Transcriptional biomarkers of toxicity - powerful tools or random noise?

An applied perspective from studies on bivalves

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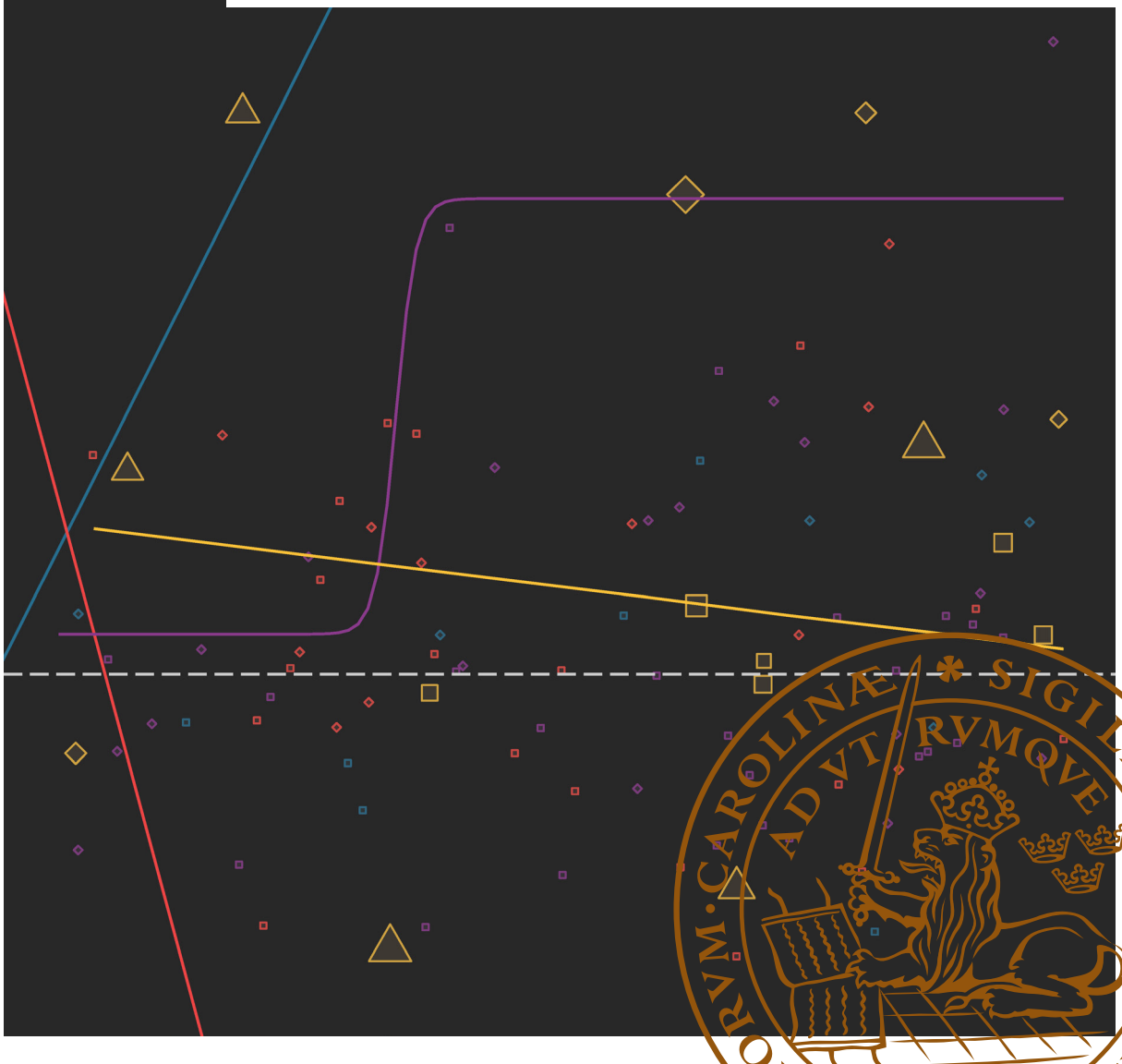

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DOCTORAL DISSERTATION

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Organization LUND UNIVERSITY Department of Biology Sölvegatan 37, 223 62 Lund, Sweden **Author**: Gustaf M.O. Ekelund Ugge	**Document name:** Doctoral dissertation **Date of issue**: 2023-02-10 **Sponsoring organization**: UNIVERSITY OF SKÖVDE

Title and subtitle: Transcriptional biomarkers of toxicity – powerful tools or random noise? An applied perspective from studies on bivalves

Abstract: Aquatic organisms are constantly at risk of being exposed to potentially harmful chemical compounds of natural or anthropogenic origin. Biological life can for instance respond to chemical stressors by changes in gene expression, and thus, certain gene transcripts can potentially function as biomarkers, i.e. early warnings, of toxicity and chemical stress. A major challenge for biomarker application is the extrapolation of transcriptional data to potential effects at the organism level or above. Importantly, successful biomarker use also requires basal understanding of how to distinguish actual responses from background noise. The aim of this thesis is, based on response magnitude and variation, to evaluate the biomarker potential in a set of putative transcriptional biomarkers of general toxicity and chemical stress.

Specifically, I addressed a selection of six transcripts involved in cytoprotection and oxidative stress: catalase (*cat*), glutathione-S-transferase (*gst*), heat shock proteins 70 and 90 (*hsp70*, *hsp90*), metallothionein (*mt*) and superoxide dismutase (*sod*). Moreover, I used metal exposures to serve as a proxy for general chemical stress, and due to their ecological relevance and nature as sedentary filter-feeders, I used bivalves as study organisms.

In a series of experiments, I tested transcriptional responses in the freshwater duck mussel, *Anodonta anatina*, exposed to copper or an industrial waste-water effluent, to address response robustness and sensitivity, and potential controlled (e.g. exposure concentration) and random (e.g. gravidness) sources of variation. In addition, I performed a systematic review and meta-analysis on transcriptional responses in metal exposed bivalves to (1) evaluate what responses to expect from arbitrary metal exposures, (2) assess the influence from metal concentration (expressed as toxic unit), exposure time and analyzed tissue, and (3) address potential impacts from publication bias in the scientific literature.

Response magnitudes were generally small in relationship to the observed variation, both for *A. anatina* and bivalves in general. The expected response to an arbitrary metal exposure would generally be close to zero, based on both experimental observations and on the estimated impact from publication bias. Although many of the transcripts demonstrated concentration-response relationships, large background noise might in practice obscure the small responses even at relatively high exposures. As demonstrated in *A. anatina* under copper exposure, this can be the case already for single species under high resolution exposures to single pollutants. As demonstrated by the meta-regression, this problem can only be expected to increase further upon extrapolation between different species and exposure scenarios, due to increasing heterogeneity and random variation. Similar patterns can also be expected for time-dependent response variation, although the meta-regression revealed a general trend of slightly increasing response magnitude with increasing exposure times.

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Key words: *Anodonta anatina*, biomarkers, bivalves, digestive gland, dose-response, ecotoxicology, gene transcription, gills, meta-analysis, meta-regression, metal, qPCR, systematic review, toxic unit, wastewater

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Author contributions

I. GEU, AJ and OB conceived the study and designed the experimental setup. GEU performed the experimental exposures and biochemical assays. GEU and RS performed and evaluated the qPCR assays, and RS designed the primers. GEU analyzed the data with support from OB, AJ and BO. GEU lead the writing, and all authors contributed with guidance and revisions of the manuscript.

II. GEU, AJ and OB conceived the study and designed the experimental setup. GEU performed the experimental work and analyzed the data. GEU lead the writing, and all authors contributed with guidance and revisions of the manuscript.

III. GEU and OB conceived the study and designed the experimental setup. GEU and AWA performed the laboratory exposures. GEU performed the qPCR assays, analyzed the data, and lead the writing. All authors contributed with guidance and revisions of the manuscript.

IV. GEU and OB conceived the study. GEU designed and performed the systematic review and data collection. GEU and US developed the meta-analytical models and analyzed the data. GEU lead the writing, and all authors contributed with guidance and revisions of the manuscript.

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List of abbreviations

AChE	Acetylcholinesterase
AOP	Adverse outcome pathway
cat	Catalase, gene or transcript
CAT	Catalase, enzyme
EC_X	X % effect concentration
ERA	Environmental risk assessment
gst	Glutathione-S-transferase, gene or transcript
GST	Glutathione-S-transferase, enzyme
hsp70/90	Heat shock protein 70/90, gene or transcript
Hsp70/90	Heat shock protein 70/90, protein
LC_X	X % lethal concentration
mt	Metallothionein, gene or transcript
MT	Metallothionein, protein
qPCR	see RT-qPCR; used interchangeably throughout thesis
RQN	RNA quality number
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
sod	Superoxide dismutase, gene or transcript
SOD	Superoxide dismutase, enzyme
TU	Toxic unit

Abstract

Aquatic organisms are constantly at risk of being exposed to potentially harmful chemical compounds of natural or anthropogenic origin. Biological life can for instance respond to chemical stressors by changes in gene expression, and thus, certain gene transcripts can potentially function as biomarkers, i.e. early warnings, of toxicity and chemical stress. A major challenge for biomarker application is the extrapolation of transcriptional data to potential effects at the organism level or above. Importantly, successful biomarker use also requires basal understanding of how to distinguish actual responses from background noise. The aim of this thesis is, based on response magnitude and variation, to evaluate the biomarker potential in a set of putative transcriptional biomarkers of general toxicity and chemical stress.

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Popular science summary

Organisms in the environment constantly encounter various natural and man-made chemicals. Many of the regular encounters are more or less safe, but depending on the intensity of the exposure, all chemicals have the potential to be harmful in different ways. Thus, in the research field of environmental toxicology, there are different ways to address questions concerning potential negative effects from chemicals. One such approach is the use of so-called *biomarkers*. In short, a biomarker is a selected biological feature or measure that (1) can be measured in or on an organism, and (2) change either upon encounters with chemicals, or from the harmful effects that can arise. Since many unwanted effects from chemicals originate at the cellular level, various molecular responses are often considered as potential biomarkers to anticipate harmful effects on the organism.

Genes are pieces of biological information carried by all organisms, and commonly contain instructions to produce proteins with functions in the cell. The first step of gene expression is known as transcription, and occurs when a gene, i.e. a certain sequence in the organism's DNA, is transcribed to temporary working copies of itself, i.e. gene transcripts made of RNA. These are in turn used as templates for cellular production of the protein that corresponds to the particular gene. Although the DNA itself is constant, the levels of different gene transcripts will vary over time depending on the current needs of the organism. For instance, certain genes are known for being involved in protective actions against chemical stressors, and are expected to be expressed at higher levels in organisms under chemical stress. By responding to exposures that might eventually be harmful, transcripts of these genes are believed to serve as early warnings before actual harm arises.

In this thesis, I address six selected stress genes and evaluate whether their gene transcripts can be used as biomarkers of toxic chemicals in general. Specifically, I used the freshwater duck mussel (*Anodonta anatina*) in a series of laboratory experiments, to test how gene transcription was affected by copper and an industrial wastewater effluent. In these studies, I addressed how sensitive and how robust the gene transcripts were for consideration as general biomarkers. In addition, I performed a study on published scientific literature, a so-called systematic review and meta-analysis, to address similar questions on a larger scale, i.e. on bivalves and metals in general.

The transcript response signals were in general both weak and variable, which limits the potential use as biomarkers. The experiments and meta-analysis together suggested that we cannot necessarily expect detectable biomarker signals simply because mussels are exposed to toxic metals. I could show in duck mussels that responses commonly increased with increasing concentrations of copper, i.e. increasing chemical stress, but such relationships were generally not detected across species and metals in the meta-analysis. In general, the background noise was so large that it risked obscuring the biomarker signal, even in cases where gene expression actually changed as a response to the chemicals. Overall, random variation both within and between species and exposures will likely limit the ability to detect biomarker signals.

One of many potential sources of variability in duck mussels was gravidness. When gravid, as occurs in nature from late summer to early spring, the background noise can be expected to increase for most of my tested biomarkers. This was however not limited to gene transcripts. To put the transcripts into a wider perspective, I also tested two additional enzymatic biomarkers. These showed similar variability and response signals as the transcripts, both in general and with regards to gravidness. On one hand, this could mean that the selected transcripts are neither substantially better nor worse than established biomarkers at detecting chemical stress. On the other hand, there would then be no advantage in using transcripts as biomarkers, as compared to molecular responses that are easier to interpret from a toxicological perspective.

In summary, clear biomarker signals could possibly be expected under specific and validated test conditions (e.g. avoiding gravidness, targeting specific species and chemicals), and in particular at high concentrations and/or after long exposure durations. In practice, conditions for environmental monitoring and risk assessment are however rarely optimal, which can critically limit the universality of these biomarkers based on general stress genes. Overall, the best way for practical implementation of transcripts in future environmental risk assessment might be a complementary approach of validated biomarkers, models that link transcripts to harmful effects on the organism, and techniques that early on can detect general changes in the organism's transcriptional patterns.

Populärvetenskaplig sammanfattning

Organismer kommer i miljön i konstant kontakt med olika naturliga och konstgjorda kemikalier. Även om de i många fall är mer eller mindre ofarliga besitter alla kemikalier förmågan att, beroende på exponeringens intensitet, vara skadliga på olika sätt. Inom forskningsfältet miljötoxikologi finns det därför olika sätt att angripa frågor kring potentiella negativa effekter från kemikalier. Ett sådant sätt är att använda så kallade *biomarkörer*. Kortfattat är en biomarkör en utvald egenskap eller ett biologiskt mått som (1) kan mätas i eller på en organism, och (2) förändras antingen vid kontakt med kemikalier, eller till följd av oönskade effekter som uppstår. Eftersom många effekter först uppstår på cellnivå brukar olika typer av molekylära förändringar betraktas som möjliga biomarkörer för att förutse skadliga effekter på organismen.

Gener utgör bitar av biologisk information som bärs av alla organismer, och innehåller i regel instruktioner för att bilda proteiner med funktioner i cellen. Det första steget i genuttrycksprocessen kallas transkription och sker när en gen, d.v.s. en specifik sekvens av organismens DNA, kopieras till tillfälliga arbetskopior av sig själv, d.v.s. gentranskript som består av RNA. Dessa används i sin tur som mallar för att i cellen bilda det protein som motsvarar den specifika genen. Medan mängden DNA är konstant varierar nivåerna av olika gentranskript över tid beroende på organismens behov för tillfället. Vissa gener är till exempel kända för att ha skyddande funktioner mot kemiska stressfaktorer, varför de förväntas uttryckas i högre grad hos organismer som utsätts för kemisk stress. Genom att svara på exponeringar som riskerar att bli skadliga längre fram kan transkript av dessa gener betraktas som tidiga varningar innan dess att verklig skada uppstår.

I denna avhandling har jag undersökt sex utvalda stressgener och utvärderat huruvida deras gentranskript kan användas som generella biomarkörer för giftiga kemikalier. Närmre bestämt använde jag sötvattensarten allmän dammussla (*Anodonta anatina*) i en rad laboratorieförsök, för att undersöka hur gentranskriptionen påverkades dels av koppar, dels av ett industriellt utsläppsvatten. I dessa försök studerade jag hur känsliga och hur robusta gentranskripten var för att kunna fungera som generella biomarkörer. Dessutom genomförde jag en studie baserad på publicerad vetenskaplig litteratur, en så kallad systematisk översikt och metaanalys (eng. ’*systematic review and meta-analysis*’), för att besvara motsvarande frågor i större skala, d.v.s. rörande musslor och metaller i allmänhet.

Svarssignalerna hos gentranskripten var i regel både svaga och varierande, vilket begränsar möjlig användning som biomarkörer. Tillsammans tyder experimenten och metaanalysen på att vi inte nödvändigtvis kan förvänta oss detekterbara biomarkörsignaler enbart för att musslor utsätts för giftiga metaller. Jag påvisade i dammusslor att svarssignalerna generellt ökade vid högre halter av koppar, d.v.s. högre kemisk stress, men dessa samband syntes i regel inte i metaanalysen vid jämförelser mellan olika arter och metaller. Bakgrundsbruset var i allmänhet så högt att det riskerade att dölja biomarkörsignalen, även i de fall där genuttrycket faktiskt förändrades som svar på kemikalierna. Sammantaget kan slumpmässig variation, både inom och mellan arter och exponeringar, troligtvis begränsa förmågan att detektera biomarkörsignaler.

En av flera möjliga källor till variabilitet i dammusslor var graviditet. När musslorna är gravida, vilket i naturen äger rum från sensommar till tidig vår, kan bakgrundsbruset förväntas öka hos de flesta av mina undersökta biomarkörer. Detta gällde dock inte enbart gentranskript. För att sätta transkripten i ett vidare perspektiv undersökte jag även två ytterligare biomarkörer baserade på enzymaktivitet. Jämfört med transkripten påvisade dessa en liknande grad av variabilitet och liknande svarssignaler, både överlag och med särskilt avseende på graviditet. Å ena sidan kan detta tolkas som att de utvalda gentranskripten varken är väsentligt bättre eller sämre än etablerade biomarkörer på att detektera kemisk stress. Å andra sidan finns det i så fall heller ingen fördel med att använda transkript som biomarkörer, jämfört med molekylära förändringar som är enklare att tolka ur ett toxikologiskt perspektiv.

Sammanfattningsvis skulle tydliga biomarkörsignaler möjligtvis kunna förväntas under specifika och validerade försöksförhållanden (t.ex. försök som utesluter gravida musslor, eller riktar sig mot specifika arter och kemikalier), särskilt vid höga koncentrationer och/eller längre perioder av exponering. I praktiken är dock förhållandena för miljöövervakning och -riskbedömning sällan optimala, vilket begränsar allmängiltigheten hos dessa markörer. Sammantaget kan det bästa sättet för att implementera gentranskript i framtida miljöriskbedömning visa sig bli en kompletterande användning av validerade biomarkörer, modeller som knyter gentranskript skadliga effekter på organismen, och tekniker som tidigt kan detektera förändringar i organismens övergripande transkriptionsmönster.

Preface

After graduating with an M.Sc. in biology and ecology in 2015, I started working for Toxicon AB, a small laboratory situated in the countryside along the Scanian west coast. A key component of the company profile was, at the time, custom environmental consulting, by offering environmental monitoring, risk assessment and standard as well as non-standard ecotoxicological testing. With hands-on experience of biochemical biomarkers, the company management increasingly directed my focus towards further incorporation of molecular methods in toxicological testing and environmental risk assessment (ERA). The main interest was to apply and develop methods that, for both practical and financial reasons, could be performed in-house. With the purchase of a reverse transcription quantitative polymerase chain reaction (RT-qPCR) machine, the idea was to develop biomarker assays based on gene transcripts. It was however clear from start that there was no scientific consensus on how to successfully incorporate these techniques into ERA, and that further research was needed. Along with collaborating researchers from the University of Skövde, the original idea was therefore further developed into the project WaterAssess.

The WaterAssess project

The project 'Multi-biomarker panel for environmental impact assessment of wastewater effluents', or WaterAssess for short, was a joint collaboration (2017-2020) between University of Skövde (project management, research, supervision), Toxicon (co-funding, experience with practical application of biomarkers), TATAA Biocenter AB (co-funding, expertise in qPCR) and Lund University (research, supervision), co-funded by the Swedish Knowledge Foundation. The overarching aim was to develop a panel of qPCR-based biomarkers for practical application in ERA of industrial wastewater effluents. A major portion of the project budget was devoted to research, predominantly performed by a prospective PhD student using existing infrastructure at the contributing companies. That is how in 2017, I was financed by the University of Skövde and enrolled for doctoral studies at Lund University, to perform experimental work at Toxicon and TATAA.

Aims of the thesis

The overall aim of this thesis was to critically evaluate the potential of applying transcriptional responses as biomarkers of general toxicity. All experimental work was performed within the WaterAssess framework, with practical application of qPCR-based biomarkers as the ultimate objective. Other molecular techniques would be available (and in some cases even preferable) to address some of the research questions from a basic science perspective. However, rather than choosing techniques based on research questions, my questions in themselves assumed application of this specific technique. Furthermore, whether or not a specific change in gene expression occurs can in itself be of great scientific interest. From an applied perspective, a more relevant question would however be to what extent that change is detectable under given circumstances. Hence, throughout the thesis, 'response' should primarily be interpreted as 'biological signal', which may or may not coincide with 'biological effect'. For this reason, I have not made the distinction whether induction of defense systems is a manifestation of toxicity or, simply, of functioning homeostatic regulation. Provided a clear correlation to exposure and/or effects, this separation would anyway not be meaningful for application of biomarkers as early warnings.

Throughout this work, I used bivalves as study organisms, a selection of six genes as transcriptional responses, and (predominantly) metals to represent general chemical stressors. Specifically, I addressed the following questions:

Q1: Should we generally expect 'stress genes' to respond to sublethal exposures of a single toxic compound (**Paper I** and **IV**) or a mixture (**Paper II**)?

Q2: Can we expect (monotonic) concentration-dependent transcriptional responses, and hence, predict response magnitude based on exposure concentration (**Paper III-IV**)?

Q3: Should we expect variability in response magnitudes with exposure time, and what are the general trends of time-dependence (**Paper IV**)?

Q4: How is biomarker sensitivity affected by individual variation in the freshwater mussel *Anodonta anatina* (**Paper I-III**)?

Q5: What is the overall biomarker potential of the selected transcripts in bivalves? Specifically, how do the transcripts compare to more established enzymatic biomarkers (**Paper I-II**)? How do the transcriptional biomarker candidates perform in *A. anatina* in terms of sensitivity and robustness (**Paper III**)? How do they perform in metal exposed bivalves in general (**Paper IV**)?

Introduction

Transcriptional responses to chemical stress

In their natural environment, aquatic organisms are at constant risk of exposure to various, potentially harmful, chemical compounds. In addition to naturally occurring toxicants, such as poisonous food sources, venomous predators and toxic algal blooms, a wide range of chemical stressors of anthropogenic origin reach aquatic ecosystems via for instance wastewater effluents and runoff (Deblonde *et al.* 2011, Müller *et al.* 2020). Upon harmful exposure to a chemical stressor, the organism's ability to maintain homeostasis is exceeded and consequently, adverse effects arise (Adiele *et al.* 2011, Zeng *et al.* 2019, Castaldo *et al.* 2020). In order to cope with variations in the natural environment, various molecular defense systems have consequently evolved to relieve general chemical stress and maintain cellular homeostasis (Kültz 2003, Sulmon *et al*. 2015, Birnie-Gauvin *et al.* 2017, Wang *et al.* 2019a). Responding to toxicant exposure, cells commonly change transcription patterns by inducing genes for toxicant metabolism and cytoprotection, and by activating or suppressing genes involved in various downstream pathways (Jennings *et al.* 2013). For instance, metal ions can cause cellular disruption by mechanisms of general toxicity and oxidative stress, which can in turn lead to induction of general stress genes to protect cellular integrity and essential functions (Navarro *et al.* 2011, Boukadida *et al.* 2017). In contrast, many organic toxicants can also act by high-affinity interaction with specific biomolecules, e.g. receptors or enzymes, exaggerating or inhibiting e.g. natural endocrine pathways (Muncke & Eggen 2006, Hayes *et al.* 2007). Thus, some stress responses are general and occur at exposures to various groups of toxicants whereas others are specific to certain chemical classes; still other transient transcriptional changes are indirect and not related to the chemical stressor itself (Martyniuk 2018).

Environmental risk assessment (ERA) based on the propagation of cellular responses to higher biological levels will require mechanistic and/or empirical links to ecologically relevant effects (Snape *et al.* 2004). For instance, transcriptomic points of departure, i.e. threshold concentrations at which changes start occurring at the whole transcriptome level, is a non-mechanistic approach that might be used for the estimation of safe, no-effect concentrations (Pagé-Larivière *et al*. 2019, Mittal *et al*. 2022). Another approach is adverse outcome pathways (AOPs), i.e. conceptual models of sequential events from the organism's exposure to a toxicant and

propagating to responses at cell, organ, organism and population levels (Ankley *et al.* 2010). Rather than necessarily focusing on detailed mechanisms of action, the purpose of an AOP is to identify key events at the molecular level that can be linked to organism or population responses across different chemical exposures. In complex response pathways, specific transcripts might vary at different stages and levels of exposure (Martinez *et al.* 2018, Granadeiro *et al.* 2019), and applying AOPs or gene network analysis can help to identify nodes, i.e. genes or groups of genes, central to certain exposure or response types (Brüggemann *et al.* 2018, Leng *et al.* 2019). A crucial step is to '*determine which changes are not meaningful (i.e. transient, indirect to the chemical itself) compared to those molecular responses indicative of the exposure*' (Martyniuk 2018). In order to do so, it is necessary to address and interpret both variability and magnitude, i.e. effect size, of transcriptional responses (Martyniuk 2018).

Effect size and concentration-dependence

Traditionally in toxicology, adverse effects have often demonstrated a monotonic, typically sigmoidal, concentration-response (or dose-response) relationship (Figure 1A), in which effect size increases with increasing exposure, i.e. concentration or time (Tsatsakis *et al.* 2018). In toxicology, measures of responses such as mortality, enzyme activity or transcription of a gene, can be referred to as endpoints. Concentration-response curves are used to calculate effect concentrations (EC_X) as the concentration of a chemical compound that induces X % of the maximal response, such as LC_{50} or LC_{10} (the 50 % and 10 % lethal concentration, respectively) (Oris & Bailer 1997). For transcriptional endpoints, concentration-response relationships have not been extensively studied at high resolution. Few transcriptional studies investigate effects at more than three or four exposure concentrations, and even fewer fit concentration-response curves. In one study, Smetanová *et al.* (2015) fitted concentration-response models to two transcriptomic response datasets over six to seven exposure concentrations. While most transcripts showed best fits in linear, U-shaped or exponential models, only 3-9 % of the transcripts showed best fits in sigmoidal models, which was suggested to result from a difficulty in reaching a maximal response (Smetanová *et al.* 2015). In contrast, when addressing transcriptomic concentration-response relationships in adult *Mytilus californianus* across eight concentrations of copper, Hall *et al*. (2020) were able to fit sigmoidal concentration-response curves to 88 % of the differentially expressed transcripts.

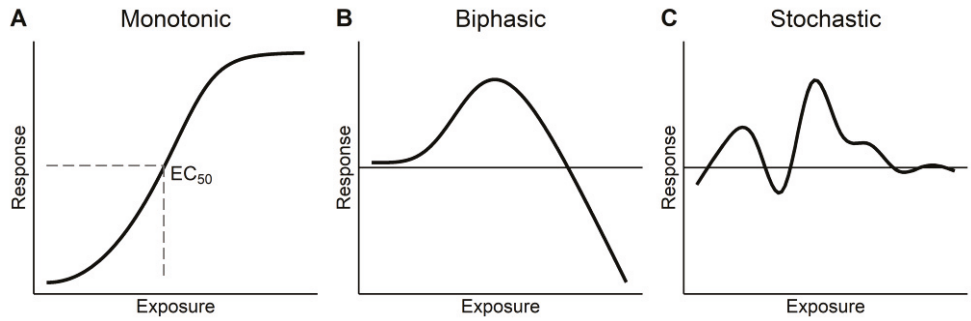

Figure 1. Different types of potential concentration-response relationships. Many adverse effects follow sigmoidal concentration-response relationships (A), which allows for estimation of for instance EC_{50} (50 % effect concentration). Other adverse effects can follow biphasic or hormetic concentration-response relationships (B), in which a response that is observed at low exposures reverses at higher exposures. Endpoints related to complex response pathways could potentially be expected to show variable and seemingly stochastic response patterns (C).

Many toxicants and response parameters do however not appear to follow monotonic relationships, but rather display biphasic (U- or J-shaped) concentration-responses (Zanuncio *et al.* 2011, Simonin *et al.* 2017). In hormetic concentration-responses (Figure 1B), the change in a biological parameter typically peaks at low levels to attenuate and eventually reverse at increasing exposure (Agathokleus & Calabrese 2019). In complex biological response systems, various non-monotonic concentration-response relationships are indeed to be expected (Conolly & Lutz 2004). Despite being based on too few doses to fit concentration-response curves, many studies do imply non-monotonic transcriptional responses relative toxicant exposures (Bigot *et al.* 2011, Arukwe *et al.* 2017, Martinez *et al.* 2018, Li *et al.* 2019, Zhang & Zhai 2020). Furthermore, transcriptional responses at different concentrations of the same toxicant often show a number of uniquely responding genes at each level (Yadetie *et al.* 2018, Wang *et al.* 2019b, Qian *et al.* 2020, Yamaguchi *et al.* 2020). For a given toxicant, multiple complex pathways can be involved the mechanisms of toxicity, providing a wide selection of genes potentially involved in different stages or different levels of toxicant exposure. Consequently, analogous to stochastic responses at the cellular level (Raj *et al.* 2006, Wang *et al.* 2019c), seemingly stochastic concentration-response relationships could potentially result for a given marker, depending on its role in the response pathway, the exposure conditions and potential confounding factors (Figure 1C).

Natural variation

Apart from toxicant exposure and chemical stress, there are numerous additional sources of variability in gene expression. For organisms in the environment, both intrinsic (e.g. sex and reproductive stage) and extrinsic (e.g. season) factors can cause fluctuation in transcript levels and variation within a population (Farcy *et al.* 2007, Navarro *et al.* 2013, Dreier *et al.* 2016). Even single cell models have demonstrated high intrinsic fluctuations in transcriptional responses to outer stimuli (Raj *et al.* 2006, Wang *et al.* 2019c). Rather than regulating amounts of transcribed RNA, eukaryotic cells modulate the frequencies of transcriptional bursts (Wang *et al.* 2019c). Even at the cellular level, instantaneous responses therefore often appear stochastic, although fluctuating RNA levels can be buffered by e.g. slow protein degradation at the cellular level (Raj *et al.* 2006). In order to function as an indicator of exposure or a predictor of adverse effects, it is of great importance that transcriptional responses can be distinguished from background noise. For meaningful interpretation of transcriptional data and distinction of actual stress responses, it is therefore key to address baseline and response variability (Bahamonde *et al.* 2016, Martyniuk 2018).

Biomarkers in ecotoxicology

In ecotoxicology, a biomarker is a measurable biological parameter, at the whole organism level or below, that responds to and can be used to indicate exposure to or adverse effects from toxicants (Sanchez & Porcher 2009). Biomarker selection is highly dependent on the intended use (e.g. what biological level of organization to address, what level of specificity/universality is required), and markers ideal for addressing certain questions might be meaningless for others. Common biomarker examples include changes in behavior, enzymatic activity, metabolites, proteins and gene transcripts (Bigot *et al.* 2011, Navarro *et al.* 2011, Gonzalez-Rey *et al.* 2014, Liu *et al.* 2014, 2016; Hartmann *et al.* 2016 Trombini *et al*. 2022). By addressing multiple biological systems and/or levels, multi-biomarker approaches can allow assessment of overall health or risk of harm under both laboratory and field conditions (Duarte *et al.* 2017, Aguilera *et al.* 2019, Baudou *et al.* 2019). The use of multiple biomarkers can partially compensate for inherent variability in the separate markers (Baudou *et al.* 2019), and given appropriate methods of response integration, evaluation methods based on overall patterns might ultimately increase the robustness of biomarker assessment (Devin *et al.* 2014, Duarte *et al.* 2017).

In this thesis, the general criteria proposed by van der Oost *et al.* (2003) were used as a basis for evaluation of biomarkers intended for use in ERA and environmental monitoring:

1. The assay should be reliable, cheap and easy to perform.

2. The response should be sensitive to toxicant exposure and/or effects.

3. Natural variability of the biomarker should be well understood to distinguish responses from baseline variation.

4. Confounding factors to biomarker responses should be well understood.

5. The mechanistic link between exposure and biomarker response should be understood.

6. The link between biomarker responses and effects on the whole organism should be established.

The use of transcripts as biomarkers

Unless specified otherwise, the concept 'biomarker' from here on refers to a single transcriptional response (that is, a single transcript), and neither to for instance a transcriptome profile (which would be considered multiple potential markers) nor enzymatic or behavioral responses (which will be specified as other types of biomarkers).

The potential to use gene transcripts as biomarkers has been both recognized (Calzolai *et al.* 2007, Piña *et al.* 2007, Poynton & Vulpe 2009) and questioned (Forbes *et al*. 2006, Fent & Sumpter 2011) for over a decade. The conceptual idea is that certain transcripts might be used as early warnings by representing how the organism copes with chemical stress before harm arises at higher organizational levels, while other transcripts might be used to gain deeper mechanistic understanding of compound/mixture toxicity or the interaction between chemical stress and other stressors. Evaluation of a transcription-based biomarker candidate will thus be highly dependent on the specific question of interest, but there is a common set of key issues that need to be addressed to ensure adequate interpretation and meaningful practical application (Table 1).

Table 1. Key issues for transcriptional biomarkers intended for use in ecotoxicology. A qualitative evaluation and summary of the current state, based on criteria proposed by van der Oost *et al.* (2003).

Criterion	Current state
Reliability	Technical methods (e.g. RT-qPCR) are largely reproducible and reliable. Multi-marker assays can be made cost efficient.
Sensitivity	In general, sensitivity evaluation of biomarker candidates would improve from concentration-response studies. Sensitivity can be variable, and biomarker evaluation needs to be based on specific questions of interest.
Natural variability	Variably studied for different biomarkers. Baseline variation often poorly studied for most markers and species.
Confounding factors	Variably studied for different biomarkers. Some potential confounding factors often identified in general stress biomarkers.
Link: exposure – response	High-throughput sequencing can give deeper insights into mechanisms at a systemic level. Links are established for many defense systems and primary response pathways, but poor quantification often limits predictability.
Link: response – effects	High-throughput sequencing techniques and AOPs can give deeper insights into pathways involved in toxic harm. Links are established for some defense systems and primary response pathways, but often poorly quantified, in particular for effects at the population level and above.

Reliability

Transcriptional biomarker assays are commonly based on RT-qPCR, which allows high-resolution quantification of selected transcripts. RT-qPCR methods however involve a series of highly sensitive steps (e.g. sample handling, extraction and quality of RNA, primer design and assay efficiency), making transparency crucial for method reproducibility (Bustin *et al.* 2013). It has been demonstrated that pre-processing, e.g. tissue sampling, is the major source of variation in contrast to the quite small technical variation of the assays (Kitchen *et al.* 2010). Thus, reporting RT-qPCR assay conditions only is not sufficient for reproducibility. Complying with reporting guidelines such as e.g. MIQE (*M*inimum *I*nformation for publication of *Q*uantitative real-time PCR *E*xperiments) improves good practice and helps to ensure technical reliability of the assay (Bustin *et al.* 2009, 2010, 2013; Remans *et al.* 2014). Given well-designed assays and carefully planned sampling, transcriptional biomarker panels can be made reliable and reproducible. Cost efficiency can for instance be achieved by automation of various processing steps and by running multiple assays in parallel.

Sensitivity

Depending on the intended use, a biomarker response can either be universal to various chemical stressors and/or biological effects, or specific to certain groups of toxicants or adverse effects. Regardless, sensitivity to exposure/effects is crucial, and a distinguishable response at relevant exposure/effect levels is a minimum requirement for use as a biomarker (van der Oost *et al.* 2003). Furthermore, for practical use in environmental monitoring and ERA the biomarker needs to give a

somewhat consistent or predictable response related to the effects or level of toxicant exposure (van der Oost *et al.* 2003). Multi-biomarker models might to some extent compensate for a higher degree of variation and unpredictability of the separate markers (Baudou *et al.* 2019). Yet, the design, application and interpretation of multi-biomarker panels can improve greatly with increasing knowledge on the separate biomarker responses.

As previously mentioned, relatively few toxicological studies measure transcriptional responses at multiple (> 3) concentrations, and even fewer fit concentration-response curves. Numerous potential biomarker genes have been demonstrated to respond under various toxicant exposures, but many are suggested to follow non-monotonic concentration-response curves (Bigot *et al.* 2011, Smetanová *et al.* 2015, Arukwe *et al.* 2017, Martinez *et al.* 2018, Li *et al.* 2019, Zhang & Zhai 2020). Depending on the intended use and specific question of interest, monotonic responses might not be absolutely necessary. It will however still be required to know what response magnitudes to expect at a certain exposure level. Regardless of the ultimate application, concentration-response testing as a standard in the evaluation process of putative biomarkers would thus appear as an efficient way to ensure a certain necessary basic understanding.

Natural variation and confounding factors

Depending on intended biomarker use, baseline and response variation might need to be established both under field and laboratory conditions. For instance, seasonal fluctuation under field conditions has been observed in both stress gene expression levels (Farcy *et al.* 2007) and transcriptome profiles overall (Navarro *et al.* 2013), confirming the need of further investigation. Furthermore, it is often insufficiently addressed to what extent baseline gene expression varies with for instance species, sex and developmental stage (Fent & Sumpter 2011, Simmons *et al*. 2015, Bahamonde *et al.* 2016).

In many cases, part of the response variability can be attributed to specific external sources that in some way interfere with responses to chemical stress. Potential confounding factors such as temperature (Liu *et al.* 2014, 2016; Boukadida *et al.* 2017, Collins *et al*. 2021), pathogens (Burki *et al.* 2012, Liu *et al.* 2014, 2016), and additional toxicants, i.e. mixtures (Bigot *et al.* 2011, Gonzalez-Rey *et al.* 2014, Shen *et al.* 2020), have indeed been demonstrated to influence expression levels of various gene transcripts. Whereas controlled laboratory conditions limit the influence from external confounding factors in a particular experiment, a lacking understanding of their influence might risk incomparable results from studies with different setups (Martyniuk 2018). Even more so in environmental monitoring, where it might be necessary to compare individuals from different sampling locations, potentially sampled at different occasions. Identifying potential

confounding factors and quantifying their effect on the biomarker signal can therefore be crucial in the evaluation of biomarker candidates.

The links between exposure, response and effects

Provided appropriate experimental design, high-throughput sequencing and AOPs can allow deeper understanding of pathways and systemic networks involved in transcriptional responses to toxic stress, hence facilitating the identification and confirmation of mechanistically relevant biomarker candidates (Poynton *et al.* 2007, Kim *et al.* 2015, Corton *et al.* 2019, Martyniuk *et al.* 2020). Mechanisms involved in various defense systems and primary response pathways have been supported by observed transcriptional responses to model toxicants (Muncke & Eggen 2006, Hayes *et al.* 2007, Poynton *et al.* 2014, Regoli & Giuliani 2014). Similarly, some transcriptional pathways show links to effects at higher biological levels (Hussainzada *et al.* 2014, Zhang *et al.* 2016, Blalock *et al.* 2018). Even without mechanistic links, transcriptomic points of departure might in some cases be extrapolated, for instance, to predict chronic effect concentrations or to estimate no-effect concentrations at the whole organism level (Pagé-Larivière *et al*. 2019, Mittal *et al*. 2022). A major critique against biomarkers is however that that they can often not be meaningfully interpreted on their own without large amounts of additional information (Forbes *et al*. 2006). Ideally, biomarker selection should therefore be based on specific responses that can be readily extrapolated to predict effects at higher biological levels. If the intended application is narrow, it might for instance be required that the biomarker candidate responds with high specificity to certain types of effects or certain groups of toxicants. In those cases, biomarker selection could be facilitated by well-developed AOPs based on specific mechanistic understanding. In contrast, for early defense biomarkers involved in general stress responses, homeostatic pathways and defense mechanisms are often relatively well understood. In those cases, effort directed towards the quantitative link to harm might be more efficient for applied biomarker use, as compared to mechanistic investigation of downstream effects.

Study system

Bivalves as study organisms in ecotoxicology

As sedentary filter feeders, bivalves are ideal bioindicators for pollution under both field and laboratory conditions. With limited possibility of migration to unpolluted areas, exposure might be unavoidable, and toxicants present in the water column, dissolved or particulate, will eventually be taken up by filtration. For survival in polluted areas, mechanisms of coping with chemical stress can thus be crucial. Consequently, various aspects of toxicant uptake and effects have been addressed in bivalves, and it has been proposed that bivalve biomarkers show promise for development of AOPs and application in ERA (Khan *et al*. 2020). Numerous species have been used as study organisms, and to represent bivalves in general, the meta-analysis (**Paper IV**) included any species presented in studies selected through systematic review. By comparison to commonly used models such as the marine *Mytilus* spp., freshwater bivalves are however underrepresented (Binelli *et al.* 2015). Thus, throughout the experimental work (**Paper I-III**), the main focus was instead a native freshwater species, the duck mussel (*Anodonta anatina*).

The duck mussel (*Anodonta anatina*, fam. Unionidae)

The non-invasive *A. anatina* (Figure 2) is a unionid mussel native to, and widely distributed across, Europe and parts of western Asia (Lopes-Lima 2014, Lopes-Lima *et al.* 2017). It is a generalist occurring under oligotrophic to eutrophic conditions in ponds, lakes and rivers, preferably with sand or gravel substrates (Lopes-Lima 2014). With a distribution throughout the country, *A. anatina* is the most abundant large freshwater mussel species in Sweden (von Proschwitz & Wengström 2021), suggesting high ecological relevance for in particular Swedish and Scandinavian freshwater systems.

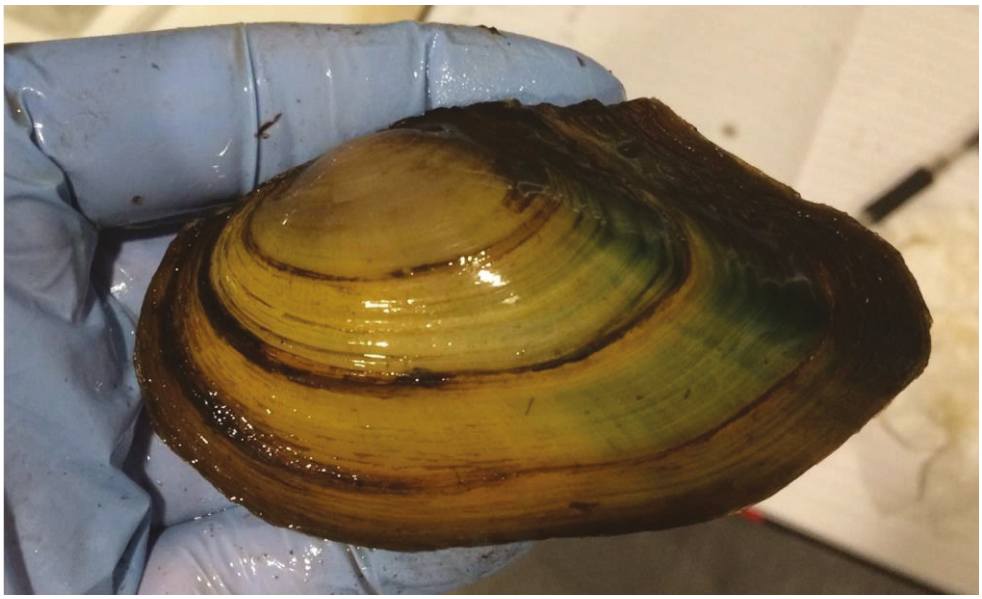

Figure 2. An adult specimen of duck mussel, *Anodonta anatina*, the most abundant species of large freshwater mussels in Sweden.

The occurrence of hermaphroditism in *A. anatina* is believed to be influenced by environmental factors, with river-dwelling populations primarily being dioecious (Hinzmann *et al.* 2013). The mussels spawn in early summer, and females (by function) brood eggs and larvae (glochidia) in their gills from late summer until late winter or early spring (Aldridge 1999, Hinzmann *et al.* 2013). Glochidia are then released into the water column and enter a parasitic life stage as they mature to juveniles in gills of host fish (Barnhart *et al.* 2008). Under laboratory manipulation, *A. anatina* glochidia have been shown to successfully infest a range of species such as perch (*Perca fluviatilis*), grass carp (*Ctenopharyngodon idella*), ide (*Leusiscus idus*) and brown trout (*Salmo trutta*) (Huber *et al.* 2019).

Although transcriptional responses have not previously been addressed in *A. anatina*, an increasing number of ecotoxicological field and laboratory studies have used the species to assess e.g. acute toxicity (Kováts *et al.* 2010, Oliveira *et al.* 2015), biochemical and cellular responses (Santini *et al.* 2011, Nugroho & Frank 2012a, 2012b; Falfushynska *et al.* 2013, 2014; Oliveira *et al.* 2015, Bielen *et al.* 2016, Abdelsaleheen *et al*. 2021), physiological and behavioral responses (Hartmann *et al*. 2016, Abramenko *et al*. 2021) and pollutant uptake (Nugroho & Frank 2011, Berglund *et al.* 2019, Abdelsaleheen *et al*. 2021, Weber *et al*. 2021).

Metal as a stressor

The main focus throughout this work has been on metals, selected to represent general toxicity. Metal toxicity commonly involves oxidative stress via generation of reactive oxygen species, with potential disruptive effects on for instance metabolism, cellular integrity and homeostasis, and immune responses (Le Saux *et al.* 2020). Specifically, copper (Cu) was used as the chemical stressor in **Paper I** and **III**. Despite being essential to biological life, excess Cu can generate oxidative stress, interact with cellular sodium homeostasis and in different ways interfere with energy metabolism (Brix *et al*. 2022). As a result, Cu is generally highly toxic to aquatic life and can cause adverse effects already in the low to moderate µg/L range (Brix *et al.* 2001, Durán & Beiras 2013).

In contrast to the other experimental studies, the exposure in **Paper II** was based on a wastewater effluent from a chemical industry, i.e. a mixture. This exposure was selected to represent a more relevant stressor from an applied perspective, and the main focus was the metal contents of the effluent.

Finally, the meta-analysis in **Paper IV** included all metals (single exposures under controlled laboratory conditions) to which transcriptional responses could be extracted from relevant scientific literature on bivalves.

Toxic unit

Toxic unit (TU) is a relative measure of toxicity that allows comparisons of different toxicants and exposures on the same scale, in particular if the toxicants have similar modes of action. It is calculated as a ratio of the exposure concentration of a specific toxicant and the corresponding effect concentration (EC_X) of a relevant endpoint, such as LC_{50}:

$$\mathrm{TU} = \frac{\text{Exposure concentration}}{\mathrm{EC_X}}$$

In **Paper II**, TU was used to estimate the cumulative (assumed additive) metal toxicity in the wastewater effluent, and to determine the relative contribution of different metals. In **Paper IV**, TU was used as a proxy for concentration in a meta-regression across studies, to address the general trends of concentration-dependent transcriptional responses in bivalves. In addition, to put all studies on the same scale for inter-study comparisons, measured water concentrations of Cu from **Paper I** and **III** were also converted to TU in the 'Main findings' section of this thesis.

Selected biomarkers

The primary focus throughout the thesis work was a set of six gene transcripts coding for proteins involved in cellular homeostasis by cytoprotection, toxicant metabolism and responses to oxidative stress: catalase (*cat*), glutathione-S-transferase (*gst*), heat shock protein 70 (*hsp70*), heat shock protein 90 (*hsp90*), metallothionein (*mt*) and superoxide dismutase (*sod*). These are all involved in some aspect of early defenses against general chemical stress (Le Saux *et al.* 2020), and have commonly been used as response genes in various types of (eco)toxicological studies on bivalves for the last decade (Bigot *et al.* 2011, Navarro *et al.* 2011, Gonzalez-Rey *et al.* 2014, Liu *et al.* 2014, 2016; Châtel *et al.* 2018, Hanana *et al*. 2018, Li *et al.* 2018, Lebordais *et al*. 2021). Selected transcripts were thus expected to respond to chemical stressors in general. Furthermore, the first two experiments (**Paper I-II**) included spectrophotometric measurements of acetylcholinesterase (AChE) and glutathione-S-transferase (GST) enzymatic activity as additional endpoints. Although not covered by the core objectives of the WaterAssess project and the thesis work, these biomarkers were added when it became apparent from the first experiment that observed transcriptional responses were generally ambiguous. Specifically, AChE and GST were selected to represent established biomarkers, and as enzymes they also represent a higher level of biological organization.

Superoxide dismutase and catalase

Superoxide dismutase (SOD) and catalase (CAT) are key enzymes for cellular protection against oxidative stress from reactive oxygen species. SOD constitutes a family of enzymes that use metals (e.g. Cu, Zn, Mn, Fe and Ni) as co-factors to scavenge superoxide radicals, resulting in a net production of hydrogen peroxide and oxygen (Zuo *et al.* 2015):

$$2\,H^+ + 2\,O_2^{\bullet -} \rightarrow H_2O_2 + O_2$$

Different isoforms of SOD are found in different cellular compartments, whereas CAT is mainly located to peroxisomes (Zuo *et al.* 2015). The latter is a heme-containing enzyme expressed in nearly all aerobic organisms, and catalyzes the decomposition of hydrogen peroxide to water and oxygen (Zuo *et al.* 2015):

$$2\,H_2O_2 \rightarrow 2\,H_2O + O_2$$

Due to their central role in regulation of oxidative homeostasis, enzymatic activities of SOD and CAT are commonly considered as biomarkers for broad application across various taxa, toxicants and exposure types (van der Oost *et al.* 2003, Rodrigues & Pardal 2014, Lehtonen *et al.* 2016, Suman *et al.* 2021, Ding *et al.* 2022, Qu *et al.* 2022, Telahigue *et al*. 2022). Similarly, gene expression of *sod* and *cat* has

been addressed in a range of (eco)toxicological studies, including various studies on bivalves (Bigot *et al.* 2011, Navarro *et al.* 2011, Gonzalez-Rey *et al.* 2014, Châtel *et al.* 2018, Li *et al.* 2018, Louis *et al.* 2021).

