Acta vet. scand. 1985, *26*, 449—460.

From the Institute of Hygiene and Microbiology, Royal Veterinary and Agricultural University, Copenhagen, Denmark.

VIBRIO ANGUILLARUM: PREVALENCE OF TYPICAL AND ATYPICAL STRAINS IN MARINE RECIPIENTS WITH SPECIAL REFERENCE TO CARBOHYDRATE POLLUTION

By

Jens Laurits Larsen

LARSEN, JENS LAURITS: *Vibrio anguillarum: Prevalence of typical and atypical strains in marine recipients with special reference to carbohydrate pollution.* Acta vet. scand. 1985, *26*, 449—460. — Primary isolates of Vibrio anguillarum-like organisms could be separated into typical V. anguillarum (VA) and atypical V. anguillarum (AVA) by biochemical tests. The prevalence of the fish pathogenic V. anguillarum was highly influenced by carbohydrate pollution as compared to the AVA. Water an sediment counts of VA generally increased at the polluted sites during April-May and persisted at a level of approx. 100/ml water and 1,000/g sediment until October-November. A further increase in VA counts could be registered locally at the time when the sugar beet processing season started (September-October). At the control site VA counts increased during June-July to a level of 10/ml persisting until August, while the only increase in sediment counts occurred in September (100/g). The maximum counts in water and sediment were at the control site 10/ml and 100/g and the polluted sites 100,000/ml and 50,000/g, respectively.

bacterial contamination; fish diseases.

The use of the designations Vibrio and Vibriosis in different papers concerning bacterial ecology in marine recipients probably indicates the presence of a great diversity of species which were only identified on a genus level (*Simidu et al.* 1971, *Simidu & Taga* 1974). This is also reflected in recent descriptions of many new species with and without any epidemiological significance for humans, fish, and shellfish (e.g. *Blake et al.* 1980, *Davis et al.* 1981, *Schiewe* 1981, *Hickman et al.* 1982, *Allen et al.* 1983, *Brenner et al.* 1983, *Tison & Seidler* 1983, *Yang et al.* 1983, *Hada et al.* 1984).

It appears from literature that pollution of coastal waters with contaminations originating from human activities or from rivers and other streams has a stimulating effect on growth of Vibrios (*Liston & Baross* 1973, *Simidu & Taga* 1974, *Kaneko & Colwell* 1978, *Shinoda et al.* 1980, *Larsen* 1982). Temperature and attachment to surfaces of phyto- or zooplanktion are decisive parameters in the ecology of these organisms (*Simidu et al.* 1971, *Kaneko & Colwell* 1975, *Larsen et al.* 1981, *Larsen* 1982, *Huq et al.* 1983).

The attachment process is in general an important step in bacterial ecology, both in the strategy of survival and as an initial step in the establishment of infections (*Costerton* 1978, *Beachey* 1981). This process is influenced by factors such as bacterial concentration, time, and motility (*Fletcher* 1979); also proteins with isoelectric points below pH 7 (*Meadows* 1971), and the presence of glucose (*Marshall et al.* 1971)) might stimulate adsorption to surfaces.

Akagi et al. (1977) found that a carbohydrate concentration exceeding 0.5 mg/l significantly affected the relationship between oligotrophic and heterotrophic bacteria in the marine environment in favour of the heterotrophic flora to which the Vibrios belong. A recent study (*Larsen* 1982) showed that the prevalence of Vibrio anguillarum-like organisms (VLO) in water and sediment was approx. 10 times higher in carbohydrate polluted marine recipients than at a control site.

