The link between shrimp farm runoff and blooms of toxic *Heterosigma akashiwo* in Red Sea coastal waters

doi:10.5697/oc.54-2.287
OCEANOLOGIA, 54 (2), 2012.
pp. 287–309.

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2012.

KEYWORDS
Haemolytic activity
Ichthyotoxic algae
Outbreak-forming algae
Raphidophytes
Red Sea

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Received 29 September 2011, revised 16 January 2012, accepted 13 February 2012.

Abstract

In May 2010 a copious bloom of the raphidophyte *Heterosigma akashiwo* was observed for the first time in Red Sea waters off the coasts of Saudi Arabia. This bloom was confined to an area where water and phytoplankton flow freely between the sea and a shrimp farm. The phytoplankton density and physico-chemical characteristics of the sea water were therefore investigated weekly at bloom and non-bloom sites in order to gain insight into the environmental factors prevailing at the bloom site and their link with the shrimp farm runoff. The bloom site showed higher nutrient concentrations than the non-bloom site, indicating the possible role of the shrimp farm in flushing nutrients into this site. The bloom appeared on 27 May, coinciding with a decrease in salinity (<30‰) and an increase in temperature (>19°C). The results of toxicological assays showed that both bloom samples and batch cultures of *H. akashiwo* were toxic to *Artemia salina* and exhibited haemolytic activity with respect to rabbit erythrocytes. Bloom samples showed a higher

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toxicity ($LC_{50} = 8.9 \times 10^4$ cells ml^{-1}) and haemolytic activity ($EC_{50} = 3.64 \times 10^4$ cells ml^{-1}) than the batch cultures ($LC_{50} = 11.6 \times 10^4$ cells ml^{-1}, $EC_{50} = 5.1 \times 10^4$ cells ml^{-1}). In the light of the results of this study, the link between *H. akashiwo* blooms and shrimp farm runoff should be considered during the monitoring of Red Sea coastal waters for the presence of harmful algal blooms.

1. Introduction

Harmful algal blooms (HABs) are increasingly becoming a global problem for human health, fisheries and the aquatic environment (Anderson 1997). *Heterosigma akashiwo* (Hada) Hada ex Hara & Chihara, a member of the Raphidophyceae, is one of the main bloom-forming phytoplankters. *H. akashiwo* causes brown or purplish red tide blooms in temperate to subtropical eutrophic coastal waters worldwide (Livingston 2007, Kempton et al. 2008, Shikata et al. 2008, Rensel et al. 2010). Considered an ichthyotoxic alga (Yang et al. 1995, Khan et al. 1996, Tomas et al. 2001), it has caused severe fish mortality with significant damage to the mariculture economy in several countries (Tiffany et al. 2001, Kempton et al. 2008). Although the exact killing mechanisms are somewhat unclear, there are several toxicity mechanisms in raphidophytes, including the production of brevetoxin-like compounds (Khan et al. 1997), reactive oxygen species such as superoxide and hydrogen peroxide (Yang et al. 1995, Oda et al. 1997, Twiner & Trick 2000), and haemagglutinating and haemolysing compounds (Fu et al. 2004, Kuroda et al. 2005, Ling & Trick 2010). Several factors, including temperature, salinity, irradiance and nutrient concentrations, may account for the increased incidence of *Heterosigma* blooms (Ono et al. 2000, Anderson et al. 2008).

Prior to 2010, only two harmful algal blooms of *Noctiluca scintillans* (Mohamed & Messad 2007) and *Gonyaulax* sp. (Zakaria A. Mohamed, King Khalid University, pers. comm.) had been documented in the Red Sea off the southern coasts of Saudi Arabia – those events took place in 2004. In May 2010, a bloom of *H. akashiwo* was observed for the first time off the Al Shouqyq region, making it the third HAB documented in South Saudi offshore waters. The bloom event was noticed as occurring at a site located in an area receiving water discharge from a nearby shrimp farm. Thus, a link is expected between *Heterosigma* bloom formation and shrimp fish runoff into this site in the Red Sea. Therefore, the aim of this study was to assess the effect of shrimp farm runoff on the formation of an *H. akashiwo* bloom by the analysis of the environmental and biological characteristics of sea water at the bloom site, which receives fish farm discharge, and at a non-bloom site far away from any aquaculture activities.

2. Material and methods

2.1. Study area and water samples

The study area comprised two sites: site 1, where the *Heterosigma akashiwo* bloom was observed – this is referred to as the 'bloom site'; site 2, located about 20 km north of site 1, where no blooms were recorded – this is the 'non-bloom site'. The two sites are located north of Al Shouqyq city on the southern Red Sea coasts of Saudi Arabia (19.65–19.80°N) (Figure 1). Site 1 (bloom site) is closed off by a large shrimp farm and thus potentially receives drainage of farm wastes, whereas there are no aquaculture operations near site 2. Sampling was started when a red tide of *H. akashiwo* was observed on 27 May 2010 and was continued every week until the bloom disappeared. Phytoplankton samples were collected from the two sites around midday (13:00 hrs) to ensure the presence of *Heterosigma* on the water surface, as this alga has a diel vertical migration reaching depths of 10 m at night (Yamochi & Abe 1984). Bloom and phytoplankton samples were taken at 1 m depth by vertical tows, using

