Influence of ionic strength and substratum hydrophobicity on the co-adhesion of oral microbial pairs

Rolf Bos, Henny C. van der Mei and Henk J. Busscher

Co-adhesion between oral microbial pairs (i.e. adhesion of a planktonic microorganism to a sessile organism adhering to a substratum surface) has been described as a highly specific interaction, mediated by stereochemical groups on the interacting microbial cell surfaces, and also as a non-specific, critical colloid-chemical interaction. In a colloid-chemical approach, microbial co-adhesion is considered as an interplay between, amongst others, hydrophobic and electrostatic interactions. The aim of this paper was to determine the influence of ionic strength on the co-adhesion of Streptococcus oralis 34 to either Actinomyces naeslundii T14V-J1 or its mutant strain 5951 adhering to glass in a parallel-plate flow chamber. To this end, the ionic strength of the suspension was varied by the addition of KCl. Another aim was to investigate whether substratum hydrophobicity affected the co-adhesion between the organisms by allowing the sessile organisms (in this case the actinomyces) to adhere either to hydrophilic or to hydrophobic, dimethyldichlorosilane (DDS)-coated glass. The kinetics of co-adhesion of S. oralis 34 to the actinomyces decreased with increasing ionic strength, expressed as the ratio, \( \chi \), between the local and non-local initial deposition rates of the streptococci in the vicinity of, or far away from, the adhering actinomyces, respectively. In a stationary end-point of co-adhesion, ionic strength appeared not to be a determinant factor for the co-adhesion of S. oralis 34 with A. naeslundii 5951, either when the actinomyces were adhering to hydrophilic glass or to hydrophobic, DDS-coated glass. However, for S. oralis 34 co-adhering in a stationary end-point with A. naeslundii T14V-J1 in the high-ionic-strength (250 mM KCI) suspension, co-adhesion was far less on hydrophobic, DDS-coated glass than on hydrophilic glass. It is possible that the hydrophobic fibrils on A. naeslundii T14V-J1 bearing the lectin responsible for co-adhesion were immobilized in the latter case by adsorption to the hydrophobic substratum, making them less available for interaction with the streptococci.

Keywords: coaggregation, co-adhesion, hydrophobicity, zeta potentials, ionic strength

INTRODUCTION

Interactions between micro-organisms and a substratum surface as well as interactions between micro-organisms are important events during the initial stages of biofilm formation and determine the microbial composition and structure of the biofilm in later stages. Physico-chemical properties of the interacting cell and substratum surfaces influence the adhesion of micro-organisms and may consequently contribute to species variability in a biofilm (Verrier et al., 1987; Pratt-Terpstra et al., 1989). In addition to cell–substratum interactions, species variability is promoted by micro-organisms that adhere initially, since these organisms may stimulate adhesion of planktonic organisms, a phenomenon known as co-adhesion. When interspecies binding occurs, a complex multispecies biofilm, as frequently observed in the oral cavity, will emerge (Gibbons & Nygaard, 1970).

A physico-chemical approach will most likely never be able to fully explain all aspects of microbial adhesion to
surfaces, including interspecies binding. Even when adhesion to inert surfaces is considered, the influences of microbial cell-surface appendages (Handley et al., 1991), of highly specific, sparsely distributed receptor sites (Busscher et al., 1992) or of biosurfactant release (Rosenberg, 1986) by adhering organisms will be difficult to incorporate. Consequently, microbial adhesion to substratum surfaces must be considered a multifactorial process, unlikely to be ever explained on the basis of one single mechanism or approach. Considering now the complexity of microbial cell surfaces, it is even more unlikely that a single approach or mechanism can be identified which covers all aspects of microbial coaggregation and co-adhesion.

