

Polyquaternium polymers cause inflammatory response and alterations of the lipidome in *Danio rerio* larvae

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ABSTRACT

Polyquaternium polymers are widely used in various applications, such as personal care products and wastewater treatment plants, and eventually end up in the aquatic environment. While polymers have been perceived of low toxicological concern due to their size, several studies have pointed towards water-soluble cationic polymers being toxic towards aquatic organisms – and that the toxicity largely is determined by the polymer charge density. The present study investigated the polyquaternium toxicological mechanism of action throughout lipidomic analysis and changes in immune-gene expression (qPCR) of zebrafish larvae exposed continuously to two water-soluble polymers; a high charge density polyquaternium-6 and a low charge density polyquaternium-10, for 5 and 12 days upon fertilization. The results showed that the investigated polyquaterniums cause both inflammatory responses and significant alterations of the zebrafish larvae lipidome. Depending on polyquaternium polymer and larvae development stage, the gene expression showed an inflammatory response (e.g. significant up-regulation of *il8*, *il1β* and *tnfα*) in the exposed zebrafish. Alterations of the lipidome were additionally observed, with severe depletion of lipids (e.g. lyso-glycerophosphocholines and ceramides) in the 12 days old larvae exposed to high charge density polymer. The findings furthermore support a hypothetical mechanism of action to be non-specific and lethality potentially to be narcosis-like driven.

1. Introduction

Polymers are large macromolecules produced in large tonnage globally with a broad variety of applications and discharged from wastewater treatment plants (Ponizovsky et al., 2022; ECCO, 2020). This diverse class of chemicals are currently exempt from EU-REACH registration-requirements and have been considered of low toxicological concern due to their size and inability to cross the cell membrane. In recent years the research on environmental hazard of solid particle polymers, such as plastics, has immensely increased, while the water-soluble polymers have been neglected (Arp and Knutsen, 2020). Studies have shown that cationic polymers (CPs) can cause adverse effects on aquatic organisms (Boethling and Nabholz, 1997; Rawlings et al., 2022; Salinas et al., 2021; Hansen et al., 2023), and are recognized as polymers of concern potentially requiring registration in upcoming REACH-revision (Almroth et al., 2020; European Commission, 2020).

Few publications are publicly available on the water-soluble CP environmental hazard, and most publications focus on acute toxicity and improving standard tests (Connors et al., 2022), while knowledge is lacking on toxicological mechanism of action, the causal relationship between CP biological action and the molecular initiating events (MIE) of an adverse outcome pathway (AOP) (Knapen et al., 2020). Generating new hypotheses on MIE could support improvement of hazard identification and environmental risk assessment or identification of sensitive organisms or life stages. Additionally, it can improve the understanding of whether water-soluble polymers share the same toxicological mechanism of action by virtue of the same functional group or other physicochemical properties.

The same cationic polymer can exist in various monomeric ratios – thus, occurring as a distribution in molecular weight and charge densities (CD, mEq/g). Cationic polymers are amphiphile surfactants, i.e. with both a hydrophobic and hydrophilic end. The biological interaction

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is the quaternary ammonium moiety contributing to the cationic charge of the polyquaternium. Studies point towards that the CPs impair aquatic organisms by surface-interactions – such as adsorption to the gills in fish (Kerr et al., 2014; Rawlings et al., 2022; Nolte et al., 2017b; Pereira et al., 2018; Connors et al., 2022; Hansen et al., 2023). Toxicity has been correlated to polymer CD (Rawlings et al., 2022; Hansen et al., 2023). This study includes a high CD polyquaternium-6's (PQ6) (a homopolymer of the quaternary ammonium diallyldimethylammonium chloride with CD of 6.2 mEq/g) with the tradename Merquat< 100 K and a low CD polyquaternium-10's (PQ10, a co-polymer of acrylamide and quaternary ammonium diallyldimethylammonium chloride with CD in ranges of appr. 0.5–1 mEq/g) with the tradename UCARE JR30M. Both investigated polymers are water-soluble. The acute toxicity towards zebrafish embryos of these polyquaternium tradenames were investigated by Rawlings et al. (2022). The 96 h-FET LC50-value of PQ6 Merquat< 100 K was 0.91 mg/L and the dechlorinated 96 h-FET LC50-value of PQ6 Merquat< 100 K was 0.80 mg/L. The 96 h-FET LC50-value of PQ10 UCARE JR30M was 10.73 mg/L and the dechlorinated 96 h-FET LC50-value of PQ10 UCARE JR30M was 10.58 mg/L (Rawlings et al., 2022). Since CPs are widely used, potentially discharged to the aquatic environment and often not readily biodegradable, they pose an environmental concern (Duis et al., 2021).