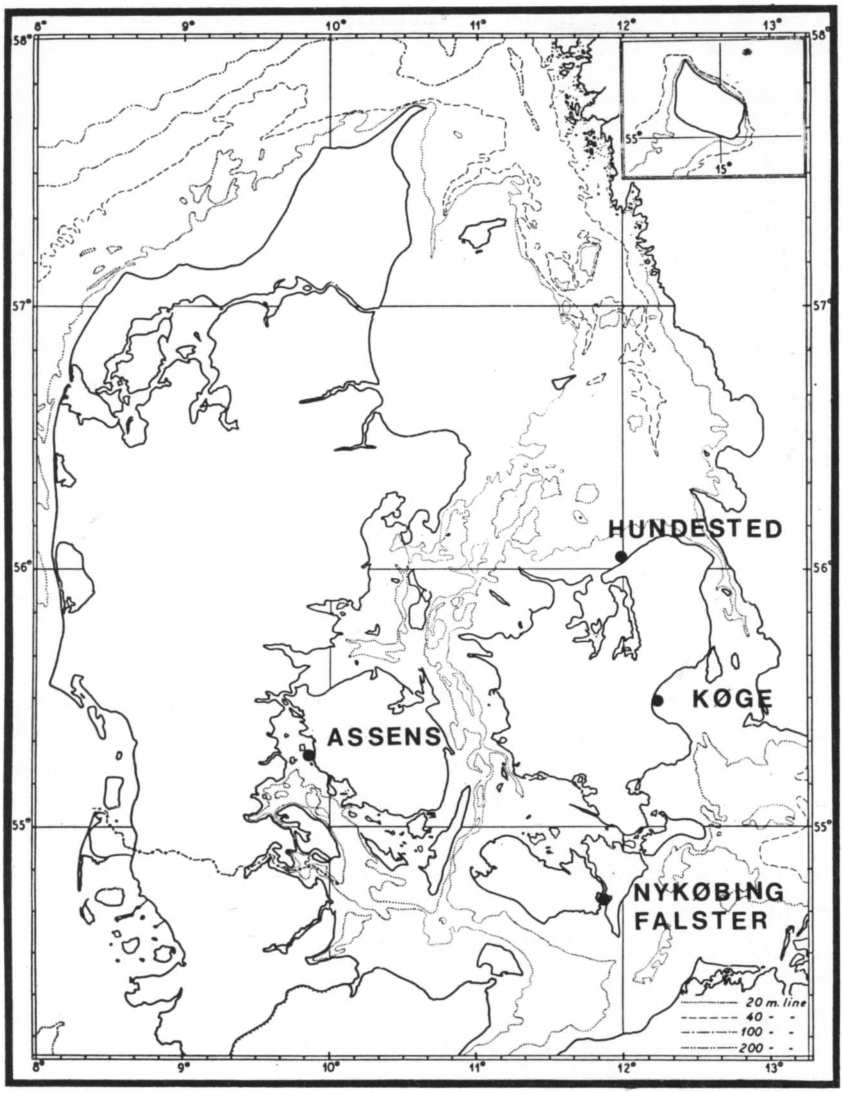
Laboratory examinations of selected colonies on TCBS agar and haemolytic colonies on blood agar made it possible to estimate the relative importance of VLO in relation to carbohydrate pollution in selected Danish marine recipients (*Larsen* 1982).

This paper concerns the prevalence of Vibrio anguillarum in these recipients. Furthermore, a group of organisms which might be impossible to distinguish from Vibrio anguillarum by primary isolation is also considered.

MATERIALS AND METHODS

Sampling sites and methods

The areas for collection of water and sediment appears from Fig. 1, and Table 1 summarizes the most important differences between the 3 carbohydrate polluted sites: Køge, Assens, and



F i g u r e 1. Sampling sites for water and sediment at 3 carbohydrate loaded marine recipients (Køge, Assens, and Nykøbing F.) and at a control location (Hundested).

Nykøbing F., and the control site: Hundested. Water was taken with a Ruttner water sampler, 0.5—1 ml from the surface, and transferred to 10 l plastic containers. A Van Veen Grab was used for sediments and only the upper 2 cm was used for the bacteriological examination. Samples were obtained from locations with-

Table 1. Parameters for characterization of 3 carbohydrate polluted areas (Køge, Assens, Nykøbing F.) and a control location (Hundested).

	Køge	Assens	Nykøbing F.	Hundested
Coastal morphology	bay	belt	sound	bay
Sugar plant	—	+	+	—
Cellulose plant	+	+	—	—
Chemical industries	+	—	—	—
Sewage	+	+	+	—
Load in p.e. = (approx.)	1,300,000	445,000	220,000	
Salinity variation	6—15	9.5—21	8—14	12—22
Temperature variation	3—17	3—19	1.5—19	3—15
Carbohydrate mg/l	3.6	3.2	3.5	1.7
Total N mg/l	2.3	2.5	2.7	1.2
Bottom	sand	sand and clay	sand, mud, and mash	sand
Depth in m	6—10	6—10	3—4	8—16
% cod with ulcers	11	32	7	0

p.e.: person equivalent

in 250—500 m from waste water outlets ("most polluted sites") and from locations 2,000—5,000 m from pollution sources ("least polluted sites"). Further details on temperature, salinity, and bacteriological analyses are given by *Larsen* (1982).

