Netherlands Journal of Sea Research
9 *(3–4) : 351–364 (1975)*

MARINE VIBRIOS ISOLATED FROM WATER ALONG THE DUTCH COAST

by

CAROL GOLTEN and W. A. SCHEFFERS
(Laboratory of Microbiology, Delft University of Technology, Delft, The Netherlands)

CONTENTS

I. INTRODUCTION

Marine vibrios deserve attention because of their rôle in food-borne disease, in tissue infections in man, fish and shellfish, as well as for their interesting morphology, physiology and ecology.

Vibrio parahaemolyticus was first described in Japan as the causative agent in numerous cases of food infection with symptoms of acute gastroenteritis, following consumption of raw or semi-preserved sea food or other food, contaminated from marine sources (see SAKAZAKI, 1969; OKABE, 1974). Subsequent studies have shown that this mildly halophilic organism is widely distributed in sea water, sediment, plankton, fish and shellfish, in warm and temperate climates in different parts of the world, particularly in coastal regions (see FUJINO, SAKAGUCHI, SAKAZAKI & TAKEDA, 1974).

Also, bacteria showing the characteristics of *V. parahaemolyticus* or of the closely related *V. alginolyticus* have been incriminated in connection with infections of wounds, ears and eyes in persons who had been in contact with marine or estuarine shore areas (TWEDT, SPAULDING & HALL, 1969); ROLAND, 1970, 1971; ZEN-YOJI, LE CLAIR, OHTA & MONTAGUE, 1973).

V. parahaemolyticus has been reported to be pathogenic in crabs (KRANTZ, COLWELL & LOVELACE, 1969) and shrimps (VANDERZANT, NICKELSON & PARKER, 1970). *V. alginolyticus*, *V. anguillarum* and other marine vibrios have been found associated with bacillary necrosis, a disease of larval and juvenile bivalve mollusks (TUBIASH, COLWELL & SAKAZAKI, 1970). Bacteria classified as *V. anguillarum* cause ulcerous lesions in marine and freshwater fish (HENDRIE, HODGKISS & SHEWAN, 1971; SHEWAN & VÉRON, 1974).

Regarding the occurrence of *V. parahaemolyticus* and *V. alginolyticus* in the Dutch coastal region, the only data available were those of KAMPELMACHER, VAN NOORLE JANSEN, MOSSEL & GROEN (1972) on samples of mussels, oysters and water, taken from the Oosterschelde estuary in the South-Western part of the country during the winter season. The purpose of our present study was to obtain additional data on the occurrence of these vibrios in sea and estuarine water along the coast of The Netherlands. It is a common experience that these mesophilic organisms are most abundant during the warm season (SAKAZAKI, 1969; BAROSS & LISTON, 1970; KANEKO & COLWELL, 1973). We collected our samples in August-September; water temperatures along the Dutch coast reach a maximum during these months (WIGGERS, 1970; MANUELS & ROMMETS, 1971). Samples were taken at 67 sites along the Dutch coast (North Sea; Wadden Sea; delta region of Zealand). A preliminary report of this work was published by SCHEFFERS & GOLTEN (1973).

Acknowledgements.—The authors are indebted to Allen & Hanburys Ltd, Ware, Herts., England, for a gift of the vibriostatic agent 0/129 (2,4-diamino-6,7-diisopropyl-pteridine; COLLIER, CAMPBELL & FITZGERALD, 1950); to the National Institute of Public Health, Bilthoven, The Netherlands, for providing the strain of *V. parahaemolyticus* isolated by KAMPELMACHER *et al.* (1970); and to Dr Willemina E. de Boer and Mrs Wilma H. Batenburg-van der Vegte for preparing the electron micrographs.

II. MATERIALS AND METHODS

Strains.—In preliminary experiments and for comparative purposes, the following collection strains were used: *Vibrio parahaemolyticus* ATCC 17802 (isolated by Fujino from a case of acute gastroenteritis), *V. parahaemolyticus* RIV (isolated by Kampelmacher, Mossel, van Noorle Jansen & Vincentie from a North Sea haddock), *V. alginolyticus* ATCC 17749 (isolated by Nakamura from spoiled fish) and *V. alginolyticus* ATCC 17750 (isolated by Takizawa from fish). Stock cultures were maintained on slopes of trypticase soy agar (TSA), consisting of: 5 g phytone (BBL), 15 g trypticase (BBL), 30 g NaCl, and 18 g Bacto agar in 1 litre deionized water, *p*H 7.3, and sterilized at 120° C. After incubation for 24 h at 37° C, cultures were kept at room temperature.