Glutathione-S-transferase

GST is a family of cytosolic and mitochondrial enzymes also involved in protection against oxidative stress. The most prominent role of GST is to deactivate/detoxify electrophilic toxicants by catalyzing conjugation to reduced glutathione, an antioxidant peptide with a nucleophilic thiol (-SH) group (Blanchette *et al.* 2007):

$$\text{Toxicant} + \text{Glutathione}-\text{SH} \rightarrow \text{Toxicant}-\text{S}-\text{Glutathione} + \text{H}^+$$

By interacting with a wide range of potential stressors, both GST activity (van der Oost *et al.* 2003, Rodrigues & Pardal 2014, Suman *et al.* 2021) and *gst* expression (Krueger *et al.* 2022, Park & Kwak 2022, Zhang *et al.* 2022) have been used as biomarkers for various taxa and exposures, including bivalves (Bigot *et al.* 2011, Navarro *et al.* 2011, Gonzalez-Rey *et al.* 2014, Lehtonen *et al.* 2016, Châtel *et al.* 2018, Li *et al.* 2018).

Heat shock proteins 70 and 90

Heat shock proteins are a family of molecular chaperones that, apart from various regulatory functions, protect cellular proteins against damage from stressors such as heat and toxicants (Lang *et al.* 2021). Heat shock proteins 70 and 90 (Hsp70 and Hp90, designated by their respective molecular weights) are key components in the heat shock protein network, and both play important roles for refolding damaged proteins to their native and functional conformation (Lang *et al.* 2021).

Due to their functions in protein homeostasis, Hsp70 and Hsp90 are considered as potential biomarkers for different types of stressors. As suggested by the name, these proteins can be induced by exposure to high temperatures or by seasonal variation in temperature (Snyder *et al.* 2001, Dimitriadis *et al.* 2012, Madeira *et al*. 2018, Nguyen *et al.* 2021). Moreover, both Hsp70 and Hsp90 have been demonstrated to respond to various chemical stressors (Snyder *et al.* 2001, van der Oost *et al*. 2003, Moniruzzaman *et al.* 2020, Pereira *et al.* 2021). Thus, numerous studies have also addressed the use of *hsp70* and *hsp90* transcripts as biomarkers of toxicity. In the case of bivalves, these transcripts have been studied in a range of species under various toxicant exposures (Navarro *et al.* 2011, Liu *et al.* 2014, 2016; Châtel *et al.* 2018, Gao *et al.* 2022).

Metallothionein

Metallothionein (MT) is a group of cysteine-rich proteins that primarily protect the cell against metal imbalances, by binding to and regulating cellular levels of essential metals (e.g. Cu and Zn), and by sequestering non-essential, toxic metals such as Cd and Hg (Klaassen *et al.* 1999, Amiard *et al.* 2006, Ruttkay-Nedecky *et al.* 2013). Common to all MTs is a high content of cysteine that provides multiple thiol (-SH) groups as binding sites for metals (Klaassen *et al.* 1999, Amiard *et al.* 2006). Furthermore, by scavenging reactive oxygen species, the thiol groups also give MT direct functions as an antioxidant (Ruttkay-Nedecky *et al.* 2013). In fact, although the induction of MT has been demonstrated in a range of taxa upon exposure to metals and metalloids (Amiard *et al*. 2006, Araujo *et al*. 2022, Yang *et al*. 2022), the same has also been demonstrated for other types of toxicants and mixtures (Araujo *et al*. 2022, Trabelsi *et al*. 2022, Trombini *et al*. 2022). Overall, this implies that MT as a biomarker might not be specific to metal exposures.

Amiard *et al*. (2006) suggested that '*bivalves are most probably the best candidates for biomarker programmes involving MT concentrations as biomarkers*', and consequently, MT content, as well as gene transcription of *mt*, have been addressed in numerous ecotoxicological studies on bivalves (Jenny *et al*. 2004, Bigot *et al.* 2011, Navarro *et al.* 2011, Châtel *et al.* 2018, Gao *et al*. 2021, Lebordais *et al*. 2021, Telahigue *et al*. 2022).

Acetylcholinesterase

Animal neurosignaling is highly dependent on chemical neurotransmitters, of which acetylcholine is the most prominent (Čolović *et al*. 2013). Upon nervous transmission, the enzyme AChE ensures acetylcholine turnover in the synaptic cleft by catalyzing hydrolysis to inactive choline and acetic acid (Bocquené & Galgani 1998, Čolović *et al*. 2013):

$$\text{Acetylcholine}^+ + H_2O \rightarrow \text{Acetic acid} + \text{Choline}^+$$

Consequently, inhibition of AChE will result in a build-up of acetylcholine, which in turn can lead to hyperstimulation, loss of muscular control and, eventually, death (Bocquené & Galgani 1998, Čolović *et al*. 2013). For instance, many pesticides are designed to act by this mode of action, and hence, AChE activity has long been used as a biomarker of pesticide exposure (Bocquené & Galgani 1998, Kaushal *et al*. 2021). However, it has been shown that AChE activity can also be affected by toxicants that do not specifically target the enzyme (Guilhermino *et al*. 1998, Vieira & Nunes 2021). In bivalves as well as other organism groups, AChE activity has therefore also been assessed as a general biomarker of toxicity and chemical stress (Gonzalez-Rey *et al.* 2014, Rodrigues & Pardal 2014, Lehtonen *et al.* 2016, Suman *et al.* 2021, Qu *et al*. 2022, Trabelsi *et al*. 2022).

Methods

This section presents the major outlines of the exposure setup and biomarker assays used across the experiments (**Paper I-III**), and of the systematic review and meta-analysis (**Paper IV**). For detailed protocols, and for minor differences in exposure setup and experimental design between studies, the reader is referred to the respective papers.

Experimental setup

All mussels used in experiments were collected in a freshwater stream, Vinne å, in southern Sweden (56°06'45" N, 13°54'35" E). The stream is without known point source pollution, but is adjacent to human settlement and a lake subject to recreational use (e.g. fishing and swimming). Mussels are abundantly occurring and can be easily collected by hand (Figure 3), e.g. using an aquascope for visual detection.

Figure 3. Vinne å, the freshwater stream from which *A. anatina* were collected for the experimental studies. The bottom substrate is largely covered in mussel shells (right) as a result of high abundance of mussels in the stream.

To distinguish *A. anatina* from the co-occurring, closely related swan mussel (*Anodonta cygnea*), species determination was performed *in situ* (mainly based on shell morphology), and confirmed after observing collected mussels in the lab (based on siphon and soft tissue morphology). After acclimatization to laboratory conditions, mussels (n = 20-40) were individually exposed to Cu or an industrial wastewater effluent for 96 h (Figure 4). Immediately after the exposure phase, mussels were dissected to preserve gills and digestive glands for biomarker assessment and chemical analyses. Gills were visually inspected for eggs/glochidia, and their presence was used to determine gravidness (occurring in **Paper I** and **II**, for which experiments were performed in autumn) (Figure 5).

Figure 4. In all experiments, mussels were individually exposed to a chemical stressor for 96 h prior to dissection of gills and digestive glands for biomarker and chemical analyses.

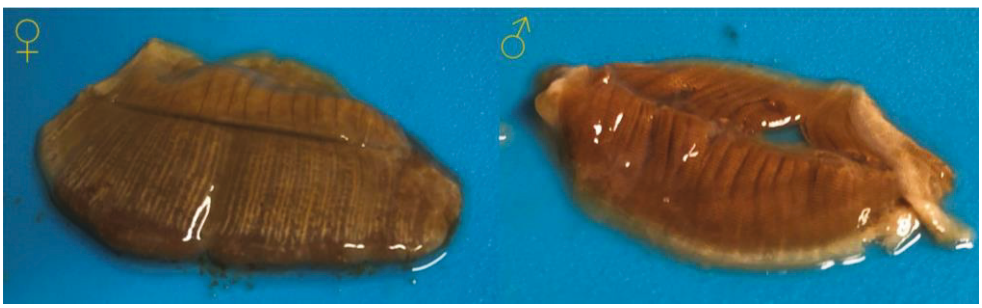

Figure 5. Gills from gravid (left) and non-gravid (right) mussels. Gravidness in *A. anatina* occurs from late summer to late winter or early spring and can be determined from gills by the swollen tissue and visible presence of eggs/glochidia.

Toxicant exposures

Exposures were in each study performed in dilutions of reconstituted freshwater (ISO 6341:2012), that was also used for negative control treatments. For **Paper I** and **III**, Cu exposures were prepared from stock solutions of $CuCl_2 \bullet H_2O$. Specifically, mussels in **Paper I** were exposed to three environmentally relevant concentrations of Cu (nominally 1, 10 and 100 µg/L). In **Paper III**, the setup was a high-resolution concentration-series covering a major portion of the entire sublethal spectrum (nominally 1 to 2 000 µg Cu/L at 2-fold increments in twelve steps). For **Paper II**, frozen samples of wastewater effluent were received from a chemical industry. Thawed samples were then mixed flow-proportionally to represent an average weekly contaminant load, and diluted to 60 % in reconstituted freshwater. After each of the experimental studies, chemical analysis laboratories were employed to analyze total metal contents in both exposure media and tissue samples (see **Paper I-III** for details).

In environmental toxicology, bioavailability can be defined as the fraction of a toxicant that is '*taken up from the environment and is available to cause a biological response*', but the term is more often used to refer to estimated amounts in the exposure matrix that would be readily available for accumulation upon encountering biota (McLaughlin & Lanno 2014). For instance, metal bioavailability from water is often estimated by incorporating various water parameters into 'biotic ligand models', to predict affinity and uptake at target sites on the organism (Niyogi & Wood 2004). Using Cu as an example, organic carbon in the water phase can act as a ligand to reduce uptake and toxicity, and water hardness and pH are commonly negatively correlated to observed Cu toxicity (Erickson *et al*. 1996, Markich *et al*. 2003, Gillis *et al*. 2008, Wang *et al*. 2009, Giacomin *et al*. 2013). Rather than estimating the bioavailable fraction based on water parameters and/or using dissolved metal concentrations, each study however measured the total concentrations in water and mussel tissues. Aware that this approach has its limitations, it was still decided to be a more sensible proxy for overall exposure due to the nature of the study organism. By filter feeding, bivalves risk exposure to both dissolved and particulate toxicants present in the water column, and bottom-dwelling species such as *A. anatina* can risk additional exposure via contaminated sediment. In fact, the organic content in sediments will influence metal partitioning, and sand (used as bottom substrate in **Paper I-II**) can adsorb metals and, to a certain extent, remove them from the water phase (Hassan *et al*. 1996). This appeared to be the case particularly for Cu in **Paper I**, and hence, the sand substrate was replaced with presumably inert glass beads in **Paper III**.

Furthermore, the wastewater effluent in **Paper II** contained a large fraction of organic carbon in addition to a mixture of various metals. Although assumed non-toxic in itself (as previously assessed by the chemical industry), the organic content certainly influenced metal speciation and bioavailability to some extent.

Consequently, the assumptions on metal toxicity in **Paper II** are simplifications to serve as a proxy for mixture toxicity overall. For proper understanding of actual bioavailability in bivalves upon metal exposures, and particularly for mixtures, it can be necessary to specifically target metal speciation as well as presence of potential ligands and competing cations in both exposure water and internal tissues, but that was beyond the scope of this thesis.

Exposure time

Ideally, at least one experiment should have been carried out over a time series of different exposure times, in order to model concentration-time-response relationships. This objective was however ultimately down-prioritized in favor of the concentration-response setup, and hence, acute exposures of 96 h were performed throughout the experiments. First, this exposure time puts the experiments in a reasonable frame of reference. Metal toxicity has previously been studied in adult *A. anatina* under 96 h exposures (Oliveira *et al*. 2015), and this is a common exposure time for acute toxicity tests on both bivalves and other aquatic taxa such as fish. Second, responses at 96 h were considered to be a trade-off between immediate, transient responses and long-term adaptations to chronic stress.

Gene expression

Transcriptional responses in *A. anatina* were assessed by RT-qPCR. RNA extracted from individual gill and digestive gland samples were used to synthesize cDNA as a template for qPCR-assays. The assays were in turn based on primers designed for amplification of the target genes in unionids (Table 2), and on SYBR® Green-based detection. For each experiment, relative gene expression of target genes was determined in each tissue by the $2^{-\Delta\Delta}$Ct method (Livak & Schmittgen 2001), using the mean of the control group for between-sample normalization, and reference genes (β-actin and 28S rRNA, and additionally 18S rRNA in **Paper III**) for within-sample normalization.

Table 2. Primer and amplicon details for RT-qPCR assays used to determine stress gene expression in *A. anatina*. Primer design was in each case based on reference sequences from at least four bivalve species, with a minimum of one unionid. Amplicon sequences were determined by Sanger sequencing of one amplicon sample from each respective assay. For the amplicon sequence, bold text indicates primer binding sites. Nucleotides that differed between forward and reverse strands are shown within parentheses, and underlined text indicates a gap in the sequence of either strand.

Gene	Primer sequence (5'-3')	Amplicon sequence (5'-3')	Reference sequences (accession number)
cat	forward: GGAAGACTGACCAGGGTAT reverse: CCTCAGCGATGGCATTGTA	**GGAAGACTGCACCAGGGTAT**(C/T)AAAAACTTAC(A/C)(A/C)GCAGAGA(A/C)AAAA(C/G)CAG(C/T)GAAATGGC(C/T)(A/G)GCA(A/T)GACCCTGG(A/T)TA(A/T)TCCATA(A/G)A(A/G)(A/G)ACCCTGG**TCAATGCCCTCGCTGAGG** (111 bp)	HQ148707.1, KU363383.1, FJ608579.1, EU145723.1, HM188565.1, EU407490.1
gst	forward: GTCCAACACCATGCTGAG reverse: GTAGTCCTCCACTCCATCAT	**GTCCAACACCATGCTGAG**GTACTT(A/G)GGAAGAAA(A/G)CA(A/T)GATCTGTACGGTGGTGACGTCAAGGAAGGTGCTCTTATTGATATGATTA**ATGATGGAGTGGAGGACTAC** (106 bp)	HQ166721.1, AY885666.1, EU145724.1, EF194203.1, AY557404.1, AJ557140.1
hsp70	forward: GGTATTGAGACGGCTGGT reverse: CACACCAGGCTGGTTGTC	**TATTCACACCCCT**CCTCC(A/G)(C/G)T(A/T)AT(A/C)ACTC(C/G)CCT(A/G)TCAACA(A/C)(A/G)(A/G)A(A/C)ACCACCATTCCCACTAAACA(C/G)AC(C/G)CA(C/G)ACCTTTACTACC(C/T)(A/C)C**TCCGACAACCAGCCT** (102 bp)	KX758099.1, HQ148706.1, KT923183.1, AF172607.1, EF526096.1, AB122063.1, KJ123764.1
hsp90	forward: TACCATTGCCAAATCTGG reverse: ACACCAAACTGCCCAATCA	**TACCATTGCCAAATACT**CGAACGAA(A/G)GC(A/T)(A/T)T(G/T)(A/T)GGAAGCCCTGCAGGCAGGTGCAGAATCTCC(A/G)**TGATTGTGGCAGTTTGGTGT** (84 bp)	HQ180224.1, GU433881.1, EF687776.1, KR633143.1
mt	forward: ATGCAACTGCCTTGAGAC reverse: ACTTTACATCCAGGACACTT	**ATGCAACTGCCTTGAGAC**CGGAGAATG(A/T)AAATGTTCAGGTGAC(A/T)GTACTACTGGGAGCCTGCAGATGTGGGGATGCCTGTAAATGTGGGGATGCCTG(A/C)**AAGTGTCCTGGATGTAAAGT** (118 bp)	EF185127.1, GQ184290.1, KJ019820.1, KJ019821.1, U67347.1, AJ577130.1, AJ577131.1, AJ243263.1
sod	forward: GCTACGGTCATTCCACTCT reverse: CCAGTTATCTCACCAGTTATGTTC	**GCTACGGTCATTCCACTCT**TTTATATAACCGA(A/T)AACGCAAGTTAGACCTGCATAAACCGCCATGTCCATTAAGGCTGTTTGCGTACTGAGGGGTGACAGT(A/G)AAGTTAAAGGAACTGTCAAG(G/T)TTTTACAAGAGGGAAGTGGTTCA(A/G)T**GAACATAACTGGTGAGATAACTGG** (171 bp)	FJ194441.1, KU363382.1, FJ608580.1, KT724303.1, EU145730.1, AY377970.2

It is often stressed that high RNA integrity is necessary for downstream applications. For instance, RNA quality or integrity numbers (RQN and RIN, respectively) of >5 have been suggested as a minimum for RT-qPCR (Fleige & Pfaffl 2006, Becker *et al.* 2010). Measures such as RQN and RIN however rely on intact 28S rRNA, and do not account for the 'hidden break' that occurs in various invertebrates and can cleave their 28S rRNA into two fragments of approximately the same size as 18S rRNA (Natsidis *et al*. 2019, Adema 2021). Consequently, such measures will potentially underestimate RNA integrity in bivalves (Adema 2021). In fact, from visual inspection of RNA integrity on gels and electropherograms, it appeared that the band or peak corresponding to 28S rRNA was consistently absent or very weak in *A. anatina* samples, which is to expect if the 28S hidden break hypothesis is true. Judging by RQN scores, it also appeared that seemingly intact RNA would at best yield scores that imply semi-intact RNA (i.e. approximately 4-6), and that partially degraded samples can yield similar scores as heavily degraded RNA (Figure 6).

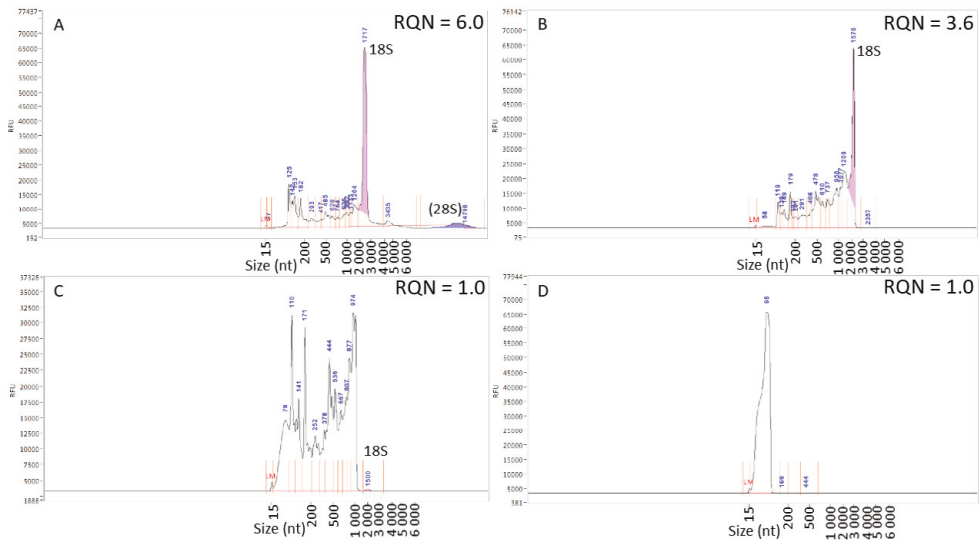

Figure 6. Electropherograms and RNA Quality Numbers (RQNs) of *A. anatina* RNA. The top panel (A, B) displays two samples of what appeared to be relatively intact RNA, with RQN scores however implying moderate (A) to relatively high (B) degradation. Both samples (A, B) showed clear 18S rRNA peaks (pink) at 1 600 – 1 700 nucleotides, and no large peaks at short fragments. In (A), a weak peak at 15 000 nucleotides was identified as 28S rRNA (purple), whereas no 28S peak was identified in (B). The bottom panel shows two samples of what appeared to be partially degraded RNA with multiple peaks at moderate fragment lengths (C), and heavily degraded RNA with a single peak at short fragments (D). Despite difference in appearance, both samples received RQN scores of 1.0, implying the same amount of heavy degradation.

RNA integrity in the *A. anatina* samples was assessed both quantitatively and qualitatively. To ensure as high sample quality as possible, re-extractions were generally performed for samples demonstrating for instance low RQN, absent 18S peaks and/or 'smear' at short fragment lengths. Despite this, the studies on

A. anatina often relied on RNA demonstrating low RQN scores (<5). If truly present, RNA degradation would indeed inflate variation in the response data, but apart from that, the influence from degradation would in our studies be reduced by the facts that (1) relative, and not absolute, gene expression was addressed, and (2) target sequences were short (<250 bp) (Fleige & Pfaffl 2006). Ultimately, RNA quality never differed notably between treatment groups in any of the experiments, and hence, any artifactual variation from potential RNA degradation was considered as inherent to the methodology.

Enzyme activity

Enzyme activities of AChE and GST were determined according to assays already established at the Toxicon laboratory where exposure experiments were performed. In short, enzyme activity was determined spectrophotometrically according to protocols modified from Bocquené and Galgani (1998) and Habig *et al.* (1974), and normalized to protein content according to the method described by Bradford (1976). To enable comparisons on the same relative scale as transcriptional responses, enzyme activities were also normalized to the mean of corresponding control treatment samples.

Meta-analysis

To evaluate the general trends of transcriptional biomarkers across bivalve species and exposure scenarios, a systematic review was performed on scientific literature to extract response data for a series of quantitative meta-analyses (**Paper IV**). The systematic review targeted published literature available from scientific databases (Web of Science and Scopus), based on specific search terms and a set of inclusion criteria. For inclusion, bivalves had to be exposed to a single metal under controlled laboratory conditions, and individual responses for one or more of the selected transcripts (*cat*, *gst*, *hsp70*, *hsp90*, *mt* and *sod*) had to be measured by RT-qPCR. A total of 22 studies were selected, from which transcriptional effect sizes (n = 396) were extracted.

Using Bayesian mixed effects models, the effects of metal exposure on transcriptional responses were tested both overall, and for the specific transcripts and tissues. Furthermore, the general trends in concentration- and time-dependence of responses were addressed by performing meta-regressions, using TU (as a proxy for concentration) and exposure time as moderators. Finally, the potential impact from publication bias in the literature was estimated, using a meta-regression-based method proposed by Nakagawa *et al.* (2022).

Main findings

Response magnitude and variation

Generally, the experimental studies (**Paper I-III**) offered little support for robust biomarker responses upon arbitrary metal exposure (Figure 7). Without subdivision by transcript, tissue or exposure, the datapoints centered approximately around the mean of corresponding control treatments, i.e. a relative response of zero (Figure 7). In fact, no single transcript in neither gills nor digestive glands responded to general exposure unless accounting for interactions with gravidness (**Paper I-II**), or considering concentration-response relationships (**Paper III**) (discussed further under 'Gravidness in *A. anatina*' and 'Concentration-dependence', respectively). The average modelled response magnitudes in *A. anatina* were consistently smaller than a 2-fold change, except for *hsp70*, *hsp90* and *mt* in gills at high Cu concentrations in **Paper III** (Figure 8C-E). Although responses were generally somewhat larger in gills than in digestive glands, the same was also true for random variation. Individual responses (i.e. single datapoints) ranged between a 9.7-fold decrease to a 20-fold increase relative the non-exposed control (Figure 7), and there was considerable individual variation in *A. anatina* transcriptional responses in relation to the generally small response magnitudes.

In contrast to the experimental studies, the meta-analysis (**Paper IV**) demonstrated an overall effect of an approximately 65 % increase, as determined by the bulk of published scientific literature on metal exposed bivalves (Figure 7). This effect was however likely inflated by a bias towards publication of positive results. Similar patterns were also observed when subdividing transcripts and tissues. That is, despite initial implications of positive effects in most transcripts and tissues, the estimated 'true' effects were consistently close to zero when adjusting for publication bias (**Paper IV**).

As compared to *individual* response magnitudes in *A. anatina*, the meta-analysis revealed an even wider range of *average* responses reported in the literature, ranging from an 18-fold decrease to a 460-fold increase (Figure 7). Considering the general lack of strong response trends across both our experimental studies and meta-analysis, it therefore appears that random variation adds substantial background noise on varying scales, potentially affecting comparisons both within specific and between different exposure scenarios (choice of study species, toxicant, exposure conditions).

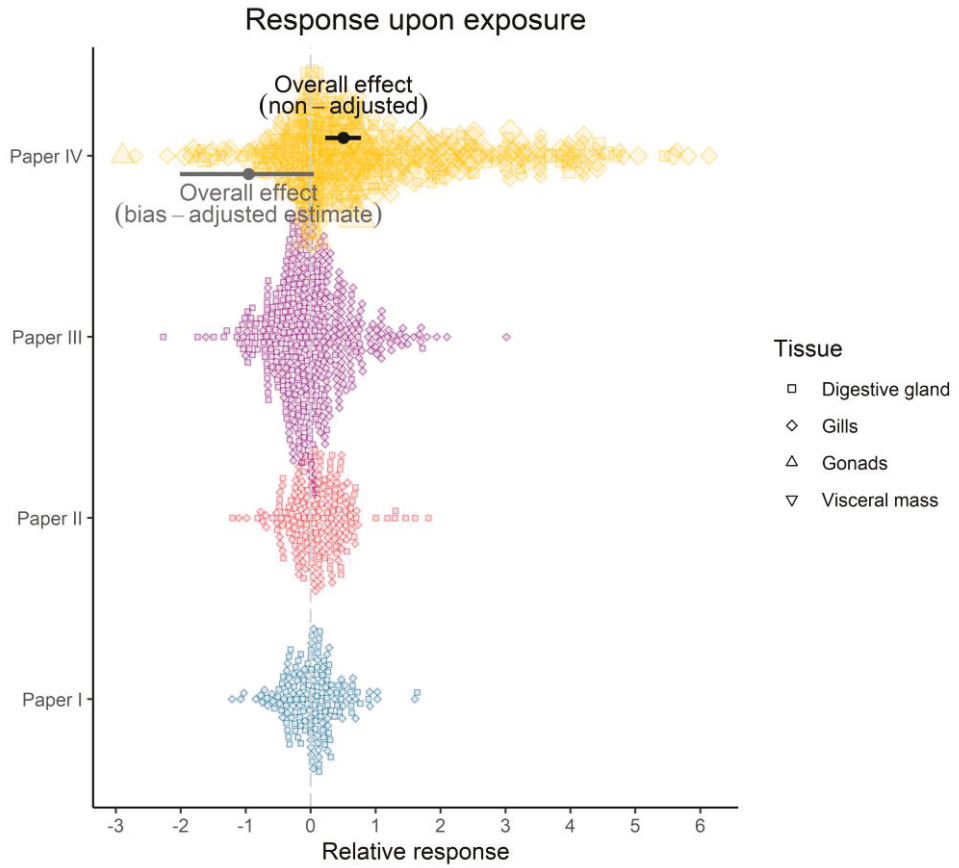

Figure 7. The transcriptional response in bivalves under exposure to metal or a wastewater effluent. The overall effect from the meta-analysis on metal exposed bivales (**Paper IV**) is denoted with 95 % credible intervals (black shows the main model estimate, grey shows an estimate adjusted for publication bias). Relative response corresponds to fold-change (ln-transformed; **Paper I-III**) or log response ratio (lnRR; **Paper IV**) relative a non-exposed control group. Each datapoint corresponds to one tissue sample from an individual *A. anatina* (control group individuals excluded; **Paper I-III**) or a mean effect extracted from literature (**Paper IV**). For **Paper IV**, point size is proportional to its relative weight in the meta-analysis.

Concentration-dependence

Due to its influence on predictability, a central question for potential biomarker application is how responses vary with exposure intensity, such as toxicant concentration. In Cu exposed *A. anatina*, all transcripts except *sod* demonstrated monotonic concentration-response relationships relative measured Cu in both water and tissue (Figure 8, **Paper III**).

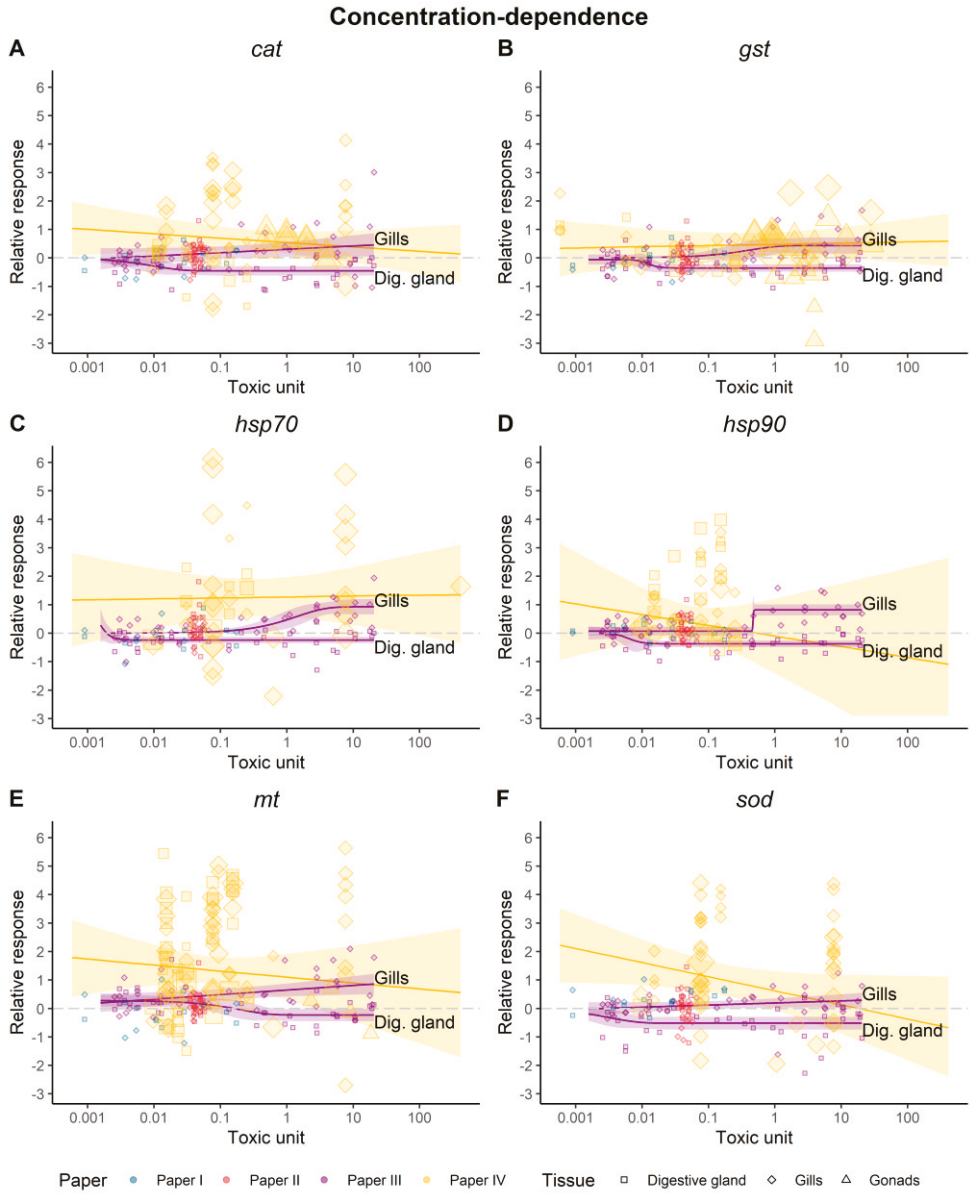

Figure 8. Concentration-dependence of transcriptional responses in metal exposed bivalves as determined by concentration-response curve-fitting (**Paper III**) and meta-regression (**Paper IV**). Although transcripts in Cu exposed *A. anatina* commonly demonstrated monotonic concentration-response relationships (**Paper III**), the relative changes in expression were generally small. On larger scales (between species, toxicants, etc.), such relationships can easily be obscured by background variation, as demonstrated by the meta-regressions (**Paper IV**). Relative response corresponds to fold-change (ln-transformed; **Paper I-III**) or log response ratio (lnRR; **Paper IV**) relative a non-exposed control group. Toxic unit was used as a proxy for concentration to normalize different metals to a common scale based on toxicity to bivalves. Each datapoint corresponds to one tissue sample from an individual *A. anatina* (control group individuals excluded; **Paper I-III**) or an effect size extracted from literature (**Paper IV**). For **Paper IV**, point size is proportional to the relative weight in the meta-regression. Datapoints from **Paper I** and **II** are included for reference only.

The general trend was that response signals in *A. anatina* gills increased with increasing water Cu, although no modelled response magnitude exceeded a 2.5-fold increase even at very high exposure concentrations (Figure 8). For instance, Cu exposed *Mytilus californianus* have previously demonstrated a similar concentration-response relationship for *hsp70* in gills, including a close approximation of EC_{50}, although saturating at a higher response magnitude of approximately a 6-fold increase (Hall *et al*. 2020). Furthermore, the exposure of *A. anatina* demonstrated implication of concentration-dependent Cu uptake into gills, and in turn, several transcripts revealed strong concentration-response relationships relative the internal gill Cu concentration as well (**Paper III**). Digestive gland transcripts on the other hand generally demonstrated only minor changes (Figure 8), possibly due to a low uptake and internal exposure in this tissue (**Paper III**).

In contrast to experiments on *A. anatina*, the meta-analysis (**Paper IV**) did not reveal any general trends of concentration-dependence, neither for metal exposed bivalves overall nor for tissue-specific responses. The same was also true for all separate transcripts except *sod*, for which the literature data suggested decreasing response magnitudes with increasing exposure (Figure 8F). Interestingly, another recent meta-analysis on earthworms demonstrated concentration-dependence of *mt* for metal exposures, but not for *hsp70* upon exposure to organic contaminants (Swart *et al*. 2022). However, measures of variation were not considered in the models used in this study, suggesting it is plausible that heterogeneity and random variability could in practice obscure or attenuate *mt* responses in earthworms as well. Regardless, the overall implication from **Paper III** and **IV** is that monotonic concentration-response relationships to toxicant exposure are not necessarily unlikely, but there is a risk of insufficient robustness for detection across scales in heterogenous datasets (Figure 8). In practice, successful detection or prediction of such responses might therefore require highly specific, high-resolution exposures of single, or small and well-defined groups of, species, tissues and toxicants.

Time-dependence

Exposure time is another important variable with potential effects on toxicity and exposure intensity, and consequently, on biomarker signal. As the work of **Paper I-II** progressed and positive results were largely absent, initial intentions to also include time-response relationships in the experimental setups were ultimately down-prioritized in favor of high-resolution concentration-response relationships. Ultimately, all of the experimental exposures were performed for 96 h, and variation of exposure time was only considered in the meta-analysis (**Paper IV**), where it was included as a moderator of response magnitude. According to the overall meta-regression, there was a slight effect from exposure time, with the overall response increasing somewhat with longer exposures (Figure 9). Similar, although commonly

weaker, trends were also observed when subdividing responses by tissues and transcripts (**Paper IV**).

Considering cross-interactions and translation of effects to higher biological levels, transcriptional time-dependence is most certainly more complex than what could be captured by these simplified models. Similar to concentration-dependence, the heterogenous data used in the meta-analysis is likely to obscure various fine-tuned response patterns that could be detected in high-resolution time-series of more specific exposure scenarios (species, toxicant, concentration). Even within single cells, mRNA numbers are prone to high fluctuation over short time spans (Wang *et al*. 2019c), which in practice introduces background noise already at the most basal levels. For biomarker application, it is ultimately crucial that the response signal is predictable, and preferably somewhat stable, over time. Previous studies have however demonstrated response peaks between a few days and a few weeks of metal exposure (Fang *et al*. 2010, Liu *et al*. 2014, Bao *et al*. 2018). From that perspective, results from the meta-regression imply that despite an inherent variability, success of detecting transcriptional responses would generally be most likely after at least a few days of exposure, as compared to short exposures of for instance a few hours.

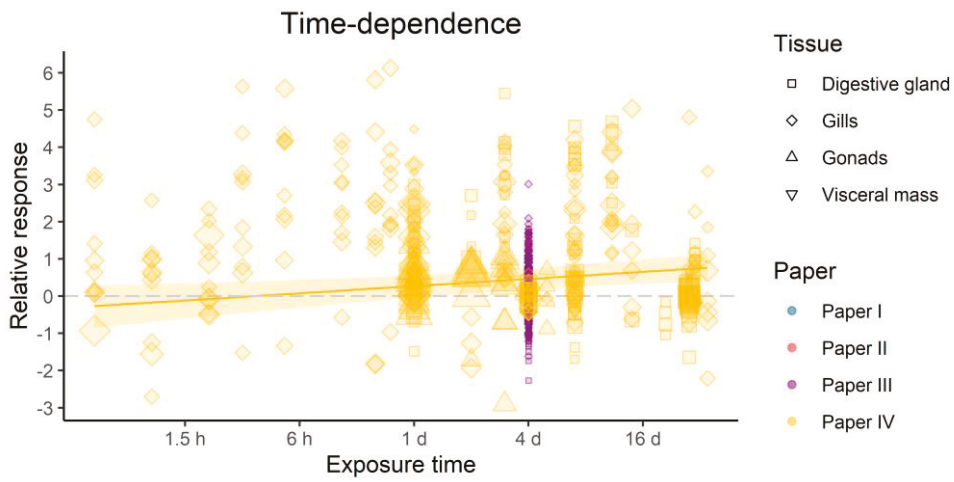

Figure 9. Time-dependence of transcriptional responses in metal exposed bivalves as determined by meta-regression in **Paper IV**. Response magnitude increased slightly with exposure time, suggesting that robust biomarker responses are somewhat more likely captured after a few days of exposure than within hours. Experimental exposures (**Paper I-III**) were all performed for 96 h, and corresponding datapoints are included for reference only. Relative response corresponds to fold-change (ln-transformed; **Paper I-III**) or log response ratio (lnRR; **Paper IV**) relative a non-exposed control group. Each datapoint corresponds to one tissue sample from an individual *A. anatina* (control group individuals excluded; **Paper I-III**) or an effect size extracted from literature (**Paper IV**). For **Paper IV**, point size is proportional to the relative weight in the meta-regression.

Variability in relation to biochemical biomarkers

In addition to transcriptional responses, **Paper I-II** included two biochemical biomarkers: enzymatic activities of AChE and GST. A major reason for including these responses was to use them as a point of reference for the biomarker evaluation of selected transcripts. With respect to random variation, the median coefficient of variation (CVs) in *A. anatina* gills was 36-46 % overall, with no apparent differences with regard to treatment or biomarker type (Figure 10A). By comparison, variation was somewhat smaller in digestive glands (overall CVs of 20-37 %). A trend of large variation was however observed in digestive gland transcripts of wastewater exposed mussels (Figure 10B). This in turn appeared to notably influence the overall variation in exposed mussels from **Paper II**. Apart from this potential effect from the wastewater effluent, the total variation (natural and technical) was however similar between transcriptional and biochemical responses in general.

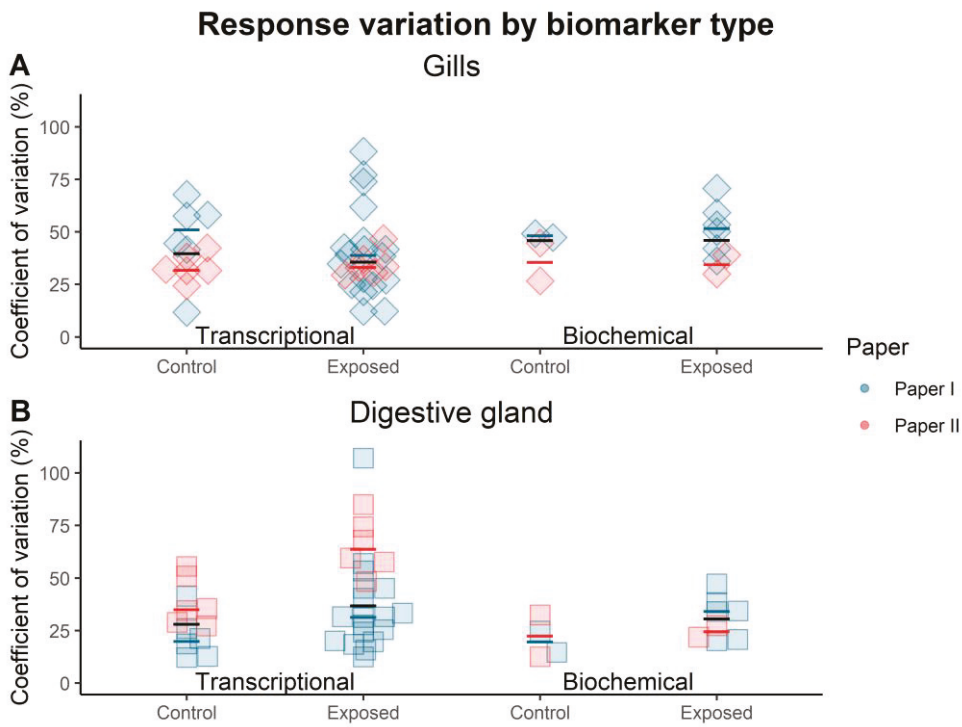

Figure 10. Variation in signal of transcriptional and biochemical biomarkers in (A) gills and (B) digestive glands of *A. anatina* exposed to Cu (**Paper I**) or an industrial wastewater effluent (**Paper II**). Variation was generally in the same order of magnitude for transcriptional and biochemical biomarkers in both tisues. Bars denote the median coefficient of variation (CV) per exposure group (black shows the overall median, blue and red show the median for **Paper I** and **II**, respectively). Each datapoint corresponds to one of six transcriptional (*cat*, *gst*, *hsp70*, *hsp90*, *mt* and *sod*) or two biochemical (AChE and GST) biomarkers in a specific exposure group.

Furthermore, gill AChE was the only biomarker demonstrated to respond to toxicant exposure (wastewater, **Paper II**) unless accounting for interactions with gravidness. Even so, the detected response magnitude was a modest 40 % increase that, in fact, even contradicted the expected inhibition, as predicted from a preliminary dose-finding experiment (**Paper II**). Moreover, both GST and AChE appeared potentially affected by gravidness in *A. anatina*, similar to most of the transcriptional responses (discussed further under 'Gravidness in *A. anatina*'). Similarly, considerable variation has previously been demonstrated for these and other biochemical biomarkers in various taxa, with regards to for instance season, lifecycle stage and external environmental factors (Domingues *et al*. 2015, Scarduelli *et al*. 2017, Benito *et al*. 2019). Consequently, from baseline expression/ activity up to at least low or moderately toxic exposures, transcriptional and enzymatic endpoints generally appear to face similar challenges of random variation potentially obscuring biomarker responses.

Gravidness in *A. anatina*

In the first two studies (**Paper I-II**), exposures were performed in fall, during which gravid *A. anatina* females nurture eggs and glochidia in compartments of their gills (Aldridge 1999, Hinzmann *et al.* 2013). Not surprisingly, potential effects from gravidness were most prominent in gills (Figure 11), although in a few cases also apparent in digestive glands (AChE in **Paper I-II**) or independent of tissue (*sod* in **Paper I**). In either of the two studies, all biomarkers apart from *gst* demonstrated effects from gravidness (Figure 11). **Paper II** even revealed a clear difference in *hsp90* between the control and exposed group, that is, an interaction between gravidness and the wastewater exposure (Figure 11E). Furthermore, there appeared to be a general tendency of slightly higher baseline signals in gravid *A. anatina*, and potentially also larger response magnitude upon exposure (Figure 11A). With the potential exception of *hsp70* (Figure 11D), this trend was however not consistent when subdividing transcripts and studies. Similar, seemingly random, patterns of variability between sexes (although not attributed to gravidness) have previously been demonstrated in for instance *Mytilus galloprovincialis*. In this species, *cat*, *gst*, *hsp70*, *mt* and *sod* have all been demonstrated to potentially differ between males and females, either in response to Cr(VI) exposures (Ciacci *et al*. 2012) or in baseline expression between seasons (Wathsala *et al*. 2021). Whether the specific results in gravid *A. anatina* represent reproducible effects or just random variation, they overall illustrate that gravidness and/or sex, unless accounted for, can introduce unwanted and potentially detrimental noise on biomarker signals in general, and particularly in gills.

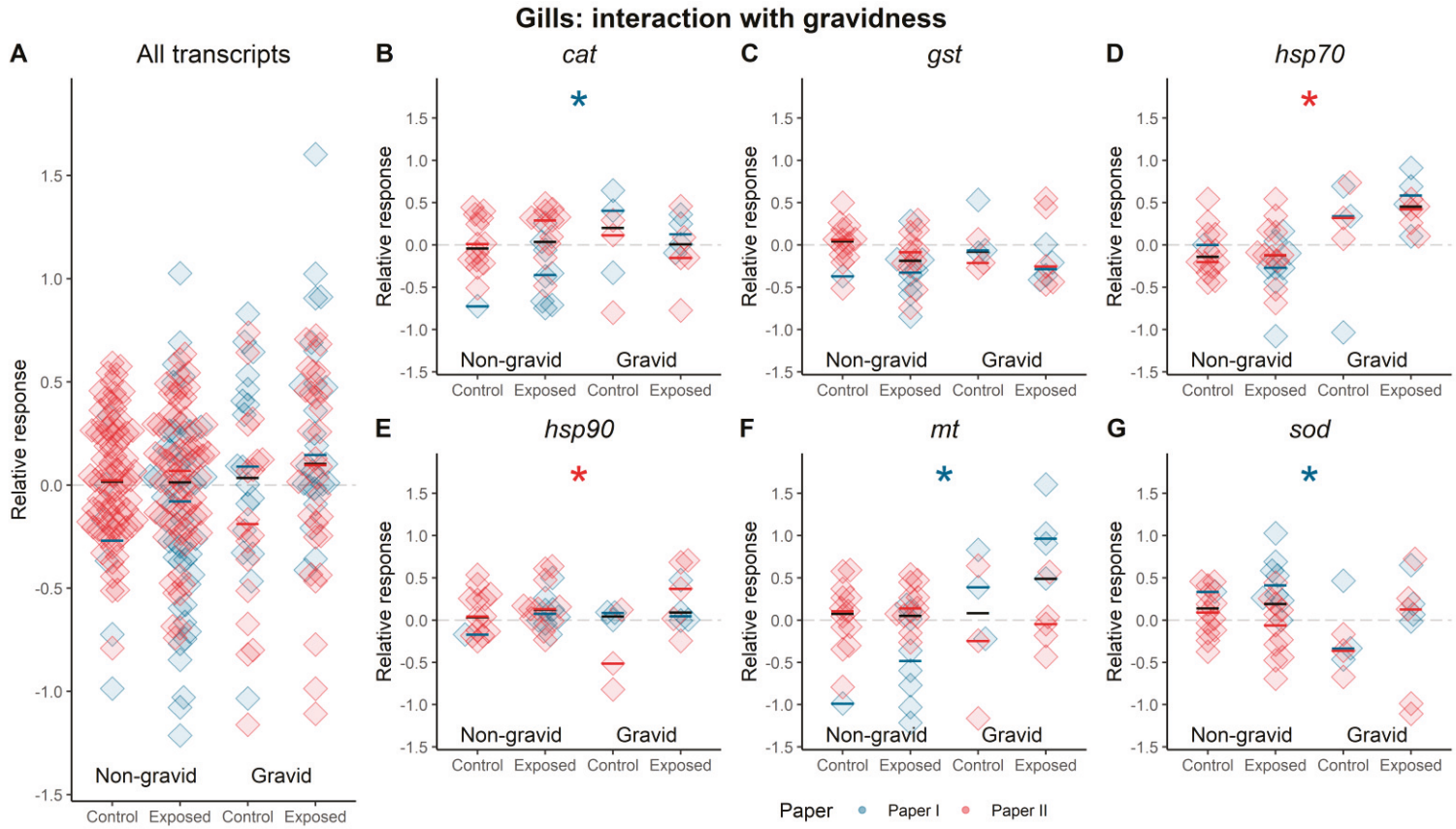

Figure 11. Transcriptional responses in gills of non-gravid and gravid *A. anatina* exposed to Cu (**Paper I**) or an industrial wastewater effluent (**Paper II**). Effects from gravidness, although inconsistent between studies, were demonstrated for most transcripts. In practice, gravidness would therefore appear as a source of increased random variation. Bars denote the median response per exposure group (black shows the overall median, blue and red show the median for **Paper I** and **II**, respectively). Relative response corresponds to ln-transformed fold-change. Each datapoint corresponds to an individual *A. anatina*. Asterisks denote that an effect from gravidness or an interaction between gravidness and treatment has been demonstrated for that specific transcript (blue and red indicate **Paper I** and **II**, respectively).

Conclusions and future perspective

Through a series of studies, I have evaluated a set of putative transcriptional biomarkers of general toxicity by addressing a set of overarching research questions, first presented in the beginning of this thesis ('Aims of this thesis'):

Q1: Should we generally expect 'stress genes' to respond to sublethal exposures of a single toxic compound or a mixture?

Q2: Can we expect (monotonic) concentration-dependent transcriptional responses, and hence, predict response magnitude based on exposure concentration?

Q3: Should we expect variability in response magnitudes with exposure time, and what are the general trends of time-dependence?

Q4: How is biomarker sensitivity affected by individual variation in the freshwater mussel *Anodonta anatina*?

Q5: What is the overall biomarker potential of the selected transcripts in bivalves? Specifically, how do the transcripts compare to more established enzymatic biomarkers? How do the transcriptional biomarker candidates perform in *A. anatina* in terms of sensitivity and robustness? How do they perform in metal exposed bivalves in general?