CPs and nanoparticles are both large of size and surface-active, and could potentially both be acting through physical impairment. Among other cytotoxic properties of graphene oxide nanomaterials such as direct physical interactions between the cell wall and sharp edges of nanomaterials (Akhavan and Ghaderi, 2010), aggregated nanomaterials trapping cells (Hashemi et al., 2014), interruption of glycolysis process and hormonal secretion in animals (Akhavan and Ghaderi, 2012; Akhavan et al., 2015), damage of DNA (Akhavan et al., 2012), metal ion release (Wang et al., 2014), ROS generation (Dutta et al., 2015) or oxidative stress (Liu et al., 2011), other nanoparticle exposure has caused inflammation and alterations in the lipidome (disruption of fatty acid biosynthesis and lipid metabolism) in young zebrafish (Marana et al., 2022). The authors also found a significant regulation of immune relevant genes, such as *tnfa*. Additionally, Polyquaternium-1 has been found to cause inflammatory effects in human corneal epithelial cells (Paimela et al., 2012). The lipid metabolism is known to be strongly correlated to inflammation and inflammation-relevant genes as for instance interleukins (*il*), interferons (*ifn*) and tumor necrosis factor (*tnf*) families (Bernadi et al., 2018; Chen et al., 2009; Lager et al., 2011; Hassen et al., 2014). While particular nanomaterials, such as for example graphene oxide, and water-soluble polymers are both to some extent hypothesized to be able to physically impair organisms, the differences between the two types of compounds' physico-chemical properties limits the comparison and interpretation of initiating molecular or physical events causing an adverse effect.

Since fish are widely used model-organisms in (eco-)toxicological studies, and most data available on acute toxicity of CPs are on fish (Connors et al., 2022; Boethling and Nabholz, 1997; Rawlings et al., 2022; Hall and Mirenda, 1991; Kerr et al., 2014; Cumming et al., 2008), the present study focuses on zebrafish larvae (*Danio rerio*).

Our initial study hypothesis is that water-soluble polymers may alter or interfere with cell membranes (lipid layers) and act via a non-specific toxic mode of action resulting in narcosis. Consequently, the study design and aims of this paper were to:

- Investigate zebrafish larvae 5 days post fertilization (dpf) and 12 dpf for changes in lipidome and of immune relevant genes
- Generate hypotheses on the toxicological mechanism of action from continuous exposure at time of fertilization of two cationic polyquaternium polymers with different physicochemical properties and acute toxicity profiles

2. Materials and methods

2.1. Test material

Continuous exposure of two water-soluble polyquaterniums (PQ's) were investigated on zebrafish (*D. rerio*). The PQs share the same quaternary ammonium functional group but differ in physicochemical properties including molecular weight and charge density: Polyquaternium-10 with the tradename JR30M (PQ10), average molecular weight of 2202 kDa and charge density of 1.03 mEq/g, and polyquaternium-6 with the tradename Merquat< 100 K (PQ6), average molecular weight of 29 kDa and charge density of 6.2 mEq/g (Table S1). Stock solutions were prepared at the day of use by weighing out polymer corrected for percentage active ingredient and diluting into 0.2 µm sterile filtered facility water (Table S2) under constant stirring for minimum 30 min.

