TRICLOSAN RESISTANCE IN ENVIRONMENTAL ISOLATES

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ABSTRACT

Triclosan is a biocide that is common in the environment and to which many bacteria have acquired resistance. The frequency and identity of triclosan resistant members of eight sampling localities was investigated. Their level of resistance to triclosan, resistance to four antibiotics with different targets, and potential for growth on and degradation of triclosan was assessed. Triclosan resistant genotypes were isolated from five of eight localities sampled. In localities in which they were detected, they occurred at high frequency. The most commonly represented genera among the isolates were *Pseudomonas* and *Vibrio*. Most isolates were highly resistant to triclosan and showed cross resistance to isoniazid, an antibiotic that like triclosan, inhibits fatty acid biosynthesis. In contrast, resistance to the other antibiotics tested was uncommon. Data also suggest that some isolates may be capable of growth on triclosan in the absence of other carbon sources. These results indicate that *Pseudomonas* and *Vibrio* may become more common in habitats exposed to high levels of triclosan, and that cross resistance to antibiotics affecting fatty acid biosynthesis may be a problem among bacterial communities subjected to selection due to triclosan exposure.

Triclosan is a biocidal agent that is commonly used in commercial products, and has been found in aquatic habitats (Kolpin et al. 2002). Although it was once thought to be a non-specific biocide (Schweizer 2001), it is now known to inhibit the synthesis of fatty acids by binding enoyl reductase, which catalyzes the final reaction of fatty acid biosynthesis (Baldock et al. 1996), the protein encoded by *fabI* in *Escherichia coli* (McMurry et al 1999). This suggests that the potential for evolution of specific resistance mechanisms, and cross-resistance to antibiotics with similar targets exists.

A number of modes of resistance to triclosan have been described. These include mutations in or increased expression of *fabI*; detoxification via a number of types of efflux pumps, many of which confer resistance to other compounds; and enzymatic degradation of triclosan (Schweizer 2001). *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* are all resistant to triclosan (Chuanchuen et al 2001; Schweizer 2001), and resistance mutants have been found within species, that are primarily susceptible, such as *Escherichia coli* and *Mycobacterium smegmatis* (Schweizer 2001, McMurry et al 1999). However because most studies of triclosan resistance focus on medically important taxa, neither the frequency of triclosan resistance nor the breadth of taxa demonstrating resistance has been explored across a range of habitats.

This study assessed triclosan resistance among environmental isolates. The frequency of resistant bacteria among those culturable on rich media plates was estimated, and the identity of the isolates was determined based on restriction fragment analysis and sequencing of 16S ribosomal DNA. Diversity among the isolates with respect to a number of traits that affect potential changes in community and population structure in response to triclosan selection was also assessed. These included level of resistance to triclosan, resistance to four antibiotics with different targets, and potential for growth on and degradation of triclosan.

METHODS

Environmental sampling and collection of isolates

Water samples, swabs, or toothpicked samples were taken from the following locations: the edge of Eel Pond, a pool in Sippiwissett Marsh, the edge of Cedar Swamp, sinks and door handles in a

restroom in the Falmouth Hospital, the pavement beside the Swope Hall dumpster (MBL), showers in Ebert Hall second floor women's bathroom (MBL), and dental plaque of two course members, one a user of triclosan containing toothpaste, another a user of toothpaste containing no triclosan. These sites were chosen to represent a range of habitat types and a range of levels of potential triclosan exposure. Samples from each site were pooled and suspended in minimal media without electron donor if not water samples. Six rich media plates, either sea water complete (SBC) for Eel Pond and Sippiwissett or low salt Luria broth (LSLB) plates for the other sites, with 50μg/mL triclosan (5-Chloro-2-(2,4-dichlorophenoxy)phenol obtained from Sigma-Aldrich) were inoculated with .2mL pooled sample per site. An additional two plates without triclosan were inoculated with .1mL pooled sample as a control. Up to eight distinct colonies were isolated from these plates for each site for which there was growth on the triclosan plates. A total of 34 triclosan resistant genotypes were isolated.