Samples.—Water samples were taken in sterile 50 ml screw-cap bottles. During the period 15 August to 13 September 1972, in total 67 samples were collected. Of these, 61 were taken from the shore: on beaches,

from piers, in creeks and harbours, along dikes and dams; 6 samples were collected from ferry boats in open water. The average temperature of the water at the time of sampling was 18.2° C; in 51 cases the temperature was between 17.0° and 19.5°C. In sampling shallow creeks, lower or higher temperatures were observed, mainly depending on the time of the day: in 8 cases the water temperature was below 17.0° C (minimum 14.0° C) and in another 8 cases above 19.5° C (maximum 23.0° C). Unless processed within 2 h, samples were stored at 6° to 10° C in a cool box during transport to the laboratory. Directly after arrival enrichment cultures were started.

Enrichment.—In order to detect vibrios, even if present in low numbers in the sample, the isolation procedure must be preceded by an enrichment culture. In preliminary experiments, the following adaptation of the method of VANDERZANT & NICKELSON (1972) was found satisfactory for our purpose: 50 ml sample was transferred to a 300 ml-erlenmeyer flask containing 50 ml double-strength enrichment broth. The broth consisted of 10 g phytone (BBL), 30 g trypticase (BBL), 2 g bile salts (Bacto, no. 3) and 110 g NaCl in 1 litre deionized water, *p*H 7.3. Mixing with the sea water sample yielded trypticase soy broth with 7% (w/v) NaCl. After 18 or 48 h stationary incubation at 41° ± 1° C, streaks were made from the enrichment culture onto the isolation medium.

Isolation.—The selective agar medium was modified thiosulphate citrate bile salts sucrose agar (TCBS agar). This was obtained by dissolving in 980 ml deionized water: 10 g peptone (Difco), 5 g yeast extract (Difco), 10 g $Na_2S_2O_3 \cdot 5H_2O$, 10 g trisodium citrate $\cdot 2H_2O$, 5 g sodium taurocholate (Fluka), 3 g sodium cholate (NBCo), 0.04 g bromthymol blue, 0.04 g thymol blue and 18 g Bacto agar; final *p*H was 8.6. After sterilization at 120° C and cooling to 50° C a solution of 20 g sucrose in 20 ml deionized water, sterilized by filtration, was added. This TCBS agar medium is a modification of the formula given by SAKAZAKI (1969) which included 5 g ox gall instead of the taurocholate and 1% instead of 3% NaCl. Subsequent to solidification, TCBS agar plates were dried for 30 min at 65° C. After inoculation, plates were incubated at 41° ± 1° C for 18 h. For identification, pure cultures from relevant colonies on TCBS agar were obtained by way of streaking on TSA; the cultures were maintained on TSA slopes.

Viable counts.—For the enumeration of viable cells in a suspension of *V. parahaemolyticus*, 0.5 ml-aliquots of serial dilutions in 3% (w/v) NaCl solution were plated out in duplicate onto TSA plates. For *V. algino-*

lyticus, trypticase soy bile salts agar (TSBA) was employed as the counting medium. TSBA was prepared by the addition of 1 g Bacto bile salts no. 3 to 1 litre TSA. Inoculated plates were incubated at 37° C for 24 h.

Most probable numbers (MPN) of the vibrios in sea water were determined as follows: from a decimal dilution series of the sample in 3% (w/v) NaCl, five 10 ml-aliquots per dilution were mixed with 10 ml double-strength enrichment medium and incubated at 41° ± 1°C for up to 48 h. TCBS agar plates were inoculated from tubes showing growth, and incubated at 41° ± 1° C for 18 h. Pure cultures obtained from characteristic colonies on these plates were further identified.

Determination of morphological characteristics.—Cell form and Gram-reaction were studied from growth on fresh maintenance slopes. For electron microscopy, specimens of cells grown in liquid culture or on solid medium were prepared as previously described (DE BOER, GOLTEN & SCHEFFERS, 1975a, 1975b).

