

# Effect of the test media and toxicity of LAS on the growth of *Isochrysis galbana*

M. C. Garrido-Perez · J. A. Perales-VargasMachuca ·  
E. Nebot-Sanz · D. Sales-Márquez

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**Abstract** In this paper, the toxicity of linear alkylbenzene sulfonate (LAS) was evaluated in the marine microalga *Isochrysis galbana* using data of growth inhibition toxicity tests at 96-h exposure time. Toxicity was examined in standard conditions and by means of the modification of two variables of the test media: (1) the dilution water and (2) the content of nutrients in the test medium. For this purpose, a total of 10 toxicity test were designed: five dilution waters, four natural marine waters and one synthetic seawater; each in two different nutritive conditions, saturated nutrient concentration (SC) by the addition of modified *f*/2 nutritive medium, and natural nutrient concentration (NC), i.e., without the addition of *f*/2. At threshold toxicity levels, the dilution waters used in the test and the nutrient concentrations did not affect the toxicity of LAS. At  $IC_{50}$  concentrations, the toxicity of LAS is influenced by both variables: under SC conditions, the toxic effect of LAS diminishes, obtaining in all the tests  $IC_{50} > 10$  mg/L LAS. Under NC conditions,  $IC_{50}$  concentrations ranging between 3.15 and 9.26 mg/L LAS have been obtained.

**Keywords** Surfactant · Linear alkylbenzene sulfonate (LAS) · Toxicity test · Microalga · *Isochrysis galbana* ·  $IC_{50}$  · Nutrient conditions

## Introduction

The xenobiotic chemicals that reach the coasts through either punctual or diffuse sources of pollution can cause important inhibitory effects on the growth, reproduction and other physiological functions of the biological communities. Determining the toxicity of these chemicals and the environmental variables that modify the thresholds of toxicity is fundamental for developing tools for the management and protection of aquatic ecosystems (Rand 1995; ECETOC 2001).

At present, linear alkylbenzene sulfonates (LAS) is the most widely manufactured and consumed surfactant (Roberts 2003). Estimated world LAS consumption in 2000 was around 2.5 million tons (Sanz et al. 2003) and the use of surfactants will likely increase in the years to come (Cavalli et al. 1999). Due to its widespread use, discharges of wastewaters to natural waters contain important loads of these compounds (León et al. 2002; Ying 2006; González et al. 2007). The monitoring of surfactants in continental and marine waters has been the subject of numerous researches in the last years (Matthijs et al. 1999; Cavalli et al. 2000; Leon et al. 2002; Lara-Martin et al. 2006). Regarding the effects of the LAS in aquatic organisms, there are numerous toxicity studies on freshwater species; see revisions in ECETOC (1993), Ramamoorthy and Baddaloo (1995), Rand (1995) and HERA (2004). However, information on marine organism is scarce, and in most of the published works, tests were carried out in standard and controlled experimental conditions, differing from those that we usually find in environmental waters.

In the marine environment, the unicellular planktonic algae are very important components of the biological communities. The importance of algal toxicity data has

M. C. Garrido-Perez (✉) · J. A. Perales-VargasMachuca ·  
E. Nebot-Sanz · D. Sales-Márquez  
Department of Chemical Engineering, Food Technologies  
and Environmental Technologies, Faculty of Marine  
and Environmental Sciences, University of Cadiz, Pol. Río San  
Pedro s/n, 11510 Puerto Real, Cadiz, Spain  
e-mail: carmen.garrido@uca.es

been recognized internationally. At this moment, there are numerous test guidelines published and incorporated in various regulatory procedures. Standardised tests of toxicity with microalgae are usually static tests, in which the algae are cultivated and exposed to different concentrations of a toxic chemical under controlled conditions of light and temperature.

In seawater tests, the medium is usually synthetic seawater enriched with salts, macro and micronutrients and vitamins up to non-limiting (saturated) concentrations. However, the seawater and nutritive formulations used in those tests are not representative of the natural marine environment. Coastal waters are considerably different from continental waters, from other adjacent coastal waters, and from oceanic waters, with regard to the concentrations of nutrients and organic and inorganic compounds. The concentrations of these compounds depend on their proximity to estuaries, direct discharges to the sea, local maritime traffic, the morphology of the zone, and the water exchange capacity (ECETOC 2001). In any case, the concentrations of nutritive elements are very much lower than those provided in any synthetic formulation for algal toxicity test. If toxicity tests are carried out in order to study the effects of chemicals on phytoplanktonic communities, the extrapolations of the results obtained under different conditions to those of the natural environment could be wrong.