In order to estimate the frequency of triclosan resistant genotypes among those capable of growth on rich plates, three samples each from Eel Pond and from the showers in an Ebert Hall bathroom were collected. Serial dilutions of these samples were plated on rich media with and without triclosan.

Identification of isolates

16S ribosomal DNA of all 34 isolates was amplified using the primers SDBact0008F20 – SDBact1492R19 (general eubacterial primers). Restriction analyses using HhaI were used to group isolates with shared restriction fragment length profiles. One isolate from per site was then sequenced for each profile type. These sequences were then compared with sequences in Genbank, and a subset was used in construction of a neighbor joining tree in the software package ARB.

Evaluation of triclosan resistance and Antibiotic cross resistance

The maximum solubility of triclosan in aqueous solution at neutral pH is low (approximately 128μg/ml (.44mM)). Therefore all isolates were grown 200 μl of saturated solution of the appropriate rich media (SWC or LSLB) in microtiter plates. At 24 hours the OD at 590nm was determined and compared that of the same genotype grown in rich media without triclosan. Two to three replicates were performed for each genotype.

Resistance to four antibiotics at 1mM concentration was evaluated as described for triclosan resistance, except that only two replicates per isolate were performed. The antibiotics isoniazid, trimethoprim, naladixic acid and spectinomycin were used. The processes affected by these antibiotics are fatty acid biosynthesis, folic acid synthesis, DNA replication and protein synthesis respectively.

Assessment of potential triclosan degradation

The ability of all isolates to grow in minimal media with triclosan as the only carbon source was assessed. The media contained FW or SW base, sodium sulfate, MOPS, trace elements, and 150μg/ml triclosan (at room temperature some of this triclosan precipitates). After two serial transfers, growth through the third transfer was assessed by plating on rich media before and after incubation. For those isolates for which dilutions yielded countable plates at both the start and end of the incubation, the Malthusian parameter was calculated: $m = \ln (N_6 \times 100^n / N_0)$, where N_0 and N_n are the initial and final population densities, and 100^6 reflects the number of 100-fold dilutions performed. This parameter is sometimes called the exponential growth rate parameter.

In order to determine whether the isolates were degrading triclosan, stationary phase cultures of isolates grown in triclosan-saturated rich media were centrifuged to remove cells and the supernatants were analyzed using high pressure liquid chromatographic (HPLC) analysis. The Agilent system with a Waters C18 column was used. The samples were eluted with 70% methanol: 30% 10mM potassium phosphate pH 2.3 with a flow rate of 1ml/minute. Triclosan was detected at an absorbance of 280nm. Triclosan concentrations were determined by comparison to a 0.5mM standard dissolved in 95% ethanol. The amount of triclosan as estimated by HPLC peak area in comparison to a standard was compared with that of the uninoculated media. In addition, concentrations of triclosan stationary phase cultures of minimal triclosan media were also estimated for a subset of the isolates.

RESULTS

Distribution of triclosan resistant genotypes

No colonies grew on the 50 μg/ml (0.167mM) triclosan plates inoculated from Cedar Swamp and either of the course members' dental plaque, despite dense growth on the control plates not

containing triclosan. In contrast, many colonies grew on all six triclosan plates inoculated from Eel Pond, Sippiwissett, Swope Hall, Ebert Hall, and the Falmouth Hospital, indicating that in habitats in which triclosan resistance occurs it may be common. Dilution plates of three samples taken from Ebert Hall showers showed that the mean frequency of triclosan resistant genotypes among cells that could form colonies on LSLB was 0.0012 (Std Dev. 0.002). The mean frequency of triclosan resistant genotypes among the three Eel Pond samples was even higher (0.0072, Std. Dev. 0.01).

