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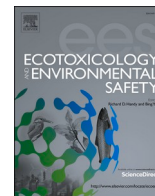
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# First evidence of lithium toxicity in the cryptic species complex of the estuarine copepod *Eurytemora affinis*

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## ABSTRACT

The development of renewable and low-carbon energy sources means that strategic elements such as lithium (Li) are increasingly being used. The data available on the effects of Li on aquatic organisms are relatively scarce. The copepod *Eurytemora affinis*, widely distributed in the brackish estuarine waters of the northern hemisphere, is a species of choice in ecotoxicology but in fact constitutes a cryptic species complex, composed of at least six cryptic species. Cryptic diversity can lead to misinterpretation and alter the reproducibility of routine ecotoxicological tests. In the present study, two cryptic species of the *E. affinis* complex from the Seine (European clade) and the St. Lawrence (North-Atlantic clade) estuaries were used to assess Li toxicity and to compare their differential sensitivity. Larvae were exposed to different concentrations of Li (0.4, 4.39, 35.36 and 80.83 mg L<sup>-1</sup>) under semi-static conditions for 96 h. Larval development stages were determined and log-logistic functions were fitted to evaluate mortality (LC<sub>50</sub>) and growth (EC<sub>50</sub>) parameters. After 96 h of exposure, the results showed that the European and North-Atlantic clades had LC<sub>50</sub> values of 55.33 and 67.81 mg L<sup>-1</sup> and EC<sub>50</sub> values of 28.94 and 41.45 mg L<sup>-1</sup>, respectively. A moderate difference in sensitivity to Li between the European and North-Atlantic clades of the *E. affinis* complex was observed. Thus, the cryptic species diversity should be considered using *E. affinis* to avoid bias in the interpretation of the data. Despite environmental concentrations of Li are expected to increase over the next years, EC<sub>50</sub> and LC<sub>50</sub> found for *E. affinis* cryptic species are largely higher than Li environmental concentrations to provoke extreme effects.

## 1. Introduction

Lithium (Li) is an alkali metal that occurs naturally in stable minerals and salts. It is currently considered to be one of the emerging pollutants of concern due to its high demand associated with the production of rechargeable batteries (Li-ion batteries) for mobile electronics, electric and hybrid vehicles as well as for green technologies applied in the energy transition (Bibienne et al., 2020). Over the last decade, Li production has intensified and is expected to increase over the next few years at an annual growth rate of between 18 % and 30 %, mainly due to the need for Li-ion batteries (Melchor-Martínez et al., 2021). Naturally present in freshwater, this element is also found in coastal and marine water at concentrations of up to several hundred µg L<sup>-1</sup> (Barbosa et al., 2023). In extreme case, high Li concentrations were found near polluted areas such as in the Donbass rivers (Ukraine) with 13.7 mg L<sup>-1</sup> due to

mine wastewater discharges (Barbosa et al., 2023). However, the effects of Li on marine and coastal organisms remain relatively unknown. The determination of these effects is especially of importance in a context where Li concentrations in the environment are expected to increase due to the strong demand for this element (Barbosa et al., 2023).

The calanoid copepod *Eurytemora affinis* is distributed in brackish and estuarine waters all over the Holarctic. It is also found in the freshwater zones of estuaries, lakes and rivers (Chambord et al., 2016; Lee, 1999; Winkler et al., 2008). *E. affinis* is a dominant zooplankton species in estuaries and occurs during most of the year with a maximal abundance in mid-spring such as in the Seine estuary (France, Mouny and Dauvin, 2002). Due to its large biomass, it occupies a central position in estuarine food webs (Dauvin and Desroy, 2005). Consequently, *E. affinis* has been widely used for ecotoxicological studies (Arcanjo et al., 2021; Cailleaud et al., 2011; Forget-Leray et al., 2005; Kadiene

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et al., 2017; Legrand et al., 2017; Lesueur et al., 2013, 2015; Stillway and Teh, 2020). Initially considered as a single species, phylogenetic analysis revealed a high level of genetic differentiation within *E. affinis* populations with a maximum sequence divergence of 10 % in the large subunit (16S) rRNA and 19 % in cytochrome c oxydase I (Lee, 1999, 2000; Lee and Frost, 2002). Thus, the existence of a cryptic complex species *E. affinis* composed of at least six clades was suggested. Subsequent morphological studies have provided evidence that the cryptic complex species *E. affinis* currently comprises at least three valid species: *Eurytemora carolleeae* from North America (Alekseev and Souissi, 2011), *Eurytemora capsica* from the Caspian Sea (Sukhikh and Alekseev, 2013) and *Eurytemora affinis* (Poppe, 1880) composed of unidentified cryptic species within the complex such as the North-Atlantic and the European clades. The existence of sympatric relation between these different species has been demonstrated. Particularly, in the St. Lawrence estuary (Canada), the North-Atlantic clade of *E. affinis* and *E. carolleeae* coexist and show partial spatial segregation (Favier and Winkler, 2014; Winkler et al., 2008). The *E. affinis* North Atlantic clade generally occupies the central part of the estuarine transition zone whereas *E. carolleeae* is found upstream and downstream from the *E. affinis* North Atlantic clade. *E. carolleeae* is known to invade both fresh and brackish waters. To date, its presence in Europe has been recorded in the Gulf of Finland, the Gulf of Riga, Amsterdam channels, the Oder River, Kiel Bight, Mecklenburg Bight, the Arkona Sea, the Bornholm Sea and the Eastern Gotland Sea (Stugocki et al., 2021; Sukhikh et al., 2013, 2019, 2020; Sukhikh and Alekseev, 2023).