The present work has been designed with the aim of studying the toxicity of LAS on the growth of *Isochrysis galbana*. With this objective, we have studied the effect of both types, saline matrix or dilution water and nutrient concentrations in the test media on the toxicity of the LAS.

## Material and methods

### Chemical tested

The chemical tested was a commercial mixture of homologues (C10–C13) and isomers of the sodium salt of dodecylbenzenesulfonate acid (LAS) (Supplier: Fluka Chemie A.G; Product N°: 44200). The compound was tested in the following concentrations of 2, 4, 6, 8 and 10 mg/L. Dissolutions of LAS were constructed using five different dilutions waters (Table 1).

### Algal culture and test inoculum

*Isochrysis galbana* is an alga of the *Haptophyceae* class, found all over the world. In recent years it has been one of the most used marine microalgae in toxicity tests (Moreno-Garrido et al. 2000; Hampel et al. 2001; Tsvetnenko and Evans, 2002; Garrido-Pérez et al. 2003; Tzovenis et al.

2004; Weiner et al. 2004; Yap et al. 2004; Satoh et al. 2005; Campa-Córdova et al. 2006; Correa-Reyes et al. 2007).

The source of the monocultures of *I. galbana* used in the toxicity tests was the Collection of Marine Microalgae Cultures of the Institute of Marine Sciences of Andalusia (Lubián and Yufera 1989).

The inoculum of *I. galbana* was cultivated under aseptic conditions in a nutritive medium composed of a synthetic seawater (USEPA 2002) and a supply of nutrients and vitamins according to the f/2 nutritive medium (Guillard and Ryther 1962) modified with double nitrate and phosphate concentrations (Huertas et al. 2000) and silicate (250 µg/L SiO<sub>2</sub>). Both the preculture and the tests were performed in an incubation chamber under controlled conditions of continuous illumination (cold white light of 11000 lux) and temperature (20 ± 1°C). The aeration to facilitate the exchange of gases was performed by means of manual shaking twice a day. Once the exponential phase of growth was reached (7–10 incubation days), the test inoculum was concentrated. For this, the culture was centrifuged at 2,000 g for 15 min. The concentrate of cells obtained was resuspended in synthetic seawater and this operation was repeated until an inoculum in synthetic seawater free of nutrients was obtained (USEPA 2002).

### Experimental design

A total of ten different toxicity tests have been performed: five dilution waters, —one synthetic seawater and four natural waters—, each one in two different nutritive conditions —saturated nutrient concentration (SC) by the addition of modified f/2, and natural nutritive concentration (NC), that is, without the addition of f/2—. Table 1 show the codes of each test. SSW\* is the test carried out in standard conditions with regard to dilution water (synthetic seawater) and the availability of nutrients (saturated condition).

The experimental protocol of the tests is according the method 1003.0 for measuring the toxicity of effluents and receiving waters with the microalga *Selenastrum capricornotum* (USEPA, 2002). The tests were performed in transparent and sterilised vials of borosilicate glass of 15 mL capacity. To allow the exchange of gases and to avoid external contamination, the vials were sealed with aluminium capsules. Volumes of 4 mL of test concentrations, and 100 microlitres of concentrated algae inoculum (with a cell concentration enough to ensure initial exponential growth) were added into each test vial. Each concentration or “treatment” and controls were tested in triplicate.