Separation of Vibrio anguillarum (VA) and atypical Vibrio anguillarum (AVA)

Dominating colonies on TCBC agar (Difco), both yellow (sucrose-positive) and greenish (sucrose-negative), and different haemolytic colonies on blood agar (Marine agar, Difco, with 5 % citrated calf blood) were selected for further characterization according to Table 2. All characterization tests were performed by standard methods (*Cowan* 1974).

RESULTS

Distribution of Vibrio anguillarum (VA) and atypical Vibrio anguillarum (AVA) among the isolates

On the basis of examination of 542 strains isolated from water and sediment it was found that 355 were VA and 187 AVA. The distribution of these isolates among the different sites is

Table 2. Identification of typical Vibrio anguillarum (VA) and atypical Vibrio anguillarum (AVA) strains from water and sediment.

	Common characteristics
Gram reaction	—
Morphology	rods
Motility	+
Catalase	+
Oxidase	+
OF test	fermentative

	Characters for differentiation: Typical Vibrio anguillarum		Atypical Vibrio anguillarum	
Typical morphology*	+	—	+	—
Typical motility**	+	—	+	—
Arginine	+	+	+	—
Lysine	—	—	+	—
Ornithine	—	—	—	—
Growth on TCBS agar	+	+	+	d
O/129 sensitivity	+	+	—	+

* *Morphology*: +: typical: short curved rods 0.5—1×1—3 μ.
—: atypical: long coarse rods 1—1.5×3—8 μ.
** *Motility*: +: typical: rapid vibrating movements, "snakes" may occur.
—: atypical: slow rotating movements.
d: diverse reactions.

Table 3. Number of identifications of Vibrio anguillarum (VA) and atypical Vibrio anguillarum (AVA).

	Køge	Assens	Nykøbing F.	Hundested
VA	104	103	120	28
AVA	40	53	38	56

listed in Table 3. The following figures are based on these identifications.

Vibrio anguillarum (VA) in marine recipients (Fig. 2 a & b)

Water: At the most polluted sites the number of VA was low from March to April, but from April to May there was a rise to approx. 10^2/ml, a level that persisted until September/October where there was a further rise in Assens and Nykøbing F. according to the season for local sugar beet processing. It should be

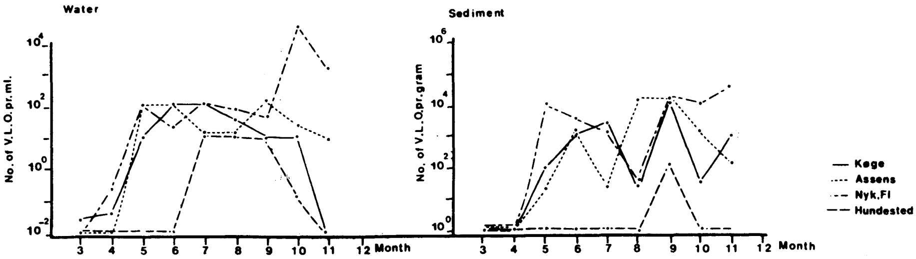


Figure 2a: Prevalence of Vibrio anguillarum at the most heavily polluted sites at the 3 stations and at the control site (Hundested).

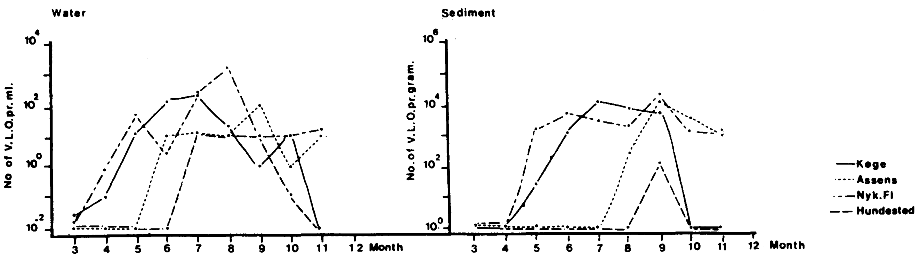


Figure 2b. Prevalence of Vibrio anguillarum at the least polluted sites at the 3 stations and at the control site (Hundested).

noticed that Nykøbing F. reached a maximum of approx. 10^5/ml. At Hundested (control site) VA was registered with low counts until July; in this and the following 2 months, the prevalence was approx. 10^1/ml.

The least polluted sites near Køge and Nykøbing F. har a rise in VA from March to May, and the prevalence fluctuated at about 10^2/ml. In Assens the rise occurred from May to June, reaching a maximum of 10^2/ml in September. A value of approx. 10^1/ml persisted in Assens and Nykøbing F. in November, where the 2 other sites had very low counts.