It is known that cryptic species may have different sensitivities to pollutants. A study of Weston et al. (2013) demonstrated that the sensitivity of the amphipod *Hyaella azteca* cryptic species to the pyrethroid insecticide cyfluthrin differed by at least 550-fold. Two different lineages of the amphipod *Gammarus fossarum* exposed to  $1.0 \mu\text{g L}^{-1}$  of thiacloprid showed a difference in feeding rate of around 50 % (Feckler et al., 2012). In the same way, two cryptic species of the nematode *Litoditis marina* exhibit different growth rate and fecundity in response to copper (Monteiro et al., 2018). These studies highlight that ignoring the cryptic diversity can lead to a misinterpretation and alter the reproducibility of routine ecotoxicological tests (Jourdan et al., 2023). Studies reporting results on differential sensitivities to pollutants within the *E. affinis* complex are very rare. Engström-Öst et al. (2017) compared the effect of toxic cyanobacteria on the *E. carolleeae* freshwater population in Lake Michigan and on *E. affinis* in the Gulf of Finland. Their results showed that the two cryptic species respond differently with lower survival of adults and nauplii size in *E. affinis* compared to *E. carolleeae* after exposure to cyanobacteria filtrate.

The objectives of the present study were 1) to determine for the first time the effect of Li on two cryptic species within the *E. affinis* estuarine complex (the European and North-Atlantic clades) using a larval-bioassay and 2) to assess whether there are differences in responses to Li contamination between these two cryptic species. Lethal concentrations (LC) and effective concentrations on growth (EC) of larvae (nauplii) from the Seine and the St. Lawrence estuaries were determined using a semi-static bioassay for 96 h.

## 2. Materials and methods

### 2.1. Experimental solutions

A stock solution ( $20,000 \text{ mg L}^{-1}$ ) was prepared by diluting LiCl (99 %, VWR) in artificial brackish water at a salinity of 5 PSU. Artificial brackish water was prepared following the protocol of Artoxkit (2003). Five nominal concentrations were chosen for the toxicity tests based on preliminary trials: 0, 1, 10, 50 and  $100 \text{ mg L}^{-1}$  of Li. Exposure solutions were prepared by serial dilution from the stock solution.

### 2.2. Life cycle of *Eurytemora affinis*

The life cycle of *E. affinis* is divided in three phases characterised by successive moults with metamorphosis. The larval phase (naupliar) is composed of six stages (N1 to N6) followed by the juvenile and adult phases. *E. affinis* is an egg-bearing copepod. Ovigerous females carry their eggs in a sac attached to the base of the prosome until hatching. N1 stages measure approximately  $100 \mu\text{m}$  (Katona, 1971).

### 2.3. Sampling procedures and acclimatisation

Due to logistic reasons, the experimental design was divided in two parts. Experiments were firstly realized in the laboratory of UQAR-ISMER in Rimouski (Canada) and then in the laboratory of SEBIO Le Havre (France) for the St. Lawrence and the Seine estuary copepods, respectively. Copepods from the St. Lawrence estuary were sampled at Saint-Jean-Port-Joli (Canada,  $47^{\circ}13'17.688''\text{N}$ ,  $70^{\circ}19'13.548''\text{W}$ ) in August 2023 and those from the Seine estuary were sampled at Villequier (France,  $49^{\circ}30'51.638''\text{N}$ ,  $0^{\circ}40'36.915''\text{E}$ ) in October 2023. Temperature and salinity conditions were relatively similar for both sites with  $19.9^{\circ}\text{C}$ , 0.9 PSU and  $15.2^{\circ}\text{C}$ , 0.3 PSU for the St. Lawrence and the Seine sampling sites, respectively. For both sites, organisms were collected below the surface using a  $250 \mu\text{m}$  plankton net, then sieved using a 1 mm mesh to eliminate large particles and predators. The samples were then transferred to isothermal containers diluted with water from the sampling site for transport to the laboratory. Upon arrival, they were placed into a 2 L beaker containing 1 L of water from the sampling site and gradually acclimatised overnight to the experimental conditions ( $15^{\circ}\text{C}$ , 5 PSU, Lesueur et al., 2013). After the acclimatisation period, the ovigerous females were incubated for 24 h (12 h L: 12 h D) into a homemade hatching chamber composed of a plastic beaker where the bottom was replaced by a  $200 \mu\text{m}$  mesh, placed into a slightly bigger beaker until hatching of nauplii. Females and the early life stages were fed *ad libitum* ( $15,000 \text{ cells mL}^{-1}$ ) with Cryptophyceae algae. A mixture of *Rhodomonas minuta* and *Rhodomonas salina* were used for the experiments conducted with nauplii from the St. Lawrence estuary population and *R. salina* for those from the Seine estuary population. Due to their high composition of polyunsaturated fatty acids, these algae are considered optimal for the growth of *E. affinis* (Dunstan et al., 2005; Vanderploeg et al., 1996).