2.2. Zebrafish exposure study

AB-wildtype zebrafish were obtained from an in-house facility at The University of Copenhagen, Denmark. Fish were bred and raised in tanks in a recirculated system (AquaSchwartz, Germany) with a light/dark cycle of 14/10 h at 27 °C., a pH of 7 and conductivity of 400 µS. Breeding pairs were randomly selected the day before the tests were initiated. The following morning eggs were collected and within 2 h, 15 eggs per exposure group in triplicates were transferred to polystyrene petri dishes containing 30 mL of the exposure or control solution. All petri dishes were pre-sorbed with the respective exposure solution for 24 h. Exposure concentrations were decided to be approximately 0.5 times the nominal LC50-value of PQ6 Merquat< 100 K from Rawlings et al. (2022) to ensure the exposure concentrations to be non-lethal. Furthermore, similar exposure concentrations were chosen for the two different PQ's to compare potency of non-lethal effects. PQ10 nominal exposure concentrations were 0.019; 0.043; 0.094; 0.207; 0.455 and 1.00 mg/L and PQ6 nominal exposure concentrations were 0.017; 0.038; 0.083; 0.182 and 0.400 mg/L. Non-exposed controls were included for each polymer. Tests were conducted as semi-static, where half of the media was renewed daily. From 5 dpf the zebrafish larvae were fed daily and any aggregated food at the bottom of the test vessel was cleaned out during the feeding and renewal regime. The experiment was conducted with a permit from The Animal Experiments Inspectorate under the Danish Ministry of Environment and Food (Permit: 2017–15–0201–01301). Visual observations of the fish were conducted daily, to evaluate health-state and physical observations linked to toxicity. After 5 dpf and 12 dpf five larvae per analysis-replicate were sampled and transferred to Eppendorf-tubes, snap-frozen (liquid nitrogen) and stored at – 80 °C.

2.3. Gene expression of immune-relevant genes

The following immune-relevant genes were targeted; *c3a*, *il10*, *ifng1*, *saa*, *tnfa*, *il1b*, *il8*, *il4–13a* and *b-actin*. For gene expression (qPCR) analysis we followed the protocol described earlier by Marana et al. (2022) without deviations. See supporting information for detailed protocol.

2.4. Untargeted lipidomics with sensitive capLC-Orbitrap MS/MS

Untargeted lipidomics was conducted on extracts following a modified Matyash lipid-extraction protocol described by Sostare et al. (2018). See supporting information for detailed protocol and analysis workflow. In short, the modified Matyash-protocol was followed and the sample extracts were evaporated to dryness and resuspended in 150 µL butanol-isopropanol-water mixture, as described by Danne-Rasche et al. (2018), and stored at – 80 °C upon analysis. All samples were filtered prior to analysis (Qiagen MB spin-columns) and analyzed using Orbitrap high-resolution tandem mass spectrometry (Q Exactive HF,

ThermoFisher Scientific) hyphenated with a capLC (Ultimate3000, ThermoFisher Scientific). Lipids were separated using a C₁₈ column (75 µm x 150 mm, 3 µm, ThermoFisher Scientific) with a biphasic mobile phase (isopropanol, acetonitrile and water) at a 1000 nL/minute-flowrate and the mass spectrometer was operated in data-dependent acquisition Top20-mode. Data was processed and analyzed individually for each polymer treatment and each sampling timepoint, respectively, to account for expected significant changes in biology between 5 and 12 dpf using the software Compound Discoverer (3.3.111, ThermoFisher Scientific), where subsequent compound prioritization, principal component analysis (PCA) and ANOVA differential analysis (p-value < 0.05) was conducted. Annotation classification or identification level was conducted according to Viant et al. (2019). Compound abundance across the tested concentrations was visualized in a heatmap in R (R-version 4.2.2) using the R-package *ph heatmap* (Kolde, 2019) with Euclidian clustering on rows and no clustering on columns, to display increasing concentration.

