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Pergamon

0273-1223(95)00010-0

Wat. Sci. Tech. Vol. 30, No. 8, pp. 295–302, 1994.
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0273–1223/94 $7·00 + 0·00

EFFECT OF MICROALGAE GROWING ON WASTEWATER BATCH CULTURE ON *ESCHERICHIA COLI* AND *VIBRIO CHOLERAE* SURVIVAL

N. Mezrioui*, B. Oudra*, K. Oufdou*, L. Hassani*, M. Loudiki* and J. Darley**

* *Université CADI AYYAD, Faculté des Sciences - Semlalia, Départment de Biologie, B.P. S/15, Marrakesh 40 000, Morocco*
** *Ministère de l'Intérieur, Direction Générale des Collectivités Locales, Division de l'eau et de l'assainissement, Rabat, Morocco*

ABSTRACT

The stabilization pond is one of the more important biological wastewater treatment systems, applied in many countries. An experiment treating wastewater by stabilization ponds under the arid climate of Marrakesh (Morocco) has been underway since 1985. The experimental installation, made from two lined stabilization ponds, received domestic sewage which carried not only the organic load but also a significant bacterial load and other microorganisms. In this new habitat, the cells' bacterial behaviour was affected by various physico-chemical and biological factors. It appears that in such treatment system, known for excessive algal production, the microalgae has evidently an influence on bacterial growth. In this paper, we proposed to appreciate how microalgae essentially: *Chlorella* (Chlorophyta), *Synechococcus* and *Synechocystis* (Cyanobacteria), can affect the behaviour, survival and temporal evolution of *Escherichia coli* and *Vibrio cholerae*. In wastewater stabilization ponds of Marrakesh high levels of *V. cholerae* and low concentrations of coliform bacteria were noted during summer periods. This period coincided with a bloom of picocyanobacteria associated with a weak relative abundance of *Chlorella*. Some interactions tests were carried out with these bacteria and these algae, using a treated wastewater batch culture. Results show that the green algae reduces *V. cholerae* (pathogenic bacteria) abundances more than *E. coli* (fecal contamination bacteria) where as better survival of this pathogenic bacteria was noted in presence of Cyanobacteria. The die-off of *E. coli* appears to be more reduced in presence of Cyanobacteria than *Chlorella*. Furthermore, the alkaline pH seems to present a more bactericidal effect on *E. coli* than on *V. cholerae*. Thus, the Cyanobacteria blooms, associated with a weak percentage of *Chlorella* abundance, occurring periodically during summer in sewage stabilization ponds of Marrakesh, will be considered as one of the major factors leading to high levels of *V. cholerae* and low abundances of fecal coliform bacteria during the hot period.

KEYWORDS

Chlorella, Cyanobacteria, *Escherichia coli*, survival, *Synechocystis*, *Synechococcus*, *Vibrio cholerae*, wastewater.

INTRODUCTION

The physiological state of *Vibrio cholerae* can by affected be various conditions during their lifetime in aquatic environments (Singleton *et al.*, 1982; Huq *et al.*, 1984; Colwell, 1986). The action of phytoplankton

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was considered as one of the most important biological factors controlling bacterial growth in aquatic habitat (Rhee, 1972; Forlani *et al.*, 1988). Some studies relevant to the biological interactions between bacteria and algae, confirm the positive or negative mutual influence (Dor and Svi, 1980 a, b). Islam *et al.* (1989) observed that *Vibrio cholerae* can extend their survival time in an artificial aquatic environment in association with a green algae, *Rhizoclonium fontanum*. In the sewage treatment system, this association is considered as the principal factor leading to biological waste purification efficiency (Oswald, 1988). Domestic sewage carry not only organic load but also a significant pathogenic bacteria load as *V. cholerae*. In wastewater, temporal evolution of *V. cholerae*, which is negatively correlated with fecal contamination indicator, reached low levels during the cold season and growth levels in warm season (Nair *et al.*, 1988; Hofer and Ernandez, 1990; Martin and Bonnefont, 1990). So, understanding of the die-off and aftergrowth characteristics of *V. cholerae* and indicator bacteria in the presence of various algal species is of design importance. The few available data don't clarify the effect of the algal species on *V. cholerae* were also noted during summer periods (Lesne *et al.*, 1991), which coincide with net blooms of picocyanobacteria observed in the same study site (Oudra *et al.*, 1994). In this investigation, the effect on growth of algae isolated from wastewater stabilisation ponds was followed for a short period, to appreciate how these algae can affect the behaviour, survival and establishment of *E. coli* and *V. cholerae* in such wastewater treatment systems.