Determination of physiological and biochemical characteristics.—Unless stated otherwise, all test media were made up with 3% (w/v) NaCl, and incubation was at 37° C. Tests were performed with cell material, obtained from TSA slopes, incubated 24 h at 37° C.

Motility was observed in the light microscope and in SIM medium (Difco). Swarming was detected on TSA after 24 h incubation.

Growth at 42° C was judged from TSB cultures, incubated in a waterbath at 42.0° ± 0.2° C. Trypticase soy broth (TSB) consisted of 5 g phytone, 15 g trypticase, and 30 g NaCl in 1 litre deionized water, *p*H 7.3, sterilized at 120° C. Salt requirement and salt tolerance were studied by comparison of growth in TSB (3% NaCl) with growth in the corresponding media, containing 0.0%, 7.0% and 10.0% (w/v) NaCl, respectively, during incubation for up to 3 days.

Oxidase activity was determined with the reagent of GABY & HADLEY (1957), applied on filter paper containing cell smears. Catalase production on TSA was tested with 10% H_2O_2.

Fermentative acid production from glucose, sucrose, L-arabinose, and mannitol was tested with marine oxidation-fermentation medium (MOF) of LEIFSON (1963), modified according to TWEDT, SPAULDING & HALL (1969). The carbohydrates, sterilized separately, were added to the basal medium in 1% concentration. Fermentation tests were performed in tubes, sealed with paraffin oil; unsealed aerobic tubes and tubes containing basal medium without carbohydrate were run as controls. Carbohydrate fermentation was observed during a 2-week incubation period. In general, positive results were obtained within

18 to 24 h. On prolonged incubation, the phenol red indicator tended to become decolorized in the anaerobic tubes.

Gas production from carbohydrates and H_2S formation were observed in triple sugar iron agar (TSI) after 18 h.

Acetoin production and methyl red reaction were tested after 5 days in MR-VP medium. Indole formation was observed in 1% tryptone (Bacto) after 48 h, using KovÁcs' (1928) reagent. Citrate utilization was demonstrated on Simmons citrate agar (Oxoid). Lysine decarboxylase activity was tested in TAYLOR's (1961) modified lysine decarboxylase broth (Oxoid). Ornithine decarboxylase and arginine dihydrolase activities were examined in media according to MØLLER (1955), incubated for up to 4 days. For detection of urease activity, the medium of CHRISTENSEN (1946) was employed. Nitrate reduction to nitrite or gas was tested according to COWAN & STEEL (1965).

Gelatinase activity was detected on a modified gelatin agar: 10 g peptone, 30 g NaCl, and 20 g agar in 1 litre water, *p*H 7.3, was sterilized at 120° C; the molten agar medium was mixed with 5 g gelatin soaked in 50 ml water, and 2 ml Teepol was added in order to suppress swarming (DE BOER, GOLTEN & SCHEFFERS, 1975b); the complete medium was sterilized at 110° C. Inoculated plates of gelatin agar, incubated for 2 days, were flooded with acid mercuric chloride solution (12 g $HgCl_2$, 80 ml water, and 16 ml concentrated HCl). Clear zones around colonies indicated gelatin hydrolysis.

Amylase activity was demonstrated on starch agar according to SKERMAN (1967), employing TSBA as the basal medium. After 3 days incubation, the plates were flooded with dilute iodine solution for detection of starch hydrolysis around colonies.

Sensitivity to novobiocin and to 0/129 was observed after 18 h incubation of surface-inoculated TSA plates, supplied with paper discs containing 5 μg novobiocin (Oxoid) and 10 μg 0/129, respectively. The latter were prepared from a solution of 2,4-diamino-6,7-diisopropyl-pteridine (Allen & Hanburys Ltd) in dioxane, according to SCHUBERT (1962).

III. RESULTS

Selection and evaluation of enrichment and isolation procedure.—From the various enrichment methods for *V. parahaemolyticus* and *V. alginolyticus* recommended in the literature we arbitrarily chose the method of VANDERZANT & NICKELSON (1972), which is based on a combined exploitation of two physiological properties of these vibrios: tolerance for sodium chloride concentrations around 7% and for temperatures around 42° C. We incubated our enrichment cultures at

41° ± 1° C for 18 h; only when no growth was visible at the end of this period, incubation was continued up to 48 h.