Generally, response magnitudes were small relative the observed variation, which overall limits the biomarker potentials (**Q1**, **Q5**). This was true specifically for *A. anatina* exposed to Cu or industrial wastewater effluent, but also in general accordance with the body of scientific literature on other bivalve species and metal stressors. A main conclusion is that although many of the responses are in fact likely to be concentration-dependent, the combination of small response magnitudes and large background noise can in practice reduce the likelihood of detection even at relatively high exposures (**Q2**). This problem is true already for single species and single pollutants, and can only be expected to increase upon extrapolation between species and exposure scenarios, as might be necessary for environmental relevance in ERA (**Q5**). Similar patterns could potentially be expected also for time-dependent response variation, although the general trend was slightly increasing response magnitude with increasing exposure times, consistent for inter-species and inter-toxicant comparisons (**Q3**). Thus, high concentration and/or long exposure duration does presumably increase the likelihood of response detection overall, but likely not

to the extent that it justifies universal application as biomarkers of general toxicity and chemical stress in bivalves (**Q2**, **Q3**, **Q5**).

During autumn, biomarker baselines in *A. anatina* were influenced by gravidness (**Q4**). Although affecting both tissues, effects were more prominent in gills than in digestive glands. However, responses were generally more robust in gills as well, presumably due to a higher uptake and internal metal exposure in this tissue. Thus, despite the apparently higher random variability, biomarker potentials of the separate transcripts generally appear higher in gills than in digestive glands. Consequently, gills would be the suggested tissue of choice for further transcriptional studies on metal exposures, for *A. anatina* in particular, but perhaps also for bivalves in general (**Q4**, **Q5**).

Furthermore, the variability in biomarker signal was overall similar for selected transcripts as for commonly used biochemical biomarkers, both in terms of response to exposure, baseline variation and potential interactions with gravidness (**Q5**). For instance, potential degradation of RNA did apparently not influence biomarker variability to a greater extent than the natural and technical variation observed in the biochemical responses. Overall, low to moderately toxic exposures of *A. anatina* (**Paper I-II**) offered little general support for biochemical biomarkers being neither more sensitive nor robust than the transcriptional responses. However, the higher level of biological organization and potential direct links to adverse effects means the inherent toxicological relevance is commonly higher in biochemical than transcriptional responses. Thus, unless transcripts provide a higher likelihood of detecting early warnings, biochemical responses will by default have a general advantage in biomarker potential (**Q5**).

As demonstrated for bivalves under metal exposure, the challenge of applying transcriptional biomarkers might not be a total absence of responses, but rather a lack of robust signals that are consistent or predictable across studies and exposure scenarios (species, toxicant, concentration, exposure time). It is fully plausible that response sensitivity and robustness varies among organism groups, and that some taxa would serve as better bioindicators of transcriptional responses than bivalves. However, if addressing overall responses across more taxonomic groups and/or toxicant types, the already substantial heterogeneity would presumably only increase further. Another, more comprehensive, systematic review and meta-analysis might be required to properly address these issues, but I find it highly unlikely that such a study would find strong support for universal biomarker potential in the selected transcripts. On the contrary, future meta-analyses on the subject should preferably focus on identifying and specifying exposure scenarios (taxon, toxicant, transcript) that are the most likely to generate robust responses, to validate specified rather than universal application of potential biomarkers.

I have not been the first (and will presumably not be the last) researcher to address transcripts such as *cat*, *gst*, *hsp70*, *hsp90*, *mt* or *sod* in the attempt of capturing

responses to general toxicity, with mixed implications. Although representing general modes of action (oxidative stress, cytoprotection), it is possible that these are neither specific nor universal enough to overcome limitations and uncertainties associated with the interpretation of early molecular responses. For instance, higher mechanistic specificity and/or more robust biomarker signal would facilitate incorporation into AOPs to put the response in a toxicologically meaningful context. On the other hand, measures such as transcriptomic points of departure would presumably be more sensitive for detecting early transcriptomic responses to chemical stressors at the systemic level, regardless of the mechanism of action or pathways involved. Consequently, although I have demonstrated important limitations to the potential use of a 'universal biomarker of general toxicity' approach, there are other applications of transcriptional responses that can indeed be of great use in ecotoxicology and environmental sciences.

In conclusion, from a strict biomarker perspective, the key to adequate practical use is likely a clearly defined and empirically validated application, regardless of organizational level of the response. It might for instance be possible to identify *specific transcripts* in *specific organisms*, that are powerful for predicting *specific effects* from *specific types of exposures*. In order to make use of the full potential of early molecular responses, it might however be necessary to also think beyond the concept of biomarkers. For instance, sequencing techniques offer approaches to target specific transcripts or pathways based on mechanism of action, as well as address the sensitivity of study organisms in terms of holistic response patterns. In future ERA and environmental monitoring of toxicants, such approaches, preferably in combination with validated biomarkers and/or other molecular targets, can prove highly useful for detecting early warnings and for predicting adverse effects.

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Paper I

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RESEARCH ARTICLE

Transcriptional and biochemical biomarker responses in a freshwater mussel (*Anodonta anatina*) under environmentally relevant Cu exposure

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Abstract
Molecular biomarkers, like gene transcripts or enzyme activities, are potentially powerful tools for early warning assessment of pollution. However, a thorough understanding of response and baseline variation is required to distinguish actual effects from pollution. Here, we assess the freshwater mussel *Anodonta anatina* as a biomarker model species for freshwater ecosystems, by testing responses of six transcriptional (*cat*, *gst*, *hsp70*, *hsp90*, *mt*, and *sod*) and two biochemical (AChE and GST) biomarkers to environmentally relevant Cu water concentrations. Mussels ($n = 20$), collected from a stream free from point source pollution, were exposed in the laboratory, for 96 h, to Cu treatments (< 0.2 μg/L, 0.77 ± 0.87 μg/L, and 6.3 ± 5.4 μg/L). Gills and digestive glands were extracted and analyzed for transcriptional and biochemical responses. Biological and statistical effect sizes from Cu treatments were in general small (mean $\log_2$ fold-change ≤ 0.80 and Cohen's $f \leq 0.69$, respectively), and no significant treatment effects were observed. In contrast, four out of eight biomarkers (*cat*, *gst*, *hsp70*, and GST) showed a significant sex:tissue interaction, and additionally one (*sod*) showed significant overall effects from sex. Specifically, three markers in gills (*cat*, *mt*, GST) and one in digestive gland (AChE) displayed significant sex differences, independent of treatment. Results suggest that sex or tissue effects might obscure low-magnitude biomarker responses and potential early warnings. Thus, variation in biomarker baselines and response patterns needs to be further addressed for the future use of *A. anatina* as a biomarker model species.

Keywords Bivalve · Gene expression · Response variability · Sex effects · Effect size · RT-qPCR

Introduction

High-resolution quantification of early molecular responses to environmental stress is recently made possible by rapid advances within omics technology. Transcriptomics can potentially be used to find biomarkers for pollution; however, gene expression is challenging to relate to whole-organism, population, or ecosystem effects. In contrast, standardized single endpoint ecotoxicity tests, such as mortality and inhibition of growth rate or reproduction (e.g., Organisation for Economic Co-operation and Development 1992, 2004, 2006, 2012), give useful insight into toxic potency of pollutants, but without giving early warnings (Connon et al. 2012). Ideally, molecular responses would be extrapolated to predict effects on higher biological levels. The link is however complicated by a lacking understanding of how baseline gene expression patterns vary with, for example, species, sexes, developmental stages, and seasons (Bahamonde et al. 2016; Fent and Sumpter 2011). Although not always addressed, understanding the variation is thus necessary to discern actual responses from the background noise, for meaningful interpretation of transcriptional data and for successful integration into molecular biomarker panels (Bahamonde et al. 2016). In this study, we have taken initial steps to address baseline and response variation of transcriptional and biochemical biomarkers within and between tissues, sexes, and treatments of a potential

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model species, the duck mussel (*Anodonta anatina*, family Unionoida).

In ecological monitoring, bivalves are commonly used as bioindicators of aquatic pollution, and various species have been used as model organisms for transcriptional biomarkers in laboratory and field studies (Bigot et al. 2011; Gonzales-Rey et al. 2014; Jaumot et al. 2015; Liu et al. 2014, 2016; Navarro et al. 2011). *Mytilus* sp. is a frequent bivalve model in marine monitoring, and the zebra mussel (*Dreissena polymorpha*) has been proposed as a potential freshwater counterpart (Binelli et al. 2015). However, the zebra mussel is invasive to many Scandinavian and European freshwater systems, which makes us suggest a naturally occurring species, *A. anatina*, as a safer and more ecologically relevant model candidate for field and laboratory studies. This species is widely distributed in Sweden as well as across large parts of Europe (Lopes-Lima 2014). Research on *A. anatina* has to date mostly focused on its phylogeny, morphology, reproduction, and seasonal behavior (Aldridge 1999; Jonsson et al. 2013; Lurman et al. 2014). *A. anatina* and other unionid mussels undergo a complex lifecycle, where gravid females brood eggs and larvae (glochidia) in their gills until parasitic glochidia are released to mature in the gills of host fish (Aldridge 1999; Barnhart et al. 2008; Hinzmann et al. 2013). *A. anatina* has already been used as a model in ecotoxicological field and laboratory studies (Bielen et al. 2016; Falfushynska et al. 2013, 2014; Hartmann et al. 2016; Nugroho and Frank 2011, 2012a, b; Oliviera et al. 2015; Santini et al. 2011), but to our knowledge, transcriptional biomarkers have not previously been studied in the species.

As a primary step to test the potential of *A. anatina* as a model species for molecular biomarkers, we quantified six transcriptional biomarkers after chemical stress. The genes were catalase (*cat*), glutathione-S-transferase (*gst*), heat shock proteins 70 and 90 (*hsp70* and *hsp90*, respectively), metallothionein (*mt*), and superoxide dismutase (*sod*). Catalase and superoxide dismutase are enzymes involved in cellular defense against reactive oxygen species (Bigot et al. 2011; Boukadida et al. 2017), whereas metallothionein plays a main role in metal homeostasis (Fabisiak et al. 1999). Heat shock proteins are involved in general cellular stress response (Liu et al. 2014, 2016) and glutathione-S-transferase in pollutant detoxification (Canesi et al. 1999). As a complement to the transcriptional biomarkers, enzymatic activity of glutathione-S-transferase (GST) was measured in addition to its transcription. Finally, enzymatic activity of acetylcholinesterase (AChE), an enzyme crucial to regulation of nerve signaling (Bocquené and Galgani 1998), was also assessed.

In order to elicit stress responses, Cu was chosen as our experimental model toxicant. In pristine freshwater systems, Cu concentrations are often in the nanogram per liter range (Álvarez-Vázquez et al. 2017; Sander et al. 2013; Vukosav et al. 2014), while in urbanized and polluted areas, in the microgram per liter range (Álvarez-Vázquez et al. 2017; Bhuiyan et al. 2015; Wilson and McMahon 1981), approaching the milligram per liter range in heavily polluted areas (Bhuiyan et al. 2015). According to Swedish environmental quality standards, an annual mean concentration of ≤ 0.5 μg bioavailable Cu/L is required for a "good" status classification (Havs- och vattenmyndigheten 2018). In the current experiment, responses of wild-caught mussels were assessed after exposure to an environmentally relevant range of sublethal Cu levels (additions of 1–100 μg/L) in the laboratory. The a priori hypotheses were that (1) biomarkers respond within the range of Cu concentrations (different relative expressions or activities as compared to the control treatment) and (2) relative response magnitudes differ between treatments and between tissues (gill and digestive gland). In addition to treatment and tissue, mussel sex was included in the model when it became apparent that gravidness affected certain biomarkers.

Material and methods

Biomarkers

Six transcriptional (*cat*, *gst*, *hsp70*, *hsp90*, *mt*, *sod*) and two biochemical (AChE, GST) responses were analyzed by reverse transcription quantitative polymerase chain reaction (qPCR) and enzymatic activity assays, respectively. Stress gene selection was based on biomarkers previously used to assess chemical stress in laboratory and field studies on bivalves (Bigot et al. 2011; Gonzales-Rey et al. 2014; Jaumot et al. 2015; Liu et al. 2014, 2016; Navarro et al. 2011). *AlleleID* software (Premier Biosoft, USA) was used for primer design, based on homologous sequences found using the NCBI nucleotide search function (Table A.1). For each gene used, sequences were found for a minimum of one unionid mussel species (order Unionoida) and a minimum of four bivalve species in total. Actual sequences from *Anodonta anatina* were only found and used for primer design for the 28S rRNA gene. The two biochemical markers, AChE and GST, have both been previously used in mussel gills and digestive glands (Lehtonen et al. 2016).

Mussel collection and maintenance

On the 13th of October 2017, 20 mussels (species *Anodonta anatina*, 83 ± 13 mm shell length) were collected in Vinne å (southern Sweden, 56° 06′ 45″ N, 13° 54′ 35″ E), a freshwater stream with no known point source pollution. Before the start of experiments, the mussels were acclimatized to laboratory conditions for 14 days. During this period, mussels were kept in a 60-L glass aquarium, with 30 L reconstituted freshwater (International organization for standardization 2012), hereafter referred to as freshwater medium, used as medium. The

freshwater medium was prepared from distilled water with additions of 294 mg/L $CaCl_2 \cdot 2H_2O$, 123.3 mg/L $MgSO_4 \cdot 7H_2O$, 64.8 mg/L $NaHCO_3$, and 5.8 mg/L KCl (laboratory reagent grade, Scharlau) and had a nominal hardness of 250 mg/L $CaCO_3$. A 5-cm siliceous sand layer was added as bottom substrate to the aquarium. The sand ("Blästersand," batch 07/17, purchased from Brogård Sand AB, Sweden) originated from Lake Vättern, Sweden, and had a grain size of 0.2–0.7 mm. Before use, the sand was thoroughly washed by hand, by repeatedly stirring it under tap water until the runoff water was clear, and subsequently rinsed with distilled water before added to the aquarium. During acclimatization, the medium was continuously aerated, and three times weekly, 10–20 L medium was renewed. At each time of medium renewal, mussels were fed by additions of *Pseudokirchneriella subcapitata* corresponding to approximately 8×10^5 cells $mussel^{-1}$ day^{-1}, except for 48 h before the start of the experiments, during which mussels were starved. During acclimatization, water temperature ranged between 16 and 20 °C, and the light cycle was 16-h light:8-h dark.

Experimental treatments

A Cu stock solution was prepared from $CuCl_2 \cdot 2H_2O$ (laboratory reagent grade, Fisher Scientific) diluted in freshwater medium to a nominal concentration of 100 mg/L. Stock solution was diluted with freshwater medium in preparation of the three exposure media, to nominal Cu water concentrations of 1, 10, and 100 μg/L, respectively. Freshwater medium without Cu was used for control treatments. One hundred micrograms per liter was selected as the highest concentration in order not to impact filtration and, thus, Cu uptake, based on a previous preliminary Cu exposure experiment with identical setup. In the pre-experiment, prolonged shell closure was observed at additions of 200 μg Cu/L. Glass aquaria with 4.5 L continuously aerated exposure medium and a 5-cm sand layer, prepared as previously described, were prepared approximately 48 h prior to the experimental start. Water (unfiltered) for determination of total Cu concentration was sampled in experimental aquaria at the experiment start, as well as in Vinne å at five time points during December 2017 to December 2018. In addition, the Cu stock solution was sampled. Samples were frozen and subsequently acidified with 1% (v/v) HNO_3 before Cu determination. Total Cu concentrations in water samples were measured by ICP-MS (*Aurora Elite*, Bruker Daltonics, Germany) for experimental treatments and environmental samples, and by ICP-OES (*Optima 8300*, Perkin Elmer, USA) for the stock solution, respectively. Measured Cu concentrations were < 0.2 μg/L both in the control treatment and after 1 μg/L addition, and on average 0.77 ± 0.87 μg/L and 6.3 ± 5.4 μg/L after addition of 10 μg/L and 100 μg/L, respectively (Table A.2). Estimated Cu sand/water partition coefficients (K_d) were 84 and 110 L/kg for the 10 and 100 μg Cu/L additions, respectively (Table A.2). The Cu concentration of the stock solution was 66 mg/L, and sampled background levels in Vinne å ranged between 0.080 and 0.71 μg Cu/L over time (Table A.2).

Mussels were treated in individual aquaria and fed daily with *P. subcapitata* solution (approximately 8×10^5 cells day^{-1}). During the experiment, medium temperature was 15 ± 1 °C and the light cycle was 16-h light:8-h dark. After 48 h, 1.5-L medium was renewed in each aquarium, and after 96 h, the exposure was interrupted and mussels were immediately dissected. Gills and digestive glands were extracted and snap frozen in liquid nitrogen. Tissues were stored at − 80 °C until RNA extraction and again until cytosol extraction for biochemical assays. The extractions were made from frozen tissue, and samples were not allowed to thaw in between. During dissection, gravid mussels (hereafter referred to simply as females) were distinguished visually by the presence of eggs or glochidia in the gills (Aldridge 1999; Hinzmann et al. 2013). Male:female sex distribution across treatments was 1:4, 2:3, 3:2, and 3:2 in control, 1, 10, and 100 μg Cu/L, respectively. Upon snap freezing, one female gill sample was lost from each treatment group, i.e., $n = 4$ per treatment remaining for gills.

After biomarker analyses, Cu concentration in remaining tissue samples (16 gill and 14 digestive gland samples, respectively) was analyzed by ICP-SFMS (*Element XR*, Thermo Scientific, Germany) according to Engström et al. (2004). Bioconcentration factors (BCFs) were estimated for each tissue sample where corresponding water concentration was determined (> LOQ).

$$BCF = \frac{\text{Tissue concentration } (\mu g/kg \text{ WW})}{\text{Water concentration } (\mu g/L)}$$

Gene expression

RNA was extracted from each sample, followed by reverse transcription synthesis of cDNA, which was in turned used in qPCR assays. RNA was extracted from gill and digestive gland samples by using the *SurePrep™ TrueTotal™ RNA Purification Kit* (Fisher Scientific, USA). The A260/A280 ratio was checked using a *NanoVue™ Plus* (GE Healthcare, USA) and was 1.9–2.1 in extracted samples. RNA integrity was assessed qualitatively by gel electrophoresis on 1% agarose gels, and a subset of samples underwent RNA quality assessment using *Fragment Analyzer* (Advanced Analytical, Austria). Based on qualitative and quantitative integrity assessments, all samples were assumed to have an RQN (RNA quality number) of ≥ 6.

For each sample, 1 μg of RNA was converted to cDNA by reverse transcription, using the *Verso cDNA Synthesis Kit* (Thermo Scientific, USA), with random hexamer primers and a reaction cycle of 42 °C for 60 min, 95 °C for 2 min, and 4 °C for 2 min. qPCR assays were performed using *TATA A SYBR® GrandMaster® Mix* (TATAA Biocenter, Sweden),

and 400 nM of the respective primer pair, on a *CFX384™ Real-Time PCR Detection System* (Bio-Rad, USA). The reaction program consisted of polymerase activation at 95 °C for 60 s, then 45 cycles of denaturation (95 °C for 5 s), annealing (58 °C for 30 s), and extension (72 °C for 10 s). A dissociation curve (from 60° to 95° C) finalized the program. Primers were purchased from Integrated DNA Technologies (Belgium).

Assay efficiencies were estimated from dilution series of pooled samples. Efficiency determination was performed on a *StepOnePlus™* (Applied Biosystems, USA), using *Maxima SYBR Green/ROX qPCR Master Mix* (Thermo Scientific, USA) for the reactions. Estimated efficiencies were 96–102% (Table A.1), and a 100% efficiency was assumed for all assays. Relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), normalizing expression by the mean expression of control samples of gill and digestive gland tissue, respectively. The mean of two reference genes, β-actin and 28S rRNA, was used for within-sample normalization.

Enzyme activity

AChE and GST activity assays were modified from Bocquené and Galgani (1998) and Habig et al. (1974), respectively. Tissue samples were mechanically homogenized on ice in 5:1 (v:w) 0.02 M Na_2HPO_4/KH_2PO_4 buffer (0.1% Triton-X, pH 7.4) for AChE assays and in 4:1 (v:w) 0.1 M KH_2PO_4 buffer (pH 7.4) for GST assays. Following homogenization, samples were centrifuged at 10,000*g* (4 °C, 20 min), and supernatants were stored at − 80° until analysis. Activities were measured for spectrophotometrically at 412 nm for 5 min and 350 nm for 2 min, for AChE and GST respectively. Assays were performed in 96-well microplates (Nunc, Denmark), and absorbance measured using a *SpectraMax 190* plate reader (Molecular Devices, USA). AChE activity was expressed as the hydrolysis rate of acetylthiocholine (Bocquené and Galgani 1998), whereas GST activity was expressed as the rate of glutathione conjugation to 1-chloro-2,4-dinitrobenzene (Habig et al. 1974). Finally, enzymatic activities were normalized by the protein concentration from each extracted tissue sample, as determined by the Bradford (1976) method, using a bovine serum albumin standard curve.

Statistics

Statistical analyses were run and figures were generated in R version 3.5.2 (R Core team 2018). Measured tissue concentrations were compared between treatment groups by ANOVA. Concentration dependence of tissue Cu concentrations was tested by correlation (Pearson) to measured Cu concentration in the exposure medium. Both concentrations were $\log_{10}$-transformed, and samples corresponding to water levels below LOQ were excluded. Gene expressions and enzyme activities were normalized relative to the mean of the respective tissue in the control group and then $\log_2$-transformed. Transformed values are hereafter referred to as responses and were visualized by principal component analysis, using the R package "factoextra" (Kassambara and Mundt 2017). Responses were then analyzed in a linear mixed model, using the R package "lme4" (Bates et al. 2015). Response was used as the dependent variable for each marker, and full models included the fixed effect terms treatment, sex, and tissue, as well as their interactions (Table A.3). Mussel ID was used as a random effect. Model selection was performed by sequential type I ANOVA analysis, where insignificant effect factors were removed one at a time until remaining factors and/or interactions were significant ($p < 0.05$). For biomarkers where the mixed model selection resulted in a singular fit, the tissues were instead analyzed by separate linear models. Residual normality for biomarker responses and tissue Cu concentrations was assessed by Shapiro-Wilk normality tests and *Q-Q* plots. Significant differences in the final models were identified with a Tukey HSD post hoc test, using the "emmeans" package (Lenth 2018). Finally, the R package "simr" (Green and MacLeod 2016) was used for power analysis by simulation in the linear mixed models, while the packages "*pwr*" (Champely 2018) and "sjstats" (Lüdecke 2018) were used for power analysis and effect size assessment of ANOVAs.

Results

Treatment groups did not differ significantly in measured tissue concentration in either gills or digestive glands ($p > 0.05$), despite gills demonstrating an approximately twofold higher mean concentration in the 100 μg/L group as compared to control (Fig. 1). Yet, the gill Cu concentrations were significantly correlated with measured water Cu concentrations (Fig. 1). In contrast, digestive gland Cu concentrations were not correlated with exposure levels (Fig. 1). Across exposures in which water Cu was > LOQ, median BCF was 3400 L/kg WW and 1500 L/kg WW in gills and digestive gland, respectively.

Overall, sex and tissue effects and/or interactions were prominent for all biomarkers except *hsp90*. No distinct responses from Cu treatments were demonstrated. Biomarker responses showed overlapping distributions in all experimental treatments, and no biomarker showed discernable differences between treatment groups, neither in gills nor in digestive glands (Figs. 2 and 3). Gill responses generally showed higher variation for all markers except *gst* and *hsp90*. Standard deviations of *mt*, AChE, and GST were consistently greater in gills than in digestive glands across treatments (Table A.4). For *mt* and GST, overall standard deviation in gills was more than twice the size of that in digestive glands. Similarly, no treatment effects could be inferred from principal component analysis of biomarker responses separated by tissue (Figs. 4 and 5). In gills however, there was a separation of

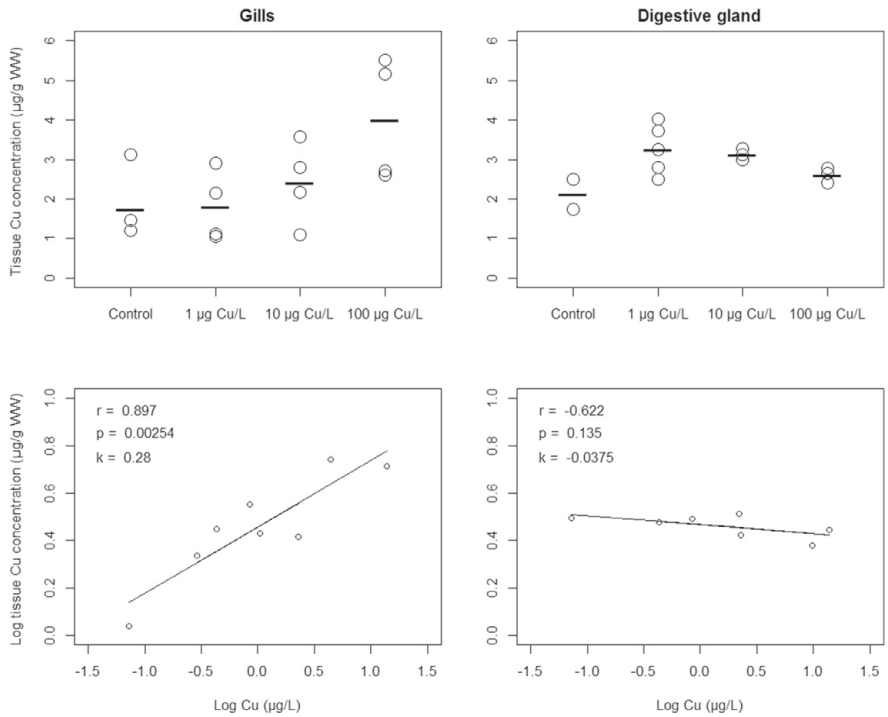

Fig. 1 Cu concentration (µg/g WW) in gill (upper left) and digestive gland samples (upper right), and their respective correlation with measured water concentration (below). For tissue sample Cu analysis, 16 gill and 14 digestive gland samples remained after transcriptional and biochemical analyses. Circles represent single samples and black bars show group means

females and males along PC1, with only little overlap (Fig. 4), implying a sex difference in expressions of most notably *cat*, *mt*, and *hsp70* (Table A.5).

Linear (mixed) models

In the full models, observed power was low for treatment effects (< 0.4) and treatment interactions (< 0.6) (Table A.3). Treatment effects were insignificant in all markers ($p > 0.05$), and only AChE showed an initially significant treatment interaction (Table A.3). Due to insignificance in a subsequent step of the AChE model selection, the treatment term was however excluded in all final models (Table 1). For *mt* and AChE, the final models were linear models separated by tissue, whereas the other markers were analyzed by linear mixed models. In *cat*, *gst*, GST, and *hsp70*, there was a significant sex:tissue interaction, whereas *sod* expression only showed significant sex effects (Table 1). Significant sex effects were also observed in gill *mt* expression and digestive gland AChE activity. In contrast, for *hsp90* expression, as well as *mt* expression in digestive gland and AChE activity in gills, there were no significant effects (Table 1). Males showed a significantly higher *sod* expression than females in general and a higher GST and AChE activity in gills and digestive gland, respectively. Females on the other hand displayed a significantly higher expression of *mt* in gills, as well as a higher gill-specific expression of *cat* compared to males (Table 1). In addition, mean *hsp70* expression was higher in females than males, and the linear mixed model implied a sex:tissue interaction that was however not significantly confirmed post hoc (Table 1). Males displayed significantly higher relative *cat* and *gst* expressions in digestive gland as compared to gills, while females showed significantly higher GST activity in digestive gland as compared to gills (Table 1).

Treatment effects

Treatment effects were further analyzed in a simplified model of tissue-separated treatment effects, completely disregarding sex effects. One-way ANOVA analyses for each marker, separated by tissue, showed that no treatment response differed significantly from control. Observed power ranged from

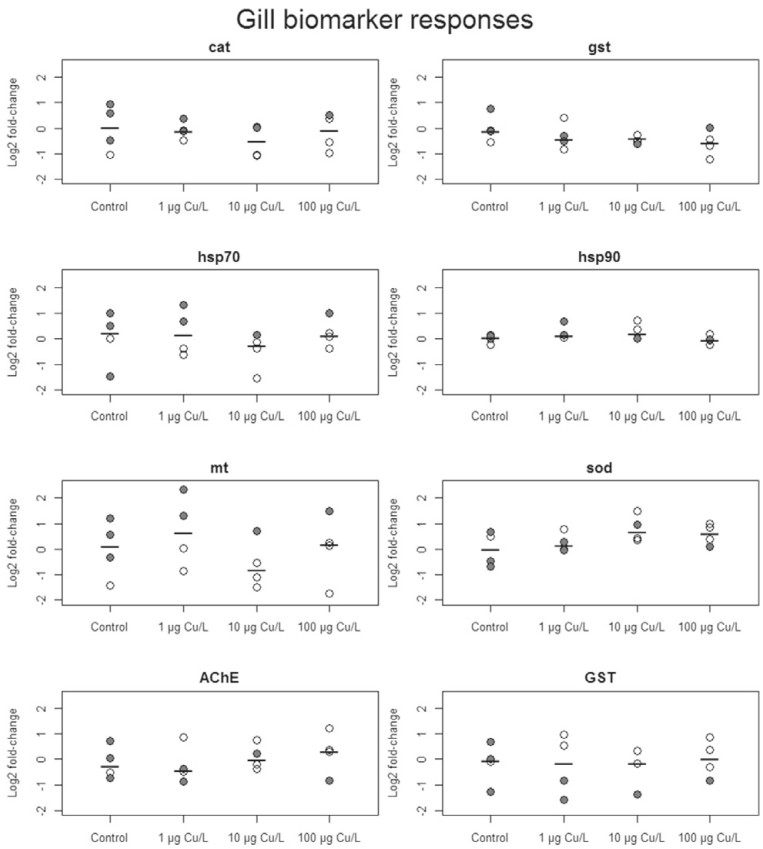

Fig. 2 Biomarker responses ($\log_2$ fold-change relative control treatment) by copper treatment, in gill tissue ($n = 4$ per treatment) from *A. anatina*. Gray and white points correspond to gravid and non-gravid mussels, respectively, while black bars show treatment median responses

0.097 to 0.55 (Table A.6), and the largest observed mean response magnitudes for each marker ranged between a $\log_2$ fold-change of 0.26–0.80 and 0.13–0.69, in gills and digestive gland, respectively (Table A.6). For certain markers (*hsp90* in gills, *sod* in gills and digestive gland, AChE in digestive gland), an approximate doubling of sample size would have given a power of 0.8 with current effect size, while others (e.g., *cat* and *gst* in digestive gland, GST in gills) would require more than a tenfold increase in sample size to achieve the requested power (Table A.6).

Discussion

Cu exposure and uptake

Bioavailability and potential uptake of Cu depends on various water parameters, and toxicity decreases with, for example, dissolved organic carbon (DOC) and water hardness (Arnold et al. 2009; Gillis et al. 2008, 2010; Wang et al. 2009; Giacomin et al. 2013). Importantly, bioavailability depends on Cu partitioning, which in turn is affected by both water parameters and sediment organic content (European Copper Institute 2008). For sandy sediments, Hassan et al. (1996) demonstrated Cu partition coefficients (K_d) ranging from 0.6 to 149 L/kg, in line with estimated K_d values from our experiment. Under current settings, the substrate is therefore assumed to be an important factor to reduce Cu bioavailability and potential stress responses, by adsorbing a major fraction of the added Cu.

Different patterns of Cu uptake and tissue distribution have been reported for various bivalve species and exposure conditions (Canesi et al. 1999; García-Navarro et al. 2017; Nugroho and Frank 2011; Sakellari et al. 2013; Serafim and Bebianno 2009; Won et al. 2016). Whole-body BCFs of 3300 L/kg WW (Potipat et al. 2015) and 576 to approximately

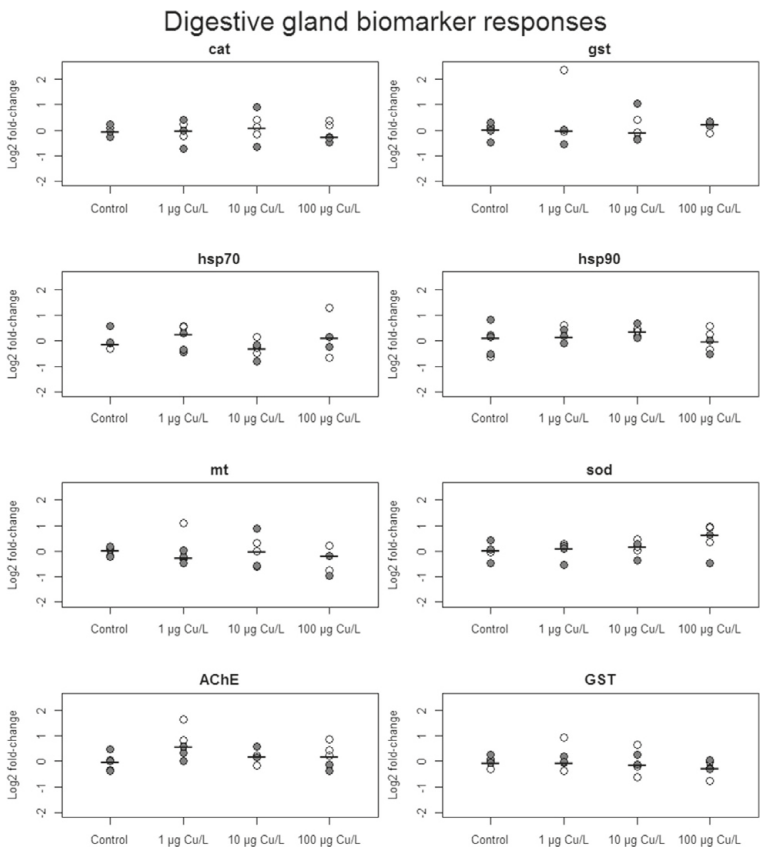

Fig. 3 Biomarker responses (log_2 fold-change relative control treatment) by copper treatment, in digestive gland tissue ($n = 5$ per treatment) from *A. anatina*. Gray and white points correspond to gravid and non-gravid mussels, respectively, and black bars show treatment median responses

15,000 L/kg DW (Le et al. 2011; Rosioru et al. 2016) have been proposed. In gills specifically, a BCF of 42 L/kg WW was modeled for *Corbicula fluminea* (Chen et al. 2010). For aquatic organisms in general, McGeer et al. (2003) reported a mean whole-body BCF of 1200 ± 1800 L/kg WW under the exposure range of 1–10 μg Cu/L. Considering the variation in reported Cu uptake, our observed tissue concentrations and BCFs are well within the expected range.

Under current settings, the gill Cu levels were positively correlated with water concentrations, implying Cu uptake under the highest exposures. Although treatment groups did not significantly differ in gill Cu concentrations, this is likely due to variation in actual exposure within and between groups. In contrast, digestive glands showed no correlation, suggesting that observed Cu concentrations rather reflect the tissue baseline. Potential, not necessarily mutually exclusive, explanations to differences between in gills and digestive glands include different accumulation and/ or elimination rates. Gills constitute a first-line defense against harmful toxicants, and gill uptake might be of importance for bivalve regulation of reactive oxygen species (ROS) under Cu stress (Won et al. 2016). However, fecal elimination, via the digestive gland, has been proposed as a major route for metal elimination in *A. anatina* (Nugroho and Frank 2011). At low exposure concentrations, an efficient Cu elimination might thus result in no net uptake to digestive glands. Also, accumulation rates may differ between tissues (Canesi et al. 1999; Serafim and Bebianno 2009). Thus, the current exposure period and/or Cu concentrations were potentially not enough for digestive gland net uptake to occur.

Biomarker responses

Experimental Cu exposures were in the lower range as compared to previous studies on bivalve transcriptional responses, and response magnitudes in our experiment were generally

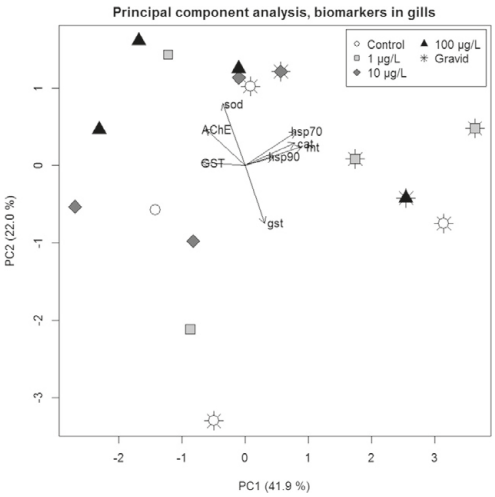

Fig. 4 Principal component analysis of eight molecular biomarker responses in gills of *A. anatina* ($n = 16$). Arrows imply the contribution of the respective biomarkers to PC1 and PC2

lower in both tissues than previously reported. All mean responses (converted back from the $\log_2$ scale) were within the range of 0.67–1.7 relative to control. With regard to the biochemical markers, *Mytilus galloprovincialis* exposed for 96 h to 5 or 15 µg Cu/L demonstrated an approximate 1.5-fold increase in GST activity (Perić and Burić 2019). Similar to our results, AChE activity was however unaffected except in

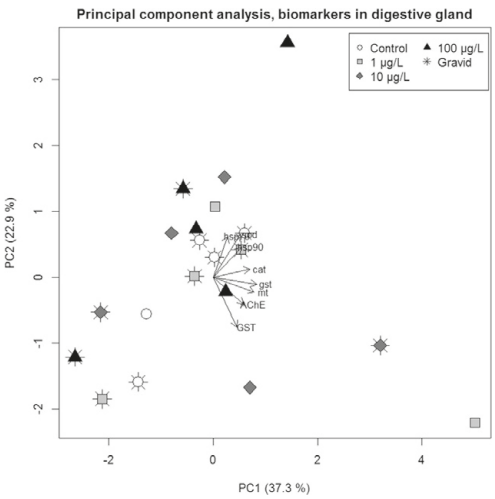

Fig. 5 Principal component analysis of eight molecular biomarker responses in digestive gland of *A. anatina* ($n = 20$). Arrows imply the contribution of the respective biomarkers to PC1 and PC2

a binary exposure of Cu and chlorpyrifos (Perić and Burić 2019). In contrast, in transcriptional markers, the freshwater mussel *Corbicula fluminea* displayed *mt*, *cat*, *sod*, and *gst* responses varying between a tenfold decrease to a fourfold increase in gills and digestive gland after acute (12 h) exposure to a nominal concentration of 10 µg Cu/L (Bigot et al. 2011). Our largest observed mean responses approximately correspond to acute *C. fluminea* response magnitudes at the nominal concentration of 50 µg Cu/L (Bigot et al. 2011), however not regarding the same genes and tissues. Even larger response magnitudes have been demonstrated in, for example, larvae and hemocytes of *Mytilus* spp. after various Cu stress exposures. After exposure to 10–20 µg Cu/L, significant upregulations ranging between two- and eightfold increases have been demonstrated across all our assessed transcriptional markers (Boukadida et al. 2017; Liu et al. 2014, 2016). Without a treatment to serve as positive control, we currently cannot be certain of what level of Cu stress would be required to induce corresponding response magnitudes in *A. anatina*.

Sex and tissue differences

Except for AChE activity, digestive glands did not demonstrate sex-specific differences. Considering that no uptake was demonstrated for this tissue, for most of the tested biomarkers, there appears to be little sex influence on baseline signal. In contrast, gill signals differed distinctly in four markers, with females showing higher *cat*, *mt*, and *hsp70* (non-significant) expressions and lower GST activity than males. In *A. anatina*, gravidness develops sequentially from early summer until glochidia are released in late winter/early spring (Hinzmann et al. 2013). Since mussels were sampled in October, observed differences in gills could potentially be explained by gravidness, by the eggs and glochidia directly interfering with biomarker signals, and/or by protective molecular mechanisms in the gravid mussel gill. Acute toxicity (48 h LC_{50}) to *A. anatina* glochidia has been demonstrated at 18.9 µg Cu/L (Kováts et al. 2010) and at 6.5–32 µg Cu/L in glochidia of various other unionid species (Wang et al. 2007). This range only overlaps the highest measured Cu concentrations of our experiment, whereas our mussels were exposed for a longer period. Still, we observed no treatment:sex:tissue interaction, suggesting that observed differences were mainly due to gravidness-induced baseline variation rather than responses to Cu exposure. Regardless of mechanistic explanation, relative sex differences in gills may possibly change over the course of the season depending on female gravidness.

In addition to tissue-specific sex differences, some markers showed a sex-specific difference in biomarker signal between tissues (*cat*, *gst*, GST). However, although Cu uptake patterns differed, no treatment:tissue or treatment:sex:tissue interaction was observed, i.e., no tissue difference in actual stress response can be concluded. In the absence of such interactions,

Table 1 Effects from sex, tissue, and their interactions in the final (mixed) linear model for analyzed biomarkers, as well as the observed power for the effects (based on 100 simulations)

Biomarker	Final model	(Fixed) effect	Effect size ($\Delta\log_2$ as compared to control)	Obs. power ($1-\beta$)	Model term sign. level	Observed differences (post hoc)
cat	Response ~ Sex*Tissue + (1|ID)	Intercept (F:Dg)	− 0.077	–	–	Sex differences:
		Sex (M:Dg)	0.17	0.32	$p = 0.089$	Dg:F < M ($p = 0.84$)
		Tissue (F:G)	0.34	0.22	$p = 0.32$	*G:F > M* (*p = 0.0087*)
		Sex:tissue (M:G)	− 0.96	*0.86*	*p = 0.0061*	Tissue differences:
						F:Dg < G ($p = 0.44$)
						M:Dg > G (*p = 0.045*)
gst	Response ~ Sex*Tissue + (1|ID)	Intercept (F:Dg)	0.060	–	–	Sex differences:
		Sex (M:Dg)	0.26	0.04	$p = 0.62$	Dg:F < M ($p = 0.73$)
		Tissue (F:G)	− 0.060	*0.94*	*p = 0.0020*	G:F > M ($p = 0.31$)
		Sex:tissue (M:G)	− 0.74	*0.82*	*p = 0.0067*	Tissue differences:
						Dg > G (*p = 0.0024*)
						F:Dg > G ($p = 0.99$)
						M:Dg > G (*p = 0.0009*)
hsp70	Response ~ Sex*Tissue + (1|ID)	Intercept (F:Dg)	− 0.11	–	–	Sex differences:
		Sex (M:Dg)	0.21	0.12	$p = 0.28$	Dg:F < M ($p = 0.86$)
		Tissue (F:G)	0.56	0.05	$p = 0.84$	G:F > M ($p = 0.067$)
		Sex:tissue (M:G)	− 1.0	0.79	*p = 0.021*	Tissue differences:
						F:Dg < G ($p = 0.25$)
						M:Dg > G ($p = 0.39$)
hsp90	Response ~ (1|ID)	Intercept	0.15	–	–	–
mt	Gill response ~ Sex +1	Intercept (F)	1.0	–	–	Sex difference:
		Sex (M)	− 1.8	*0.99*	*p = 0.00051*	*F > M* (*p = 0.00051*)
	Dig. gland response ~ 1	Intercept	− 0.086	–	–	–
sod	Response ~ Sex + (1|ID)	Intercept (F)	0.054	–	–	Sex difference:
		Sex (M)	0.44	0.75	*p = 0.011*	*F < M* (*p = 0.012*)
AChE	Gill response ~ 1	Intercept	0.0083	–	–	–
	Dig. gland response ~ Sex +1	Intercept (F)	0.088	–	–	Sex difference:
		Sex (M)	0.43	0.56	*p = 0.049*	*F < M* (*p = 0.049*)
GST	Response ~ Sex*Tissue + (1|ID)	Intercept (F:Dg)	0.022	–	–	Sex differences:
		Sex (M:Dg)	− 0.13	0.49	$p = 0.066$	Dg:F > M ($p = 0.95$)
		Tissue (F:G)	− 0.76	0.19	$p = 0.29$	*G:F < M* (*p = 0.0031*)
		Sex:tissue (M:G)	1.1	*0.89*	*p = 0.0028*	Tissue differences:
						F:Dg > G (*p = 0.025*)
						M:Dg < G ($p = 0.39$)

Significance level is presented for all model terms, and differences were tested post hoc for significant model terms ($p < 0.05$). Italicized entries imply observed powers ≥ 0.8 and p values < 0.05. *Dg* digestive gland, *G* gills, *F* females, *M* males

remaining tissue differences rather reflect overall variation within and between sexes.

Tested biomarkers

The currently tested biomarkers represent a subset of general stress responses and have all previously been shown to respond to Cu exposure in bivalves (Bigot et al. 2011; Boukadida et al. 2017; Goswami et al. 2014; Liu et al. 2014, 2016; Perić et al. 2017). Apart from metallothionein, which is cytoprotective by binding to metals (Fabisiak et al. 1999), the biomarkers were however not expected to respond specifically to Cu exposure. Rather, responses would mainly reflect mechanisms of cellular protection from, for example, oxidative stress (Bigot et al. 2011; Boukadida et al. 2017) or protein damage (Liu et al. 2014, 2016). The primary objective was to identify biomarkers responding to general chemical stress in *A. anatina*, rather than to respond specifically to Cu exposure. Since we failed to detect stress responses at current elevated Cu concentrations, additional markers, specific to Cu or other target pollutants, might be needed before *A. anatina* can be successfully used in, for example, environmental monitoring.

An ideal biomarker for early warnings of chemical stress should be one that gives a strong response at levels below, or

time points before, responses translate to whole-organism effects. Despite low exposure concentrations, Cu in our experiment was approaching water levels at which we previously observed prolonged shell closure in preliminary experiments (unpublished). That is, further concentration increases would risk higher-level effects by affecting mussel behavior. In practice, the window for detecting early warnings might thus be quite narrow for acute Cu stress, further increasing the need of a strong biomarker response.

In our experiment, response magnitudes were generally small, but an approximate doubling of the sample size would have substantially increased the power for *hsp90*, *sod*, and AChE in one or both tissues. However, most of the markers would require drastically increased sample sizes to improve power. Ideally, large effects should be obtained biologically by high response magnitude and/or statistically by low natural variation, even at small sample sizes. At current Cu stress, *A. anatina* background variation however obscures potential low-magnitude responses, and the tested markers appear to require quite drastically increased sample sizes for detection of early warnings. Thus, no successful *A. anatina* biomarker candidate can be singled out, and further search for and/or evaluation of biomarkers is necessary Alternatively, successful assessment of low-level stress may ultimately depend on response pattern analysis of multiple stress biomarkers.

Conclusion

Either larger response magnitudes or substantially larger sample sizes would have been required to quantify molecular stress responses under the current Cu concentrations. Our results, specifically displayed by sex differences in gills, illustrate how potential low-magnitude stress responses, and potentially early warnings, might be obscured by variations in baseline biomarker expression/activity. However, for successful development of environmentally relevant biomarker models, natural variation should preferably be addressed rather than avoided. Being abundant, sessile, and ecologically relevant, we suggest further studies on *Anodonta anatina* as a biomarker model species. A better understanding of response magnitudes, variation, and links to higher biological levels is needed to realize the potential of *A. anatina* as a freshwater biomarker model.

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Compliance with ethical standards

Conflict of interest This study is part of the project WaterAssess, which is a collaboration between University of Skövde, Lund University, ALS Scandinavia Toxicon AB, and TATAA Biocenter, and the work is partially funded by commercial companies (ALS Scandinavia Toxicon AB, Landskrona, Sweden, and TATAA Biocenter, Gothenburg, Sweden). Project outlines have been determined jointly, but experimental design and data analysis was carried out largely without involvement of the funding sources. Where present, commercial interests have been judged not to bias the scientific. Gustaf Ekelund Ugge is employed by ALS Scandinavia Toxicon AB, but is currently on a leave of absence for pursuing a PhD. Robert Sjöback is employed and in the board of TATAA Biocenter.

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Appendix

Transcriptional and biochemical biomarker responses in a freshwater mussel (*Anodonta anatina*) under environmentally relevant Cu exposure

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Table A.1. Primer and assay details for qPCR assays of *Anodonta anatina* stress gene expression. Primer sequences, efficiency, standard curve slope, intercept and R^2. Also presented is the estimated amplicon length, and accession numbers for gene sequences used for primer design. fwd = forward primer, rev = reverse primer.

Gene	Sequence (5'-3')	Efficiency (%)	Slope	Intercept	R^2	Amplicon size (bp)	Sequence references for primer design (accession number)
β-actin (*β-act*)	fwd: CAAACTGGGATGATATGGA	101	-3.32	23.5	0.894	128	KT923181.1, HM045420.1, AF082863.1, AF172606.1, AF157491.1
	rev: CATCTTTTCTCTGTTGGC						
28S rRNA (28S)	fwd: ATCCTTGCTCGTCACGAC	98.5	-3.37	13.1	0.999	75	KX822588.1, KC703865.1, MF414393.1, MF414392.1, MF414391.1, MF414390.1, KX822589.1, KX822630.1, U82350.1
	rev: GTACCAACCCTTCCTACG						
Catalase (*cat*)	fwd: GGAAGACTGACCAGGGTAT	96.0	-3.43	21.5	0.997	108	HQ148707.1, KU363383.1, FJ608579.1, EU145723.1, HM188565.1, EU407490.1
	rev: CCTCAGCGATGGCATTGTA						
Glutathione-S-transferase (*gst*)	fwd: GTCCAACACCATGCTGAG	99.2	-3.35	21.5	0.998	106	HQ166721.1, AY885666.1, EU145724.1, EF194203.1, AY557404.1, AJ557140.1
	rev: GTAGTCCTCCACTCCATCAT						
Heat shock protein 70 (*hsp70*)	fwd: GGTATTGAGACGGCTGGT	102	-3.30	21.8	0.997	111	KX758099.1, HQ148706.1, KT923183.1, AF172607.1, EF526096.1, AB122063.1, KJ123764.1
	rev: CACACCAGGCTGGTTGTC						
Heat shock protein 90 (*hsp90*)	fwd: TACCATTGCCAAATCTGG	101	-3.31	24.8	0.995	84	HQ180224.1, GU433881.1, EF687776.1, KR633143.1
	rev: ACACCAAACTGCCCAATCA						
Metallothionein (*mt*)	fwd: ATGCAACTGCCTTGAGAC	100	-3.32	22.5	0.997	114	EF185127.1, GQ184290.1, KJ019820.1, KJ019821.1, U67347.1, AJ577130.1, AJ577131.1, AJ243263.1
	rev: ACTTTACATCCAGGACACTT						
Superoxide dismutase (*sod*)	fwd: GCTACGGTCATTCCACTCT	97.4	-3.39	24.9	0.995	171	FJ194441.1, KU363382.1, FJ608580.1, KT724303.1, EU145730.1, AY377970.2
	rev: CCAGTTATCTCACCAGTTATGTTC						

Table A.2. Concentrations of total Cu in sampled water of experimental treatments, Cu stock solution and environmental samples. The limit of quantification (LOQ) was 0.2 µg/L.