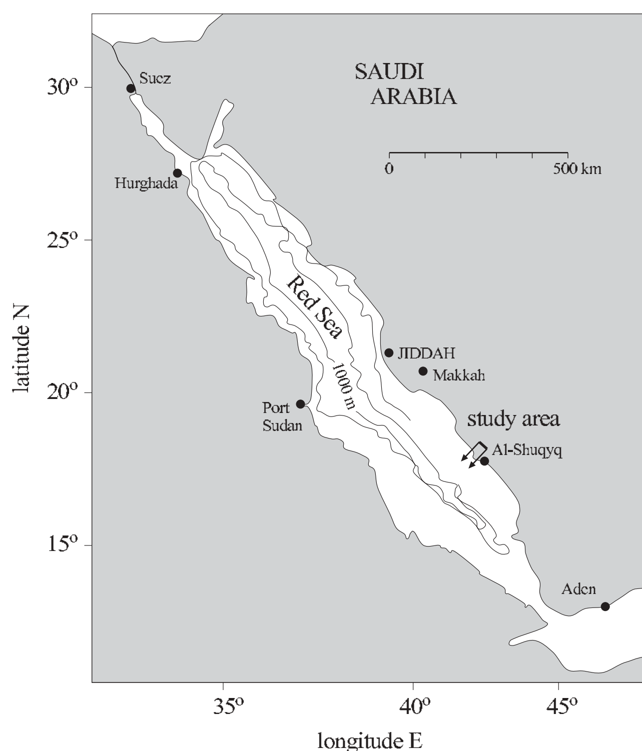

Figure 1. Map showing the location of the two study sites off the southern Saudi coasts of the Red Sea

a plankton net of mesh size 10 μm. Concentrated by plankton tows, phytoplankton cells were sieved through a 60 μm mesh to eliminate larger organisms and then divided into three parts. One part was fixed with 1% Lugol's solution and preserved in a brown bottle – this was used for the identification and counting of phytoplankton; the second part was placed in a 100 ml polyethylene bottle and used for testing the toxicity of the *Heterosigma* bloom; the third part was placed in a 250 ml polyethylene bottle and used for the isolation and culturing of *Heterosigma*. Meanwhile, water samples for the analysis of nutrients were collected from the same sites as the phytoplankton samples using a Van Dorn water sampler. Phytoplankton and water samples were transported to the laboratory in an icebox for chemical and biological analysis.

2.2. Measurement of physical and chemical parameters

Water temperature, salinity (conductivity) and pH were measured in situ using a multipurpose-probe meter (WTW Digit 88), and dissolved oxygen with an O_2-meter. Light intensity was measured at the surface and 1 m depth using an underwater light photon meter (ALW-CMP, Alec Electronics). Concentrations of nutrients, including ammonium, nitrate and phosphate, were determined in GF/C filtered water samples by the standard analytical methods as approved by the American Public Health Association (APHA) (APHA 1995). All chemical variables were determined in triplicate.

2.3. Identification and counting of *Heterosigma akashiwo* and other phytoplankton

Heterosigma akashiwo and other dominant species of phytoplankton were counted in the Lugol-preserved samples and freshly collected samples (less than 5 hours after sampling) using Utermöhl's technique (Utermöhl 1958) under an Olympus binocular light microscope equipped with a digital camera. Identification was based on morphological characteristics according to Hallegraeff & Hara (1995), Throndsen (1997), Hasle & Syversten (1997) and Steidinger & Tangen (1997), and with the aid of the floristic paper by Band-Schmidt et al. (2004). Chlorophyll *a* was determined by filtering an aliquot of phytoplankton samples onto GF/C glass fibre filters. The filters with adhering algal cells were extracted in methanol (95%), and the absorbance was read at 653 and 666 nm on a UV/visible spectrophotometer (UV-1601 PC, Shimadzu Corporation, Kyoto, Japan). The amount of chlorophyll *a* was calculated according to the formulas of Lichtenthaler & Wellburn (1985).

2.4. Isolation and culturing of *Heterosigma akashiwo*

An aliquot (10 ml) of *Heterosigma akashiwo* bloom samples was inoculated into a 250 ml flask containing 100 ml sterilized sea water (through a 0.22 μm filter) enriched with F/2 medium without silica (Guillard 1975). Vegetative cells of *H. akashiwo* were isolated with micropipettes under a Carl Zeiss inverted microscope. The cells were transferred individually to 96-well assay plates, previously filled with modified F/2 medium (20‰ salinity) and maintained at 25±2°C, with 60 μE m^{-2} s^{-1} of cool white fluorescent light and a 12:12 light:dark (LD) cycle. Cultures from the wells were transferred into 100 ml culture flasks containing 50 ml modified F/2 medium and incubated under the above conditions for 10 days. The cell concentration was monitored every two days using a haemocytometer; the motility was also observed. All glassware, polycarbonate bottles and the pipettes used for culturing, storing enriched sea water and sampling were soaked in 1.2 N HCl ($\geq$24 h), rinsed copiously with Milli-Q1 water, and microwave-sterilized (heated for 10 min on high power) prior to use.

2.5. Ichthyotoxicity of *Heterosigma akashiwo* by *Artemia salina*

The brine shrimp *Artemia salina* was used to test the toxicity of *Heterosigma akashiwo* according to Yan et al. (2003). A known volume of bloom samples or batch cultures of *H. akashiwo* was centrifuged (1000 × g for 10 min at 4°C). The algal pellets were resuspended in 10 ml of sterilized sea water for the aqueous extracts or in 10 ml methanol (95%) for the methanol extract, and vigorously agitated by sonication. The lysate was centrifuged at 12 000 × g for 10 min at 4°C, after which the supernatant was withdrawn and stored at −20°C until use. The methanol extract was evaporated to dryness, and the dried extract dissolved in an aliquot of filtered sea water. Bloom extracts, culture extracts and the medium of batch cultures (extracellular exudates) were diluted with sterilized sea water to give a dilution series of 1, 2, 3, 5, 10, 20, 50 and 100%. Sterilized sea water was used as the control. 500 μl of each dilution was added to a 5 ml culture tube containing 25 nauplii of 48 h-hatched cysts of *A. salina*. The tubes were incubated at 20°C under a continuous light flux of 90 μmol photons m^{-2} s^{-1}. After 48 h, the percentage mortality of nauplii was calculated compared to controls. The LC_{50} value was determined by probit analysis (Finney 1963).

