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Salinization of freshwater coastal ecosystems: possible impacts on the genetic diversity of zooplankton populations

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DEDICATION

To my mom, dad, and brother

Thank you for your blessings, love, and care

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RESUMO

As previsões feitas no âmbito das alterações climáticas, apontam para a ocorrência do aumento do nível médio da água do mar e o aumento da frequência de eventos extremos (ex. secas prolongadas). Associado a estes eventos é expectável que ocorram intrusões de água do mar em regiões costeiras, nomeadamente sistemas aquáticos costeiros dulçaquícolas, provocando a sua salinização. Este aumento de salinidade pode comprometer a resiliência do biota que habita estas regiões. De facto, já foram publicados vários estudos que reportam os efeitos provocados por aumentos na salinidade quer a nível sub-individual que individual. No entanto, poucos são os trabalhos que avaliaram os efeitos de salinização na diversidade genética de populações de organismos dulçaquícolas. Sabe-se que a diversidade genética é um componente chave para as populações poderem lidar com perturbações ambientais. Deste modo, se uma exposição a salinização provocar um decréscimo na diversidade genética (por exemplo, através do morte dos gentótipos mais sensíveis) irá também causar uma diminuição na plasticidade evolutiva e resiliência dessa população a perturbação ambientais futuras. De acordo com o exposto, o presente trabalhado pretendeu compreender os efeitos de salinização na diversidade genética de populações do cladócero *Daphnia lonhispina* e do rotífero *Brachionus calyciflorus*, sob a influência de diferentes temperaturas. Para atingir este objectivo foram selecionadas seis linhagens clonais de *D. longispina* e seis linhagens clonais de *B. calyciflorus* com diferentes sensibilidades letais a salinização. Cada linhagem clonal foi exposta a um controlo (ASTM) e ao valor de conductividade correspondente ao $LC_{70,48h}$ para *D. longispina* e ao $LC_{70,24h}$ para *B. calyciflorus*. A exposição e cada um destes dois tratamentos decorreu 17, 20 e 23ºC. A densidade populacional foi monitorizada diariamente durante todo o período de exposição. Baixos níveis de salinidade (4.8 to 9.7 mS/cm, comparativamente a valores de conductividade da água do mar de aprox. 35 mS/cm) provoracam reduções significativas na densidade populacional das diferentes linhagens clonais após curtos períodos de exposição, provocando a extinção de algumas das linhagens após longos períodos de exposição. As temperaturas extremas de 17ºC e 23ºC induziram efeitos mais intensos na densidade populacional que a temperatura de 20ºC. Verificou-se ainda uma alteração na ordem de sensibilidade entre as linhagens clonais quando comparando respostas a períodos de curta ou longa duração de exposição. Estes resultados e as suas consequências a nível populacional são discutidos no âmbito da hipótese de erosão devida a selecção natural.

Palavras-chave: Altreações Climáticas – Efeitos a longo prazo – Recuperação populacional - Aclimatação – Erosão Genética

ABSTRACT

Under the context of climate changes, it is forsee a global sea level rise and an increase in the frequency of extreme weather events. Associated with such events (namely sea level rise and periods of intense drought) it is expected to occur the intrusion of seawater into coastal low-lying freshwater ecosystems, causing its salinization. Such salinization may compromise the resilience of natural populations inhabiting these coastal regions. Actually, several studies already reported adverse effects at sub-individual and individual level caused by exposure to increased salinity, but, only a few addressed the effects that salinization may cause in the genetic diversity of freshwater biota. Genetic diversity is a key for populations to be able to cope with environmental changes. Thus, if exposure to salinization leads to a decrease of a population's genetic diversity (e.g. through the death of the most sensitive genotypes), it will as well reduce the population's evolutionary plasticity and resilience under future perturbations. Accordingly with the mentioned previously, this work intended to understand the effects of salinization on the genetic diversity of populations of the cladoceran *Daphnia longispina* and rotifer *Brachionus calyciflorus*, under different temperature regimes. For this, six lineages of *D. longispina* and six lineages of *B. calciflorus*, differing in their lethal sensitivity to salinity were selected. Lab-populations of each clonal lineage were maintained under optimal conditions until reaching a steady state. At this point, they were exposed to low salinity levels (corresponding to the $LC_{70,48h}$ and $LC_{70,24h}$, respectively for the most toleranbt clonal lineage of *D. longispina* and *B. calyciflorus*) at 17, 20 or 23ºC, for at least 30 days. The population densities were monitored every day until the end of the experiment. Low levels of salinization (4.8 to 9.7 mS/cm, when comparing with conductivities of seawater approx. 35 mS/cm) affected survival and reproduction of the two tested species, leading to the extirpation of some genotypes after long-term exposures. Different ranks of sensitivity were observed for clonal lineages when comparing short-term and long-term responses,most probably due to acclimation-driven population recovery. Extreme temperatures (17^oC and 23^oC) induce negetive effct of salinity on genetic diversity of zooplankton.These results and its consequences at the population level are discussed in light of the genetic erosion hypothesis, through natural selection (due to small within genotype variability).

Keywords:

Climate Change - Long-Term Exposure - Population Recovery - Acclimation - Genetic Erosion

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1. Introduction

1.1 Climate change

Increased perception about climate variability, global warming, and its consequences on ecosystems has growing concern among the public (Lineman et al., 2015). The Intergovernmental Panel on Climate Change (IPCC) defines climate change as, "change of the state of the climate that can be identified (e.g. by using statistical tests) by changes in the mean and/or the variability of its properties, and that persists for an extended period, typically decades or longer. It refers to any change in climate over time, whether due to natural variability or as a result of human activity" (Pachauri et al., 2014). The Hartmann (2014) further refers that changes in the climate are more unequivocal than the past and increased global average air/water temperature, changes in atmospheric composition, variations of the radiation budget, hydrological cycle, prominent extreme events and changes in atmospheric circulation provide significant evidence for global climate change.

Reasons for climate change are two-folded involving both natural and anthropogenic phenomena. Prior to the industrial revolution, climate change was mainly governed by natural forces such as volcanic eruptions, changes in solar irradiance (Crowley, 2000). A large amount of gases (CO_2, SO_2 and water vapor) and dust particles entered into atmosphere abruptly with the volcanic eruptions. One scenario that can occur with a volcanic eruption, which can lead to climate change, is that the spewed particles can cool the planet by shading incoming solar radiance for days, months even for years. On the other hand, volcanic eruptions may release a large amount of greenhouse gases at once which can contribute to global warming. Other than volcanism, fluctuations in the solar cycle impacts on global temperature of Earth by about 0.1^oC increase in solar maximum and decrease during solar minimum (Pielke, 2005).

However, industrial revolution influenced anthropogenic activities, which had a significant impact on global climate change over past decades. Transition to the industrial revolution and associated larger economic and population growth around the globe has significantly consumed fossil fuels (Olivier et al., 2012). Increased emission of greenhouse gases through the combustion of these fossil fuels has become a dominant casual factor in climate change since 1950 (Fig. 1). Carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and water vapor (H_2O) are concerned as main greenhouse gases which absorb solar energy and slowing

or preventing the loss of heat to space. In 2005, CO_2 concentration almost exceeded the natural range over the past 650,000 years (185-300 ppm) and it is estimated that global CO_2 emissions will increase from current 385ppm level to 450-600 ppm level by the end of this century (Solomon et al., 2009). Fossil fuel use (burning coal and liquid fuels) and land use change (deforestation, agriculture) are main causes of this elevated CO_2 concentrations (Stocker, 2014). In 2011, it was recorded a global annual mean concentration of CH_4 as 1803.2 ppb (Stocker, 2014) (Fig. 1). It is about a 150% increment comparing with CH_4 concentration in the year of 1750. Stocker (2014) also recorded a global annual mean level of N_2O as 324.3 ppb in 2011 (Fig. 1). Naturally, N_2O is emitting to the atmosphere as part of Earth's nitrogen cycle through different biological processes and further human activities such as agriculture, fossil fuels, wastewater management and other industrial processes are inducing it (IPCC, 2007). The elevated CO_2 and other greenhouse gases will have a great impact on intensifying the climate change, specially in global warming (Flato et al., 2013).

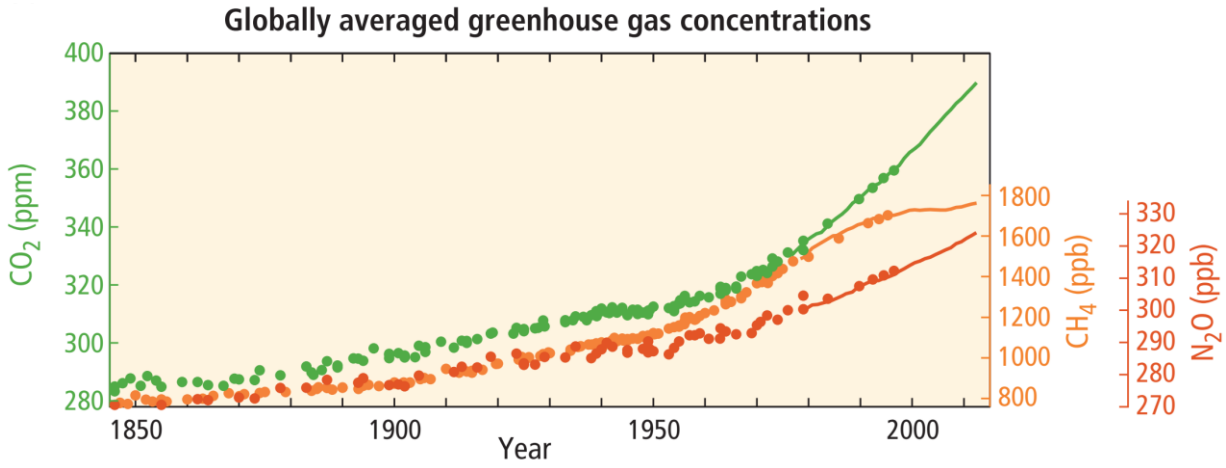


Fig. 1: Atmospheric concentrations of the greenhouse gases carbon dioxide (CO_2, green), methane (CH_4, orange) and nitrous oxide (N_2O, red) determined from ice core data (dots) and from direct atmospheric measurements (lines) (Source: IPCC, 2014).

The IPCC (2014) stated that, within the context of climate change, the last three decades were consecutively warmer comparatively to any former decade prior 1850. Earth has warmed by about 0.85°C over the period of 1880 to 2012. Average ocean temperature in the first 70 m from surface raised by 0.11^{o}C per decade, from 1971 to 2010. As consequences of climate change, global average sea level has increased 0.12 to 0.22 m and continuous oceanic uptake of CO_2 has resulted in 26% increase in acidity of the ocean; the pH of ocean surface water

has decreased by 0.1. Also Arctic and Antarctic sea ice extent reduced by 3.5 - 4.1% and 1.2 – 1.8% per decade respectively in between 1979-2011.

Projections of climate change are based on different models from simple to intermediate, comprehensive climatic models and earth system models. According to IPCC (2014), worst case scenario modeled as Representation Concentration Pathways 8.5 (RCP8.5) which based on approximate total radiative forcing in the year 2100 relative to 1750 as 8.5 Wm^{-2} predicted future changes in climate as below.

By the year 2100,

- CO_2 concentration will reach 936 ppm while expecting a combined increase with CH_4 and N_2O to 1313 ppm.
- The average global surface temperature will increase 2.6°C to 4.8°C. Interannual-to-decadal variability and regional inequality will increase.
- The variability in precipitation between wet and dry regions and between wet and dry seasons will continue to increase.
- Frequency and magnitude of extreme events will increase.

RCP8.5 in IPCC (2014) further predict the magnitude of consequences of climate change such as the rise of global mean sea level from 0.45 to 0.82 m, a decrease of global glacier volume 35% to 85% and a decrease of ocean surface pH 0.30 to 0.32 end of 21st century.

1.2 Freshwater ecosystems and climate change

Ocean and saline bodies occupy about 97% of global water while the rest being freshwater, in the forms of solid, liquid and vapor. Among all freshwater sources, 69% are in polar regions as glaciers and 30% stuck as groundwater. Rivers, streams, lakes, and reservoirs only contain 0.3% of global freshwater (Liu et al., 2011). An estimated 126,000 documented species of fishes, molluscs, reptiles, insects, plants, and mammals inhabit in these ecosystems (IUCN, 2015). However, species richness of these fresh water ecosystems may be increased to over one million with unidentified species. The importance of freshwater ecosystems to humankind and other species are numerous and well documented (Allan and Flecker, 1993, Dudgeon et al., 2006). For example, human exploit freshwater ecosystems for agriculture, irrigation, recreation, industrial and domestic uses (Wetzel, 2009) and other species have an advantage on freshwater ecosystems as their habitat and niche which serve as breeding, feeding, and nursery grounds (Liu et al., 2011).