For isolation of the vibrios from enrichment cultures, VANDERZANT & NICKELSON used a salt-starch medium. However, this medium is liable to yield many false-positive isolates, as was also concluded by VANDERZANT, NICKELSON & HAZELWOOD (1974). Likewise, TCBS agar (SAKAZAKI, 1969), although widely in use as a selective medium for vibrios, may have limited selectivity. VANDERZANT & NICKELSON (1972) found that on TCBS agar many suspect colonies, obtained from sea foods, were false-positives, the isolates being oxidase-negative or producing H_2S or gas. Since higher selectivity may be expected to result from increasing the salt content as well as the incubation temperature of TCBS agar plates, we used TCBS agar with 3% (w/v) instead of 1% NaCl, incubated at 41° instead of 37° C. In preliminary experiments, collection strains of *V. parahaemolyticus* and *V. alginolyticus* grew well under these conditions and formed colonies of normal appearance. On prolonged incubation, the yellow-brown colour of *V. alginolyticus* colonies vanished, and the colour of the surrounding medium changed back from yellow to bluish-green; thus, *V. alginolyticus* colonies gradually became indistinguishable from *V. parahaemolyticus* colonies (a situation which also is encountered when following the original version of the TCBS procedure; even on plates stored in the refrigerator, *V. alginolyticus* colonies loose their characteristic colour). On the basis of these results, we decided to employ as the isolation medium TCBS agar with 3% (w/v) NaCl, incubated at 41° ± 1° C for 18 to 24 h.

In order to assess the reliability of the selected procedure for enrichment and isolation, recovery of *V. parahaemolyticus* from artificially inoculated sea water was examined. Samples of 50 ml fresh, natural sea water were inoculated with various dilutions of *V. parahaemolyticus* collection strains in 3% (w/v) NaCl solution. Enrichment cultures from inoculated samples were incubated and subsequently streaked onto the isolation medium. Suspect colonies were isolated and identified. Noninoculated aliquots of the same sea water were run in parallel in order to establish that no *V. parahaemolyticus* was detectable in the samples proper. Concomitantly, the number of viable cells of *V. parahaemolyticus* in the inocula was determined on TSA counting plates. From the results of these recovery experiments (Table I) we conclude that 1 to 20 viable cells of *V. parahaemolyticus* in 50 ml sea water are detectable with reasonable probability and that more than 20 viable cells in 50 ml sea water are detectable with high probability by the procedure described.

Similar experiments were performed on the recovery of *V. alginolyticus* collection strains (ATCC 17749 and ATCC 17750). In these

TABLE I

Detectability of *Vibrio parahaemolyticus* in sea water, following our procedure for enrichment and isolation: + means isolation of *V. parahaemolyticus*, confirmed by identification; — means *V. parahaemolyticus* not isolated, overgrown by *V. alginolyticus* from sea water.

Strain	*Number of viable cells inoculated into 50 ml sea water*	*Result of enrichment and isolation procedure*
V. parahaemolyticus ATCC 17802	240–280	+, +, −, +
	24–28	+, +, +, +
	12–16	+, +
	2–6	+, −, +, −
	0	−, −, −
V. parahaemolyticus RIV	120–200	+, +, +
	12–20	+, −, +
	1–2	+, +
	0	−, −, −

experiments, the natural sea water was sterilized by filtration prior to inoculation with the test strains, in order to eliminate any *V. alginolyticus* present in the natural flora. Enumeration of viable cells in the inocula was performed on TSBA counting plates; the bile salts in this medium serve to suppress swarming of *V. alginolyticus* (DE BOER, GOLTEN & SCHEFFERS, 1975b) which otherwise would interfere with colony counting. With our procedure of enrichment and isolation, 3 to 20 viable cells of *V. alginolyticus* could be recovered from 50 ml sea water.