Identification of isolates

Restriction digests of 16S DNA sequences revealed eight fragment length phenotypes among 27 isolates (the other seven isolates did not yield visible bands after digestion). Isolates with shared pattern types were from similar types of habitats (e.g. Eel Pond and Sippiwissett Marsh or Ebert and Swope Halls) (Table 1). Using 16S DNA sequence, 19 isolates could be placed within a general taxonomic group based either on results of NCBI-BLAST results and or BLAST results and results of a neighbor-joining tree constructed in ARB. All but two of the remaining isolates could be placed within a broad taxonomic group based on shared restriction pattern with a sequenced isolate. All 32 of these isolates fell within the gamma Proteobacteria (Table 1). The two most common groups were *Pseudomonas* species, which made up 75% of the fresh water isolates, and *Vibrio* species, which made up 71% of the salt water isolates (Table 1). In addition, isolates of species not expected to occur communally with humans, such as *Halomonas* and *Shewanella* were also observed.

Evaluation of triclosan resistance and Antibiotic cross resistance

Although the isolates were identified using plates containing a moderate triclosan concentration (50μg/ml or .44mM), all but two genotypes grew to optical densities at 590nm within 80 of the rich media controls in triclosan-saturated rich media (Figure 1). These two isolates were *Stenotrophomonas maltophilia* isolated from Falmouth Hospital.

All isolates but one grew to high ODs in the presence of 1mM isoniazid, the fatty acid biosynthesis inhibiting antibiotic (Fig. 1). In contrast, most did not grow in the presence of the antibiotics inhibiting other processes (trimethoprim inhibiting folic acid synthesis, naladixic acid, inhibiting DNA replication, and spectinomycin, inhibiting protein synthesis). The exceptions were the *Halomonas* isolate from Sippiwissett, which were only marginally resistant to isoniazid,

the two *Stenotrophomonas maltophilia* isolates from Falmouth Hospital, which were marginally resistant to both trimethoprim and spectinomycin, and three salt water isolates, one *Vibrio* and two unidentified isolates, which showed resistance to all antibiotics tested.

Assessment of potential triclosan degradation

HPLC analysis of supernatants from triclosan-saturated rich cultures was used to assess the amount of triclosan removed by the bacteria in the presence of alternative carbon sources. Since only one replicate was analyzed for each sample, these data have been grouped qualitatively into four classes ranging from low (darker) to high (lighter) (Fig. 2). Supernatants from cultures of two isolates, a *Pseudomonas aeruginosa* and a *Vibrio* showed substantial decreases in triclosan concentrations relative to uninoculated media (Fig.2).

The isolates were serially transferred on minimal media with triclosan as the only carbon source for three transfer cycles. After two transfers, no cells were detected in cultures of five isolates, and after three transfers this number had increased to seven, resulting in two negative estimated Malthusian parameters (Fig. 2). An additional nine isolates had sufficiently high growth rates that the number of cells recovered on plates for all dilutions exceeded the countable range. Growth assays of four isolates were lost due to contamination. The remaining 14 isolates whose growth rates could be estimated had Malthusian parameters ranging from 3.2 to 5.9 (Fig, 2). High growth rates on triclosan minimal media were observed in the *Pseudomonas* species with restriction fragment profiles and/or sequences that differed from those of *Pseudomonas aeruginosa*, and in *Vibrio*-like isolates with sequences most similar to *Vibrio parahhaemolyticus* (Fig. 2). HPLC analysis of supernatant after removal of cells at stationary phase showed more evidence of reduced triclosan concentrations among the *Pseudomonas* isolates that grew in this minimal media than did supernatant from the *Vibrio* cultures (Fig. 2).

DISCUSSION

Distribution of triclosan resistant genotypes

The data presented here indicate that in habitats where triclosan resistance occurs it is relatively common. Although no more than eight isolates were analyzed from each of the five localities in which resistance was found, 10s to 100s more resistant colonies grew on the initial plates, and in

two localities the frequency of triclosan resistance among culturable genotypes was estimated to be one in 100 to 1000 cells. Triclosan resistance was found not only in the habitats of human commensals but also in both saltwater habitats, and may be more common in the latter than the former based on frequency estimates and qualitative comparisons of isolation plates and controls (data not shown).