2.6. Haemolytic activity test

Haemolytic activity was tested by erythrocyte lysis assay (ELA) according to Eschbach et al. (2001) and its modification by Ling & Trick (2010). ELA was carried out on bloom samples, on algal cells and on extracellular

exudates of exponentially growing cultures (6 days after inoculation) of *H. akashiwo*. An aliquot with a known number of *Heterosigma* cells was centrifuged ($6000 \times$ g for 10 min at 4°C), and the supernatant containing extracellular exudates following filtration through a 0.45 μm pore size GF/C filter was collected. Algal samples were prepared following the protocols of Eschbach et al. (2001), modified by Ling & Trick (2010). The cells of bloom samples (10 ml) and pellets of centrifuged cultures were ruptured in ELA buffer, prepared as described by Eschbach et al. (2001) (150 mM NaCl, 3.2 mM KCl, 1.25 mM $MgSO_4$, 3.75 mM $CaCl_2$ and 12.2 mM TRIS base; pH adjusted to 7.4 with HCl) by sonication for 60 s at 20°C in a bath-type sonicator. Complete cell rupture was confirmed by microscopic observation. Ultrasonicated algal samples and supernatants were kept in the freezer until use. The dry methanol extract of *H. akashiwo* cells prepared for the *Artemia salina* assay was re-dissolved in ELA buffer before use in ELA.

2.7. Erythrocyte lysis assay

Blood freshly collected from a rabbit was immediately mixed with 0.1 ml 10% sodium citrate to prevent it from coagulating. For the ELA, erythrocytes were harvested from the blood by centrifugation in a 1.5 ml microcentrifuge tube at $1500 \times$ g for 5 min at 4°C. The pelleted erythrocytes were washed twice with ELA buffer by vortexing and centrifugation at $1500 \times$ g for 5 min at 4°C. Erythrocyte suspensions were adjusted to the appropriate cell density (5×10^6) in ELA buffer with a haemocytometer. The ELA method was basically that of Eschbach et al. (2001) with modifications by Ling & Trick (2010). Briefly, 0.5 ml of erythrocyte suspension and 0.5 ml of cell extract or extracellular exudates of *H. akashiwo* were added to 1.5 ml polypropylene microcentrifuge tubes. For negative controls, erythrocytes were incubated in ELA buffer. For positive controls, representing complete lysis, erythrocytes were sonicated at the same setting as for the sonicated algal samples and examined microscopically to verify complete lysis of erythrocytes. Parallel sets of algal samples incubated in ELA buffer served as controls to account for background absorbance of algal samples. Tubes containing cell-erythrocyte or cell-extract-erythrocyte mixtures were incubated for 6 h at 20°C under a continuous light flux of 90 μmol photons m^{-2} s^{-1}. Each set of samples was pipetted in triplicate. All pipetting steps were performed under dimmed light conditions. Following incubation, tubes were centrifuged at $2000 \times$ g for 5 min at 20°C and 200 μl of each supernatant was transferred to a 96-well microtitre plate. Absorption was read at 414 nm with an ELX800 Microplate Reader (Biotek, USA). The haemolytic activity of each algal sample was expressed as the percentage haemolysis relative to both the positive and negative controls,

according to the following equation: % haemolysis relative to control = (100%) $\times$ $E_{414} - A_{414} - N_{414}/P_{414}$, where E_{414}, A_{414}, N_{414} and P_{414} are the absorption at 414 nm of the experimental sample (algal sample incubated with erythrocytes), algal sample, negative control and positive control respectively. Algal samples with a percentage haemolysis greater than zero were considered haemolytic, whereas algal samples with a percentage haemolysis at or below zero were considered non-haemolytic. To compare our data with data from other haemolytic assays, saponin (Sigma-Aldrich) was used as a reference.

2.8. Statistical analysis

Differences in algal density and environmental parameters between bloom site and non-bloom site were compared by ANOVA using the Excel data analysis tool at the 0.05 significance level. Correlations among algal density, environmental parameters and bloom toxicity in bloom site were measured using Spearman rank correlation coefficients using the Excel data analysis tool.

3. Results

3.1. Environmental and biological factors associated with *Heterosigma* blooms

During the field study, a purplish slick was observed on 27 May 2010 in the Red Sea off the Al Shouqyq coasts, southern Saudi Arabia. Microscopic examination of samples collected from this bloom revealed a motile, golden

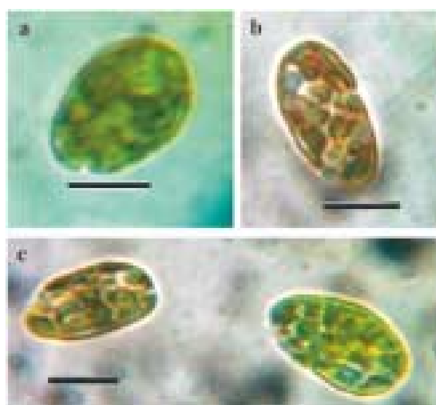

Figure 2. Micrograph of *Heterosigma akashiwo* forming blooms in the Red Sea off the southern coast of Saudi Arabia: a) cells of batch cultures; b, c) cells of bloom samples. Scale bar: 10 μm

brown microflagellate alga. The cells, 20–23 μm in length and 10–17 μm in width, are slightly flattened dorsoventrally with two subequal flagella arising from the anterior of the cells, one of which appeared dynamic, and the other almost rigid. The cell periphery has 8 to 16 discoid chloroplasts, brown or yellow brown in colour (Figure 2). Based on Hara & Chihara (1987), the species was identified as *Heterosigma akashiwo* (Hada) Hada ex Hara & Chihara. The *H. akashiwo* bloom was confined to site 1, located near a shrimp farm; it was not detected at site 2. The bloom event followed an increase in water temperature from 17 to 19°C and an abrupt decrease in salinity from 37.3 to 29‰.