### 2.4. Toxicity test

The toxicity test used in this study was adapted from Lesueur et al. (2013), (2015). Exposures were performed in 6-well microplates (Greiner Bio-One) which were saturated 24 h before the beginning of the experiments with 10 mL of exposure solutions to avoid Li adsorption. Hatched nauplii were randomly distributed in six replicates per exposure concentrations (10 nauplii per replicate). Microplates were covered to prevent evaporation and nauplii were maintained under semi-static conditions for 96 h (12 h L: 12 h D). Experimental medium was renewed after 48 h by transferring the nauplii to another 6-well plate that had been saturated beforehand. The nauplii were fed at 0 h and 48 h of exposure with an algal suspension concentrated in the exposure solution ( $15,000 \text{ cells mL}^{-1}$ ). Survival rate was determined by counting the number of livings nauplii. The nauplii were considered dead when no movement was observed for at least 10 s. At 96 h, live nauplii were fixed in 95 % ethanol and stored at  $-20^{\circ}\text{C}$  until growth analysis and staging using a stereomicroscope (Olympus SZX16 or Leica MZ10F) and image software (cellSens standard 3.2 program or Image J). Larval growth was calculated by subtracting the mean body length of 50 nauplii measured at the start of the experiment from that of live larvae at 96 h. Ontogenetic development of the nauplii was determined at 0 h and 96 h using the method of Katona (1971).

## 2.5. Cryptic species identification

Given the possible co-occurrence of *E. affinis* (North-Atlantic or European clade) and *E. carolleae* in our sampling sites and the difficulty of establishing morphological discrimination between cryptic species when they are alive, *a posteriori* identification was conducted. Briefly, a subsample of 20 females were isolated and stored at  $-20^{\circ}\text{C}$  in 95 % ethanol after each sampling. Prosome length (PL), width of the anterior (W1) and posterior (W2) parts of the genital somite were measured on females for each experiment (Fig. 1). The genital double-somite ratio (W1/W2) is generally larger in *E. carolleae* than in the North-Atlantic or European clades of *E. affinis* (Alekseev and Souissi, 2011; Sukhikh et al., 2013; Winkler et al., unpublished). The combined PL/W1 and W1/W2 ratios were used as predictor of cryptic species identity based on previous results from Winkler et al. (unpublished).

The morphological identifications were confirmed by the genetic analysis as described in Avila et al. (2023). Briefly, females were passed through two consecutive baths of TRIS (1 M, pH 8.0, VWR) for 5 min to remove 95 % ethanol before DNA extraction with QuickExtract™ Solution 1.0 (Biosearch Technologies, UK). DNA was amplified using specific primers of *E. affinis* North-Atlantic clade and *E. carolleae* (Table 1), using the following PCR protocol: initial denaturation at  $95^{\circ}\text{C}$  for 10 s, 5 cycles (denaturation at  $90^{\circ}\text{C}$  for 30 s, annealing at  $45^{\circ}\text{C}$  for 10 s and elongation at  $72^{\circ}\text{C}$  for 60 s), followed by 28 cycles (denaturation at  $90^{\circ}\text{C}$  for 30 s, annealing at  $55^{\circ}\text{C}$  for 45 s and elongation at  $72^{\circ}\text{C}$  for 60 s) and finally and elongation at  $72^{\circ}\text{C}$  for 5 min. PCR products were visualised on 1.2 % agarose gels by electrophoresis (90 V, 20 min) and species identified by length of the PCR products with the Gel Imaging System (ChemiDoc™ XRS; BIORAD).

## 2.6. Verification of lithium exposure concentrations

Water samples were taken from each testing medium for each experimental condition after 48 h exposure, before medium renewal, and at 96 h. They were immediately stored at  $-80^{\circ}\text{C}$  until analysis. Water samples were diluted with ultrapure water acidified with nitric acid (1 %) to fall within the calibration range ( $0.1\text{ mg L}^{-1}$  to  $10\text{ mg L}^{-1}$ ). Lithium Standard solution for ICP  $1\text{ g L}^{-1}$  (Supelco reference: 12292) was used as standard. Water samples were analysed in triplicates to determine experimental Li concentrations by ICP-AES (ICAP 6300 Thermo) equipped with the Qtegra software at  $460.286\text{ nm}$ . The limit of quantification (LOQ) was  $0.12\text{ mg L}^{-1}$ .