Often interspecies binding is mediated by lectin or lectin-like carbohydrate interactions (Kolenbrander, 1989). The fundamental forces in lectin–carbohydrate interactions are Lifshitz–van der Waals’ forces, electrostatic interactions and hydrogen bonding. Stereospecificity is achieved mainly through the highly directional hydrogen bonds (Quiocho, 1986). Although these short-range interactions (separation distances < 5 nm) are of key importance to obtain specificity, the overall surface characteristics of the interacting micro-organisms are equally important. Often, micro-organisms are unable to approach each other close enough, due to, for instance, electrostatic repulsion (separation distances 10–20 nm), for stereochemical combinations between highly localized molecular groups to occur. Therefore, in order to adequately describe interspecies binding, overall, long-range and non-directional interaction forces between organisms must also be taken into consideration. Recently, we showed that lectin-mediated interspecies binding between Streptococcus oralis 34 and Actinomyces naeslundii T14V-J1 or its mutant strain 5951 could be described in colloid–chemical terms as a critical balance between attractive Lifshitz–van der Waals’ forces and localized electrostatic attraction on the one hand and overall electrostatic repulsion on the other (Bos et al., 1996).

Study of such a delicate force balance requires an experimental system in which other forces such as hydrodynamic shear can be well controlled. With the aid of a parallel-plate flow chamber (Sjölem et al., 1989) equipped with in situ observation and image analysis facilities, it is possible to study the kinetics as well as the spatial arrangement of microbial adhesion under controlled hydrodynamic conditions. Quantification of co-adhesion in a parallel-plate flow chamber can be done by monitoring the deposition of one microbial strain (e.g. a streptococcal strain) in the vicinity of an already-adhering but different strain (e.g. actinomyces), while distinguishing between the strains on the basis of their times of arrival at the substratum surface. When, for instance, this methodology was employed to study the role of calcium ions in the co-adhesion between S. oralis 34 and A. naeslundii 5951, we observed that in buffer with added calcium ions the local initial deposition rate near preadhering actinomyces was maximal and only limited by mass transport. In buffer supplemented with magnesium or barium, however, the local initial deposition rate was 10-fold lower (Bos et al., 1996) than in buffer with calcium added, whereas the non-local initial deposition rate, i.e. streptococcal deposition to the bare substratum, was not influenced by the type of cation added.

Several investigators have shown that substratum hydrophobicity, charge and the ionic strength of the microbial suspension have a pronounced effect on the adhesion of micro-organisms, but their effect on co-adhesion is unknown. Therefore, it is the aim of this paper to study the influence of ionic strength and substratum hydrophobicity on co-adhesion of two pairs of coaggregating oral microbial strains. To this end, co-adhesion experiments were carried out in a parallel-plate flow chamber, using hydrophilic glass and silanized hydrophobic glass as substrata. The ionic strength of the suspension was varied by the addition of KCl.

**METHODS**

**Bacterial strains, culture conditions and harvesting.** Two pairs of coaggregating streptococci and actinomyces were used which showed exclusively lectin-mediated coaggregation: Streptococcus oralis 34 (formerly called S. sanguis 34) with either Actinomyces naeslundii T14V-J1 or 5951. A. naeslundii T14V-J1 bears two types of fibrils, mediating the interaction with salivary proteins (type 1 fibrils) and lectin-like fibrils (type 2 fibrils), responsible for the interaction with sugars; A. naeslundii 5951 only possesses type 2 fibrils (Cisar et al., 1988). All strains were cultured in Schaedler’s broth supplemented with 0·01 g haemin 1–1; S. oralis 34 was incubated in ambient air while the actinomyces were incubated in an anaerobic cabinet (DW Scientific) in an atmosphere of H2/N2/CO2 (10:85:5, by vol.) at 37 °C (strains were kindly provided by Dr P. E. Kolenbrander and Dr J. O. Cisar, NIH, Bethesda, USA). For each experiment, strains were inoculated from blood agar in a batch culture. This culture was used to inoculate a second culture which was grown for 16 h prior to harvesting.

Bacteria were harvested by centrifugation (5 min at 10000 g), washed twice with demineralized water and resuspended in the appropriate buffer. To break bacterial chains and aggregates, cells were sonicated for 30 s at 30 W (Vibra Cell model 375; Sonics and Materials). Sonication was done intermittently while cooling in an ice/water bath. These conditions were found not to cause cell lysis in any strain, nor did the supernatant of pelleted sonicated cells cause coaggregation of the partner cells. Actinomyces were suspended in buffer (2 mM potassium phosphate, 50 mM potassium chloride and 0·5 mM calcium chloride, pH 6·8) to a density of 1 x 108 ml–1. Streptococci were suspended in buffer, with the appropriate amount of KCl added, to a density of 3 x 108 ml–1.