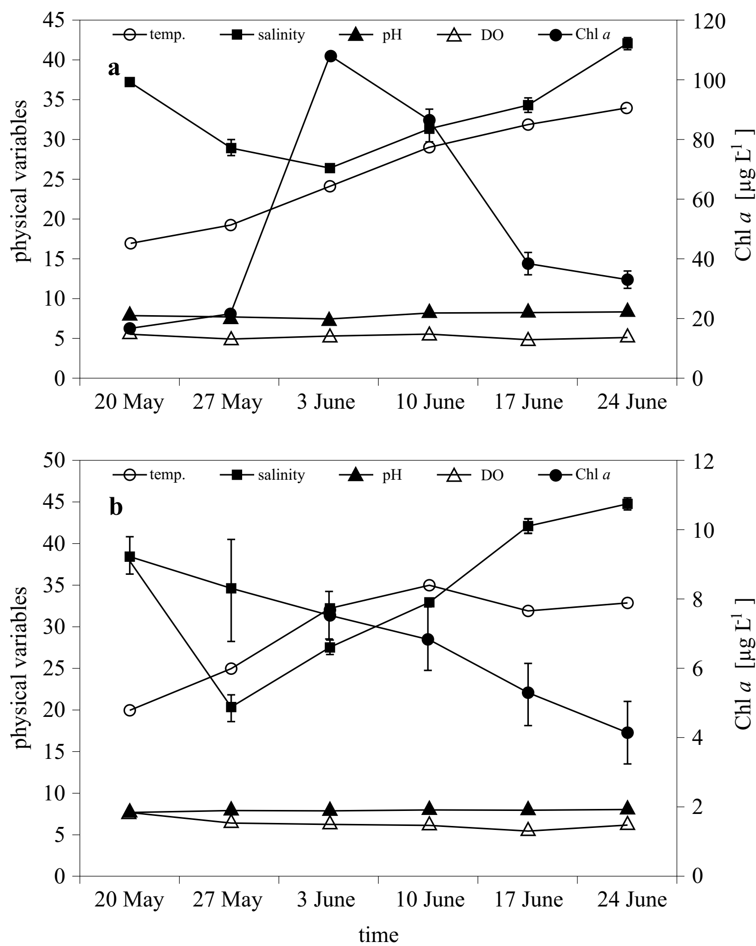

Figure 3. Variations in water temperature, pH, salinity, dissolved oxygen and chlorophyll *a* at the bloom site (a) and the non-bloom site (b) off southern Saudi coasts of the Red Sea during the study period

During this study, some physico-chemical parameters of sea water were determined at the bloom site (site 1) and the non-bloom site (site 2) in order to discover the prevailing environmental parameter(s) favouring bloom formation at the bloom site. The results show that there was no significant difference in temperature ($F = 3.2$, $P = 0.09$), pH ($F = 3.1$, $P = 0.09$) or salinity ($F = 0.1$, $P = 0.8$) between the two sites during the study period. The surface water temperature at both sites increased gradually during the study period, whereas salinity decreased sharply until reaching the lowest level (26.5‰) on 3 June, coincident with the highest peak of *H. akashiwo* cells at site 1 (Figure 3). The salinity rose again to more than 31‰ during the remaining part of the study period. In contrast, dissolved oxygen ($F = 329.9$, $P < 0.001$), NO_3 ($F = 2748.7$,

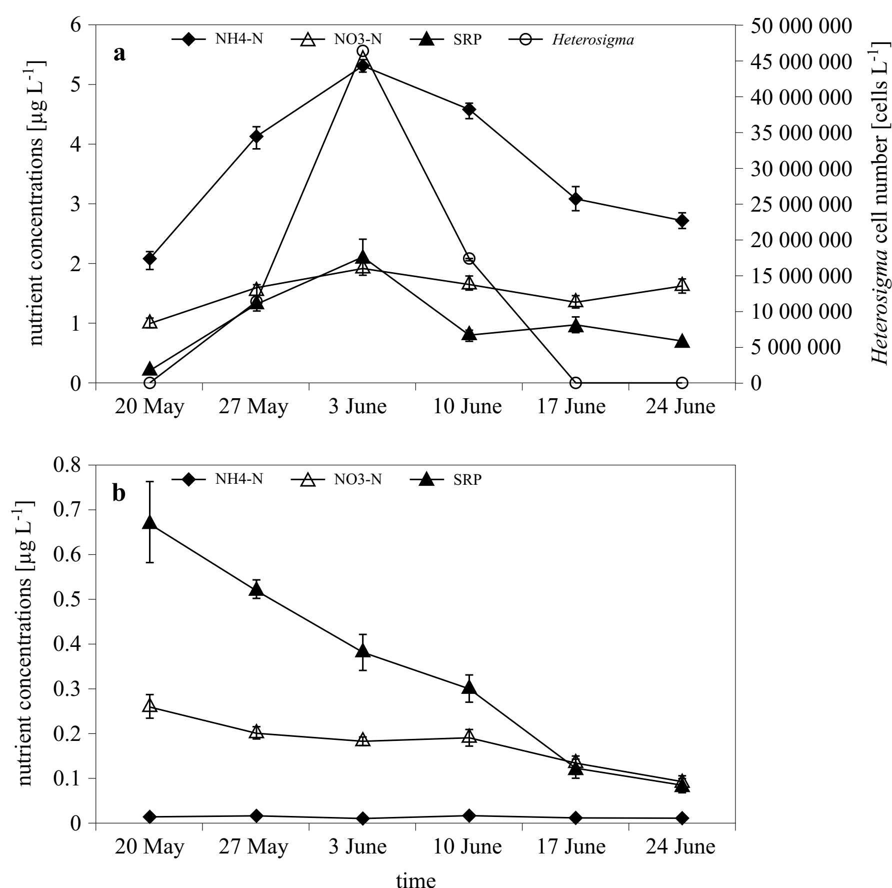

Figure 4. Variations in NH_4, NO_3 and PO_4 concentrations, and the cell density of *Heterosigma akashiwo* at the bloom site (a) and the non-bloom site (b) off southern Saudi coasts of the Red Sea during the study period

$P<0.001$), NH_4 ($F=1031$, $P<0.001$) and phosphate ($F=385.9$, $P<0.001$) concentrations varied significantly between the two sites. In general, nutrient concentrations (NH_4, NO_3 and PO_4) were higher at the bloom site than at the non-bloom site (Figure 4), indicating their possible promotion of *H. akashiwo* bloom formation at the bloom site.