Freshwater ecosystems are highly vulnerable to climatic changes (Hays et al., 2005, Schindler et al., 2005). Most of the freshwater ecosystems are geographically isolated in terrestrial landscape and heavily exploited by humans (Woodward et al., 2010). The synergetic effect of anthropogenic heavy exploitation and climatic changes may cause drastic reduction of biodiversity in these ecosystems (Mooney et al., 2009). Increased evaporation, subjecting to severe droughts and floods, low flow regimes and salinization are major threats to freshwater ecosystems. Apart from that increased rate of melting ice, changes in the flow regimes of rivers and streams increased hypolimnetic water temperature and effects upon the water cycle also visible with climate change (Hershkovitz et al., 2014). These changes are visible in most of the freshwater ecosystems and especially, Arctic ecosystems have strong influence by climatic change. These threats will result in negative impacts on population structure, interactions, food webs and communities within the ecosystem (Hart et al., 1991, Nielsen et al., 2003).

1.3 Climate change induced salinization of freshwater ecosystems

Apart from temperature and the pH, salinity is a key environmental factor that is affected by the predicted climate dynamics (Harley et al., 2006). Especially freshwater ecosystems suffer from increased salinization caused by the sea-level rise, increased evaporation, and extreme weather conditions such as severe droughts and storms. (Nielsen et al., 2003, Ghazy et al., 2009). Sea level rise and heavy storms will allow seawater flooding into coastal freshwater bodies. Alternatively, increased evaporation together with drought conditions may lower flow level and groundwater level in freshwater bodies which cause seawater intrusions into it (Nielsen et al., 2003). In both cases, salinity in the freshwater ecosystem will increase. This salt water influx would affect biogeochemistry of the freshwater ecosystem. Concerning world average values for aquatic ecosystems, freshwater ecosystems have salinity less than $3gL^{-1}$ and seawater has salinity $35gL^{-1}$ concerning world average values for these ecosystems (Boulton et al., 2014). Significant negative effects specifically on species richness and growth of freshwater biota are visible when salinity reaches 1 gL^{-1} (Hart et al., 1991).

Increased salinity may affect the freshwater biota in different scales. Depending on individual salinity tolerance ability, plants categorized inhabitants of freshwater ecosystems as glycophytes and salt tolerant (halophytes). Freshwater fauna, called as stenohaline (non-saltwater tolerant) and euryhaline (saltwater tolerant) depends on their sensitivity to salinity (Nielsen et al., 2003). It is based on individual organism's inability to change its physiological status with increasing salinity specially their osmoregulation ability.

In terms of microbial communities (virus, bacteria, fungi, protozoans, and lichens), increasing salinity may affect their physiology, function and community composition at the level of 10 gL^{-1} (Hart et al., 1991). Increasing salinity in freshwater ecosystems may cause the shifting of methanogenic bacteria to sulfate reducing organisms and increased denitrification (Capone and Kiene, 1988). However, some of the microorganisms such as *Anabaena* sp. and cyanobacteria have an ability to acclimatize increasing salinities of more than 7 gL^{-1} (Hart et al., 1991).

Nielsen et al. (2003) reported that most of the algae species do not tolerate salinity increases over 10 gL^{-1}. However, exceptions occur with some of the algal species being tolerant to salinization by exhibiting the ability to produce cysts. Most of the macrophytes show reduced growth, inhibition of reproduction and lethal effects on germination (Nielsen et al., 2003). It

is recorded 4 gL^{-1} as an upper salinity tolerance limit for freshwater macrophytes (Hart et al., 1991).

Increasing salinity and its effect on aquatic invertebrates are well documented (Brock, 1982, Campbell, 1994). A large range of microinvertebrates including rotifers, microcrustaceans, and protozoans are negatively affected by salinity levels of 2 gL^{-1}. It includes a general decrease of species richness, the effect on different life stages among others. For example, decrease of rotifer species recorded in Australian lakes with subjected to salinities above 2 gL^{-1}. Vertebrates such as fish and amphibians are likely to have high salinity tolerance comparatively other freshwater biota. As an example, adult Macquarie perch (*Macquaria australasica*) can survive in 30 gL^{-1} salt level but it's eggs vitality will be impaired at 4 gL^{-1} (O'Brien and Ryan, 1997). As an example of amphibians, *Lithobates sphenocephalus* can tolerate salinity level up to 11 ppt and *Hyla cinerea* observed in even 23.4 ppt (Albecker and McCoy, 2017).

1.4 Zooplankton in freshwater aquatic ecosystems

Freshwater ecosystems comprise with three major groups of zooplankton: cladocerans, rotifers, and copepods. The role of zooplankton in aquatic ecosystems is well documented (Keister et al., 2012, Varadharajan and Soundarapandian, 2013). Zooplankton play a significant role in energy transfer in the aquatic ecosystems, providing a major link between the base of the food web/chain and other higher trophic levels of the ecosystem. On the other hand, zooplankton can affect biomass, production, and assemblage of the phytoplankton in the ecosystem. Effect on nutrient dynamics in aquatic ecosystems by zooplankton pivotal for phytoplankton growth (Keister et al., 2012). Zooplankton are ideal model species group to study the effect of salinization on freshwater biota due to their high sensitiveness to environmental gradients such as light, temperature, salinity, and turbidity (Bonnet and Frid, 2004, Hays et al., 2005, Marques et al., 2008).

Cladocerans are restricted to freshwater environments with salinity lower than 1 gL^{-1} (Hart et al., 1991) or conductivity less than 500 μS/cm (Hebert et al., 2002). Rotifers are known as a highest salt tolerant group among other freshwater zooplankton being present even above 2.7 ppt. (Schallenberg et al., 2003). These salt concentrations may also be affected by other abiotic parameters such as temperature (Grzesiuk and Mikulski, 2006). Effects of increasing salinity on different zooplankton species are well studied (Ghazy et al., 2009, El-Gamal et al., 2014). Salinity is limiting dispersal ability of zooplankton species (Grzesiuk and Mikulski,

2006), change zooplankton community composition (Schallenberg et al., 2003), effect on survival, life history traits, growth and reproduction [*Branchipus schaefferi*: Sarma et al. (2005); *Daphnia magna*: Gonçalves et al. (2007); *Daphnia longispina*: El-Gamal et al. (2014): Gonçalves et al. (2007); *Moina micrura*: Santangelo et al. (2008)].

1.5 Genetic diversity

Natural populations are genetically variable. Genetic diversity is a key for populations being able to respond to changes in the environment. Genes regulate body size, shape, physiological processes, behavioral traits, reproductive characteristics, tolerance to environmental extremes, dispersal and colonizing ability, the timing of seasonal and annual cycles (phenology), disease resistance, and many other traits (Raven and Peter, 1986).

Populations consist of numerous individuals bearing different genotypes. Therefore their sensitivity for a particular stress will differ from each other. If this within genotype variability is higher in a population, the ability to survival under stresses and spreading across the wide geographical range is often possible. However, environmental conditions may rapidly change, thus, if a particular population has narrow within genetic variability, it has a high risk of being (locally) extinct under unprecedented environmental conditions/stresses.

The importance of genetic diversity for sustainability of population is well documented in several studies specially with reference to conservation of endangered species (Pereira et al., 2013, Fasola et al., 2015). In the current context, the immediate importance of genetic diversity is well recognized. For example, Convention on Biological Diversity (CBD) (2013) initiates to implement policies for conserve genetic diversity and prevent genetic erosion of populations.

1.6 Genetic erosion

In population genetics perspective, genetic erosion can be defined as the loss of genotypes that determine a specific trait or a set of traits (Bijlsma and Loeschcke, 2012). When under high selective pressures, the most sensitive genotypes of a population may disappear, leading to the genetic erosion in that particular population (Ribeiro and Lopes, 2013). The causes for genetic erosion mostly recorded as genetic drift, gene flow and natural selection (Ribeiro and Lopes, 2013).

Natural selection is a process how a population can adapt to their environment. In evolutionary perspective species with favorable traits to existing environmental conditions have a higher success of survival, reproduction and pass those characteristics to the next generation. In genetic perspective, "Natural selection is a consistent difference in survival and reproduction between different genotypes, or even different genes, in what we could call reproductive success"(Futyama, 2004). Therefore genetic diversity is the result of survival and adaptation success through natural selection. When a population has low within genotypes variability, sensitive genotypes to a perturbation have a high risk to be eliminated from the original population under the process of natural selection (Fasola et al., 2015). In other words, if current population lose their original genotypes due to stresses in the natural environment that compromise with the resilience and adaptations for future stresses and ultimately can lead to the extinction of that population (Venâncio et al., 2016).

Genetic drift is defined as the change in relative frequency of alleles in a population by random event or chance. Smaller populations are more vulnerable to genetic drift as allele frequencies are relatively low. A significant difference between the natural selection and genetic drift is that genetic drift is a completely random process. Bottleneck events (eg. sudden changes in the environment) can result genetic drift. It results in genetic erosion if particular population loose genotypes of it's original population after bottleneck event (Chen et al., 2012, Dong et al., 2012, Fasola et al., 2015).

Consequences of genetic erosion are well studied as loss of alleles in population, reduce the fitness, reduce reproduction rate, increased susceptibility to pathogens, absence or alterations of protective or co-tolerance mechanisms, reduce environmental plasticity, and increase vulnerability of extinction with the future stresses (Green, 2003, Lopes et al., 2009, Fasola et al., 2015). Population can overcome these detrimental effects if they can adapt and increase natural population's tolerance to stresses through (i) acclimation ability of individuals (physiological changes induced by environment which does not cause any change in genetic structure of population); (ii) spread of tolerance genes through mutations or immigration which allow a rise of tolerant genotypes (can alter genetic structure of population) (Maxwell et al., 2014, Fasola et al., 2015).

1.7 Knowledge gap

There are several studies conducted to find the effect of climate change induced salinization on freshwater ecosystems. The effect of salinity on coastal marine ecosystems comes from desalinization, e.g. the freshening of seawater, mainly caused by increased freshwater inflow through pronounced melting of glaciers in polar regions (Arendt et al., 2011). The effects of desalinization on the physiology, behavior (Zajaczkowski and Legezynska, 2001, Eiane and Daase, 2002) and the genetic diversity (Lowe et al., 2005, Markert et al., 2010) of marine planktonic communities are well studied.

Conversely, freshwater ecosystems suffer from increased salinization caused by the sea-level rise, increased evaporation, and extreme weather conditions such as severe droughts and storms with climate change. ((Nielsen et al., 2003, Ghazy et al., 2009). Most studies on the effects of increased salinity on freshwater communities have focused on their physiology and behavior (Sarma et al., 2006, Ghazy et al., 2009, Kearney et al., 2016, Whitney et al., 2016), biodiversity (Hart et al., 1991) and ecosystem-level consequences (Nielsen et al., 2003, Schallenberg et al., 2003). However, the effects of salinization on the genetic diversity of freshwater communities have received less attention.

As salinization induces physical stress and behavioral changes (Kearney et al., 2016, Whitney et al., 2016), it is crucial to study how these affect the genetic diversity of freshwater biota. Also, it is not well known to what extent salinization can cause genetic erosion of freshwater communities. Since environmental variables are known to often interact, it is interesting to investigate how the combined effects of temperature and salinity can affect the genetic diversity and contribute to genetic erosion.

1.8 Research objectives

The objective of the present study is to understand the effect of salinization on the genetic diversity of zooplankton populations, under different temperature regimes. To attain this major goal, following questions were addressed:-

(i) Can salinization cause genetic erosion in zooplankton population?

(ii) If so, can extreme events enhance the salinization driven genetic erosion (e.g. summer droughts, winter sea flooding)?

To obtain these specified objectives, two freshwater species of zooplankton were selected: the cladoceran; *Daphnia longispina* and the rotifer; *Brachionus calyciflorus*.

2. Materials and Methods

2.1 Artificial sea water

Artificial sea salt (Ocean Fish™) was acquired from PRODAC International®, Cittadella, Italy. A stock solution of artificial seawater (33 gL^{-1}) was prepared by dissolving 33 g of artificial sea salt in 1 L of ultra-pure water (Milli-Q Academic system; Millipore, MA, USA). This stock solution was subsequently diluted (with the culture medium used for each test species) to obtain the salinity levels used to perform the experiments.