**Substrata.** Glass plates (7·5 x 5 cm), constituting top and bottom plates of the parallel-plate flow chamber, were used as substrata and were cleaned as described previously by Sjölem et al. (1989), yielding a 0° water contact angle. Hydrophobic substrata were obtained by silanization [dimethyldichlorosiloxane (DDS)] of clean glass plates as previously described by Ruardy et al. (1995), yielding a hydrophobic surface with a water contact angle of 92°.

**Microelectrophoresis.** Electrophoretic mobilities of the bacteria in the appropriate buffers were measured with a Lazer Zee Meter 501 (PenKem), which uses scattering of incident laser light to enable detection of the bacteria at relatively low magnifications. Aliquots of the bacterial suspensions were added
to a density of approximately $1 \times 10^6$ ml$^{-1}$ to buffer with various amounts of added KCl. The voltage difference across the electrophoresis chamber was set to 150 V and the electrophoretic mobilities measured were converted to apparent zeta potentials through the use of the Smoluchowski equation (Hiemenz, 1977). All zeta potentials reported are the mean values of measurements on two different cultures.

Streaming potential. Streaming potentials of the substratum surfaces were measured in the appropriate buffers employing rectangular platinum electrodes (5.0 x 25.0 mm) located at both ends of a parallel-plate flow chamber (Van Wagener & Andrade, 1980). Two glass slides separated by a 0.2 mm Teflon gasket constituted the top and bottom plates of the chamber. Streaming potentials were measured at ten different pressures ranging from 37.5 to 150 Torr (5-20 kPa) and each pressure was applied for 10 s in both directions. Subsequently, zeta potentials were derived from the pressure dependence of the streaming potentials, neglecting surface conductivity.

Parallel-plate flow chamber system and image analysis. The parallel-plate flow chamber and image analysis procedures employed have been described in detail previously (Sjollema et al., 1993; Bos et al., 1994), but will be briefly repeated for completeness. Microbial adhesion was observed in situ on the bottom plate of the parallel-plate flow chamber (7.5 x 3.8 cm) with a channel height of 0.06 cm. Observations were done with a CCD-MXR camera (High Technology), mounted on a phase-contrast microscope (Olympus BH 2) equipped with a x 40 ultra-long working distance objective (Olympus U.WD-CD Plan 40 PL). The camera was connected to an image analyser (TEA; Difia).

Live images were Laplace-filtered after subtraction of an out-of-focus image. Thereafter, adhering bacteria were discriminated from the background by single-grey-value thresholding. This yielded binary black-and-white images which were subsequently stored on disk. In the setup, an image covers a surface area of 0.016 mm$^2$.

Deposition protocol. Co-adhesion experiments were carried out at 22°C. Microbial suspensions were circulated through the system by means of hydrostatic pressure at a wall shear rate of 10 s$^{-1}$, which yields a laminar flow with a Reynolds number of 0.6.

The actinomyces suspension was circulated first through the system until a surface density of $10^6$ cm$^{-2}$ was reached. Thereafter, the flow was switched to buffer with an appropriate concentration of KCl to remove all unattached actinomyces from the flow chamber and the tubes. The co-adhesion phase started when the flow was switched to the streptococcal suspension and image acquisition was set in progress for 4 h.