## 2.7. Data analysis

Statistical procedures were performed using R studio (R Development Core Team, 2011). The normality of the data was verified using the Shapiro-Wilk test and the homoscedasticity of the variance was verified



Fig. 1. Photo showing the measurements taken on a female *E. affinis* (PL: prosome length, W1: width of the anterior part of the genital somite, and W2: width of the posterior part of the genital somite).

Table 1

Primers from the cytochrome c oxidase subunit I (COI) used by Avila et al. (2023) for the identification of the two *Eurytemora affinis* cryptic species found in the St. Lawrence estuary.

Species	Forward (5'-3')	Reverse (5'-3')
<i>E. affinis</i> North-Atlantic clade	ACCTTAGGGAAGTTCGAGC	ACAAAATCGGATCTCCGCC
<i>E. carolleae</i>	CTCACGCAGGTAGGTCTGTC	CCCCTCCACTTGCCTCATAA

using the Levene's test. Concentration-response curves were fitted to each data set. Lethal concentrations (LC) and effect concentrations (EC) were determined using the R-package drc (Ritz et al., 2015). The 3-parameters log-logistic function (Eq. 1) was chosen as it provided the best fit based on AIC scores.

$$f(y) = 0 + \frac{d - 0}{1 + \exp(b(\log(x) - \log(e)))} \quad (1)$$

Where  $y$  is the response of endpoint measured (survival or growth);  $d$  the response of endpoint measured at  $x = 100$ ;  $b$  the slope parameter;  $x$  the Li concentration; and  $e$  the  $\text{EC}/\text{LC}_x$ . A  $z$ -test implemented by the compParm() function of the R-package drc was used to determine  $P$ -values and compare  $\text{EC}/\text{LC}_{50}$  values between the two populations.

We adopted the language of evidence to discuss our results rather than in traditional significance (Muff et al., 2022). Statistical evidence is based on a range of  $p$ -values as following: very strong ( $< 0.001$ ), strong ( $< 0.01$ ), moderate ( $< 0.05$ ), weak ( $< 0.1$ ) and little or no evidence ( $> 0.1$ ).

## 3. Results and discussion

### 3.1. Species identification

The prosome length (PL), the width of the anterior (W1) and the posterior (W2) parts of the genital somite were measured on a subsample of 20 females for each sampling. The PL/W1 ratio ranged from 5.32 to 7.65 and from 5.47 to 6.38 for the females from the Seine and the St. Lawrence estuary, respectively (Fig. 2). For the W1/W2 ratio, values ranged from 1.09 to 1.37 and from 1.07 to 1.34 and for the females sampled in the Seine and in the St. Lawrence estuary, respectively. Based on this morphological analysis, we can assume that only the *E. affinis* European clade and the North-Atlantic clade were found in the Seine and in the St. Lawrence estuaries respectively during our sampling.

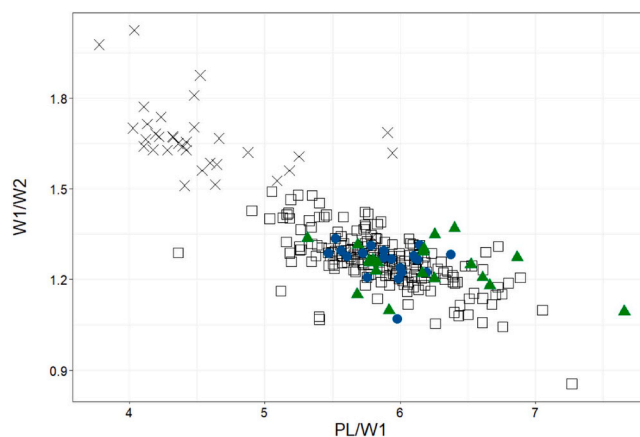


Fig. 2. Morphological indexes PL/W1 and W1/W2 used for discriminating the two *Eurytemora affinis* cryptic species. Cross represent *E. carolleae* and squares *E. affinis*. Data are from Winkler G. (unpublished data). They confirm cryptic species identity using COI genotyping (Avila et al., 2023). Individuals from the present study are represented in green triangles for the Seine estuary and blue dots for the St. Lawrence estuary females, respectively.

### 3.2. Chemical characterisation of exposure concentrations

Copepod nauplii were exposed to nominal concentrations of 1, 10, 50 and 100 mg L<sup>-1</sup>. Testing medium for each experimental concentrations were sampled at 48 h and 96 h exposure to obtain an average exposure value over the duration of the bioassay. ICP-AES analysis revealed that the Li concentration for the stock solution was 95.5 % of the nominal concentration (Table 2). The mean Li concentrations measured were all below the nominal concentrations and ranged from 40 % (1 mg L<sup>-1</sup>) to 80.83 % (100 mg L<sup>-1</sup>) of nominal concentrations. The Li concentration for the water in control condition was below the limit of quantification (0.12 mg L<sup>-1</sup>).