The high frequency of resistance does not necessarily indicate a direct influence of triclosan on these communities. This is because resistance may be common due to evolution in response to triclosan exposure and subsequent changes in community structure, or due to pleiotrophic effects of genes that were selected for some other function. The latter may have occurred in the saltwater localities, where frequent exposure to high triclosan concentrations is less likely than in habitats such as the Ebert Hall bathroom. However the absence of triclosan resistant genotypes from three sampled habitats indicates that resistance is either not universally beneficial, or is not an easily evolvable trait in all environments.

Identification of isolates

Both the freshwater and saltwater localities were dominated by a single group. *Pseudomonas* made up 71% of the freshwater isolates, and *Vibrio* represented 75% of the saltwater isolates (Table 1). The high frequency of *Pseudomonas* among triclosan resistant isolates was expected, because *Pseudomonas aeruginosa* is known to possess multiple resistance mechanism (Chuanchuen et al. 2001) and is common in areas of human habitation such as the ones from which these isolates were collected. Similarly, although triclosan resistance has not been described to date in *Stenotrophomonas maltophilia*, since some isolates of this organism possess multidrug resistance due to efflux pumps (Li et al. 2002), it is not unexpected. However triclosan resistance has not been reported to date in *Vibrios*. *Vibrios* have been shown to possess antibiotic resistance through a variety of mechanisms (Morita et al 2000; Petroni et al. 2002); uncovering the mechanism(s) of resistance used by the isolates in this study may aid in explaining the high frequency of triclosan resistance in these saltwater habitats.

Evaluation of triclosan resistance and Antibiotic cross resistance

With the exception of the two *Stenotrophomonas maltophilia* isolates from Falmouth Hospital, all isolates were resistant to the maximum triclosan concentration that is soluable in aqueous solution (Fig. 1). This level of resistance may indicate that the mechanism of resistance in many

of these strains is not highly concentration dependent one. Alternatively, these genotypes may be selected on substantially higher concentrations of triclosan than those used in this assay, since the presence of detergents in many triclosan-containing products may change its solubility or its entry into the cell. Regardless of the cause, this high level of resistance indicates that if triclosan exposure was to increase in these habitats, a change in community composition resulting in even higher frequencies of these already resistant genotypes is more likely than the appearance of triclosan resistance in currently susceptible members of the community.

The antibiotic cross resistance profiles reported here suggest that cross resistance to drugs that target pathways other than fatty acid biosynthesis is not common among these environmental isolates (Fig. 1). If multidrug efflux pumps are the mechanisms by which these strains achieve resistance to triclosan, these pumps are not currently useful for the elimination of the other antibiotic compounds tested in this study. In contrast, in most of the isolates resistance to isoniazid, which targets enoyl reductase (Baldock et al. 1996) was observed at 1mM concentrations in liquid culture (Fig. 1). This suggests that co-resistance to other drugs targeting fatty acid biosynthesis may be a problem in triclosan resistant bacteria. Mechanisms of resistance that are specific to the mode of action of triclosan such as mutations to *fabI* or changes in its regulation are most consistent with this observed pattern of antibiotic resistance. However an *Escherichia coli* strain not known to be resistant showed some growth in 1mM isoniazid, and MIC assays conducted on agar plates with filters soaked in antibiotics detected both resistance and susceptibility to all four antibiotics among these same isolates (data not shown). This emphasizes the assay-dependence of antibiotic resistance phenotypes, and the importance of testing in conditions as close as possible to those in which the drug is used.