Artificial seawater was chosen for this study rather than natural seawater or commonly used salt (NaCl) because:- (i) sodium (Na^+) and chloride (Cl^-) are major ions in seawater but there are many other minor ions which can be toxic chronically and are neglected by using solely NaCl solutions. (ii) natural seawater has the risk of contamination with other pollutants and thus it would be difficult to discriminate the toxicity due to increased salinity or to other contaminants.

2.2 Culture of the test species

Two zooplankton species were selected as model species for these experiments: the cladoceran *Daphnia longispina* (O.F. Muller,1785) and the rotifer *Brachionus calyciflorus* (Pallas, 1766). *Daphnia longispina* is the most ubiquitous cladoceran in Europe (Ma et al., 2014). They show differential physiological tolerance to ionic variations (e.g. Mg^{2+}) and are well known as good indicator species for salinity (Amsinck et al., 2003, Bos et al., 1996). Also, their reproduction occurs by cyclic parthenogenesis, which allows maintaining identical clones for several generations under laboratory conditions. *Brachionus calyciflorus* is one of the widely distributed rotifer species and population oscillations were recorded in many studies due to climate change (Binzer et al., 2012). Commercially available cyst allows establish cultures of clonal lineages in the laboratory.

Six clonal lineages of *D. longispina*, with differential sensitivity to the lethal level of salinization, were selected to perform this study: E84, E89, E99, N35, N37 and N91 (Leitão et al., 2013, Venâncio, 2017) (Table 1).

Table 1: Median effective conductivities ($LC_{50,48h}$) (with 95% confidence interval) for natural seawater of selected clonal lineages of *Daphnia longispina* (values retrieved from Venâncio, 2017)

Clonal lineage	$LC_{50,48h}$ (95% CL) (mS/cm) in natural seawater
N35	4.99 (3.31 - 8.69)
N37	4.78 (4.63 - 4.94)
N91	2.68 (1.63 – 3.37)
E84	2.73 (1.68 – 3.42)
E89	4.65 (4.48 – 4.83)
E99	4.45 (3.96 – 5.02)

These clonal lineages were originated from two wild populations: (i) from a reference site and (ii) from a historically-impacted site with acid mine drainage (AMD). Both sites are located at the aquatic system of an abandoned cupric-pyrite mine: São Domingos mine, Southeast Portugal. Ore exploitation of this mine ended more than fifty years ago, after an active use of hundreds of years. Continuous oxidation of mine tailings produces acidic and metal contaminated effluent. Therefore, historically contaminated aquatic system continuously exposed to hydrogen ions (pH = 2.1) and metal ions (Fe, Al, Zn, Cu, Mn, Co, Ni, Cd, Pb, Cr, As in decreasing order) present in AMD (Leitão et al., 2013). These six clonal lineages were cultured in the laboratory for more than 500 generations. They were cultured under temperature 20±1^{o}C and photoperiod 16:8 h L:D in ASTM hard water (American Society for Testing and Materials) (OECD, 2004), with the addition of vitamins (7.5 mgL^{-1} B1, 1 mgL^{-1} B12 and 0.75 mgL^{-1} biotin) and standard organic extract Marinure 25 from the algae *Ascophyllum nodosum* (Pann Britannica Industries Ltd, Waltham Abbey, UK) (Baird et al., 1989). Organisms were fed everyday with the green algae *Raphidocelis subcapitata* at a concentration of $1.5x10^5$ cells/mL. Culture medium was renewed every other day.

Commercial cysts of *B. calyciforus* were acquired from RotoxKit F™ (MicroBio Tests Inc, Gent, Belgium) and hatched following the standard procedure in RotoxKit F™ (MicroBio Tests, 1998). Since cysts are the result of sexual reproduction, individuals from different cyst exhibit different genotypes. Sixteen clonal lineages were isolated from sixteen cysts. Neonates were cultured under a controlled condition at a temperature of 20±1^{o}C and

photoperiod 16:8 h L:D, in reconstituted freshwater (ASTM medium) which was prepared according to Rotoxkit F™ (MicroBio Tests, 1998) procedure. Organisms were fed with the green algae *R. subcapitata* ($0.5x10^5$ cells/mL) every day. Culture medium was renewed every other day.

2.3 Lethal toxicity assays

Since the sensitivity of the rotifer clonal lineage to salinization was not known, firstly, lethal toxicity assays carried out with the sixteen clonal lineages to characterize their lethal sensitivity to increased salinity. Therefore, from the cultures mentioned in section 2.2, neonates of the sixteen clonal lineages of *B. calyciforus* were isolated and exposed to a range of increased conductivities as 3.50, 4.90, 6.86 and 9.60 mS/cm according to standard procedure described on Acute RotoxKit F™ (MicroBio Tests, 1998). Five neonates from each clonal lineage were exposed per replicate. Three replicates for each treatment and corresponding negative controls (ASTM medium) were carried out simultaneously in 16mL wells filled with 8mL of test solution in 6 wells plastic plate. This experiment was conducted under the same culture conditions mentioned in section 2.2 without the addition of algae. Organisms were exposed for a period of 24 h and at the end of this period, the number of immobile organisms (organisms that did not exhibit any movement for 15 s after gentle prodding) was registered at each replicate. This lethal toxicity assay allowed selecting six clonal lineages according to their differential sensitivity to salinity. These six clonal lineages were used to carry out long-term assays.

2.4 Long-term assays

Long-term assays were conducted by exposing organisms to increased salinity under 3 temperatures: (i) 17 ± 1^oC to mimic a scenario of salt water intrusion during winter; (ii) 20 ± 1^oC was set as the optimal temperature and served as the control, since this is the temperature at which organisms were cultured in the laboratory; (iii) and 23 ± 1^oC to simulate seawater intrusion in freshwater ecosystems during summer.

2.4.1 Long-term assays with *Daphnia longispina*

For each temperature, 10 neonates less than 24h-old and from third to fifth brood, of each clonal lineage of *D. longispina* were introduced per replicate. Each replicate consisted in a 1 L glass vessel containing 800 mL of ASTM or ASTM plus salt (at desired concentration). Six replicates per clonal lineage (to use 3 as controls and 3 as treatments) per temperature were

reared under relevant temperature regime and photoperiod 16:8 h L:D until each population reached their carrying capacity (23 d for 17^{o}C, 21 d for 20^{o}C and 19 d for 23^{o}C). In that period, organisms were fed with the green algae *R. subcapitata* (1.5 x 10^{5}cells/mL) every day and culture medium was renewed every other day. The population density was monitored daily to check whether each population reaches its carrying capacity.

After reaching the carrying capacity, three replicates from each clonal lineage were exposed to the concentrations of artificial seawater that caused 70% mortality in the most tolerant clonal lineage (E89) ($LC_{70,24h}$:- 4.80 mS/cm) and to corresponding negative control (ASTM hardwater). Exposure occurred in the same conditions as described in the previous paragraph. Three replicates for treatment and three corresponding controls were run at each temperature. Same photoperiod conditions, feeding, and renewing practices were maintained as in laboratory cultures (as mentioned in section 2.2 of Materials and Methods) throughout this experiment. Immobilization of organisms (here considered as mortality; organisms remaining immobile during 15 s after gentle prodding) being checked every 24 h until half of clonal lineages totally disappear (47 d for 17^{o}C, 41 d for 20^{o}C, and 31 d for 23^{o}C), time at which the experiment ended. The population density was calculated for each population with time.

2.4.2 Long-term assays with *Brachionus calyciflorus*

Six clonal lineages of *B. calyciflorus* (G, P, D, H, N, F) were chosen to carry out this experiment based on the results obtained from the lethal toxicity assays previously described in section 2.3 of Materials and Methods. These six clonal lineages were selected for this study according to their range of genetically determined sensitivity to lethal levels of artificial sea water (G, P, D – sensitive and H, N, F – tolerant) (Table 2).

Table 2: Effective conductivities: $LC_{20,24h}$; $LC_{50,24h}$ and $LC_{70,24h}$ (with 95% confidence interval) for artificial seawater of selected clonal lineages of *B. calyciflorus*

Clonal lineage	$LC_{20,24h}$ (95% CL) mS/cm	$LC_{50,24h}$ (95% CL) mS/cm	$LC_{70,24h}$ (95% CL) mS/cm
D	4.19[a]	6.04[a]	7.60[a]
G	4.19 (3.18-4.89)	6.06 (5.26-7.11)	7.64 (6.60-9.82)
P	5.34 (4.56-5.89)	6.56 (5.95-7.30)	7.47 (6.78-8.66)
N	6.95 (5.88-7.63)	8.28 (7.55-9.25)	9.24 (8.43-10.93)
F	6.33 (5.19-7.15)	8.34 (7.39-10.00)	9.89 (8.61-13.24)

H	9.01 (8.74-9.28)	9.41 (9.13-9.68)	9.66 (9.38-9.95)

[a] – 95% confidence interval could not be computed.

For rotifer assays, 5 neonates less than 24h-old, of each clonal lineage of *B. calyciflorus*, were introduced per replicate to 16 mL wells containing 8 mL ASTM in 6 wells plastic plates. Six replicates per clonal lineage (to use 3 as controls and 3 as treatments) and per temperature were maintained under relevant temperature regimes and photoperiod 16:8 h L:D until they reached their carrying capacity (13 d for 17^{o}C, 10 d for 20^{o}C and 8 d for 23^{o}C). In that period, organisms were fed with green algae *R. subcapitata* in the concentration of 0.5 x 10^5cells/mL every day and culture medium was renewed every other day. Number of individuals in each replicate was daily counted to check whether each population reached its carrying capacity. After achieving the carrying capacity, populations were exposed to the concentrations of artificial seawater that caused 70% of mortality in the most tolerant clonal lineage (H) ($LC_{70,24h}$:- 9.66 mS/cm) and to the negative control (ASTM medium). All individuals of each clonal lineage were exposed simultaneously in 16 mL wells in 6 wells plastic plate filled with 8 mL of test solution. Three replicates for treatment and three corresponding controls were run at each temperature. Same photoperiod conditions, feeding, and renewing practices were maintained as in laboratory cultures (as mentioned in section 2.2 of Materials and Methods) throughout this experiment. Immobilization (mortality) of organisms was being checked every 24 h until half of the genotypes totally died (34 d). The population density was measured for each population with time.

Conductivity (Wissenchaftlich Technische Werkstatten, F330 conductivity meter, Weilheim, Germany), pH (Wissenchaftlich Technische Werkstatten 330 pH meter Weilheim, Germany), and dissolved oxygen (Wissenchaftlich Technische Werkstatten OX330 oxygen meter Weilheim, Germany) were measured in each treatment at the new and old medium during renewal. These parameters were controlled in the desired ranges throughout study period as mentioned in Table 3.

Table 3: Maintained culture conditions - pH, Dissolved oxygen and Conductivity

Test species	Control/ Treatment	pH	Dissolved oxygen (mgL^{-1})	Conductivity (mS/cm)
D. longispina	Control	7.60 - 8.11	7.53 - 9.75	0.58 – 0.75
	Treatment (LC_{70}	7.80 – 8.21	6.53 - 8.56	4.68 – 6.64

	= 4.8 mS/cm)			
B. calyciflorus	Control	7.74 - 8.46	7.72 – 9.74	0.21 – 0.25
	Treatment (LC_{70} = 9.7 mS/cm)	7.48 - 8.17	7.40 - 8.43	9.79 – 11.09

2.5 Data analysis

Lethal concentrations causing 70% of mortality of population (LC_{70}), were computed for both species through the PriProbit© software (Sakuma, 1998). Lethal concentration causing 50% of mortality in population (LC_{50}) and lethal concentration resulting 20% mortality in population (LC_{20}) computed further for *B. calyciflorus.* Lethal time resulting 50% and 90% of mortality of population (LT_{50} and LT_{90}) were computed with exponential, logistic and Gompertz models using STATISTICA 7.0 and the best-fitted value was chosen according to smallest relative spread, for the different times of exposure: *B. calyciflorus*:- 72 h, 96 h (short-term) and 816 h (long-term); *D. longispina* :- 72 h, 96 h (short-term) and 1128 h at 17^{o}C, 984 h at 20^{o}C and 744 h at 23^{o}C (long-term).

$$Relative\ spread = \frac{Upper\ confidence\ limit - Lower\ confidence\ limit}{LT_{50}\ or\ LT_{90}\ (respective\ value)}$$

To assess if the strict and temporal tolerance of each clonal lineages to salinization were related, the values of LT_{50} and LC_{50} (for 20^{o}C) were compared. The LT_{50} values in short term exposures (72 h and 96 h) were compared with LT_{50} value in long term exposure (corresponding time that end the experiment under different temperatures (e.g. *B. calyciflorus*: 816 h in all 3 temperatures; *D. longispina*: 17^{o}C – 1128 h, 20^{o}C – 984 h, 23^{o}C – 744 h) to identify any difference in responses according to time scale, or to identify any recovery ability under continuous exposure for lethal concentration. The LT_{90} values at different temperatures were compared to study extinction risk for each clonal lineage under particular temperature. One way ANOVA was performed to identify significant differences among LT_{90} values under different temperature regimes. Data sets were tested for normality with Kolmogorov-Smirnov test and for homoscedasticity (Bartlett test).