3. Results and discussion

3.1. Inflammatory response

The daily visual observations revealed that the highest test concentration of PQ6 (0.4 mg/L) negatively affected the fish. They had labored movement, and a few were estimated to be in such distress, that they were euthanized due to animal welfare concerns. Consequently, less animals were available for analysis in this exposure group and no gene expression analysis was made.

The gene expression data showed an inflammatory response in the exposed larvae to the investigated polymers (Fig 1). The immune

relevant genes of interest were altered in expression and did not unambiguously follow a dose-dependent response. For the 5 and 12 dpf zebrafish larvae exposed continuously to the PQ6 *il10* (Fig 1 m), *il4-13α*, (Fig 1 a) *ifnγ1* (Fig 1 i) and *c3* (Fig 1 k) were not affected or not sufficiently expressed for analysis. Also, *il1β* (Fig 1 o) was significantly down-regulated at 0.017 mg/L and significantly up-regulated at 0.4 mg/L at 5 dpf and significantly down-regulated at 0.083 mg/L at 12 dpf. Moreover, *il8* (Fig 1 g) was unregulated or not sufficiently expressed at 5 dpf, but was significantly down-regulated at 0.083 mg/L at 12 dpf. *tnfa* (Fig 1 e) was significantly up-regulated in the highest concentration of 0.4 mg/L at 5 dpf, and not significantly regulated at 12 dpf. *saa* (Fig 1 c) was significantly up-regulated in 0.038, 0.083 and 0.4 mg/L at 5 dpf, and not significantly regulated at 12 dpf.

The regulations observed in the PQ6-exposed fish were only partially conclusive due to limited significance in regulation with indications of dose-dependent regulation. Interestingly, a *saa* acute phase response was upregulated in several concentrations in the 5 dpf fish. A similar tendency has been observed in zebrafish during a parasite infection in fins, demonstrating polymer effects to cause outer physical tissue damage and lead to inflammatory conditions (Jørgensen et al., 2018). Generally, the inflammatory effects were more pronounced in 5 dpf compared to 12 dpf. For the 5 and 12 dpf zebrafish larvae exposed to PQ10 the classical anti-inflammatory gene *il10* (Fig 1 n) and the inflammatory gene *ifnγ1* (Fig 1 j), as well as the acute phase protein serum amyloid A (*saa*) (Fig 1 d) were unchanged or not expressed. The interleukin *il1β* (Fig 1 p) was significantly up regulated in 0.46 and 1 mg/L at 5 dpf, whereas there was no significant regulation at 12 dpf. Moreover, *il4-13α* (Fig 1 b) was unregulated at 5 dpf, but significantly down-regulated at 12 dpf at concentrations 0.043, 0.46 and 1 mg/L. Similarly, *tnfa* (Fig 1 f) was unchanged at 5 dpf, however significantly