Assessment of potential triclosan degradation

The positive growth parameters estimated for many of these isolates suggests that the ability to grow on minimal triclosan may be common (Fig. 2). However it is possible that some isolates were able to grow on the vitamins provided in the minimal media and control inoculations on minimal media without triclosan were not conducted. Additional replicates performed on separately prepared media with this additional control will be necessary to confirm this surprising result. The results of HPLC analyses suggest that more triclosan is consumed in minimal media than in rich media (Fig. 2). This is to be expected since alternate carbon sources

are available in the latter. Correspondence between growth rates as estimated from colony counts, and triclosan removal from minimal media as estimated from HPLC analysis cannot be analyzed statistically with only a single replicate. However although greater replication is clearly needed, these data suggest that some of these isolates, especially some of the *Pseudomonas* and *Vibrio* isolates may be capable of degrading and growing on triclosan. This is consistent with (Meade et al. 2001), who report that an isolate of *Pseudomonas putida* degrades triclosan.

Conclusions

Although some habitats sampled contained no detectable triclosan resistant bacteria using the isolation techniques reported here, in others triclosan resistant bacteria occur at relatively high frequencies. Most are *Pseudomonas* and *Vibrio* genotypes that are highly resistant to triclosan. This suggests that if triclosan exposure increases in the environment, these bacteria will increase in frequency in the communities, and other non-resistant genotypes may be lost. Antibiotic resistance profiles suggest that cross resistance to antibiotics targeting fatty acid synthesis is more common among these isolates than is cross resistance to antibiotics targeting other pathways. The data also suggest that some of these isolates may be capable of degrading and growing on triclosan.

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Table 1. Identification of triclosan resistant isolates based on HhaI restriction digests and sequence data from 16S DNA. – indicates no data available.

GROUP	Site	Restriction pattern	GENBANK best match/es	E- Val
Enterobacteriaceae (3)	Hospital	1	*Pectobacterium sp.*	10^{-21}
	Swope Hall	1	*Citrobacter*	0.0
	Ebert Hall	-	*Serratia*	10^{-108}
Pseudomonadaceae (15)	Ebert Hall	2	*Pseudomonas aeruginosa*	0.0
	Ebert Hall	2	*Pseudomonas aeruginosa*	0.0
	Ebert Hall	2	-	-
	Ebert Hall	2	-	-
	Hospital	3	*Pseudomonas aeruginosa*	10^{-27}
	Hospital	3	-	-
	Hospital	4	-	-
	Hospital	4	-	-
	Swope Hall	4	*Pseudomonas sp.*	10^{-15}
	Swope Hall	4	-	-
	Swope Hall	4	-	-
	Swope Hall	4	-	-
	Swope Hall	4	-	-
	Ebert Hall	5	*Pseudomonas putida*	0.0
	Ebert Hall	5	*Pseudomonas putida*	0.0
Vibrionales (10) *parahaemolyticus*	Eel Pond 10^{-28}	6	*Vibrio alginolyticus/V.*	
	Eel Pond	6	-	
	Sippiwissett	6	*Vibrio parahaemolyticus / Vibrio sp.*	10^{-126}
	Sippiwissett	6	-	-
	Sippiwissett	6	-	-
	Eel Pond	7	*Marine gamma/Vibrio sp.*	10^{-67}
	Eel Pond	7	-	-
	Sippiwissett	7	*Vibrio splendidus / Vibrio sp.*	0.0
	Sippiwissett	-	*Vibrio splendidus / Vibrio sp.*	0.0
	Eel Pond	-	*Marine gamma/Vibrio sp.*	10^{-48}
Halomonadaceae (1)	Sippiwissett	-	*Halomonas*	10^{-168}
Alteromonadaceae (1)	Sippiwissett	-	*Shewanella /Aeromonas*	10^{-26}
Xanthomonadales (2)	Hospital	8	*Stenotrophomonas maltophilia*	0.0
	Hospital	8	*Stenotrophomonas maltophilia*	0.0
Unidentified (2)	Eel Pond	-	-	-
	Sippiwissett	-	-	

