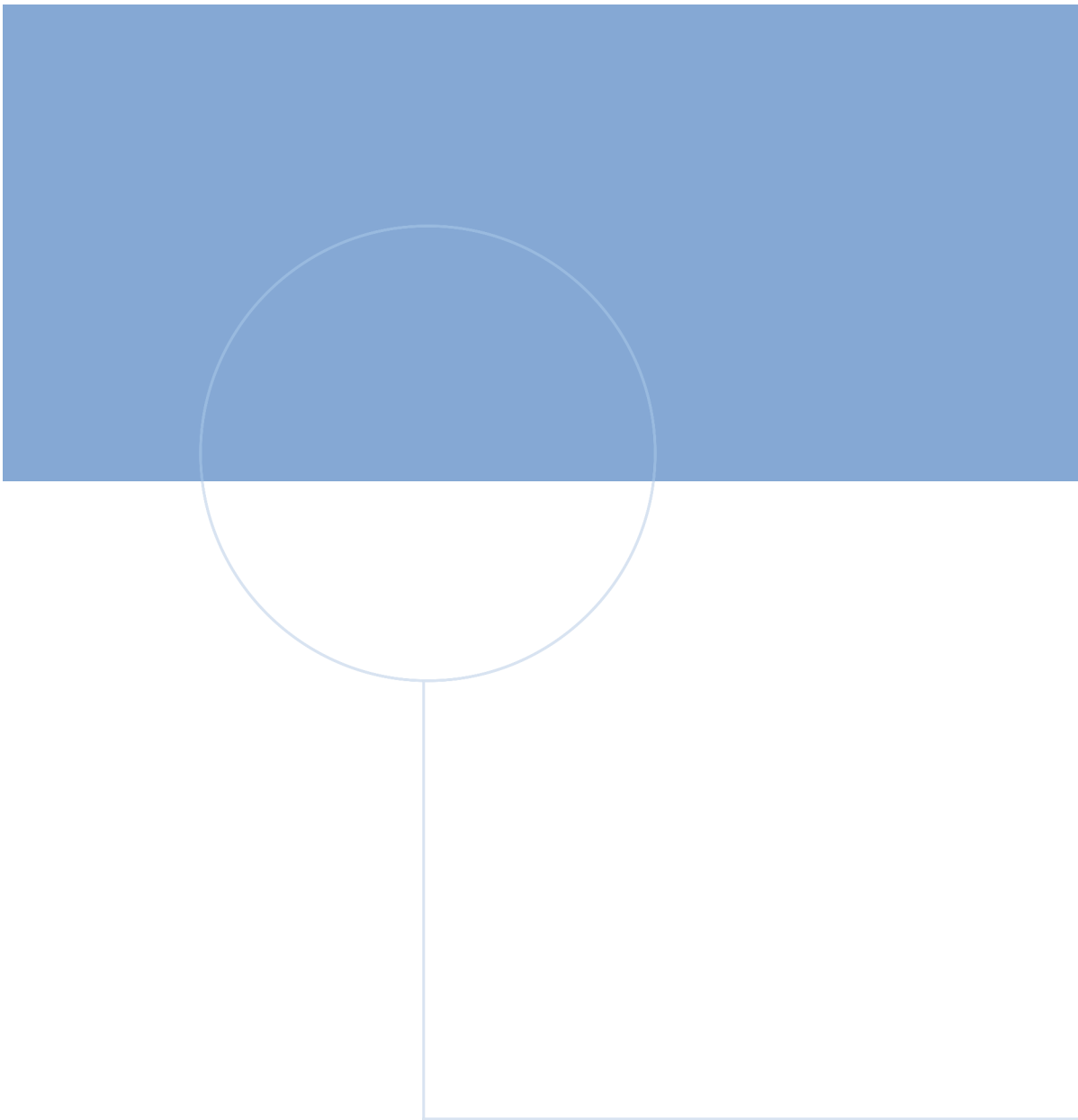


Figure A3: Correlations biomarkers (per mg protein) and weight in the ibuprofen exposure experiment individuals.



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