The abundance of *H. akashiwo* at the bloom site increased markedly during the study, with the highest density (46×10^6 cells L^{-1}) obtained on 3 June (Figure 4); it began to decline on 10 June and eventually crashed on 24 June, coinciding with the salinity increase up to 40‰. The cell density of *H. akashiwo* correlated negatively with salinity ($r = -0.83$) and pH ($r = -0.7$), and positively with NH_4 ($r = 0.88$), NO_3 ($r = 0.78$) and PO_4 ($r = 0.86$). The cell density of this alga was only weakly correlated with water temperature ($r = 0.2$), as the temperature did not vary significantly during the last three periods of the study (Figure 3a). Chlorophyll *a* concentrations were higher at the bloom site than at the non-bloom site and correlated positively with *H. akashiwo* cell density ($r = 0.87$) at the bloom site.

In addition to *H. akashiwo* cells, the bloom site contained 17 other algal species, but with low cell densities (Table 1). Most of these algae are potentially toxic species of dinoflagellates (e.g. *Alexandrium*, *Dinophysis*, *Gymnodinium*), raphidophytes (e.g. *Chattonella*) and cyanobacteria (e.g. *Trichodesmium*). Remarkably, all of these species except *Chattonella* had been recorded at this site before the *H. akashiwo* bloom appeared, and began to disappear gradually as the cell density of *H. akashiwo* increased (Table 1). Thereafter, these species re-appeared at the site when the bloom collapsed on 24 June. In contrast, the raphidophyte *Chattonella* was associated with the *Heterosigma* bloom during the study period.

3.2. Toxicity of *Heterosigma akashiwo* bloom and cultures

During this study, the raphidophyte *H. akashiwo* was toxic to *A. salina*. As shown in Table 2, both the aqueous and methanol extracts of *H. akashiwo* blooms were toxic towards *A. salina* with a significant difference in LC_{50} values ($F=15.2$–62.5, $P=0.01$–0.001): the methanol extracts were more toxic ($LC_{50} = 9.14$–9.8×10^4 cells ml^{-1}) than the aqueous ones ($LC_{50} = 9.7$–11.3×10^4 cells ml^{-1}) (Table 2). The toxicity to *A. salina* varied between bloom samples collected in different study periods for both the methanol ($F=7.91$, $P=0.0088$) and the aqueous extracts ($F=26.6$, $P=0.0002$). The methanol extract of the 3 June bloom exhibited the highest toxicity ($LC_{50} = 8.9 \times 10^4$ cells ml^{-1}), whereas the aqueous extract of the 27 May bloom ($LC_{50} = 9.8 \times 10^4$ cells ml^{-1}) was the least toxic. In contrast to the bloom

Table 1. The phytoplankton and their cell densities (cells L^{-1}) in water samples collected from sites containing and not containing *Heterosigma akashiwo* blooms in the Red Sea off the southern coast of Saudi Arabia during the present study

Species	Site containing *H. akashiwo* bloom						Site not containing *H. akashiwo* bloom					
	20 May	27 May	3 June	10 June	17 June	24 June	20 May	27 May	2 June	10 June	17June	24 June
Raphidophyceae												
Heterosigma akashiwo	320	11.4×10^6	46×10^6	17.3×10^6	1120	0	0	0	0	130	86	0
Chattonella sp.	0	0	142	88	12	0	0	33	0	16	0	0
Dinophyceae												
Alexandrium sp.	38	0	0	0	0	143	45	89	83	114	121	146
Ceratium sp.	334	26	0	0	0	586	366	238	398	210	330	205
Dinophysis sp.	0	0	0	0	0	0	43	62	87	60	74	70
Gymnodinium sp.	0	0	0	0	0	0	252	293	265	278	286	282
Gyrodinium sp.	348	0	0	0	0	65	320	339	328	0	0	0
Bacillariophyceae												
Nitzschia sp.	658	121	0	0	156	281	541	260	290	113	95	58
Navicula sp.	131	0	0	0	0	0	180	90	120	78	83	61
Pseudonitzschia sp.	28	12	0	0	0	0	0	0	0	0	0	0
Chaetoceros sp.	188	68	0	0	0	46	250	98	134	0	0	30
Thalassiosira sp.	472	162	81	0	0	0	543	330	412	190	48	51
Skeletonema sp.	1284	835	38	0	0	25	1830	890	1186	238	108	120
Gyrosigma sp.	410	328	88	0	0	30	968	854	912	808	458	490
Prymnesiophyceae												
Chrysochromulina sp.	35	8	0	0	0	0	0	0	0	0	0	0
Chlorophyceae												
Dunaliella sp.	720	184	0	0	0	0	870	912	938	860	885	925
Cyanophyceae												
Trichodesmium sp.	54	13	0	0	0	0	0	0	0	0	0	0
Synechochoccus sp.	185	90	0	0	0	0	250	568	387	870	840	1090

Each value is the mean of three readings.