Isolates from water samples.—From 67 samples (50 ml) of sea water, estuarine water, and other saline or brackish inshore water, collected along the coast of The Netherlands, 10 samples yielded no vibrios (Fig. 1). In these cases, the enrichment cultures developed a slight turbidity only or remained completely clear, and streaks onto selective agar yielded no colonies. The other 57 samples gave turbid enrichment cultures, yielding colonies on the TCBS agar. Nearly all colonies were of the *V. alginolyticus* type. Pure cultures, obtained from representative colonies were identified. The results are summarized in Table II and Fig. 1. *V. parahaemolyticus* was not isolated from our samples. In one case, a suspect colony type (sucrose-negative, however only 1 mm in diameter) was found, which belonged to a nonfermentative, halotolerant rod, insensitive to 0/129 (not presented in the Table). With few exceptions, the other isolates were identical to *V. alginolyticus*. The exceptions, presented in Table II, were 2 arabinose-positive strains and

Fig. 1. Sampling sites along the Dutch coast with no vibrios isolated from sample (○) and with *Vibrio parahaemolyticus* biotype 2 *(alginolyticus)* isolated from sample (●). None of the samples yielded *V. parahaemolyticus* biotype 1 *(parahaemolyticus)*.

4 methyl red-positive strains, which in all other characters corresponded to *V. alginolyticus*. In view of the weight, attached to arabinose fermentation and methyl red reaction in current taxonomy of these vibrios (SHEWAN & VÉRON, 1974), these strains might be considered as intermediates between *V. alginolyticus* and *V. parahaemolyticus*.

All our isolates were motile and swarmed on TSA. In a number of strains, the type of flagellation was studied by electron microscopy. In general, all strains investigated showed sheathed polar flagella when grown in liquid culture (Plate I). When grown on agar media, the cells had lateral flagella (Plate II). These modes of flagellation correspond to those described by BAUMANN, BAUMANN & MANDEL (1971) and ALLEN & BAUMANN (1971) in *Beneckea*. Sometimes we observed

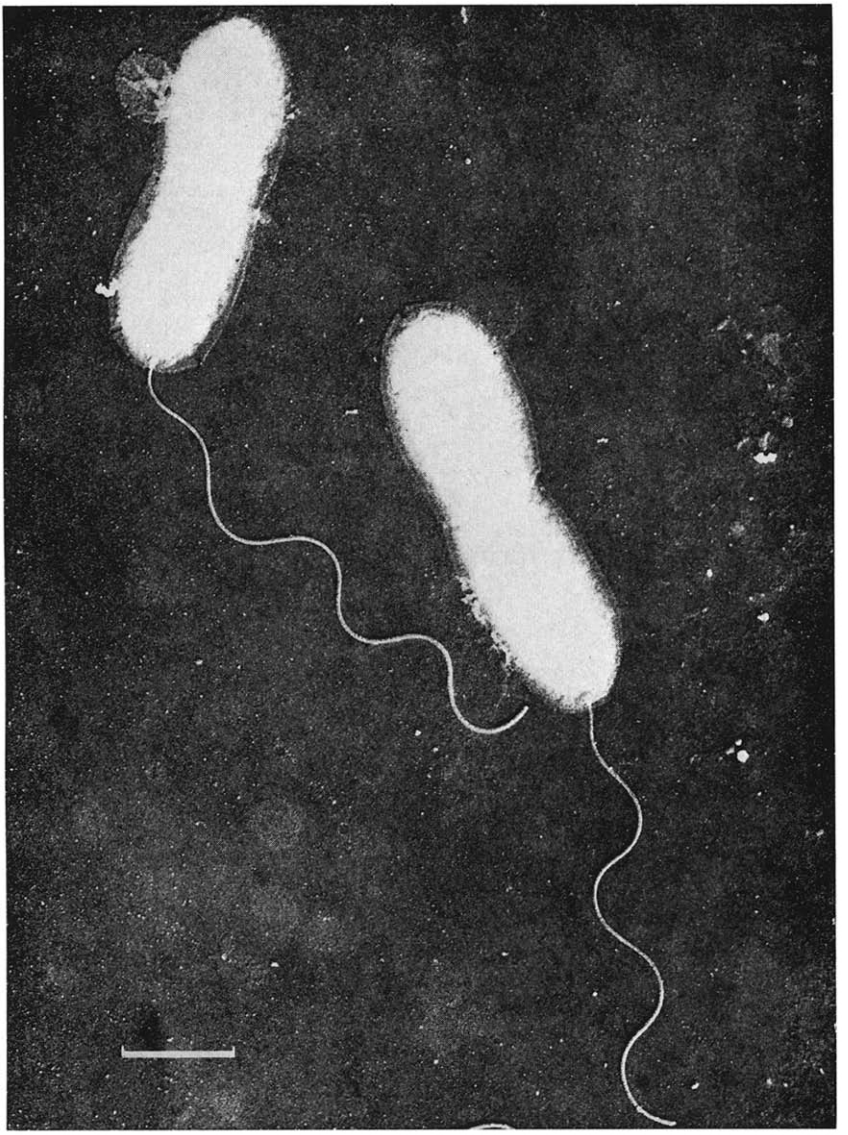

PLATE I

Vibrio alginolyticus 31 from broth culture. One sheathed, polar flagellum per cell. Marker: 1 μm.