Treatment	Samples (n)	Mean Cu concentration (g/L) (min – max)	Estimated K_d[a] (L/kg) (min – max)
Control	3	<LOQ	-
1 µg Cu/L	3	<LOQ	-
10 µg Cu/L	5	$7.7 * 10^{-7}$ ($7.2 * 10^{-8} – 2.2 * 10^{-6}$)	84 (22 – 1 000)
100 µg Cu/L	5	$6.3 * 10^{-6}$ ($1.0 * 10^{-6} – 1.4 * 10^{-5}$)	110 (42 – 690)
100 mg Cu/L (stock solution)	1	$6.6 * 10^{-2}$	-
Background (environmental samples)	5[b]	$3.9 * 10^{-7}$ ($8.0 * 10^{-9} – 7.1 * 10^{-7}$)	-

[a] K_d was estimated by the formula $K_d = C_s/(C_w/S.C.)$ (Hassan *et al*. 1996). C_w is the measured water concentration (µg/L) and S.C. is the sand to medium ratio (kg/L) in the aquarium. C_s is the concentration (µg/kg) assumed to adsorb to the sand, and was calculated as $C_s = (66\ 000\ µg/L * V_{stock} – 5\ L * C_w)/1.5$ kg. 66 000 µg/L was the measured Cu concentration of the stock solution, and V_{stock} is the volume (L) of stock solution added to the respective treatments. Each aquarium contained 5 L water and approximately 1.5 kg of sand.

[b] Background levels were sampled every three months over the course of a year, in December 2017 and March, June, September and December 2018.

Table A.3. Full linear mixed models for investigated biomarkers. Observed power (based on 100 simulations) and significance level are presented for model fixed effect terms.

Biomarker	Full model	Fixed effect	Obs. power ($1 - \beta$)	Significance level (p)
cat	Response ~ Treatment*Sex*Tissue + (1|ID)	Treatment	0.02	0.93
		Sex	0.20	0.17
		Tissue	0.14	0.39
		Treatment:Sex	0.06	0.73
		Treatment:Tissue	0.19	0.58
		Sex:Tissue	0.64	0.019
		Treatment:Sex:Tissue	0.12	0.74
gst	Response ~ Treatment*Sex*Tissue + (1|ID)	Treatment	0.05	0.92
		Sex	0.04	0.74
		Tissue	**0.92**	0.0026
		Treatment:Sex	0.21	0.34
		Treatment:Tissue	0.50	0.099
		Sex:Tissue	0.79	0.016
		Treatment:Sex:Tissue	0.25	0.32
hsp70	Response ~ Treatment*Sex*Tissue + (1|ID)	Treatment	0.23	0.27
		Sex	0.11	0.39
		Tissue	0.07	0.83
		Treatment:Sex	0.03	1.0
		Treatment:Tissue	0.08	0.92
		Sex:Tissue	0.78	0.017
		Treatment:Sex:Tissue	0.49	0.21
hsp90	Response ~ Treatment*Sex*Tissue + (1|ID)	Treatment	0.30	0.12
		Sex	0.05	0.79
		Tissue	0.08	0.81
		Treatment:Sex	0.26	0.20
		Treatment:Tissue	0.07	0.98
		Sex:Tissue	0.06	0.79
		Treatment:Sex:Tissue	0.27	0.38
mt	Response ~ Treatment*Sex*Tissue + (1|ID)	Treatment	0.21	0.26
		Sex	0.77	0.0053
		Tissue	0.18	0.37
		Treatment:Sex	0.12	0.82
		Treatment:Tissue	0.39	0.25
		Sex:Tissue	**1.0**	0.00027
		Treatment:Sex:Tissue	0.19	0.73
sod	Response ~ Treatment*Sex*Tissue + (1|ID)	Treatment	0.37	0.22
		Sex	0.31	0.14
		Tissue	0.43	0.062
		Treatment:Sex	0.07	0.88
		Treatment:Tissue	0.28	0.25
		Sex:Tissue	0.12	0.72
		Treatment:Sex:Tissue	0.14	0.50
AChE	Response ~ Treatment*Sex*Tissue + (1|ID)	Treatment	0.14	0.44
		Sex	0.54	0.038
		Tissue	0.47	0.066
		Treatment:Sex	0.52	0.045
		Treatment:Tissue	0.41	0.19
		Sex:Tissue	0.06	0.99
		Treatment:Sex:Tissue	0.14	0.66
GST	Response ~ Treatment*Sex*Tissue + (1|ID)	Treatment	0.07	0.96
		Sex	0.53	0.039
		Tissue	0.18	0.30
		Treatment:Sex	0.27	0.27
		Treatment:Tissue	0.23	0.50
		Sex:Tissue	**0.90**	0.0041
		Treatment:Sex:Tissue	0.25	0.70

Table A.4. Biomarker response standard deviations in gills and digestive glands, by treatment and overall across treatments.

Biomarker	Gills (*n*=4 per treatment)					Digestive gland (*n*=5 per treatment)				
	Control	1 µg Cu/L	10 µg Cu/L	100 µg Cu/L	Overall	Control	1 µg Cu/L	10 µg Cu/L	100 µg Cu/L	Overall
cat	0.918	0.346	0.628	0.713	0.642	0.184	0.433	0.583	0.359	0.390
gst	0.547	0.527	0.178	0.516	0.472	0.296	1.14	0.592	0.192	0.625
hsp70	1.08	0.912	0.747	0.579	0.818	0.337	0.488	0.356	0.717	0.495
hsp90	0.177	0.286	0.346	0.175	0.272	0.590	0.267	0.224	0.448	0.409
mt	1.14	1.40	0.959	1.33	1.19	0.179	0.632	0.624	0.473	0.498
sod	0.675	0.364	0.527	0.415	0.554	0.314	0.332	0.300	0.578	0.411
AChE	0.644	0.741	0.498	0.836	0.646	0.343	0.611	0.309	0.484	0.494
GST	0.808	1.19	0.727	0.755	0.807	0.213	0.487	0.489	0.314	0.389

Table A.5. Biomarker weights in principal components 1 and 2, in *A. anatina* gills and digestive gland, respectively.

Biomarker	Gills		Digestive gland	
	PC1	PC2	PC1	PC2
cat	0.430	0.215	0.406	0.0947
gst	0.166	-0.565	0.482	-0.0805
hsp70	0.437	0.325	0.160	0.458
hsp90	0.243	0.0764	0.311	0.338
mt	0.491	0.182	0.455	-0.164
sod	-0.194	0.602	0.300	0.482
AChE	-0.342	0.356	0.339	-0.303
GST	-0.376	0.0276	0.262	-0.556

Table A.6. Observed mean responses and power analysis of copper effects in gills and digestive glands of *A. anatina*. Required sample size is rounded off to integers to give a power of approximately 0.8.

Biomarker	Gills (n=4 per treatment)				Digestive gland (n=5 per treatment)			
	Largest obs. mean response ($\Delta\log_2$)	Treatment effect size (Cohen's f)	Obs. power (1-β)	Req. Δn (1-β≈0.8)	Largest obs. mean response ($\Delta\log_2$)	Treatment effect size (Cohen's f)	Obs. power (1-β)	Req. Δn (1-β≈0.8)
cat	-0.51 (10 µg/L)	0.33	0.13	+23 (575%)	0.13 (10 µg/L)	0.22	0.099	+53 (1 060%)
gst	-0.58 (100 µg/L)	0.52	0.29	+7 (175%)	0.37 (1 µg/L)	0.21	0.097	+55 (1 100%)
hsp70	-0.48 (10 µg/L)	0.40	0.18	+14 (350%)	-0.32 (10 µg/L)	0.42	0.25	+12 (240%)
hsp90	0.27 (10 µg/L)	0.64	0.42	+4 (100%)	0.36 (10 µg/L)	0.43	0.27	+11 (220%)
mt	0.69 (1 µg/L)	0.43	0.21	+11 (275%)	-0.37 (100 µg/L)	0.36	0.19	+17 (340%)
sod	0.80 (10 µg/L)	0.69	0.48	+3 (75%)	0.47 (100 µg/L)	0.52	0.38	+6 (120%)
AChE	0.26 (100 µg/L)	0.30	0.12	+26 (650%)	0.69 (1 µg/L)	0.64	0.55	+3 (60%)
GST	-0.33 (10 µg/L)	0.17	0.071	+90 (2 250%)	-0.26 (100 µg/L)	0.40	0.23	+13 (260%)

Paper II

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RESEARCH ARTICLE

Molecular biomarker responses in the freshwater mussel *Anodonta anatina* exposed to an industrial wastewater effluent

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Abstract
Using a selection of molecular biomarkers, we evaluated responses in freshwater mussels (*Anodonta anatina*) exposed to effluent from an industrial wastewater treatment facility. The aims of this work were to (1) assess biomarkers of general toxicity under sublethal exposure to an anthropogenic mixture of chemicals, represented by an arbitrary effluent, and (2) evaluate the potential of *A. anatina* as a bioindicator of pollution. Adult mussels (n = in total 32; 24 males and 8 females) were exposed (96 h) in the laboratory to a fixed dilution of effluent or to a control treatment of standardized freshwater. Metal concentrations were in general higher in the effluent, by an order of magnitude or more, compared to the control. Toxic unit estimates were used as proxies of chemical stress, and Cu, Ni, and Zn were identified as potential major contributors (Cu> Ni > Zn). Six transcriptional (*cat*, *gst*, *hsp70*, *hsp90*, *mt*, *sod*) and two biochemical (AChE, GST) biomarkers were analyzed in two tissues, gills, and digestive glands. Out of the 16 responses (eight biomarkers × two tissues), 14 effect sizes were small (within ± 28 % of control) and differences non-significant ($p > 0.05$). Results did however show that (1) AChE activity increased by 40% in gills of exposed mussels compared to control, (2) *hsp90* expression was 100% higher in exposed female gills compared to control, and (3) three marker signals (AChE in both tissues, and *hsp70* in gills) differed between sexes, independent of treatment. Results highlight a need for further investigation of molecular biomarker variability and robustness in *A. anatina*.

Keywords Bivalve · Effect size · Mixture toxicity · RT-qPCR · Sex effects · Wastewater

Introduction

Chemically complex pollution from anthropogenic activities is a major concern in environmental protection and has gained considerable attention in ecotoxicology and environmental sciences. As a result of daily use in human activities, for instance, agriculture, industrial production, and use of, e.g., pharmaceuticals and personal care products, a variety of natural and synthetic compounds may eventually enter the environment (Anliker et al. 2020; Herrero-Hernández et al. 2020; Su et al. 2020; Vareda et al. 2019). Industries and households, via wastewater effluents or runoff, constitute major sources of complex pollution to aquatic recipients (Chen et al. 2020; Ellis and Butler 2015; López-Pacheco et al. 2019). Although separate pollutants in, e.g., treated wastewater often occur at low concentrations (Farkas et al. 2020; Vareda et al. 2019; Wang et al. 2018), they may interact and contribute to additive or synergistic biological effects when in mixtures, causing adverse effects in exposed organisms (Aronzon et al. 2020; Cedergreen 2014; Mebane et al. 2020; Wang et al. 2019). Therefore, in anthropogenic mixtures, such as wastewater effluents, with few conspicuous chemical parameters or without prior knowledge of the chemical composition, general biomarkers of chemical stress might be useful for detection of sublethal mixture toxicity. Furthermore, early biomarker responses can potentially be used under both laboratory and field settings to anticipate harmful effects from pollutant exposure and may, in the long term, improve strategies of monitoring of sensitive ecosystems and protection of recipients (e.g., van der Oost et al. 2003).

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Potential responses in organisms under toxic exposures include changes in molecular parameters, such as enzyme activity or transcript levels, some of which are commonly used as biomarkers to detect general chemical stress (Lehtonen et al. 2016; Perić and Burić 2019; Tsangaris et al. 2016). By definition, biomarkers are used to detect deviations from a normal state (e.g., van der Oost et al. 2003), which is often defined by a control group. However, there is a lack of data describing variabilities in responses and baseline signals, making it difficult to distinguish stress responses from background noise, i.e., normal variation. For molecular markers, it is often unclear how response magnitudes (i.e., effect sizes) vary with interacting internal (e.g., tissue, sex) and external factors (for instance, chemical composition, toxicant concentration, exposure time) (e.g., Bahamonde et al. 2016).

In this study, we assessed biochemical and transcriptional responses that represent commonly used biomarkers of general toxicity and chemical stress. For instance, the enzyme acetylcholinesterase (AChE) is involved in neurosignaling, and its activity in bivalves may respond to different types of mixture exposures (e.g., Aguirre-Martínez and Martín-Díaz 2020; Perić and Burić 2019; Tsangaris et al. 2016). Heat shock proteins protect cellular integrity and respond to a wide range of both chemical and physical stressors (Ferreira-Rodríguez et al. 2018; Liu et al. 2014, 2016). Catalase (CAT) and superoxide dismutase (SOD) protect against oxidative stress, and their activities as well as transcript levels may respond to mixture exposure (Bigot et al. 2011; Gonzalez-Rey et al. 2014; Lehtonen et al. 2016; Turja et al. 2013). Metallothionein (MT) is involved in maintaining cellular metal homeostasis and responds to various metal stressors (Bigot et al. 2011; Mourgaud et al. 2002), and glutathione-S-transferase (GST) is an enzyme important in toxicant metabolism and detoxification, responding to various stressors and mixtures (Bigot et al. 2011; Lehtonen et al. 2016; Perić and Burić 2019; Turja et al. 2013). Using the freshwater duck mussel (*Anodonta anatina*), we measured enzyme activities of AChE and GST, while *cat*, *gst*, heat shock protein 70 (*hsp70*), heat shock protein 90 (*hsp90*), *mt*, and *sod* were measured on the transcriptional level.

A. anatina is native to and widely distributed in Scandinavian and many European freshwater ecosystems (Lopes-Lima 2014). Bivalves are likely exposed to toxicants occurring in their (natural or laboratory) environment due to sessility and filtration feeding, and *A. anatina* could serve as an ecologically relevant freshwater model in ecotoxicology. Previous studies cover, e.g., pollutant uptake (Berglund et al. 2019; Nugroho and Frank 2011), molecular and behavioral biomarkers (Bielen et al. 2016; Falfushynska et al. 2013; Hartmann et al. 2016; Oliviera et al. 2015), and mortality (Kováts et al. 2010; Oliviera et al. 2015). The reproductive cycle of *A. anatina* includes a gravid stage during autumn/winter (Aldridge 1999; Hinzmann et al. 2013), potentially increasing variability in transcriptional and biochemical biomarkers (Ekelund Ugge et al. 2020).

Our objectives were to (1) evaluate selected responses as biomarkers of sublethal exposure to chemically complex, anthropogenic pollution and (2) assess *A. anatina* as a bioindicator species. An industrial wastewater effluent, i.e., a complex mixture, was used to represent an arbitrary anthropogenic stressor. While constituting a mixture of organic and inorganic substances, the main focus was, for practical reasons, limited to evaluation of metals. The selected biomarkers were assessed in *A. anatina* after acute (96 h) laboratory exposure to either a single effluent concentration or a control treatment of standardized freshwater. We hypothesized that (1) biomarker signals in digestive glands and gills would differ between effluent exposed and non-exposed mussels and that (2) gravid mussels would show different baseline signals (fixed effects) and/or response magnitudes (treatment interactions) compared to non-gravid.

Material and methods

Mussel collection and maintenance

Adult mussels (length 92 ± 17 mm) were collected on September 19, 2018, in Vinne å (Southern Sweden, 56° 06′ 45″ N, 13° 54′35″ E). The location is adjacent to human settlement and subject to, e.g., recreational fishing, but free from point sources of pollution. After being brought to the laboratory, the mussels were acclimatized to laboratory conditions for 26 days. During this period, a preliminary range-finding experiment was performed on a different subset of mussels (details presented in appendix A). Acclimatizing mussels were kept in two 40 L aquaria containing 30 L continuously aerated standardized freshwater (ISO 6341: 2012) with a nominal water hardness of 250 mg/L CaO_3. As bottom substrate, each aquarium contained an approximately 5 cm sand layer (0.2–0.7 mm grain size). Standardized freshwater and bottom substrate were both prepared as previously reported (Ekelund Ugge et al. 2020). Three times weekly, 15–20 L medium was renewed, and main experiment mussels were randomly re-distributed between aquaria to avoid tank effects on acclimatization. Additions of *Pseudokirchneriella subcapitata* were made to feed the mussels, corresponding to approximately 3.2×10^6 cells × mussel^{-1} × day^{-1}. No food was added within 48 h prior to the start of the experiment. During acclimatization and experimental periods, water temperature was 21±1° C, and the light cycle was 16 h light: 8 h dark.

Experimental treatment

Frozen samples of treated effluent water were obtained from an industrial wastewater treatment facility. The facility receives process and sanitary wastewater, as well as runoff, from an industrial area where mainly organic chemical products are

manufactured. Compounds such as organic acids, phenols, and aldehydes may occur at high concentrations in incoming wastewater but are efficiently removed in the treatment process (personal communication). Phenol and aldehyde samples from the sampling period measured <0.05 mg/L and <0.5 mg/L, respectively, as reported by the industry. Other plausible organic contaminants from raw materials and manufactured products have previously been analyzed but not detected and are therefore not routinely monitored. The industry reported an effluent total organic carbon (TOC) content ranging between 31 and 36 mg/L for the period when water was sampled (median 33 mg/L). Based on previous evaluation, this is assumed to largely consist of non-toxic microbial degradation products (personal communication). Organic pollutants were therefore not measured in this study, and instead, metal contamination was selected as the main focus and used as a proxy to represent the chemical complexity. Effluent metal concentrations are continuously monitored by the industry, and contamination is believed to result mainly from corrosion and erosion of, e.g., metal piping, galvanized steel, and stainless steel equipment, in the processing of organic compounds. A minor fraction is believed to result directly from manufacturing of metal containing products, and in addition, there might be metal contamination from raw materials and runoff from loading areas (personal communication).

Eight effluent samples, representing 8 consecutive days of effluents from the treatment facility, were thawed and mixed flow proportionally. During exposure, mussels were kept individually in glass containers (Ø 12 cm) of 1 L aerated medium (effluent or standardized freshwater), with 0.3 L sand added as bottom substrate. Light, temperature, and feeding conditions were the same as during acclimatization. Acute exposures of 96 h were performed as a trade-off between capturing immediate responses and allowing for potential time-dependent uptake of pollutants. After 96 ± 0.5 h, mussels were dissected. Gravid mussels were distinguished visually by the presence of immature glochidia in the gills (Figure A.1, appendix A). Gill and digestive gland tissues were dissected, and subsamples were immediately snap frozen in liquid nitrogen and subsequently stored at −80°C for biochemical assays and chemical analysis or submerged in RNA-Later (Invitrogen, USA) and stored at −20°C for gene expression analyses.

A preliminary range-finding experiment was performed to select an effluent concentration for the main exposure experiment (details presented in appendix A). A dilution to 60% of the initial concentration was selected, as this was the highest test concentration that did not appear to impair mussel filtration (roughly estimated by daily visual inspection of valve opening). In addition, this exposure, although overlapping with control treatment variation and not being replicated, showed implication of AChE inhibition (Figure A.2, appendix A).

In the main experiment, mussels were exposed to 60% industrial effluent water (n = 16) or a control treatment of standardized freshwater (n = 16). Glass containers with exposure media were prepared and kept under aeration approximately 24 h prior to experimental start. Before the addition of mussels, each container was sampled for chemical analysis of exposure media. pH and oxygen were monitored at 0 h, 48 h, and 96 h. Initial pH was 7.8 (± 0.04) and 8.2 (± 0.03) in control and effluent treatments respectively, steadily decreasing to 7.1 (± 0.3) and 7.7 (± 0.2) after 96 h. Oxygen saturation was consistently ≥90 % (7.9–9.1 mg O_2/L) in all containers, except for one effluent container in which saturation was 80 % (7.1 mg O_2/L) at 48 h. After the experiment was ended, distribution of gravid and non-gravid mussels was determined to be 3:13 and 5:11 in the control and effluent treatment, respectively.

Chemical analysis

A number of elements were analyzed in exposure media sampled (non-filtered) at the experimental start (Table 1) and in water samples from Vinne å (Table A.1, appendix A). Samples were kept frozen (−20°C) prior to analysis, and one control treatment sample was lost during freezing. Upon arrival to the chemical analysis laboratory, the water samples were acidified by addition of nitric acid (1% v/v), then analyzed by inductively coupled plasma sector field mass spectrometry (ICP-SFMS) (*Element*, Thermo Scientific, Germany), inductively coupled plasma atomic emission spectrometry (ICP-AES) (*Agilent ICP-OES 725*, Agilent, USA), and atomic fluorescence spectrometry (AFS) (*PSA Millennium Merlin*, P S Analytical, UK) according to standards from the International Organization for Standardization and the US Environmental Protection Agency (ISO 17852:2006, 11885:2007, 17294-2:2016. U.S. EPA 1994a, 1994b). Metal content was also determined in snap frozen tissue samples remaining after biochemical assays. Tissues were subject to nitric acid/hydrogen peroxide digestion, and metals were analyzed by ICP-SFMS (*Element 2*, Thermo Scientific, Germany) (ISO 17294-2: 2016; U.S. EPA 1994b) (Table 1).

Estimation of chemical stress

Toxic units (TUs) were calculated for sublethal organism effects (behavioral/ growth/ physiological/cellular/biochemical endpoints) and mortality, respectively (TU EC_{50} and TU LC_{50}), to quantitatively estimate the chemical stress imposed by effluent exposure. TUs were determined for elements measured in water, a priori excluding the non-metals P and Si as well as macrominerals Ca, K, Na, and Mg. Elements for which toxicity data was not found were simply reported as "not applicable" (NA) (Table A.2, appendix A). Mollusk 96-h 50%

Table. 1 Median concentration (min-max) of elements measured in in water and tissue samples (n = 16 per group).

		Ca	K	Mg	Na	Al	As	Ba	Cd
Water (μg/L)	Control*	77 000 (74 000–81 000)	3 200 (3 000–3 500)	10 000 (10 000–11 000)	19 000 (18 000–21 000)	25 (13–57)	0.061 (0.050†–0.094)	53 (25–63)	0.0052 (0.0020†–0.014)
	Effluent	40 000 (36 000–42 000)	14 000 (10 000–15 000)	6 000 (4 400–6 100)	370 000 (330 000–380 000)	48 (38–97)	0.23 (0.15–0.36)	82 (57–110)	0.023 (0.016–0.033)
Dig. gland (mg/kg WW)	Control	290 (130–610)	410 (370–680)	NA	230 (180–360)	NA	1.1 (0.49–1.3)	NA	0.056 (0.035–0.089)
	Effluent	260 (110–520)	480 (360–1 500)	NA	310 (230–560)	NA	1.0 (0.56–1.3)	NA	0.056 (0.028–0.095)
Gills (mg/kg WW)	Control	35 000 (12 000–71 000)	510 (360–750)	NA	420 (240–1 300)	NA	0.89 (0.30–2.4)	NA	0.14 (0.023–0.29)
	Effluent	31 000 (11 000–71 000)	550 (170–1 100)	NA	530 (200–670)	NA	0.89 (0.35–1.9)	NA	0.15 (0.042–0.25)
		Co	**Cr**	**Cu**	**Fe**	**Hg**	**Mn**	**Mo**	
Water (μg/L)	Control*	0.027 (0.0059–0.072)	0.023 (0.010†–0.054)	0.68 (0.48–0.89)	4.5 (0.59–41)	<0.0020‡	2.5 (1.5–5.9)	0.27 (0.17–0.33)	
	Effluent	0.21 (0.17–0.33)	3.1 (2.3–3.7)	7.7 (5.1–13)	28 (16–54)	<0.0020‡	6.5 (2.8–9.2)	9.1 (7.9–9.5)	
Dig. gland (mg/kg WW)	Control	0.078 (0.045–0.28)	0.032 (0.030†–0.070)	3.2 (2.3–4.5)	73 (42–120)	0.027† (0.014–0.033)	14 (1.8–40)	NA	
	Effluent	0.079 (0.058–0.20)	0.040 (0.030†–0.13)	3.2 (2.2–5.2)	69 (27–120)	0.023 (0.011–0.051)	10 (1.8–35)	NA	
Gills (mg/kg WW)	Control	0.17 (0.033–1.0)	0.26 (0.048–0.55)	1.3 (0.71–3.4)	470 (89–1500)	0.013 (0.010†–0.023)	2 300 (500–5 600)	NA	
	Effluent	0.19 (0.052–0.62)	0.23 (0.11–0.76)	1.3 (0.83–8.6)	510 (120–1800)	0.013 (0.010†–0.017)	2 200 (1 000–5 300)	NA	
		Ni	**P**	**Pb**	**Si**	**Sr**	**V**	**Zn**	
Water (μg/L)	Control*	0.061 (0.050†–0.085)	7.2 (3.4–14)	0.028 (0.010†–0.061)	960 (600–1 300)	68 (50–80)	0.15 (0.075–0.22)	0.54 (0.34–1.4)	
	Effluent	16 (12–19)	120 (93–140)	0.11 (0.038–0.18)	2 200 (2 000–2 300)	69 (58–77)	0.43 (0.33–0.63)	8.4 (4.4–18)	
Dig. gland (mg/kg WW)	Control	0.073 (0.053–0.12)	NA	0.040† (0.040–0.10)	NA	NA	NA	12 (8.4–15)	
	Effluent	0.13 (0.072–0.29)	NA	0.040† (0.040–0.20)	NA	NA	NA	12 (8.8–14)	
Gills (mg/kg WW)	Control	0.078 (0.040†–0.18)	NA	0.055 (0.040†–0.11)	NA	NA	NA	130 (24–290)	
	Effluent	0.10 (0.040†–0.26)	NA	0.058 (0.040†–21)	NA	NA	NA	130 (36–290)	

*n = 15

†≥ 1 sample <LOR, assumed to equal LOR

‡All samples <LOR

effect concentrations (EC_{50}) and 96-h 50% lethal concentrations (LC_{50}) from laboratory experiments were retrieved from the US EPA ECOTOX database (https://cfpub.epa.gov/ecotox/) on September 7–8, 2020 (details presented in appendices B and C, deposited at https://data.mendeley.com/datasets/jc469bc5mv/1). Toxic units (TU EC_{50} and TU LC_{50}) were calculated for each metal as TU XC_{50} = Measured conc. /Mollusk 96 h XC_{50}, with measured concentration and effect concentration represented by the respective median. TUs for all metals were added, as $TU_M XC_{50} = \sum TU\ XC_{50}$, to represent additive mixture toxicity (TU_M EC_{50} and TU_M LC_{50}) for each treatment (Table A.2, appendix A).

As an additional estimate of relative contributions to stress, measured metal concentrations were, when applicable, converted to fraction of the respective environmental quality standards (EQSs) for inland surface waters, as Fraction of EQS = Measured conc. /EQS. Measured concentrations were represented by the median of measured total concentration, and EQSs by the respective annual mean EQS from European and Swedish legislation (European Parliament and Council 2013; Havs- och vattenmyndigheten 2019) (Table A.2, appendix A).

Biomarker assays

The biomarker selection consisted of eight molecular biomarkers. The enzymatic assays for AChE and GST activities were based on the rates for hydrolysis of acetylthiocholine (Bocquené and Galgani 1998) and glutathione conjugation to 1-chloro-2,4-dinitrobenzene (Habig et al. 1974), respectively. Samples were prepared in phosphate buffers, and spectrophotometric analyses (using a *SpectraMax 190* plate reader (Molecular Devices, USA)) were performed, all according to previous description (Ekelund Ugge et al. 2020). All enzyme activities were normalized, first by sample protein concentration (Bradford 1976) and second to the mean activity of control samples of the respective tissue.

For transcriptional markers (*cat*, *gst*, *hsp70*, *hsp90*, *mt*, and *sod*), relative transcript levels were measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR). RNA was extracted using the *Norgen's Total RNA Purification Kit* (Norgen, Canada), including 40 μM of DL-dithiothreitol (DTT, Promega, USA) in the lysis buffer. Tissues were homogenized using a *TissueLyser II* (Qiagen, Germany) and 5-mm stainless steel beads (Qiagen, Germany). RNA amounts and A260/A280 ratios (showing 1.9–2.1) were measured using a *NanoDrop 2000* spectrophotometer (Thermo Scientific, USA). Before cDNA synthesis, the RNA was DNAse treated using the *Heat&Run gDNA removal kit* according to instructions (ArcticZymes, Norway), and the RNA integrity was assessed in randomly selected subset of samples using a *Fragment Analyzer* (Advanced Analytical, Austria). Since our interest was relative rather than absolute gene expression, and only short sequences were targeted (<200 bp, Ekelund Ugge et al. 2020, appendix), reverse transcription was performed despite apparent RNA degradation (RNA quality numbers (RQN) of 1.8–2.3 and 2.8–4.5 in the gills and digestive glands, respectively). cDNA was synthesized by reverse transcription of 200 ng and 100 ng RNA for digestive gland and gills, respectively, using the *TATAA GrandScript cDNA* synthesis kit (TATAA Biocenter AB, Sweden). The qPCR assays were performed as previously described (primer sequences are presented in Table A.3, appendix A, and assay details are found in Ekelund Ugge et al. 2020). Gene expression was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), where expressions were normalized by the mean of control samples of the gill and digestive gland tissue, respectively, and then internally for each individual sample by the mean of two reference genes, *β-actin* and *28S* rRNA.

Statistics

Statistical analyses were run and figures were generated in R version 4.0.2 (R Core Team 2020). For the principal component analysis (PCA), water and tissue concentrations of measured metals were normalized as percentage of the respective mean from control samples and $\log_{10}$-transformed. For various chemical parameters, there were samples showing concentrations below the levels of reporting (LOR). Unless that was true for the majority (≥ 50 %) of samples, concentrations <LOR were assumed to equal the respective LOR. If, however, a majority of samples displayed concentrations <LOR, as for Hg in water and Pb in digestive glands, the parameter was removed completely from the respective PCA. For elements measured in both tissue and water samples, correlations (Pearson) were determined for measured concentrations ($\log_{10}$-transformed), excluding datapoints for which either concentration (tissue or water) was <LOR. Biomarker responses ($\log_2$-transformed) were analyzed by linear models and separated by tissue. Treatment, sex, and the treatment/sex interaction were used as fixed factors in the full models. Model selection for linear models was based on lowest AIC scores after sequential ANOVA analysis, where least significant ($p > 0.05$) effect factors were removed one at a time. Residual normality for biomarker responses was assessed by Shapiro-Wilk normality tests and Q-Q plots. Significant differences ($\alpha = 0.05$) in the final models were identified with a Tukey HSD post hoc test, using the "emmeans" package (Lenth 2020). Treatment effects on overall variation were assessed by a paired *t*-test, in which the coefficient of variation (CV) of each biomarker/tissue pair was compared between control and exposed mussels. CV was calculated by dividing each biomarker/tissue standard deviation by corresponding mean response (non-log-transformed). Finally, the "ggplot2" package (Wickham 2016) was used to produce all figures.

Results

With regards to chemical composition, digestive gland control and effluent samples were slightly separated along principal component 2 of the PCA (Figure 1A), while there was no apparent difference in gills (Figure 1B). The control and effluent exposure media were however highly separated along principal component 1 (Figure 1C). Most concentrations were higher in the effluent medium than in the control medium, many by an order of magnitude (e.g., Na, Co, Cu, Fe, Mo, P, and Zn) or more (e.g., Cr and Ni) (Table 1). The only elements at higher concentration in the control medium were Ca and Mg, with roughly twice as high concentrations as in the effluent exposure. In contrast, tissue levels were overall similar between treatments (Table 1). The most notable difference was an approximately 2-fold higher digestive gland concentration of Ni in exposed mussels compared to control, positively correlated to exposure concentration (Table 1, Figure A.3, Table A.4, appendix A). In addition, digestive gland Na levels were also positively correlated to exposure concentration, while no correlation was detected between tissue and water concentrations of As, Ca, Cd, Co, Cr, Cu, Fe, K, Mn, Pb, and Zn (Figure A.3, Table A.4, appendix A).

The effluent and control TU_M EC_{50} were 0.15 and 0.020, respectively (Table A.2, appendix A). This corresponds to approximately 1/7 and 1/50 of estimated median effect concentration for sublethal organism effects to occur. With regards to mortality, the effluent TU_M LC_{50} was 0.039 (1/26 of the estimated median lethal metal exposure), while the control TU_M LC_{50} was 0.0028 (1/360 of the median lethal exposure) (Table A.2, appendix A). Based on TU estimations, Cu contributed the most to metal toxicity, followed by Ni, Zn, and Al (Al>Zn for sublethal organism effects, Zn>Al for mortality). Finally, total concentrations of Cu, Ni, and Zn in the effluent exposure exceeded their respective EQS for bioavailable concentration in inland surface waters, by factors of 15, 4.1, and 1.5, respectively, while Cu in the control exceeded the EQS by a factor of 1.4 (Table A.2, appendix A).

Only two of the eight biomarkers, AChE and *hsp90*, showed a significant treatment effect and interaction,

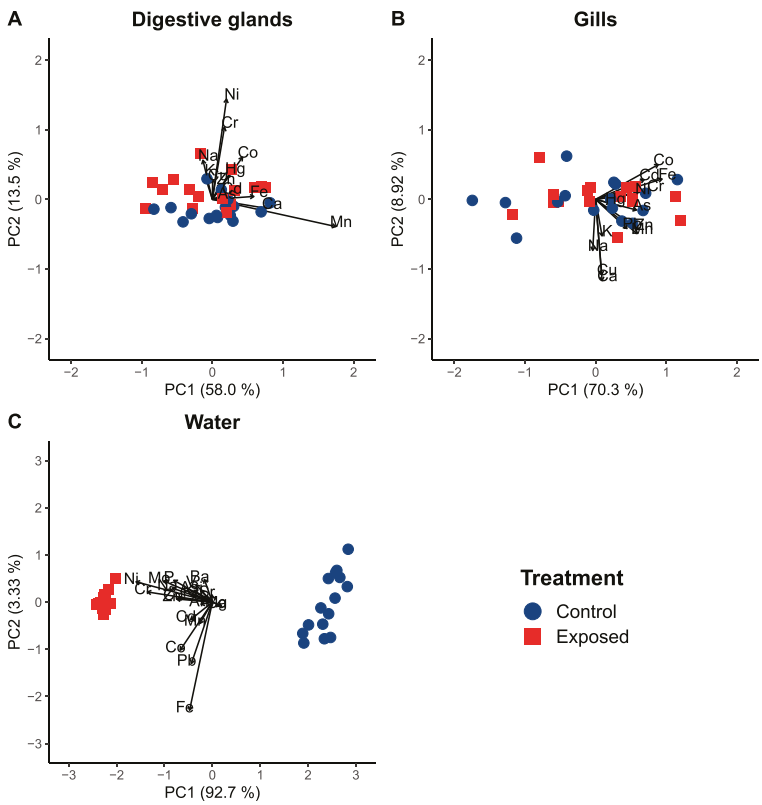

Figure 1 Principal component analyses of metal content (as well as P and Si content in water) in **A** digestive glands and **B** gills of *Anodonta anatina* after 96-h exposure to industrial wastewater effluent and **C** initial concentrations in water. Arrows show relative weights of each measured parameter and are scaled to the length of the plot axes

respectively, and only in gills. Other biomarker responses to the effluent exposure were within ± 28 % of the respective control (log_2 fold changes ranging from −0.19 to 0.36), across both gills and digestive glands (Table 2, Figures 2 and 3). AChE activity in exposed mussels was 40% higher than control (log_2 fold change = 0.48) (Table 2, Figure 2). For *hsp90*, a treatment/sex interaction revealed a higher expression in both exposed females (100% higher, log_2 fold change = 1.0) and males (79 % higher, log_2 fold change = 0.84) compared to control females (Table 2, Figure 2).

Two gills and one digestive gland biomarker showed sex differences, independent of treatment. AChE activity and *hsp70* expression were 61 % higher and 36 % lower, respectively, in male gills compared to females (log_2 fold changes = 0.69 and −0.63, respectively, Table 2, Figure 2). In digestive glands, males demonstrated a 37% higher baseline AChE activity than females (log_2 fold change = 0.46, Table 2, Figure 3).

Biomarker CVs ranged from 13 to 85% (Figure A.4, appendix A). Assessed pairwise across biomarkers and tissues; variation increased with effluent exposure in eleven out of 16 biomarker/tissue pairs, and decreased in five (Figure A.4, appendix A). The median CV increased from 32 in control treatments to 38 % in the effluent group (p = 0.017, Figure A.4, appendix A).

Discussion

Exposure and chemical stress

The selected industry mainly produces organic chemical products (personal communication). Consistently, monitoring data from the industry showed TOC levels around 30 mg/L in the undiluted effluent, and we assumed elevated TOC for effluent exposures relative the control. By comparison, our daily feeding of the mussels would have added a negligible amount of up to approximately 100 μg organic carbon L^{-1} day^{-1} in each exposure tank, assuming an algal carbon content in the range of 5–30 pg/cell (e.g., Pérez-Morales et al. 2015). In routine monitoring performed by the industry, phenol and aldehyde levels in the effluent were typically below detection limits, and when analyzed, other plausible organic pollutants have not been detected. Therefore, without dismissing potential impact from or interactions with organic and other inorganic substances, the focus of this study was, however, narrowed down to metal toxicity, as a proxy of chemical stress from mixture toxicity.

The effluent metal content was in general, when adjusted for dilution, within the orders of magnitude previously reported by the industry (personal communication). The exceptions were Ca and Mg, both occurring at approximately twice as high concentration in the control as in the exposure medium. As essential components in the standardized freshwater, they are assumed non-toxic at current concentrations. Remaining metals occurred at higher concentration compared to the control treatment, suggesting that the effluent exposure might, by comparison, be viewed as a complex chemical stressor, even without considering the potential contribution from organic or other inorganic compounds.

Effluent TU_M EC_{50} and TU_M LC_{50} were both an order of magnitude higher than the control, further implying a higher level of chemical stress. Three of the metals contributing most to toxicity, Cu, Ni, and Zn, showed levels at least an order of magnitude higher as compared to the control. Total concentrations of these metals exceeded their respective EQS for inland surface waters, although environmental impact assessment is to be based specifically on the bioavailable fraction. For instance, metal bioavailability can decrease with, e.g., water hardness and dissolved organic carbon (Bourgeault et al. 2010; Shoults-Wilson et al. 2010; Wang et al. 2009), implying bioavailability below 100% of the total concentration under current settings. On the other hand, effluent concentrations of Cu, Ni, and Zn, but also e.g. Cr, were all within ranges that separately may trigger various molecular responses in bivalves upon acute (72–96 h) exposures (e.g., Ciacci et al. 2012; Franzellitti et al. 2020; Li et al. 2018; Potet et al. 2016). We therefore argue that the current effluent exposure represents a sublethal acute stressor to which molecular responses, albeit not whole-organism effects, would be expected.

Metal uptake in bivalve soft tissues may be observable within hours (e.g., Cai and Wang 2019; Lee and Lee 2005), suggesting that 96-h exposure would be sufficient for uptake to occur. Yet, apart from Ni and Na in digestive glands, we could detect no correlation between external exposure and tissue concentration. The body burden depends on uptake from water, dietary uptake, and elimination rates, all of which are variable (Luoma and Rainbow 2005), and even with sufficient time, uptake might be limited by bioavailability. On the other hand, dietary sources might contribute substantially to total metal uptake (Lee et al. 2015; Luoma and Rainbow 2005). Filter feeding would thus be a potential exposure route of metals associated with algal cells or present in particulate forms (Hull et al. 2013; Lee et al. 2015). Considering the static exposure and moderate concentrations of each separate metal, elimination rates after 96 h might have been high enough to balance potential uptake (King et al. 2005; Nugroho and Frank 2011). Another potential explanation is that the effluent might trigger avoidance behavior to reduce the actual exposure (Hartmann et al. 2016). Avoidance was however not tested and not specifically noted upon visual inspection (e.g., prolonged valve closure), except for the 100 % effluent exposure in the preliminary experiment. Regardless, the overall implication, based on measured water and tissue concentrations, is that metal uptake was in most cases balanced or exceeded by excretion.

Table 2 Final model for biomarker endpoints and effect sizes of treatment responses, sex differences and treatment/sex interactions. F statistics, degrees of freedom, model term *p* values and significant differences (post hoc) are presented for the linear models. Mean treatment responses are presented for all biomarkers except when there was a significant treatment/sex interaction. Mean sex differences and treatment/sex interactions are presented when included in the final model

Endpoint		Final model	Factor	F	d.f.	*p*	Significant differences (post hoc)	Treatment response (log_2 fold change in WW compared to C)	Sex difference (log_2 fold change in M compared to F)	Treatment/sex interaction (log_2 fold-change compared to mean C)
Dig. gland	*cat*	Resp ~ 1	–	–	0, 31	–	–	0.157	–	–
	gst	Resp ~ 1	–	–	0, 31	–	–	0.205	–	–
	hsp70	Resp ~ 1	–	–	0, 31	–	–	0.357	–	–
	hsp90	Resp ~ Treat	Treat	2.17	1, 30	0.151	–	0.298	–	–
	mt	Resp ~ 1	–	–	0, 31	–	–	0.244	–	–
	sod	Resp ~ Sex	Sex	2.44	1, 30	0.129	–	0.191	–0.480	–
	AChE	Resp ~ Sex	Sex	7.69	1, 30	0.00944	**F<M** (p = 0.00944)	0.0736	**0.456**	–
	GST	Resp ~ 1	–	–	0, 31	–	–	0.0376	–	–
Gills	*cat*	Resp ~ Sex	Sex	2.33	1, 30	0.137	–	0.137	0.311	–
	gst	Resp ~ 1	–	–	0, 31	–	–	–0.142	–	–
	hsp70	Resp ~ Sex	Sex	13.3	1, 30	0.000983	**F>M** (p = 0.000983)	0.104	**–0.634**	–
	hsp90	Resp ~	Treat	3.84	1, 28	0.0600	**C:F<WW:F** (p = 0.0222)	–	–	**–0.581 (C:F)**[a]
		Treat ×	Sex	1.01	1, 28	0.323	**C:F<WW:M** (p = 0.0396)			0.134 (C:M)[a, b]
		Sex	Treat/Sex	5.61	1, 28	0.0249				**0.442 (WW:F)**[b]
										0.257 (WW:M)[b]
	mt	Resp ~ 1	–	–	0, 31	–	–	0.165	–	–
	sod	Resp ~ Sex	Sex	2.66	1, 30	0.114	–	–0.189	0.399	–
	AChE	Resp ~ Treat +	Treat	5.09	1, 29	0.0317	**C<WW** (p = 0.0118)	**0.484**		–
		Sex	Sex	9.88	1, 29	0.00384	**F<M** (p = 0.00384)		**0.690**	
	GST	Resp ~ 1	–	–	0, 31	–	–	0.158	–	–

F females, *M* males, *C* control, *WW* wastewater effluent. For treatment/sex interactions, significant differences (p<0.05) are indicated by different letters

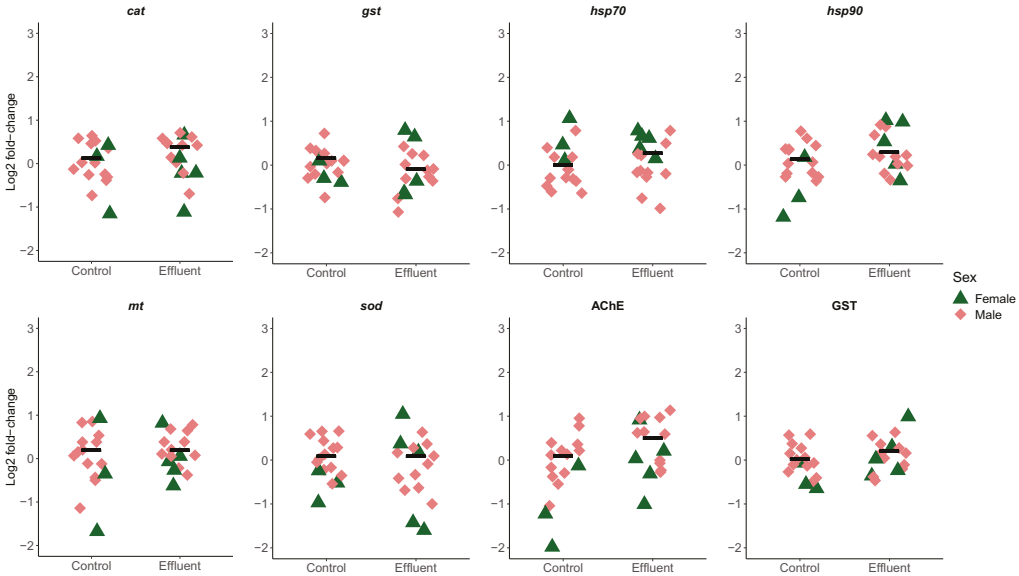

Figure 2 Biomarker responses (log_2 fold change relative control) in gills of Anodonta anatina exposed to a control treatment (n= 16) or an industrial wastewater effluent (n = 16) for 96 h. Bars correspond to median responses

Treatment effects

Stress proteins (here *hsp70*, *hsp90*, and *mt*) and markers of redox homeostasis (here *cat*, *gst*, GST, and *sod*) have been suggested as two key groups of biomarkers for general metal toxicity (Le Saux et al. 2020). Even low metal concentrations have been demonstrated to increase bivalve expression and activity of *cat*, *gst*, GST, *hsp70*, *mt*, and *sod* by ≥ 50 % (e.g., Ciacci et al. 2012; Franzellitti et al. 2020; Li et al. 2018; Perić and Burić 2019). In contrast, effect sizes from the effluent exposure were overall small. The mussel gill is the first organ in contact with waterborne pollutants, which may explain the responses in AChE and *hsp90*. However, apart from AChE and *hsp90*, all gill biomarker signals in the effluent exposure were within ± 12 % of the control. Hence, potential responses were not distinguishable from baseline noise. In digestive glands, all biomarkers responded to the effluent by ≤ 28 % increases, consistently non-significant despite elevated tissue levels of, e.g., Ni. One possible explanation could be a certain level of general metal tolerance, as the experimental mussels had previously been exposed to higher concentrations of, e.g., Fe, Mn, and Al (Table A.1, appendix A). Adaptation to metal exposure might for instance cause inter-population differences in transcriptional response patterns (Milan et al. 2016). This would affect the predictability of, e.g., biomarker effect sizes, potentially reducing the general sensitivity to relevant changes in the environment. Furthermore, it is possible that larger effect sizes would have been observed in immediate or long-term responses, but simply not captured by the 96-h static exposure. In order for a biomarker to be robust in, e.g., environmental monitoring, responses also require a certain degree of stability over time. Thus, results suggest that selected biomarkers, with potential exceptions of gill AChE and *hsp90*, were separately not robust and/or not sensitive enough to detect the effluent exposure in particular and perhaps not low to moderate stress in general.

AChE activity is quite commonly inhibited by chemical stressors (Bocquené and Galgani 1998). For instance, AChE inhibition has been demonstrated in *Anodonta cygnea*, a close relative to *A. anatina*, after acute exposure to low levels of a complex metal mixture (Butrimavičienė et al. 2019). While performed without replication of the effluent treatments, implication of AChE inhibition was also observed in our preliminary experiment, however, overlapping with the response range in the control group. In contrast, the main experiment demonstrated a clear 40 % increase in AChE gill activity in the effluent exposure. These seemingly contradictive results likely reflect high AChE variability and insufficient replication in the preliminary experiment. In fact, increased activity of AChE and other cholinesterases has been previously observed in other taxa after acute metal exposures (Brahma and Gupta 2020; Dahms-Verster et al. 2020; Oliva et al. 2019). Taken together, this suggests that AChE in *A. anatina* is quite

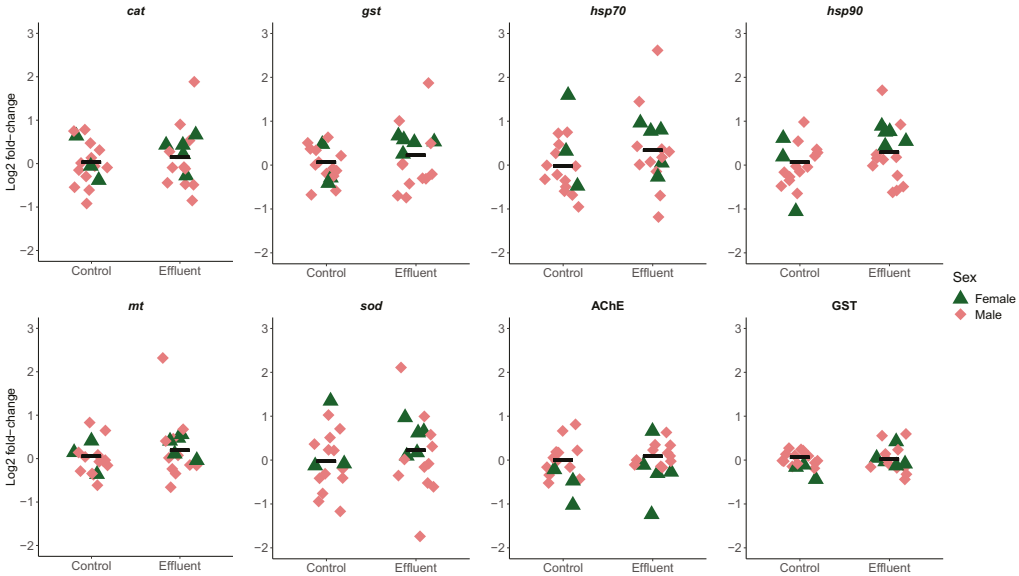

Figure 3 Biomarker responses (log_2 fold change relative control) in digestive glands of Anodonta anatina exposed to a control treatment (n= 16) or an industrial wastewater effluent (n= 16) for 96 h. Bars correspond to median responses

variable, and that this enzyme might be less robust as a biomarker than what is often assumed.