**Table 1** Test codes and concentration of nutrients in (a) NC test, (b) SC test

(a)					
Elements/Test code	SSW Synthetic seawater	H Huelva Estuary	C Bay of Cadiz	I Iro River	Al Bay of Algeciras
C (mg/L)	24.306	27.450	31.170	44.030	30.210
N (mg/L)	<0.010	0.172	0.109	2.402	0.302
P (mg/L)	<0.002	0.057	0.013	0.463	<0.002
Si (mg/L)	<0.020	0.046	0.120	0.372	0.070
(b)					
Elements/Test code	SSW* Synthetic seawater + f/2	H* Estuary of Huelva + f/2	C* Bay of Cadiz + f/2	I* Rio Iro + f/2	Al* Bay of Algeciras + f/2
C (mg/L)	24.306	27.450	31.170	44.030	30.210
N (mg/L)	24.724	24.896	24.833	27.126	25.026
P (mg/L)	1.985	2.042	1.998	2.448	1.985
Si (mg/L)	0.117	0.163	0.237	0.489	0.187

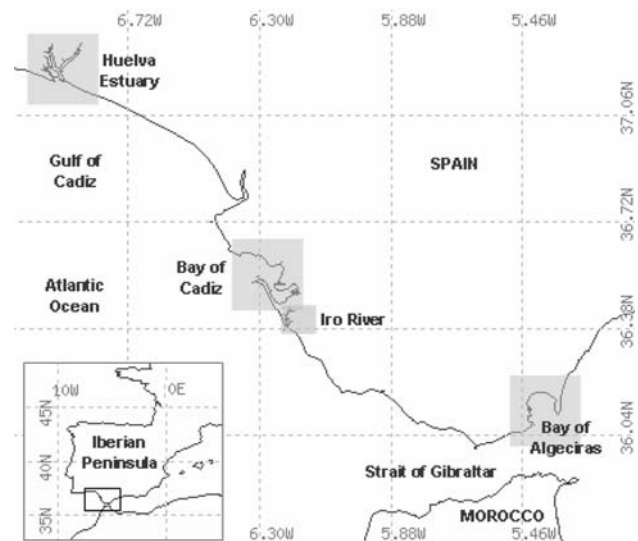
### Dilution seawaters

Five different saline dilution waters were used in this work: one synthetic seawater from USEPA formulation (USEPA 2002) and four natural waters collected along the coast of Gulf of Cadiz (Southern Spain) (Fig. 1). In the selected littoral area, three great industrial and urban areas are located: Huelva Estuary, Bay of Cadiz, and Bay of Algeciras (Table 2). For several decades, the industrial and urban effluent discharges from these areas have had a direct impact on those coasts, adversely affecting the quality of the waters. The fourth location is the estuary of the Iro River, integrated in a system of salt marshes of high ecological value in the inner lands of the Bay of Cadiz. This estuary is strongly affected by the effluent of an urban wastewater treatment plant. The processes of dilution and renewal in this estuary are very limited due to the geomorphology and the tidal regime, so the concentration of nutrients and chemicals are very significant.

In each of these coastal areas, water sampling was performed at several different points distributed homogeneously according to the morphology of the sampling area. A composite representative sample was constructed from the subsamples and the concentrations of the main nutrients (N, P, C and Si) were analysed (Table 1a).

### Response observed and data analysis

30 min after the inoculation of the algae ( $t = 0$ ) and after 96 h exposure time, biomass concentration of the treatments was measured in terms of optical density (USEPA, 2002) at a wavelength of 690 nm in a colorimeter adapted for direct measurements of the test vials (Nannocolor®



**Fig. 1** Map of the location of the study zones: Huelva Estuary, Bay of Cadiz, Iro River and Bay of Algeciras

PT-3 MACHERY-NAGEL). For the analysis of the toxicity data, net values of biomass were calculated by the following expression:

$$\text{Net biomass}(B') = \text{Biomass}_{t=96\text{ h}} - \text{Biomass}_{t=0}$$

Various statistical tests recommended in the toxicity protocols of USEPA (2002) were applied as quality control of the results. The condition of *normality* was determined by the Shapiro-Wilk test. The *homogeneity of the variance* of the triplicate has been determined by the Barlett test. For the exposure period of 96 h, both conditions were found in all the tests. The software IcPIN provided by the USEPA (Norberg-King, 1988) was used to estimate different  $IC_p$

**Table 2** Characteristics of the study zones: number of inhabitants recorded in the census in the area of study and discharges catalogued (CMAJA 1996)