MATERIALS AND METHODS

– Bacteriai strains

Bacterial strains tested were *E. coli*, isolated from stabilisation ponds influent, and *V. cholerea* E1 tor ogawa serotype, isolated from a patient (Sanitary Institut–Rabat). These purified bacterial strains were grown on trypticase-soya agar at 37°C for 18 hours. Cells from the exponential phase were suspended in physiological water (0.9% of NaCl) and used as inoculum.

– Algae strains

The cultures used were unialgal but not axenic and were as follow: *Chlorella sorokiniana* SOROKIN & KRAUSS (green algae) isolate from the wastewater stabilization ponds of Marrakesh (Morocco) and maintained on culture on a solidified mineral medium suggested by Dauta (1982). The cyanobacteria strains: *Synechococcus elongatus* NAGELI (Sync) and *Synechocystis parvula* PERIFILIEW (Synst) were also isolated from the same study site and were cultivated on BG 13 medium (Ferris and Hirsch, 1991). These microalgae cells were maintained in the exponential phase by systematic repicage (each 3 days) in a specific growth medium. In order to have a synchronized inoculum, the algal cells were suspended in a few ml of filtered wastewater and stored in the dark for 24 hours.

– Experimental procedure

In order to study interactions between bacteria and algae in wastewater habitat, the reaction medium used was a treated wastewater, filtered (Glass fiber filter, Whatman GF/C), autoclaved and supplemented with nitrogen by Ca $(NO_3)_2$ and phosphorus by K_2HPO_4, to obtain a final concentration respectively of 3.41 mg N/l and 1.78 mg P/l. Such enrichment avoids the cells' growth nutrient limitation over the experimentation time. To reach the investigation objective, a series of tests were carried out; the microbial cells were grown in flasks containing 140 ml of a reaction medium, as specified above, under three different conditions.

(i) Medium 1: reaction medium and bacteria (*E. cole* and *V. cholerae*) incubated in a light/dark cycle.

(ii) Medium 2: reaction medium seeded with both tested bacteria and algae (*Chlorella* or *cyanobacteria*) and incubated under light/dark cycle.

(iii) Medium 3: The same reaction medium as medium 2 but kept completely dark.

The erlenmeyer flask cultures (medium 1 and 2) were incubated exactly in a 14:10 light/dark regime (provided by 40W white fluorescent tubes) at an intensity of 60 $\mu E\ m^{-2}s^{-1}$. The room temperature was kept at 26°C±1°C. The culture flasks were agitated as appropriate on a table shaker set at 100 rev./min.

The algal growth was estimated as increase in cells number by direct counting using an Haemacytometer-Malassez, 0.2 mm deep (Sournia, 1978). The growth rates were determined by expression $\mu = Ln\ N_1/N_0$, in

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which No was the initial number of cells and N1 was number of cells after 24 h. of incubation the doubling time (dt) was calculated from $dt = 0.69 / \mu$ (Guillard, 1973).

Enumeration of bacteria was done by indirect count of colonies forming units (CFU) on selective media: TCBS agar (Difco) incubated at 37°C for 24 hours (*V. cholerae*) and TTC-Tergitol 7 agar incubated at 44.5°C for 24 h. (*E. coli*). The bacterial and algal growth counts were daily done twice after each clear and dark period. The pH was simultaneously measured, using ORION Research, model 601A pH meter.

RESULTS AND DISCUSSION

Bacterial abundances of *E. coli* and *C. cholerae* in filtered and autoclaved wastewater stabilization pond effluent, seeded or not with algae, and exposed to light or kept dark, are illustrated in figure 1.