Expression of *hsp90* demonstrated a treatment/sex interaction in gills. In this general stress marker, effluent exposure induced a 79–100% higher expression in both males and females but only compared to control females. This suggests that at least in gravid females, current stress levels were enough to induce a clearly detectable biomarker response. Still, it must be noted that both treatment groups consisted of 70–80% males, which for *hsp90* would obscure this effect if not including sex in the model. Therefore, without consideration of sex interactions, only a single biomarker (AChE in gills) showed a distinguishable treatment response under current exposure.

Sex effects and response variability

Consistent with previous findings in *A. anatina* (Ekelund Ugge et al. 2020), we found higher *hsp70* expression in gills and lower AChE activity in digestive glands of gravid females, compared to males. In addition, the current study detected a sex effect in gill AChE activity as well as the *hsp90* treatment/sex interaction (discussed under the "Treatment effects" section), while there were no differences in, e.g., *cat*, *mt*, or GST as described previously (Ekelund Ugge et al. 2020). The different observations could result from random variation or differences between experiments (e.g., experiments carried out at different temperatures, mussels potentially collected or exposed at different stages of gravidness). Overall, the results therefore highlight biomarker variability, suggesting sex, and in particular gravidness, as potential confounding factors.

Responses to chemical stress may to a certain extent be buffered by various biological and ecological processes, and responses on one level often do not translate proportionally to adverse effects at higher organizational levels (e.g., Forbes and Calow 2002; Geist et al. 2007). Conversely, even when the mean response of an ecotoxicological endpoint remains unaffected by stress, underlying variation can potentially increase, and thus, response variability has in itself been suggested as a relevant toxicological endpoint (Nikinmaa and Anttila 2019). In addition to variability introduced by sex differences, we demonstrated an increase in variation for a majority of markers in *A. anatina* from the effluent exposure. In, e.g., risk assessment and environmental monitoring, biological responses to chemical stress should preferably be approached both by using multiple biomarkers (ideally in multiple tissues) and by incorporating variability measures in such biomarker panels.

Conclusions

The small effect sizes suggest an inability of the chosen biomarkers to reliably indicate exposure to anthropogenic effluents in *A. anatina*. Only two biomarkers, one biochemical and one transcriptional, responded to exposure. Furthermore,

despite increased tissue concentration of Ni in digestive glands, treatment responses were only observed in gills. This is further complicated by the confounding factor gravidness, which mainly appears to affect gill responses. An overall increase in variation across markers after the effluent exposure suggests that multi-biomarker approaches may potentially increase robustness for detection of chemical stress, despite (and potentially due to) high inherent variability of the separate markers. In future research, we propose continued assessment of multi-biomarker approaches as well as inter- and intra-population variability, both in terms of confounding effects and marker variation as a potential endpoint in itself.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-021-15633-4.

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Exposure treatments and biochemical assays were performed in the ALS Scandinavia Toxicon AB laboratory facilities. RNA extraction was performed in the Aquatic Ecology DNA lab and the Molecular Ecology and Evolution Lab (MEEL), Department of Biology, Lund University (Lund, Sweden). The cDNA synthesis, RNA fragment analysis, and qPCR assays were performed at TATAA Biocenter AB. Chemical analyses were performed by ALS Scandinavia.

Author contribution Gustaf Ekelund Ugge conceptualized and designed the study with support and supervision from Olof Berglund and Annie Jonsson. Experiments and analyses were carried out by Gustaf Ekelund Ugge, who also wrote the original draft. All authors contributed to and approved the final version of the manuscript.

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TATAA Biocenter AB was involved in designing and performing the transcriptional biomarker assays, and ALS Scandinavia (Luleå, Sweden; parent company of Toxicon AB) performed the chemical analyses of tissue and water samples. Staff from ALS Scandinavia Toxicon AB occasionally assisted in laboratory maintenance (feeding mussels and measuring water pH/O_2). Parts of the manuscript were discussed with a representative from the anonymous chemical company before submission to agree on technical descriptions of the industry and the effluent. Otherwise, no funding sources were involved in designing the experiments, collecting data, or writing the manuscript.

Availability of data and materials Experimental datasets, as well as literature datasets used for toxic unit (TU) calculations, can be found at https://data.mendeley.com/datasets/jc469bc5mv/1.

Declarations

Ethics approval and consent to participate The work was carried out in accordance with Swedish legislation and mussels were collected in agreement with the fishing holder of Vinne å.

Consent for publication The chemical company providing effluent water has consented to publication but has requested to remain anonymous. We ask readers to stay aware that our results represent a static laboratory exposure, and as such they should not be interpreted as representative of conditions in the actual recipient.

Competing interests Gustaf Ekelund Ugge is employed by ALS Scandinavia Toxicon AB, but is on a leave of absence for pursuing a PhD.

In order to ensure anonymity as well as technical correctness, a representative of the anonymous chemical company was allowed to read a draft of the manuscript prior to submission, to comment on descriptions of the industry, the wastewater treatment and routine measurements performed by the company.

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Appendix A

Molecular biomarker responses in the freshwater mussel *Anodonta anatina* exposed to an industrial wastewater effluent

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Appendix A

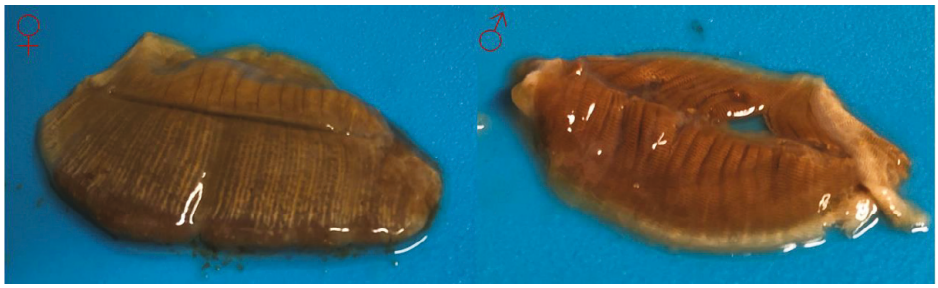

Figure A.1 Extracted gills of gravid (left) and non-gravid (right) *Anodonta anatina.*

Appendix A

Preliminary dose-finding experiment

Mussels for the preliminary experiment were collected the same date and at the same location as the main experiment mussels (19th of September 2018 in Vinne å, Southern Sweden). They were kept in a separate 40 L aquarium and acclimatized to laboratory conditions for 16 days. During acclimatization and experimental exposure, conditions were the same as in the main experiment.

For the experiment, effluent water was stepwise diluted with standardized freshwater into seven concentrations (4.7-100 %), using a dilution factor of $1\frac{2}{3}$. Mussels (length 69 ± 9 mm) were exposed for 96 h to effluent (n = 1 per concentration) or to a control treatment of standardized freshwater (n = 3), and gill AChE activities were assessed (see *Biomarker analysis* section under main article M&M).

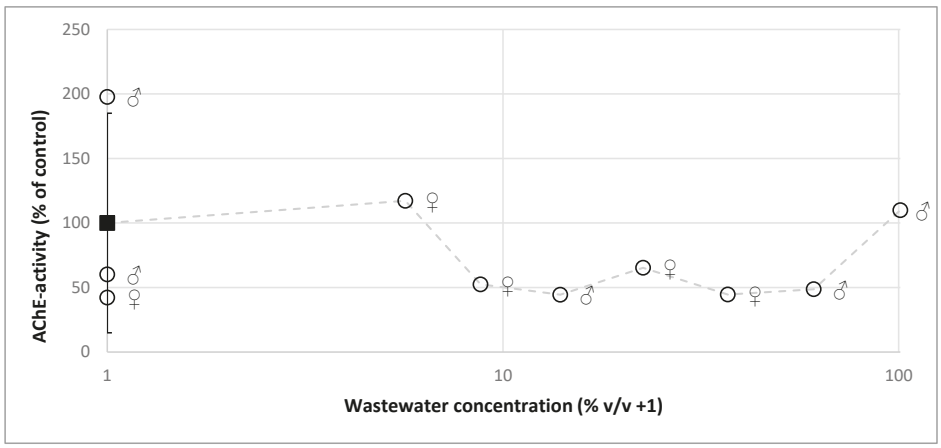

Figure A.2 Relative acetylcholinesterase (AChE) activity in gills of *Anodonta anatina* after 96 h exposure to industrial wastewater effluent. Results are part of a preliminary dose-finding experiment consisting of a control treatment (n = 3) and industrial wastewater in the range of 4.7 – 100 % (volume/volume) of the initial effluent concentration (n = 1 per treatment, in total seven concentrations). Circles represent individual mussels, while the black square and error bars show control group mean and SD. Symbols ♀ and ♂ denote gravid and non-gravid individuals.

Appendix A

Table A.1 Chemical composition of Vinne å water (n = 7). Water (unfiltered) was sampled between January and July 2018 (January 5th and 30th, February 21st, April 5th and 27th, May 27th and July 13th). † ≥ 1 sample <LOR.

Element	Median (µg/L)	Min – max (µg/L)
Ca	56 000	34 000 – 78 000
K	1 900	1 700 – 2 300
Mg	2 200	1 600 – 2 700
Na	11 000	8 500 – 15 000
Al	94	22 – 210
As	0.27	0.23 – 0.32
Ba	34	22 – 44
Cd	0.016	0.0033 – 0.034
Co	0.19	0.077 – 0.23
Cr	0.21	0.055 – 0.32
Cu	2.4	0.95 – 3.4
Fe	463	100 – 690
Hg	0.0035	0.002† – 0.0081
Mn	60	45 – 68
Mo	0.28	0.22 – 0.38
Ni	0.84	0.31 – 1.5
P	33	19 – 43
Pb	0.22	0.061 – 0.34
Si	3 800	2 800 – 4 400
Sr	120	77 – 180
V	1.0	0.81 – 1.2
Zn	4.0	0.85 – 5.1

Appendix A

Toxic unit (TU) estimations

Data based on eggs, embryos and larvae were excluded from each metal dataset, and so were entries with unclear effect concentrations (e.g. denoted by 'NR', '~', '<' or '>'). Juveniles were included only from studies that did not include adults. Consequently, if both adults and juveniles of the same species were examined in a single study, only the adult data were extracted. In studies reporting multiple effect concentrations for the same species (e.g. due to different exposure setups), all entries except the highest concentration were excluded in order not to overestimate the stressor. After the selection above, one entry for each species per study was used to identify the median mollusk 96 h EC_{50} and LC_{50} of each metal (Table A.2, full datasets and references presented in appendices B and C).

Appendix A

Table A.2 Metal toxic units in the control and wastewater treatments, and measured concentrations as fractions of respective environmental quality standard (EQS). Toxic units are based on mollusk 96 h 50 % effect concentration of sublethal organism effects (EC_{50}) and mortality (LC_{50}). Metal toxicity is assumed additive in the mixture toxic units (TU_M) of the control and wastewater exposures. Toxicity data were retrieved from the U.S. EPA ECOTOX database (appendices B and C).

Metal	TU EC_{50}		Median 96 h EC_{50} (µg/L)	TU LC_{50}		Median 96 h LC_{50} (µg/L)	Fraction of EQS		EQS (annual average; µg/L)
	Control	Wastewater	(# of EC_{50} entries)	Control	Wastewater	(# of LC_{50} entries)	Control	Wastewater	
Al	$1.0 * 10^{-2}$	$2.0 * 10^{-2}$	2 400 (1)	$4.4 * 10^{-4}$	$8.7 * 10^{-4}$	55 500 (5)	-	-	-
As	$5.5 * 10^{-7}$	$2.1 * 10^{-6}$	110 000 (1)	$2.5 * 10^{-6}$	$9.6 * 10^{-6}$	24 500 (3)	$1.2 * 10^{-1}$	$4.7 * 10^{-1}$	0.50[1]
Ba	-	-	NA (0)	-	-	NA (0)	-	-	-
Cd	$3.0 * 10^{-6}$	$1.3 * 10^{-5}$	1 700 (4)	$2.4 * 10^{-6}$	$1.0 * 10^{-5}$	2 200 (78)	$2.1 * 10^{-2}$	$9.0 * 10^{-2}$	0.25[1,2,3]
Co	-	-	NA (0)	-	-	NA (0)	-	-	-
Cr	-	-	NA (0)	$1.5 * 10^{-6}$	$2.0 * 10^{-4}$	15 200 (18)	$6.8 * 10^{-3}$	$9.0 * 10^{-1}$	3.4[1]
Cu	$9.2 * 10^{-3}$	$1.0 * 10^{-1}$	74 (13)	$2.2 * 10^{-3}$	$2.4 * 10^{-2}$	315 (112)	**1.4**	**15**	0.5[1,4]
Fe	$7.1 * 10^{-5}$	$4.4 * 10^{-4}$	64 (2)	$4.4 * 10^{-7}$	$2.7 * 10^{-6}$	10 300 (2)	-	-	-
Hg	$\leq 3.0 * 10^{-6}$	$\leq 3.0 * 10^{-6}$	670 (3)	$\leq 1 * 10^{-5}$	$\leq 1 * 10^{-5}$	200 (43)	-	-	-
Mn	-	-	NA (0)	$5.5 * 10^{-5}$	$1.4 * 10^{-4}$	45 600 (3)	-	-	-
Mo	-	-	NA (0)	-	-	NA (0)	-	-	-
Ni	$7.9 * 10^{-5}$	$2.1 * 10^{-2}$	770 (1)	$4.3 * 10^{-5}$	$1.1 * 10^{-2}$	1 440 (19)	$1.5 * 10^{-2}$	**4.1**	4[1,2,4]
Pb	-	-	NA (0)	$1.4 * 10^{-5}$	$1.1 * 10^{-5}$	10 300 (19)	$2.3 * 10^{-2}$	$9.2 * 10^{-2}$	1.2[1,2,4]
Sr	-	-	NA (0)	-	-	NA (0)	-	-	-
V	-	-	NA (0)	-	-	NA (0)	-	-	-
Zn	$5.4 * 10^{-4}$	$8.4 * 10^{-3}$	1 000 (5)	$1.2 * 10^{-4}$	$1.9 * 10^{-3}$	4 510 (61)	$9.8 * 10^{-2}$	**1.5**	5.5[1,4]
TU_M	**$2.0 * 10^{-2}$**	**$1.5 * 10^{-1}$**		**$2.8 * 10^{-3}$**	**$3.9 * 10^{-2}$**				

[1]Havs- och vattenmyndigheten 2019
[2]European parliament and council 2013
[3]Based on water hardness ≥ 200 mg/L $CaCO_3$
[4]Bioavailable concentration

Appendix A

Table A.3 Primer sequences used in the RT-qPCR assays. Assay details are presented in Ekelund Ugge *et al*. 2020.

	Sequence (5'-3')	
Gene	Forward	Reverse
β-actin (*β-act*)	CAAACTGGGATGATATGGA	CATCTTTTCTCTGTTGGC
28S rRNA (28S)	ATCCTTGCTCGTCACGAC	GTACCAACCCTTCCTACG
Catalase (*cat*)	GGAAGACTGACCAGGGTAT	CCTCAGCGATGGCATTGTA
Glutathione-S-transferase (*gst*)	GTCCAACACCATGCTGAG	GTAGTCCTCCACTCCATCAT
Heat shock protein 70 (*hsp70*)	GGTATTGAGACGGCTGGT	CACACCAGGCTGGTTGTC
Heat shock protein 90 (*hsp90*)	TACCATTGCCAAATCTGG	ACACCAAACTGCCCAATCA
Metallothionein (*mt*)	ATGCAACTGCCTTGAGAC	ACTTTACATCCAGGACACTT
Superoxide dismutase (*sod*)	GCTACGGTCATTCCACTCT	CCAGTTATCTCACCAGTTATGTTC

Appendix A

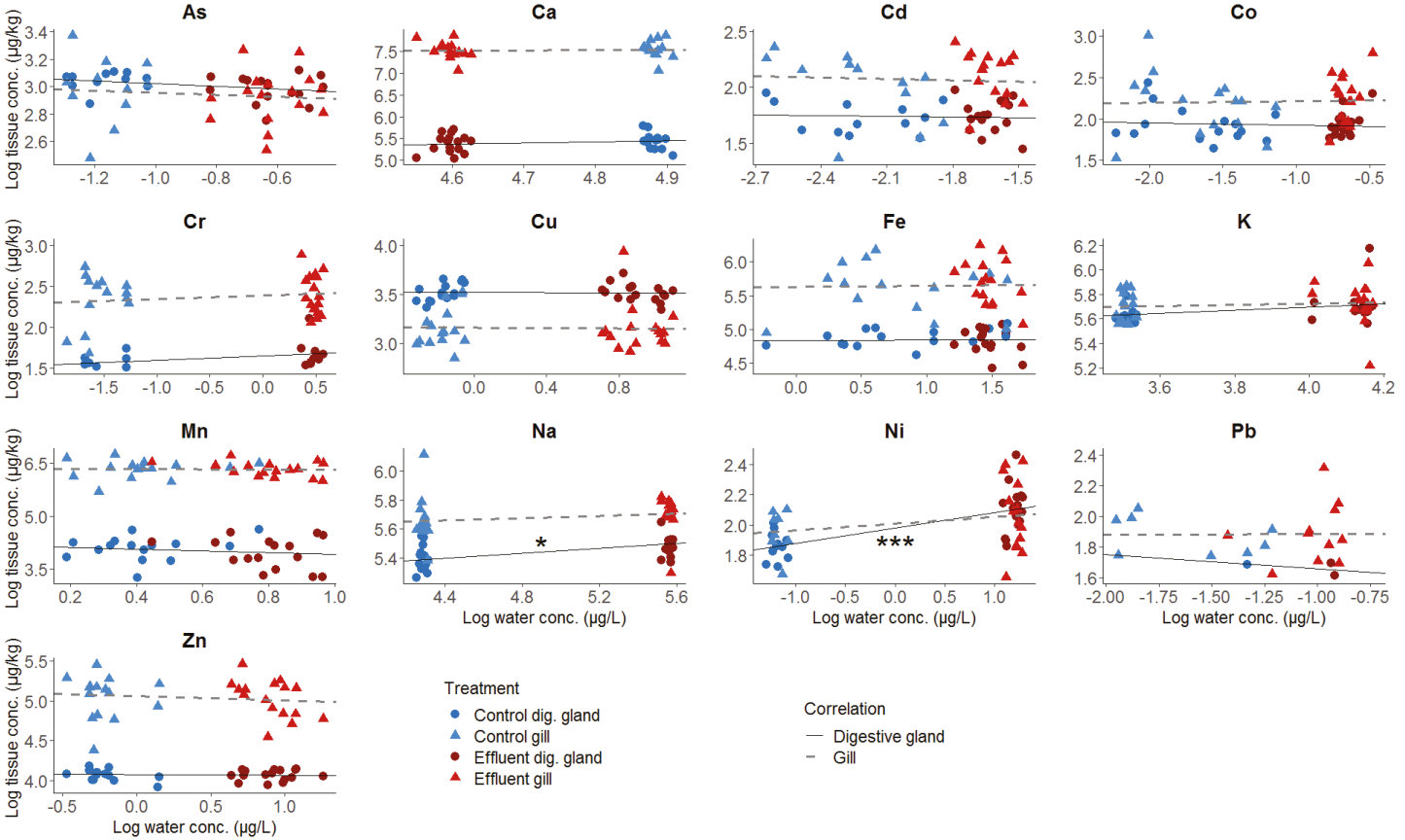

Figure A.3 Tissue and water concentrations (µg/kg WW and µg/L, respectively) and their correlation in *Anodonta anatina* experimentally exposed to industrial effluent water for 96 h. Significance of correlations is implied by * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$.

Appendix A

Table A.4 Correlation between tissue and ambient water concentration in *A. anatina* experimentally exposed to industrial effluent water. For the correlation analysis, any sample was excluded for which measured water and/or tissue concentration was below the level of reporting (LOR). Hg was analyzed in both tissue and water samples, but all water samples showed levels <LOR and were thus excluded.

	Digestive gland			Gill		
Element	Corr. coeff. (r)	Significance (p)	Slope (k)	Corr. coeff. (r)	Significance (p)	Slope (k)
As	-0.318	0.106	-0.0994	-0.106	0.599	-0.0777
Ca	0.169	0.363	0.225	0.0416	0.824	0.0530
Cd	-0.0574	0.772	-0.0229	-0.0577	0.771	-0.0430
Co	-0.0771	0.680	-0.0246	0.0295	0.875	0.0172
Cr	0.399	0.0811	0.0525	0.160	0.400	0.0436
Cu	-0.0333	0.859	-0.00526	-0.0232	0.901	-0.00901
Fe	0.0151	0.936	0.00472	0.0240	0.898	0.0162
Hg	-	-	-	-	-	-
K	0.336	0.0645	0.121	0.104	0.578	0.0487
Mn	-0.156	0.401	-0.258	-0.0454	0.808	-0.0440
Na	0.527	***0.00230***	0.0853	0.181	0.329	0.0384
Ni	0.685	$\boldsymbol{8.20 * 10^{-5}}$	0.101	0.242	0.279	0.0433
Pb	-0.474	0.686	-0.0912	0.00705	0.977	0.00307
Zn	-0.0999	0.593	-0.0112	-0.118	0.528	-0.0494

Appendix A

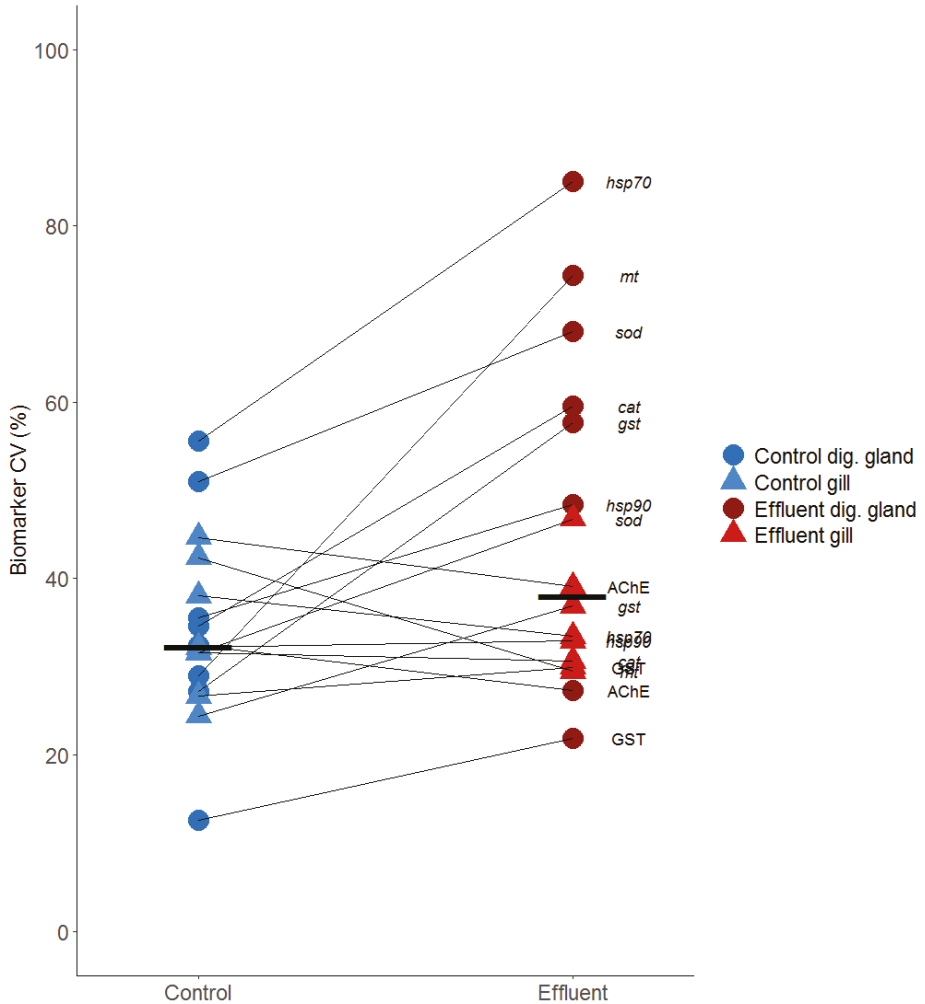

Figure A.4 The coefficient of variation (CV) across biomarkers and treatments. Bars show median CV.

Paper III

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Evaluation of transcriptional biomarkers using a high-resolution regression approach: Concentration-dependence of selected transcripts in copper-exposed freshwater mussels (*Anodonta anatina*)

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ABSTRACT

We tested concentration-dependence of selected gene transcripts (*cat*, *gst*, *hsp70*, *hsp90*, *mt* and *sod*) for evaluation as biomarkers of chemical stress. Contrary to the common approach of factorial designs and few exposure concentrations, we used regression across a high-resolution concentration series. Specifically, freshwater mussels (*Anodonta anatina*) were acutely (96 h) exposed to Cu (13 nominal concentrations, measuring 0.13–1 600 μg/L), and transcripts were measured by RT-qPCR. In digestive glands, *cat*, *hsp90* and *mt* decreased with water Cu ($p < 0.05$), but response magnitudes saturated at < 2-fold decreases. In gills, *gst*, *hsp70*, *hsp90* and *mt* increased with water Cu ($p < 0.05$). While *hsp70*, *hsp90* and *mt* exceeded 2-fold increases within the exposure range, high Cu concentrations were required (38–160 μg/L). Although gill responses were generally more robust compared to digestive glands, overall small response magnitudes and moderate sensitivity may set limit for potential application as general biomarkers of chemical stress.

1. Introduction

It has long been suggested that gene transcripts, related to specific mechanisms of action or responding to cellular stress in general, may provide important insights into biological responses to toxicants (Calzolai et al. 2007, Piña et al. 2007, Poynton & Vulpe, 2009). For instance, transcriptomic benchmark dose analysis has shown promise in toxicity screening and regulatory toxicology, for estimating chronic effect levels (Pagé-Larivière et al. 2019) and for identifying pathways sensitive to various pollutants (Martínez et al. 2019, 2020). Another approach is the use of transcriptional responses as early warnings of exposure and adverse effects in environmental risk assessment (Calzolai et al. 2007). The term *biomarker* can, in the context of ecotoxicology and environmental risk assessment, be defined as a measurable biological change in response to exposure to and/or effects from chemical pollution (van der Oost et al. 2003). Various molecular responses, such as enzyme activities and metabolites, have shown promise as biomarkers (van der Oost et al. 2003, Turja et al. 2013, Perić & Burić 2019), suggesting that selected transcriptional responses, separate or as part of integrated biomarker responses, may similarly provide a powerful tool in environmental risk assessment and biomonitoring of pollution.

Organisms have evolved various ways of coping with potentially stressful exposures, and measures commonly include regulation of cellular processes or cytoprotective proteins (Kültz 2003, Birnie-Gauvin et al. 2017). For instance, many stress proteins and enzymes involved in cellular redox homeostasis are considered to respond to general chemical stress, such as non-specific toxicity from metal stressors (Le Saux et al. 2020). Various metal and mixture exposures may induce changed transcription of e.g. metallothionein (*mt*), heat shock proteins 70 and 90 (*hsp70* and *hsp90*, respectively), catalase (*cat*), glutathione-S-transferase (*gst*) and superoxide dismutase (*sod*) (Bigot et al. 2011, Navarro et al. 2011, Liu et al. 2014, 2016; Boukadida et al. 2017). Based on mechanistic understanding, and in some cases empirical data, these and other genes of similar function are suggested to respond to a broad range of chemical stressors, and are therefore considered general biomarker candidates of chemical stress.

For practical use of any biomarker, a basic understanding of response magnitude and variation is crucial (van der Oost et al. 2003, Bahamonde et al. 2016). In order to decide appropriate application of transcriptional responses, assessment across multiple exposure concentrations (among

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other things) may therefore be required (Fent & Sumpter, 2011, Bahamonde et al. 2016). Concentration-response testing is of particular importance for biomarkers intended for quantification of pollutant exposure and/or effects, as well as biomarkers for comparative evaluation of e.g. wastewaters. Using setups of six to ten exposure concentrations, monotonic transcriptional up- and downregulations have been demonstrated in a few aquatic organisms, such as watermilfoil (*Myriophyllum spicatum*), water fleas (*Daphnia magna*), California mussels (*Mytilus californianus*) and Chinook salmon (*Oncorhynchus tshawytscha*) (Osachoff et al. 2013, Smetanová et al. 2015, Hall et al. 2020). In contrast, there is also implication of non-monotonic (for instance U-shaped) concentration-responses of various transcripts, with a seemingly strong up- or downregulation at low exposure and effects diminishing at higher toxicant concentrations (Bigot et al. 2011, Osachoff et al. 2013, Smetanová et al. 2015). Thus, non-monotonic responses may limit the exposure range under which meaningful changes can be detected, which can certainly restrict the biomarker potential. In our experience, many transcriptional studies in environmental sciences and toxicology primarily focus on specific threshold exposures, rather than adopting regression approaches across multiple exposure concentrations. By comparison, experiments covering more than four concentrations are rare even among studies that imply concentration- or dose-dependence of responses (Figure A.1). Consequently, current evaluation of transcriptional biomarker candidates may generally suffer from low resolution and narrow ranges of chemical stress.

In this study, we used regression to test concentration-dependence of six transcripts (*cat*, *gst*, *hsp70*, *hsp90*, *mt* and *sod*) in a freshwater mussel (*Anodonta anatina*), using a high-resolution concentration-response setup of acute (96 h) copper exposures. Our hypothesis was that response magnitude, i.e. induction or inhibition relative baseline expression, increases monotonically with increasing Cu exposure via water. The aim was to assess sensitivity and robustness of each response, for evaluation of biomarker potential. For this purpose, we here define sensitivity by the exposure concentration at which a given response occurs, and robustness by the response magnitude at a given exposure or range of exposures. By our definition, a sensitive biomarker is one that responds at low and/or environmentally relevant exposure concentrations, while a robust biomarker gives a predictable, clearly distinguishable response upon exposure. For practical application, the ideal biomarker is both sensitive and robust, demonstrating clear responses to the relevant exposures of interest. Finally, we therefore qualitatively evaluated the overall biomarker potential of the selected transcripts.

2. Material and methods

2.1. Mussel collection and maintenance

We used the European freshwater duck mussel (*A. anatina*) as an environmentally relevant study organism. This generalist species occurs naturally in lakes and rivers across most of Europe and parts of Asia (Lopes-Lima, 2014), and specifically, it is the most common large freshwater mussel in Sweden (von Proschwitz & Wengström 2021). Adult mussels ($n = 40$) of similar size (86 ± 13 mm) were collected on the 10th of April 2019 in Vinne å (Southern Sweden, 56°06'45" N, 13°54'35" E). For 15 days prior to the experiment, mussels were acclimatized to laboratory conditions in continuously aerated reconstituted freshwater (ISO, 6341:2012), hereafter referred to as freshwater medium. To reduce carry-over of sediment from the field, mussels were rinsed under tap water and subsequently under deionized water. Before being transferred to aquaria for long-term acclimatization, mussels were contained for 48 h in a single 60 L aquarium with 40 L freshwater medium and no bottom substrate. During this period, freshwater medium was renewed daily. After 48 h, mussels were randomly subdivided into two 40 L aquaria, each with 30 L freshwater medium and a 5 cm layer of siliceous sand (0.2–0.7 mm) as bottom substrate. The sand was of the same origin and was washed as has been previously described (Ekelund Ugge et al. 2020). Three times weekly, all freshwater medium was renewed, upon which mussels were randomly re-distributed between aquaria to reduce any tank effects on acclimatization. The mussels were fed by additions of a *Pseudokirchneriella subcapitata* culture, corresponding to approximately 8×10^5 cells × mussel^{-1} × day^{-1}, except no food was added within 48 h of the experiment start. During acclimatization and experimental periods, water temperature was 20 ± 1 °C, and the light cycle was 16 h light: 8 h dark.

2.2. Experimental exposures

We used Cu as our model stressor, aiming to cover a large fraction of sublethal Cu stress with high resolution. A Cu stock solution was made by dissolving $CuCl_2 \bullet 2\ H_2O$ (Fisher Scientific, USA) in deionized water, to a nominal concentration of 100 mg Cu/L. A concentration series was prepared by dilution of the stock solution in freshwater medium. Using 2 000 µg Cu/L as our highest concentration and a dilution factor of 2, we prepared exposure media at nominal concentrations of 0 (control), 1, 2, 3.9, 7.8, 16, 31, 63, 125, 250, 500, 1 000 and 2 000 µg Cu/L. A total of 40 glass containers (Ø 12 cm) were prepared with 1 L of respective exposure medium 48 h prior to experimental start (four containers as control and three containers each for remaining nominal concentrations). In each container, a 5 cm layer of glass marbles (Ø 16 mm) was added as substrate, and the exposure medium was continuously aerated. At the start of experiments, mussels ($n = 40$) were randomly assigned to the exposure containers, with one individual in each. During the exposure, *P. subcapitata* was added daily at the same amount as during acclimatization. Oxygen and pH were monitored in each container at 0, 48 and 96 h (Appendix B). At the start of the experiment, oxygen saturation was 99 – 100% (9.0 – 9.2 mg/L) and pH 7.7 – 8.0 in all containers. O_2 was stable over time in most containers, but decreased slightly in some after 96 h (88 – 100%, 7.9 – 9.2 mg/L). While pH generally decreased over time (≥ 6.3 at 96 h in control, 1 – 125 µg Cu/L), it increased slightly in the highest Cu exposure groups (≤ 8.3 at 96 h in 250 – 2 000 µg Cu/L). After 96 h, exposure was ended, and mussels dissected to extract gills and digestive glands. Tissue aliquots from each mussel were immediately submerged in RNA-Later (Invitrogen, USA) for transcriptional analyses, and subsequently stored at − 20 °C. Remaining tissue was immediately frozen in liquid nitrogen, stored at − 80 °C, and subsequently used for chemical analysis.

2.3. Chemical analysis

Medium was sampled from each exposure container at the experimental start and frozen (−20 °C) prior to chemical analysis. Samples were acidified by addition of nitric acid (1% v/v), and total Cu concentration was measured in unfiltered samples by inductively coupled plasma sector field mass spectrometry (ICP-SFMS) (*Element*, Thermo Scientific, Germany) (ISO 17294–2:2016, U.S. EPA 1994a). Measured and nominal Cu concentrations were strongly correlated (Fig. 1), and measured concentrations were used for further analyses.

Twelve previously analyzed water samples from Vinne å (Ekelund Ugge et al., 2020, 2022) were included as background references of Cu concentrations (Appendix C). Also, one additional water sample was collected from Vinne å on the 10th of April 2019 and immediately frozen (−20 °C) upon arrival to the laboratory. After acidification by nitric acid (1% v/v), total concentrations of a number of elements (Al, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, P, Pb, Si, Sr, V, and Zn) were analyzed in this sample by ICP-SFMS (Element, Thermo Scientific, Germany), inductively coupled plasma atomic emission spectrometry (ICP-AES) (Agilent ICP-OES 725, Agilent, USA) and atomic fluorescence spectrometry (AFS) (PSA Millennium Merlin, P S Analytical, UK) (ISO 17852:2006, ISO 11885:2007, ISO 17294–2:2016. U.S. EPA 1994a, 1994b). This water sample was used both as a Cu background reference, and to ensure overall low metallic contamination in the sampling site. Using Swedish environmental quality standards (EQS) of As, Cd, Cr, Cu,

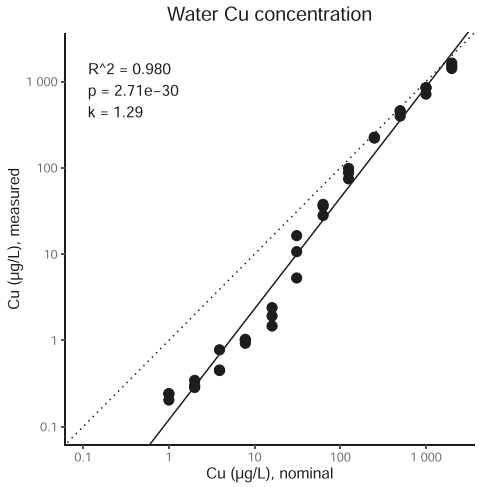

Fig. 1. Measured water concentration of total Cu relative nominal concentrations. Water was sampled at the start of experiments, 48 h after preparation of exposure medium and experimental containers. Points represent exposure containers to which Cu was added. The black line shows the fitted linear regression, while the dashed line illustrates a 1:1 relationship.

Hg, Ni, Pb and Zn (Havs- och vattenmyndigheten, 2019), background metal contamination was determined low overall. Measured total Cu slightly exceeded the EQS (although set for the bioavailable fraction only), while remaining metals measured below the respective EQS (Appendix C).

Cu concentrations were also measured in an aliquot of the snap frozen gill and digestive gland from each individual, except for one digestive gland sample that was accidentally lost (Appendix D). In addition, gill and digestive gland samples from three mussels were analyzed as biological background references for Vinne å. These tissues were dissected in the field on the 27th of April 2018, and stored at − 20 °C since. Prior to analysis, the tissue samples were digested with nitric acid/hydrogen peroxide, and metal concentrations were measured by ICP-SFMS (*Element 2*, Thermo Scientific, Germany) (ISO 17294–2:2016, U.S. EPA 1994a). In addition to Cu, tissue concentrations of Ca, Na and K were measured to give an overview of potential effects on metallic electrolytes (Appendix D). Additional trace elements and contaminants (As, Cd, Co, Cr, Fe, Hg, Mn, Ni, Pb, Zn) were also measured, to ensure low metallic contaminant loads from the sampling location (Appendix D). For each tissue, principal component analyses (PCAs) were performed to visually explore patterns of different metal content with different Cu-exposure, and to reveal potential effects on tissue electrolytes (Figure A.2).

2.4. Biomarker candidates

Six genes (*cat*, *gst*, *hsp70*, *hsp90*, *mt* and *sod*) were selected as transcriptional biomarker candidates of general toxicity and chemical stress. Relative gene expression levels were measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR). RNA was extracted using the *SurePrep™ TrueTotal™ RNA Purification Kit* (Fisher Scientific, USA) or the *Norgen Total RNA Purification Kit* (Norgen, Canada) (Appendix B). A final concentration of 1% (v/v) β-mercaptoethanol (Sigma Aldrich, USA) was added to the lysis buffer to protect RNA from degradation by RNases. Tissues were homogenized using a *TissueLyser LT* (Qiagen, Germany) and 5 mm stainless steel beads (Qiagen, Germany). DNase treatment was performed using the *Norgen RNase-Free DNase I Kit* (Norgen, Canada) or *Invitrogen DNA-Free™ Kit* (Invitrogen, USA) (Appendix B). After extraction, RNA amounts were measured using the *Qubit™ RNA HS Assay Kit* on a *Qubit 4* fluorometer (Invitrogen, USA) and A260/A280 ratios were measured using a *NanoDrop 2000* spectrophotometer (Thermo Scientific, USA). RNA integrity was assessed using a *Fragment Analyzer* (Advanced Analytical, Austria). Reverse transcription was performed despite generally low RNA quality numbers (Appendix B). This measure is however likely biased towards underestimating integrity of mussel RNA, by not accounting for the invertebrate 28 S RNA hidden break (Natsidis et al. 2019, Adema, 2021). Regardless, the targeted sequences were short (<200 bp, Ekelund Ugge et al. 2020, appendix) and the objective was assessing relative rather than absolute gene expression, which reduces the risk of downstream impacts from RNA degradation (Fleige & Pfaffl, 2006). cDNA was synthesized by reverse transcription of 200 ng RNA, using the *TATAA GrandScript cDNA Synthesis Kit* (TATAA Biocenter AB, Sweden). The cDNA was diluted a 4-fold with nuclease-free water, and 2 µl was then used in each qPCR assay. qPCR was performed on an *Applied Biosystems™ QuantStudio™ 12 K Flex* (Applied Biosystems, USA), using *TATAA SYBR® GrandMaster® Mix Low Rox™* (TATAA Biocenter AB, Sweden). The assays were performed as previously described (Ekelund Ugge et al. 2020). Relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), where expressions were normalized internally for each individual sample by the mean of the three reference genes *β-actin*, *18 S* rRNA and *28 S* rRNA (Figure A.3), and then by the mean of control samples of gill and digestive gland tissue, respectively.

Similar to previous transcriptional concentration-response assessment (Smetanová et al. 2015), a model selection was performed for each separate transcript from various regression models (linear, sigmoidal, exponential and hormetic). Relative expression, separated by tissue, was fitted to measured Cu concentrations in water, as well as to measured tissue Cu concentration. In order to evaluate biomarker potential, response sensitivity and robustness were evaluated from selected models. First, for all models reaching a response plateau (i.e. a maximal model response magnitude, Δ_{max}), a 50% effect concentration (EC_{50}) was determined. In this case, EC_{50} was defined as the concentration corresponding to a response magnitude of 50% relative the Δ_{max} (Hall et al. 2020). Each EC_{50} was in turn used as a measure of sensitivity and the corresponding response magnitude as a measure of robustness. Second, we assessed the sensitivity from a set response magnitude. In early transcriptional studies by microarrays, a 2-fold change in expression (100% increase relative the control) has commonly been used as a cutoff for transcriptional responses (e.g. McArthy & Smyth, 2009). Here, we set each response magnitude to a $|1|$ $\log_2$ fold-change (100% increase or 50% decrease relative the control), and in turn extracted corresponding exposure concentrations from each model. In turn, sensitivity was determined by the respective threshold concentration and robustness by the 95% confidence intervals (CIs) of the $|1|$ $\log_2$ fold-change. Finally, estimated sensitivity and robustness were used for a qualitative overall evaluation of biomarker potentials for detection of environmentally relevant Cu exposures.

2.5. Statistics

Statistical analyses were run in R version 4.0.5 (R Core Team, 2021), using the 'ggplot2' and 'ggpubr' packages (Wickham, 2016, Kassambara, 2020) for figure production. Log_2-transformed relative gene expressions, separated by tissue, were fitted to measured water and tissue concentrations of Cu by a selection of regression models. Linear models were fitted by linear regression on the linear and $\log_{10}$-transformed concentration scale, respectively. The 'drc' package (Ritz et al. 2015) was used to fit log-logistic models, Weibull models (type I and II), asymptotic regression models, exponential decay models, and hormesis

models (Brain-Cousens hormesis models and Cedergreen-Ritz-Streibig models). For each response variable, the respective model with the lowest Akaike information criterion (AIC) score was selected as the best fit. For models fitted with the 'drc' package, the 'ED' function was used for estimation of EC_{50}. The significance ($\alpha = 0.05$) of each selected model was tested against a linear regression model with a slope of zero. Finally, we fit regression models to tissue concentrations of Cu and Na relative water Cu concentration. Tissue Cu was modeled on the basis of our experimental design to test the concentration-dependence of Cu net accumulation upon acute exposure. Tissue Na was modeled on the basis of exploratory PCAs on tissue metals (Figure A.2), to test the trend of decreasing tissue Na with increasing water Cu observed for both gills and digestive glands. As neither Cu uptake nor Na dynamics were our primary focus, we used a statistical rather than mechanistic modeling approach to test correlations. The model selection was performed by the same procedure as for transcripts, where tissue concentrations were treated as responses, analogous to relative gene expression.

3. Results

Transcripts were, in general, negatively related to water concentrations of Cu in digestive glands, and positively related in gills (Fig. 2). Three digestive gland (*cat*, *hsp90* and *mt*) and four gill transcripts (*gst*, *hsp70*, *hsp90* and *mt*) demonstrated significant monotonic concentration-response relationships ($p < 0.05$), implying transcript inhibition and induction, respectively with increasing water Cu concentration (Fig. 2, Table A.1).

According to the fitted concentration-response models, response magnitudes were in general larger in gills than in digestive glands. Three gill transcripts demonstrated robust responses by exceeding $|1|$ $\log_2$ fold-changes within the tested Cu range (*hsp70* at 160, *hsp90* at 38 and *mt* at 160 μg Cu/L, respectively) (Table 1). For *gst*, *hsp70* and *hsp90* in gills, the EC_{50} was 23, 78 and 37 μg Cu/L, respectively, with corresponding response magnitudes of 0.31, 0.67 and 0.64 $\log_2$ fold-changes, respectively. The *gst* and *hsp90* CIs however included 0 at EC_{50}. In contrast, no transcript in digestive glands was robust as to reach a $|1|$ $\log_2$ fold-change within the measured range (Table 1). Apart from *mt* (EC_{50} = 11 μg Cu/L), digestive gland EC_{50} values were < 1 μg Cu/L (Table 1). Response magnitudes in digestive gland transcripts were in general small, and CIs consistently included 0 at EC_{50}.

In gills, responses relative tissue Cu concentration were similar to responses relative water Cu, while digestive glands demonstrated mixed trends. In general, responses in gills increased monotonically with tissue Cu concentrations ($p < 0.05$ for all transcripts except *sod*) (Fig. 3, Table A.2). In digestive glands, we observed trends of both increasing and decreasing expressions, but *hsp70* and *hsp90* increased monotonically with tissue Cu concentration ($p < 0.05$) (Fig. 3, Table A.2). All gill transcripts exceeded a $|1|$ $\log_2$ fold-change under the measured tissue Cu ranges, and except for *cat*, no CI included 0 (Table 2). In digestive glands, only *sod* exceeded a $|1|$ $\log_2$ fold-change under the measured tissue Cu ranges, but the CI included 0 (Table 2). Digestive gland *hsp70* and gill *hsp90* were the only transcripts that both demonstrated significant monotonic increases and for which EC_{50} values were within the measured tissue Cu ranges (3 900 and 4 900 μg/kg WW, respectively) (Table 2). The corresponding response magnitudes (−0.34 and 0.61 $\log_2$ fold-change, respectively) were however not significantly different from 0.

Across all samples, the measured digestive gland and gill Cu concentrations ranged from 2 500–6 100 μg and 1 200–35,000 μg/kg wet weight (WW), respectively (Appendix D). Mean Cu concentrations in the background samples were 1.6 μg/L for water (ranging from 0.080 to 3.4 μg/L, Appendix C), 3 000 μg/kg in digestive glands (2 900 – 3 000 μg/kg WW, Appendix D) and 1 700 μg/kg WW in gills (1 200 – 2 100 μg/kg WW, Appendix D). In the experimental mussels, tissue concentration of Cu increased with water concentration, both in digestive glands and gills (Fig. 4, Table A.3). Digestive gland concentrations reached a plateau within the measured range of water Cu concentrations (Fig. 4A), while the Weibull model fitted to gill Cu suggested further increasing tissue Cu beyond the scope of tested water concentrations (Fig. 4B). In contrast, there was a negative correlation between water Cu and Na concentrations in both digestive gland and gill tissue, with Na declining from background levels of approximately 250 – 300 mg/kg WW down to the 100 mg/kg WW range at high exposures (Fig. 5, Table A.3). With regards to overall metal content of the tissues, measured concentrations were in general similar between tissues, except for Ca and Mn, for which concentrations were approximately two orders of magnitude higher in gills (g/kg WW) than in digestive glands (mg/kg WW) (Appendix D). Non-essential elements were present in both tissues in the low to intermediate μg/kg WW range (Cd, Hg, Pb), or approaching low mg/kg WW levels (As) (Appendix D). Remaining metals were in the intermediate μg/kg WW (Co, Cr, Ni) or mg/kg WW (Fe, K, Zn) ranges (Appendix D).

4. Discussion

4.1. Cu stress exposure

In freshwater ecosystems, Cu concentrations range from approximately a few hundreds of nanograms per liter in pristine environments to hundreds of micrograms per liter or more in polluted areas (Vukosav et al. 2014, Bhuiyan et al. 2015, Álvarez-Vázquez et al. 2017). Although toxic at high concentrations, Cu is essential to biological life and a theoretical metabolic requirement of 26 300 μg Cu/kg dry weight (DW) has been proposed for mollusks (White & Rainbow, 1985). Naturally occurring soft tissue Cu content in the range of 11 000 – 34,000 μg/kg DW has been reported for various species of freshwater mussels (Kraak et al. 1992, Le et al. 2011, Bertucci et al. 2017). Based on WW to DW conversion factors of 6.9–10.5 for bivalve soft tissue (Ricciardi & Bourget, 1998), the data would suggest a background range of approximately 1 000 – 5 000 μg Cu/kg WW, consistent with concentrations measured in our control mussels and background mussels sampled directly from Vinne å. Thus, this range appears to represent natural background Cu levels in *A. anatina* tissues, suggesting 5 000 μg/kg WW as an approximate threshold for indication of elevated tissue levels.