Coastal zone	Population (N° inhabitants, 2004) <sup>a</sup>	N° Industrial discharges		N° Urban discharges		Morphology and exchange/dilution capacity
		>1,000 m <sup>3</sup> d <sup>-1</sup>	<1,000 m <sup>3</sup> d <sup>-1</sup>	>1,000 m <sup>3</sup> d <sup>-1</sup>	<1000 m <sup>3</sup> d <sup>-1</sup>	
Huelva Estuary	189.109	16	10	3	40	Artificial channel Medium-low exchange
Bay of Cadiz	602.809	4	7	9	76	Natural bay with mean depth of 10 m Medium-high exchange
River Iro	61.028	0	1	0	12	Estuary under strong tidal influence Very low exchange
Bay of Algeciras	229.078	8	5	6	22	Natural bay with mean depth of 400 m High-very high exchange

<sup>a</sup> <http://www.ine.es>

(and the 95% confidence range) by means of linear interpolation method.

## Results and discussion

Plots in Fig. 2 show the temporal evolution of the biomass concentrations of the toxicity tests carried out in this work. SSW\* graphic shows exponential growth of the controls under saturated nutrient concentrations (SC). Comparing the evolution of the treatments under both nutritive conditions for the same dilution water, it is observed that the growth rate of the tests under SC (SSW\*, H\*, C\*, I\* and Al\*) is higher than the one under natural nutritive concentration (NC). In the same way, it is observed how both nutritive conditions undergo a lag phase during the first 24 h in the tests carried out with the dilution water from the Bay of Cadiz (C\* and C). In the case of the dilution waters from Iro River and Bay of Algeciras (I, I\*, Al and Al\*), a decrease of the populations is observed instead of the aforementioned lag phase. These phases could be due to the differences between the culture media of the inoculum (synthetic seawater + modified f/2) and the toxicity media. Because of this, in the case of the SSW, this lag phase does not take place. This effect does not depend on the nutrient concentration as it is observed both in the enriched media (SC) and in the natural ones (NC).

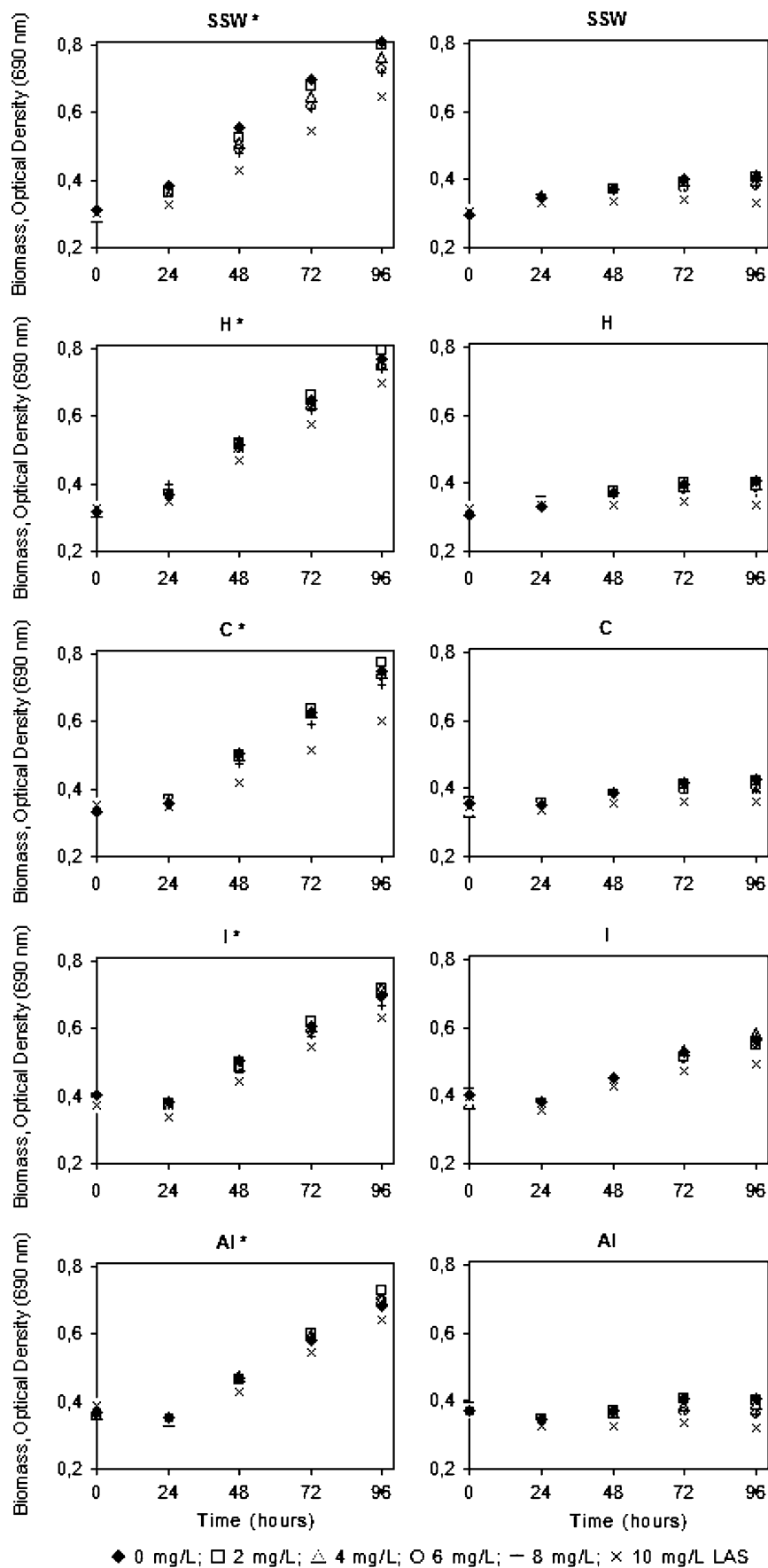
The Fig. 3a and Table 3 show the calculated IC<sub>p</sub> concentrations of the test in standard experimental conditions (SSW\*). IC<sub>50</sub> concentration is higher to 10 mg/L and it has the same order of magnitude that the one calculated by Hampel et al. (2001) for *Isochrysis galbana* and for others marine microalgae (Table 4). Comparing the results obtained in standard conditions (IC<sub>50</sub> > 10 mg/L) with the