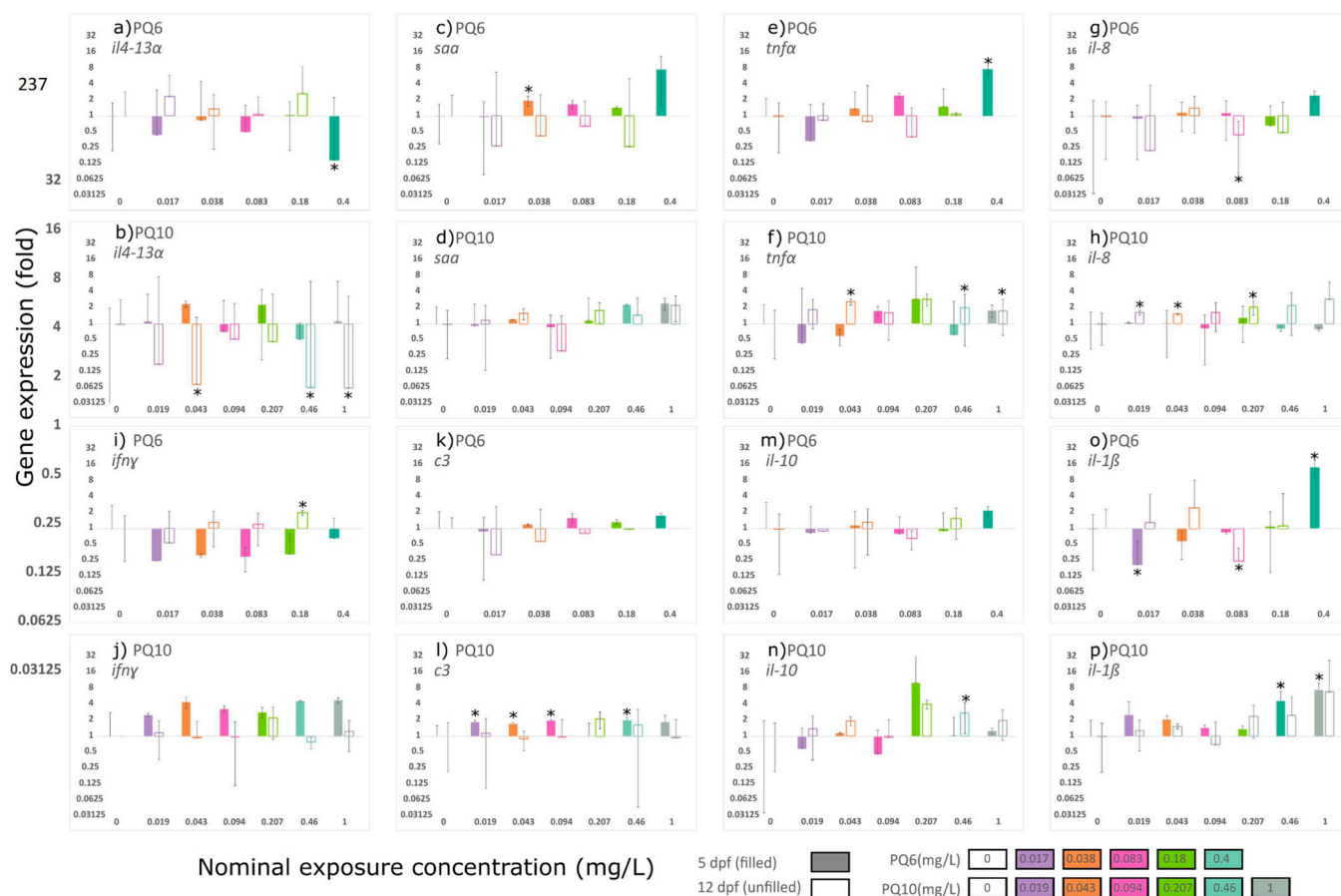


Fig. 1. Caption.

up-regulated at 12 dpf at concentrations 0.043, 0.46 and 1 mg/L. Also, *il8* (Fig 1 h) was also only significantly up-regulated at 12 dpf at 0.21 mg/L. Lastly, the complement factor *c3* (Fig 1 l) was significantly up-regulated at concentrations of 0.019, 0.043, 0.094 and 0.46 mg/L at 5 dpf, but not significantly regulated at 12 dpf.

PQ10 caused significant up-regulation of *il1 β* , *il8* and *tnfa*, at 12 dpf, but not at 5 dpf. These gene expression changes are signs of inflammation and are associated with oxidative stress and ROS-production (Qiao et al., 2019). These indications of inflammation are further supported by the significant down-regulation of the Th2 associated interleukin *il4–13a*. Expression of *il4–13a* is correlated with balancing responses to pathogens and suppressing inflammation. Whilst *il4–13a* gene expression was found to be more pronounced in 5 dpf fish exposed to PQ6, the opposite was observed for PQ10 exposures. The inflammatory responses occurring after 12 days of exposure indicates potential chronic inflammation in the fish, a status known to be associated with adverse negative effects (Nathan and Ding, 2010). Inflammation can occur due to several impacts, e.g., cell damage and several lipids are linked to inflammatory responses, for instance ceramide signaling, supported by the results in the coming section (Chiurchiù et al., 2018).