The difference between nominal and measured concentrations are probably due to multiple factors. First, the stock solution was 95.5 % of nominal concentrations, explaining that measured concentrations were below nominal concentrations for the exposure solutions. On the other hand, it might be also due to bioaccumulation of Li by copepods and algae during the exposure time. A recent study demonstrated the capacity of *E. affinis* to bioaccumulate metals (copper, nickel, lead, and arsenic) at lethal or sublethal concentrations from polluted sediments after 96 h of exposure (Das et al., 2022). Kadiene et al. (2019) showed that *E. affinis* accumulates dissolved cadmium and nickel by oral pathway. It is also known that algae can bioaccumulate metals (Arunakumara and Zhang, 2008) such as *R. salina* with copper (Debelius et al., 2009).

### 3.3. Li effects on naupliar growth, development, and survival

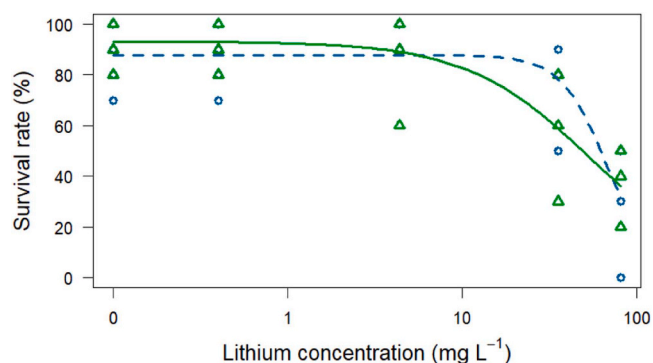
Nauplii hatched from wild-caught ovigerous females from two different *E. affinis* cryptic species that were used to assess Li effects on survival and growth for 96 h. Mean survival rate in controls was 90 ± 3.16 % and 83.33 ± 4.22 % for the nauplii from the European and the North-Atlantic clades, respectively (Fig. 3). No changes in survival rates were observed from 0.40 to 4.39 mg L<sup>-1</sup> for both clades. Survival rate decreased at the highest concentrations (35.36 mg L<sup>-1</sup> and 80.83 mg L<sup>-1</sup>) until it reached 36.67 ± 3.16 % for the European clade and 31.67 ± 7.49 % for North-Atlantic clade nauplii. The LC<sub>50</sub> was 55.33 ± 1.18 mg L<sup>-1</sup> for the European clade and 67.81 ± 1.09 mg L<sup>-1</sup> for the North-Atlantic clade after 96 h of exposure (Table 3).

This is the first study to report LC<sub>50</sub>, 96 h values (55.33 and 67.81 mg L<sup>-1</sup>) for Li in a European and North-Atlantic estuarine crustacean, respectively. Acute toxicity tests have been previously used to assess the Li effects on different aquatic taxa with various ranges of LC<sub>50</sub> (Table 4). For the cyprinid *Tanichthys albonubes*, a wide range of LC<sub>50</sub> values from 9.2 to 62 mg L<sup>-1</sup> after 48 h of exposure was found (Aral and Vecchio-Sadus, 2008). The bivalve *Dreissena polymorpha* appears to be the most resistant freshwater taxa with LC<sub>50</sub> values ranged from 185 to 232 mg L<sup>-1</sup> at 24 h (Aral and Vecchio-Sadus, 2008). Chen et al. (2024) found LC<sub>50</sub> values for the freshwater cladoceran *Daphnia magna* under static conditions of 10.2 and 4.1 mg L<sup>-1</sup> at 24 h and 48 h, respectively. Compared to our results, *D. magna* is more sensitive to Li than *E. affinis*. However, there is still little information available on acute toxicity of Li on marine and coastal organisms. Fraga et al. (2022) reported LC<sub>50</sub> of

**Table 2**  
Concentrations of Li in experimental water.

Li concentrations (mg L <sup>-1</sup> )		
Nominal	Measured (± SD)	% of nominal concentration
20,000 (stock solution)	19,098.00	95.5
0	< QL <sup>a</sup>	100
1	0.40 (± 0.04)	40
10	4.39 (± 0.23)	43.9
50	35.36 (± 2.59)	70.7
100	80.83 (± 9.73)	80.83

<sup>a</sup> Quantification limit (QL) = 0.12 mg L<sup>-1</sup>.



**Fig. 3.** Concentration-response curve for the survival of the European (green triangles and full line) and the North-Atlantic (blue dots and dotted line) clade nauplii after 96 h of exposure to different concentrations of Li.

**Table 3**

Lethal (LC<sub>50</sub>) and effective (EC<sub>50</sub>) concentration values (mg L<sup>-1</sup>) and 95 % confidence intervals (95 % CI) calculated for the two *E. affinis* cryptic species exposed 96 h to different Li concentrations (0.4, 4.39, 35.36 and 80.83 mg L<sup>-1</sup>).