Sediments: The most polluted sampling sites in Køge, Assens and Nykøbing F. had VA counts of approx. 10^0/g in March/April, then there was an increase during May and June to approx. 10^3/g, and through the whole season the values fluctuated from 10^2/g to 10^4/g. For the control site, only one registration exceeded 10^0. At the least polluted sites at Køge and Nykøbing F., a similar tendency was found, but with rather stable values of approx. 10^3—10^4/g. Some delay was found at Assens, where the density increased in July and reached a maximum of 10^4/g in September.

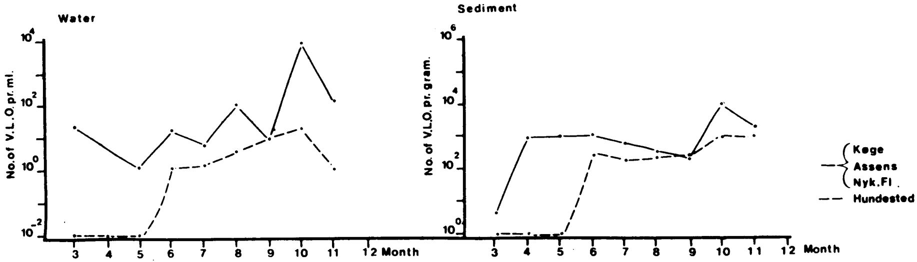


Figure 3a. Prevalence of atypical Vibrio anguillarum (AVA) at the most heavily polluted sites at the 3 stations and at the control site (Hundested).

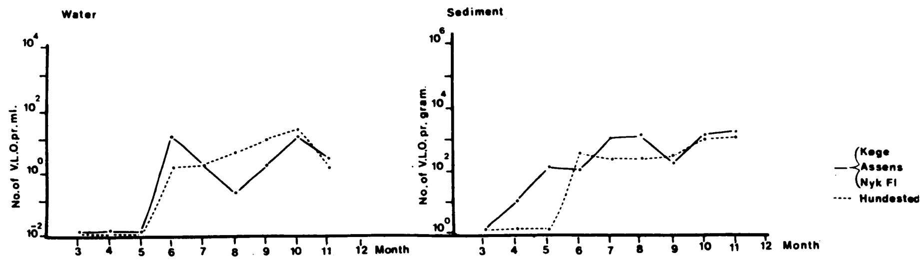


Figure 3b. Prevalence of atypical Vibrio anguillarum (AVA) at the least polluted sites at the 3 stations and at the control site (Hundested).

Atypical Vibrio anguillarum (AVA) in marine recipients (Fig. 3 a & b)

The comparisons of the mean values representing AVA should naturally be made with some caution because of the sparse material. Generally the prevalence of AVA was highest at the heaviest polluted sites, while no pronounced differences were found at the sites far from the sources of pollution.

For the heaviest contaminated areas the prevalence in water most frequently exceeded 10^1/ml (also in March) and reached a maximum in October of approx. 10^4/ml. On the contrary, low counts were registered at the control site from March to June, and the highest values in October were approx. 5×10^1/ml.

In sediment, AVA was found in March and for the rest of the year the prevalence was 10^3—10^4/g; for the control site low counts were registered until June where an increase to approx. 10^2—10^3/g occurred.

DISCUSSION

Evaluation of the relative importance of Vibrio anguillarum in Danish marine carbohydrate polluted recipients was based on counts made on Blood Agar plates (BA) and Thiosulphate-Citrate-Bile Salts-Sucrose-Agar (TCBS).

The use of haemolytic properties as an indication of Vibrios has been made previously by *Baross & Liston* (1970), and TCBS is a well-known selective and indicative medium for the preliminary isolation of Vibrio species (*Kobayashi et al.* 1963). The specificity of TCBS has recently been questioned (*Lotz et al.* 1983), and it was evident in the present study that some Enterobacteriaceae might mimic Vibrios. These strains could, however, easily be separated by the oxidase test when they were cultured on a non-selective medium.

When more experience was obtained, the colony-morphological differences on TCBS made it possible to recognize Vibrios.

An inhibitory effect of TCBS agar on Vibrio anguillarum grown in broth cultures was observed, but comparing counts on TCBS and BA the results were rather identical, which might be ascribed to a bacterial association with 'particles' from which the growth could be initiated. This theory was supported by the fact that it might cause some problems to obtain pure cultures of the strains if special precautions were neglected.