Table 2. Haemolytic activity (EC_{50}, cells ml^{-1}) and results of *Artemia salina* assay (LC_{50}, cells ml^{-1}) for cells and supernatant (medium) of both blooms and batch cultures of *Heterosigma akashiwo* from Saudi coastal waters

Bloom/Strain	Erythrocyte lysis assay		
	*EC_{50} Aq. extract (cell no.)	EC_{50} Medium (cell equiv.)	EC_{50} Meth. extract (cell no.)
27 May bloom	4.8×10^4	12.3×10^4	4.72×10^4
3 June bloom	4×10^4	14.37×10^4	3.64×10^4
10 June bloom	4.5×10^4	13.83×10^4	3.9×10^4
17 June bloom	4.92×10^4	9.61×10^4	4.82×10^4
H. akashiwo strain 1	6.03×10^4	ND	5.13×10^4
H. akashiwo strain 2	6×10^4	ND	5.12×10^4
H. akashiwo strain 3	5.58×10^4	ND	5.1×10^4
H. akashiwo strain 4	5.97×10^4	ND	5.09×10^4
	A. salina assay		
	LC_{50} Aq. extract (cell equiv.)	LC_{50} Meth. exctract (cell equiv.)	Medium (cell equiv.)
27 May bloom	11.3×10^4	9.8×10^4	*ND
3 June bloom	9.7×10^4	8.9×10^4	ND
10 June bloom	10.1×10^4	9.1×10^4	ND
17 June bloom	10.8×10^4	9.5×10^4	ND
H. akashiwo strain 1	14.4×10^4	12.3×10^4	ND
H. akashiwo strain 2	14.8×10^4	11.9×10^4	ND
H. akashiwo strain 3	14.7×10^4	11.6×10^4	ND
H. akashiwo strain 4	14.5×10^4	12.2×10^4	ND

*EC_{50} – Effective concentration causing 50% haemolysis.
*ND – Non-detectable.

samples, neither the methanol nor the aqueous extracts of *H. akashiwo* strains isolated from different blooms during the present study showed any significant variation in toxicity to *A. salina* (F=3.1, P=0.08 & F=1.95, P=0.2 respectively). However, the methanol extracts of these strains did exhibit a greater toxicity towards *A. salina* than the aqueous extracts, with LC_{50} values varying significantly between the two extracts (F=132.1–640, P=0.000001–0.0003). On the other hand, the cell-free medium of these strains and the supernatants of the centrifuged bloom samples did not cause mortality in *A. salina* (Table 2).

The results of the erythrocyte lysis assay (ELA) showed that both methanol and aqueous extracts of the *H. akashiwo* bloom exhibited haemolytic activity with respect to rabbit erythrocytes. The activity differed significantly between the aqueous and the methanol extracts (F=89.1–178.8,

$P<0.000001$). In general, the methanol extracts of these bloom samples caused higher haemolytic activity ($EC_{50} = 3.64–4.82 \times 10^4$ cells ml^{-1}) than the aqueous extracts ($EC_{50} = 4–4.92 \times 10^4$ cells ml^{-1}) (Table 2). Moreover, the haemolytic activity varied significantly among bloom samples collected in different periods of the present study ($F=17.1–1531.1$, $P= 0.01–0.00009$). The highest haemolytic activity was elicited by the methanol extract of the 3 June bloom ($EC_{50} = 3.64 \times 10^4$ cells ml^{-1}), whereas the lowest activity was recorded in the aqueous extract of the 17 June bloom ($EC_{50} = 4.92 \times 10^4$ cells ml^{-1}). The *H. akashiwo* strains isolated from these blooms also displayed haemolytic activity with EC_{50} values that did not vary significantly among these strains ($F=2.37–2.74$, $P=0.1$). However, the haemolytic activity of these strains did show a significant variation between the methanol and aqueous extracts ($F=1024.9–6288.1$, $P<0.001$). The methanol extracts exhibited a higher haemolytic activity than the aqueous extracts (Table 2). The cell-free culture supernatants of these strains did not cause any haemolytic activity. However, the cell-free water of the different blooms produced a haemolytic activity that varied among the bloom samples with the highest activity ($EC_{50} = 9.61 \times 10^4$ ml^{-1} cell equivalents) obtained for the bloom samples of 17 June, when the bloom density began to decrease (one week before the bloom collapse).

4. Discussion

4.1. Environmental and biological factors associated with *Heterosigma* blooms

This is the first report of a HAB of *Heterosigma akashiwo* in Red Sea coastal waters off Saudi Arabia. The ultimate source of these new HAB taxa in Saudi waters is enigmatic, but they could have been transported in ship ballast water, which is thought to be an important means by which HAB taxa move around the world (Marshall et al. 2005, Smayda 2007). However, even if the inoculation of the seed population of an organism into the water column does occur, these species do not bloom unless environmental conditions are favourable to their growth. In the case of *H. akashiwo*, the development and formation of blooms in specific locations worldwide have been linked to cultural eutrophication (Anderson et al. 2008, Rensel et al. 2010), along with other abiotic factors including temperature, salinity, irradiance and day length (Martinez et al. 2010). In the present study, the *H. akashiwo* bloom occurred only at site 1 (the bloom site), which is located near a shrimp farm, but was not detected at site 2 (the non-bloom site), which is about 20 km distant from site 1 and not exposed to aquaculture discharge. As site 1 exchanges water with the adjacent shrimp farm, it is

possible that some nutrients derived from this farm could have contributed to the formation of *H. akashiwo* blooms at this site. This hypothesis was tested during the present study by plotting the physico-chemical properties of sea water at the bloom site against those of the non-bloom site. The two sites showed significant differences in nutrient concentrations (NO_3, NH_4, PO_4) rather than other variables (e.g. temperature, pH, salinity). The concentrations of these nutrients were higher at the bloom site than at the non-bloom site. These very high nutrient concentrations at site 1 presumably occurred because of the fish farm discharge into this site making it eutrophic. The worldwide increase in aquaculture is considered a part of the eutrophication problem, and has been blamed for pollution of the ecosystem (Stewart 1997). Such eutrophic conditions could have favoured the formation of the *H. akashiwo* bloom at site 1, in line with previous studies reporting that blooms of *H. akashiwo* have often been associated with or stimulated by fish pens or shellfish aquaculture operations (Taylor & Haigh 1993, Smayda 1998, Peperzak 2002).