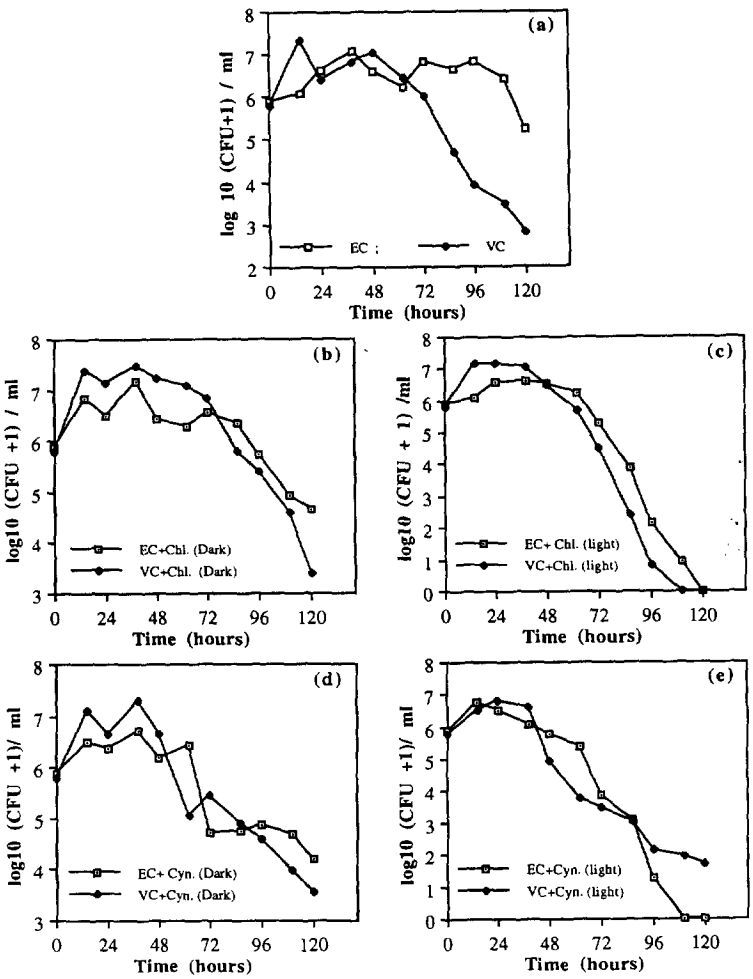

Fig. 1. Comparison of temporal evolution of *E. coli* and *V. cholerae* incubated in light and dark, mixed or not with *Chlorella* (chl.) and Cyanobacteria (Cyn.).

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In order to compare the behaviour of both studied bacteria in this microcosm study, the bacterial die-off coefficients (k) were calculated from data obtained under experimental conditions. The computation of k derives from the theoretic equation of exponential decline of bacterial abundance: $Nt = No\ e^{-kt}$ where No is the initial cells concentration, Nt is the concentration of bacteria after incubation time, t: time of incubation (in hours) and k: die-off coefficient (in h^{-1}) (Chamberlin and Mitchell, 1978; Burdyl and Post, 1979). Table 1 gives the bacteria survival, estimated by k calculation in presence and absence of algae.

TABLE 1. Bacteria Survival in Terms of Die-Off Coefficient (k Expressed by h^{-1}, n=3 Repetitions) in Presence or Absence of Algae in Light and Dark Microcosms

Bacterial strains	Bacteria only (light)*	Bacteria + *Chlorella* (light)*	Bacteria + *Chlorella* (dark)	Bacteria + Cyn. (light)*	Bacteria + Cyn. (dark)
E. coli	0.0144 ± 0.0014	0.0656 ± 0.0011	0.0191 ± 0,0006	0.0731 ± 0.0005	0.0226 ± 0.0002
V. cholerae	0.0375 ± 0.0012	0.0876 ± 0.0008	0.0388 ± 0.0002	0.0536 ± 0.0009	0.0321 ± 0.0004

Cyn. : Cyanobacteria = Synechocystis (Synst.) + Synechococcus (Sync.)
* 14 : 10 light / dark cycle.