Whilst Polyquaternium-1 has been found to cause inflammatory effects in a previous study (Paimela et al., 2012), effects on immune relevant genes were to some extent expected. However, no previous studies have investigated differences in inflammatory effects caused by polyquaternium polymers of varying physicochemical properties. The acute toxicity is known to vary significantly between polymers of varying charge density, such as PQ6 and PQ10 (Rawlings et al., 2020; Hansen et al., 2023). The results of the present study show inflammatory effects caused even by the polymer of low acute toxicity, PQ10.

3.2. Lipidomics

After data processing of the untargeted lipidomic profiles obtained from 5 dpf larvae exposed to PQ6, this analysis revealed 7679 unique chemical entities. By applying strict filtering criteria, 6602 substances were omitted from the dataset (filtering criteria detailed in SI) and the remaining 1077 compounds were subjected to differential analysis to focus on metabolites with significantly differentiating concentrations relative to the control group. This revealed 18 compounds that differed significantly (p -value < 0.05) in one or more of the exposed groups compared to the control group (Table S3). For the 5 dpf larvae exposed to PQ10, the analysis revealed 9768 compounds, of which 8738 were filtered out, and of the 1030 remaining metabolites, 13 were found to be significantly differentiated in abundance in one or more of the exposed groups compared to the control group (Table S4). Compounds significantly differentiated in both polymer-exposures (18 and 13) in 5 dpf larvae generally suggested several fatty acid structural similarities (cf. Tables S3 and S-4). Fatty acids are linked to inflammatory responses by the eicosanoid family acting as inflammatory mediators decreasing production of e.g. *il1 β* and *tnfa*, and are formed from 20-carbon polyunsaturated fatty acids from cell membrane phospholipids (Calder, 2006).

For the 12 dpf larvae exposed to PQ10, the untargeted lipidomic analysis revealed 15,603 compounds of which 14,882 were subtracted. Of the 721 remaining metabolites, only two compounds (myristamide and ecalcidene) had significantly different concentrations compared to the control across all exposure doses (Table S4). Myristamide is a primary polyunsaturated fatty acid amide, supporting inflammatory observations (Ezzili et al., 2010).

For the 12 dpf larvae exposed to PQ6 the results were entirely different. The untargeted lipidomic analysis (revealed 9310 compounds and 8362 were filtered out) yielded 948 metabolites for further functional analysis. The differential analysis revealed that the highest dose had the largest effect on the lipidome of 12 dpf larvae and revealed a completely different pattern compared to the data for the 5 dpf data (cf. heatmaps in Fig. S1). The differential analysis (for 12 dpf larvae)

revealed 562 lipids to have a significantly (p < 0.05) lower concentrations in the highest PQ6 dose relative to the control. These compounds were further filtered, to only include 79 entities with level 2 annotation (Table S5). Phospholipids, ceramides, sphingolipids and other lipid classes were found to have a significantly lower concentration in the group exposed to 0.4 mg/L of PQ6 relative to the control. These were groups of Lyso-glycerophosphocholines (21 substances), glycerophosphocholines (34), Lyso-glycerophosphoethanolamines (4), Glycerophosphoethanolamines (6), Ceramides (6), Sphingomyelins (5), Glycerophosphoserines (1), Palmitoyl sphingomyelin (1) and Diacylglyceryl-trimethylhomo-Serine (1).