Tissue Cu concentrations were positively correlated to the Cu concentration in water, both for digestive glands and gills. While concentration- and time-dependent uptake into mussel soft tissues is to be expected upon water exposure to Cu (Won et al. 2016, Le et al. 2021), small increases of essential elements could potentially fall within the background noise from baseline level variation. For instance, we only observed slightly elevated digestive gland Cu levels (≤ 6 100 μg/kg WW). In contrast, multiple gill samples exceeded 5 000 μg Cu/kg WW, reaching up to 35,000 μg/kg WW. Assuming time-dependent uptake, it might not be surprising that high acute Cu exposure can result in similar accumulation as longer exposure to lower concentrations. For instance, the two highest exposure groups of our study demonstrated gill concentrations in the same range as *A. anatina* after 15 days of laboratory exposure to 120 – 360 μg Cu/L (Sohail et al. 2016). Similar gill concentrations have also been reported for the freshwater mussel *Diplodon chilensis* after long-term (30–60 days) *in situ* exposure at moderate to high Cu concentrations (Yussepone et al. 2020). For acute exposures, there are kinetic two-compartment models that, under various exposure settings, may explain higher short-term Cu uptake in the bivalve gill than in remaining soft tissues (Sánchez-Marín et al. 2016, Le et al. 2021). Similar differences might also be expected for non-essential metals, such as Cd (Cooper et al. 2010). Considering the bivalve gill is commonly the tissue in first contact with foreign compounds, metal uptake into gills, and subsequent molecular responses, may simply reflect a first line defense against acute stressors (Won et al. 2016). In contrast, digestive gland metal accumulation can on the short term be more prominent upon e.g. dietary exposure (Cooper et al. 2010, Sánchez-Marín et al. 2016). Overall, while observed differences in digestive gland Cu were

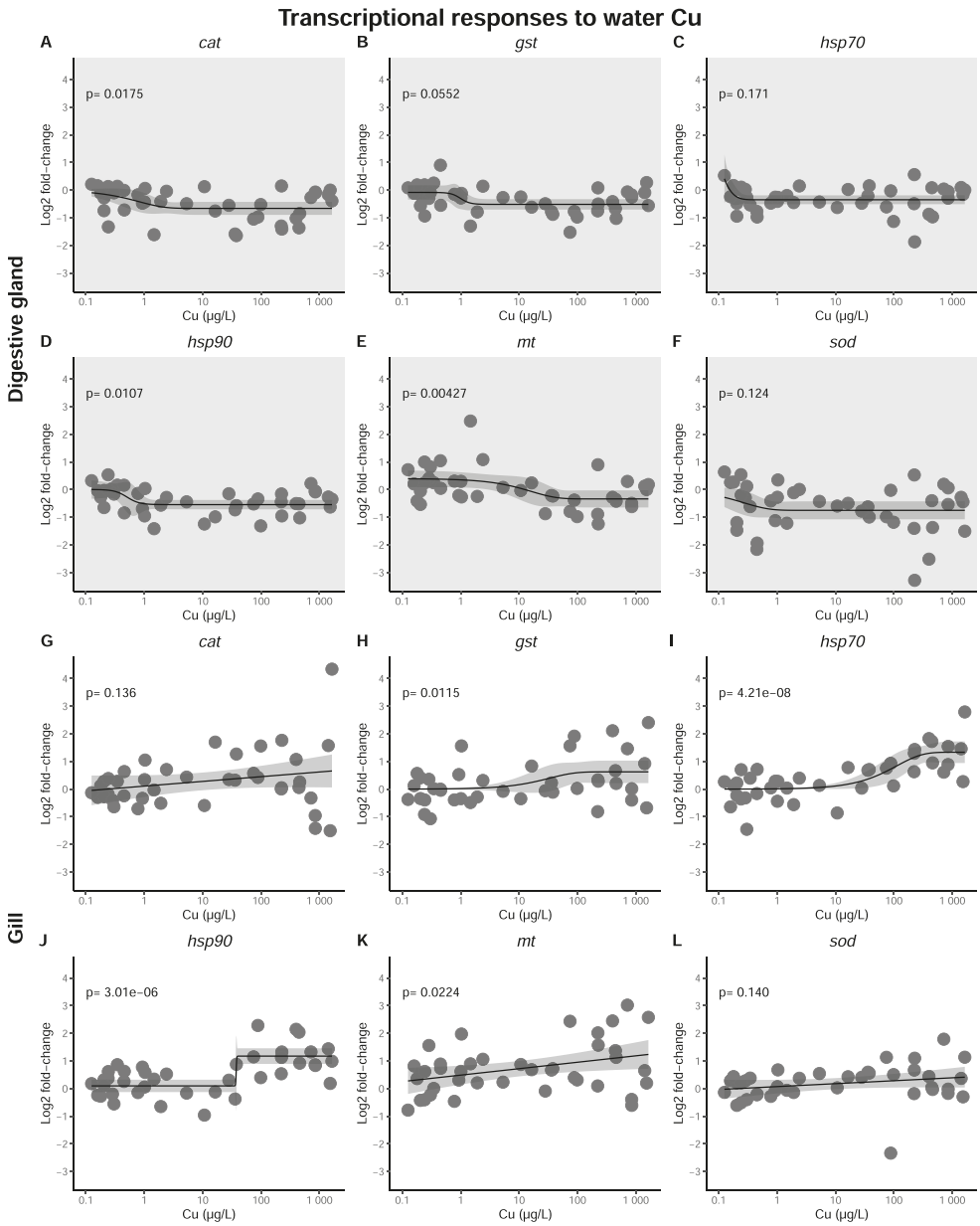

Fig. 2. Concentration-response relationships of transcriptional biomarker candidates in digestive glands (A-F) and gills (G-L) of *A. anatina* ($n = 40$) acutely exposed to Cu via water (96 h). Black lines correspond to fitted regression models and shaded areas represent model 95% confidence intervals. In digestive glands, asymptotic regression models were fitted for *cat* (A), *hsp70* (C) and *sod* (F), log-logistic models for *gst* (B) and *hsp90* (D), and an exponential decay model for *mt* (E). In gills, linear-log models were fitted for *cat* (G), *mt* (K) and *sod* (L), asymptotic regression models for *gst* (H) and *hsp70* (I), and a log-logistic model for *hsp90* (J). Model details and parameters are presented in Table A.1.

Table 1
Fitted transcriptional responses in *A. anatina* to acute (96 h) Cu exposure via water. Modeled EC_{50} values are presented with corresponding response magnitudes (log_2 fold-change). Also presented is the modeled threshold concentration for exerting a response of $\geq |1|$ (log_2 fold-change; i.e. changes corresponding to ≥100% increase or ≥50% reduction) and response confidence intervals (CIs) at this concentration.

Tissue	Transcript	EC_{50} (μg/L)	Response magnitude (log_2 fold-change) (95% CI)	Threshold for log_2 fold-change ≥ |1| (μg/L)	CI (95%) at log_2 fold-change = |1|
Digestive gland	*cat*	0.56	−0.33 (−0.68 to 0.024)	–[c]	–[c]
	gst	0.99	−0.30 (−0.68 to 0.089)	–[c]	–[c]
	hsp70	0.028[a]	8.4 (−28 to 45)	0.10[a]	−1.2 – 3.2
	hsp90	0.51	−0.27 (−0.81 to 0.28)	–[c]	–[c]
	mt	11	0.031 (−0.40 to 0.46)	–[c]	–[c]
	sod	0.185	−0.37 (−0.79 to 0.040)	–[c]	–[c]
Gill	*cat*	–[b]	–[b]	170,000[a]	−0.0098 – 2.0
	gst	23	0.31 (−0.12 to 0.75)	–[c]	–[c]
	hsp70	78	0.67 (0.22 to 1.1)	160	0.56 – 1.4
	hsp90	37	0.64 (−0.58 to 1.9)	38	0.13 – 1.9
	mt	–[b]	–[b]	160	0.63 – 1.4
	sod	–[b]	–[b]	520,000,000[a]	−0.12 – 2.1

[a] Outside the measured range.
[b] Not applicable for linear models.
[c] Maximum modeled response magnitude < |1| log_2 fold-change.

small, our results imply concentration-dependent Cu accumulation in gills under current water exposure.

Generally, our study demonstrated little overall effects from Cu on tissue metal content in *A. anatina*. In addition to tissue Cu, there were however also exposure-dependent differences in tissue Na. Interestingly, Na in both gills and digestive glands was negatively correlated to measured water Cu. It has been demonstrated that Cu uptake can partially occur by Na-dependent pathways (Grosell & Wood, 2002, Nadella et al. 2007), and the external Na concentrations may affect Cu uptake by mussels (Le et al. 2021). Contrary, interference with Na uptake, for instance via inhibited Na^+/K^+-ATPase activity, could in itself occur as an effect from Cu exposure (Pelgrom et al. 1995, Giacomin et al. 2013, Zimmer et al. 2014). Exposure to Cu has previously been linked to decreasing soft-tissue Na concentrations in freshwater mussels (Jorge et al. 2013), and it has been proposed that Na turnover rate is an important predictor for Cu toxicity in freshwater animals (Grosell et al. 2002). Despite lower tissue Cu and overall smaller transcriptional responses in digestive glands than in gills, our study revealed similar relationships between tissue Na and water Cu for both tissues. This implies that Cu exposure in gills could eventually translate to downstream effects on whole-organism Na balance. While further investigation of Cu and Na fluxes would be required for mechanistic conclusions, our observations are overall consistent with previous findings, suggesting concentration-dependent osmoregulatory stress from Cu exposure.

4.2. Transcriptional concentration-response relationships

We assessed concentration-response relationships for six transcriptional biomarker candidates in *A. anatina*, and found monotonic responses for three transcripts in digestive glands and four in gills. A key aspect of practical biomarker application is to establish the relationship between an exposure and corresponding response (van der Oost et al. 2003). Consequently, it has been argued that concentration-response testing is crucial for assessing transcriptional responses to pollution (Fent & Sumpter, 2011). Many transcriptional biomarker candidates might not respond to given exposures of interest, while others may display for instance U-shaped response curves across exposure concentrations (Osachoff et al. 2013, Smetanová et al. 2015). While different response types could potentially be considered for biomarker use, monotonic relationships (e.g. linear, sigmoidal or exponential) may facilitate response prediction, and hence, practical application. Thus, establishing concentration-response relationships is necessary to determine potential applications, as well as limitations, of transcriptional biomarker candidates.

Transcripts in gills and digestive glands consistently showed opposite response directions relative water Cu. Most levels decreased in digestive glands with increasing water exposure, and corresponding transcripts increased in gills. Furthermore, some digestive gland responses showed opposite trends relative water and tissue Cu concentration. For instance, digestive gland *hsp90* increased with tissue Cu despite decreasing with water Cu. Since measured concentrations suggested only minor Cu accumulation in digestive glands, response patterns may behave differently under e.g. dietary or long-term water exposure. As compared to digestive glands, gills displayed a wider range of tissue Cu concentrations, and response trends were consistent relative both water and tissue Cu concentration for all transcripts. In general, results therefore imply that molecular responses upon acute Cu exposure, and perhaps metal stress in general, are more predictable and easier to interpret in gills.

Using RNASeq, a range of monotonic responses has been demonstrated in the marine California blue mussel, *M. californianus*, upon acute water exposure to Cu (Hall et al. 2020). Although transcripts involved in e.g. the cell cycle were proposed as the most sensitive biomarkers to acute Cu stress, there was evidence of concentration-dependent induction of genes related to oxidative stress and protein chaperoning (Hall et al. 2020), similar to our observations in *A. anatina*. For instance, *hsp70* and *gst* isoforms (GST A2, GST Mu4, GST omega-1, Hsp70B2) were upregulated in gills of adult *M. californianus*, with EC_{50} values in the range of 69 – 93 μg/L (Hall et al. 2020). These response patterns are comparable to *gst*, *hsp70* and *hsp90* in *A. anatina* gills. Therefore, although other pathways may prove more sensitive to Cu exposure and metal stress, observed gill responses in exposed bivalves do imply monotonically increasing expression of various genes involved in oxidative stress and general cytoprotection.

4.3. Biomarker application

All biomarker candidates selected for this study are related to general toxicity (oxidative stress and cellular disruption), suggesting that altered transcription can reflect coping measures against chemical stress. It has been proposed that integration into mechanistic models might be required for practically useful prediction of adverse effects (Forbes et al. 2006). In practice, incorporation into predictive models would assume that sensitivity and robustness of the potential biomarker are established and validated, which is often not the case. Hence, our main focus at this stage was response sensitivity and robustness to exposure, rather than effect prediction or further mechanistic elucidation. In our study, three gill transcripts (*gst*, *hsp70* and *hsp90*) were fitted to non-linear functions, demonstrating EC_{50} values at relatively high Cu concentrations (23–78 μg/L), indicating only moderate sensitivity. For digestive glands, we could extract EC_{50} values for all responses. Except for *mt*, EC_{50} values

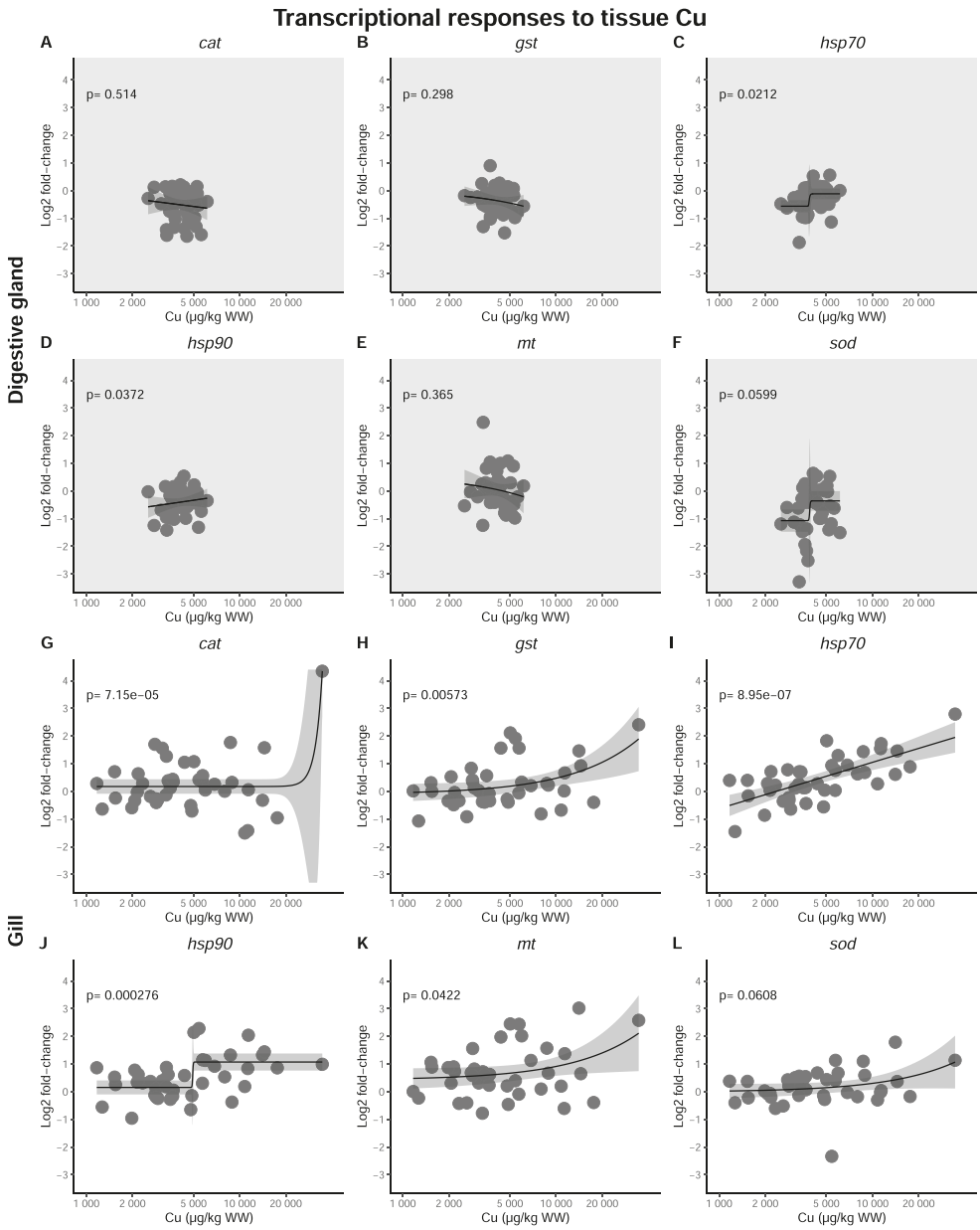

Fig. 3. Transcriptional responses in digestive glands ($n = 39$) (A-F) and gills ($n = 40$) (G-L) of Cu-exposed (96 h) *A. anatina* relative tissue Cu concentration. Black lines correspond to fitted regression models and shaded areas represent model 95% confidence intervals. In digestive glands, linear-log models were fitted for *cat* (A) and *hsp90* (D), linear models for *gst* (B) and *mt* (E), a Weibull type 1 model for *hsp70* (C) and a log-logistic model for *sod* (F). In gills, a log-logistic model was fitted for *cat* (G) and *hsp90* (J), linear models for *gst* (H), *mt* (K) and *sod* (L), and a linear-log model for *hsp70* (I). Model details and parameters are presented in Table A.2.

Table 2
Fitted transcriptional responses in *A. anatina* relative internal tissue Cu concentrations. Modeled EC_{50} values are presented with corresponding response magnitudes (log_2 fold-change). Also presented is the modeled threshold concentration for exerting a response of $\geq |1|$ (log_2 fold-change; i.e. changes corresponding to ≥100% increase or ≥50% reduction) and response confidence intervals (CIs) at this concentration.

Tissue	Transcript	EC_{50} (µg/kg WW)	Response magnitude (log_2 fold-change) (95% CI)	Threshold for log_2 fold-change $\geq \|1\|$ (µg/kg WW)	CI (95%) at log_2 fold-change $= \|1\|$
Digestive gland	*cat*	–[a]	–[a]	20,000[b]	−2.5 – 0.52
	gst	–[a]	–[a]	11,000[b]	−2.2 – 0.23
	hsp70	3 900	−0.34 (−1.6 to 0.94)	–[c]	–[c]
	hsp90	–[a]	–[a]	230,000[b]	−2.1 – 4.1
	mt	–[a]	–[a]	12,000[b]	−3.3 – 1.3
	sod	3 900	−0.71 (−3.3 to 1.9)	3 900	−2.0 – 0.036
Gill	*cat*	49,000[b]	65 (−1 300 to 1 500)	29,000	−3.8 – 5.8
	gst	–[a]	–[a]	19,000	0.43 – 1.6
	hsp70	–[a]	–[a]	9 300	0.73 – 1.3
	hsp90	4 900	0.61 (−1.2 to 2.4)	5 000	0.34 – 1.7
	mt	–[a]	–[a]	12,000	0.61 – 1.4
	sod	–[a]	–[a]	32,000	0.12 – 1.9

[a] Not applicable for linear models.
[b] Outside the measured range.
[c] Maximum modeled response magnitude $< |1|$ log_2 fold-change.

were consistently < 1 µg/L in this tissue, suggesting higher sensitivity than in the gill. On the other hand, response magnitudes were generally larger for gill than for digestive gland transcripts, implying higher robustness. Importantly, all confidence intervals of digestive gland responses at EC_{50} included 0, while *hsp70* in gills did not. Therefore, gill *hsp70* was the only biomarker giving a significant, robust response at its EC_{50}, while digestive gland responses in general appeared to saturate at background, or slightly elevated, water Cu levels.

Within the tested concentration range, all digestive gland transcripts reached their respective maximal response magnitudes at $< |1|$ log_2 fold-change. Again, this suggests that concentration-dependent responses were not very robust in this tissue. Some digestive gland transcripts could potentially be applied for detection of low concentration Cu exposure (or moderate in the case of *mt*), but this would likely require large sample sizes considering the generally small response magnitudes relative baseline variation (Ekelund Ugge et al. 2020). With regards to the separate digestive gland transcripts, practical applicability, if any, could ultimately be limited to 'on/off' response detection at selected, low water concentrations.

In gills, *hsp70*, *hsp90*, and *mt* demonstrated robust responses of ≥ 1 log_2 fold-changes, i.e. 2-fold increases, at the high end of environmentally relevant Cu concentrations (38–160 µg/L). In contrast, *cat* and *sod* would, based on extrapolation from the respective linear models, require extremely high Cu levels (170 mg/L – 520 g/L) for $\geq |1|$ log_2 fold-changes to occur. Overall, this would suggest higher importance of general cytoprotection when coping with Cu stress, as compared to pathways involved in protection against oxidative damage. In fact, considering that concentrations in the mg/L range are often lethal to bivalves (Okazaki, 1976, Ong & Din, 2001, Watters et al. 2013), the results suggest that even mussel mortality could be a more sensitive endpoint than a $\geq |1|$ log_2 fold-change of *cat* or *sod*. Furthermore, no response magnitudes reached $|2|$ log_2 fold-changes within the tested Cu range. By comparison, maximal observed response magnitudes in *M. californianus* gills ranged from $< |1|$ up to $\geq |4|$ log_2 fold-changes, despite exposures up to only 120 µg/L (Hall et al. 2020). Although potentially robust enough for detection of acute exposure to high Cu concentrations, gill *hsp70*, *hsp90* and *mt* in *A. anatina* were limited by high variation, small response magnitudes (considering the wide range of tested Cu concentrations) and a moderate sensitivity. Whether or not sufficient for practical use as biomarkers will ultimately depend on the intended application.

4.4. Conclusions

We have demonstrated concentration-dependent, monotonic responses in a range of transcriptional stress biomarker candidates in *A. anatina*. In gills, both Cu levels and most of the tested transcripts increased monotonically. In contrast, both Cu and transcripts in digestive glands generally demonstrated a substantial overlap with the background variation, even at high water Cu concentrations. Based on

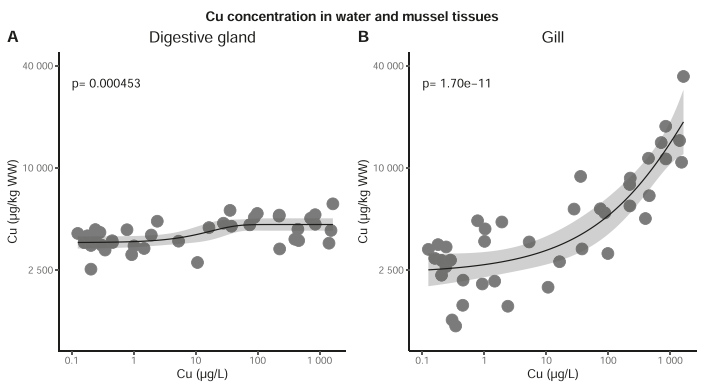

Fig. 4. Tissue Cu concentration relative water Cu in digestive glands ($n = 39$) (A) and gills ($n = 40$) (B) of mussels (*Anodonta anatina*). Black lines correspond to the fitted regression models and shaded areas represent model 95% confidence intervals. An asymptotic regression model was fitted for digestive glands (A), and a Weibull type 2 model for gills (B). Model details and parameters are presented in Table A.3.

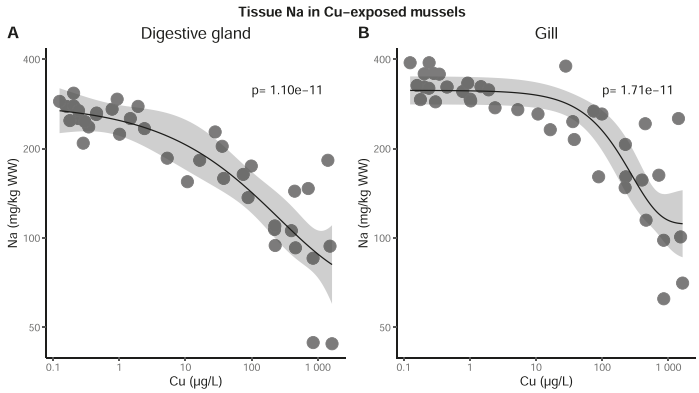

Fig. 5. Digestive gland ($n = 39$) (A) and gill ($n = 40$) (B) concentration of Na in mussels (*Anodonta anatina*) acutely exposed to Cu via water (96 h). Black lines correspond to the fitted regression models and shaded areas represent model 95% confidence intervals. A Weibull type 1 model was fitted for digestive gland (A), and an asymptotic regression model for gills (B). Model details and parameters are presented in Table A.3.

response sensitivity and robustness, we suggest that biomarker potential is overall higher for gill than for digestive gland transcripts. Still, even in gills, robust responses were limited to Cu exposures at relatively high concentrations. While biomarker predictions of adverse effects remain subject for future research, an important first step has been to quantitatively describe concentration-response relationships. Whether intended for use as simple biomarkers of exposure or for incorporation into complex mechanism-based models, effective application will ultimately require critical evaluation of biomarker potential, for which concentration-response testing should be considered standard.

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CRediT authorship contribution statement

Gustaf Ekelund Ugge: Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Annie Jonsson**: Project administration, Funding acquisition, Supervision, Writing – review & editing. **Anders Walstad**: Investigation. **Olof Berglund**: Supervision, Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Gustaf Ekelund Ugge and Anders Walstad are employed by ALS Scandinavia Toxicon AB. Gustaf Ekelund Ugge is on a leave of absence for pursuing a PhD.

Data statement

Data are available on Mendeley (Ekelund Ugge, Gustaf Magnus Oskar; Jonsson, Annie; Walstad, Anders; Berglund, Olof (2021), "Data for: Evaluation of transcriptional biomarkers using a high-resolution regression approach: Concentration-dependence of selected transcripts in copper-exposed freshwater mussels (*Anodonta anatina*)", Mendeley Data, V1, doi: 10.17632/mkd8fw935c.1).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2021.103795.

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Appendix A

Evaluation of transcriptional biomarkers using a high-resolution regression approach: Concentration-dependence of selected transcripts in copper-exposed freshwater mussels (*Anodonta anatina*)

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Appendix A

A literature search ('All fields') was performed in Web of Science (WoS) Core Collection on the 19th of August 2021. The search term used was '*(concentration-response OR concentration-depend* OR "concentration response" OR "concentration depend*" OR dose-response OR dose-depend* OR "dose response" OR "dose depend*") AND (transcript* OR "gene expression") AND (toxic* OR pollut*)*'. Out of 6 393 original articles, the 100 most recent publications were selected for screening. These articles were screened in order to extract the reported number of concentration/doses of a treatment, for which transcriptional responses were assessed. When possible, the number of concentrations/doses were extracted from the article abstracts, otherwise this was done from the original paper. Papers for which this information was not available (e.g. if transcription was not assessed as a response to a treatment) were labeled 'NA'. Finally, all entries were here categorized as 'Environmental sciences and toxicology' (WoS categories 'Environmental sciences' and 'Toxicology'), 'Environmental siences' (WoS category 'Environmental sciences', but not 'Toxicology'), 'Toxicology' (WoS category 'Toxicology', but not 'Environmental sciences') and 'Other' (neither categorized as 'Environmental sciences' nor 'Toxicology' by WoS).

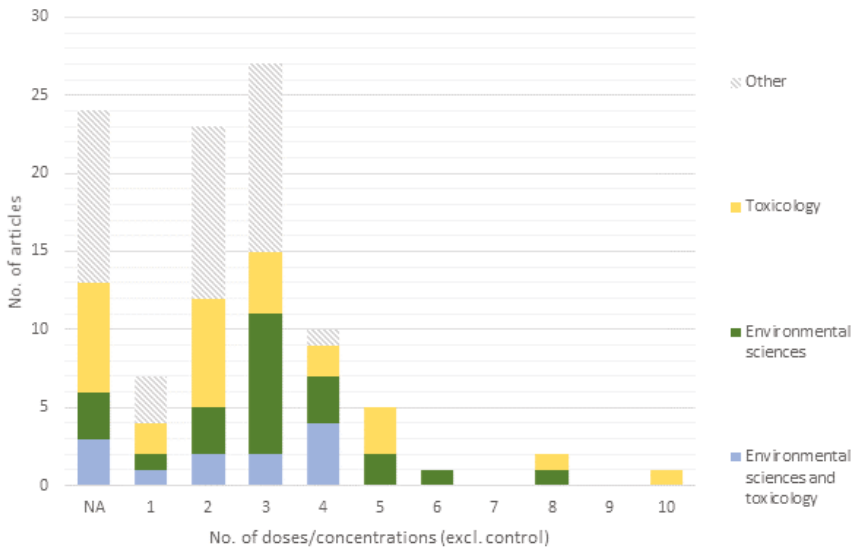

Figure A1. Distribution of number of exposure doses/concentrations used in transcriptional studies that imply dose-dependent responses. The literature screening was based on the 100 most recent (as of the 19th of August 2021) original articles found in Web of Science Core Collection.

Appendix A

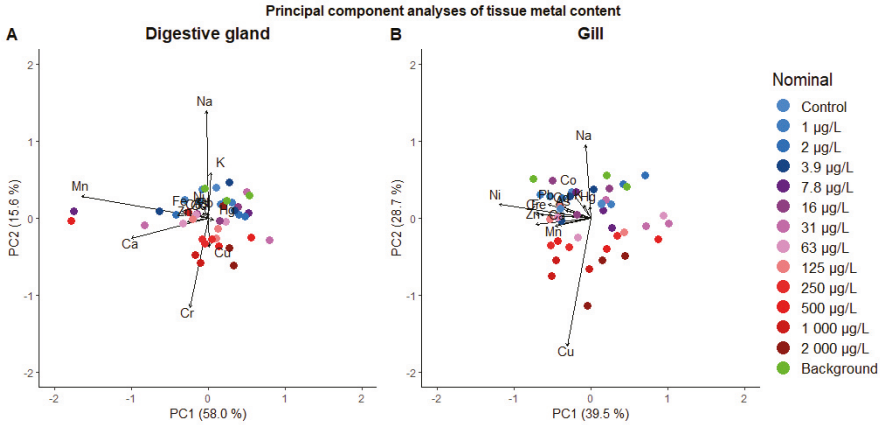

Figure A.2. Principal component analyses (PCAs) of metal content in digestive gland (A) and gill (B) of mussels (*Anodonta anatina*) acutely exposed to Cu via water (96 h), or sampled directly from Vinne å ('background'). The color coding represents the different nominal exposures according to the right panel. PCAs were performed on measured tissue concentrations, normalized as percentage of control group mean and then $\log_{10}$-transformed. Arrows are scaled to the lengths of the axes.

Appendix A

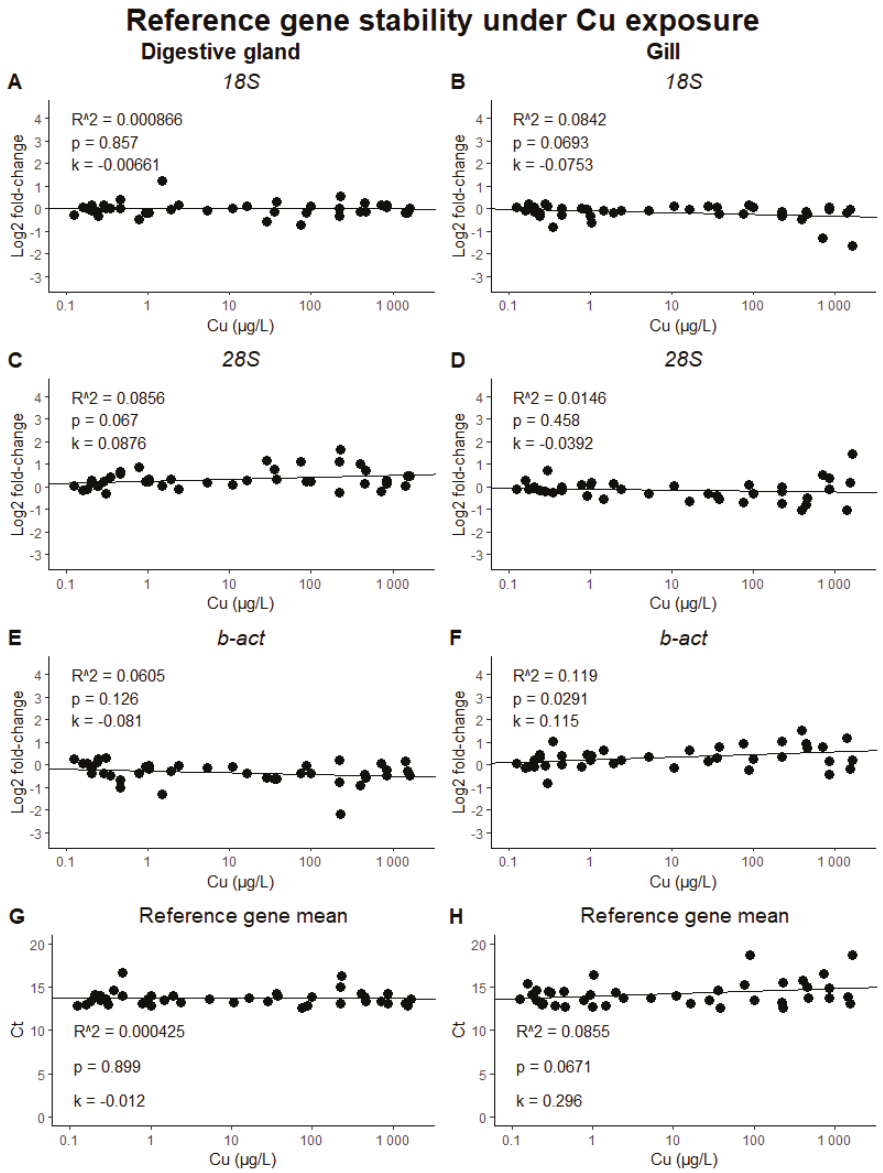

Figure A.3. Stability of the used reference gene expressions in digestive glands (A, C, E, F) and gills (B, D, F, H) of *Anodonta anatina* exposed to Cu (96 h). The subplots represent the $\log_2$ fold-changes of 18S (A, B), 28S (C, D) and *β-actin* (E, F), as well as the mean RT-qPCR cycle threshold (C_t) of the above three reference genes (G, H), relative measured water Cu.

Appendix A

Table A.1. Fitted models for transcriptional responses (log_2 fold-change) in freshwater mussels (*Anodonta anatina*) acutely exposed to Cu via water (96 h).

Tissue	Response	Model type	Model function[†]	Parameter estimates
Digestive gland	*cat*	Asymptotic regression	f(x) = d × (1 − exp(−x/e))	d = -0.660 e = 0.815
	gst	Log-logistic	$f(x) = c + \frac{d-c}{1 + exp(b(log(x) - log(e)))}$	b = 5.30 c = -0.514 d = -0.0816 e = 0.991
	hsp70	Asymptotic regression	f(x) = c + (d − c) × (1 − exp(−x/e))	c = 17.2 d = -0.348 e = 0.0398
	hsp90	Log-logistic	$f(x) = c + \frac{d-c}{1 + exp(b(log(x) - log(e)))}$	b = 4.08 c = -0.542 d = 0.00524 e = 0.505
	mt	Exponential decay	f(x) = c + (d − c) × (exp(−x/e))	c = -0.336 d = 0.398 e = 16. 2
	sod	Asymptotic regression	f(x) = d × (1 − exp(−x/e))	d = -0.749 e = 0.267
Gill	*cat*	Linear-log	f(x) = α+ β × log(x)	α = 0.109 β = 0.171
	gst	Asymptotic regression	f(x) = d × (1 − exp(−x/e))	d = 0.625 e = 33.1
	hsp70	Asymptotic regression	f(x) = d × (1 − exp(−x/e))	d = 1.34 e = 113
	hsp90	Log-logistic	$f(x) = c + \frac{d-c}{1 + exp(b(log(x) - log(e)))}$	b = -101 c = 0.0994 d = 1.18 e = 37.5
	mt	Linear-log	f(x) = α+ β × log(x)	α = 0.489 β = 0.233
	sod	Linear-log	f(x) = α+ β × log(x)	α = 0.0738 β = 0.106

[†]x = µg total Cu/L in water exposure

Appendix A

Table A.2. Fitted models for transcriptional responses (log_2 fold-change) in freshwater mussels (*Anodonta anatina*) relative internal tissue concentration of Cu.

Tissue	Response	Model type	Model function[†]	Parameter estimates
Digestive gland	*cat*	Linear-log	$f(x) = \alpha + \beta \times \log(x)$	α = 2.05 β = -0.710
	gst	Linear	$f(x) = \alpha + \beta \times x$	α = 0.0539 β = -0.000100
	hsp70	Weibull type 1	$f(x) = c + (d - c) \times \exp(-\exp(b(\log(x) - \log(e))))$	b= -112 c = -0.561 d = -0.113 e = 3 860
	hsp90	Linear-log	$f(x) = \alpha + \beta \times \log(x)$	α = -3.31 β = 0.806
	mt	Linear	$f(x) = \alpha + \beta \times x$	α = 0.574 β = -0.000127
	sod	Log-logistic	$f(x) = c + \frac{d-c}{1 + exp(b(log(x) - log(e)))}$	b= -201 c = -1.07 d = -0.356 e = 3 900
Gill	*cat*	Log-logistic	$f(x) = c + \frac{d-c}{1 + exp(b(log(x) - log(e)))}$	b = -10.1 c = 0.176 d = 131 e = 48 500
	gst	Linear	$f(x) = \alpha + \beta \times x$	α = -0.103 β = 0.0000577
	hsp70	Linear-log	$f(x) = \alpha + \beta \times \log(x)$	α = -5.64 β = 1.67
	hsp90	Log-logistic	$f(x) = c + \frac{d-c}{1 + exp(b(log(x) - log(e)))}$	b = -250 c = 0.155 d = 1.07 e = 4 940
	mt	Linear	$f(x) = \alpha + \beta \times x$	α = 0.426 β = 0.0000488
	sod	Linear	$f(x) = \alpha + \beta \times x$	α = -0.00998 β = 0.0000313

[†]x = µg total Cu/kg WW in tissue

Appendix A

Table A.3. Fitted models for tissue Cu and Na concentration (µg/kg WW and mg/kg WW, respectively, $\log_{10}$-transformed) in freshwater mussels (*Anodonta anatina*) acutely exposed to Cu via water (96 h).

Response variable	Model type	Model function[†]	Parameter estimates
Digestive gland Cu	Asymptotic regression	f(x) = c + (d − c) × (1 − exp(− x/e))	c = 3.56 d = 3.67 e = 17.5
Gill Cu	Weibull type 2	f(x) = c + (d − c) × (1 − exp(− exp(b(log(x) − log(e)))))	b = 0.426 c = 3.38 d = 5.13 e = 3 610
Digestive gland Na	Weibull type 1	f(x) = c + (d − c) × exp(− exp(b(log(x) − log(e))))	b = 0.415 c = 1.84 d = 2.45 e = 264
Gill Na	Asymptotic regression	f(x) = c + (d − c) × (1 − exp(− x/e))	c = 2.50 d = 2.05 e = 279

[†]x = µg total Cu/L in water exposure

Appendix B

		Mussel properties			Exposure conditions									RNA extraction							
					0 h			*48 h*			*96 h*			*Digestive gland*				*Gill*			
Sample ID	Treatment (nominal)	Shell length (mm)	Shell height (mm)	Wet weight (g)	pH	O2 saturation (%)	O2 (mg/l)	pH	O2 saturation (%)	O2 (mg/l)	pH	O2 saturation (%)	O2 (mg/l)	Extraction	DNAse	A260/ A280	RQN	Extraction	DNAse	A260/ A280	RQN
01	Control (0 µg Cu/l)	83	52	69,77	7,89	99,5	9,06	7,53	100,1	9,16	6,88	100,0	9,18	Norgen	Invitrogen	2,0	5,2	SurePrep	Norgen	2,0	2,8
02	1 µg Cu/l	87	49	57,21	7,99	100,0	9,07	7,66	98,7	8,94	7,59	99,4	9,02	SurePrep	Norgen	2,1	1,0	Norgen	Norgen	2,1	3,4
03	2 µg Cu/l	87	47	67,52	7,88	99,8	9,05	7,43	89,1	8,05	7,39	99,0	8,97	SurePrep	Norgen	2,0	5,8	Norgen	Norgen	2,0	3,7
04	3.9 µg Cu/l	92	60	90,03	7,86	99,8	9,07	7,22	97,9	8,87	6,35	97,7	8,85	SurePrep	Norgen	1,9	1,0	Norgen	Norgen	1,8	3,0
05	7.8 µg Cu/l	99	56	88,89	7,84	99,8	9,04	7,31	99,6	9,01	6,97	99,7	9,04	SurePrep	Norgen	2,0	1,5	Norgen	Norgen	1,9	3,0
06	16 µg Cu/l	84	54	77,03	7,76	99,4	9,05	7,62	100,0	9,07	7,32	99,0	9,00	Norgen	Invitrogen	2,2	3,0	Norgen	Norgen	2,1	2,0
07	31 µg Cu/l	76	46	45,55	7,79	99,6	9,03	7,45	100,2	9,09	6,59	98,3	8,92	Norgen	Invitrogen	2,0	4,9	SurePrep	Norgen	1,7	1,0
08	63 µg Cu/l	79	51	58,6	7,78	99,4	9,03	7,83	99,9	9,09	7,56	99,6	9,07	Norgen	Invitrogen	2,0	5,0	Norgen	Norgen	2,0	3,6
09	125 µg Cu/l	99	58	109,98	7,74	99,6	9,02	7,80	99,9	9,06	7,59	99,7	9,06	Norgen	Invitrogen	2,0	2,5	Norgen	Norgen	2,0	3,1
10	250 µg Cu/l	83	50	71,14	7,77	99,8	8,97	7,92	97,6	8,79	8,04	97,5	8,78	SurePrep	Norgen	1,7	1,0	Norgen	Norgen	2,0	4,9
11	500 µg Cu/l	84	48	72,95	7,74	99,3	9,06	7,98	99,6	9,14	8,13	99,4	9,15	SurePrep	Norgen	2,0	2,7	SurePrep	Norgen	2,0	1,9
12	1 000 µg Cu/l	80	49	54,39	7,80	99,6	9,04	8,09	98,9	9,02	8,30	96,6	8,77	Norgen	Invitrogen	2,0	5,7	SurePrep	Norgen	2,1	2,8
13	2 000 µg Cu/l	86	53	78,71	7,68	99,5	9,06	8,06	97,3	8,86	8,20	89,4	8,11	SurePrep	Norgen	2,0	3,7	SurePrep	Norgen	2,0	2,8
14	Control (0 µg Cu/l)	83	50	62,47	7,87	99,3	9,19	7,55	99,8	9,22	6,97	99,8	9,26	Norgen	Invitrogen	2,1	5,7	SurePrep	Norgen	2,0	3,3
15	1 µg Cu/l	85	54	78,44	7,99	99,4	9,05	7,48	97,1	8,86	7,05	97,0	8,84	SurePrep	Norgen	2,0	2,4	SurePrep	Norgen	1,9	3,2
16	2 µg Cu/l	96	58	95,04	7,87	99,4	9,04	7,38	98,8	9,02	6,29	98,6	9,01	Norgen	Invitrogen	2,0	3,2	SurePrep	Norgen	2,1	3,9
17	3.9 µg Cu/l	98	52	75,84	7,89	99,7	9,07	7,46	97,6	8,90	7,06	97,5	8,88	SurePrep	Norgen	2,0	4,8	SurePrep	Norgen	2,0	3,7
18	7.8 µg Cu/l	79	47	51,28	7,77	99,3	9,14	7,51	99,5	9,16	6,64	99,8	9,22	Norgen	Invitrogen	1,9	1,0	SurePrep	Norgen	2,0	3,3
19	16 µg Cu/l	81	49	58,61	7,76	99,5	9,10	7,69	99,1	9,08	7,36	99,3	9,12	Norgen	Invitrogen	1,9	6,8	SurePrep	Norgen	1,9	3,6
20	31 µg Cu/l	87	52	85,31	7,81	99,5	9,05	7,55	99,2	9,01	7,10	98,3	8,96	Norgen	Invitrogen	2,0	5,0	SurePrep	Norgen	2,0	1,0
21	63 µg Cu/l	75	49	48,02	7,75	99,4	9,06	7,83	99,6	9,09	7,56	99,6	9,09	SurePrep	Norgen	1,7	1,0	SurePrep	Norgen	2,0	3,4
22	125 µg Cu/l	85	52	66,69	7,89	99,5	9,03	7,82	94,4	8,57	7,77	96,2	8,71	Norgen	Invitrogen	1,8	6,0	SurePrep	Norgen	2,0	5,6
23	250 µg Cu/l	96	54	89,29	7,85	99,4	8,98	7,87	92,0	8,35	7,78	87,5	7,91	SurePrep	Norgen	2,0	4,5	SurePrep	Norgen	2,0	4,0
24	500 µg Cu/l	83	51	64,37	7,76	99,5	9,03	8,03	99,1	9,03	8,11	96,0	8,72	SurePrep	Norgen	2,0	1,0	SurePrep	Norgen	2,0	5,9
25	1 000 µg Cu/l	80	50	54,13	7,73	99,5	9,05	8,03	99,3	9,05	8,24	98,9	8,98	SurePrep	Norgen	2,1	1,5	SurePrep	Norgen	2,2	2,9
26	2 000 µg Cu/l	80	47	55,84	7,70	99,7	9,06	7,97	98,9	9,01	8,22	97,7	8,87	Norgen	Invitrogen	2,0	1,0	SurePrep	Norgen	1,8	1,0
27	Control (0 µg Cu/l)	80	48	53,44	7,97	99,5	9,09	7,56	96,7	8,84	7,43	99,8	9,12	Norgen	Invitrogen	1,9	1,0	SurePrep	Norgen	2,0	4,0
28	1 µg Cu/l	95	57	96,32	7,88	99,6	9,11	7,41	99,1	9,07	6,59	99,2	9,10	SurePrep	Norgen	2,0	4,1	SurePrep	Norgen	2,0	4,9
29	2 µg Cu/l	80	46	53,84	7,88	99,6	9,09	7,59	99,2	9,07	7,04	99,4	9,10	Norgen	Norgen	1,9	1,7	SurePrep	Norgen	1,8	1,0
30	3.9 µg Cu/l	84	51	60,41	7,80	99,5	9,10	7,51	99,7	9,11	6,37	99,5	9,14	Norgen	Norgen	2,0	1,0	SurePrep	Norgen	2,0	2,9
31	7.8 µg Cu/l	86	53	77,3	7,83	99,4	9,02	7,60	98,3	8,92	7,08	98,2	8,92	SurePrep	Norgen	2,1	1,7	SurePrep	Norgen	2,0	4,3
32	16 µg Cu/l	86	53	72,76	7,80	99,7	9,03	7,72	100,1	9,10	7,48	98,5	8,92	Norgen	Norgen	2,0	3,6	SurePrep	Norgen	1,8	2,5
33	31 µg Cu/l	77	45	48,42	7,79	99,6	9,03	7,65	99,5	9,04	7,13	99,5	9,00	SurePrep	Norgen	2,0	5,1	SurePrep	Norgen	2,0	3,3
34	63 µg Cu/l	95	61	85,47	7,75	99,6	9,02	7,56	98,8	8,98	6,72	99,3	8,99	SurePrep	Norgen	1,8	2,4	SurePrep	Norgen	2,0	3,1
35	125 µg Cu/l	96	54	89,39	7,78	99,7	9,02	7,80	97,1	8,80	7,57	96,5	8,70	SurePrep	Norgen	2,0	5,5	SurePrep	Norgen	2,0	1,0
36	250 µg Cu/l	76	45	53,75	7,77	99,7	9,01	7,95	98,4	8,93	8,05	99,0	8,95	Norgen	Norgen	2,0	4,6	SurePrep	Norgen	2,0	3,3
37	500 µg Cu/l	90	61	97,2	7,76	99,6	9,03	7,99	99,3	9,04	8,12	97,8	8,88	SurePrep	Norgen	2,1	2,2	SurePrep	Norgen	2,0	1,0
38	1 000 µg Cu/l	93	55	82,09	7,71	99,6	9,06	8,07	99,6	9,08	8,29	99,2	9,04	SurePrep	Norgen	2,0	3,5	SurePrep	Norgen	2,0	3,0
39	2 000 µg Cu/l	87	50	81,83	7,69	99,7	9,05	7,92	99,8	9,08	8,14	99,1	9,01	SurePrep	Norgen	2,0	2,1	SurePrep	Norgen	1,7	1,0
40	Control (0 µg Cu/l)	85	49	64,18	7,97	99,4	9,07	7,68	98,3	8,95	7,45	98,7	9,00	Norgen	Norgen	1,9	1,7	SurePrep	Norgen	2,0	4,0

Appendix C

Vinne å metal concentrations (non-filtered)