Clades	Survival		Growth	
	LC <sub>50</sub> 96 h ± SE (mg L <sup>-1</sup> )	95 % CI	EC <sub>50</sub> 96 h ± SE (mg L <sup>-1</sup> )	95 % CI
European	55.33 ± 1.18	39.57 – 77.37	28.94 ± 1.10	23.88 – 35.07
North-Atlantic	67.81 ± 1.09	57.32 – 80.21	41.45 ± 1.16	31.14 – 55.19

**Table 4**

Comparison of LC<sub>50</sub> values for Li found for different taxa.

Species	LC <sub>50</sub> (mg L <sup>-1</sup> )	Duration test (h)	Reference
<i>E. affinis</i> (North-Atlantic clade)	67.81	96	This study
<i>E. affinis</i> (European clade)	55.33	96	This study
<i>Mytilus galloprovincialis</i>	1665.42	96	Fraga et al. (2022)
<i>Tanichthys albonubes</i>	9.2–62	48	Aral and Vecchio-Sadus (2008)
<i>Daphnia magna</i>	4.1	48	Chen et al. (2024)
<i>Daphniopsis tibetana</i>	325.4	48	Huo et al. (2011)
<i>Dreissena polymorpha</i>	185–232	24	Aral and Vecchio-Sadus (2008)
<i>Daphnia magna</i>	10.2	24	Chen et al. (2024)

1665.42 mg L<sup>-1</sup> at 96 h for the bivalve *Mytilus galloprovincialis* under semi-static conditions. The noted LC<sub>50</sub> value decrease to 153.78 mg L<sup>-1</sup> at 9 days of exposure. Huo et al. (2011) established that LC<sub>50</sub> value at 48 h was 325.4 mg L<sup>-1</sup> for the cladoceran *Daphniopsis tibetana*. Values found in the literature for marine and coastal organisms are largely higher than the LC<sub>50</sub> found for *E. affinis* cryptic species. All these studies highlight that the sensitivity to Li is species-specific. Based on the few existing studies, freshwater species are likely to be more sensitive to Li than coastal and marine species due to an antagonist effect of sodium on Li toxicity as suggested by Kszos and Stewart (2003). This would position the cryptic species of the *E. affinis* complex as estuarine species in the middle between freshwater and marine species in terms of sensitivity to Li.

Measurement of nauplii size provides additional information on the potential sublethal effects of Li on the growth of early developmental stages. The mean growth of nauplii in controls was estimated at 118.58 ± 4.44 μm and 42.83 ± 4.17 μm after 96 h for the European and the North-Atlantic clades, respectively (Fig. 4). No effect on growth was observed at the two lowest exposure concentrations (0.4 and

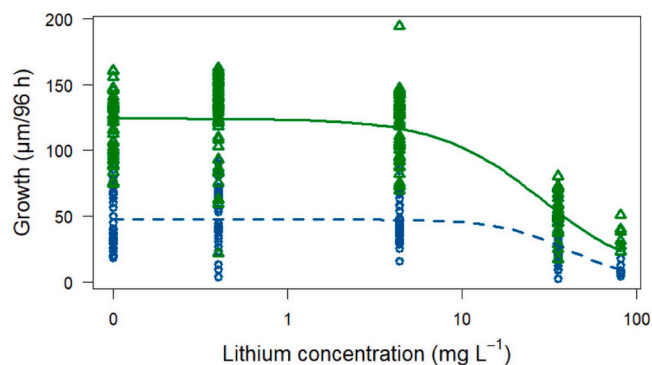


Fig. 4. Concentration-response curve for the growth of the European (green triangles and full line) and the North-Atlantic (blue dots and dotted line) clade nauplii nauplii after 96 h of exposure to different concentrations of Li.

4.39 mg L<sup>-1</sup>) for the two clades. However, an inhibition of approximately 70 % of growth compared to the control was observed at the highest concentration of 80.83 mg L<sup>-1</sup>. The concentrations for which a 50 % inhibition of nauplii growth was observed (EC<sub>50</sub>, 96 h) were 28.94 ± 1.10 mg L<sup>-1</sup> for the European clade and 41.45 ± 1.16 mg L<sup>-1</sup> for the North-Atlantic clade after 96 h exposure to Li (Table 3).

The effects on nauplii larval development were assessed through the percentage of larval development stages at 0 h and 96 h. Populations were composed of 80 % and 21 % of naupliar stage N1 and 20 % and 79 % of naupliar stage N2 for the European and the North-Atlantic clades, respectively, before exposure to Li (0 h) (Fig. 5A). The number of moults observed in individuals exposed to Li was different compared to controls for both clades at the two highest exposure doses. For the European clade, nauplii exposed to the lowest exposure concentrations underwent two to three moults, one to two moults at 35.36 mg L<sup>-1</sup> and zero to one moult in the case of exposure to the highest Li concentration (80.83 mg L<sup>-1</sup>; Fig. 5B). At least 50 % of the living nauplii reached the stage N5 until 4.39 mg L<sup>-1</sup>, whereas at highest concentrations (80.83 mg L<sup>-1</sup>) they only reached the stage N2. For the North-Atlantic clade, the number of moults varied from one to two up to 4.39 mg L<sup>-1</sup> and up to one moult for the highest concentrations (Fig. 5C). In contrast to nauplii of the European clade, the maximum development stage reached by nauplii of the North-Atlantic clade was lower with N4 at the lowest concentrations, despite the higher N2 proportion at 0 h.