3. Results

3.1 Acute test results of *Brachionus calyciflorus*

The median lethal conductivity values ($LC_{50,24h}$) computed for rotifers ranged from 5.74 to 9.41 mS/cm. Clonal lineages H, F and N showed the highest tolerance towards salinity, with $LC_{50,24h}$ of 9.41, 8.34 and 8.28 mS/cm respectively (Fig. 2). On the other hand D, G and P were the clonal lineages that showed to be the least tolerants, after a 24 h exposure to lethal levels of artificial sea water. The $LC_{50,24h}$ values computed for D, G and P were 6.04, 6.06 and 6.56 mS/cm respectively (Fig. 2). These 6 clonal lineages (G, P, D & H, N, F) were selected for subsequent long-term exposure assays due to their difference in salt sensitivity.

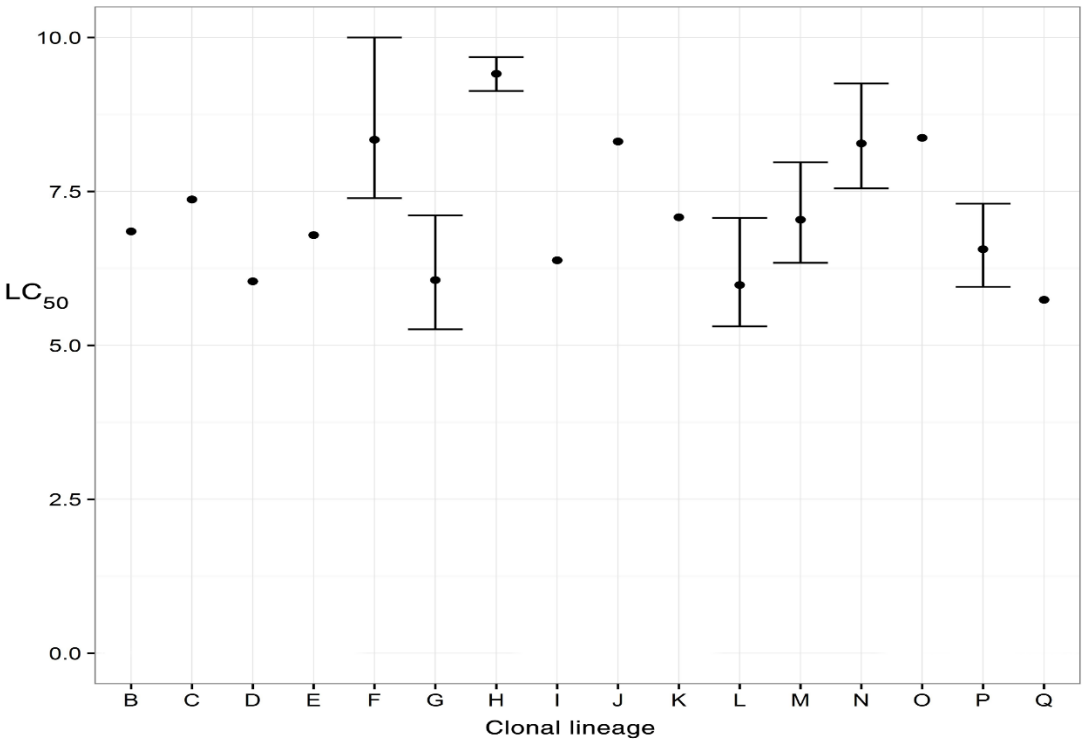


Fig. 2: Median lethal conductivity values ($LC_{50,24h}$) computed for the 16 clonal lineages of the freshwater *B. calyciflorus.* Vertical bars indicate 95% confidence intervals. No confidence limit could be computed for B, C, D, E, I, J, K, O and Q clonal lineages.

3.2 Results of long-term assays

3.2.1 *Daphnia longispina*

The population density of controls and treatments (exposed to LC_{70}) were changed throughout the experiment period under different temperature regimes as in Fig. 3. In these graphs population density illustrated as pooled running average. The population densities of controls were stable throughout the study period. Populations exposed to LC_{70} were declined exponentially in all 3 temperatures (Fig. 3). All experiments were continued until at least 3 clonal lineages disappeared. Time taken to disappear at least 3 clonal lineages was different according to temperature (17^oC – 1128 h, 20^oC – 984 h and 23^oC – 744 h). With increasing temperature reduction of lethal time was observed or in other words, higher the temperature faster disappearance of clonal lineages was noted.

Under 17^oC E84, E99 and N35 clonal lineages were the first three clones that disappeared in 1080 h, 1104 h and 1128 h respectively. Same three lineages; N35, E99, and E84 disappeared under 20^oC while taking 864 h, 888 h and 984 h. At 23^oC, other than same three clonal lineages (N35, E84, and E99), N37 also disappeared in the same time at the end of 744 h. Under 23^oC, N35 and E84 took 624 h and 696 h to disappear respectively while both E99 and N37 showed same average time to died out as 744 h.

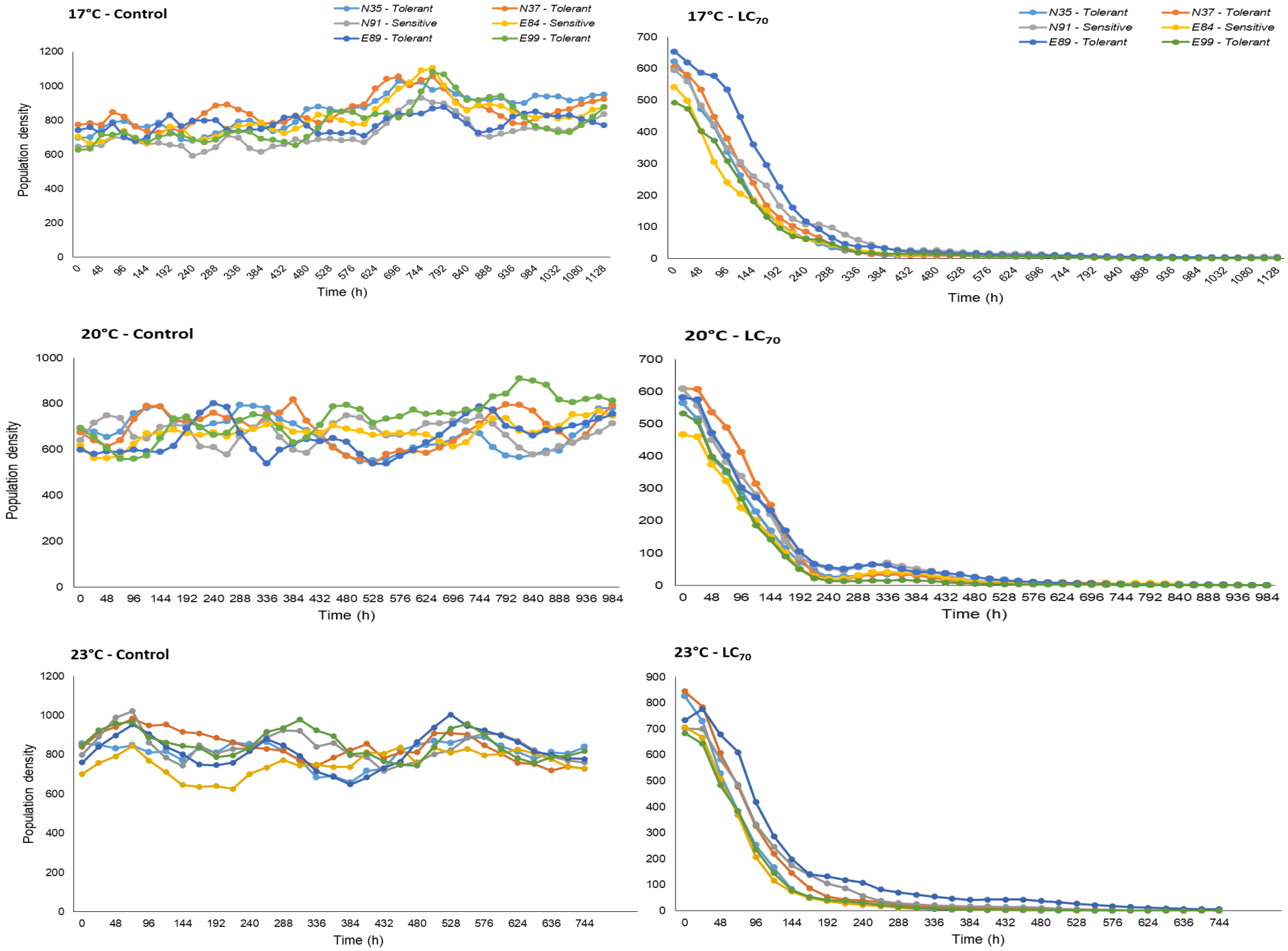


Fig. 3: Population density as pooled running averages in control (left) and exposed to LC_{70} (right) at 17°C, 20°C and 23°C for six clonal lineages of *Daphnia longispina*.

3.2.2 *Brachionus calyciflorus*

Figure 4 shows fluctuations of *B. calyciflorus* population density in controls and treatments (exposed to LC_{70}) throughout the experiment period at 3 different temperatures. The population density of controls slightly fluctuated in the study period. Populations exposed to LC_{70} in 17^{o}C were declined exponentially and 3 clonal lineages were disappeared with in 600 h of continuous exposure. F, H, and P were the clonal lineages disappeared first under 17^{o}C. These three clonal lineages (F, H, and P) took time to disappear in average as 432 h, 480 h and 600 h respectively. Under 17^{o}C, at 744 h all six clonal lineages were totally disappeared. Shortest time to initiate disappearance of clonal lineages and fastest disappearance of clones recorded under 17^{o}C.

Under 20^{o}C, it took 816 h to disappear 3 clonal lineages and population recovery could observe in D and P sensitive lineages. H, N, and G are the first three clonal disappeared at 20^{o}C showing the average time to disappear as 600 h, 696 h and 816 h respectively (Fig. 4). P clonal lineages show a dramatic reduction of population in first 72 h and then population density increased again. At the end of 816 h of continuous exposure, still, it could manage 17% of the population survive compare to an initial population which subjected to exposure. D clonal lineage lost it's 34% of the initial population in first 48 h of exposure. But the population was increased bit again and maintained its population density about 20% compared to initial population even at the end of 816 h of continuous exposure. Longest time to start disappearance of clonal lineages could observe under 20^{o}C.

As shown in fig.4 in 23^{o}C, it took 720 h to disappear 3 genotypes and attempts to population recovery could observe in 120 h – 360 h. First clone: F, disappeared at 552 h under 23^{o}C. Then N and G clonal lineages disappeared at 696 h and 720 h respectively. So, initiation of the disappearance of clonal lineages disappearance and rate of losing clones were intermediate at 23^{o}C compare to other 2 temperatures. All genotypes except G, showed attempts to increase its population in between 120 h – 360 h of continuous exposure.