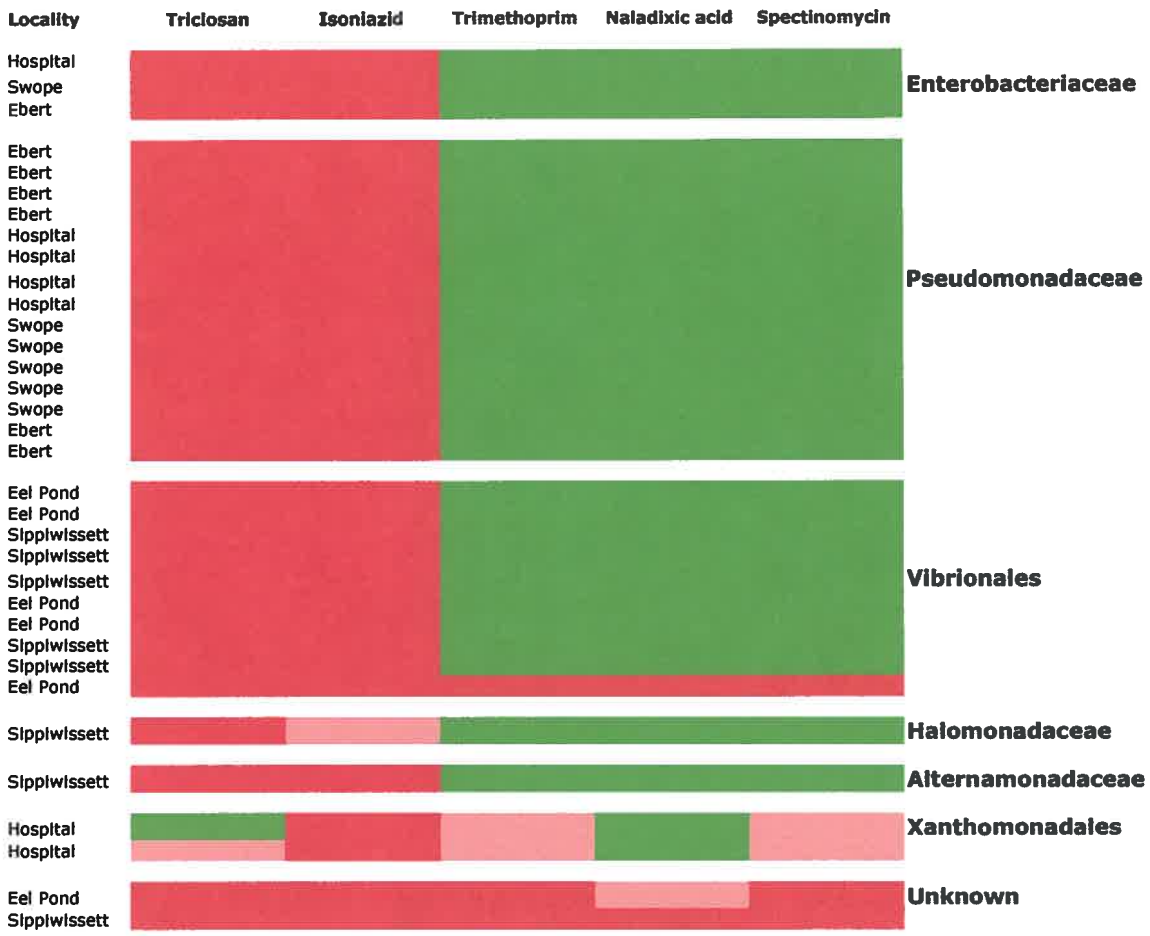

Figure 1. Resistance profiles of triclosan resistant isolates. Triclosan tested at saturation (approx 0.44mM) in rich media, all antibiotics tested at 1mM in rich media. Red indicates growth greater than 80% that observed in biocide/antibiotic free media (resistant). Pink indicates growth between 50-80% of controls (marginal resistance). Green indicates growth less than 50% of control (susceptible). All designations based on average ODs at 590 nm of 2-3 replicates.