Li inhibits the growth and the development of *E. affinis* cryptic species above 35 mg L<sup>-1</sup>. As there are very few studies reporting growth

inhibition related to Li exposure in crustaceans (Chen et al., 2024; Martins et al., 2022), comparisons are difficult. A growth inhibition of 39 % compared to the control was found for *Daphnia magna* at 750 µg L<sup>-1</sup> after 21 d of exposure (Chen et al., 2024). Li is a neurotoxic substance known to inhibit acetylcholinesterase activity (Chen et al., 2024; Oliveira et al., 2011; Viana et al., 2020), interfere with Na<sup>+</sup>/K<sup>+</sup> ATPase activity (Chen et al., 2024; Kszos and Stewart, 2003; Tkatcheva et al., 2015) and affect the synthesis of neurotransmitters such as dopamine (Nagato et al., 2013). These neurotoxic effects can lead to changes in osmoregulatory processes, behaviour, growth, and reproduction (Martins et al., 2022; Wilder et al., 2000). Observation of live nauplii revealed more restricted mobility after 24 h of exposure to 35.36 and 80.83 mg L<sup>-1</sup> compared to the lower concentrations. Impaired swimming behaviour observed at higher Li concentrations might be the result of damage to the nervous system and might have impaired effectiveness of food intake resulting then in a lack of energy. Furthermore, nauplii might devote part of their energy to detoxification processes resulting in a reduction of growth.

The LC<sub>50</sub> and EC<sub>50</sub> found for cryptic species of *E. affinis* are too high to cause extreme effects based on the Li concentrations reported in the literature for the Seine and the St. Lawrence estuaries. Li levels in water ranging from 17.4 to 124 µg L<sup>-1</sup> (Stoffyn-Egli, 1982) and from 15 to 35 µg g<sup>-1</sup> in sediments (Loring, 1990) have been reported from the St. Lawrence estuary. A more recent study in the Seine estuary reported higher values of around 40 µg g<sup>-1</sup> in sediments (Menet-Nedelec et al., 2016). Natural events and anthropogenic activities, such as dredging, can lead to the remobilisation of pollutants into the water column. This can make a pollutant, such as Li, bioavailable to organisms. It is recognised that Li concentrations in the environment are likely to increase over the next few years due to the high demand for this element, particularly for Li-ion batteries, and could reach high levels in aquatic systems. Even in extreme cases, such as in the Donbass River with 13.7 mg L<sup>-1</sup>, the values for EC<sub>50</sub> and LC<sub>50</sub> determined in this study are even higher. Despite the lack of evidence of extreme effects of Li at environmentally relevant concentrations on marine and coastal organisms, sublethal effects such as reduced population fitness, histopathological alteration and biomarkers should not be underestimated (Chen et al., 2024; Fraga et al., 2022; Martins et al., 2022).

#### 3.4. Sensitivity of species within the cryptic complex to Li

There was no evidence that the survival rate differs between the two clades in response to Li exposure based on their LC<sub>50</sub> values (z-test, *P* = 0.28). However, our data revealed that there was moderate evidence

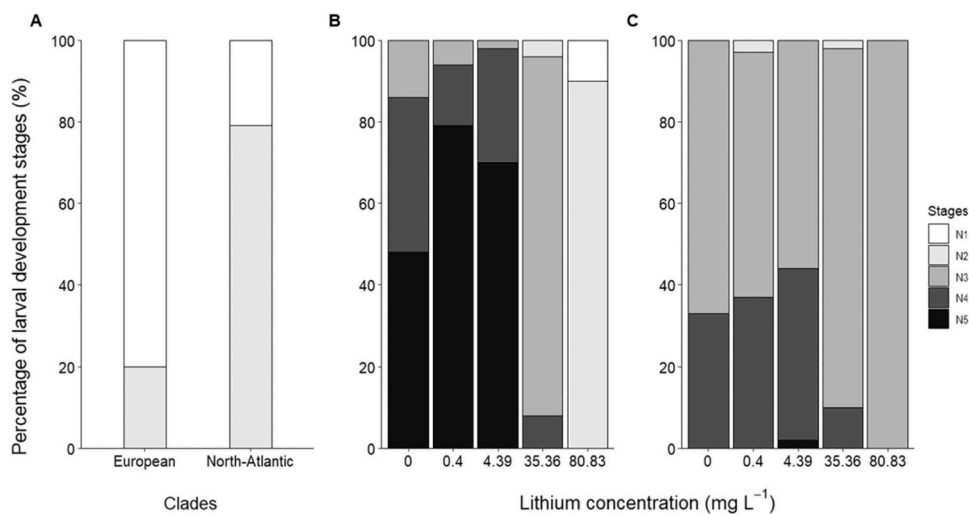


Fig. 5. Percentage of larval development stages (%) at 0 h for the two clades (A) and at 96 h of Li exposure of nauplii from the European (B) and the North-Atlantic (C) clades.

that the EC<sub>50</sub> values for the European clade are lower than those for the North-Atlantic clade (z-test,  $P = 0.041$ ).