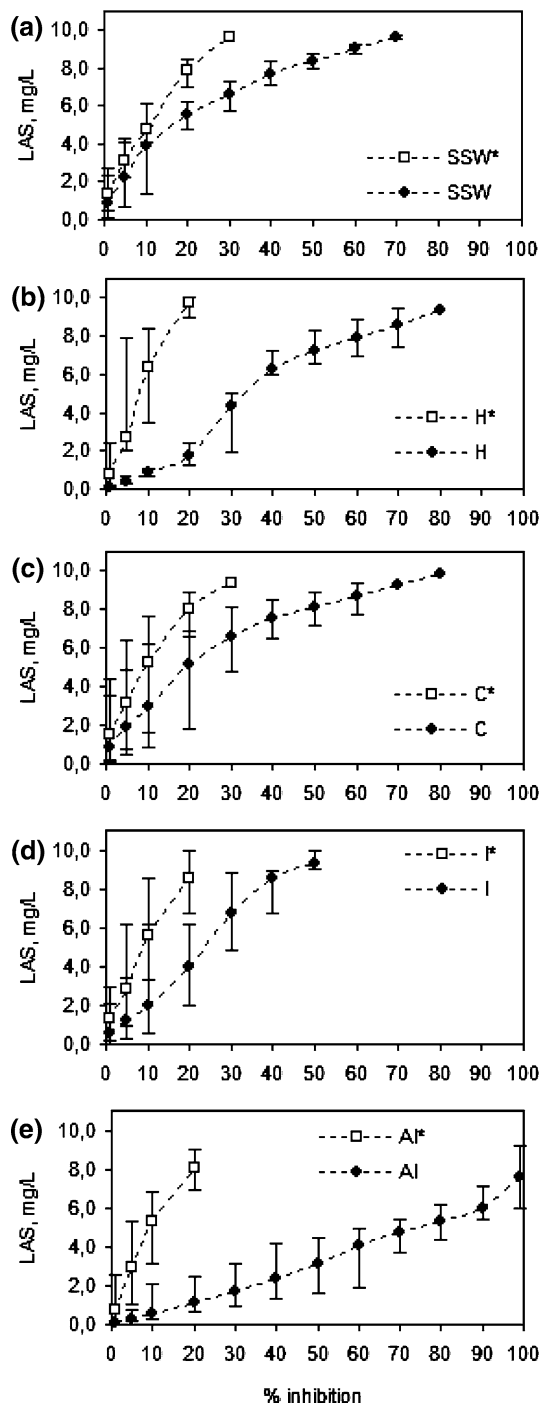
ones published for freshwater microalgae (ECETOC, 1993), it is observed that *Isochrysis galbana* shows a sensitivity to the LAS similar to *Dunaliella primolecta* and *Microcystis aeruginosa*, although it is more resistant than *Navicula Peliculosa*. Regarding other marine organisms (Table 4) and in the case of the marine crustaceans, the EC<sub>50</sub> concentrations calculated for other authors (Painter and Zabel 1988; Liwarska-Bizukojk 2005) are higher (from 40.4 to 66 mg/L). With respect to molluscs, *Isochrysis galbana* it has a similar sensibility to the ones observed by several authors. Finally, regarding the group of the marine fishes, the bibliographical data show that these fishes are more sensitive to the surfactant than the microalga.