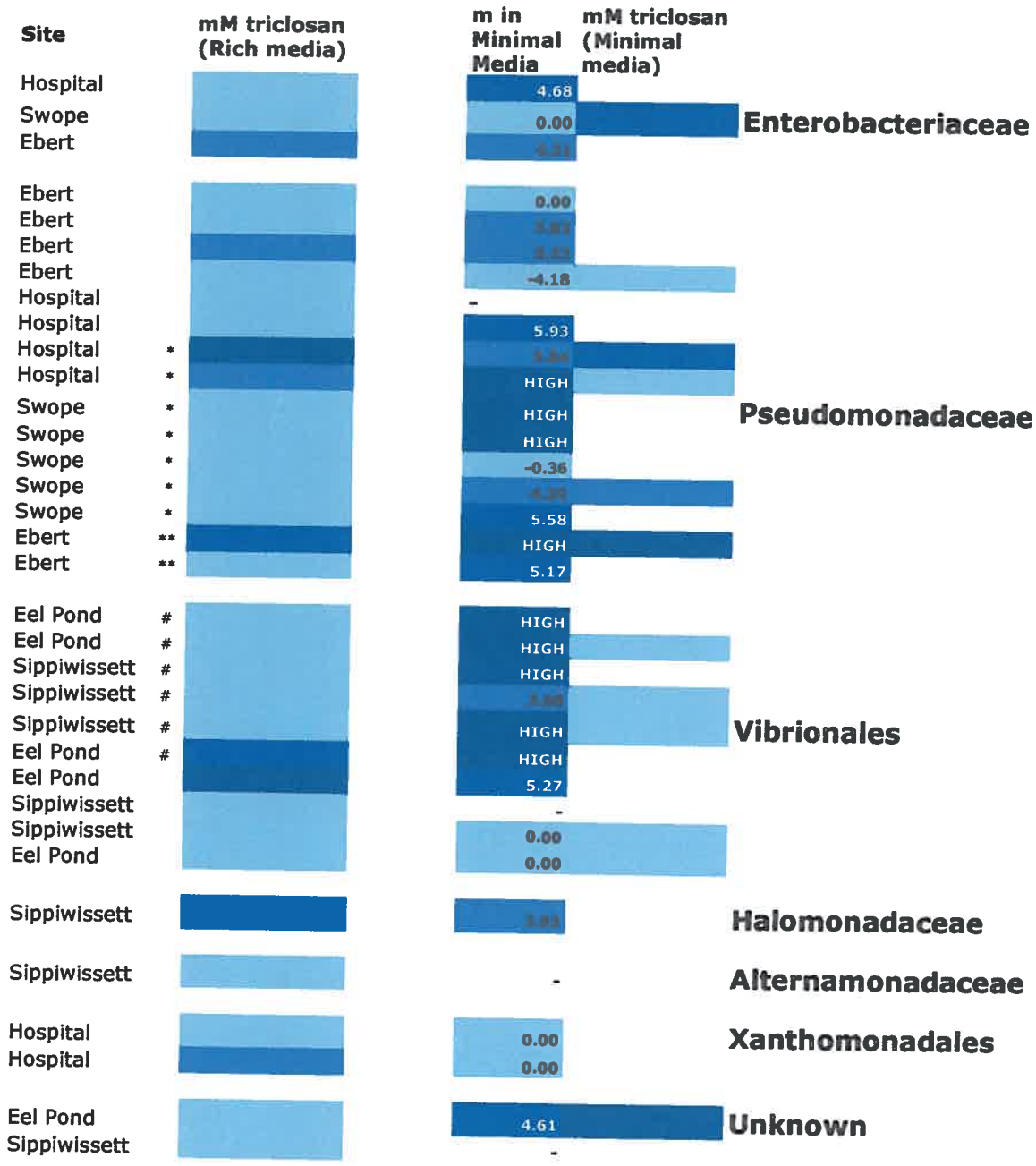

Figure 2 . Degradation of triclosan by environmental isolates. Columns 1 and 3: triclosan remaining in media after growth to stationary phase as estimated by HPLC analysis, where darker color indicates lower triclosan concentration. Column 2: Malthusian growth parameter estimated from colony counts from plating before and after incubation on a third serial transfer in minimal triclosan media. All data shown are one replicate per isolate.