During the first two days of experiment, we note an increasing of bacterial abundance. Beyond 62 hours bacterial reduction is observed particularly when bacteria were cultivated with algal cells (figure 1). In fact, the bacterial die-off rate (k), at light is well expressed in presence of algae (Table 1). In a control flask (bacteria without algae) the coefficients (k) evaluated for *E. coli* and *V. cholerae* were respectively 0.0144 and 0.0375 h^{-1}. When the same reaction medium was seeded with *Chlorella* or Cyanobacteria and exposed to light, survival of both bacteria was significantly reduced in comparison with the control flask or microcosm containing bacteria and algae incubated in the dark. The die-off rate of *E. coli* in light is 0.0656 and 0.0731 h^{-1} in presence respectively of *Chlorella* and Cyanobacteria. The mortality of *V. cholerae* is also more pronounced in light when it's associated with algae; the coefficient k is 0.0876 h^{-1} with *Chlorella* and 0.0536 h^{-1} with Cyanobacteria.

In absence of algal activity (incubation in the dark), survival of *E. coli* and *V. cholerea* is increased over that measured in the same microcosm but exposed to light (Table 1). The die-off rate of *E. coli* associated with *Chlorella*, which is 0.0656 h^{-1} in light became 0.0191 h^{-1} in the dark. The same patterns are observed with *V. cholerae*. In the presence of *Chlorella*, *V. cholerae* survives better in the dark (k=0.0388 h^{-1}) than in light (k=0.0876 h^{-1}). Therefore, the die-off of *E. coli* and *V. cholerae* was faster when exposed with algae to light than compared with the control microcosm (bacteria cultivated without algae) or the microcosm containing algae and incubated in the dark.

This increasing bacterial die-off in presence of algae in light conditions can be explained in part by the increasing pH values which reached 9.9 (figure 2). On the other hand, the bacteria reduction can be explained by the algal activity which is different in light and dark microcosms. In fact, the bacterial reduction is correlated with increasing exponential phase of algal growth (figure 3). In light batch cultures, a higher growth of algae is observed than in the dark microcosms. The global growth rate for *Chlorella* is 4.44 day^{-1} in light and 0.48 day^{-1} in the dark. For Cyanobacteria, the two species *Synechococcus* and *Synechocystis* show equally a high growth in light. The global growth rate is respectively 1.47 and 0.99 day^{-1} in light and in the dark; we note a significant reduction in cells numbers (mortality) (Table 2). In batch algal culture kept in the dark, the pH remains stable during incubation-time (figure 3) and the algal activity is less intense. Therefore the bacteria die-off is attenuated.

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However, if we compare the survival of pathogenic bacteria (*V. cholerae*) and indicator pollution bacteria (*E. coli*), we note under these experimental conditions that the behaviour of these two species is different. In presence of *Chlorella* incubated in light, *E. coli* survives better ($k = 0.0656\ h^{-1}$) than *V. cholerae* ($k = 0.0876\ h^{-1}$). An inversion of bacterial survival is noted with Cyanobacteria. The die-off coefficient is about 0.0536 h^{-1} for *V. cholerae* against 0.0731 h^{-1} for *E. coli*.

Considering what number of algae would affect what number of bacteria, we note that during the experimentation time, the Cyanobacteria strains, with an average global growth of $\mu = 1.23\ d^{-1}$, lead to an accentuated mortality of *E. coli* ($k = 0.073\ h^{-1}$) compared with that observed with *V. cholerae* ($k = 0.0536\ h^{-1}$). No cells of *E. coli* are detected after 120 hours of incubation (fig. 1-e). In presence of *Chlorella*, with a global rate of 4.44 d^{-1}, we note a more significant mortality of *V. cholerae* ($k = 0.0876\ h^{-1}$) than that found for *E. coli* ($k = 0.0656\ h^{-1}$).

TABLE 2. Computation of Microalgae Global Growth Rate (μ in d^{-1}) and Global Doubling Time (dt in h), Associated with Bacteria in Light and Dark Microcosms, During the Experimental Time (5 Days)

Interval, day	Light * (1-5)		Dark (1-5)	
	μ (d-1)	dt (h)	μ (d-1)	dt (h)
Chlorella	4.44	0.16	0.48	1.46
Synechcoccus	1.47	0.48	- **	- **
Synechocystis	0.99	0.70	- **	- **

* 14 : 10 light / dark cycle.
** negative values : reduction of cells number.