Plots in Fig. 3 compare the calculated IC<sub>p</sub> concentrations obtained in each nutritive conditions for the different dilution waters selected in this study. It is observed that in all the tests, there are differences between the effects of the LAS on the microalga under SC and NC. In SC tests, LAS toxicity is lower. In fact, in those tests, the maximum percentage of inhibition calculated for the range of concentrations tested, did not reach 50% in any case. On the other hand, under NC conditions, it was possible to calculate the IC<sub>50</sub> for all the dilution waters. The differences among the IC<sub>p</sub> concentrations are more noticeable insofar as the calculated effect is bigger. Thus, for all the dilutions waters, the differences between IC<sub>20</sub> under SC and NC are higher than the ones obtained for the IC<sub>1</sub> (Table 3). These differences are more noticeable in the tests carried out with dilution water from the Huelva Estuary (Fig. 3b) and from the Bay of Algeciras (Fig. 3e).

Regarding the IC<sub>20</sub>, for 4 out of the 5 types of dilution water (except the Bay of Cadiz, Fig. 3c) there are meaningful differences between both nutritive conditions (there is no overlapping of the confidence ranges). These

**Fig. 2** Temporal evolution of biomass during the toxicity tests





**Fig. 3** Inhibition concentrations ( $IC_p$ , 96-h) obtained in the toxicity tests of LAS and *Isochrysis galbana*

differences are maximized in the tests with dilution water from the Bay of Algeciras (decrease of an 85.8% of the value of the  $IC_{20}$  under NC, Fig. 3e) and they are minimized in the tests with synthetic seawater (decrease of the 30%, Fig. 3a).

In SC tests, the microalgae resist the toxic effect of the LAS in better physiological conditions than when facing

the toxic effect in NC, where the alga is subjected to, at least, two sources of stress: limitation of nutrients and presence of a toxic chemical. In the case of the test in NC with dilution water from the Iro River (Fig. 3d) the alga is more resistant to LAS with respect to the other tests in NC because the higher nutrient natural concentrations in that waters (Table 1a). In terms of toxicity, the inhibition concentrations for  $p \geq 50$  demonstrate that the alga is more resistance to LAS in “enriched” waters, since the alga is capable of absorbing the toxic shock in better conditions than in “more limited in nutrients” natural waters.

In order to analyse the effect of the dilution water in the toxicity of LAS, the results of the  $IC_p$  calculated for the different waters employed in this work are shown separately in Fig. 4. There are no meaningful differences in the results obtained from waters of different sources under SC (Fig. 4a). However, under NC, differences are found in the results, being those obtained from the Huelva Estuary and from the Bay of Algeciras more sensible (Fig. 4b).

Table 2 summarises the data of urban and industrial activities in the origin areas of the dilution waters. The Huelva Estuary and the Bay of Algeciras are highly populated areas with a strong urban and industrial activity. Nowadays both zones are considered as the most anthropized areas in southern Spain. Pollutants loadings and the wide range of chemicals that reach the environmental waters throughout the discharges are very high. Thus, the differences obtained in the tests of these waters under NC could be due to the joint (synergic) action with other chemicals and LAS, as described in some works (Abel and Axiak 1991; Broderius et al. 1995; Jensen and Sverdrup 2002). In those cases, it should be necessary to carry out a whole characterization of the main pollutants present in those waters with the aim to obtain more concrete conclusions.