Data analysis. Data analysis was done as previously described (Sjollema & Busscher, 1990; Bos et al., 1995), distinguishing adhering streptococci from actinomyces on the basis of their times of arrival. Co-adhesion was quantified by determining the number of streptococci adhering in a shell around the centre of the pre-adhering actinomyces and to other parts of the substratum. Based on this analysis, the total initial deposition rate, $j_{0}$, i.e. the number of organisms initially adhering per unit time and area, can be written as

$$j_{0} = \phi j_{0e} + (1 - \phi) j_{0n}$$

in which $j_{0e}$ and $j_{0n}$ represent the initial deposition rate within the shells immediately around pre-adhering actinomyces ('local initial deposition rate') and the initial deposition rate outside these shells on the bare substratum ('non-local initial deposition rate'), respectively. $\phi$ is the fraction of the substratum surface contained within the shells around the pre-adhering actinomyces. For the present experiments, the radius of the shells was chosen to be 1.0 $\mu$m and $\phi$ can be calculated to be 0.07 based on the density and the dimensions of the pre-adhering actinomyces. For a co-adhering pair, the ratio between local and non-local initial deposition rate, $\chi$, can become up to 19 times unity, whereas for a non co-adhering pair $\chi$ is equal or smaller than unity (Bos et al., 1995).

Co-adhesion in a stationary end-point was quantified by means of radial pair distribution functions, revealing the relative prevalence of streptococci adhering in the immediate vicinity of the pre-adhering actinomyces as compared to the mean density of adhering streptococci. The term 'stationary end-point' was chosen to denote the end-point of the co-adhesion process rather than to use the term 'equilibrium co-adhesion', because the use of the word 'equilibrium' implies reversibility, which has not been established in microbial co-adhesion. Also in this respect, the expression 'stationary state of co-adhesion' was considered inappropriate to use since it might cause confusion with the expressions used to designate microbial growth states. Local densities of adhering streptococci were determined in shells with thickness $dr$ (0-16 $\mu$m) at distances $r$ from the centre of the pre-adhering actinomyces. This procedure was performed for each adhering actinomyces. The local density of streptococci around pre-adhering actinomyces was normalized with respect to the mean density of the streptococci, yielding the distribution function $g_{21}(r)$. When streptococci are randomly distributed over the entire substratum surface, $g_{21}(r)$ equals unity. However, if there is preferential adhesion around actinomyces, then $g_{21}(r) > 1$. Such regions must of course be compensated for by regions where $g_{21}(r) < 1$.

Details about the exact calculations of the radial pair distribution function have been given elsewhere (Sjollema & Busscher, 1990).

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**Fig. 1.** Zeta potentials of *S. oralis* 34 (——△——) and *A. naeslundii* T14V-J1 (——●——) and 5951 (——■——). In a 2 mM potassium phosphate buffer with 0.5 mM CaCl$_2$ added, as a function of ionic strength, i.e. the amount of KCl added. Experiments were done in duplicate with separate cultures, yielding standard deviations of ± 1.5 mV over six readings of the zeta potential, which equals the variation between two cultures.
RESULTS

Fig. 1 presents the zeta potentials of the strains used as a function of the KCl concentration. All strains clearly displayed less negative zeta potentials with increasing ionic strength.

The zeta potentials of the hydrophilic and hydrophobic, DDS-coated glass were similar in the low (15 and 50 mM KCl)-ionic-strength buffers and amounted to −23 mV on average. In the 250 mM KCl buffer, however, the hydrophilic glass became less negative (−14 mV), while the zeta potential of the hydrophobic, DDS-coated glass remained constant (−23 mV).

Fig. 2 presents examples of the deposition kinetics of streptococci from a low- and a high-ionic-strength buffer to hydrophilic glass with pre-adhered A. naeslundi2 5951.

A quantitative analysis of streptococcal deposition far away from pre-adhering actinomyces ('non-local' data) is given in Table 1, showing that the non-local initial deposition rates of *S. oralis* 34 as well as the non-locally adhering numbers of micro-organisms after 4 h varied both with ionic strength and with substratum hydrophobicity. The non-local initial deposition rates decrease with increasing ionic strength on the hydrophilic substratum but not on hydrophobic, DDS-coated glass. In the low- and intermediate-ionic-strength suspensions, both non-local initial deposition rates as well as numbers of organisms adhering after 4 h are lower on a hydrophobic substratum than on a hydrophilic substratum. However, in the high-ionic-strength suspension both the initial deposition rates and the numbers adhering after 4 h are similar or higher on the hydrophobic substratum.