Element	*Sampling date* 2017-12-09	2018-01-05	2018-01-30	2018-02-21	2018-03-20	2018-04-05	2018-04-27	2018-05-27	2018-06-20	2018-07-13	2018-09-19	2018-12-05	2019-04-10	Environmental quality standards (HVMFS 2019:25) *Based on filtered (0.45 µm) concentration* Annual average (µg/L)
Ca (mg/L)		36,9	34,4	56,4		46,8	61,5	77,5		73,5			69,3	
Fe (mg/L)		0,606	0,685	0,463		0,643	0,393	0,163		0,1			0,16	
K (mg/L)		1,77	1,77	1,88		1,65	1,95	2,25		2,05			2,12	
Mg (mg/L)		1,71	1,6	2,21		1,94	2,25	2,69		2,73			2,6	
Na (mg/L)		8,76	8,5	11		10,8	11,9	13,4		14,6			12,2	
Si (mg/L)		3,39	3,88	4,31		4,42	3,8	2,8		3,83			3,56	
Al (µg/L)		208	203	93,6		129	64,6	36,9		21,6			37,1	
As (µg/L)		0,316	0,317	0,231		0,266	0,248	0,277		0,273			0,258	0,5
Ba (µg/L)		27,2	21,7	33,5		24,9	36	43,6		34,7			42,2	
Cd (µg/L)		0,0281	0,0337	0,0155		0,0212	0,0136	0,00692		0,00336			0,00814	0,08
Co (µg/L)		0,214	0,229	0,191		0,219	0,134	0,107		0,0766			0,0884	
Cr (µg/L)		0,317	0,264	0,18		0,218	0,208	0,138		0,0549			0,165	3,4
Cu (µg/L)	0,707	2,36	2,6	2,14	0,529	2,08	3,36	2,98	0,433	0,953	0,080	0,875	2,00	0,5
Hg (µg/L)		0,00772	0,00805	0,00293		0,00729	0,00349	0,002		0,002			0,002	0,07
Mn (µg/L)		45	49,8	59,8		64,3	62,5	68		53,6			42,9	
Mo (µg/L)		0,244	0,219	0,284		0,235	0,324	0,373		0,381			0,471	
Ni (µg/L)		0,844	1,03	0,624		0,602	0,861	1,47		0,306			0,574	4
P (µg/L)		33,4	43,4	35,2		34,5	27	23,1		19			23,8	
Pb (µg/L)		0,254	0,344	0,147		0,316	0,216	0,168		0,0609			0,0808	1,2
Sr (µg/L)		83	77	122		103	133	174		176			153	
V (µg/L)		1,15	1,11	1,01		1,00	1,00	0,932		0,806			0,887	
Zn (µg/L)	1,051	4,13	5,08	2,65	0,445	4,00	3,95	3,5	0,923	0,854	0,875	1,111	1,57	5,5

Samples 2017-12-09, 2018-03-20, 2018-06-20, 2018-09-19 and 2018-12-05 previously described in Ekelund Ugge et al. 2020.
Samples 2018-01-05, 2018-01-30, 2018-02-21, 2018-04-05, 2018-04-27, 2018-05-27 and 2018-07-13 previously described in Ekelund Ugge et al. 2022

Color coding
<LOR, set to LOR
Bioavailable fraction
Maximal permitted concentration (no annual average EQS)

Appendix D

Sample ID	Cu in water, nominal (µg/L)	Cu in water, measured T0 (µg/L)	Cu in water, measured T96 (µg/L)	As in gill (mg/kg)	Ca in gill (mg/kg)	Cd in gill (mg/kg)	Co in gill (mg/kg)	Cr in gill (mg/kg)	Cu in gill (mg/kg)	Fe in gill (mg/kg)	Hg in gill (mg/kg)	K in gill (mg/kg)	Mn in gill (mg/kg)	Na in gill (mg/kg)	Ni in gill (mg/kg)	Pb in gill (mg/kg)	Zn in gill (mg/kg)
1	0	0,204	1,35	1,67	29300	0,255	0,328	0,194	2,33	430	0,0332	306	2590	323	0,0996	0,064	196
2	1	0,24	1,14	0,837	22200	0,14	0,16	0,2	2,62	381	0,0261	298	1750	320	0,047	0,0619	98,6
3	2	0,345	1,33	0,93	14000	0,186	0,358	0,141	1,17	250	0,0317	276	1030	357	0,036	0,0263	55,8
4	3,9	0,454	2,61	1,3	30500	0,196	0,151	0,332	2,18	551	0,0248	286	2990	323	0,108	0,0565	158
5	7,8	1,01	2,52	0,906	17000	0,231	0,222	0,118	3,69	312	0,0249	316	1500	294	0,117	0,0337	97,9
6	16	1,47	1,75	1,73	21100	0,365	0,59	0,408	2,15	637	0,0406	296	1780	324	0,182	0,0658	119
7	31	16,4	7,16	0,672	14600	0,108	0,0686	0,101	2,8	133	0,0169	272	1410	232	0,0305	0,0176	59,1
8	63	37,8	7,26	0,751	12000	0,244	0,321	0,0967	3,33	159	0,0293	271	906	215	0,0203	0,0235	59,4
9	125	98,8	9,87	1,41	33600	0,199	0,241	0,484	3,13	578	0,0226	258	2940	262	0,0826	0,104	156
10	250	227	42,5	1,02	30100	0,175	0,151	0,21	8,71	493	0,0228	249	2250	161	0,123	0,0584	200
11	500	399	66,5	0,644	16400	0,154	0,111	0,0389	5,03	236	0,0303	256	1530	157	0,0385	0,0294	48,6
12	1000	851	272	0,759	24800	0,28	0,267	0,26	11,3	333	0,0266	185	2280	62,5	0,0671	0,0369	142
13	2000	1640	435	1,15	33300	0,148	0,1	0,144	34,6	397	0,0262	205	3180	70,6	0,0668	0,0428	154
14	0	0,159	1,37	1,15	24300	0,183	0,219	0,213	2,93	283	0,0283	300	1770	327	0,0709	0,0367	118
15	1	0,204	1,43	1,16	37300	0,458	0,261	0,378	2,85	651	0,036	337	3840	358	0,175	0,0768	187
16	2	0,285	1,64	1,27	23800	0,388	0,164	0,272	2,86	552	0,0321	288	2390	359	0,161	0,0575	146
17	3,9	0,449	1,79	0,877	33600	0,173	0,147	0,134	1,55	563	0,0275	301	2690	323	0,0686	0,0625	155
18	7,8	1,03	1,58	0,627	26200	0,161	0,0684	0,203	4,37	266	0,0203	304	2290	290	0,0548	0,0392	128
19	16	1,92	1,31	0,853	26900	0,259	0,294	0,254	4,8	259	0,0298	316	2250	316	0,0945	0,0666	185
20	31	10,7	4,73	1,05	28300	0,176	0,154	0,217	1,98	422	0,0272	306	2100	262	0,0424	0,0491	128
21	63	35,9	7,82	1,37	21700	0,248	0,189	0,353	8,91	246	0,0253	328	2020	247	0,107	0,0428	136
22	125	88,3	13,9	0,865	15200	0,186	0,229	0,185	5,43	304	0,0321	252	1330	161	0,0469	0,0395	52,7
23	250	222	35,2	0,931	37500	0,224	0,192	0,399	7,99	693	0,0232	244	3620	148	0,0967	0,0758	258
24	500	460	46	1,09	21700	0,116	0,131	0,305	6,86	494	0,0278	228	1620	115	0,0427	0,052	82,6
25	1000	848	172	1,21	42300	0,173	0,112	0,446	17,6	811	0,0234	251	3390	98,4	0,0996	0,0779	152
26	2000	1530	274	1,13	22800	0,171	0,157	0,24	10,8	312	0,0298	335	1570	101	0,0604	0,0457	87
27	0	0,179	0,979	1,16	34200	0,253	0,238	0,224	3,53	628	0,0282	305	3310	293	0,113	0,0667	204
28	1	0,242	2,03	1,23	30600	0,327	0,343	0,181	3,42	518	0,0339	340	2600	390	0,223	0,0695	201
29	2	0,302	1,13	0,801	20500	0,175	0,212	0,234	1,27	296	0,0298	319	1550	288	0,0424	0,0381	86,1
30	3,9	0,779	1,38	1,02	33800	0,235	0,11	0,269	4,88	734	0,0219	305	3000	312	0,119	0,076	162
31	7,8	0,928	3,39	1,58	24900	0,175	0,219	0,375	2,07	507	0,0245	308	2430	333	0,0946	0,0498	133
32	16	2,4	2,77	1,01	24300	0,197	0,202	0,277	1,53	431	0,0303	301	1540	275	0,0458	0,0459	91,9
33	31	5,3	2,88	0,685	24700	0,134	0,0752	0,127	3,66	260	0,0258	270	1900	271	0,0193	0,0394	89,1
34	63	28,1	6,4	1,33	32800	0,195	0,199	0,407	5,72	498	0,0261	573	2130	380	0,152	0,0375	191
35	125	74,6	9,6	1,52	30900	0,193	0,199	0,424	5,74	655	0,0216	634	2260	268	0,186	0,0711	114
36	250	224	33,3	1,13	22800	0,164	0,143	0,175	5,97	628	0,0214	516	1820	207	0,03	0,03	85,8
37	500	447	44,1	1,4	18000	0,214	0,19	0,324	11,4	501	0,0218	448	1310	243	0,292	0,0342	132
38	1000	717	159	0,971	29500	0,177	0,114	0,285	14,1	589	0,0155	424	2300	163	0,184	0,0503	188
39	2000	1420	420	1,03	19300	0,0978	0,163	0,206	14,5	370	0,0272	632	1500	253	0,028	0,03	75,1
40	0	0,125	1,19	1,14	19400	0,215	0,187	0,194	3,31	481	0,0208	432	1490	390	0,0747	0,0426	77
1B				0,984	22400	0,0932	0,108	0,26	1,2	471	0,0239	499	1830	487	0,0653	0,0721	71,7
2B				1,09	19200	0,0962	0,0873	0,174	1,75	317	0,0244	515	1450	530	0,0495	0,0523	62,9
3B				1,25	29800	0,189	0,177	0,325	2,09	793	0,0279	603	2240	503	0,421	0,0894	190

Background mussels

<LOR, set to LOR

Appendix D

Sample ID	As in dig. gland (mg/kg)	Ca in dig. gland (mg/kg)	Cd in dig. gland (mg/kg)	Co in dig. gland (mg/kg)	Cr in dig. gland (mg/kg)	Cu in dig. gland (mg/kg)	Fe in dig. gland (mg/kg)	Hg in dig. gland (mg/kg)	K in dig. gland (mg/kg)	Mn in dig. gland (mg/kg)	Na in dig. gland (mg/kg)	Ni in dig. gland (mg/kg)	Pb in dig. gland (mg/kg)	Zn in dig. gland (mg/kg)
1	1,11	338	0,0876	0,106	0,05	2,53	83,1	0,0416	432	26,8	308	0,0606	0,0247	14,3
2	0,801	192	0,0648	0,122	0,0311	4,33	64,3	0,0382	446	7,72	253	0,0782	0,0247	13,1
3	0,946	177	0,0839	0,108	0,0541	3,28	75,1	0,0457	374	5,74	237	0,0699	0,034	12,1
4	0,961	182	0,0742	0,0979	0,0133	3,63	65,9	0,0443	427	9,84	264	0,0728	0,0346	15,1
5	1,2	166	0,0608	0,0657	0,0403	3,47	55,1	0,0276	402	4,86	224	0,0447	0,0278	13
6	1,06	193	0,0636	0,0903	0,038	3,35	78	0,0404	418	6,13	253	0,0545	0,0296	12
7	0,955	200	0,0312	0,0506	0,078	4,45	31,1	0,0387	359	2,23	183	0,0384	0,0245	14,6
8	0,854	265	0,0564	0,0696	0,0393	4,53	60	0,0357	405	8,76	159	0,0336	0,0261	12,6
9	0,875	249	0,0657	0,103	0,0631	5,39	61,8	0,0361	335	10,4	175	0,0709	0,0284	12,9
10	0,955	369	0,0517	0,0561	0,0566	3,33	56,8	0,0322	352	16,2	94,5	0,0462	0,0229	13,4
11	0,712	191	0,0824	0,0542	0,0472	3,8	61,6	0,0378	323	4,2	106	0,0512	0,0247	10,2
12	0,973	339	0,0779	0,0766	0,0679	4,66	76,6	0,0468	255	15,4	44,5	0,0799	0,0313	14,4
13	0,939	299	0,0979	0,0654	0,0547	6,14	65,2	0,0351	270	5,24	44,1	0,0538	0,0387	17
14	0,819	210	0,0456	0,0833	0,0328	3,63	52	0,0442	381	10,6	278	0,0701	0,0208	13,4
15	1,31	293	0,0571	0,0688	0,0432	3,49	74,6	0,0336	516	18,4	279	0,0651	0,0266	15,2
16	0,991	428	0,074	0,0577	0,0586	4,17	111	0,0426	439	30,9	209	0,0736	0,0413	15,5
17	1,13	184	0,0768	0,0945	0,0476	3,72	58,5	0,0436	412	6,96	261	0,066	0,03	14,9
18														
19	1,08	321	0,0847	0,095	0,0703	4,02	72,2	0,0441	386	17,6	278	0,0853	0,033	14,1
20	0,71	883	0,0501	0,127	0,0506	2,77	73,2	0,0311	220	69,9	155	0,0443	0,0192	13,2
21	1,13	424	0,133	0,179	0,076	5,62	54,3	0,0354	426	23,5	204	0,0774	0,03	14,6
22	0,879	294	0,0603	0,0558	0,0726	5,1	93,3	0,0437	305	9,72	137	0,0797	0,0303	12,3
23	0,916	308	0,0811	0,0822	0,0607	5,2	90,5	0,0493	359	10,7	107	0,0594	0,0317	15,1
24	0,932	287	0,0674	0,114	0,0666	3,73	92	0,0406	261	8,3	92,8	0,0579	0,0265	12,2
25	1,02	451	0,0567	0,0886	0,107	5,3	77,7	0,033	343	13,9	85,6	0,101	0,0349	17,5
26	1,13	392	0,0845	0,0779	0,0753	4,28	70,4	0,0519	413	4,61	94	0,0937	0,0272	19
27	1,15	302	0,095	0,103	0,02	3,98	84,2	0,0413	471	16,6	249	0,089	0,0439	16,3
28	1,07	223	0,0702	0,0759	0,0415	3,73	70,5	0,0447	472	10,3	269	0,0653	0,0311	14,7
29	1,09	165	0,0752	0,1	0,0594	3,51	62,4	0,0382	417	4,71	247	0,0774	0,022	14
30	1,13	589	0,0728	0,0563	0,0646	4,32	143	0,0362	388	42,5	272	0,0867	0,038	16,4
31	1,5	2830	0,0742	0,136	0,092	3,08	145	0,0295	467	293	294	0,057	0,0203	28,2
32	1,08	250	0,0767	0,0727	0,0603	4,85	88,8	0,0426	329	9,07	234	0,0606	0,0314	14,3
33	0,815	195	0,0425	0,0543	0,01	3,7	65,7	0,0393	343	5,95	186	0,0521	0,0293	12,5
34	1,26	368	0,0608	0,0977	0,0534	4,72	64,4	0,0397	438	17,5	228	0,0661	0,0261	16,6
35	1,02	395	0,0688	0,0714	0,0386	4,62	101	0,0344	296	19,6	164	0,079	0,0266	12,3
36	1,1	286	0,0765	0,0548	0,0808	5,27	119	0,0396	309	13,3	110	0,0713	0,0326	14,3
37	0,934	3290	0,102	0,0859	0,052	4,35	142	0,0345	368	324	144	0,0751	0,0395	36,9
38	0,777	512	0,07	0,0524	0,026	5,05	112	0,0423	425	20,6	147	0,0825	0,0329	14,3
39	1,26	438	0,0664	0,0698	0,0239	3,6	76	0,0452	544	6,42	183	0,0625	0,0275	18,4
40	0,967	285	0,0548	0,0701	0,02	4,11	67,6	0,0473	539	12	289	0,0863	0,0477	14,4
1B	0,856	225	0,0894	0,0573	0,0484	2,88	91,2	0,0343	690	7,96	281	0,0929	0,0253	11,3
2B	0,889	195	0,113	0,0495	0,03	2,95	80,5	0,0326	636	4,18	277	0,0712	0,04	11,5
3B	1,09	312	0,103	0,0551	0,0296	3,01	123	0,0298	895	13,7	259	0,108	0,03	14,7

Background mussels

Paper IV

Transcriptional responses as biomarkers of general toxicity: A systematic review and meta-analysis on metal-exposed bivalves

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ABSTRACT

Through a systematic review and a series of meta-analyses, we evaluated the general responsiveness of putative transcriptional biomarkers of general toxicity and chemical stress. We targeted metal exposures performed on bivalves under controlled laboratory conditions, and selected six transcripts associated with general toxicity for evaluation: catalase (*cat*), glutathione-S-transferase (*gst*), heat shock proteins 70 and 90 (*hsp70*, *hsp90*), metallothionein (*mt*) and superoxide dismutase (*sod*). Transcriptional responses (n = 396) were extracted from published scientific articles (k = 22) and converted to log response ratios (lnRRs). By estimating toxic units (TUs), we normalized different metal exposures to a common scale, as a proxy of concentration. Using Bayesian hierarchical random effect models, we then tested the effects of metal exposure on lnRR, both for metal exposure in general and in meta-regressions using TU and exposure time as independent variables. Corresponding analyses were also repeated with transcript and tissue as additional moderators. Observed patterns were similar for general as for transcript- and tissue-specific responses. The expected overall response to arbitrary metal exposure was a lnRR of 0.50, corresponding to a 65 % increase relative a non-exposed control. However, when accounting for publication bias, the estimated 'true' response showed no such effect. Furthermore, expected response magnitude increased slightly with exposure time, but there was little support for general monotonic concentration-dependence with regards to TU. Altogether, this work reveals potential limitations that need consideration prior to applying the selected transcripts as biomarkers in environmental risk assessment.

KEYWORDS

aquatic toxicology; Bayesian statistics; ecotoxicology; meta-regression; mollusk toxicology; publication bias; risk assessment; toxic unit

INTRODUCTION

In ecotoxicology, a biomarker is considered a measurable biological change that can be used as an indicator of chemical exposure and/or a predictor of adverse effects (van der Oost *et al.* 2003). In the context of environmental risk assessment (ERA) of chemicals, understanding of both mechanistic and quantitative links between exposures and relevant biological effects are crucial to predict harm on biota and ecosystems (van der Oost *et al.* 2003, Martin *et al.* 2019). Consequently, it is of great importance that ERAs are supported by robust scientific evidence (Martin *et al.* 2019). For practical application of biomarkers, empirical support can therefore be required to show that a specific marker candidate is both sensitive, by responding at relevant exposures, and robust, by large and predictable response magnitudes.

Molecular biomarkers such as gene transcripts have been proposed to capture responses upstream of adverse effects on the organism level (Calzolai *et al.* 2007, Piña *et al.* 2007). Some transcripts may be specific to certain toxicants or biological effects while others, including responses involved in toxicant metabolism, oxidative stress and general cytoprotection, are considered potential biomarkers of general toxicity and chemical stress (Sulmon *et al.* 2015, Le Saux *et al.* 2020). Many studies on transcriptional biomarker candidates are however based on single or few exposure groups and/or pooled samples from multiple individuals. Such exposure setups may provide important understanding of the molecular mechanisms involved in the responses that may guide identifying markers likely to respond to a chemical stressor. However, substantial empirical support of response effect sizes and variability is required for successful detection and appropriate interpretation of biomarker responses. The evaluation of a putative biomarker can therefore suffer greatly if, for instance, concentration-dependence and individual variation are insufficiently addressed (Fent & Sumpter 2011, Bahamonde *et al.* 2016). Furthermore, experimental setups in ecotoxicological research often differ in study species, biomarker candidates (transcripts and/or tissues) and exposure conditions (chemicals, concentrations and/or exposure durations) (Martin *et al.* 2019). As a result, it can be difficult to put single transcriptional studies in a relevant frame of reference within the body of scientific literature. Therefore, out of context, even results standing out as highly significant may on their own offer little information on the general potential and practical applicability of a biomarker candidate in ERAs.

Bivalve mollusks are common study organisms used for studying various aspects of aquatic pollution (Zhou *et al.* 2008, Binelli *et al.* 2015, Beyer *et al.* 2017). One important feature among bivalves is that they are sessile, which greatly facilitates both site-specific *in situ* assessments and field-collection for laboratory studies (Zhou *et al.* 2008, Binelli *et al.* 2015, Beyer *et al.* 2017). Due to filter-feeding, they are continuously exposed to large volumes of water, and consequently, pollutants present in the water column (Binelli *et al.* 2015, Beyer *et al.* 2017). Also, since many bivalves are bottom-dwellers (Kraan *et al.* 2010, Zieritz *et al.* 2014), sediment is often an additional plausible exposure route. Furthermore, bivalves occupy various aquatic habitat types, which can allow selection of relevant study species on a case by case basis, rather than having to rely on laboratory model species. In general, their role as sentinel species and the high availability of ecotoxicological studies make bivalves candidates for further evaluation of transcriptional biomarkers of pollution.

In this study, we performed a systematic literature review to synthesize published research on transcriptional responses to toxicants, and subsequently a series of meta-analyses to quantify expected responses to toxicant exposure. Due to their ecological relevance and practical use in ERAs and biomonitoring, we targeted responses in bivalves. In a previous literature review, Miao *et al.* (2015) identified glutathione-S-transferase (*gst*), heat shock proteins 70 and 90 (*hsp70*, *hsp90*), metallothionein (*mt*) and superoxide dismutase (*sod*) among the genes most frequently reported among bivalves to respond to pollutant exposures in general. By addressing response trends both to general and continuous exposures (concentration and time), our objective was to evaluate the overall responsiveness of transcriptional biomarker candidates of general toxicity and chemical stress. Specifically, we selected metal exposures to represent general toxicity and a specific set of transcripts (catalase (*cat*), *gst*, *hsp70*, *hsp90*, *mt* and *sod*) that represent common biomarkers of non-specific chemical stress (Miao *et al.* 2015, Sulmon *et al.* 2015, Le Saux *et al.* 2020). To account for individual variation while also reducing the variability of experimental exposures, we limited the analysis to include controlled laboratory studies where transcriptional responses were measured on the individual level. Specifically, we asked whether available data can generally support that (1) transcript levels respond to metal exposure, (2) responses show monotonic concentration-dependence and (3) response magnitudes increase or decrease with exposure time. For each of these questions, we evaluated general responses as well as responses in transcript- and tissue-specific subsets. For transparency and reproducibility, we used the guidelines specified by O'Dea *et al.* (2021) as a basis for the reporting of our study (see checklist in Supporting Information).

MATERIALS AND METHODS

Systematic review

Literature searches were performed in two databases, Web of Science and Scopus (Table 1). The searches were initially performed on the 15th of May 2019, followed by an updated search on the 13th of September 2021. In the first search, we included all publications to date, while we excluded publications indexed before 2019 in the second search. In addition, for articles subsequently selected for inclusion in the meta-analysis, a backward citation search was performed to identify additional potentially relevant literature not captured in the database searches. For this purpose, we used the reference indexing functions in both Web of Science and Scopus.

Table 1. Databases and search terms used in the literature search for the systematic review. The search was initially performed on the 15th of May 2019, and updated on the 13th of September 2021.

Database	Search terms	Search hits
Web of Science (search for 'Topic' within 'All databases')	(*transcript* OR *pcr OR (gene NEAR/1 expression)) AND (mollus* OR mussel* OR bivalv* OR clam*) AND (pollut* OR *toxic* OR xenobiot* OR (stress* NEAR/3 chemic*)) AND (*toxic* OR stress* OR respons* OR biomarker*) AND (aquat* OR fresh* OR limn* OR marine)	2 151 (15th of May 2019) + 653 (13th of September 2021)
Scopus (search for 'Title, Abstract, Keywords')	(*transcript* OR *pcr OR (gene W/1 expression)) AND (mollus* OR mussel* OR bivalv* OR clam*) AND (pollut* OR *toxic* OR xenobiot* OR (stress* W/3 chemic*)) AND (*toxic* OR stress* OR respons* OR biomarker*) AND (aquat* OR fresh* OR limn* OR marine)	478 (15th of May 2019) + 183 (13th of September 2021)

The same screening procedure was performed for all articles, whether found directly from database searches or subsequently from the backward citation search (Figure 1, Table S.1). The screening and selection procedures were performed by one and the same person (GEU) for all searches. Duplicates, review articles and conference abstracts were removed, leaving original research articles for which we screened all titles and abstracts. In the screening process, we first removed articles not based on chemical exposures and articles on irrelevant topics (for instance parasitology, immunology, phylogenetics and human toxicology). Second, articles were excluded if based on other study organisms than bivalves. Third, we removed articles in which other types of responses or biomarkers, but not transcripts, were mentioned in the abstract (for instance proteins, metabolites, enzyme activity or histopathology). Fourth, we required that exposures were performed *in vivo* under controlled

laboratory settings, and based on single compounds in water. Specifically, *in situ* and *in vitro* studies were excluded, as were experiments in which chemical exposure was performed via for instance diet or sediment, or by injection. Similarly, we also excluded studies on nano- and microparticles or chemical mixtures, and studies on environmental stressors typically considered outside of ecotoxicology (for instance pH, nutrients, radiation). After screening for eligible ecotoxicological studies, we performed an additional selection step to narrow the range from all pollutants and transcripts to general toxicity and general stress responses. As a proxy of general toxicity, we selected to include studies (1) based on metal exposures and (2) testing one or more biomarker candidates from the selected set of transcripts (*cat*, *gst*, *hsp70*, *hsp90*, *mt* and *sod*), all of which are representative of general cytoprotection and oxidative stress defense.

After removal of duplicates representing overlap between literature searches ($k = 31$), a total of 122 articles were selected for full-text assessment of experimental setups. To ensure a sufficient level of understanding of experimental setups, we excluded articles not written in English ($k = 4$). Also, one article that was previously not identified as a review article was excluded for not presenting original data. When evaluating experimental designs, we required that (1) a negative control exposure had been performed parallel to metal exposures, (2) transcriptional responses were assessed on the individual level, by quantitative polymerase chain reaction (qPCR), (3) exposure setups were unambiguous and replicated, and that (4) criteria specified for title and abstract screening were still met after full-text evaluation. In case an article contained multiple experiments, exposure groups and/or transcriptional responses, all subsets fulfilling the criteria were included (see Data extraction). Articles not fulfilling all criteria were excluded from the meta-analysis for non-eligible experimental design/ non-applicable methodology ($k = 79$). Consequently, we excluded for instance studies that only used a 0 h exposure as control group and studies that pooled tissue or RNA samples from multiple individuals prior to qPCR. For data extraction, we required that measures of response effect size, variation and sample size were presented for each exposure group, including the negative control. In case any essential piece of information was unclear or lacking from an article at this stage, authors were contacted, initially via e-mail. If no author response was received after first contact, or if the response left unclarities, requests were clarified and repeated at least once. Repeated requests were made both via email and, when possible to track the author profile, via ResearchGate (www.researchgate.net). Studies for which available information remained insufficient ($k = 16$) were ultimately excluded from further analysis to avoid uncertain assumptions of missing data or unclear exposures. In the end, a remaining 22 studies (listed separately in the References section) were left for inclusion in the meta-analysis.

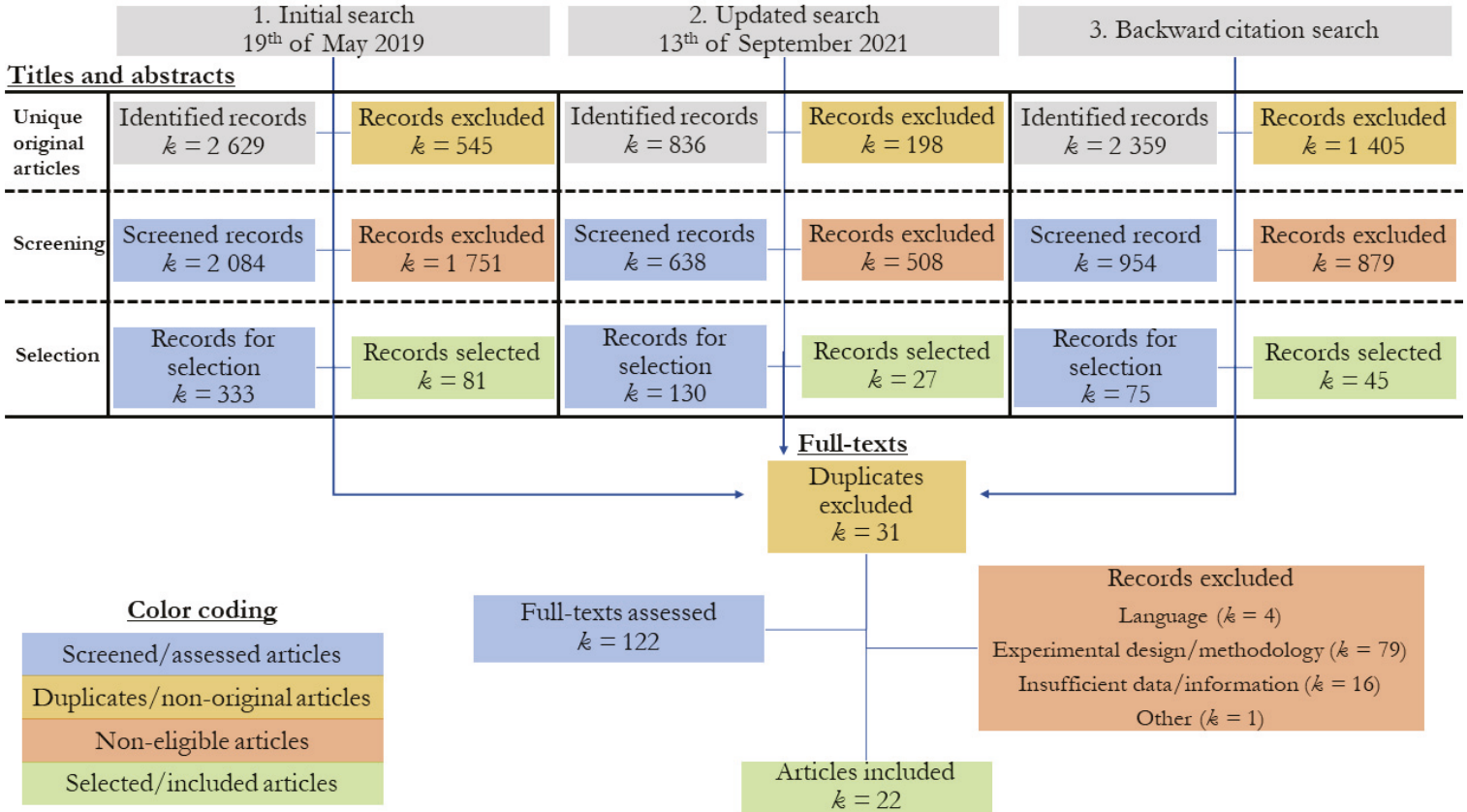

Figure 1. A PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) flowchart summarizing the screening and selection processes for articles included in the final meta-analysis. The number of studies (*k*) is presented for each screening and selection step. For details on the evaluation procedure, see text and Table S.1.

Dataset

Data extraction

Data were extracted for relevant responses (transcript × tissue) in all relevant exposure groups (toxicant × concentration × exposure time), including negative control treatments. All data extractions were performed by one and the same person (GEU). In case a study included additional pollutant exposures (for instance mixtures or nanoparticles), we included any exposure group corresponding to single metal exposure via water, but omitted remaining groups. If interactions with other environmental stressors were investigated (for instance different temperatures or CO_2 levels), we extracted data only from exposure groups representing normal or background conditions of that stressor.

In a few cases, datasets were provided directly by the authors. Otherwise, all data were collected from original articles and supplementary materials. Unless presented in text or tables, we used the software Graph Grabber version 2.0.2 (Quintessa, England) for graphical data extraction. Specifically, we extracted or calculated response mean, standard deviation and sample size for each available biomarker candidate (*cat*, *gst*, *hsp70*, *hsp90*, *mt*, and *sod*) in all analyzed tissues and species. Different isoforms of the transcripts were treated as replicates of the same biomarker candidate as a way to retain as much data as possible, since transcript isoform was not specified in all studies. In case presented sample sizes were non-specified (e.g. presented as ranges) or inconsistent (e.g. different *n* in text and figure legend), the smallest presented sample size was assumed for all exposure groups of that study, unless specific sample sizes were provided upon request to the authors.

Toxic units

As a way to normalize different metals to a common scale, we used toxic units (TUs) as a proxy of exposure concentration. For simplicity, the term 'concentration' will hereafter be used to also include relative measures of exposure such as TU. Specifically, we used the 'standartox' package in R to obtain metal toxicity data from the United States Environmental Protection Agency ECOTOX Knowledgebase (Scharmüller *et al.* 2020). For each metal represented in the meta-analysis dataset, acute toxicity data (72 – 96 h 50 % lethal concentrations, LC_{50}) for bivalve species were retrieved on the 15th of December 2021. The choice of using mortality as an endpoint for normalization was largely based on data availability. Additionally, although not directly linked to the transcriptional responses of interest, mortality from metal exposure was considered a measure of general chemical stress. No bivalve acute toxicity data were available for As(V), Gd, Sm and Y, and these

exposures were excluded from TU determination. In the downstream analyses, corresponding datapoints (n = 58) were however excluded only from models based on TU. For remaining metals, an LC_{50} was retrieved for every available bivalve species. In case of more than one LC_{50} datapoint for a metal × species combination, only the lowest LC_{50} was retained, as a conservative estimate of species sensitivity. In turn, the median $\log_{10}(LC_{50})$ was selected across species to represent general bivalve sensitivity, and for normalization of TU. The reported metal concentration ($\log_{10}$-transformed) was then used to determine logTU for each (applicable) entry in the meta-analysis dataset, according to Equation 1.

$$\text{logTU} = \log_{10}(\text{Reported concentration}) - \text{Median}\log_{10}(\text{LC}_{50})$$

(Equation 1)

Non-independence and effect size calculation

In order to account for non-independence of multiple effect measurements from the same study, we (1) split the control group sample size between exposure treatments, and (2) included a variance-covariance matrix in our models. Prior to effect size calculations, the control group sample size was in each case divided by the number of corresponding exposure groups (toxicant × concentration), which is one approach to adjust for non-independence from multiple comparisons (Higgins *et al.* 2021). The adjusted control group sample size was then in each case used for calculation of response effect size. The variance-covariance matrix was generated using the R package 'metaAidR' (Lagisz *et al.* 2021), and was included in our models to account for non-independence of multiple effect measures within the same exposure group (see section Meta-analyses). Here, we assumed a correlation factor of 0.5 for effects from the same exposure group (study × toxicant × concentration).

As the majority of studies presented results on the linear scale, all data presented on a log-scale were back-transformed prior to effect size calculations. Log response ratios (lnRRs) and corresponding variances (vLRR) were determined for each extracted response (Rosenberg, Rothstein & Gurevitch 2013), according to Equations 2 and 3. Response represents response magnitude, SD standard deviation and n (adjusted) sample size. Exposed and control groups are denoted by a subscript E and C, respectively.

$$\text{lnRR} = \ln\frac{\text{Response}_{\text{E}}}{\text{Response}_{\text{C}}}$$

(Equation 2)

$$\mathrm{vLRR} = \frac{{\mathrm{SD_E}}^2}{n_\mathrm{E} \times {\mathrm{Response_E}}^2} + \frac{{\mathrm{SD_C}}^2}{n_\mathrm{C} \times {\mathrm{Response_C}}^2}$$

(Equation 3)

Meta-analyses

To address our questions, the response variable lnRR was assessed under different combinations of categorical (transcript and tissue) and continuous (TU and exposure time) moderators, resulting in the nine models summarized in Table 2. Linear regression was used to evaluate the general trends of concentration- and time-dependence of response magnitudes. One tissue, visceral mass, was only included in a single study, and due to the low replication, the corresponding datapoints (n = 3) were excluded from analyses using tissue as a moderator. To account for heterogeneity between and within studies, we included a random effect that grouped measurements by the respective study × species × transcript × tissue × time combination. For each specific model, the random effect was however modified to omit any grouping factor also occurring as a moderator (transcript, tissue and time). Also, I^2 was calculated for each model as a measure of heterogeneity between the groups. First, a matrix (P) was defined for each model according to Equation 4, where X denotes the model matrix for the respective model, and W corresponds to the inverse of the variance-covariance matrix (see section Non-independence and effect size calculation) (Viechtbauer 2022).

$$\mathrm{P} = \mathrm{W} - \mathrm{WX}(\mathrm{X'WX})^{-1}\mathrm{X'W}$$

(Equation 4)

This was in turn used to calculate I^2, according to Equation 5. Here, $\hat{\tau}^2$ corresponds to the estimated between-group variance (extracted from the posterior distribution), k to the number of observations and p to the number of columns in the respective X matrix (Viechtbauer 2022).

$$I^2 = 100\% \times \frac{\hat{\tau}^2}{\hat{\tau}^2 + \frac{k-p}{\mathrm{tr}[\mathrm{P}]}}$$

(Equation 5)

The meta-analyses were implemented as Bayesian hierarchical random effect models in the 'brms' R package (Bürkner 2017). The variance-covariance matrix was incorporated into the models using the 'fcor' function (Table 2). Parameters were estimated from the posterior sample derived by Markov Chain Monte Carlo sampling in 'Stan' (Stan Development Team 2021), with 2 000 iterations and four chains, using a burn-in of 2 000 iterations. Prediction intervals of the effect sizes were estimated for models with no or categorial moderators only, using the posterior sample and assuming normal distributions for study effects (IntHout *et al.* 2016). All models were checked for convergence. The 'Rhat' statistic did in no case exceed the critical threshold (1.05), and simulation effective sample sizes (ESS) for the effect parameters were judged as sufficiently large (Table 2).

Table 2. Summary of model structure used for the nine meta-analyses of bivalve transcriptional responses to metal exposure. The group random effect represents the study × species × transcript × tissue × time combination, modified to exclude any grouping factor used as a moderator in the specific model. The same variance-covariance matrix (vcv_matrix) was used for all models. Simulation effective sample sizes (ESS) for effect size parameters are reported for each model.

Moderator		Continuous		
		None	**Toxic unit (log_{10}TU)**	**Exposure time (log_2Time (h))**
Categorical	**None**	**Overall response (intercept model)** ~ (1|group[a]) + fcor(vcv_matrix) ESS: 1 660	**Overall concentration-dependent response** ~ log_{10}TU + (1|group[a]) + fcor(vcv_matrix) ESS: 1 605 – 2 751	**Overall time-dependent response** ~ log_2Time + (1|group[b]) + fcor(vcv_matrix) ESS: 2 332 – 2 809
	Transcript	**Transcript overall response** ~ Transcript + (1|group[c]) + fcor(vcv_matrix) ESS: 1 306 – 2 428	**Transcript-specific concentration-dependence** ~ log_{10}TU × Transcript + (1|group[c]) + fcor(vcv_matrix) ESS: 2 217 – 3 325	**Transcript-specific time-dependence** ~ log_2Time × Transcript + (1|group[d]) + fcor(vcv_matrix) ESS: 2 114 – 2 811
	Tissue	**Tissue overall response** ~ Tissue + (1|group[e]) + fcor(vcv_matrix) ESS: 1 724 – 1 815	**Tissue-specific concentration-dependence** ~ log_{10}TU × Tissue + (1|group[e]) + fcor(vcv_matrix) ESS: 1 623 – 2 142	**Tissue-specific time-dependence** ~ log_2Time × Tissue + (1|group[f]) + fcor(vcv_matrix) ESS: 2 211 – 2 806

[a] study × species × tissue × transcript × time

[b] study × species × tissue × transcript

[c] study × species × tissue × time

[d] study × species × tissue

[e] study × species × transcript × time

[f] study × species × transcript

Sensitivity analysis

To test the influence of the assumed 0.5 correlation factor in the variance-covariance matrix, all models were repeated using variance-covariance matrices based on correlation factors of 0.1 and 0.9, respectively. We also assessed publication bias towards reporting positive results, using a funnel plot and meta-regressions based on effective sample size of the response data, such as suggested for datasets with many non-independent effects (Nakagawa *et al.* 2022). The effective sample size ($4\tilde{n}_i$) was calculated according to Equation 6, where n_E represents the sample size of the exposure group, and n_C represent the control group sample size (adjusted for multiple comparisons).

$$4\tilde{n}_i = \frac{4n_{Ei}n_{Ci}}{n_{Ei} + n_{Ci}}$$

(Equation 6)

The meta-regressions were performed by adding effective sample size as an independent variable to the models without continuous moderators. The intercept from such meta-regression models has been suggested to function as an estimate of a 'true' effect size, adjusted for bias at infinite sample sizes (Nakagawa *et al.* 2022). First, the square root of the inverted effective sample size was included as an independent variable. In cases where the modeled intercept overlapped 0, this intercept was used as an estimate of the 'true' effect size. If the intercept did not overlap 0, the inverse effective sample size was instead used for the corresponding model (Nakagawa *et al.* 2022). Also, the correlation between effective sample size and TU/ exposure time was assessed to estimate the potential influence from publication bias on the slopes of corresponding models (Figure S.1).

Software

The statistical analyses were performed using R version 4.0.5 (R Core Team 2021). The packages 'brms' version 2.16.1 (Bürkner 2017), 'brmstools' version 0.5.3 (Vuorre 2018), 'metaAidR' version 0.0.0.9000 (Lagisz *et al.* 2021), 'openxlsx' version 4.2.4 (Schauberger & Walker 2021) and 'standartox' version 0.0.1 (Scharmüller *et al.* 2020) were used for statistical analyses and dataset manipulation, while 'dplyr' version 1.0.7 (Wickham *et al.* 2021), 'ggbeeswarm' version 0.6.0 (Clarke & Sherrill-Mix 2017), 'ggplot2' version. 3.3.5 (Wickham 2016), 'ggpubr' version 0.4.0 (Kassambara 2020) and 'tidybayes' version 3.0.1 (Kay 2021) were used for producing figures.

RESULTS AND DISCUSSION

Study characteristics

A total of 396 effect sizes were extracted from the 22 included studies. The most abundant transcript was *mt* (27 %), followed by *cat* (18 %), *gst* (18 %), *sod* (16 %), *hsp90* (10 %) and *hsp70* (9.3 %). Most effect sizes corresponded to measurements in gills (54 %), followed by digestive gland (36 %), gonads (9.3 %) and visceral mass (0.76 %). For more detail, see Transcript-specific effects and Tissue-specific effects, respectively. Furthermore, 13 different bivalve species were represented in the dataset: *Dreissena polymorpha* (27 %, n = 108; Navarro *et al.* 2011, Hanana *et al.* 2017, 2018; Louis *et al.* 2021), *Crassostrea gigas* (20 %, n = 81; Choi *et al.* 2008, Jo *et al.* 2008, Cong *et al.* 2012, 2013; Metzger *et al.* 2012), *Cerastoderma glaucum* (20 %, n = 80; Karray *et al.* 2015), *Mytilus galloprovincialis* (8.6 %, n = 34; Piscopo *et al.* 2016, Jimeno-Romero *et al.* 2017, Rocha *et al.* 2018), *Anodonta anatina* (6.1 %, n = 24; Ekelund Ugge *et al.* 2020), *Geloina coaxans* (6.1 %, n = 24; Guo *et al.* 2020), *Ruditapes philippinarum* (4.0 %, n = 16; Chen *et al.* 2018), *Crassostrea virginica* (2.5 %, n = 10; Götze *et al.* 2014, Lebordais *et al.* 2021), *Mytilus edulis* (1.8 %, n = 7; Poynton *et al.* 2014), *Mercenaria mercenaria* (1.0 %, n = 4; Götze *et al.* 2014), *Meretrix meretrix* (1.0 %, n = 4; Gao *et al.* 2021), *Mactra chinensis* (0.76 %, n = 3; Zhang *et al.* 2016) and *Cerastoderma edule* (0.25 %, n = 1; Desclaux-Marchand *et al.* 2007). Finally, Cd was the most common metal exposure (53 %, n = 210, k = 14), followed by Cu (14 %, n = 55, k = 5), Cr(VI) (6.1 %, n = 24, k = 1), Hg (5.1 %, n = 20, k = 1), Gd (4.0 %, n = 16, k = 1), Sm (4.0 %, n = 16, k = 1), As(V) (3.5 %, n = 14, k = 2), Y (3.0 %, n = 12, k = 1), Ag (2.0 %, n = 8, k = 1), As(III) (2.0 %, n = 8, k = 1), Ni (1.5 %, n = 6, k = 1), Pb (1.0 %, n = 4, k = 1) and Zn (0.76 %, n = 3, k = 1).

Overall effects

By addressing responses to general metal exposure, and by using concentration and time as continuous predictors, the objective of the current meta-analyses was to assess the general responsiveness of transcriptional biomarker candidates in bivalves. We demonstrated an overall relative increase of the tested transcriptional responses upon exposure to metal stressors (Figure 2A), suggesting that the

transcripts are in fact sensitive to general metal stress. Without separation of transcripts and tissues, the average lnRR from metal exposure was 0.50. For an arbitrary metal exposure and a random transcript × tissue combination, this would translate to an expected 65 % increase relative a negative control treatment. By comparison, recent meta-analyses on pesticide-exposed fish demonstrated similar (although inverted) overall effect sizes for cholinesterase activity (Santana *et al.* 2021), but smaller effect sizes for enzymes involved in antioxidant defense and biotransformation (Santana *et al.* 2022). Similar effect sizes were also demonstrated in a meta-analysis on cortisol in fish exposed to various contaminants (Rohonczy *et al.* 2021). On the one hand, this could suggest that the robustness of transcriptional responses is comparable to that of other molecular biomarkers. However, an expected lnRR of 0.5 appears small considering the large variability (95 % prediction intervals ranging from approximately -1 to 2) and high heterogeneity (I^2 = 97 %). The overall response would therefore suggest only a moderate robustness of the selected biomarker candidates.

In contrast, there was no implication of concentration-dependence (Figures 2B, 2D), giving no support of an overall monotonic response relative the estimated amount of stress. In a meta-analysis on cortisol levels in fish, Rohonczy *et al.* (2021) were similarly unable to demonstrate concentration-dependence relative the contaminant-exposure, despite positive overall responses. While one explanation could simply be a lack of concentration-response relationships, it could also result from comparing different toxicants on a common scale. In our study, it is possible that the TU approach does not provide high enough resolution, and/or that the between-group heterogeneity (I^2 = 97 %) or other sources of unaccounted variability obscure concentration-dependence that could perhaps be demonstrated in wide-range concentration-response setups, using single species and single toxicants (Ekelund Ugge *et al.* 2022). On the other hand, the current dataset covers a wide range of both response effect sizes and estimated stress exposures. If it were universally true that the biomarker candidates are highly sensitive to the relative amount of stress exposure, the applied meta-analytical models would most likely have captured a rough estimate of the concentration-dependence. Consequently, on larger scales and in heterogenous datasets, metal stress appears to be a stronger predictor of transcriptional responses when assessed as a binary variable (exposed vs. non-exposed) than when treated as a continuous one (for instance TU).

Furthermore, we observed an overall time-dependence, with response magnitudes increasing with longer exposure periods (Figures 2C, 2D). Although the slope was shallow, the credible interval (CI) did not overlap 0. Previous studies on single bivalve species (for instance Fang *et al.* 2010, Liu *et al.* 2014, Bao *et al.* 2018) have demonstrated how the selected transcripts peak after three to 15 days of metal exposure. In line with these findings, our results therefore suggest that exposures for at least a few days are generally more likely to capture transcriptional responses than exposures of a few hours.

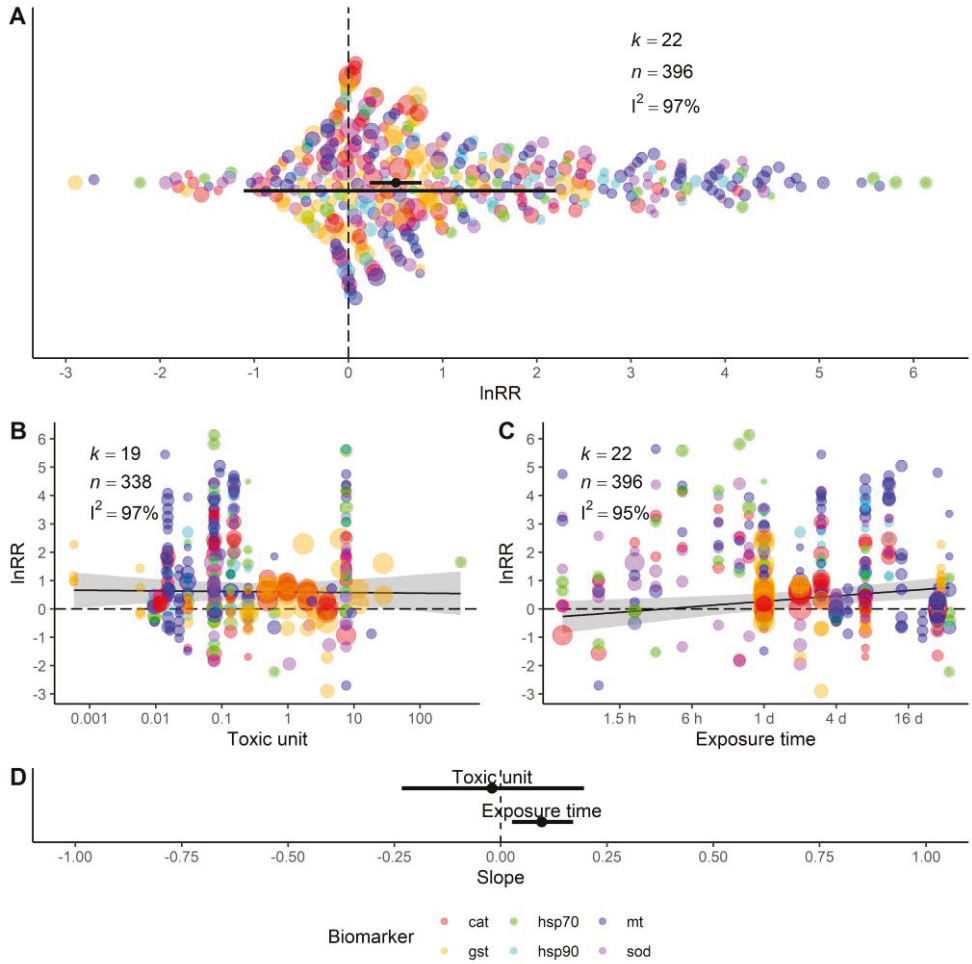

Figure 2. Effect of metal exposure on selected transcripts in bivalves. Effect size is expressed as log response ratio (lnRR), determined according to Equation 2. The sub-plots demonstrate the overall effects of metal exposure (A), concentration-dependence (B, D) and time-dependence (C, D), determined by Bayesian hierarchical random effect models. The overall effect was determined by an intercept model without moderators, while concentration- and time-dependence were determined by meta-regressions using toxic unit and exposure time as moderators. Each point represents an extracted effect size. Colors represent the different transcripts and the point size represents the relative weight (inverted standard deviation). Shaded areas (B, C) and bars (A, D) represent 95 % credible intervals, and a 95 % prediction interval is represented by a horizontal line (A). Each plot shows the number of studies (*k*) and effect sizes (*n*) represented in the respective analysis, as well as corresponding heterogeneity (I^2).