Two of the main applications of the algal toxicity tests are the environmental risk assessment (ERA) of chemicals in waters (e.g., in European Union: EEC 1993, EEC 1996) and the derivation of environmental quality criteria (e.g., in European Union: EEC 2000). In the case of the ERA, the parameter used for the determination of the PNEC (predicted no-effect concentration) is the NOEC (no-observed effect concentration). As seen in this work, insofar as the inhibition percentage diminishes, the differences between the dilution waters and the nutritive conditions are minimized. Thus, the usage of the NOEC obtained from the algal toxicity tests in standard conditions (SSW\*) can be extrapolated to the environment. But, in the procedure for deriving environmental quality criteria, it is possible to use the  $IC_{50}$  in the calculation procedure. In this case, toxicity test with microalgae carried out in standard experimental conditions could underestimate the toxicity found in natural conditions.

**Table 3** IC<sub>1</sub>, IC<sub>20</sub>, IC<sub>50</sub> and 95% confidence ranges (min. and max.) (mg/L, LAS) obtained in the toxicity tests of *I. galbana* exposed to different concentrations of LAS at 96 h

	IC <sub>1</sub>		IC <sub>20</sub>		IC <sub>50</sub>	
	SC	NC	SC	NC	SC	NC
Synthetic seawater	1.34 (0.45–2.34)	0.83 (0.13–2.68)	7.91 (7.02–8.43)	5.54 (4.81–6.24)	<10	8.41 (7.94–8.79)
Huelva Estuary	0.75 (0.24–2.40)	0.08 (0.07–0.11)	9.76 (8.99–10.00)	1.73 (1.25–2.37)	<10	7.25 (6.56–8.34)
Bay of Cadiz	1.54 (0.16–4.42)	0.85 (0.09–3.53)	8.07 (6.60–8.93)	5.20 (1.83–6.90)	<10	8.13 (7.16–8.91)
Iro River	1.30 (0.18–2.92)	0.58 (0.01–2.13)	8.57 (6.78–9.98)	3.95 (1.99–6.21)	<10	9.26 (8.98–9.99)
Bay of Algeciras	0.73 (0.21–2.53)	0.07 (0.03–0.19)	8.09 (6.95–9.03)	1.15 (0.64–2.46)	<10	3.15 (1.58–4.49)

**Table 4** EC<sub>50</sub>/IC<sub>50</sub> concentrations for LAS in some groups of aquatic species

Species	Type of water	Exposure Time (h)	LAS (mg/L)	Reference
<b>Microalga</b>				
<i>Rhodomonas salina</i>	Saltwater	72	C <sub>11</sub> (4.43); C <sub>13</sub> (0.36)	Hampel et al. (2001)
<i>Isochrysis aff. Galbana</i>	Saltwater	72	C <sub>11</sub> (7.70); C <sub>13</sub> (0.53)	Hampel et al. (2001)
<i>Tetraselmis suecica</i>	Saltwater	72	C <sub>11</sub> (13.37); C <sub>13</sub> (1.23)	Hampel et al. (2001)
<i>Nannochloropsis gaditana</i>	Saltwater	72	C <sub>11</sub> (1.38); C <sub>13</sub> (0.18)	Hampel et al. (2001)
<i>Dunaliella primolecta</i>	Freshwater	48	8.62	ECETOC (1993) <sup>a</sup>
<i>Microcystis aeruginosa</i>	Freshwater	96	5.0	ECETOC (1993) <sup>a</sup>
<i>Navícula Peliculosa</i>	Freshwater	96	1.4	ECETOC (1993) <sup>a</sup>
<b>Crustacean</b>				
<i>Mysidopsis bahia</i>	Saltwater	96	1,420	Painter and Zabel (1988)
<i>Penaeus duororum</i>	Saltwater	96	66	Painter and Zabel (1988)
<i>Artemia salina</i>	Saltwater	24	40.4	Liwarska-Bizukojc (2005)
<i>Acartia tonsa</i>	Saltwater	48	1.11	Christoffersen et al. (2003)
<b>Mollusc</b>				
<i>Crassostrea</i> sp.	Saltwater	48	7.4	Painter and Zabel (1988)
<i>Tapes phillipinarum</i>	Saltwater	96	10.5	ECETOC (1993) <sup>a</sup>
<i>Physa acuta</i>	Saltwater	24	16.65	Liwarska-Bizukojc (2005)
<b>Fish</b>				
<i>Gadus morhua</i>	Saltwater	96	1	Swedmark et al. (1971)
<i>Pleuronectes flesus</i>	Saltwater	96	1.5	Swedmark et al. (1971)
<i>Pleuronectes platessa</i>	Saltwater	96	1	Swedmark et al. (1971)
<i>Pomatochistus microps</i>	Saltwater	96	2.6	ECETOC (1993) <sup>a</sup>
<i>Sparus aurata (embryos)</i>	Saltwater	24	0.1–7.7 (2.25, n = 6)	Hampel et al. (2001)