The same results are also noted when we compare the bacterial survival in light and dark microcosms. In presence of *Chlorella*, it appears that *V. cholerae* was slightly more subject to a reduction of survival rate than *E. coli*. We note a difference in mortality in light and dark cultures of 0.0488 h^{-1} for *V. cholerea* against 0.0456 h^{-1} for *E. coli*. The same difference, evaluated in presence of Cyanobacteria, becomes reversed. *V. cholerae* show a difference in mortality (light/dark) of only 0.0215 h^{-1} in contrast to *E. coli* which present a great difference of 0.0363 h^{-1}.

Furthermore, if *E. coli* usually survives longer than *V. cholerae* in our experimental study, the survival of *V. cholerae* ($k = 0.0536\ h^{-1}$) becomes higher than that of *E. coli* ($k = 0.0731\ h^{-1}$) in presence of Cyanobacteria.

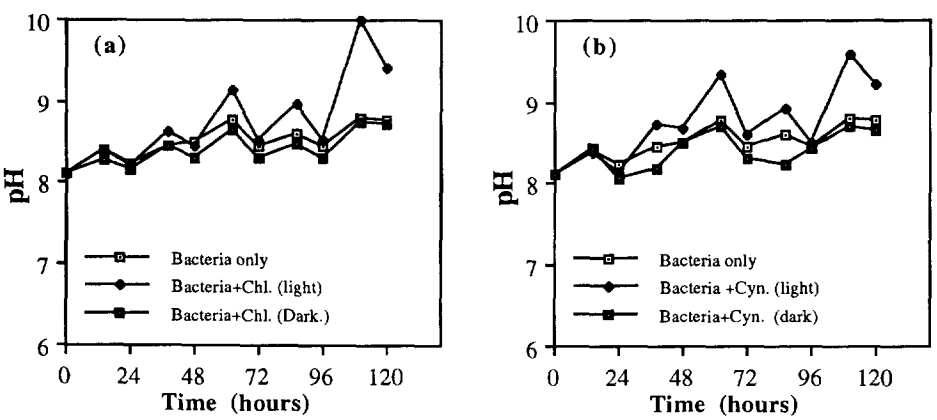

Fig. 2. Temporal evolution of pH in batch cultures containing bacteria and/or algae incubated in light and dark.

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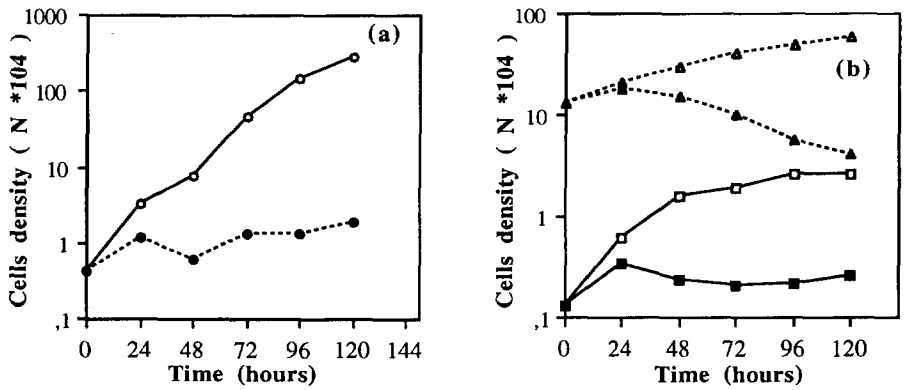

Fig. 3. Growth behaviour of *Chlorella* and Cyanobacteria (sync. and Synst.) incubated with bacteria in a light / dark regime and in complet dark (logarithmic scale).
—o— : *Chlorella* (Light) ; ---◆--- : *Chlorella* (Dark); ---▲--- : *Synst* (Dark)
---☆--- : *Synst* (Light) ; —■— : *Sync* (Dark) ; —□— : *Sync* (Light)