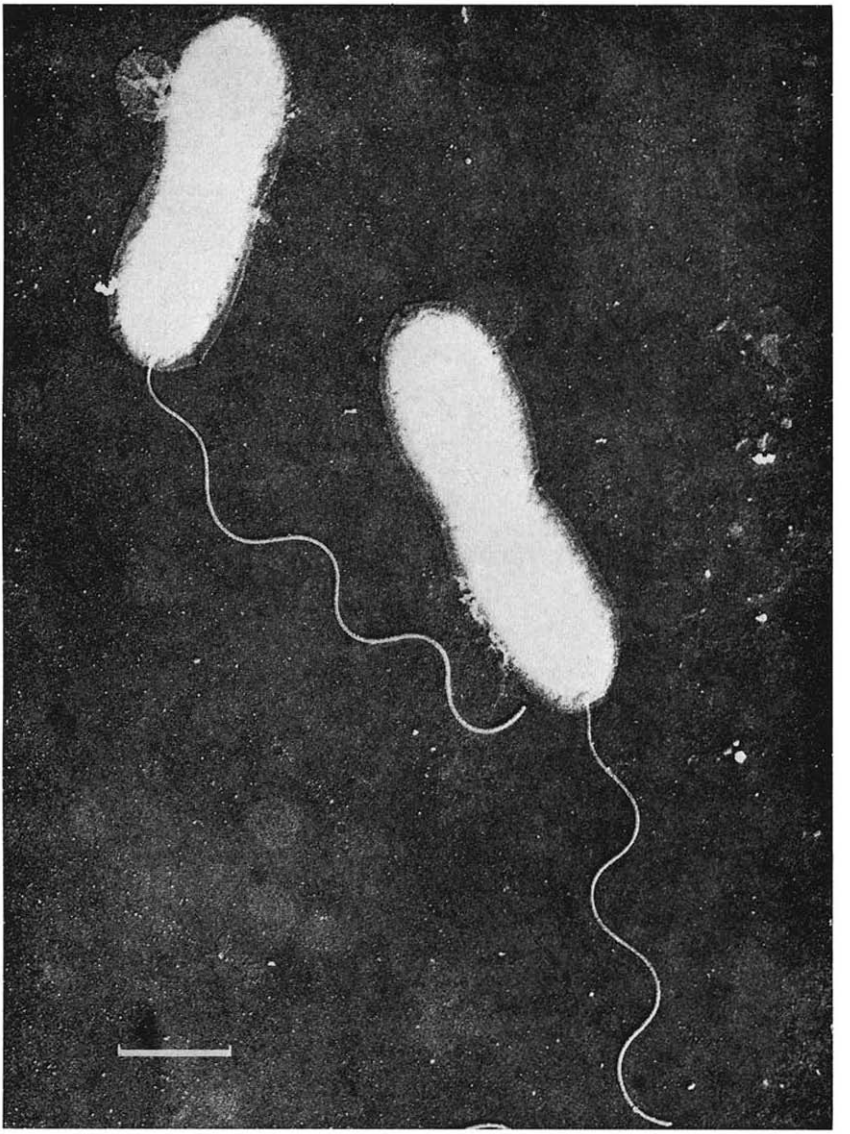

PLATE I

V. alginolyticus 31 from agar culture. Numerous unsheathed, lateral flagella per cell. Cell in centre with additional sheathed, polar flagellum. Cell on left side has broken polar flagellum with partly deteriorated sheath, core visible. Polar and lateral flagella have different wavelengths. Marker: 1 μm.

TABLE II

Characteristics of *Vibrio* isolates from water samples and of reference strains.