Transcript-specific effects

With regards to separate transcriptional responses, five out of six transcripts demonstrated average positive responses to exposure treatments (Figure 3). The implication would therefore be that the responsive transcripts *cat*, *gst*, *hsp70*, *mt* and *sod* indeed have some potential as transcriptional biomarkers in bivalves. Despite a trend of positive responses, the CI of *hsp90* overlapped 0 (Figure 3D), and insufficient robustness is likely to limit the potential biomarker use of this transcript. Additionally, there was a general lack of concentration-dependence for separate transcripts (Figure 4). Despite positive responses relative arbitrary metal exposure, there was a trend of decreasing response magnitudes with increasing TU. Slopes were however shallow with five out of six CIs overlapping 0. For *sod*, the upper confidence bound was just below 0, and the slope was also the steepest for this transcript (Figure 4F-G). In contrast, the general trend of time-dependent increases of response magnitudes was persistent in all biomarker candidates, although *gst*, *hsp70* and *hsp90* CIs overlapped 0 (Figure 5). Heterogeneity was consistently high (I^2 = 83 – 99 %), with all transcript-specific models following the general pattern *gst* ≥ *cat* > *sod* > *mt* ≥ *hsp90* > *hsp70*. In summary, *cat*, *gst*, *hsp70* and *mt* closely followed the trends of the overall effects, while *hsp90* CIs overlapped 0 for all moderators, and *sod* demonstrated a negative concentration-response relationship not observed in the other transcripts.

Tissue-specific effects

Two out of three tissues demonstrated positive average responses to exposure treatments (Figure 6). For the overall effect, digestive gland and gill CIs did not overlap 0 (Figure 6A, 6D), in contrast to gonads (Figure 6G). The potential for detecting responses therefore appears higher in gills and digestive glands, as could be expected from potential uptake and metabolism of metals in these tissues (Bonneris *et al.* 2005, Won *et al.* 2016). The general lack of concentration-dependence was consistent in all tissues (Figure 6B, 6E, 6H, 6J), but interestingly, time-dependence was weak for the separate tissues (Figure 6C, 6F, 6I, 6J). Specifically, gills and gonads showed a trend of responses increasing with time (Figure 6F, 6I), while digestive gland responses were largely unchanged (Figure 6C). Considering that all CIs overlapped 0, the general trend of time-dependence however appears driven by other factors than tissue. Finally, heterogeneity was high across tissues in all models (I^2 = 91 – 99 %), and consistently highest in gonads.

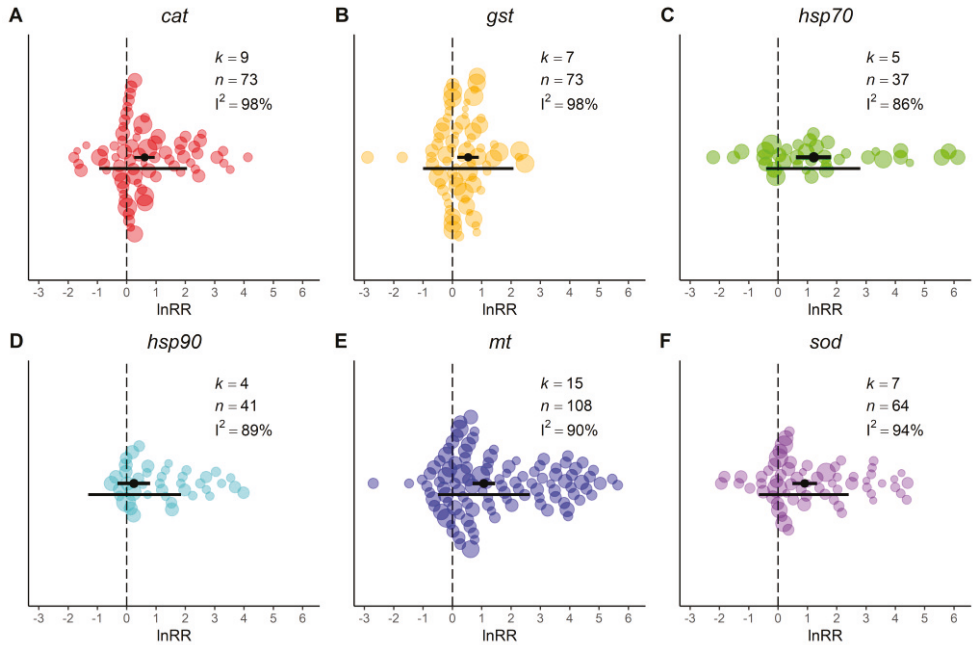

Figure 3. Effect of metal exposure on specific transcripts in bivalves. The sub-plots demonstrate the effects of arbitrary metal exposure on *cat* (A), *gst* (B), *hsp70* (C), *hsp90* (D), *mt* (E) and *sod* (F) expression. Effects (log response ratio, lnRR) were determined by Bayesian hierarchical random effect models using transcript as moderator. Each point represents an extracted effect size and the point size represents the relative weight (inverted standard deviation). Bars represent 95 % credible intervals, and horizontal lines below represent 95 % prediction intervals. Each plot shows the number of studies (*k*) and effect sizes (*n*) represented in the respective subset, as well as corresponding heterogeneity (I^2).

Sensitivity analysis and limitations of the current meta-analysis

Generally, there was little influence from changing the correlation factor in the variance-covariance matrix to 0.1 or 0.9 (Figure S.2). In a few specific cases, CIs could change from just overlapping 0 to not doing so or vice versa, such as for overall effects in gonads, concentration-dependence in *sod* or time-dependence in *gst*, *hsp70* and *mt* (Figure S.2). There is however no indication that a changed correlation factor would generally exaggerate or suppress effects in a way that would impact the general conclusions. Therefore, the results would support that our assumption of a 0.5 correlation factor represents a reasonable middle ground for addressing non-independence in our dataset.

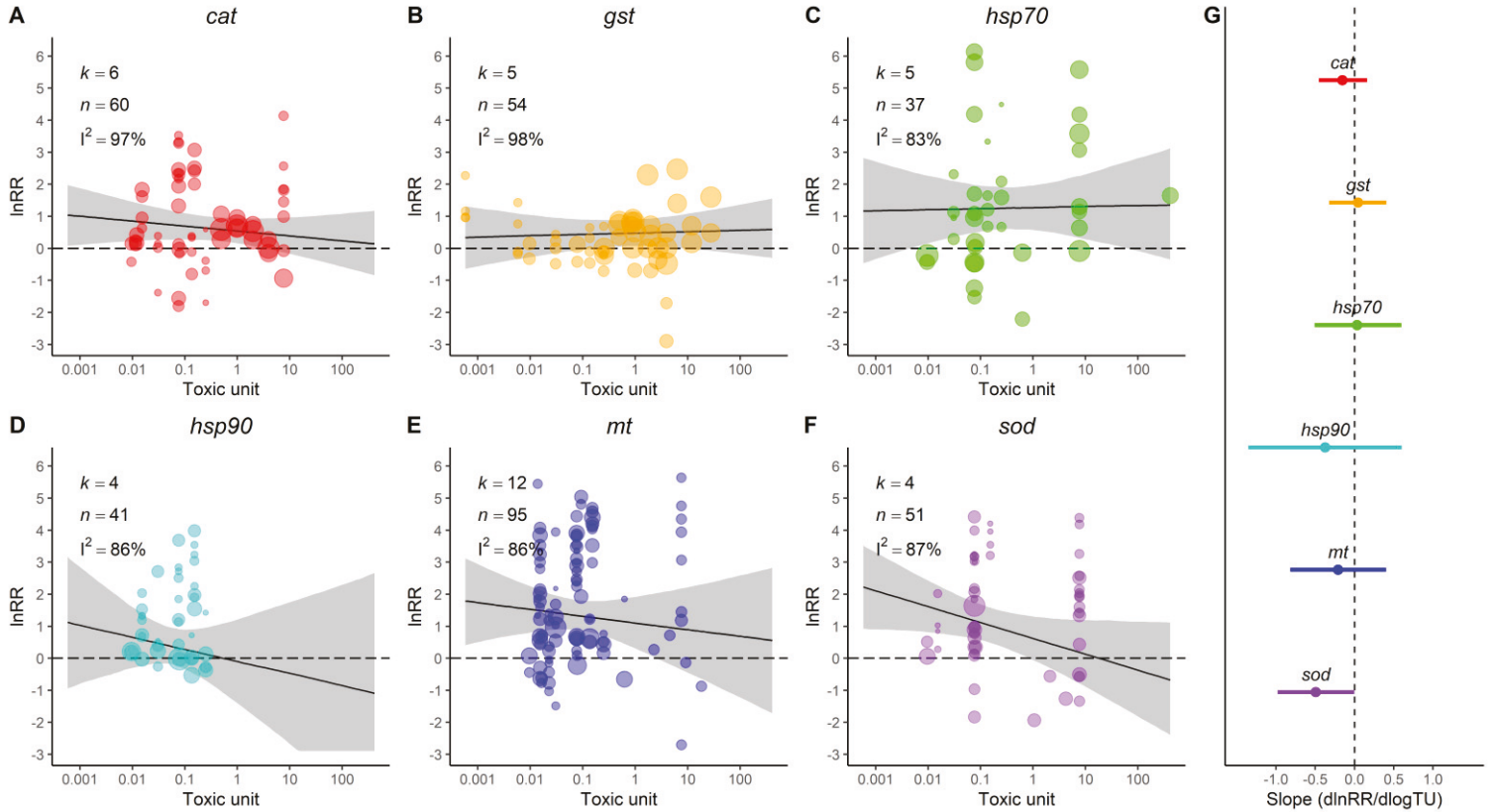

Figure 4. Concentration-dependence of specific transcripts in metal-exposed bivalves. The sub-plots demonstrate the effects on *cat* (A), *gst* (B), *hsp70* (C), *hsp90* (D), *mt* (E) and *sod* (F) expression. Effects (log response ratio, lnRR) were determined by Bayesian hierarchical random effect models using transcript and toxic unit as moderators. The model slopes are summarized in (G). Each point in plot A-F represents an extracted effect size and the point size represents the relative weight (inverted standard deviation). Shaded areas (A-F) and bars (G) represent 95 % credible intervals, and each plot shows the number of studies (*k*) and effect sizes (*n*) represented in the respective subset, as well as corresponding heterogeneity (I^2).

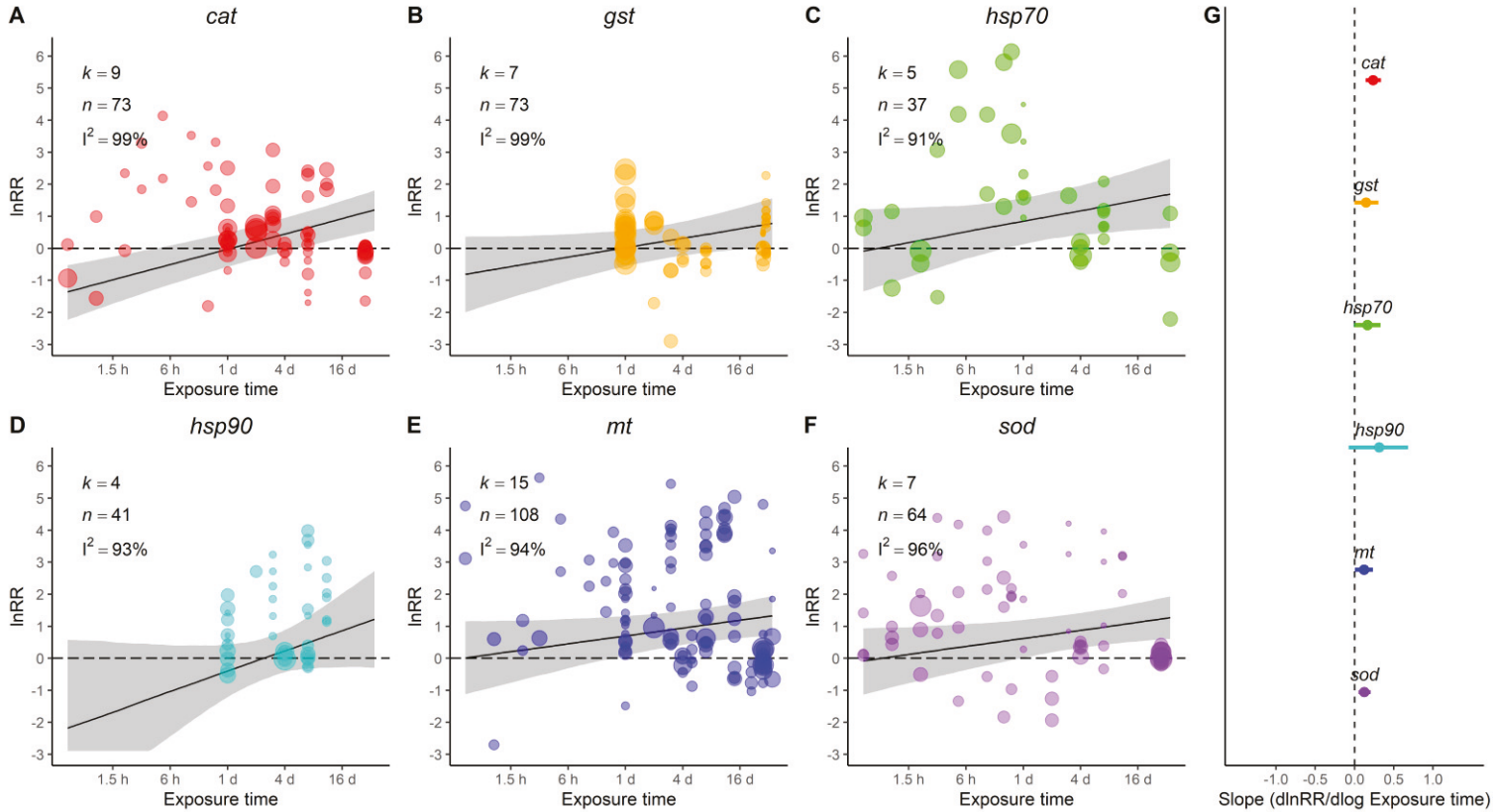

Figure 5. Time-dependence of specific transcripts in metal-exposed bivalves. The sub-plots demonstrate the effects on *cat* (A), *gst* (B), *hsp70* (C), *hsp90* (D), *mt* (E) and *sod* (F) expression. Effects (log response ratio, lnRR) were determined by Bayesian hierarchical random effect models using transcript and exposure time as moderators. The model slopes are summarized in (G). Each point in plot A-F represents an extracted effect size and the point size represents the relative weight (inverted standard deviation). Shaded areas (A-F) and bars (G) represent 95 % credible intervals, and each plot shows the number of studies (*k*) and effect sizes (*n*) represented in the respective subset, as well as corresponding heterogeneity (I^2).

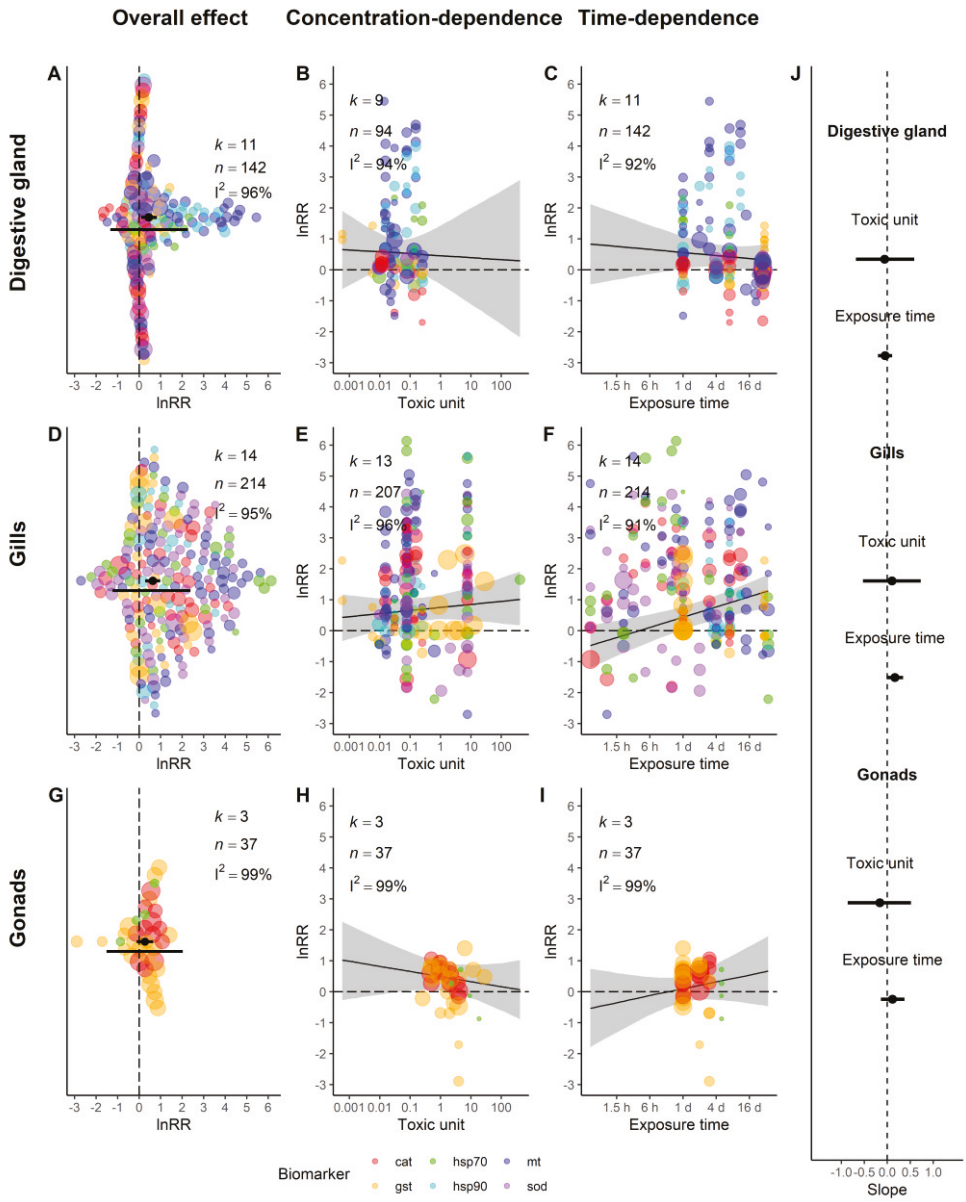

Figure 6. Effect of metal exposure on selected transcripts in three bivalve tissues. The sub-plots demonstrate the overall effects (log response ratio, lnRR) of metal exposure in digestive glands (A), gills (D) and gonads (G), the concentration-dependence of responses in digestive glands (B), gills (E) and gonads (H), and the time-dependence of responses in digestive glands (C), gills (F) and gonads (I), determined by Bayesian hierarchical random effect models. The overall effects were determined using only tissue as moderator, while concentration- and time-dependence were determined by meta-regressions using toxic unit and exposure time as additional moderators. The model slopes are summarized in (J). Each point in plot A-I represents an extracted effect size. Colors represent the different transcripts and the point size represents the relative weight (inverted standard deviation). Shaded areas (B, C, E, F, H, I) and bars (A, D, G, J) represent 95 % credible intervals, and horizontal lines (A, D, G) represent 95 % prediction intervals. Each plot shows the number of studies (*k*) and effect sizes (*n*) represented in the respective subset, as well as corresponding heterogeneity (I^2).

Transcriptional studies commonly present multiple effect sizes, and for instance, there were only two studies in the dataset used for the current meta-analysis from which we extracted a single effect size relative control. As publication bias can be driven by the reporting of positive results such as 'statistically significant' differences (e.g. Nakagawa *et al.* 2022), the likelihood of such detection, and hence, publication, would increase with an increasing number of responses (transcripts, tissues) and/or exposure treatments (toxicants, concentrations, exposure durations). Indirectly, this could however also result in publication of negative results observed within the same study, that would perhaps not be published on their own. It could therefore be possible that multi-biomarker approaches in transcriptional studies might partially counteract the impacts of publication bias.

For the current dataset, funnel plots revealed a slightly right-skewed distribution of effect sizes (Figure S.3A-B), which can be indicative of publication bias. Performing meta-regressions based on the inverted effective sample size, we also estimated new effect sizes that were adjusted for potential publication bias (Figure S.3C). Since there was no implication of dependence between effective sample size and TU or exposure time, respectively (Figure S.1), we assumed that potential interactions with model slopes were negligible, and that potential publication bias mainly affected estimates of model intercepts. That is, we would expect potential influence on the absolute effect size, but not on the change relative TU or exposure time. Not surprisingly, adjusted effect size estimates were consistently smaller than non-adjusted ones for our intercept-models (overall or separated by transcript or tissue), with CIs consistently overlapping 0 (Figure S.3C). Despite this apparent overestimation of the effect sizes by our original models, it is worth noting that even non-adjusted effect sizes were generally small. Also, underestimation of effect size variation would appear to bias the analyses to a larger extent than underestimated effect sizes. Generally, the sensitivity analysis therefore suggests that (1) our dataset (presumably extending to the bulk of scientific literature) is biased, and (2) on a large scale, the expected transcriptional responses to arbitrary metal exposure are seemingly not distinguishable from 0, even when approaching infinite sample sizes.

In addition to the impact from publication bias, there are some other important limitations to the current dataset. For instance, different isoforms were in some cases grouped together to represent a single transcript. This is in many ways analogous to using multiple species to represent 'bivalves' or multiple compounds to represent 'metals'. Ultimately, it increases the generality of the results although decreasing the specificity. Furthermore, the datapoints were not evenly distributed across neither TUs nor exposure timepoints, in particular for certain subsets of the data. For *hsp90* and gonads, the coverage over both exposure concentrations and exposure time was rather narrow, which results in greater uncertainties of the respective meta-regressions. Similarly, there was not sufficient replication or representation of all combinations for us to consider transcript × tissue interactions. Provided sufficient data, such analyses would give a higher resolution and could

help specify what transcript to analyze in which tissue for highest biomarker potential.

With regards to the meta-analyses themselves, one important limitation is the non-independence of multiple datapoints from the same studies. This has been presented as a common phenomenon in meta-analyses on ecology and evolution (Nakagawa *et al.* 2022), and would in many cases likely extend to the adjacent research fields of ecotoxicology and environmental science. By taking measures to adjust the data and models (see section Non-independence and effect size calculation), we ultimately assume that non-independence has been accounted for. Another important limitation is the use of toxic unit as a measure of relative concentration and/or chemical stress. The way we use it, TU is a rough measure that assumes equal tolerance within the whole taxonomic group of bivalves. The transformation of a toxicant × concentration combination to TU therefore adds uncertainty to each datapoint. Consequently, it might not be a suitable approach in for instance mechanistic response modeling. However, we argue that normalization of different toxicant exposures to a common scale makes it possible to better represent the general trends that we are currently addressing. Consequently, if a strong relationship between general metal stress and transcriptional responses were present, it should be detectable by meta-regression even when using a rough estimate such as TU as moderator.

Finally, we once again acknowledge some underlying limitations that affect both the generality and specificity of our results. Our objective was to identify the general trends of biomarker potentials rather than representing a fine-tuned mechanistic approach. Our results thus simply suggest what responses to expect from arbitrary exposure, as supported by available data. Still, there were important limitations to the scope of the study. We only used bivalves to represent potential, environmentally relevant bioindicators, and metal exposure as a proxy for general chemical stress. In addition, we limited the evaluation of biomarkers to six transcripts. Even so, between-group heterogeneity was consistently high ($I^2 \geq 83\%$), and it is plausible that other taxon × toxicant × transcript combinations would yield different results. Hence, for different setups and/or very specific exposure conditions, our results may be of limited use for prediction of specific responses. In that case, setups focusing on for instance single species/genera and/or single compounds can offer a higher resolution (Ekelund Ugge *et al.* 2022), which could potentially be necessary to address more specific questions. On the other hand, such setups would tend to be even less appropriate for extrapolations and for addressing more general questions. We have no apparent reason to believe that another selection of taxa, compounds and/or genes would better fit our research questions. Therefore, we suggest that our results offer a fair representation of the general biomarker potentials of (assumed) stress genes for metal-exposed bivalves in particular, and to some extent pollutant-exposed organisms in general.

Conclusions

Based on the published scientific literature, there was support for slight positive responses of the assessed transcriptional biomarker candidates at arbitrary metal exposure, both overall and (with the exception of *hsp90*) when assessed separately. The same was also true for the overall responses in gills and digestive glands. However, there was also implication of publication bias in favor of positive effect sizes, likely leading to a general overestimation of biomarker responsiveness. Predicted effect sizes from arbitrary metal exposure should therefore be interpreted with caution, as it is not unlikely that the 'true' effects in most cases would be close to 0. Taken together, this suggests low sensitivity and robustness of the biomarker candidates.

There was a slight increase in expected response with exposure time, although this effect was weaker for the transcript and tissue subsets than for the overall response. The general implication would be that sensitivity increases with time, and that the probability of detecting differences is likely higher after days or weeks than after hours of exposure.

Finally, except for a slight decrease in *sod*, there was little support of concentration-dependence of the responses with regards to TU, neither for overall responses nor for transcript- or tissue-specific effects. As discussed, this could partially be due to low resolution resulting both from the various species × transcript × toxicant combinations and from the uncertainties around TU. Nonetheless, it gives a clear implication that on a large scale, there is no universal concentration-response relationship for stress-related transcripts in metal exposed mussels. Consequently, in the absence of species-, toxicant- and/or tissue-specific data, robust responses should not necessarily be expected even at high exposure concentrations.

This work illustrates a number of limitations of the selected transcriptional responses in bivalves, which would likely be true for a range of other taxa, transcripts and toxicant exposures. Prior to potential application of transcriptional biomarkers in ERA, it will therefore be crucial to further address e.g. concentration-dependence, time-dependence and individual variation. Provided sufficient mechanistic understanding and/or empirical support, transcripts may have great potential for various approaches in ERA, such as adverse outcome pathways, multi-biomarker models or transcriptional points of departure. Whether or not there are transcripts that on their own can function as biomarkers of general toxicity and chemical stress however remains a question for future research.

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Gustaf Ekelund Ugge is employed by ALS Scandinavia Toxicon AB, but is currently on a leave of absence for pursuing a PhD.

Data availability statement

Data and code to reproduce the results are publicly available on GitHub (https://github.com/gmoeu/transcriptional-biomarkers-metaanalysis), and the full dataset is publicly available on Mendeley (https://doi.org/10.17632/83jc4yv35h).

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Transcriptional responses as biomarkers of general toxicity: A systematic review and meta-analysis on metal-exposed bivalves

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Data and code to reproduce the results are publicly available on GitHub (https://github.com/gmoeu/transcriptional-biomarkers-metaanalysis), and the full dataset is publicly available on Mendeley (https://doi.org/10.17632/83jc4yv35h).

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Table S.1. Summary of the procedure and criteria used for sequential evaluation and inclusion/exclusion of titles, abstracts and full-texts from the systematic review.

	Evaluation step	Criteria	Examples of excluded studies
Unique articles	Original papers	Unique articles presenting original data; published in scientific journals	Duplicates (between databases, between searches). Review articles, conference contributions, books.
Screening (abstracts)	Ecotoxicology and environmental science	Organisms exposed to abiotic stressor	Articles on immunology/parasitology, physiology, phylogeny, technical methodology.
	Bivalve studies	Study performed on bivalves	Articles on fish, mammals, crustaceans.
	Transcriptional studies	Transcriptional responses quantified	Studies mentioning other types of biomarkers (proteins, enzymatic assays, metabolites, histopathology) but not transcripts
	Controlled *in vivo* water exposures	*In vivo* exposure; water exposure to single compound; laboratory conditions	*In vitro* and *in situ* studies, exposures to mixtures and particles, alternative exposure routes such as diet, injection. Studies on environmental stressors such as CO_2, radiation, pH.
Selection (abstracts)	Metal exposure	Metal exposure	Articles not mentioning metal exposures in title or abstract.
	Selected transcripts	Catalase (*cat*), glutathione-S-transferase (*gst*), heat shock protein 70/90 (*hsp70*, *hsp90*), metallothionein (*mt*) and/or superoxide dismutase (*sod*)	Articles not mentioning any of the selected transcripts in title or abstract.
Full-text assessment	Experimental design	Negative control exposure parallel to toxicant exposures; responses assessed in ≥ 3 individuals per exposure group; qPCR; exposure setups unambiguous	Studies only including a T0 group as control; studies on pooled samples from multiple individuals; studies based on other methodologies than quantitative polymerase chain reaction (qPCR).
	Presented data	Mean, standard deviation and sample size presented for exposure groups and control groups, or possible to extract	Studies in which required information was not presented or sufficiently clear, unless clarification was provided upon request to the authors.

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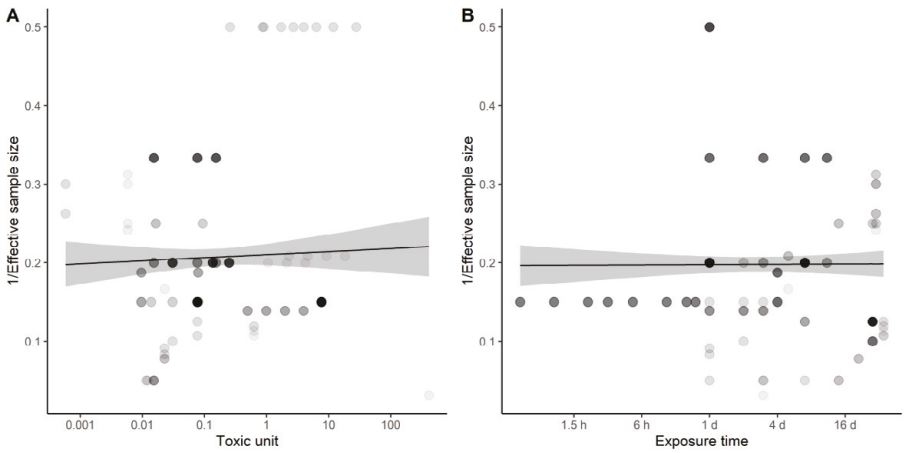

Figure S.1. Inverse effective sample size plotted against toxic unit and exposure time. Color intensity of the points correlates to the number of effect sizes representing the particular toxic unit/exposure time × effective sample size combination.

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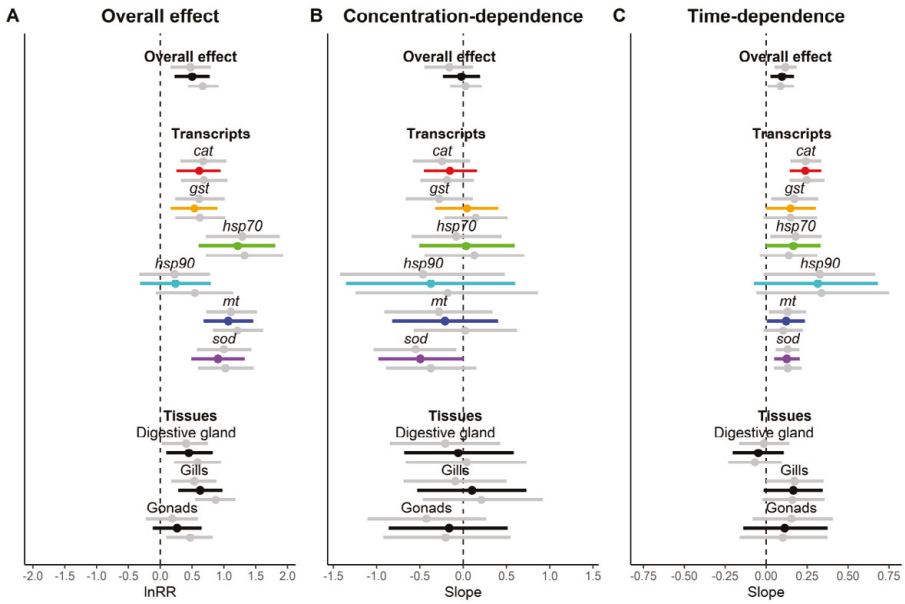

Figure S.2. The influence from applying different correlation factors in variance-covariance matrices used for meta-analyses of bivalve transcriptional responses to metal exposure. A variance-covariance matrix was applied to all models, assigning correlation factors of 0.1, 0.5 or 0.9 to all responses from the same exposure group (study × toxicant × concentration). The sub-plots demonstrate overall effects (log response ratio, lnRR) of metal exposure (A), concentration-dependence of responses (B) and time-dependence of responses (C), determined by Bayesian hierarchical random effect models. Black and color-coded points correspond to the assumed correlation factor of 0.5, while grey points above and below represent correlation factors of 0.9 and 0.1, respectively. Bars represent 95 % credible intervals.

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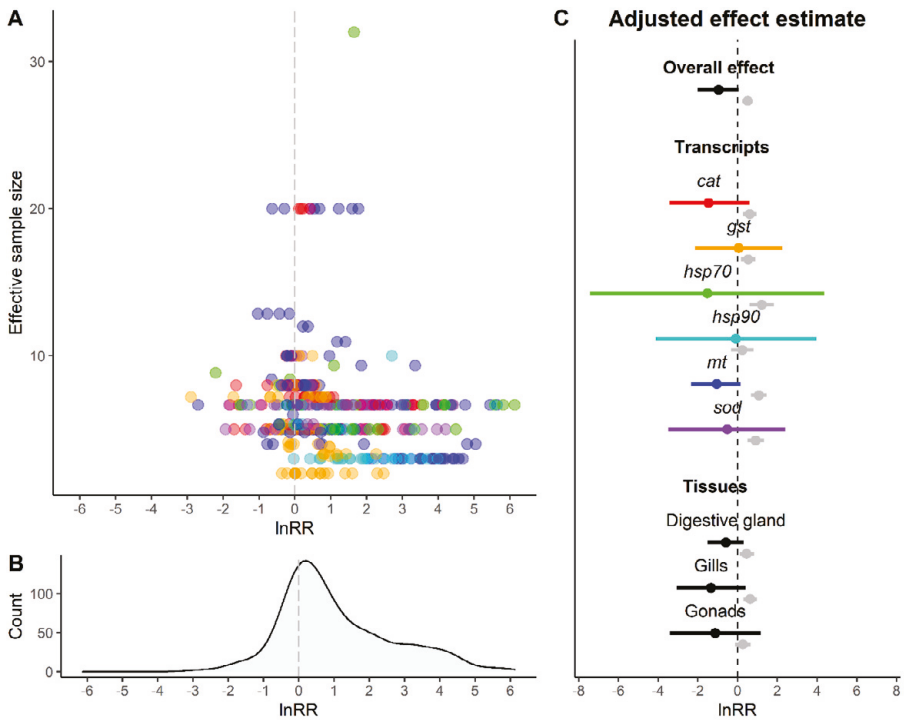

Figure S.3. Evaluation of publication bias in the dataset used for the meta-analyses. A funnel plot (A) shows the response effective sample sizes plotted against effect size (log response ratio, lnRR), and a corresponding density plot (B) shows the distribution of effect sizes in the dataset. Adjusted effect sizes (C) were estimated from the intercepts of meta-regressions based on effective sample sizes (see main text). Black and colored points correspond to adjusted effect estimates, while original estimates are shown beneath in grey. Bars represent 95% credible intervals. Colors in (A) correspond to the different transcripts as presented in (C).

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Checklist item	Sub-item number	Sub-item	Reported by authors?	Notes
Title and abstract	1.1	Identify the review as a systematic review, meta-analysis, or both	Yes	Stated in both the title and the abstract.
	1.2	Summarise the aims and scope of the review	Yes	Summarized in the abstract.
	1.3	Describe the data set	Yes	Summarized in the abstract.
	1.4	State the results of the primary outcome	Yes	Summarized in the abstract.
	1.5	State conclusions	Yes	Briefly summarized in the abstract.
	1.6	State limitations	Yes	Briefly summarized in the abstract.
Aims and questions	2.1	Provide a rationale for the review	Yes	Follows from the first paragraphs of the introduction.
	2.2	Reference any previous reviews or meta-analyses on the topic	Yes	Only one available review on a similar topic; however, addressing different questions.
	2.3	State the aims and scope of the review (including its generality)	Yes	Stated in the last paragraph of the introduction.
	2.4	State the primary questions the review addresses (e.g. which moderators were tested)	Yes	Stated in the last paragraph of the introduction.
	2.5	Describe whether effect sizes were derived from experimental and/or observational comparisons	Yes	Described in M&M > 'Systematic review', and in Table S.1.
Review registration	3.1	Register review aims, hypotheses (if applicable), and methods in a time-stamped and publicly accessible archive and provide a link to the registration in the methods section of the manuscript. Ideally registration occurs before the search, but it can be done at any stage before data analysis.	No	The review was not registered.
	3.2	Describe deviations from the registered aims and methods	–	Not applicable.
	3.3	Justify deviations from the registered aims and methods	–	Not applicable.

Checklist item	Sub-item number	Sub-item	Reported by authors?	Notes
Eligibility criteria	4.1	Report the specific criteria used for including or excluding studies when screening titles and/or abstracts, and full texts, according to the aims of the systematic review (e.g. study design, taxa, data availability)	Yes	Described in M&M > 'Systematic review', and in Table S.1.
	4.2	Justify criteria, if necessary (i.e. not obvious from aims and scope)	Yes	Described in M&M > 'Systematic review'.
Finding studies	5.1	Define the type of search (e.g. comprehensive search, representative sample)	Yes	Described in M&M > 'Systematic review', first paragraph.
	5.2	State what sources of information were sought (e.g. published and unpublished studies, personal communications)	Yes	Described in M&M > 'Systematic review', first paragraph.
	5.3	Include, for each database searched, the exact search strings used, with keyword combinations and Boolean operators	Yes	Presented in Table 1.
	5.4	Provide enough information to repeat the equivalent search (if possible), including the timespan covered (start and end dates)	Yes	Described in M&M > 'Systematic review', first paragraph, and in Table 1.
Study selection	6.1	Describe how studies were selected for inclusion at each stage of the screening process (e.g. use of decision trees, screening software)	Yes	Described in M&M > 'Systematic review', and in Table S.1.
	6.2	Report the number of people involved and how they contributed (e.g. independent parallel screening)	Yes	Described in M&M > 'Systematic review', second paragraph.

Checklist item	Sub-item number	Sub-item	Reported by authors?	Notes
Data collection process	7.1	Describe where in the reports data were collected from (e.g. text or figures)	Yes	Described in M&M > 'Dataset' > 'Data extraction'.
	7.2	Describe how data were collected (e.g. software used to digitize figures, external data sources)	Yes	Described in M&M > 'Dataset' > 'Data extraction'.
	7.3	Describe moderator variables that were constructed from collected data (e.g. number of generations calculated from years and average generation time)	Yes	This applies to the calculations of toxic units, which is described in M&M > 'Dataset' > 'Toxic units'.
	7.4	Report how missing or ambiguous information was dealt with during data collection (e.g. authors of original studies were contacted for missing descriptive statistics, and/or effect sizes were calculated from test statistics)	Yes	Described in M&M > 'Systematic review', last paragraph.
	7.5	Report who collected data	Yes	Described in M&M > 'Dataset' > 'Data extraction'.
	7.6	State the number of extractions that were checked for accuracy by co-authors	No	Extractions were only checked by the extracting author.
Data items	8.1	Describe the key data sought from each study	Yes	Described in M&M > 'Dataset' > 'Data extraction'.
	8.2	Describe items that do not appear in the main results, or which could not be extracted due to insufficient information	No	Additional descriptive data (e.g. habitat type and life stage of the mussels, food source and temperature during exposures) are however available in the full dataset on Mendeley. These items were not intended for inclusion in the models, but were extracted just in case they would be needed later on.
	8.3	Describe main assumptions or simplifications that were made (e.g. categorising both 'length' and 'mass' as 'morphology')	Yes	Described in M&M > 'Dataset' > 'Data extraction'.
	8.4	Describe the type of replication unit (e.g. individuals, broods, study sites)	Yes	Mentioned in introduction; described in more detail under M&M > 'Systematic review', and in Table S.1.

Checklist item	Sub-item number	Sub-item	Reported by authors?	Notes
Assessment of individual study quality	9.1	Describe whether the quality of studies included in the systematic review or meta-analysis was assessed (e.g. blinded data collection, reporting quality, experimental *versus* observational)	No	Studies meeting the inclusion criteria were assumed to be of sufficiently high quality, and there was no further assessment of study quality.
	9.2	Describe how information about study quality was incorporated into analyses (e.g. meta-regression and/or sensitivity analysis)	–	Not applicable.
Effect size measures	10.1	Describe effect size(s) used	Yes	Described in M&M > 'Dataset' > 'Non-independence and effect size calculation'.
	10.2	Provide a reference to the equation of each calculated effect size (e.g. standardised mean difference, log response ratio) and (if applicable) its sampling variance	Yes	Presented in M&M > 'Dataset' > 'Non-independence and effect size calculation'.
	10.3	If no reference exists, derive the equations for each effect size and state the assumed sampling distribution(s)	–	Not applicable.
Missing data	11.1	Describe any steps taken to deal with missing data during analysis (e.g. imputation, complete case, subset analysis)	Yes	Described in M&M > 'Systematic review', last paragraph; unless authors provided the data needed, those studies were excluded from further analysis.
	11.2	Justify the decisions made to deal with missing data	Yes	Described in M&M > 'Systematic review', last paragraph.
Meta-analytic model description	12.1	Describe the models used for synthesis of effect sizes	Yes	Described in M&M > 'Meta-analyses'.
	12.2	The most common approach in ecology and evolution will be a random-effects model, often with a hierarchical/multilevel structure. If other types of models are chosen (e.g. common/fixed effects model, unweighted model), provide justification for this choice	–	Not applicable; only random-effects models were used.
Software	13.1	Describe the statistical platform used for inference (e.g. *R*)	Yes	Described in M&M > 'Software'.
	13.2	Describe the packages used to run models	Yes	Described in M&M > 'Software'.
	13.3	Describe the functions used to run models	Yes	Described in M&M > 'Meta-analyses' and Table 2.
	13.4	Describe any arguments that differed from the default settings	Yes	Described in M&M > 'Meta-analyses'.
	13.5	Describe the version numbers of all software used	Yes	Described in M&M > 'Software'

Checklist item	Sub-item number	Sub-item	Reported by authors?	Notes
Non-independence	14.1	Describe the types of non-independence encountered (e.g. phylogenetic, spatial, multiple measurements over time)	Yes	Described in M&M > 'Dataset' > 'Non-independence and effect size calculation'.
	14.2	Describe how non-independence has been handled	Yes	Described in M&M > 'Dataset' > 'Non-independence and effect size calculation'.
	14.3	Justify decisions made	Yes	Described in M&M > 'Dataset' > 'Non-independence and effect size calculation'.
Meta-regression and model selection	15.1	Provide a rationale for the inclusion of moderators (covariates) that were evaluated in meta-regression models	Yes	Described in the introduction.
	15.2	Justify the number of parameters estimated in models, in relation to the number of effect sizes and studies (e.g. interaction terms were not included due to insufficient sample sizes)	Yes	Discussed in 'Results and Discussion' > 'Sensitivity analysis and limitations of the current meta-analysis', fourth paragraph.
	15.3	Describe any process of model selection	–	Not applicable; there was no model selection process. All the models are presented.
Publication bias and sensitivity analyses	16.1	Describe assessments of the risk of bias due to missing results (e.g. publication, time-lag, and taxonomic biases)	Yes	Described in M&M > 'Sensitivity analysis'.
	16.2	Describe any steps taken to investigate the effects of such biases (if present)	Yes	Described in M&M > 'Sensitivity analysis'.
	16.3	Describe any other analyses of robustness of the results, e.g. due to effect size choice, weighting or analytical model assumptions, inclusion or exclusion of subsets of the data, or the inclusion of alternative moderator variables in meta-regressions	Yes	Described in M&M > 'Sensitivity analysis'.
Clarification of *post hoc* analyses	17.1	When hypotheses were formulated after data analysis, this should be acknowledged.	–	Not applicable; no *post hoc* analyses were performed.

Checklist item	Sub-item number	Sub-item	Reported by authors?	Notes
Metadata, data, and code	18.1	Share metadata (i.e. data descriptions)	Yes	Available on GitHub and Mendeley.
	18.2	Share data required to reproduce the results presented in the manuscript	Yes	Available on GitHub.
	18.3	Share additional data, including information that was not presented in the manuscript (e.g. raw data used to calculate effect sizes, descriptions of where data were located in papers)	Yes	Available on Mendeley.
	18.4	Share analysis scripts (or, if a software package with graphical user interface (GUI) was used, then describe full model specification and fully specify choices)	Yes	Available on GitHub.
Results of study selection process	19.1	Report the number of studies screened	Yes	Described in Table 1 and Figure 1.
	19.2	Report the number of studies excluded at each stage of screening	Yes	Described in Figure 1.
	19.3	Report brief reasons for exclusion from the full text stage	Yes	Described in M&M > 'Systematic review', in Figure 1 and in Table S.1.
	19.4	Present a Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)-like flowchart (www.prisma-statement.org).	Yes	Presented in Figure 1.
Sample sizes and study characteristics	20.1	Report the number of studies and effect sizes for data included in meta-analyses	Yes	Presented in 'Results and Discussion' > 'Study characteristics' and Figures 2-6.
	20.2	Report the number of studies and effect sizes for subsets of data included in meta-regressions	Yes	Presented in Figures 2-6.
	20.3	Provide a summary of key characteristics for reported outcomes (either in text or figures; e.g. one quarter of effect sizes reported for vertebrates and the rest invertebrates)	Yes	Presented in 'Results and Discussion' > 'Study characteristics'.
	20.4	Provide a summary of limitations of included moderators (e.g. collinearity and overlap between moderators)	Yes	Discussed in 'Results and Discussion' > 'Sensitivity analysis and limitations of the current meta-analysis', fourth and fifth paragraph.
	20.5	Provide a summary of characteristics related to individual study quality (risk of bias)	–	Not applicable; study quality not assessed apart from the inclusion criteria.

Checklist item	Sub-item number	Sub-item	Reported by authors?	Notes
Meta-analysis	21.1	Provide a quantitative synthesis of results across studies, including estimates for the mean effect size, with confidence/credible intervals	Yes	Presented in Figure 2.
Heterogeneity	22.1	Report indicators of heterogeneity in the estimated effect (e.g. I^2, tau^2 and other variance components)	Yes	Presented in Figures 2-6.
Meta-regression	23.1	Provide estimates of meta-regression slopes (i.e. regression coefficients) and confidence/credible intervals	Yes	Presented in Figures 2D, 4G, 5G and 6J.
	23.2	Include estimates and confidence/credible intervals for all moderator variables that were assessed (i.e. complete reporting)	Yes	Presented in Figures 2-6.
	23.3	Report interactions, if they were included	Yes	Presented in Figures 4-6.
	23.4	Describe outcomes from model selection, if done (e.g. R2 and AIC)	–	Not applicable; model selection was not performed.
Outcomes of publication bias and sensitivity analyses	24.1	Provide results for the assessments of the risks of bias (e.g. Egger's regression, funnel plots)	Yes	Presented in Figure S.3.
	24.2	Provide results for the robustness of the review's results (e.g. subgroup analyses, meta-regression of study quality, results from alternative methods of analysis, and temporal trends)	Yes	Presented in Figure S.2.

Checklist item	Sub-item number	Sub-item	Reported by authors?	Notes
Discussion	25.1	Summarise the main findings in terms of the magnitude of effect	Yes	Discussed for the various models in 'Results and Discussion'.
	25.2	Summarise the main findings in terms of the precision of effects (e.g. size of confidence intervals, statistical significance)	Yes	Discussed for the various models in 'Results and Discussion'.
	25.3	Summarise the main findings in terms of their heterogeneity	Yes	Discussed for the various models in 'Results and Discussion'.
	25.4	Summarise the main findings in terms of their biological/practical relevance	Yes	Discussed for the various models in 'Results and Discussion'.
	25.5	Compare results with previous reviews on the topic, if available	No	No available reviews (with comparable results) on the topic. Results are however compared to reviews on other topics, asking similar questions (biomarker potentials of other response types and other organism groups).
	25.6	Consider limitations and their influence on the generality of conclusions, such as gaps in the available evidence (e.g. taxonomic and geographical research biases)	Yes	Discussed in 'Results and Discussion' > 'Sensitivity analysis and limitations of the current meta-analysis'.
Contributions and funding	26.1	Provide names, affiliations, and funding sources of all co-authors	Yes	
	26.2	List the contributions of each co-author	Yes	
	26.3	Provide contact details for the corresponding author	Yes	
	26.4	Disclose any conflicts of interest	Yes	
References	27.1	Provide a reference list of all studies included in the systematic review or meta-analysis	Yes	Briefly presented in 'Results and Discussion' > 'Study characteristics'. A full list is provided in Supporting Information.
	27.2	List included studies as referenced sources (e.g. rather than listing them in a table or supplement)	Yes	Both included as referenced sources and listed separately in Supporting Information.

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