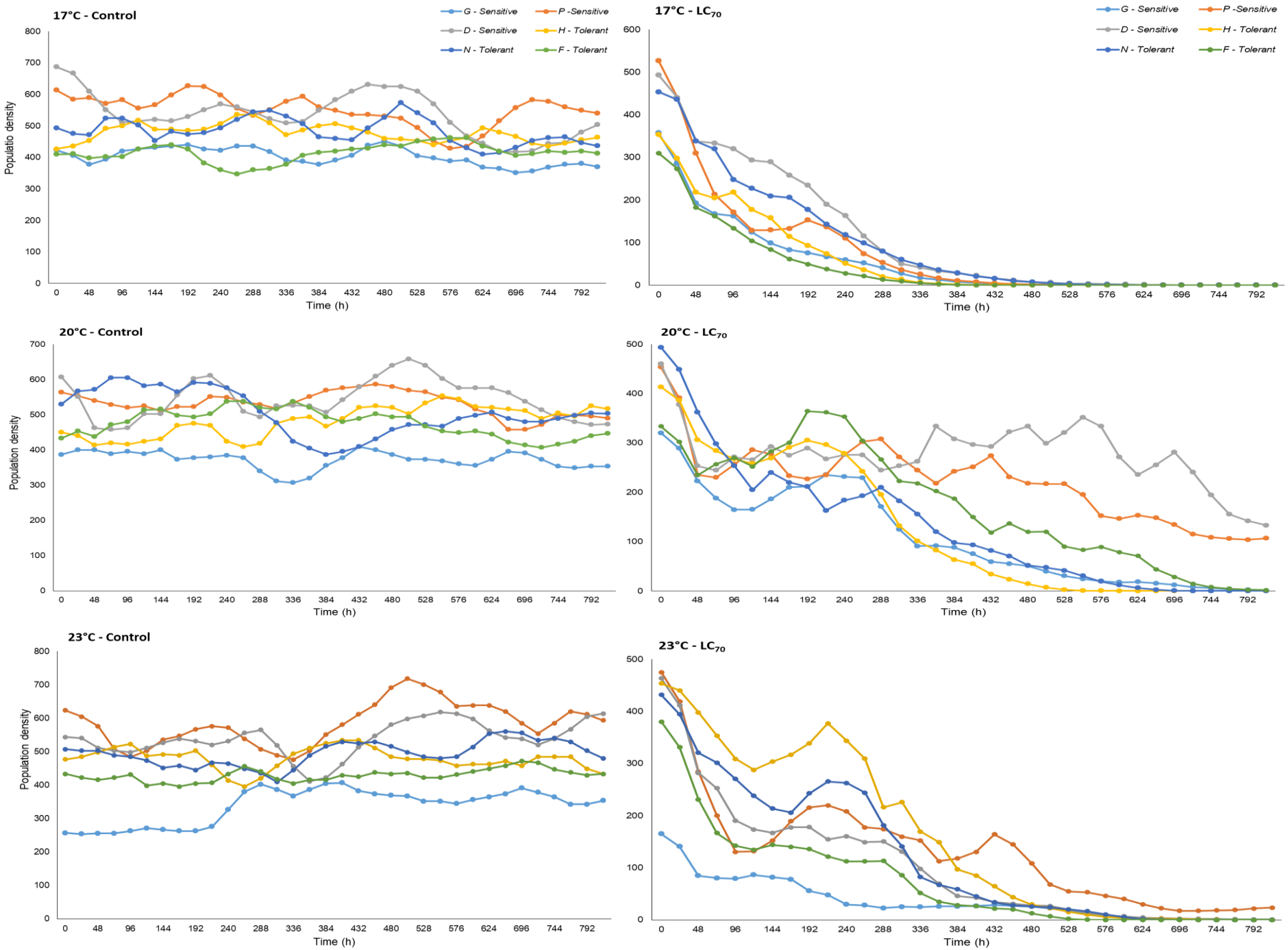


Fig. 4: Population density as pooled running averages in control (left) and exposed to LC_{70} (right) at 17°C, 20°C and 23°C for six clonal lineages of *Brachionus calyciflorus*

3.3 Determine the relationship in between LT_{50} and LC_{50} at 20°C

In the case of both species, it is expected to have high lethal time (LT) in clonal lineages which are more tolerant (high LC). But in our results, there is lack of correspondence in between LC_{50} and LT_{50} values in both species (Fig. 5 & 6).

3.3.1 *Daphnia longispina*

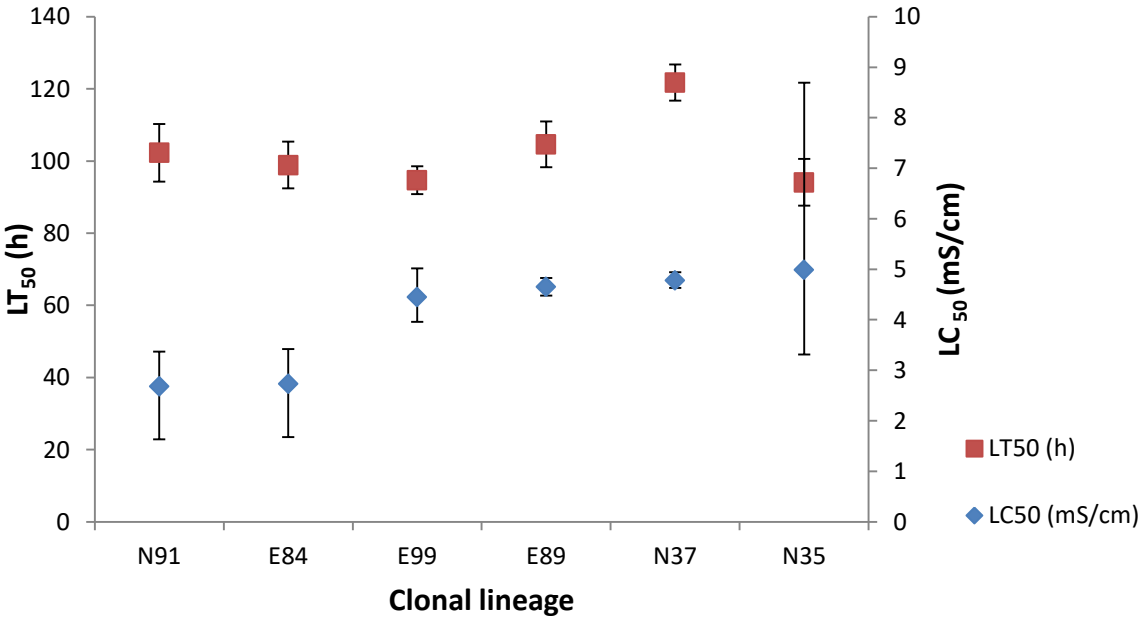


Fig. 5: Overall best-fitted LT_{50} value (comparing outputs of LT_{50}:72 h, LT_{50}:96 h and LT_{50}:816 h) with LC_{50} of *D. longispina*. Error bars are indicating 95% confidence level.

N91 and E84 have lowest $LC_{50,\ 48h}$ while N35 and E99 show the least values for $LT_{50.}$ N35 is the most tolerant species according to standard acute test for *D. longispina* which has highest $LC_{50,48h}$ as 4.99 mS/cm. But when we consider, the time taken to death which is another indication of tolerance, lowest LT_{50} value under same temperature showing by N35 claiming that N35 as the most sensitive genotype. Therefore there is lack of correspondence in LC_{50} and LT_{50} values obtained in our experiment.

3.3.2 *Brachionus calyciflorus*

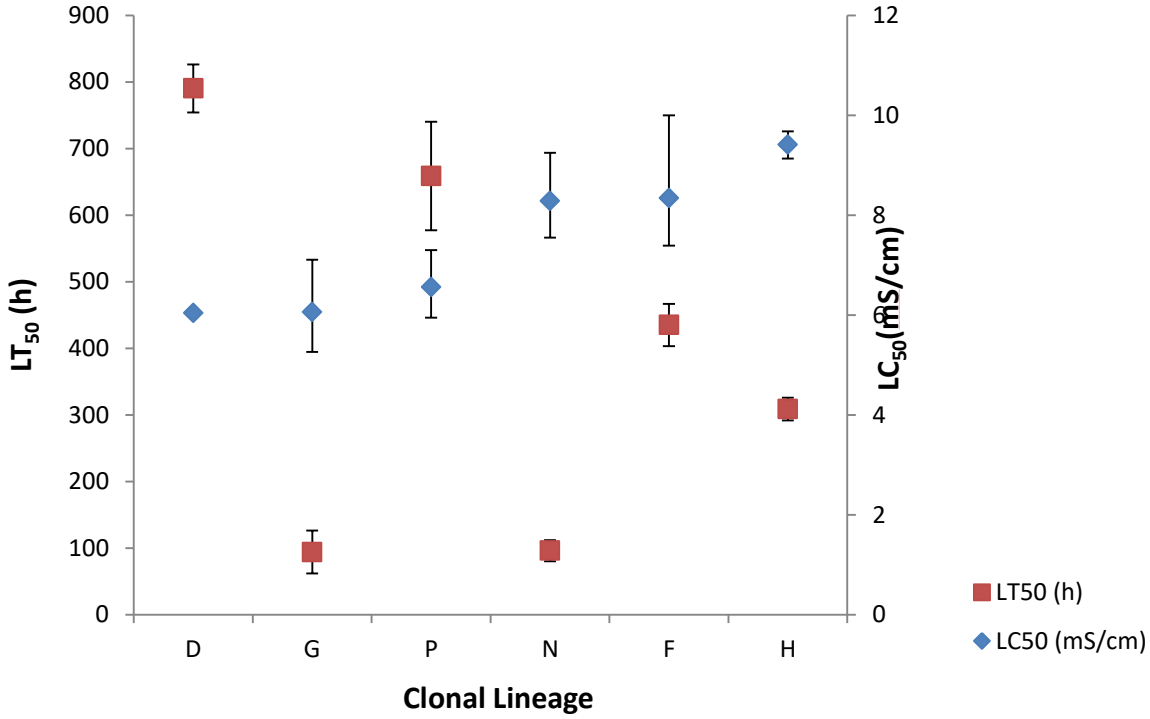


Fig. 6: Overall best-fitted LT_{50} value (comparing outputs of LT_{50}:72 h, LT_{50}:96 h and LT_{50}:816 h) with LC_{50} of *B. calyciflorus*. Error bars are indicating 95% confidence limit.

There is no any relationship can observe in between standard acute concentration for rotifers ($LC_{50,24h}$) and LT_{50} values resulted in rotifer experiment. Again with *B. calyciflorus*, most sensitive genotype according to $LC_{50,24h}$ was D genotype which indicates as highest tolerant genotype with the LT_{50} values. This again confirms lack of correspondence in LC_{50} and LT_{50} values.

3.4 Determination of recovery ability in short and long time scales

Results obtained for LT_{50} in short-term exposure (72 h, 96 h) and long-term exposures under different temperature regimes for both species are compared in below.

3.4.1 *Daphnia longispina*

- Comparison of LT_{50} in short-term exposure (72 h, 96 h) and long-term exposure (17^oC- 1128 h; 20^oC- 984 h; 23^oC- 744 h)

There is no observable difference in LT_{50} values derived from short-term and long-term exposure to 3 different temperatures (Fig. 7). It indicates that continuous exposure to LC_{70} salt concentration is suppressing recovery of all 6 genotypes in *D. longispina* regardless the temperature. Population density is continuously subjected to an exponential decay with continuous exposure to high salinity level.

As shown in Fig. 7, LT_{50} values of all lineages except E89 overlapped in both short-term and long-term exposure under 17^oC. E89 shows higher LT_{50} value in short-term (820 h) exposure but under long-term it shows low (160 h) which include in the same range of LT_{50} value as other lineages (80 h-160 h) in long-term. Both in short and long-term exposure, almost five genotypes out of six show very low within genotype variability under 17^oC.