Surprisingly, these results differ from most studies that have focused on cryptic species in ecotoxicology, which have shown a marked differential sensitivity among cryptic species (Feckler et al., 2012; Monteiro et al., 2018; Rocha-Olivares et al., 2004; Soucek et al., 2013; Weston et al., 2013). The differential sensitivity found for growth between the two clades might be explained by genetics. It has been previously demonstrated that some mutations might lead to a differential tolerance to pollutant. Weston et al. (2013) sequenced the primary pyrethroid target site in *Hyaella azteca* and found point mutations that led to the differential tolerance to pyrethroid among cryptic species. Furthermore, chemical quality of an environment can induce differential sensitivity. However, results found in the literature are sometimes divergent. Organisms originated from polluted sites tend to be more resistant than those from sites where anthropogenic activities are scarce (Olima et al., 1997). This phenomenon can be explained by a previous acclimation to pollutants of the organisms sampled in sites more prone to anthropogenic activities (Olima et al., 1997) or by the acquisition of resistance during several generations (Jansen et al., 2011). Comparing the contamination levels of the two estuaries, the Seine estuary is more polluted than the St. Lawrence estuary regarding polycyclic aromatic hydrocarbons, polychlorobiphenyls and some metals (Delile et al., 2022). Hence, we would expect the nauplii from the St. Lawrence to be more sensitive to the pollutant, but this was not the case. Alternatively, heightened sensitivity can result from a prior exposure of organisms to multiple stressors such as food limitation or chemicals (Liess et al., 2001). This is consistent with our results as we found that the European clade is more sensitive than the North-Atlantic clade regarding the growth. As such, chemical quality of a sampling site may increase or decrease sensitivity, which can make interpretation of results more complex.

The comparison between both clades highlights that there was a noticeable difference in the development. Although the proportion of N2 stages at 0 h was higher in the North-Atlantic clade, the nauplii of the European clade reached the higher developmental stage and larger size after 96 h of exposure. Such differences might be due to genetic differences between the two clades. Beyrend-Dur et al. (2009) showed differential development time between the *E. affinis* European clade and *E. carolleae* (*E. affinis* Atlantic clade). However, there is no other study comparing the *E. affinis* North-Atlantic and the European clades.

To confirm our results, experiments on other contaminants with different action modes should be conducted. It would also be interesting to examine other cryptic species within the *E. affinis* complex, especially *E. carolleae*, who shares some habitats with the North-Atlantic clade in the St. Lawrence estuary. As an invasive species, *E. carolleae* co-occurs now with the European clade in some estuaries in Europe (Sługocki et al., 2021; Sukhikh et al., 2013, 2019, 2020; Sukhikh and Alekseev, 2023). Until the results of the present study are confirmed by other experiments, we recommend verifying the identity of cryptic species in laboratory cultures of *E. affinis* at regular time intervals. This recommendation is even more valid for wild-caught organisms, as is the case in the present study, especially if several cryptic species co-occur in the same habitat. In the case of multiple cryptic species present in the same experiments, this variable should be considered in the numerical and statistical analyses as a covariate.

#### 4. Conclusion

The present study provides the first evidence of the effects of Li on the survival, growth, and development rates of the early life stages of the estuarine copepod cryptic species complex *E. affinis*. The EC<sub>50</sub> and LC<sub>50</sub> values found for the cryptic species of *E. affinis* that affect growth and survival are far above the Li environmental concentrations, even though these are expected to increase in the coming years due to the high demand for Li-ion batteries. The European clade appears to be moderately

more sensitive than the North-Atlantic clade, showing slower growth. This highlights the relevance of considering a sublethal parameter such as the growth in ecotoxicological studies. Further experiments with other contaminants and on other cryptic species of *E. affinis* should be carried out to ensure that routine ecotoxicological tests are adequate for the different cryptic species and to avoid bias in the interpretation of the data.

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#### CRediT authorship contribution statement

**Joëlle Forget-Leray:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Nathalie Giusti:** Investigation. **Ellia Roy:** Investigation. **Gesche Winkler:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Quentin Peignot:** Writing – original draft, Software, Methodology, Investigation, Formal analysis, Conceptualization.

#### 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

The data that has been used is confidential.

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