The *H. akashiwo* bloom appeared in Saudi waters when the water temperature increased from 17 to 19°C and the salinity decreased from 37.3 to 29‰, following the rainfall that usually occurs at this time of the year. These results are consistent with previous field studies, showing that *H. akashiwo* bloom formation occurs at temperatures above 15°C (Taylor & Haigh 1993, Imai & Itakura 1999, Almeda et al. 2011) in waters of lesser salinity (Hershberger et al. 1997, Kempton et al. 2008). However, the extent and intensity of the *Heterosigma* bloom in Saudi waters correlated negatively with salinity over a narrow range (26.3–34.2‰) but did not significantly change within the temperature range (19–31.4°C). The salinity and temperature ranges at which the *H. akashiwo* bloom occurred in Saudi coastal waters are close to those ranges associated with *H. akashiwo* blooms elsewhere in the world. Yamatogi et al. (2006) recorded the appearance of *H. akashiwo* in Isahaya Bay at water temperatures of 18.1–31.5°C and salinities of 23.60–34.78‰. Lee & Kim (2008) found *H. akashiwo* in Wonmun bay at temperatures of 19.5–29.8°C and salinities of 22.4–31.81‰. The absence of a correlation between temperature and the *Heterosigma* bloom in the present study is reliable, as the water temperatures throughout the study period were within the optimal range ($\geq$15°C) for *Heterosigma* growth. Therefore, it is unlikely that water temperature was a major factor regulating fluctuations of *H. akashiwo* during the present investigation period. That the disappearance of *H. akashiwo* blooms from Saudi waters followed the increase in salinity to more than 40‰ indicates that this strain of *Heterosigma* could not tolerate or adapt to such high salinities. The disappearance of a *H. akashiwo* bloom following a salinity increase

was previously investigated in Hakata Bay, Japan (Shikata et al. 2008). This observation is also consistent with other studies reporting that the highest salinity level at which the lowest level of growth of *H. akashiwo* is attained is 40‰ (Haque & Onoue 2002, Lee et al. 2005). In this regard, it has been stated that *Heterosigma* strains have the physiological ability to adapt exceptionally quickly to the range of salinities characteristically encountered in their natural environments (Honjo 2004). However, salt stress could affect the physiology of Raphidophyceae (Zhang et al. 2006), as has been reported for cyanobacteria, through iron imbalances and/or induced nutrient deficiencies (Shukla et al. 1997). In addition to salinity, the decline of *H. akashiwo* blooms can be attributed to the attack of specific bacteria and viruses (Lawrence et al. 2001, Tomaru et al. 2004) and to grazing by ciliates and heterotrophic dinoflagellates (Jin Jeong et al. 2003).

Of greater interest in this study is that the abundance of *H. akashiwo* showed a strong positive correlation with nutrient concentrations of NH_4, NO_3 and PO_4. This finding supports the hypothesis that bloom stimulation by nutrients may be a general feature of HAB taxa (Heisler et al. 2008). Specifically, *H. akashiwo* abundance is favoured over competing co-occurring phytoplankton under conditions of enhanced PO_4, NH_4 and NO_3 (Zhang et al. 2006). Remarkably, no algal species except *Chattonella* was found during the *H. akashiwo* bloom in Saudi waters during the present study. Previously, *Heterosigma* blooms had been found as monospecies in the Salish Sea (Rensel et al. 2010), and this may be due to the allelopathic activity of *Heterosigma* inhibiting or even excluding co-occurring phytoplankton and other organisms (Yamasaki et al. 2007, Yamasaki et al. 2009). The co-occurrence of *Chattonella* with *Heterosigma* blooms, as recorded here in Saudi waters, has been noted elsewhere in the world (Demir et al. 2008, Handy et al. 2008). In this regard, Smayda (1998) noted that the raphidophyte suite of *Heterosigma*, *Fibrocapsa* and *Chattonella* often co-occur, and speculated that a global niche may be opening up for this HAB group.

4.2. Toxicity of *Heterosigma akashiwo*

Besides studying the prevailing environmental conditions in Saudi waters favouring *Heterosigma akashiwo* blooms, the toxicity evaluation of this species was also a major point of interest. In this study, both aqueous and methanol extracts of *Heterosigma* blooms and batch cultures were toxic towards the brine shrimp *Artemia salina*, indicating the general toxicity of this species. Previously, it had been reported that *H. akashiwo* strongly inhibited the swimming activities of *A. salina* (Yan et al. 2003, 2004). *H. akashiwo* produces polysaccharide-protein complexes (APPCs),

analogous to a glycocalyx, which has allelopathic effects on phytoplankton and zooplankton communities (Yamasaki et al. 2009). The inhibitory effect of APPCs has been attributed to the fact that they cause *H. akashiwo* cells to adhere to the zooplankton body, strongly impairing swimming ability and consequently, decreasing food ingestion, development, reproduction and survival (Yan et al. 2003, Wang et al. 2006, Xie et al. 2008, Yu et al. 2010). Although we did not test the toxicity of *H. akashiwo* on other aquatic animals, these could well be affected in the same way as *A. salina*. Other studies have reported the negative effects of *H. akashiwo* on the survival, feeding, growth and/or reproduction of some species of copepods (Yu et al. 2010), rotifers (Xie et al. 2008) and on early stages of invertebrate larvae (Wang et al. 2006, Almeda et al. 2011). The negative effects of *H. akashiwo* on invertebrates may have potential impacts on benthic recruitment and energy transfers to higher trophic levels in marine food webs. Additionally, the inhibitory effects of *Heterosigma* on zooplankton abundance may contribute to the reduction of grazing pressure on harmful algal blooms (Almeda et al. 2011), leading to an increase in the extent and intensity of these blooms in the aquatic environment.