In 20^oC, comparatively N37 show higher LT_{50} value (120 h-130 h) while all other lineages are having close LT_{50} values range from 90 h – 110 h. There is no observable difference in LT_{50} derived for short-term and long-term exposures for each particular genotype also among six genotypes at 20^oC (Fig. 7). Under 23^oC, LT_{50} values derived from both short-term and long-term exposure are narrowed to the range of 60 h - 115 h. Differences are negligible in LT_{50} of both short-term and long-term exposure under 23^oC. (Fig. 7)

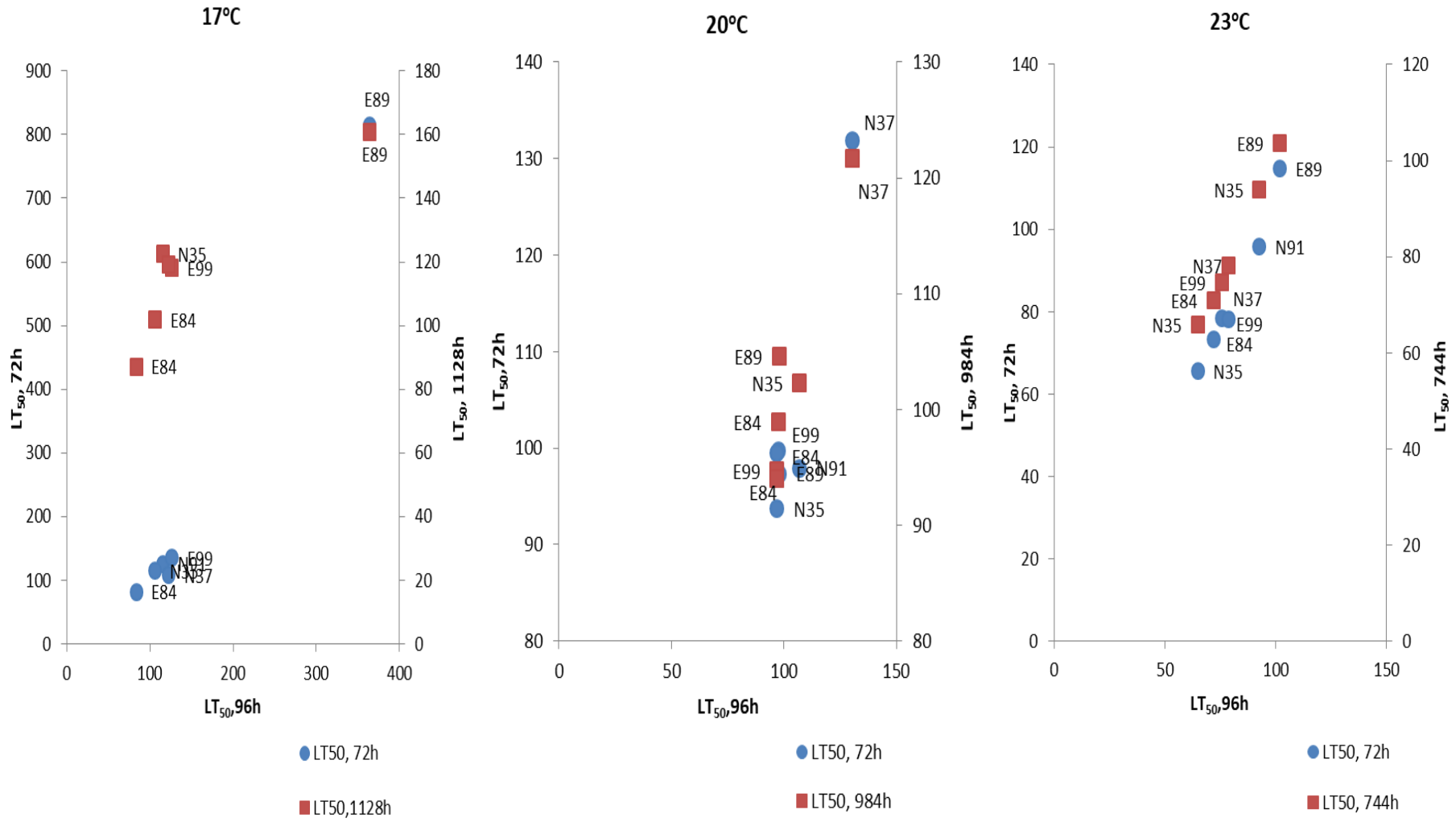


Fig. 7: Short-term (72 h) and respective long-term LT_{50} values in 17°C, 20°C and 23°C plotted against the short-term (96 h) LT_{50} of 20°C for six clonal lineages of *Daphnia longispina*.

3.4.2 *Brachionus calyciflorus*

- Comparison of LT_{50} in short-term exposure (72 h, 96 h) and long-term exposure (816 h) under different temperature regimes.

In overall, *B. calyciflorous* always show higher LT_{50} values under long-term exposure than short-term exposure at all studied temperatures. This implies even high mortality occurs within first 96 h, they have the ability to recover under continuous long-term exposure which is LC_{70} (9.7 mS/cm) (Fig. 8).

Under 17^{o}C clonal lineages of P, G, and F shows low LT_{50} (below 100 h) while N, H, and D show higher LT_{50} values (100 h -200 h) in overall (Fig. 8). All six genotypes show at least slightly high LT_{50} values comparing long-term and short-term exposure. F clonal lineage shows least difference in LT_{50} values in short and long time frames as 73.11 h and 73.21 h while D shows the highest difference as 159.24 h – 194.83 h respectively. Compared with other temperatures (20^{o}C and 23^{o}C) lowest LT_{50} values range for long-term exposure recorded in 17^{o}C (all genotypes show LT_{50} below 250 h). Also, the difference in between LT_{50} values in short-term and long-term exposure is low compared with other 2 temperatures.

Fig. 8 show high difference in LT_{50} resulted with short-term and long-term exposure under 20^{o}C. All genotypes were recorded LT_{50} value range from 60 h to 220 h in short term while their LT_{50} ranged from 150 h to 800 h in long-term concerns. D and P sensitive genotypes show high recovery with long-term exposure in 20^{o}C than other genotypes. In overall highest LT_{50} values for long-term exposure recorded under 20^{o}C comparing other 2 temperatures (D - 800 h; P - 700 h). Therefore 20^{o}C may result the least toxicity condition under continuous exposure to increased salinity (LC_{70}) for *B. calyciflorous* populations.

All genotypes show a doubling of its LT_{50} value from short-term exposure to long-term exposure under 23^{o}C. H and N tolerant clonal lineages show higher LT_{50} values in long-term exposure at 23^{o}C. Compare to other two temperatures, the intermediate range of LT_{50} values can be seen under 23^{o}C where all clonal lineages show LT_{50} in the range of 50 h to 150 h in short-term and 80 h to 350 h in long- term time frame (Fig. 8).

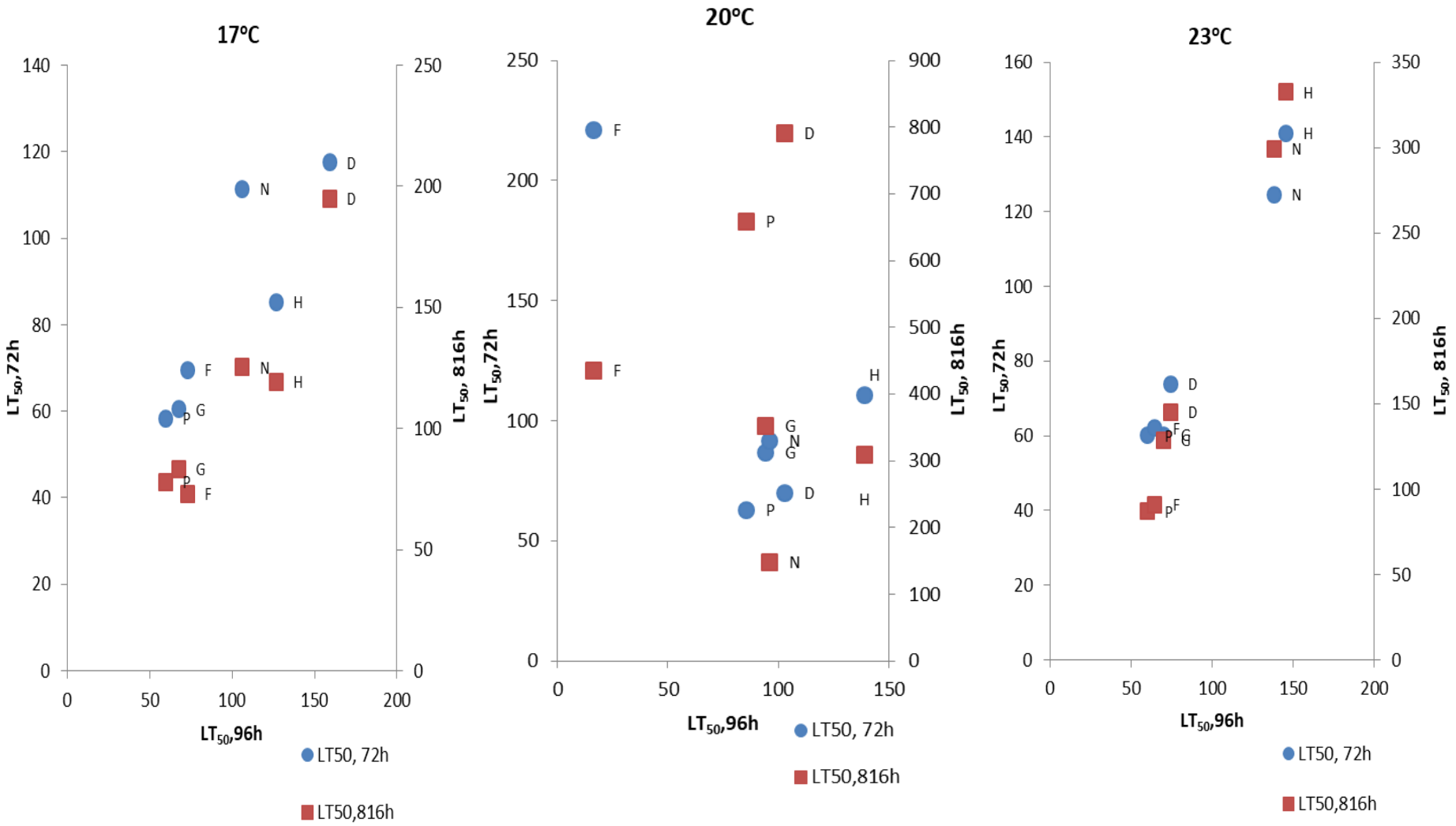
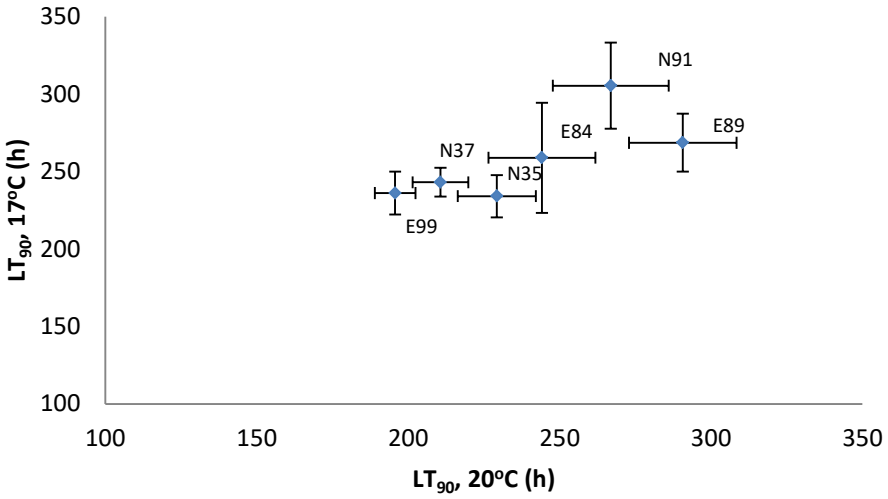


Fig. 8: Short-term (72 h) and respective long-term LT_{50} values in 17°C, 20°C and 23°C plotted against the short-term (96 h) LT_{50} of 20°C for six clonal lineages of *Brachionus calyciflorus*.

3.4.3 Probability of extinction in different genotypes under different temperature regimes (Comparison of LT_{90} in 17°C, 20°C, and 23°C)

LT_{90} is the best estimate to have an understanding about probable extinction among genotypes where almost 90% of the population died off and have the minimum possibility to recover back.

3.4.4 *Daphnia longispina*



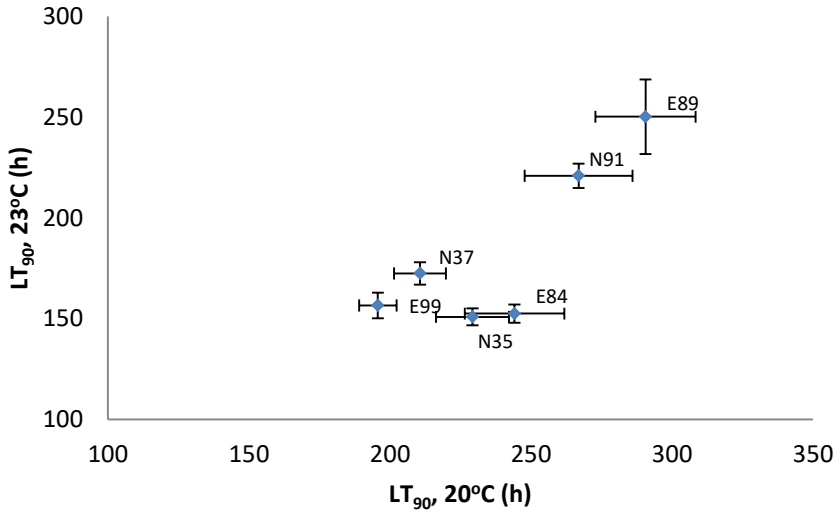


Fig. 9: LT_{90} values in 17°C and 23°C plotted against the LT_{90} of 20°C for six clonal lineages of *Daphnia longispina*. Error bars represent the confidence limits at 95%.