This significant impact on numerous lipid classes clearly shows alterations in the lipidome due to PQ6 exposure. While significantly lower concentrations were detected only in the highest exposure concentration of 0.4 mg/L, the heatmap visualization (Fig. 2) shows a tendency of dose dependency. A depletion of phospholipids in the group exposed to PQ6 at 0.4 mg/L combined with the little effect observed in the immune gene expression in the same exposure group does not point towards an inflammatory status at this exposure status. Sphingolipids are known to play an essential role in inflammation modulation, and have been identified as bioactive signaling molecules involved in regulated cell death, for instance sphingomyelin hydrolysis and ceramide generation have been implicated in a signal pathway mediating effects of e.g. *tnfa* (Simon et al., 2020; Obeid et al., 1993). Additionally, phospholipids are the main constituents of the cell membrane lipid bilayer. Cationic surfactants have shown to disrupt the cell membrane (Kwasniewska et al., 2020), which can lead to cell death (Zhang et al., 2018).

Rawlings et al. (2022) showed dechorionated embryos to have a very steep dose-response curve, indicating changes in sensitivity post hatching. This is consistent with our results of significant decrease of phospholipid concentrations in the highest exposure and the indications of steep dose-response pattern of these lipids. These results align with visual observations of labored movement of larvae and some of the larvae being removed from the study due to clinical signs of distress in the highest PQ6-dose. An adverse effect was expected to be caused by the high PQ6 concentration, since the PQ6 is lethal in concentration ranges close to the tested concentrations. However, PQ10 has a much lower acute toxicity (10-fold higher than the highest tested concentrations), and the significant inflammatory responses observed are of particular interest. The results point toward polyquaternium potentially causing adverse effects at much lower concentrations than lethal doses. The results further indicate that the functional group and structural similarities of the cationic polymer are not reflected in toxicological mechanisms of actions. However, the results of the two polymers compared indicate that physicochemical properties such as charge density affect the biological response.

The test exposure concentration was nominal and due to feeding of larvae the potential adsorption of polymers hereto, may have led to a decreasing exposure over time. Additionally, polymer adsorbing to particulate matter from food pellets could change the exposure route over the time of the experiment, enabling an oral route of exposure by addition of food from day 5 (dpf). Future work should include investigations on analytical procedures to determine actual exposure of polyquaternium polymers.

4. Conclusions

This study shows that polyquaterniums cause an inflammatory status in zebrafish larvae. The polymers may target specific organs (e.g. gills), however our findings also support a hypothetical mechanism of action to be non-specific and the lethality could be narcosis-like driven. This work implicates initial assessments of polyquaternium AOP development showing both inflammatory responses and significant alterations of the zebrafish lipidome. This suggests that polyquaternium AOPs could be further developed from this offset and supports the hypothesis of surface-impairment leading to long-term negative effects. Furthermore,