It is visible then that Cyanobacteria promotes survival of *V. cholerae* (k= 0.0536 h^{-1}) better than does *Chlorella* (k = 0.0876 h^{-1}). Whereas *E. coli* survives better with *Chlorella* (k = 0.0656 h^{-1}) than when it's associated with Cyanobacteria (k = 0.0731 h^{-1}). This higher bacterial reduction of *V. cholerae* observed with *Chlorella* can be attributed principally to substances released by this green algae which have a greater effect on *V. cholerae* than on *E. coli*. The difference in survival between *E. coli* and *V. cholerae* in presence of Cyanobacteria in light microcosms can be attributed to several factors. the pH, which is influenced positively by algal activity, seems to be an important factor stimulation the growth of *V. cholerae* (Hiq *et al.*, 1984; Colwell, 1986). Nair *et al.* (1988) who have studied the ecology of *V. cholerae* in some aquatic environments in India, have shown that the summer peaks of *V. cholerae* abundances are correlated with alkaline pH. Islam (1990) has also reported that the algal blooms and alkaline pH are associated with cholera proliferation period in Bangladesh.

In contrast for *E. coli*, it is widely described in the literature that alkaline pH presents a bactericidal effect towards this species (Pearson *et al.*, 1987; Mezrioui and Baleux, 1992). Equally this difference in survival can be attributed to toxin substances released by this alga in aquatic environments. It's known that these algal populations (Cyanobacteria) are able to synthesise toxic substances which have antibacterial properties (Himberg *et al.*, 1989; Repavich *et al.*, 1990; Yasumo and Sugawa, 1991). These substances seem to have a greater effect on *E. coli* than on *V. cholerae*.

This phenomenon was also observed when the bacterial survival was compared in the microcosm incubated without algae and the microcosm containing algae incubated in the dark. In this last medium, we observed that *V. cholerae* survives longer in the dark particularly with Cyanobacteria (k=0.0321 h^{-1}) than in control flask (k=0.0375 h^{-1}). Whereas *E. coli* survives longer in control microcosm (k=0.0144 h^{-1}) than in batch cultures kept in the dark (k=0.0191 h^{-1} with *Chlorella* and k=0.0226 h^{-1} with cyanobacteria). The relative better behaviour of *V. cholerae* observed with Cyanobacteria in dark than for *E. coli* can be attributed to the availability of *V. cholerae* to nutrients. Organics and other nutrients contained in algal protoplasm may serve as the nutrient source as described elsewhere (Augier, 1972; Soeder *et al.*, 1986). It's also probable that darkness-incubated algae which are stressed can release antibiotic substances in the medium. These substances seem to have a more toxic effect on *E. coli* than on *V. cholerae*.

These results are inclusively in concordance with the observations in stabilization ponds and particularly in those of Marrakesh. The phytoplanktonic (picocyanobacteria) blooms observed during summer (Oudra *et al.*, 1994) can be considered as one of the factors explaining abundance evolution of *V. cholerae* which are negatively correlated with those of fecal coliforms during stabilization treatment of wastewaters of

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Marrakesh. Fecal coliforms show low levels in summer (Hassani, 1993) against *V. cholerae* which presents a high summer concentration (Lesne *et al.*, 1991).

CONCLUSION

These experimental studies showed that microalgae growing in wastewater batch laboratory cultures have a net influence on bacterial behaviour and establishment. It appears that *Chlorella* reduces *V. cholerae* (pathogenic bacteria) abundances more than *E. coli* (fecal contamination bacteria). This green algae seems to secrete products which have a greater toxic effect on *V. cholerae* than on *E. coli*. Whereas better, survival of this pathogenic bacteria was observed in presence of Cyanobacteria. These algae induce an alkaline pH which promotes the survival of *V. cholerae* and reduced *E. coli* abundances, and secrete substances which seem to lead to a greater die-off of *E. coli* than *V. cholerae*. Therefore, the phytopanktonic (Cyanobacteria) blooms during summer can be considered among the most important factors influencing the temporal distributions of these bacteria with a strong abundance of *V. cholerae* and weak concentrations of coliform bacteria during stabilization treatment of wastewaters of Marrakesh.

In this work, we have also shown that the survival of the pathogenic bacteria (*V. cholerae*) does not correlate usually with the indicator pollution bacteir (*E. coli*). Consequently, measuring abundance of fecal coliform is not a good way to obtain information for predicting abundance of pathogens like *V. cholerae* in a reliable way in wastewater stabilization ponds which contain appreciable concentrations of algae as Cyanobacteria, especially during summer periods.

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