Genotypes of *D. longispina* that we used in our study were cultured and maintained in the laboratory under 20°C more than 500 generations. Therefore above graphs are showing how LT_{90} values differ in 17°C and 23°C with respective to 20°C (control temperature). In all 3 temperatures, E89 and N91 lineages show higher tolerance to salinity and deviated from other four clonal lineages. Other four lineages, E84, E99, N35, and N37 show very close LT_{90} values to each other in all 3 temperatures. E99 tend to be the first clonal lineage to disappear regardless the temperature. Then N37, N35, and E84 clonal lineages will disappear respectively. E89 and N91 have high potential to survive in all 3 studied temperatures. All studied genotypes presented lowest LT_{90} value or highest toxicity under 23°C. And on the other hand, highest LT_{90} or lowest toxicity for salinity recorded in 17°C in all studied clonal lineages except E89.

It is observed increased temperature reduce the LT_{90} values in *D. longispina* which indicate temperature rise induce toxicity of studied *D. longispina* genotypes. LT_{90} values at 17°C ranged from 230 h - 310 h while LT_{90} values under 20°C differ from 190 h to 290 h. Under 23°C, the range of LT_{90} values is 150 h – 250 h. In 23°C, it starts disappearance of genotypes earliest as about 150 h, which indicate 23°C is most lethal temperature for *D. longispina.* Then first 3 genotypes (E99, N35, and E84) disappeared sooner with about 2 h and 4 h time lag among them to disappear. Also, all genotypes reduced or narrowed its own confidence level at 23°C compared to other 2 temperatures. In other words within genotype variability is lowest at 23°C. The significance of LT_{90} values of *D. longispina* among 3 different temperatures further recorded in table 4.

Table 4: LT_{90} for different clonal lineages of *D. longispina* under different temperatures

Clonal Lineage	LT_{90} - 17°C (h)	LT_{90} - 20°C (h)	LT_{90} - 23°C (h)
E84	258.9 ± 35.49^{a}	244.2 ± 17.66^{a}	152.4 ± 4.53^{b}
E89	268.6 ± 18.72	290.7 ± 17.75	250.2 ± 18.45
E99	249.9 ± 13.84^{a}	202.4 ± 6.67^{b}	162.9 ± 6.34^{c}
N35	234.1 ± 13.68^{a}	229.3 ± 12.92^{a}	150.9 ± 4.19^{b}
N37	243.1 ± 9.26^{a}	210.7 ± 9.1^{b}	172.4 ± 5.54^{c}
N91	305.4 ± 27.77^{a}	266.9 ± 19.16^{ab}	220.9 ± 6.07^{b}

Mean ± SD, values with different superscript letters in the same row are significantly different ($p<0.05$)

As shown in Table 3, the majority of the studied genotypes except for E99 and N37, there is no significant difference among genotypes for salt sensitivity under lower temperatures (17^oC and 20^oC). E89 didn't show any difference in salt tolerance throughout studied temperatures. 23^oC was almost most unfavourable temperature for all genotypes which is having the additive effect of toxicity with salinity and temperature.

3.4.5 *Brachionus calyciflorus*

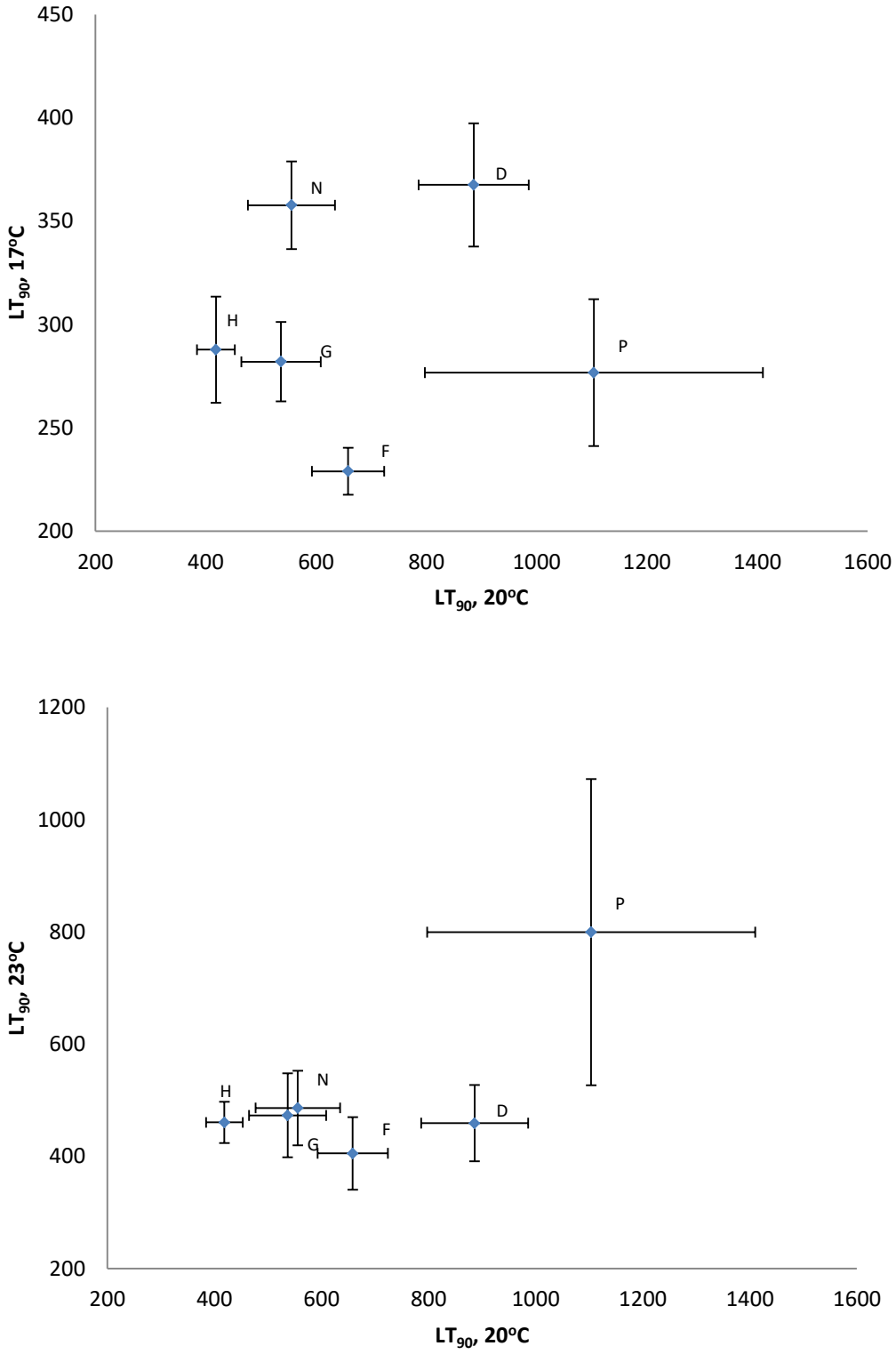


Fig. 10: LT_{90} values in 17°C and 23°C plotted against the LT_{90} of 20°C for six clonal lineages of *Brachionus calyciflorus*. Error bars represent the confidence limits at 95%.

In *B. calyciflorus* populations, LT_{90} values at 17°C ranged from 220 h - 370 h while LT_{90} values under 20°C differ from 420 h to 1100 h (Fig. 10). Under 23°C, the range of LT_{90} values is 400 h – 800 h (Fig. 10). For rotifers, 17°C is the most unfavorable temperature which results lowest LT_{90} values among all 3 temperatures. Different genotypes show different responses depend on temperature. Under 17°C, N and D genotypes show higher LT_{90} than other genotypes while D and P become more tolerant genotypes under 20°C. In 23°C, the only P could show higher salt tolerance by having high LT_{90} value.

Distribution of LT_{90} values of studied six genotypes (Fig. 10) shows the possibility of genetic erosion of each genotype which we can observe with LT_{90} value itself and narrowing or widening the spread of confidence interval among different temperatures. In overall, 17°C show lowest LT_{90} values which indicate the highest toxicity for salinity. Also the lowest within genotype variability for salinity recorded at the same temperature. Therefore we can expect higher probability for genetic erosion or extinction under 17°C for *B. calyciflorus.* In contrast, it is recorded highest LT_{90} values for all studied genotypes except H under 20°C. Also, LT_{90} values spread over a wide range for *B. calyciflorus* under 20°C compared to other 2 temperatures. Having highest LT_{90} values and highest range of LT_{90} at 20°C make it is less probable to occur genetic erosion at 20°C. In concern of 23°C, it is showing intermediate LT_{90} values compare with other 2 temperatures. Statistical significance of LT_{90} values with response to temperature under continuous exposure to LC_{70} of *B. calyciflorus* is shown in table 5.

Table 5: LT_{90} for different clonal lineages of *B. calyciflorus* under different temperature regimes

Clonal Lineage	LT_{90} – 17°C (h)	LT_{90} – 20°C (h)	LT_{90} – 23°C (h)
D	367.6 ± 29.78^{b}	885.9 ± 99.95^{a}	458.9 ± 68.0^{b}
G	282.2 ± 19.19^{b}	536.6 ± 71.82^{a}	472.6 ± 74.77^{a}
P	276.7 ± 35.53^{c}	1103.7 ± 306.52^{a}	799.2 ± 272.8^{b}
N	357.7 ± 21.20^{b}	555.7 ± 78.90^{a}	485.9 ± 66.52^{b}
F	229.0 ± 11.35^{c}	658.3 ± 65.44^{a}	405.1 ± 64.77^{b}
H	287.8 ± 25.68^{b}	418.5 ± 34.35^{a}	460.2 ± 36.81^{a}

Mean ± SD, values with different superscript letters in the same row are significantly different ($p<0.05$)

In overall *D. longispina* showed an increase of toxicity with increasing temperature. Therefore potential of genetic erosion is increased with temperature rise. In contrast, results of *B. calyciflorus* experiments are suggesting that both 17^{o}C and 23^{o}C have a negative impact on *B. calyciflorus* populations compare to 20^{o}C the control temperature. Among 17^{o}C and 23^{o}C temperatures, for *B. calyciflorus* most disastrous effect of salinity occur at 17^{o}C.

4. Discussion

In this study $LC_{50, 24h}$ for *B. calyciflorus* genotypes ranged between 5.74 and 9.41 mS/cm with artificial sea salt. $LC_{50, 24h}$ of other studies with NaCl also recorded as 3.75 (±0.04) gL^{-1} (7.42 mS/cm) which also lay in the same range with our study (Peredo-Álvarez et al., 2003). Miracle and Serra (1989) reported that *B. calyciflorus* populations decrease its survival as the salinity rises from 2 to 10 gL^{-1} (3.95 mS/cm to 19.35 mS/cm) the negative slope being progressively steeped. Further with field observations, it is recorded family Brachionidae nearly absent when salinity exceeds 2 gL^{-1} (3.9 mS/cm) and in laboratory cultures of *B. calyciflorus* started with individuals of hatched resting eggs obtained high salt tolerance up to 5 g/L (9.9 mS/cm) (Greenwald and Hurlbert, 1993, Snell et al., 1991). According to Venâncio (2017), *D. longispina* genotypes used for this study show the $LC_{50, 48h}$ ranged from 2.68 to 4.99 mS/cm. In the study of Gonçalves et al. (2007) reported that $LC_{50, 48h}$ for *D. longispina* recorded as 2.2 gL^{-1} (4.35 mS/cm) to 2.9 gL^{-1} (5.74 mS/cm). El-Gamal et al. (2014) recorded LC_{50} for same species as 2.3 gL^{-1} (4.55 mS/cm) – 2.53 gL^{-1} (5.01 mS/cm). Four genotypes (except N91 and E84) used in our study are having similar salt tolerance level as Gonçalves et al (2007) and El-Gamal et al. (2014). Possible causes for poor survival of freshwater zooplankton under salt stress can be listed as a failure and high energy demand in osmoregulation, low swimming rate, low reproduction ability, mass mortality of neonates, biochemical changes and possible affects on algae (food) survival (Peredo-Álvarez et al., 2003, Smirnov, 2017).