Tables 2 and 3 summarize the quantitative analysis of local streptococcal deposition in the close vicinity of the pre-adhering actinomyces, i.e. co-adhesion. With regard to the kinetics of local *S. oralis* 34 deposition, it can be noted from Table 2 that, for both microbial pairs, the local initial deposition rates decrease with increasing ionic strength. The influence of substratum hydrophobicity on local initial deposition rates is more pronounced, however, for *S. oralis* 34 co-adhering with *A. naeslundi2* T14V-J1 than with 5951. Similar trends can be seen when co-adhesion kinetics are quantified by the ratio, *γ*, between local and non-local initial deposition rates.

Table 3 summarizes the characteristics of the radial pair distribution functions in a stationary end-point of co-adhesion. There is no significant effect of either ionic strength or substratum hydrophobicity on the screening distance *r*<sub>c</sub>, indicating that streptococci and actinomyces can become closely associated. The effects of ionic strength and substratum hydrophobicity are also less upon co-adhesion in a stationary end-point, as judged from the radial distribution function **g**<sub>sd</sub>(*r*<sub>p</sub>) than for the kinetics of co-adhesion. Interestingly, co-adhesion in a stationary end-point decreases only with ionic strength for the pair *S. oralis* 34 and *A. naeslundi2* T14V-J1 on a hydrophobic substratum, but not on a hydrophilic substratum.

DISCUSSION

In this paper, we studied the influence of ionic strength of microbial suspensions and substratum hydrophobicity upon the interaction between two micro-organisms, one of which is adhering to a substratum surface. Ionic strength directly determines the range and magnitude of the electrostatic interactions between the micro-organisms and consequently the co-adhesion between the microbial pairs studied is influenced by the ionic strength of the microbial suspension. At the onset of this study, we anticipated little effect of substratum hydrophobicity upon co-adhesion, because we felt that substratum hydrophobicity would not be of direct influence on the interaction between two micro-organisms, one of which is adhering to a substratum surface. The observation that co-adhesion between *S. oralis* 34 and *A. naeslundi2* T14V-J1 from the high-ionic-strength buffer in a stationary end-point occurred on hydrophilic glass, but hardly on hydrophobic, DDS-coated glass was surprising to us.

According to the classical DLVO theory (developed by and named after Derjaguin, Landau, Verwey and Overbeek) for the interaction between micro-organisms and a substratum (Rutter & Vincent, 1980), increasing ionic strength minimizes both repulsive and attractive electrostatic interaction forces as well as their range. As most natural surfaces, including microbial cell surfaces, are negatively charged, microbial adhesion generally occurs despite electrostatic repulsion between the interacting surfaces. In the classical DLVO theory, it is envisaged that attractive Lifshitz–van der Waals’ forces overcome the electrostatic repulsion and mediate microbial adhesion. Although such a simplified approach of microbial adhesion has proven merits (Van Loosdrecht & Zehnder, 1990), classical DLVO theory does not include acid–base interactions as accounted for in an extended DLVO approach developed by Van Oss et al. (1986). Possibly, the hydration forces described in the literature (Pasbely & Israelachvili, 1984) as well as ‘hydrophobic interactions’ (Van Oss, 1995) may be considered as a corollary of these acid–base interactions. These hydration forces are repulsive for hydrophilic interacting surfaces and increase with increasing ionic strength (Pasbely & Israelachvili, 1984). Several studies have implicated a role of hydration forces in the adhesion of inert particles (Llimelech, 1990) and micro-organisms to substratum surfaces (Sjollema et al., 1988).

There are a number of trends in the ‘non-local’ adhesion data that are in line with the above described (extended) DLVO theory. First, the decrease in non-local initial deposition rates with increasing ionic strength is contrary to expectations on the basis of classical DLVO theory, which predicts an increase in deposition rate through the reduction in electrostatic repulsion. However, this reduction in deposition rate as experimentally observed for high KCl concentrations is in line with the existence of repulsive hydration forces, as described in the extended DLVO theory and occurring on a hydrophilic substratum. Second, on hydrophobic, DDS-coated glass, the hydration forces are attractive, but are counterbalanced by
Local initial deposition rates of streptococci are less strongly decreased with increasing ionic strength of the microbial suspension, through the influence of repulsive hydration forces or a reduction in localized electrostatic attraction. Alternately, it is suggested that potassium adsorption may prevent calcium bridging, as a mechanism contributing to co-adhesion between microbial pairs (Bos et al., 1996).