	Vibrio parahaemolyticus ATCC 17802 and RIV	*Isolates 27 and 39b*	*Isolates 7, 31, 41 and 59*	*All other isolates*	*Vibrio alginolyticus* ATCC 17749 and ATCC 17750
Cell form	rod	rod	rod	rod	rod
Gram stain	−	−	−	−	−
Motility	+	+	+	+	+
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Growth in 0.0% NaCl	−	−	−	−	−
Growth in 7.0% NaCl	+	+	+	+	+
Growth in 10.0% NaCl	−	+	+	+	+
Growth at 5° C	−	−	−	−	−
Growth at 42° C	+	+	+	+	+
Gas from glucose	−	−	−	−	−
Acid from glucose	+	+	+	+	+
Acid from sucrose	−	+	+	+	+
Acid from L-arabinose	+	+	−	−	−
Acid from mannitol	+	+	+	+	+
Indole	+	+	+	+	+
Methyl red	+	−	+	−	−
Acetoin	−	+	+	+	+
Citrate utilization	+	+	+	+	+
Hydrogen sulphide	−	−	−	−	−
Arginine dihydrolase	−	−	−	−	−
Lysine decarboxylase	+	+	+	+	+
Ornithine decarboxylase	+	+	+	+	+
Urease	−	−	−	−	−
Nitrite from nitrate	+	+	+	+	+
Gas from nitrate	−	−	−	−	−
Gelatinase	+	+	+	+	+
Amylase	+	+	+	+	+
0/129 sensitivity (10 μg)	+	+	+	+	+
Novobiocin sensitivity (5 μg)	+	+	+	+	+
Luminescence	−	−	−	−	−
Swarming	−	+	+	+	+

lateral flagella and a sheathed polar flagellum on the same cell (Plate II). Occasionally, polar flagella were partly or completely unsheathed. Flagellation in our isolates was considerably affected by a number of physical and chemical factors (DE BOER, GOLTEN & SCHEFFERS, 1975a, 1975b).

Enumeration of *V. alginolyticus* in sea water.—In a few cases, we estimated the number of *V. alginolyticus* in sea water samples by the MPN technique. Three samples taken from beaches near The Hague when the water temperature was 16° C had MPN values of 23, 13, and 23 *V. alginolyticus* per 100 ml. One additional sample, taken in October when the water temperature had fallen to 13° C yielded an MPN value of 5 per 100 ml. In all cases, isolates from positive tubes were identified as *V. alginolyticus*.

IV. DISCUSSION

We employed highly selective conditions for detection of *Vibrio parahaemolyticus*: enrichment in 7% (w/v) NaCl at 41° ± 1° C and isolation on TCBS agar with 3% (w/v) NaCl at 41° ± 1° C. Nevertheless, productivity of our procedure (see Table I) did not compare unfavourably with the efficiency of other methods. On fish swabs, the lowest detectable inoculum of *V. parahaemolyticus* by various techniques, reported by KAMPELMACHER, MOSSEL, VAN NOORLE JANSEN & VINCENTIE (1970) was 30 cells per 10 ml enrichment fluid. It may be noted that we used TCBS agar without ferric citrate, in correspondence with the formula given by SAKAZAKI (1969); apparently this omission had no detrimental effect on the development of test strains and isolates on the medium.

The reasonably good selectivity of our detection procedure, as evidenced by the recovery experiments, is further illustrated by the fact that only in one case false-positive colonies were observed in our survey of 67 water samples. In 10 of our samples no vibrios were detected (Fig. 1). These negative results show no obvious correlation with location of sampling site, water temperature, or salinity. The other 57 samples (85%) yielded isolates, which were identified as *V. alginolyticus*, i.e. *V. parahaemolyticus* biotype 2 *(alginolyticus)* according to SHEWAN & VÉRON (1974). Of these, 4 strains were methyl red-positive and 2 strains arabinose-positive. These strains might be considered as intermediates between *V. alginolyticus* and *V. parahaemolyticus*; they differ from intermediates I and II, described by ROTTINI, TAMARO, CINCO & MONTI-BRAGADIN (1974), as well as from the new biotype, introduced by THOMSON & THACKER (1974). We did not isolate *V. parahaemolyticus*, i.e. *V. parahaemolyticus* biotype (*parahaemolyticus*) according to SHEWAN & VÉRON (1974), from any of our samples. Isolation of *V. anguillarum* by our procedure could not be expected, since this organism does not tolerate 7% NaCl and is incapable of growth at 41° C.