This study relies on *D. longispina* (Cladoceran) and *B. calyciflorus* (rotifer) which has a parthenogenetic mode of reproduction, short life span and convenient in laboratory culturing can assure continuous passage of genetic information from founding mother to progeny (Loureiro et al., 2012). Six clonal lineages of *D. longispina* was initiated from laboratory cultures which maintained for more than 500 generations and six clonal lineages of *B. calyciflorus* started with isolated commercial cysts and all different clonal lineages of both species cultured for several generations until they reach stable populations under strictly control laboratory conditions (as mentioned in table 3 in 2.4 section of Materials and Methods). Therefore maternal and environmental effects were eliminated throughout this study. This leads to assume that differences observed in population occur due to their genetically determined variations. Further, observed population density fluctuations are the response of population solely to triggered selective pressure, in here salinity.

Sensitivity for salinity or any other selective pressure can be expressed as the lethal concentration that population can bear (e.g. LC_{50} – lethal concentration responsible for 50% mortality of population) or as lethal time (e.g. LT_{50} – time taken to result 50% of mortality of population after exposure). It is expected, clonal lineages representing high LC_{50} may express high LT_{50} due to their initial high tolerance ability showed in their standard toxicity assays. In contrary, our experiments regarding both species showed lack of correspondence in between LC_{50} and LT_{50} values. Similarly, Loureiro et al. (2012) also observed no compatibility in between $LC_{50,48h}$ and survival time (ST) in their experiments with Cladocera, *Simocephalus vetulus*. In the study of Lopes et al. (2005) obtained the positive relationship between LC_{50} and LT_{50} by exposing *D. longispina* to copper, but not for other ions they studied (Zinc, Cadmium, and Hydrogen). Hoffmann and Parsons (1991) stated that high-stress levels induce fast response genes which regulating hormones and protective enzymes, and then result behavioral and physiological changes. In the case of artificial seawater, zooplankton may be die-out due to other complicated series of reactions (which are not assist by fast response genes) rather than ionoregulatory failure. This will be a cause for lack of correspondence with regarding LC_{50} and LT_{50} obtained in our study.

Population genetic structure is the output of both past events that population went through and current evolution process (Fasola et al., 2015). Today many studies are established a direct relationship with individual tolerance limits with gene expression, then the relationship in between gene expression and phenotypic plasticity and further connectivity of plasticity and adaptation to novel environments (Latta et al., 2012). For example, It is observed genetic structure and diversity are important factors in invertebrate populations which are having limited dispersal capabilities (Scheffer et al., 2006). Many studies suggest that response to the salinity of zooplankton is strongly depend on genotype (Miracle and Serra, 1989, Anitha et al., 2015). According to Weider and Hebert (1987), it is further confirmed under salinity like selective pressures, even with a slight increase of salinity which is progressive and long-term, faunal populations can be subjected to subtle impacts such as arise or appearance of locally-selected races or “ecotypes”. In their study, they defined ecotypes as physiologically divergent individuals in particular species. Therefore these visible changes in populations are solely a response of a natural genetic variability in the population.

Comparative study of LT_{50} values obtained in short-term exposure (concerning exposure duration as 72 h and 92 h) with LT_{50} values of long-term exposure (*D. longispina* – 1128 h at 17^{o}C, 984 h at 20^{o}C and 744 h at 23^{o}C; *B. calyciflorus* – 816 h) showed no difference in our study of *D. longispina*. *D. longispina* did not show any recovering ability rather than a continuous decrease in population density. Similarly, Coldsnow et al. (2017) observed poor survival in highest concentrations, 1.9 $gCl^{-1}L^{-1}$ (3.76 mS/cm) and evolution of tolerance to moderate salinity levels, 1.3 $gCl^{-1}L^{-1}$ (2.57 mS/cm) within 2.5 months or 5-10 generations in their study with *Daphnia pulex*. Lethal salinity levels that used in over study for *D. longispina* is much higher, 4.80 mS/cm with artificial sea water than highest exposure level in the study of Coldsnow et al. (2017). Unbearable higher salt concentration may be a cause for not showing any recovery of *D. longispina* in our study.

In contrast, short-term LT_{50} values were very low comparatively to long –term LT_{50} values for *B. calyciflorus*. It shows the ability of *B. calyciflorus* for recovering in long-term exposure even population experienced deadly effects in initial short-term phase. Individuals of *B. calyciflorus* may have the ability to acclimate for studied salinity level (9.7 mS/cm) and population can adapt to this increased salinity level. Family Brachionidae concerned as much higher resistant species for salinity up to 9.9 mS/cm. Observed recovery may be confirmed again populations can recover under their intermediate lethal salinity levels (Coldsnow et al., 2017). There are many other studies based on zooplankton further demonstrated adaptability and differences between populations and genotypes to salinity (*Simocephalus vetulus* : Loureiro et al., 2012). High survival of *B. calyciflorus* was observed with gradual acclimation for salinity in some studies (Nagata, 1985). Possible reasons for this observed long-term recovery in *B. calyciflorus* populations may be due to their "phenotypic plasticity" which allows some genotypes to generate different phenotypes (physiological changes) to cope with changing the environment (Fasola et al., 2015). If not these observations may be solely due to chance or randomness.

Genetic diversity is a crucial factor of a population which prevents loss of potential on evolution in particular species (Ribeiro & Lopes, 2013; Lopes et al., 2009, Chen et al., 2012). Understand the potential risk of genetic erosion of zooplankton populations under different temperature regimes was a primary concern of our study. Different clonal lineages of both species showed different sensitivity under different temperatures. Comparing controls and treatments (LC_{70}) in both test species, always population density in controls was stable throughout study period while treatments show the continuous decay of population density

except very few cases (*B. calyciflorus* – P and D genotypes under 20^{o}C; *B. calyciflorus* – P genotype under 23^{o}C). It confirmed salinity has a negative effect on population density regardless the temperature difference. Ultimately continuous exposure for lethal salt concentration resulted disappearance of at least half of genotypes at the end of exposure duration. This confirmed increased salinity levels can eliminate sensitive genotypes of zooplankton population or it can cause genetic erosion in zooplankton populations. There are many studies confirmed that loss of genetic diversity or occurrence of genetic erosion in populations due to environmental pollution (Medina et al., 2007, Lopes et al., 2009, Ribeiro and Lopes, 2013, Fasola et al., 2015). Populations that expose to intense lethal pulses of contaminant can subject to microevolution due to genetic drift bottlenecks, mutations, loss of alleles and natural selection (with the disappearance of sensitive genotypes) (Ribeiro and Lopes, 2013). Both species in our study show loss of sensitive genotypes with continuous exposure to lethal salt concentration. Therefore it is an evidence for natural selection driven genetic erosion which results with a loss of sensitive genotypes of the population. Fasola et al. (2015), reviewed that loss of genetic diversity may further have an effect on fitness, environmental plasticity, co-tolerance and tradeoff mechanisms of the population and ultimately lead to the extinction of species.

Other than salinity, the temperature is a key factor for poikilothermic organisms like zooplankton which drive their body heat from the environment (Anderson-Carnahan, 1994**).** Temperature has a direct effect on their life history and population (Miracle and Serra, 1989; El-Gamal et al., 2014; Anitha et al., 2016). The combined effect of temperature and salinity was studied in our study to predict how temperature can change (enhance or suppress) toxicity of salinity. This can be used to predict effects of extreme events induced salinization, that can occur in summer and winter conditions (extreme temperature) on genetic diversity of zooplankton populations.

D. longispina showed a significant effect of temperature on their mortality. Other than most tolerant genotype (highest $LC_{50,24h}$ - E89), all other genotypes disclosed significant effect with studied temperatures. Similarly, El-Gamal et al. (2014) also showed there is the influence of temperature on toxicity of salinity. We observed the trend of increasing mortality with increasing temperature with the clonal lineages of *D. longispina*. Similarly, it is expected to have an inverse relationship with temperature and longevity which result longer life span in a colder environment in zooplankton studies (MacArthur and Baillie, 1929)**.** In contrast, the study of El-Gamal et al. (2014) reported the best survival of *D. longispina* at

25°C (90%), following 20°C (74%), 30°C (36%) and 15°C (30%) survival in their chronic assay. Possible reasons for observed increased mortality in highest studied temperature (23°C) may be due to increased respiration and other metabolic activities which allow them to intake more toxicant into their body. In contrast, low temperatures may cause a low intake of toxicant due to reducing body activity (Bullock, 1955). According to obtained results with *D. longispina* salinization events occur in summer higher temperatures may have a high risk of genetic erosion on *D. longispina* than winter sea flooding.

Temperature has a direct effect on intrinsic growth rate of rotifer populations (Miracle and Serra, 1986). In our study, we observed under 20°C have less probability to occur genetic erosion than other 2 temperatures on rotifer population. Among 17°C and 23°C, under 17°C rotifers have the highest probability to occur genetic erosion. Anitha et al. (2016) reported that highest reproductive success and instantaneous growth rate (r) (population growth rate in prescribed time) at 29°C in their study with *Brachionus angularis*. Effects of temperature mainly regulate developmental rates and metabolic activities of organisms. Life span and temperature has an inverse relationship where high temperatures result acceleration of development rates. Miracle and Serra (1986) further stated that temperature (within suitable values for the normal functioning of the organisms) influences the timing, but not fertility (e.g. the number of neonates per individual). Therefore an increase of instantaneous growth rate of rotifers is the results from the acceleration of development. Since our study also conducted in 20±3°C, may be 20°C is the optimum temperature for many rotifer genotypes we studied. Lower LT_{90} values of particular genotypes may be due to shorter life span at 17°C and high metabolic activity which increased toxic intake at 23°C. Comparing both model species used in our study, there is high potential to occur genetic erosion under 17°C and 23°C than 20°C. In other words, zooplankton populations there is high risk to occur genetic erosion with the climate change induce salinization events which occur in winter and summer conditions or with extreme events.

To sum up the overall discussion, Climate change scenarios already predicted, increasing salinity of freshwater systems (IPCC, 2014). Nielsen et al. (2003) reported that freshwater ecosystems in Australia may increase salinity to the range of 500 mg/L (1 mS/cm) - 10000 mg/L (20 mS/cm) by next 50 years. In overall with this study we observed; genetic erosion or elimination of sensitive genotypes in zooplankton populations is possible due to increased salinity levels. Also, summer and winter temperatures have a high probability to enhance this negative effect on genetic diversity. Reasons for the observed genetic erosion of tested

zooplankton species in this study can be explained with the aid of genetic erosion hypothesis (Ribeiro and Lopes, 2013). Populations that expose to intense lethal pulses of contaminant can subject to microevolution due to genetic drift bottlenecks, mutations, loss of alleles and natural selection (with the disappearance of sensitive genotypes) (Ribeiro and Lopes, 2013). Results of our study are an evidence for natural selection driven genetic erosion which results loss of sensitive genotypes of the population. This may increase the susceptibility of the population for future stresses and ultimately it may lead to the extinction of particular species (Ribeiro and Lopes, 2013; Venâncio et al., 2016).

5. Conclusion

Climate change induced salinization events are evident in freshwater ecosystems (Jiménez Cisneros, 2014). Also, extreme events that can cause increased salinity in freshwater ecosystems such as summer droughts and winter sea flooding are increasing in magnitude and frequency (Hartmann, 2014). Laboratory experiments conducted with populations of rotifer, *B. calyciflorus* and cladoceran, *D. longispina* exposing to lethal concentration (LC_{70}) of artificial sea water continuously under different temperature regimes, we observed that;

- There is lack of correspondence with lethal concentration (LC_{50}) and lethal time (LT_{50}) in studied zooplankton populations.
- Both studied zooplankton species show exponential decay of population density in short term (72 h, 96 h) while only few genotypes of *B. calyciflorus* show recovery ability or adaptability to studied intense high salinity level (9.7 mS/cm), which is twice the exposure level of *D. longispina* (4.8 mS/cm) in long-term scale.
- There is a loss of sensitive genotypes or genetic erosion with continuous exposure to lethal salinity level in both species.
- *D. longispina* showed induced trend of genetic erosion with increasing temperature while *B. calyciflorus* suggest high risk of genetic erosion in both studied extreme temperatures (17^oC and 23^oC)

Bearing in mind the objectives of this work, the obtained results suggest:

- ✓ Climate change induce salinization can cause genetic erosion specially through loss of sensitive species or with natural selection in freshwater zooplankton populations.

- ✓ Under extreme temperatures that use to mimic conditions of climate change induced extreme events such as summer droughts (23^oC) and winter sea flooding (17^oC); can enhance genetic erosion further in *B. calyciflorus* while for *D. longispina* only summer high temperatures induce negative effects on genetic diversity.

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