Vibrio anguillarum appears to be the predominant species belonging to the genus Vibrio in Danish coastal areas with outlet of carbohydrate-containing waste-water. The counts are in general high from April and throughout the year at the polluted sites, while noticeable counts at the control site were observed from July in water and from September in sediment. Obviously the sediment counts showed significant differences, while the variation in water densities was most evident during spring and autumn.

The difference was pronounced at the most polluted sites, but was also reflected in counts at the stations situated some kilometers from the outlet. Coastal morphology might naturally influence the counts. Thus, the belt of Assens permitted better exchange of water at the least polluted sites as compared to the situations in Køge and Nykøbing F. The shallow water at the last-mentioned area furthermore permitted a significant increase in VA numbers (to 10^5/ml) when the local sugar beet processing started.

The AVA strains showed rather identical prevalence in both water and sediment remote from the pollution source, while some difference was observed close to the outlet. It should, however, be emphasized that this sparse material could not give sufficient basis for conclusions concerning the atypical Vibrio anguillarum. Minor interest concerns this group as these organisms are infrequently involved in infectious diseases among fish.

For Vibrio anguillarum, previous studies have shown that there were some relationships between high prevalences and the occurrence of fish diseases (*Larsen & Jensen* 1982). Furthermore, comparisons of environmental, fish pathogenic and reference strains have shown the existence of biochemical, agglutinating and serological similarities, which in turn indicate the risk of pollution-induced proliferation of Vibrio anguillarum. It remains, actually, to be verified whether these environmental strains harbour the virulence plasmids recognized among fish pathogenic strains (*Crosa et al.* 1977, *Crosa* 1980).

The appearance of many new taxa within the genus Vibrio, as mentioned previously, shows that coastal areas are populated by a variety of Vibrio species. Different climatic conditions permit different species to proliferate, but in internal Danish waters Vibrio anguillarum occurs with highest prevalence, while Vibrio alginolyticus and especially Vibrio parahaemolyticus are of minor importance (*Larsen et al.* 1981).

For the public health authorities it is, however, important to obtain some information on the risk which could be ascribed to these organisms, especially which levels could be acceptable and used in the monitoring of water quality with respect to its safety for humans and for higher aquatic organisms.

This accentuates the necessity for a more exact identification and characterization of the strains, involving serology, haemagglutination typing, determination of plasmid contents, etc. Progress in the research outlined above is very important for the increasing aquaculture industry from two points of view: Firstly, to identify the environmental source from which the pathogenic Vibrios attacking fish and shellfish in aquaculture are recruited, and to determine how this might be avoided e.g. by technical means in the construction of the production units. Secondly, do the Vibrios implicated in disease outbreaks of aquaculture influence the disease conditions among feral fish living close to the aquaculture. The latter point might be important when the authorities are going to give permission for the establishment of new aquaculture systems.

ACKNOWLEDGEMENTS

The tehcnical assistance of Kirsten Kaas is greatly appreciated. The work was supported by the Danish Agricultural and Veterinary Research Council.

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SAMMENDRAG

Vibrio anguillarum: Prevalens af typiske og atypiske stammer i kulhydratforurenede marine recipienter.

Primære isolater af Vibrio anguillarum-lignende organismer (VLO) opdeltes efter biokemiske kriterier i typiske V. anguillarum (VA) og atypiske V. anguillarum (AVA). Prævalensen af den fiskepatogene bakterie V. anguillarum (VA) påvirkedes i høj grad af kulhydratforurening i modsætning til AVA.

Stigninger i den numeriske forekomst af VA i vand og sediment på de forurenede lokaliteter fandt sted i april-maj i relation til stigende vandtemperaturer. De typiske værdier for vand var ca. 100/ml vand og for sediment 1000/g. Dette niveau holdt sig indtil oktober-november. En yderligere stigning i VA tallene registreredes i relation til sukkerkampagnens start (september-oktober).

På kontrollokaliteten steg VA-tallene i løbet af juni-juli til ca. 10/ml men faldt igen i august. Den eneste stigning i sedimenttallene påvistes i september (100/g). De maksimale forekomster af VA fandtes på kontrollokaliteten at være 10/ml vand og 100/g sediment, medens de tilsvarende tal på de forurenede steder var 100.000/ml vand og 50.000/g sediment.

(Received June 22, 1985).

Reprints may be requested from: Jens Laurits Larsen, the Institute of Hygiene and Microbiology, Royal Veterinary and Agricultural University, 13 Bülowsvej, DK-1870 Frederiksberg C, Denmark.