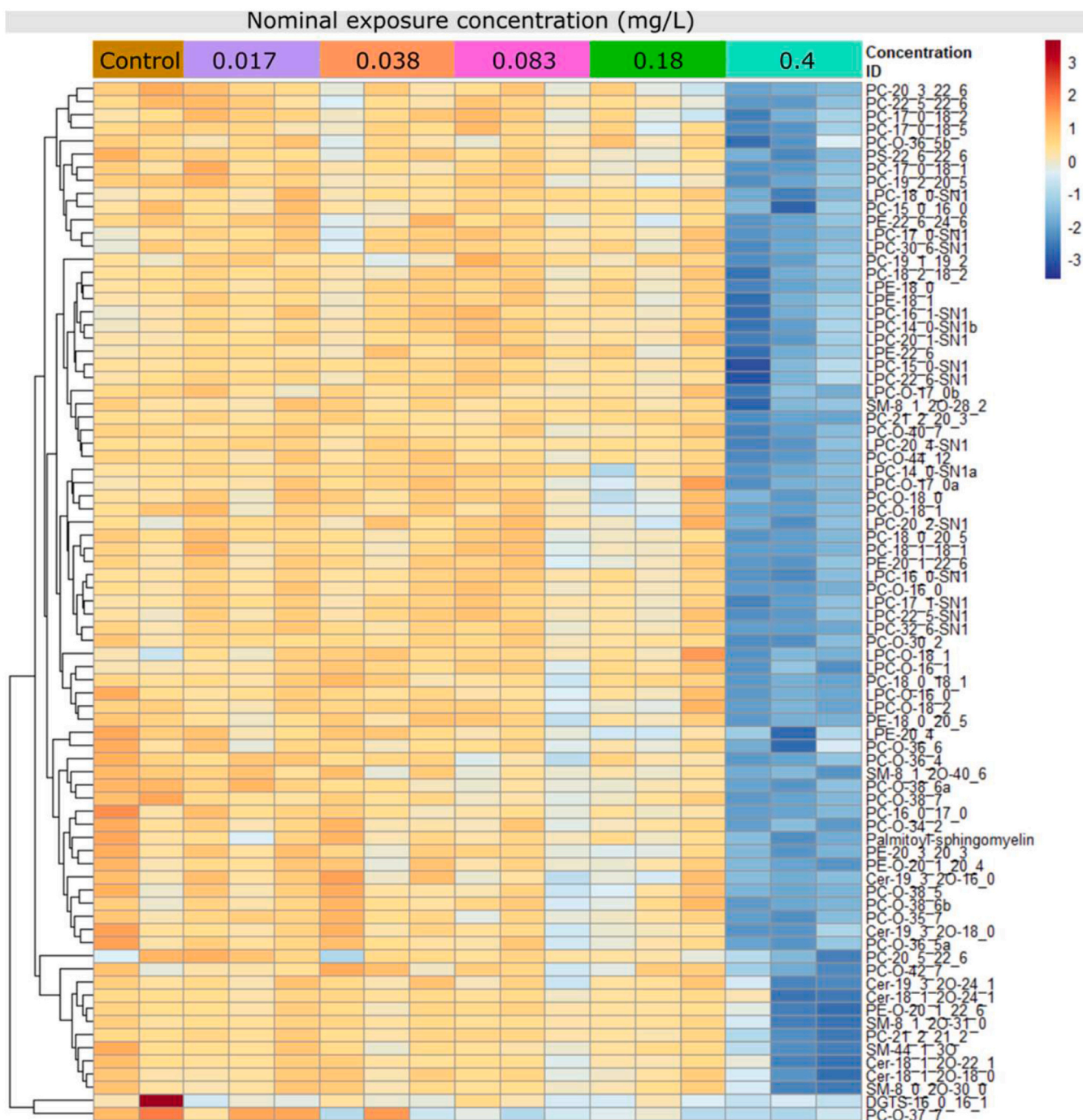


Fig. 2. Heatmap of the 79 lipids in 12 dpf zebrafish from PQ6 dose-response exposure, that were annotated with level 2 ID. Clustering is visualized on the left, compound-ID listed on the right, and the exposure concentration is visualized by color and labelling at the top of the figure. Differentiation from the overall mean is reflected in the z-score (-3 to 3), a negative or positive z-score correlates to the lipid concentration in the sample being lower or higher, respectively, than the average mean lipid concentration. Compound ID nomenclature, e.g. first line "PC-20_3_22_6" corresponds to phosphatidylcholine (PC) 20:3/22:6.

the results indicate that polyquaterniums can cause non-lethal adverse effects in exposure concentrations significantly lower than lethal doses. As a result, our understanding of the detrimental effects of polyquaterniums has been deepened, along with the advancement in methods for testing and assessing their impact.

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CRediT authorship contribution statement

Hansen Anna Magdalene Brun: Conceptualization, Data curation,

Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Poulsen Rikke:** Conceptualization, Data curation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **von Gersdorff Jørgensen Louise:** Conceptualization, Funding acquisition, Supervision, Writing – original draft. **Hansen Martin:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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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.hazl.2023.100095](https://doi.org/10.1016/j.hazl.2023.100095).

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