In addition to being toxic to *A. salina*, *H. akashiwo* exhibited marked haemolytic activity towards rabbit erythrocytes. The production of haemolytic substances is the most probable mechanism of fish kill by *H. akashiwo* and other ichthyotoxic raphidophytes (Landsberg 2002, Fu et al. 2004, Kuroda et al. 2005, Ling & Trick 2010). These compounds have been identified as polyunsaturated fatty acids (PUFAs) (Marshall et al. 2003, Pezzolesi et al. 2010). In this study, we report the powerful haemolytic activity of bloom samples and batch cultures of *H. akashiwo*. However, we have been unable to identify the substances responsible for the haemolytic activity in *H. akashiwo* extracts. Therefore, further study is needed to identify and characterize these haemolytic agents. Potent haemolytic activity was detected only in ultrasonic-ruptured *H. akashiwo* cells and in cell-free suspensions of blooms, but not in the cell-free medium of batch cultures. This may be explained by the hypothesis that the haemolytic agents of raphidophytes are located in certain intracellular compartments, and leakage or release of these haemolytic agents from algal cells occurs only as a consequence of cell damage and does not take place during normal growth (Kuroda et al. 2005, Ling & Trick 2010). This hypothesis is also supported by our results, indicating that the haemolytic activity of a cell-free suspension of bloom samples increased with decreasing *Heterosigma* cell numbers in the bloom, reaching its maximum when the bloom began to collapse.

Given that a concentration of 3 μg saponin ml^{-1} induced 50% haemolysis in the present study (data not shown), the haemolytic activities of Saudi *H. akashiwo* blooms (3.64–4.92 $\times$ 10^4 cells ml^{-1}) and batch cultures (5.97–6.03 $\times$ 10^4 cells ml^{-1}) are in accordance with the ranges reported for raphidophytes in other studies. Ling & Trick (2010) found that 50% haemolysis was observed for sonicated extracts of *H. akashiwo* at concentrations of 1.5–6 $\times$ 10^4 cells ml^{-1} and 2.5 μg ml^{-1} saponin. For *Fibrocapsa japonica*, the EC_{50} values ranged between 1.7–6.3 $\times$ 10^4 cells ml^{-1} (de Boer et al. 2004) and 0.4–1.9 $\times$ 10^4 cells ml^{-1} (de Boer et al. 2009) at EC_{50} of 4.5 μg ml^{-1} saponin as a reference. The present study also revealed a higher haemolytic activity in bloom extracts than in batch culture extracts of *H. akashiwo.* This finding could be due to the exposure of the bloom to many stresses such as salinity and nutrient limitation in the natural environment, which induces the algal cells to produce more toxins, as reported in previous studies (Ono et al. 2000, Haque & Onoue 2002, de Boer et al. 2004). This is in contrast to the cells of batch cultures, which mostly grow under optimal conditions.

Furthermore, the haemolytic activity, particularly of methanol extracts, differed significantly among bloom samples collected at different periods from Saudi coastal waters during the present study. Interestingly, the highest haemolytic activity (low EC_{50}) was associated with lower salinities and higher nutrient concentrations. These results are in accordance with previous studies regarding the negative correlation between salinity increase and toxin production by *H. akashiwo* (Haque & Onoue 2002) and *F. japonica* (de Boer et al. 2004). On the other hand, the correlation of haemolytic activity of *Heterosigma* blooms with nutrient concentrations contrasts with the results of many studies stating that toxin production is induced by nutrient limitation in dinoflagellates (Anderson et al. 1990, Simonsen et al. 1995), *H. akashiwo* (Bruyant et al. 2005) and prymnesiophytes (Johansson & Granéli 1999a,b). However, our results coincide with those obtained by de Boer et al. (2004), who found that the raphidophyte *F. japonica* produced haemolytic compounds despite the nutrient-limiting conditions. The discrepancy in the results between our study and other previous studies can be explained by the hypothesis that the effects of different environmental conditions on the production of toxins by harmful algae can vary substantially and are likely to be species-specific (Johansson & Granéli 1999b).

5. Conclusions

This is the first report of the presence of a harmful bloom of *Heterosigma akashiwo* in Saudi coastal waters. The study found a close relationship

between the formation of the *Heterosigma* bloom and nutrient discharge from a nearby shrimp farm into the bloom site. The appearance of the *Heterosigma* bloom at this site coincided with a rise in temperature (up to 24°C) and a decrease in salinity to below 30‰ as a consequence of rainfall during this time of the year. Our results also showed that the intensity (cell density) of the *H. akashiwo* bloom differed significantly between bloom samples collected during the study period, and correlated positively with nutrient (NO_3, NH_4, PO_4) concentrations but inversely with salinity. Interestingly, only the raphidophyte *Chattonella* was associated with *H. akashiwo* during the bloom period, indicating the allelopathic activity of *Heterosigma* towards co-occurring phytoplankton from other groups. Both the bloom and isolated strains of *H. akashiwo* were toxic to *Artemia salina*. The results of ELA revealed the haemolytic activity of Saudi *H. akashiwo*; this activity was statistically correlated with low salinity and high nutrient concentrations. Even though no fish mortality was reported in the study region during the present study, the literature records that such a haemolytic raphidophyte may cause ichthyotoxicity and mortality in fish in the sea and in shrimps in local aquacultures. Although the *H. akashiwo* bloom had crashed and disappeared from Saudi coastal waters by the end of June 2010, the potential recurrence of such a bloom in this or other locations along Saudi Red Sea coasts cannot be ruled out. Therefore, Saudi coastal waters, particularly those areas adjacent to aquacultures, where water and HAB populations can be exchanged with them, should be regularly monitored for the presence of such harmful algal blooms.

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