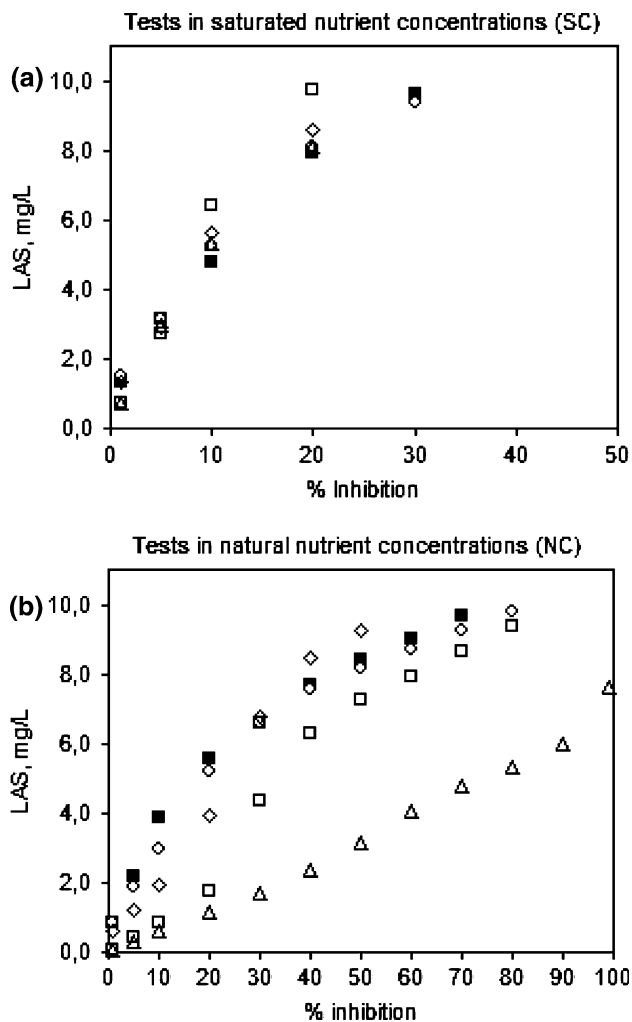
<sup>a</sup> Data obtained from the ECETOC toxicity database

Species with two or more references, range of EC<sub>50</sub> (average and N° of data) is included

## Conclusions

In this paper, results of LAS toxicity were obtained on the marine microalga *Isochrysis galbana* in standard experimental conditions and in variable conditions of nutrients and

dilution water. The results obtained indicate that the composition and the nutritive conditions of the test medium are determining factors in the determination of the IC<sub>50</sub> of LAS in *Isochrysis galbana*. The alga is more sensible in nutrient natural concentrations and in seawaters from polluted zones.



**Fig. 4** Inhibition concentrations ( $IC_p$ , 96-h) obtained in the tests under saturated nutrient concentration (SC) and natural nutrient concentration (NC). ■—Synthetic seawater (SSW); □—Huelva Estuary (H); ○—Bay of Cadiz (C); ◇—Iro River (I); △—Bay of Algeiras (Al)

Finally, and according to the particular results obtained in this work, it is advisable to carry out new toxicity tests with other chemicals on different microalga species and test media in order to obtain general conclusions about the extrapolation of toxicity results with microalga to the environment.

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