In view of the efficiency of our detection method (see Table I), the

MPN values for *V. alginolyticus*, observed in a few samples of sea water, should be considered as minimum values.

Although *V. parahaemolyticus* (biotype 1) was not isolated from our 50 ml-samples, occurrence of this potential pathogen in Dutch coastal waters is not discounted. Indeed, KAMPELMACHER, VAN NOORLE JANSEN, MOSSEL & GROEN (1972) found *V. parahaemolyticus* present in 200 ml-samples of water, rich in sediment, taken from the Oosterschelde estuary during January-March 1971. Also, in view of the similarity in physiology and ecology between biotypes 1 and 2 of *V. parahaemolyticus*, the high incidence in our samples of the biotype *alginolyticus* indicates that the biotype *parahaemolyticus* may also be expected to occur. To our knowledge, up to the present no cases of gastroenteritis or other pathogenic effects, ascribed to *V. parahaemolyticus*, have been reported in The Netherlands.

In Europe, *V. parahaemolyticus* has been isolated abundantly from the Baltic Sea and the Black Sea, and quite frequently from the Mediterranean, but rarely from the North Sea, near the Danish and, German coasts (LEISTNER & HECHELMANN, 1974). In 407 samples of fish and shellfish, originating from the North Sea and marketed in The Netherlands, KAMPELMACHER, MOSSEL, VAN NOORLE JANSEN & VINCENTIE (1970) found only one fish to contain *V. parahaemolyticus*. In Britain, the organism has been isolated from fish and shellfish (BARROW & MILLER, 1972). Up to now, conclusive evidence for pathogenic effects of *V. parahaemolyticus* in European countries is scarce (LEISTNER & HECHELMANN, 1974).

V. alginolyticus has been detected on 48% of fish from a German North Sea market in August, and on 4% in December (NAKANISHI, LEISTNER, HECHELMANN & BAUMGART, 1968). In The Netherlands, *V. alginolyticus* has been found by KAMPELMACHER, VAN NOORLE JANSEN, MOSSEL & GROEN (1972) in 4% of mussels, 7% of oysters, and 6% of water samples (200 ml) in the Oosterschelde estuary during January-March.

The results of our survey indicate a high incidence of *V. alginolyticus* in Dutch coastal waters during the warm season. According to general experience, *V. alginolyticus* and *V. parahaemolyticus* are inhabitants of coastal seas and estuaries, and are scarce in the high seas. The organisms have been found to be closely associated with zooplankton (COLWELL, LOVELACE, WAN, KANEKO, STALEY, CHEN & TUBIASH, 1973; KANEKO & COLWELL, 1973, 1975a, 1975b). Preference of the vibrios for coastal waters has been ascribed to the high organic content of inshore waters (BAROSS & LISTON, 1970). The question remains to be answered, whether organic matter in general or more specifically the concentration of plankton determines development of the vibrios;

eutrophication in polluted coastal water might result in a proliferation of the vibrios, associated with zooplankton.

V. SUMMARY

Water samples, collected along the Dutch coast, were examined for the presence of marine vibrios. The procedure included enrichment in trypticase soy broth with 7% (w/v) NaCl at 41° ± 1° C for 18 to 48 h, followed by isolation on modified thiosulphate citrate bile salts sucrose agar (TCBS agar), containing 3% (w/v) NaCl and incubated at 41° ± 1° C for 18 to 24 h. The procedure procured moderate recovery from 1 to 20 viable cells, good recovery from more than 20 viable cells of *Vibrio parahaemolyticus* in 50 ml-samples of sea water, and good recovery from 3 to 20 viable cells of *V. alginolyticus* in 50 ml-samples.

From 67 samples (50 ml) of sea water, estuarine water, and other saline or brackish inshore water, collected along the coast in a period of maximum water temperatures, 10 samples yielded no vibrios. From all other samples (85%), *Vibrio parahaemolyticus* biotype 2 *(alginolyticus)* was isolated; 4 of these isolates were methyl red-positive, and 2 other isolates were arabinose-positive. *Vibrio parahaemolyticus* biotype 1 *(parahaemolyticus)* was not detected in any of the samples.

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