Local initial deposition rates of S. oralis 34 near pre-adhering A. naeslundii on hydrophobic, DDS-coated glass are significantly lower than on hydrophilic glass. The lectins on the actinomyces, which mediate the interaction with carbohydrates on the streptococcal cell surfaces, reside on hydrophobic fibrils, which can be up to 1 µm long (Cisar & Vatter, 1979). Thermodynamically, it is likely that these hydrophobic fibrils become immobilized by adsorption to hydrophobic, DDS-coated glass (Absolom et al., 1983; Busscher et al., 1984), thus making them less available for the interaction with planktonic streptococci and yielding reduced local initial deposition rates. Co-adhesion in a stationary end-point is not affected by ionic strength, except for the pair S. oralis 34 and A. naeslundii T14V–J1 on hydrophobic, DDS-coated glass, which is probably related again to the fact that the fibrils bearing the lectin responsible for co-adhesion on A. naeslundii T14V–J1 are immobilized by adsorption to hydrophobic, DDS-coated glass.

In summary, the results of this paper demonstrate that the kinetics of co-adhesion of two oral microbial pairs decrease with increasing ionic strength of the microbial suspension, through the influence of repulsive hydration forces or a reduction in localized electrostatic attraction. Substratum hydrophobicity influenced the co-adhesion between S. oralis 34 and A. naeslundii, possibly due to adsorption of the fibrils present on the actinomyces cell surface.
Table 1. Non-local initial deposition rates and numbers of adhering S. oralis 34 after 4 h for hydrophilic and hydrophobic DDS-coated glass with pre-adhering A. naeslundii T14V-J1 or 5951

Experiments were carried out in duplicate with separately cultured bacteria and results varied by less than 20%.

<table>
<thead>
<tr>
<th>A. naeslundii strain</th>
<th>KCl concn (mM)</th>
<th>Hydrophilic</th>
<th>Hydrophobic</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>$f_{ad}$ ($\text{cm}^{-2} \text{s}^{-1}$)</td>
<td>$10^{-4} \times n_{ad}$ ($\text{cm}^{-2}$)</td>
</tr>
<tr>
<td>T14V-J1</td>
<td>15</td>
<td>580</td>
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<td>T14V-J1</td>
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<tr>
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<td>5951</td>
<td>15</td>
<td>606</td>
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<td>854</td>
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<tr>
<td>5951</td>
<td>250</td>
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<td>6.7</td>
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Table 2. Kinetics of co-adhesion expressed as local initial deposition rates and as ratios, $x$, between local and non-local initial deposition rates of S. oralis 34 to hydrophilic and hydrophobic (DDS-coated) glass with pre-adhering A. naeslundii T14V-J1 or 5951

Experiments were carried out in duplicate with separately cultured bacteria and results varied by less than 20%.

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<tr>
<td></td>
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<td>$f_{ad}$ ($\text{cm}^{-2} \text{s}^{-1}$)</td>
<td>$x$</td>
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<td>1000</td>
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Table 3. Characteristic features of the radial pair distribution functions for the co-adhesion of S. oralis 34 with A. naeslundii T14V-J1 and 5951 pre-adhering to hydrophilic or hydrophobic (DDS-coated) glass, including the screening distances, $r_s$, and the radial distribution function at the distance of preferential co-adhesion, $g_2(r_p)$

Experiments were carried out in duplicate with separately cultured bacteria and results varied by less than 20%.

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<td>$r_s$ ($\mu$m)</td>
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ACKNOWLEDGEMENTS

We are greatly indebted to Mrs Marion Schakenraad-Dolfin for manuscript preparation and to Mr Joop de Vries for performing the streaming potential measurements.

REFERENCES


